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The Interictal State In Epilepsy And Behavior

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THE INTERICTAL STATE IN EPILEPSY AND BEHAVIOR

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DEDICATION

This work is dedicated both to my family, furry and otherwise, without whose support I would not be where I am, and to the many rats whose lives were sacrificed for this work.

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CHAPTER 1

Introduction

Epilepsy is a disease defined by the presence of recurrent seizures, where a seizure is a period of excessive, synchronous neural activity in the brain. Symptoms of seizures vary depending on the location of the initiating site (seizure focus) and whether it spreads across other areas of the brain, but can range anywhere from brief staring episodes ('absence seizures') to sensing unusual odors to death from sustained, generalized convulsions. As most types of seizures involve a loss of consciousness, even mild forms of epilepsy are debilitating for patients since they prevent many common daily activities such as driving a car or bathing alone. Epilepsy is also one of the most common neurological disorders occurring in up to 1.0% of the world population (1). Approximately two million people in the United States have epilepsy and three percent will develop epilepsy at some point in their lives.

Despite epilepsy's prevalence, severity, and the fact that it has been recognized for millennia (2), very little is known about its underlying cause. While single gene defects in ion channels or neurotransmitter receptors are associated with some forms of inherited epilepsy (3-5), these mutations account for only a very small proportion of epilepsy cases. Most cases of epilepsy result from a focal brain region of enhanced excitability, which can depolarize rhythmically and cause excitability to spread through the brain, leading to generalized convulsions. Nearly any type of brain lesion can produce epilepsy, including trauma, tumor, and cerebrovascular disease, but there is often no consistently identifiable pathology (6). The heterogeneity of causes, combined with the fact that most cases have no discernable etiology, have made studying and treating the

disease difficult. Current pharmacological treatments for epilepsy target only the symptom of seizures in a general manner, but do not address the underlying brain abnormality that generates them (7). This means that patients must be on medications for life, often with severe side-effects due to their lack of specificity for the epileptic areas of brain. In approximately 30% of patients, even these medications cannot adequately reduce their seizure frequency (8).

In recent years, electrical mapping and imaging techniques have allowed for surgical resection of epileptic foci in patients with intractable epilepsy which does not respond to medications. Resection is often curative (9), which implies a fundamental difference between regions that initiate seizures and nearby areas that do not. Although there are numerous causes of epilepsy, all cases exhibit a similar syndrome of increased, coordinated activity, so there may in fact be a final, common, causal pathway. With this in mind, previous studies in our laboratory compared gene expression levels between areas of seizure onset and areas not involved in seizures within individual patients (10,11). When these results were compiled across multiple patients with different etiologies, a group of approximately 140 genes were found to be commonly differentially expressed at seizure onset zones, no matter what originally caused each patient's epilepsy. Further, many of these genes were localized to cortical layers II and III, and synaptic density was increased in these layers as well. Discovery of these common molecular changes will allow animal models of epilepsy to be verified as true molecular mimics of the human disease so that targeted therapies developed in them are more likely to translate into viable treatments.

Interictal spikes in human epilepsy

In patients with epilepsy, abnormal electrical discharges occur between seizures, referred to as "interictal spikes". These interictal spikes are small, abnormal discharges from focal areas of brain. Like seizures, they are the product of a population of inappropriately-synchronized neurons firing together. Unlike seizures, they do not spread across large areas of the brain and do not cause any recognized symptoms. However, because interictal spikes occur much more often than seizures and develop long before actual seizures, there has been a growing interest in the molecular roles of interictal spiking in epilepsy (8). The relationship of interictal spikes to seizures is still controversial, as they increase in frequency after rather than before seizures (12-15). Still, they are most frequent in the areas of brain that initiate seizures in a majority of patients, and are used to identify epileptic brain regions for resection surgeries (16-23). Some studies have shown that removing areas of high spike activity in the temporal lobe is associated with good surgical outcome (19,24,25). Furthermore, a recent double-blind study that used add-on therapy to reduce interictal spiking showed behavioral improvements in patients with epilepsy, raising the possibility that interictal spiking itself is deleterious to normal brain functioning (26).

These observations suggest that interictal spikes play an important role in human epilepsy, yet reveal the paucity of knowledge about them. One of the reasons interictal spikes have been so poorly studied is the difficulty and subjectivity involved with quantifying them. Spikes are typically marked manually by trained electroencephalographers, but thousands of spikes can occur in just a 10-minute intracranial electroencephalogram (EEG) sample and there is no strict definition of a

spike on intracranial EEG (27-30). Additionally, spiking patterns within the same patient vary greatly over time, so a tremendous amount of EEG would need to be reviewed to gain an accurate picture of spiking in each patient (17). A more objective, automated method of spike detection would solve these problems and allow better studies of interictal spiking. Studies based on a reliable detection method might reveal important characteristics of interictal spiking that have previously been masked by reviewer variability.

Despite these limitations, previous work in our laboratory supports an important role for interictal spikes in epilepsy. In Rakhade 2007 (31), it was shown that expression levels of several genes induced in epileptic regions of brain correlated precisely with the frequency and amplitude of interictal spikes measured in the regions of cortex from which the samples were taken. No such correlation was found for any aspects of seizures, such as frequency, duration or type. These results suggest that interictal spikes may be able to induce activity-dependent gene expression, and may be necessary for either creating or maintaining an epileptic focus. Experiments to determine whether this is actually true would have to be carried out in an animal model, however.

Animal models of epilepsy and interictal spikes

Animal models have been used to investigate epilepsy for decades (32), and most of them have been reported to generate interictal spikes in addition to seizures. Rarely, however, are interictal spikes the focus of study and their contribution to both pathology and epileptogenesis is difficult to disentangle from that of seizures. An ideal model for the study of clinically-important interictal spikes in animals would be to mimic the

human phenomenon as closely as possible. This would require interictal spikes in the absence of frank seizures that are similar to those observed in humans, are chronic, spontaneous, and are not associated with significant neuronal toxicity or death.

Both acute and chronic interictal spikes have been observed in animal models. In hippocampal slice culture, gamma-aminobutyric-acid-A ($GABA_A$) blockade (33-35), low extracellular magnesium concentration (36), low extracellular calcium (37,38), kainic acid (39,40), and 4-aminopyridine (41) have all been used to generate interictal-like activities (42). The advantage of these methods is that interictal activity without ictal discharges can be generated in order to study the electrophysiological properties of interictal spikes independently from seizures. Disadvantages are that the relationship of in vitro discharges to those generated in intact animals is unclear and nothing can be said about 'higher order' phenomena such as behavior with in vitro models. Acute in vivo models are better suited to address these problems. Systemic administration of GABA-blocking substances, kainic acid, or pilocarpine produces interictal spikes, but these can also be injected focally. After penicillin injection into the cortex, focal interictal spikes as well as seizures occur and can last for six to eight hours (43-45). Kainic acid or pilocarpine given systemically produce acute status epilepticus that can last for several hours, after which interictal spikes are observed (46-51). Pentylenetetrazol is generally used as a convulsant agent, but in the right dose can produce spikes with no detected seizures and produces minimal tissue damage (52). One question that remains with all of these acute models of epileptiform activity is whether they are truly identical to the spontaneous interictal spikes generated chronically in patients with epilepsy.

Chronic models of epilepsy are generally produced by an initial lesion, followed

by a latent period, and the subsequent development of spontaneous epileptiform activities (42). Most models focus on the hippocampus, and commonly used methods of modeling human hippocampal epilepsy are electrical kindling, or systemic administration of pilocarpine or kainic acid. Kindling involves repeated electrical stimulations which cause seizures. If done enough times, kindling can lead to the production of spontaneous interictal spikes and seizures (12,53,54). Status epilepticus induced by either pilocarpine or kainic acid causes significant neuronal damage in the hippocampus, but after a latent period, large interictal spikes mixed with spontaneous seizures occur chronically in a subset of animals (46-51). For chronic neocortical epilepsy, the most common techniques are application of either toxic metals or tetanus toxin. When metals, such as cobalt, are directly applied to the cortex, spontaneous interictal and ictal activities are observed and can persist for one or more weeks, depending on the method of application (12,53,54). Limitations of using metals are that the epileptiform activity requires continued presence of the irritant and extensive tissue destruction is seen at the site. This is likely different than lesions that produce epileptic foci in humans, such as brain injury, which are usually single, initial insults (1,55,56).

Tetanus toxin injected directly into either the neocortex or hippocampus produces a variety of transient excitatory patterns dependent on the injection site (57-60). It is a good model of chronic epilepsy because it does not produce significant neuronal toxicity as many of the other focal models do and does not produce the tissue destruction observed after prolonged status epilepticus. Tetanus toxin preferentially binds to the gangliosides of inhibitory neurons, where it is taken up by receptor-mediated endocytosis and cleaves synaptobrevin (VAMP-2), which prevents the release of vesicles containing

inhibitory neurotransmitters (61-63). An initial injection of tetanus toxin is followed by a latent period, after which interictal spikes and spontaneous seizures can persist almost indefinitely (>7 months) (60,64,65). Most intriguingly, Brener et. al. noted that, unlike injections into hippocampus or motor cortex that produce seizures early on, injection of small amounts of tetanus toxin into the somatosensory cortex results predominantly in interictal spikes with only infrequent seizures (60). This model seems to offer the ideal characteristics to study interictal spikes in animals since it is a chronic, spontaneous in vivo model, without seizures early on and minimal neuronal cell loss.

Psychiatric and behavioral comorbidities of the interictal state

While interictal spikes do not have any widely-accepted symptoms, epilepsy is associated with a broad range of comorbid conditions. Patients with epilepsy are more likely to have attention deficit hyperactivity disorder (ADHD) (66-72), depression (68,73-78), anxiety disorders (68,73,75,77-79), and other psychiatric conditions (68,71,78-82). Since these patients are usually in the interictal state between seizures, it is possible that interictal spikes may contribute to these pathologies. Interictal spikes in the absence of seizures are not widely thought to have definite symptoms, but they have not been thoroughly studied and there is some preliminary evidence that they may actually have subtle effects. Interictal spikes are best studied on intracranial EEG, as only the largest 1% of spikes are even detectable on scalp EEG (83), but it is clearly unethical to place intracranial grids on anyone not being considered for epilepsy surgery. As such, it is very difficult to study interictal spikes in humans outside of their role in epilepsy. Nevertheless, EEG abnormalities have been noted in a wide range of

psychiatric disorders for decades. In many of these studies, however, all EEG abnormalities were lumped into a single 'abnormal EEG' description and were not categorized specifically as interictal spikes. Several studies describe a high incidence of EEG abnormalities in anxiety disorders, panic disorders, and obsessive-compulsive disorder (84,85). However, one large community study by Bridgers found that epileptiform discharges, specifically, were correlated with symptoms of anorexia nervosa, depression, mania, personality disorders, suicidality without depression, nonpsychotic explosive behavior, and the effects of psychotropic medications (86). Individuals displaying antisocial and aggressive tendencies have also been found to display a wide range of EEG abnormalities, including epileptiform discharges (87-91). Given the wide array of psychiatric symptoms and the comorbidity of psychiatric diseases with epilepsy, long term human recordings and focused behavioral studies in animals will be needed to establish any causality between these abnormal brain activities and behaviors.

More direct associations of interictal spikes with specific cognitive behaviors have also been noted on scalp EEG. A recent double-blind study that reduced interictal spiking using lamotrigine as an add-on seizure therapies showed significant behavioral improvements in patients with epilepsy raising the possibility that interictal spiking is deleterious to normal brain functioning (26). Some studies suggest an association between high frequency interictal spikes and cognitive and behavioral decline in children with autism (92,93). Inui, et. al. found that spike variants were significantly higher among patients with mood-incongruent psychotic mood disorder (33%), schizoaffective disorder (33%), and schizophreniform disorder (30%) as compared with patients with nonpsychotic mood disorder (3.2%) and schizophrenia (0%) (94). Interictal spikes have

even been associated with self-injurious behavior among tuberous sclerosis patients (95). With a potential role in such a wide range of disorders, it would be advantageous to develop a better understanding of interictal spikes through animal models.

Genomic perspective on epilepsy

While this project will focus largely on specific protein-coding genes and pathways in epilepsy, modern techniques now allow for broader analyses to be done, such as investigating epigenetic modifications, genomic instability and genome-wide association studies. Many genetic association studies have been conducted in epilepsy, but most have been underpowered and have not yielded consistent susceptibility genes. [for review see (96)]. Recently though, results of the first large-scale, genome-wide association study in epilepsy were released (97). This study compared 3445 patients with partial (focal) epilepsies to 6935 controls in order to identify common patterns of genetic susceptibility. Even with a study this large, however, the authors found no significant genome-wide association and were forced to conclude that common genetic variation plays only a minor role in predisposition to partial epilepsies. They instead propose that epilepsy be considered under the "common disease-rare variant" hypothesis (98), where many rare genetic variants underlie a common disease. Limitations of this study are that it was carried out in an entirely European population and that types of epilepsy were ignored. It is therefore possible that a similar study in a different population or which focuses on a specific type of partial epilepsy may have more success in identifying common susceptibility variants. Still, these initial results argue against a productive role

for these types of association studies in epilepsy and leave acquired, focal models of epilepsy as the best choice for modeling what is known of the human disease.

Epigenetic modifications have been implicated in Alzheimer's disease (99-102), Huntington's disease (103-106), and many other neurological disorders [for review see (107)], so epigenetics may play a role in epilepsy as well. The first evidence of epigenetic involvement in epilepsy came from one of the older anti-seizure medications still in use today, valproic acid. In addition to enhancing GABAergic function, valproic acid can also inhibit histone deacetylases (HDACs) at therapeutic concentrations (108). Kainic acid, one of the most commonly used compounds for modeling epilepsy in vivo,induces a rapid phosphorylation of histone H3 in dentate gyrus neurons and a more widespread and sustained acetylation of histone H4 in hippocampal neurons (109,110). Three hours after the induction of a seizure, histones H4 on the BDNF promoter show hyperacetylation which correlates with increased mRNA levels. The promoter of the metabotropic glutamate receptor (GluR2) also shows epigenetic regulation, with histones H3 and H4 rapidly deacetylated after seizures and corresponding to mRNA downregulation (111). In the electroconvulsant model of epilepsy, these findings from the kainic acid model were not only recapitulated, but also showed changes in histones H3 and H4 on the CREB promoter (112). Given that the few studies investigating epigenetic modifications in epilepsy thus far have already shown positive results and that two genes with known alterations in epilepsy, BDNF and CREB, have been implicated, epigenetics is likely to play an important role in epilepsy and merits further study.

Recent studies have shown that genomic instability, such as copy number variants (CNVs), are associated with neurological disorders like ADHD (113,114) and autism

(115), so it is reasonable to conclude that genomic instability may be involved in epilepsy as well. One large study using Illunima genome-wide genotyping arrays followed by comparative genomic hybridization (CGH) found deletions at 16p13.11 in 23 of 3812 epilepsy patients, while none of the 1299 controls showed deletions larger than 16kb (116). Another study identified a microdeletion at 15q13.3 in 12 of 1,223 (1%) of idiopathic, generalized epilepsy cases using array comparative genomic hybridization (aCGH) (117) while a different study found this same deletion in 7 of 539 (1.3%) cases and then showed that it seems to behave as a susceptibility variant (118); it is found in some but not all family members with epilepsy and some unaffected members also carry the deletion. The same group then extended their search to additional large, recurrent microdeletions which were known to occur more frequently in schizophrenia, psychotic disorder, autism and mental retardation (119). This study used single nucleotide polymorphism (SNP) arrays, followed by validation with quantitative polymerase chain reaction (qPCR) and aCGH, and yielded positive results for the microdeletions at 15q11.2 (12/1234 cases versus 6/3022 controls) and 16p13.11 (6/1234 cases versus 2/3022 controls). Finally, seizures induced in the kainic acid animal model of epilepsy show instability of the mitochondrial genome (120). In this model, acute status epilepticus increased oxidative mtDNA damage, which coincided with mitochondrial H_2O_2 production and a transient decrease in mtDNA repair capacity. In the chronic phase of this model, up to three months after status epilepticus, mitochondria showed increased reactive oxygen species production, accumulation of mtDNA damage and impaired mtDNA repair capacity. Overall, genomic instability does not appear to be involved in

most cases of epilepsy, but few studies in this area have been done and more prevalent stability changes may be found in the future.

Project overview

In this project, we first show the high variability between professional reviewers in marking interictal spikes on intracranial EEG, and then develop and test an automated detection method to solve this problem. Next, we use this automated detection algorithm to identify spikes on intracranial EEG in both tumor and non-tumor patients in order to determine the best spiking parameters to identify the seizure onset zone in each group. We then develop and characterize an animal model of chronic, neocortical interictal spiking to test our observations previously made in human epilepsy and to have a molecularly-accurate model on which to test new therapeutics. Finally, we show that interictal spikes are associated with behavioral changes in this animal model and that a targeted inhibitor can both prevent the development of a spiking focus and normalize behavior.

CHAPTER 2

High inter-reviewer variability of spike detection on intracranial EEG addressed by an automated multi-channel algorithm

INTRODUCTION

Interictal spikes are abnormal discharges that occur between seizures in patients with epilepsy. They are frequently generated in the same regions of the brain that initiate seizures (16,17), but their exact role in epileptogenesis and the induction or protection from seizures remains controversial and requires further study. A significant barrier to such studies comes from the laborious nature of quantifying the thousands of interictal spikes that can occur in a single recording, and the fact that spiking rates vary greatly over time (17) within patients, meaning that many samples have to be marked per patient to get an accurate picture of the spiking pattern. There is also tremendous variability between EEG reviewers on exactly what constitutes a spike on subdural electrocorticography (ECoG) (29,121).

To address these issues, we first conducted a study of inter-reviewer variability in marking interictal spikes in ECoG and, second, compared these results to an automated spike detection algorithm. While many algorithms have been developed for the detection of interictal spikes, most are designed for scalp recordings (122), and those few that were developed specifically for ECoG were either run on single channels or had humans validate spikes after they were already detected by an algorithm (29,121,123,124). Here, we validated a novel automatic spike detection algorithm that emulated human reviewing methods by analyzing multi-channel ECoG sections obtained from 10 patients. We compared its performance to three independent human reviewers.

METHODS

Intracranial EEG recordings for this study were obtained from 10 patients undergoing two-stage surgical resections at the Comprehensive Epilepsy Program at Wayne State University (#0860000MP2F, Wayne State University Human Investigation Committee). These patients represent an equal mix of children (8 months-14 years) and adults (20-54 years), and were selected to include a wide range of activity levels and spike morphologies. Recordings were obtained with a Stellate Harmonie digital recorder (Stellate Inc., Montreal, PQ, Canada) sampled at 200Hz, and 10-minute sections were selected from periods of quiet wakefulness separated by at least six hours from a seizure. The average number of channels recorded per patient was 95.9 (SD ± 16.5; range 65-128). Three trained encephalographers independently reviewed these sections in the same referential montage (each recording electrode compaired to a single, designated 'reference' electrode outside the brain surface). Detections which occurred on the same channel within 115ms of another reviewer's mark were taken as detections of the same spike. This distance was chosen to cover the variation in manual marking of spikes without picking up marks that may occur on adjacent spikes. The use of distances 10ms above and below this value produced identical results. As there is no true 'gold standard' for comparison, each reviewer was compared to the set of all detections by the other two reviewers to calculate sensitivity (true positives/(true positives+false negatives)) and precision (true positives/(true positives+false positives)) for each patient. Finally, we used each reviewer's spike detections to generate a ranking of channels from highest spike frequency to lowest. We then compared these rankings between reviewers in each patient by calculating Kendall's coefficient of concordance (W). Spearman's rank

correlation coefficient was also calculated for each reviewer pair, as this is a more common measure of non-parametric correlation and so human reviewer performance could be compared to algorithm performance.

The spike detection method was developed as a Matlab program which interacts directly with the Stellate ECoG files (Algorithm overview in Figure 1A). It extracts successive one-minute blocks of data from the ECoG recording, with each block containing the data for all the recorded channels during that minute. First, channels whose average slopes are more than 10 standard deviations outside the mean for all channels are eliminated as artifactual, after which the data are bandpass filtered between 20 and 50Hz in order to identify interictal spikes (Figure 1B, Similar to Brown, et al. 2007). Peaks with amplitudes outside four standard deviations of the channel mean are noted as potential spike locations for further consideration. The original data are then filtered at 1-35Hz and all channels are scaled by a single scaling factor as an entire block. This method of scaling all channels as one block was chosen because it most closely approximates how a human reviewer adjusts sensitivity to review a recording and because scaling individual channels tends to cause over-marking of less active channels and under-marking of very active channels (Figure 1C). Once the data have been scaled, the amplitude and slope of each halfwave of the potential spikes identified previously are calculated and the values are compared to static thresholds. Waveforms that exceed amplitude and slope thresholds are marked as interictal spikes. All development and testing of the algorithm was done on a separate set of ECoG samples from the testing set used in this study (31).

Algorithm performance was evaluated both by the ability to correctly identify individual spikes marked by human reviewers and the ability to correctly rank channels by spike frequency. We did the same comparison using the commercial spike detector included with Stellate's Harmonie software. Optimal thresholds for each were determined prior to their use in this study on a different group of patients. Sensitivity and precision were calculated separately for spikes marked by at least one reviewer, at least two reviewers, and all three reviewers. To evaluate the performance of the two automatic algorithms (ours and Stellate's) in ranking channels by spike frequency, Spearman's rank correlation coefficient was calculated between the channel ranking of each method and the average channel rank from the three human reviewers.

Finally, to determine whether spikes detected by the algorithm but no human reviewers were truly false positives, ten randomly-selected 'incorrect' marks per patient were later shown to each of the three reviewers who were asked to decide whether these detected spikes were real or not.

RESULTS

A total of 78,743 spike detections were made by human reviewers from the 10 different patients. Surprisingly, only 23.7% of these were marked by at least two reviewers and just 3.1% by all three reviewers (Table 1). Overall, human reviewers performed poorly in regard to identifying each other's spikes (Table 2A. Sensitivity: 27.7±12.5%; precision: 50.7±18.5%), but showed good agreement in many (but not all) patients when simply ranking channel activity from highest to lowest spiking (Table 2B. Kendall's W: 0.752±0.12). Spearman's rank correlation coefficient, a similar measure of

ranking agreement for pairs of data, gave an average value of 0.660±0.02 when calculated for all reviewer pairs across the 10 patients.

To improve quantitative interictal analyses, we developed an automatic spike detection algorithm that uses a combination of frequency filtering and examination of entire blocks of channels (Figure 1). We compared the performance and channel rankings to the three human reviewers as well as a commercial spike detection program included with our EEG software. Consistent with and somewhat better than we found between reviewers, the algorithm gives an overall average correlation coefficient of 0.750±0.16 for channel ranking, while the commercial detector's was 0.574±0.27 (Table 2B). While the algorithm detected more spikes than any single reviewer (50,315 vs. 39,802 for highest reviewer), it performed with similar sensitivity and precision for each of the three human reviewers (Table 2C), and yielded better results than the commercial detector (Table 2D).

To determine whether spikes picked up by the algorithm, but none of the human reviewers were genuinely false positives or simply missed by the reviewers, 10 randomly-selected spike detections for each of the 10 patients were presented in the context of the entire recording to each of the three reviewers. They were then asked to decide whether these detected spikes were real or not. The number of spikes originally detected only by the algorithm, but then later validated by human reviewers varied widely among the three reviewers (Table 3, Mean: 31.3±30.4%; Reviewer 1: 65%; Reviewer 2: 6%; Reviewer 3: 23%) This indicates that many of the ‘false positives’ generated by the algorithm may actually be 'real' spikes.

DISCUSSION

This is perhaps the largest study of inter-reviewer variability in the identification of interictal spikes in intracranial EEG. There was surprisingly poor agreement (3.1±2.7%; range 0-7.4% for different patients; Table 1) between three human reviewers in the identification of specific interictal spikes, despite all being professional encephalographers and two of them (R1 and R3) training at the same institution. These results are in line with previous studies which compared reader variability using shorter files or individual channels (28,121,124-126). While there was significant disagreement on the identification of individual spikes for a given patient, the reviewers generally agreed on the ranking of channels by spike frequency. This is important because determination of the highest-spiking channels is often the main clinical use for quantifying interictal EEG. These findings also underscore the need for automated methods of spike detection which function reliably across a wide range of ECoG types (28-30,121).

Our detection algorithm was able to identify spikes with similar sensitivity and precision as the human reviewers, and did an excellent job of ranking channels based on interictal spiking activity (Figure 1D, Table 2B). Key advantages to this algorithm are that it requires no human intervention or tweaking of parameters, is fast enough to be run 'on-line' as the ECoG is being recorded, and its performance was validated in a completely 'real world' environment. Previous detection algorithms have been tested only against a small set of pre-extracted spikes or single channels from each patient, while here we show that our algorithm performs well when reviewing entire segments in a way that mimics how a human reviewer would see the ECoG. A reliable spike

detection algorithm with good agreement between different reviewers could improve both patient care and research on interictal spiking.

CHAPTER 3

Morphological parameters of interictal spikes to identify seizure onset zones in lesional and non-lesional patients

INTRODUCTION

Interictal spikes are often used to assist in the identification of seizure onset zones for epilepsy resection surgeries. Frequency of spiking is often the main aspect examined, and while this method has positive results (19,24,25), it is far from perfect. Depending on the study, the electrode of maximum spike frequency is located in the seizure onset zone in anywhere from 50-100% of patients examined (16,17,22). In this chapter, we attempt to improve the ability of quantitative interictal electrocorticography (ECoG) to identify seizure onset zones by examining how several additional morphological parameters of interictal spikes relate to seizure onset areas.

Next, we examine the use of electrocorticography and interictal spike detection in patients with epilepsy caused by brain tumors. Brain tumors are a common cause of epilepsy in adults, with more than one-third of all adults with brain tumors developing epilepsy, and more than 50% in cases that involve the cerebral hemispheres (127,128). Rarely, however, is a two-stage surgery with placement of electrocorticographic grids done in these patients to identify seizure onset areas. This is partially due to the additional stress and invasiveness that would be inflicted on patients who may not have a good prognosis even after surgery and partially due to an assumption that removal of the tumor should allow the epilepsy to be cured. If this assumption is not correct though, the use of intracranial electrocorticography to identify areas of seizure onset outside of the

tumor may improve outcome in these patients. Further, it may be possible to identify parameters of interictal spiking which could help detect tumor margins for resection.

METHODS

Patient clinical and electrocorticographical data

Intracranial EEG recordings for this study were obtained from 16 non-lesional patients and 11 lesional patients undergoing two-stage surgical resections at the Comprehensive Epilepsy Program at Wayne State University (#0860000MP2F, Wayne State University Human Investigation Committee). Lesional patients ranged in age from 22-54 years (Mean 40.7±8.5), and included a variety of tumor types (Table 4), while non-lesional patients ranged in age from 17-56 years (Mean 36.7±11.3). The placement of intracranial grids on lesional patients was a clinical decision and was not influenced by this study. Recordings were obtained with a 128-channel Stellate Harmonie Digital recorder (Stellate Inc., Montreal, PQ, Canada) sampled at 200Hz. Recording electrodes were given one of three "Seizure Categories" by a clinical encephalographer: Seizure Onset, Seizure Spread, or Control. Seizure Onset zones were identified by sustained rhythmic changes on EEG that were clearly distinct from background rhythms and associated with the patient's seizure type, while Seizure Spread electrodes are those that pick up these rhythmic discharges within seven seconds of seizure onset. Control electrodes are not involved in seizures.

For interictal analysis, three 10-minute sections of ECoG were selected for each patient from periods of quiet wakefulness separated by at least six hours from a seizure.

Interictal spikes were then detected using the automated method validated previously in Chapter 2. The detected spikes were broken into halfwaves by a Matlab (MathWorks, R2008b, Natick, MA) script as described previously (31) so that amplitude, slope and duration could be measured for each halfwave of each spike (Amplitude 1, Duration 1, Slope 1, Amplitude 2, Duration 2, Slope 2), as well as spike power (frequency * (Amplitude 1 + Amplitude 2)). The average value of each of these parameters was then calculated for each channel in a recording and then averaged across the three recordings per patient.

Three dimensional brain reconstructions and tumor distance calculation

Three dimensional reconstructions of each patient's brain, including the location of recording electrodes and tumors if present, were generated from magnetic resonance imaging (MRI) and computed tomography (CT) scans. These scans were done as part of normal pre-surgical evaluation and treatment. First, a pre-operative T1-weighted MRI sequence was imported using BrainSuite09 (David W. Shattuck, Laboratory of Neuroimaging, UCLA, http://www.loni.ucla.edu/Software/BrainSuite), and the included tools were used to strip away the skull and leave only the underlying brain. After creating and exporting a three-dimensional surface rendering of the patient's brain, this surface was subsequently imported into the custom software program, Smap (Dr. Jing Hua and Darshan Pai, Graphics and Imaging Laboratory, Wayne State University Computer Science Department). Using this software, a post-implantation CT showing the patient's electrode grid locations was aligned and coregistered to the pre-operative MRI. An intensity threshold for the CT was then manually chosen so that only the metal

electrodes were visible. The software was then able to overlay the CT onto the MRI of the patient's brain and extrapolate electrode positions. All electrodes were then manually verified using intraoperative photographs to ensure correct placement.

In lesional patients, tumor locations were manually outlined on each slice of a pre-operative MRI by a neurologist, and these markings were then turned into three-dimensional renderings by the Smap software. Finally, with the reconstruction of the patient's brain, the electrode locations, and the tumor location, linear distance to tumor was calculated for each electrode in each patient using the Smap software.

Correlation of spiking parameters to seizure category and tumor distance

To investigate which interictal spiking parameters best correspond with seizure category, morphological parameters of interictal spikes were first normalized to Z-scores (forcing the data to have a mean of 0 and standard deviation of 1) within each patient so that the data from multiple patients could be combined. Taking seizure category as an ordinal variable where Seizure Onset is more 'epileptic' than Seizure Spread, which is in turn more epileptic than Control, we then calculated Spearman's rank correlation coefficient for each spiking parameter. The non-normal distribution of the measured spiking parameters also required us to use the non-parametric Spearman's correlation for calculating correlations with tumor distance as well. Correlations with p values greater than 0.01 after Bonferroni correction for multiple comparisons were discarded.

Unordered relation of spiking parameters to seizure category and tumor distance

Based on the results of the above study and because the assumption that seizure category can be treated as an ordinal variable may not be correct, we also examined whether any of the three seizure category groups differed from each other in terms of morphological spiking parameters. Due to the non-normal distribution of data, the Kruskal-Wallis test was used to look for differences between the seizure category groups. If this test was significant, it was then followed with a Mann-Whitney U-test to determine which pairs within the three categories significantly differed from each other.

RESULTS

Correlation of spiking parameters to seizure category

In non-lesional patients, seizure category was significantly correlated ($p<0.01$ after Bonferroni correction) with five parameters of interictal spiking: frequency, amplitude of the first halfwave, slope of the first halfwave, amplitude of the second halfwave and spike power (Table 5). All these correlation coefficients were positive, indicating that more 'epileptic' areas of brain have more frequent, larger and steeper spikes than more normal areas. In lesional patients, seizure category was positively correlated with frequency, duration of the second halfwave and spike power. It was inversely correlated with tumor distance, meaning that brain areas closer to tumors are more likely to generate seizures.

Correlation of spiking parameters to tumor distance

Electrode distance from tumor was significantly positively correlated with spike frequency, duration of the first halfwave, and spike power. It was inversely correlated with amplitude of both halfwaves and the slope of both halfwaves. Together, these results would mean that brain areas closer to tumors spike less frequently than farther areas, but have larger and steeper interictal spikes.

Unordered relation of spiking parameters to seizure category and tumor distance

While the above correlations are statistically significant, the actual correlation coefficients are extremely small, which casts doubt on their biological relevance. One issue may be the assumption that the three electrode seizure categories 'seizure onset', 'seizure spread' and 'control' can be treated as ordered. Therefore, we analyzed the same dataset again, this time treating seizure category as a nominal variable. Significant results from the Kruskal-Wallis test (non-parametric equivalent of one-way ANOVA) were followed up with pairwise Mann-Whitney U tests, holding overall α to less than 0.05. Under these conditions, control electrodes in non-lesional patients showed less frequent spiking, smaller amplitude of both halfwaves, smaller slope of the first halfwave and less total spike power. In all cases, control electrodes were significantly different from both seizure onset and seizure spread electrodes, while no difference was seen when comparing seizure onset to seizure spread electrodes. The similarity of seizure onset to seizure spread electrodes likely explains the poor values for correlation coefficients seen previously.

In lesional patients, control electrodes demonstrated a lower spike frequency, shorter duration of the second halfwave, less total spike power and a greater distance

from the patient's tumor. Once again, control electrodes significantly differed from both seizure onset and seizure spread electrodes, while seizure onset and seizure spread electrodes never differed from each other.

Best parameters to identify seizure onset zones

A commonly reported measure of the ability of interictal spike frequency to identify the seizure onset zone is the percentage of patients in which the highest spiking electrode is contained within the seizure onset area. We therefore conducted this survey with all the individual parameters in our dataset to see which was most reliably maximal in seizure onset areas (Table 6). The typical measure of spike activity, frequency, was maximal in seizure onset electrodes in 4/16 patients, and was never at a minimum in seizure onset electrodes. Slope of the first halfwave and spike power also identified seizure onset electrodes in as many patients as spike frequency, while the other parameters did not perform as well (Figure 2). These three parameters were also significantly correlated to seizure category and significantly distinguished control electrodes from those involved in seizures by the Kruskal-Wallis test, suggesting they may all be equally good measures of identifying seizure onset zones in non-lesional patients.

In contrast to non-lesional patients, spike frequency was only maximal in seizure onset electrodes in 2/11 lesional patients. In this group, duration of the second halfwave was maximal in seizure onset electrodes in 4/11 patients and was also significant by the Kruskal-Wallis test. Adding in the fact that it also had a higher Spearman's rank

correlation coefficient to seizure category, duration of the second halfwave is a superior marker of seizure onset zones to spike frequency in lesional patients (Figure 3).

Seizure onset zones lie outside the tumor area in the majority of patients

While areas near tumors are more likely to be seizure-generating than distant areas, many patients have additional seizure foci outside the immediate area of the tumor as well. In 7/11 patients in this study, seizure onset electrodes were located greater than two centimeters away from the nearest tumor margin, sometimes on entirely different lobes of the brain (Figure 3). The type of tumor may influence the chance that a seizure onset zone will occur outside of the immediate tumor area as well, since 4/4 patients with 'high grade' tumors (WHO Grade >=3) had distant seizure onset zones, compared to only 3/7 patients with 'low grade' tumors (WHO Grade <=2). The small sample number does not give this study enough power to determine whether a true difference exists though ($p=0.214$, two-tailed t-test for difference in proportions). Overall, the average distance to the farthest seizure onset electrode across all lesional patients was 3.45cm (SD±1.76; range 1.29-6.86), suggesting that a simple "lesion-ectomy" is unlikely to remove the entire seizure onset zone in many patients.

DISCUSSION

Seizure onset and spread zones differ from non-epileptic brain

This study showed rather poor correlation between spike parameters and electrode seizure category. While statistically significant, the Spearman rank correlation

coefficients between spiking parameters and seizure category were very low and may not be biologically relevant. However, this is likely due to seizure category being treated as an ordinal variable, expecting seizure onset electrodes to be more epileptic than seizure spread electrodes. Comparing spike parameters between each seizure category showed that control electrodes consistently differed from seizure onset and seizure spread electrodes, but onset electrodes did not differ from spread electrodes. In the parameters examined here then, onset electrodes were not distinguishable from spread electrodes.

Spiking parameters that identify seizure onset zones differ in lesional and non-lesional patients

Putting together the results from the correlations, the Kruskal-Wallis tests, and the number of patients where highest parameter value was located in a seizure onset electrode brings out different measures of spiking as best for identifying seizure onset in lesional and non-lesional patients. In non-lesional patients, frequency, slope of the first halfwave and spike power all performed similarly and were maximal at seizure onset electrodes in 25% of the non-lesional patients examined. Although this is poorer than the rates reported in other recent studies which range from 50-100% (16,17,22), examining a single electrode per patient is not a terribly robust measure. Interestingly, results from Spearman correlation and Kruskal-Wallis tests in lesional patients only overlap in spike frequency and power with non-lesional patients. In lesional patients, duration of the second halfwave stands out as the best measure for identifying seizure onset areas, with twice as many patients showing maximal duration in a seizure onset electrode as with spike frequency. Additionally, the correlation coefficient for duration is nearly twice that

of spike frequency, again suggesting that this is a better measure in lesional patients than spike frequency. Quantitative interictal electrocorticography has not previously been examined in tumor patients, so the results of this study are important because they show that past research in non-lesional patients cannot necessarily be applied to lesional patients.

Proximity to tumor is not the best predictor of seizure zones

Perhaps the most interesting result of this study is that areas of seizure onset in tumor patients are often not directly adjacent to tumors. In most patients (n=7/11), seizure onset zones exist far from the actual tumor, and this may be more likely in more aggressive tumors. In fact, duration of the second halfwave was a better predictor of seizure onset zone than distance from the tumor. The areas immediately surrounding tumors seem to have low interictal activity as well, with higher spike frequency correlating well with distance from tumor. These results could help guide the critical clinical decisions involved in deciding which areas of the brain to resect in tumor patients with comorbid epilepsy.

CHAPTER 4

Characterization and pharmacologic inhibition of a neocortical model of interictal spiking

INTRODUCTION

Previous chapters have focused on interictal spiking in human epilepsy, and prior studies in our lab have implicated interictal spikes as having a potentially important role in epilepsy (31). However, other studies both in humans (14,15,129) and in vitro [For review see: (130)] suggest that interictal spikes may actually even be protective against seizures. To settle these discrepancies would require an animal model of chronic, neocortical interictal spiking. At the start of this work, no such models existed. Other models to study epilepsy typically focus on acute, severe seizures and have not been verified to act through the same molecular pathways as human epilepsy. Not surprisingly, antiepileptic drugs developed using these models simply reduce the likelihood of seizures occurring, but do not correct the underlying brain abnormality that allows seizures to occur. If a model of chronic interictal spiking could be developed and is shown to activate the same molecular pathways that are involved in human epilepsy, it would not only provide new insight into a poorly-understand phenomenon but also allow for the testing of novel, molecularly-targeted therapeutics.

In this chapter, we develop one such model. Based on the work of Jeffreys on the tetanus toxin model of chronic epilepsy and the observation by Brener (60) that injection of tetanus toxin into the rat somatosensory cortex produces predominantly interictal spikes with minimal seizures, we focused on the use of tetanus toxin in the somatosensory cortex to create a model of chronic, neocortical interictal spiking. Tetanus

toxin creates a focus of excitation by preferentially binding to gangliosides of inhibitory neurons, where it is taken up and prevents vesicle exocytosis through cleavage of synaptobrevin (VAMP-2) (61-63). After determining an appropriate dose which generates frequent interictal spikes without observed seizures (131), we characterize how this spiking focus develops and progresses over time. We show that a single injection of tetanus toxin produces a spiking focus which progresses in frequency and amplitude over time and that spikes progressively cluster together, weeks after the tetanus toxin is cleared from the brain. We show that interictal spiking alone is enough to induce the activity-dependent gene expression changes seen in our human patients in some rats, and that these genes are activated in the same cortical layers as in human epilepsy. Finally, after our work in human patients implicated the MAPK-CREB pathway as central to epilepsy, we demonstrate that a selective inhibitor of the activation of MAPK can prevent the development of a spiking focus.

METHODS

Surgery and electrode implantation

All studies were carried out with approval by Wayne State's Animal Investigation Committee (AIC protocol A01-09-06). Male Sprague-Dawley rats at four months old were kept on a 12-h light/dark cycle with free access to food and water. Rats were implanted with recording electrodes and tetanus toxin was stereotactically injected as previously described with modifications (59,60). Rats were anesthetized with pentobarbital, placed in a stereotactic frame and holes were drilled in the skull over each

hemisphere at AP +4mm, -1mm and -6mm, L3.5mm relative to the bregma (Figure 4). A seventh hole was drilled at the midline over the nasal sinus. Tetanus toxin (Sigma, catalog# T3194) was reconstituted with sterile water to 100 ng/μL in 0.01M sodium phosphate and injected into the left somatosensory cortex (AP-1mm, L 3.5mm, as measured from bregma) at a depth of 1.5 mm over four minutes. As tetanus toxin lots varied in potency, the dose injected was modified to produce the same amount of spiking when a new lot was purchased. The rats used in the *in situ* hybridization time course (described below) received 65 ng, while all others in this study received 100 ng. The needle was left in place for 10 minutes to allow diffusion and binding of the toxin. Small screws were placed in each drilled hole and wired to a plastic connector to serve as electrodes, with the nasal sinus screw used as a common reference. For the *in situ* hybridization time course, parts were obtained from Plastics1 (Parts MS363 and E363), while for all other rats, the screws were from Small Parts, Inc (TX2-4-1C) and the connector from Molex (Part#0430450829). Connector types did not have any discernable effect on any measurements made in this study. The apparatus was secured with dental cement and the skin closed. Recordings were made using a 32-channel Stellate Harmonie system recording at 200Hz for one-hour periods at the same time of day over the course of three weeks.

Evolution of the interictal spiking focus over time

To study the initiation and development of a chronic, neocortical interictal spiking focus over time, an initial group of tetanus-injected (n=7) and vehicle-injected (n=5) animals were followed over the course of three weeks. The four 3-week time point rats

from the *in situ* hybridization time course described below were added to this initial dataset to give a total of 11 tetanus-injected rats for this study. Recordings were reviewed in a referential montage (recording electrodes subtracted from the nasal sinus reference) and the location of spikes was marked manually by a single, blinded reviewer. Quantitative parameters about the spikes, including frequency, duration, amplitude, slope and field distribution on each recorded day were calculated for these marked locations using custom Matlab scripts (MathWorks, R2008b, Natick, MA). For the measurement of spike clustering, spikes occurring within 500 ms of each other were considered members of the same spike cluster. Three-dimensional plots of data on rat brains were generated using software developed for us by the Graphics and Imaging Lab (Dr. Jing Hua and Darshan Pai) of Wayne State University's Computer Science Department.

Activity dependent gene expression by *in situ* hybridization

Previous studies of human neocortex in our lab (10,31) suggested that interictal spiking may drive activity-dependent gene expression through CREB phosphorylation in human epilepsy. To determine whether interictal spiking alone can drive activity-dependent gene expression, groups of rats were sacrificed at multiple time points after initiation of a spiking focus and subsequently examined via *in situ* hybridization. Four rats per time point were sacrificed at one week, two weeks and three weeks after tetanus injection, with an additional three surgery-naïve rats used as controls. Each rat was sacrificed via a CO_2 chamber and its brain was immediately dissected and fixed in 4% paraformaldehyde/PBS at 4ºC. After subsequent equilibration in 30% sucrose in PBS, brains were cryosectioned at 20 μm. *In situ* hybridizations were performed using ^{35}S-

labeled RNA probes as previously described (11). Full-length human cDNA clones (NARP: 5198692; BDNF: 5193877; GAPDH: 95132246CA2) or expressed sequence tags (EGR1: 6188360, truncated 3' untranslated region) (Open Biosystems) were sequence verified and confirmed to share >=87% identity with the rat sequence. Despite the availability of FANTOM clones (132), these clones were chosen because they had previously been used in our human neocortical sections and contained high sequence identity so that rat neocortical *in situ* hybridizations could be directly compared to the previous human ones. Additionally, these clones had been tested previously in our rat sections and showed the expected distributions of signal, while antisense controls showed no signal. Immunohistochemical staining for phosphorylated CREB was also performed on sections adjacent to those used for *in situ* hybridization.

Prevention of interictal spiking focus development using a MAPK pathway inhibitor

Because the MAPK pathway was the pathway most strongly implicated in human epilepsy (10,11), we asked whether inhibition of this pathway could prevent the development of a spiking focus in the tetanus toxin model. The compound SL327 was chosen for this study because it selectively inhibits a member of the MAPK pathway, mitogen-activated protein kinase kinase (MEK), in organotypic slice cultures, blocks ERK1/2 phosphorylation in a dose-dependent fashion and crosses the blood-brain barrier (133,134). SL327 was obtained from Sigma-Aldrich (St. Louis, MO, catalog# S4069). First, a dosing regimen had to be determined because this compound had never been used in a chronic manner in any reported literature. SL327 was dissolved in DMSO at a concentration of 25 mg/mL and injected intraperitoneally into three rats at a dose of 50 mg/kg. To determine plasma half-life and speed of brain penetration, the three rats were

sacrificed at 1 hour, 4 hours, and 24 hours after injection and their brains were removed and frozen at -80ºC. Prior to sacrifice, blood samples were obtained via orbital bleeding at 15 minutes, 30 minutes, 1 hour, 4 hours 8 hours and 24 hours after injection up until the sacrifice time of each rat. Blood samples were centrifuged at 800 g for 10 minutes and then frozen at -80ºC until the time of analysis. Drug concentrations in all samples were determined using high-performance liquid chromatography with tandem mass spectrometric detection (HPLC-MS/MS) by our collaborator in Pharmacology, Dr. Jing Li. Pharmacokinetic parameters such as terminal elimination half-life, volume of distribution and clearance were estimated using non-compartmental analysis in the software WinNonlin Professional 5.2 (Pharsight Corp., Cary, NC).

Once a dosing regimen that would maintain drug levels similar to those reported to be effective acutely had been determined, SL327 was tested for its ability to prevent CREB phosphorylation in vivo in our tetanus toxin model. Four rats were given i.p. injections of 25 mg/kg SL327 dissolved at 25 mg/kg in DMSO twice daily for a week following tetanus injection, while another group of three rats were given only an equivalent volume of DMSO. After one week, rats were sacrificed and their brains examined for CREB phosphorylation by immunohistochemical staining.

Once the ability of SL327 to prevent CREB phosphorylation in the tetanus toxin model was established, its ability to prevent the development of an interictal spiking focus was tested. Three rats were injected with tetanus toxin and electrodes were implanted in the skull, as part of the usual protocol. For the week following surgery though, they were each treated with SL327 and monitored for interictal discharges for a total of four weeks.

RESULTS

Spikes increase in frequency, amplitude and slope over time

The injection of tetanus toxin into the rat somatosensory cortex successfully generates 'interictal' discharges with no initiating seizure. Spikes can be detected on EEG starting around days three to five after injection, and then increase in frequency with time (Figure 5A). Vehicle animals show significantly less spiking at the first week ($p=0.029$, one-tailed t-test, Bonferroni correction; 79.1±18.1 and 9.4±2.9 spikes/ hour, ± sem), which persists at two weeks ($p=0.020$, one-tailed t-test, Bonferroni correction; 101.3±19.5 and 9.5±2.9 spikes/ hour, ± sem) and three weeks ($p=0.040$, one-tailed t-test, Bonferroni correction; 178.33±45.3 and 8.6±4.2 spikes/hour±sem). Spike amplitude increases initially, but then appears to plateau by 10 days (Figure 5B; Best fit model: $y=a*x^b+c$; $R^2=0.667$). Spike slope increases progressively with time (Figure 5C; $R^2=0.530$; Linear regression slope=0.307μV/day, $p=0.0004$), while duration does not appear to change (Figure 5D; $R^2=0.148$; Linear regression slope=-0.299ms/day, $p=0.104$).

Spike clustering

Spikes were observed to progressively cluster together over time (Figure 6). While spikes initially appeared as individual, isolated discharges, 'doublets' often appeared by a week after injection and became progressively more common. As time went on, longer runs appeared and became more common, so that by three weeks over

30% of the spikes in a recording occurred clustered with other spikes (R^2=0.492; Linear regression slope=0.75%/day, p=0.0025). A single animal with frequent repetitive bursts was also observed to develop a focal seizure from this focus, but the exact relationship between spike clustering and seizures cannot be drawn from this data, given that long term recordings were not part of this project.

Spike field expansion

The placement of six epidural recording electrodes allowed us to not only detect interictal discharges, but to track their electrical field and spread over time (Figure 7). Rats varied in their patterns of field expansion so that while most were restricted to the injection site and left anterior electrode (Figure 7A,B), a small number of rats developed a spiking focus restricted to the right hemisphere, contralateral to the injection site (Figure 7C,D). This finding is consistent with other studies using tetanus toxin in hippocampi, where injection in one hemisphere sometimes leads to epileptic activity initiated in the opposite hemisphere (60,135). Combining all surveyed rats together, however, shows that the most common pattern of expansion was to begin with small spikes at the injection site, spread to involve the left anterior electrode, and then eventually involve the right middle electrode (Figure 7E). Interestingly, spikes never spread posteriorly from the injection site, suggesting that the pattern of interictal spike spread is closely related to normal anatomic barriers that separate cortical areas of different function.

Activity-dependent gene expression

Previous results from our laboratory showed that activity-dependent gene expression changes correlate with interictal spiking. Further, these expression changes could be localized to specific lamina of the neocortex, predominantly layers II and III. To ask whether interictal spiking alone could induce changes in activity dependent gene expression, we performed *in situ* hybridizations on multiple animals sacrificed at one, two and three weeks after tetanus toxin injection. The genes examined in this study were chosen because they had all been previously shown as robustly differentially expressed in human epilepsy and represented several different functional categories. Egr1 and Bdnf are immediate early genes (136-141) and Narp (Nptx2) clusters AMPA-type glutamate receptors at excitatory synapses (142,143).

Three of the four animals sacrificed after one week of spiking showed a small region of suppression at the injection site, with one also showing a focus of strong gene induction at the center (Figure 8). This focus showed Egr1, Narp, and Bdnf expression in exactly the same area on adjacent sections. This pattern was virtually identical to the pattern of Egr1 protein distribution in another study using tetanus toxin in the motor cortex (144). By two weeks, rats that were sacrificed during a time of recent increases in spike frequency and amplitude showed increased gene expression in the left, spiking hemisphere than the right, non-spiking hemisphere. By three weeks, however, most rats had stabilized in spike frequency and/or amplitude and no longer showed lateralized differences in gene expression, even when spike field was well-restricted to one hemisphere. Normal, surgery-naïve rats and 0.01M vehicle-injected rats showed even hemispheric gene expression throughout, and antisense probes showed no signal.

In rats with lateralized gene expression changes, these changes were consistently in layers II/III of the neocortex, just as we previously found in human epileptic tissue (10,11). Egr1 generally filled layers II and III, while Narp and Bdnf were restricted to thin bands in the superficial part of layer II. Again, these results parallel our previous findings in human neocortex. Despite the varying patterns of expression in the rats surveyed, all rats were internally consistent in that all the genes detected were upregulated in the same areas as the others on adjacent sections.

MAPK pathway inhibition prevents the development of a spiking focus

MAPK-CREB signaling was by far the pathway most implicated in the human epileptic transcriptome (10,11), so it seemed a logical target for therapeutic intervention in our animal model. In this experiment, we therefore determined a dosing regimen for the MAPK pathway inhibitor SL327, verified that it was able to prevent CREB phosphorylation after one week of treatment, and then tested its ability to prevent an interictal spiking focus from developing. One week after the induction of a spiking focus, CREB is usually phosphorylated in the left, spiking hemisphere relative to the right, non-spiking one (Figure 9A; n=5/7 rats). Using plasma and brain concentrations of SL327 over time (Figure 9B), Dr. Jing Li calculated the half-life to be 6.8 hours and recommended dosing the rats twice daily with 25 mg/kg. Rats treated with this dose for one week following tetanus toxin injection showed markedly reduced CREB phosphorylation relative to DMSO-injected controls (Figure 9C). Treatment of rats with SL327 during the first week after tetanus injection significantly reduced spiking rate (Figure 9D) by the first week ($p=0.005$, one-tailed t-test, Bonferroni correction; 79.1 ±

18.2 and 7.0 ± 3.6 spikes/ hour, ± sem) that persisted at 2 weeks (p=0.044, one-tailed t-test, Bonferroni correction; 100.5 ± 27.3 and 18.0 ± 9.0 spikes/ hour, ± sem) and 3 weeks (p=0.013, one-tailed t-test, Bonferroni correction; 178.3 ± 45.3 and 30.7 ± 7.6 spikes/ hour, ± sem), suggesting that MAPK is necessary for the development of interictal discharges. SL327 treated animals did not significantly differ from vehicle animals at any of these time points.

DISCUSSION

Interictal spikes may be a precursor for epilepsy and seizures

In this chapter, we characterized a rat model of chronic, neocortical interictal spiking for the first time. The advantages of this model are that it is essentially 'non-lesional' in that it does not cause the extensive neuronal loss and gliosis associated with other models like kainic acid and pilocarpine, and it has a chronic time course that better parallels the development of human epilepsy (55,56,145,146). Spikes develop after an initial latent period of several days and then steadily progress in frequency and amplitude with time, despite tetanus toxin being cleared from the brain by then (147). This implies that once initiated, a spiking focus is both self-sustaining and expands over time. Combining this observation with the fact that spikes progressively cluster together with time raises the intriguing hypothesis that interictal spikes may be an essential precursor to seizures and an integral part of epileptogenesis (148). This conclusion is supported by a recent study which shows that both the frequency of interictal spikes and the presence of

spike clusters predict which animals will develop spontaneous seizures following status epilepticus in the hippocampal kainic acid model (149).

Layer-specific changes in activity-dependent gene expression are associated with interictal spiking

Interictal spikes without observed seizures were able to induce activity-dependent gene expression changes in genes previously confirmed to be upregulated in human epileptic foci. All changes in gene expression were observed in layers II and III of the cortex, exactly as we have seen in our previous human studies and in contrast to most other work in animal models of epilepsy (42). Not all rats showed lateralized gene expression changes consistent with lateralized spiking, but those that did were the animals that were increasing in frequency and amplitude at the time of sacrifice, which may mean that only neurons undergoing active remodeling show detectable differences in these plasticity-associated genes. No rats showed expressed changes opposite their spike field and surgery-naïve rats all showed homogenous gene expression. EGR1 (150-152) and BDNF (153) are regulated by CREB, and while the control of NARP transcription has not been well studied, its promoter contains a CREB binding site (154,155). While additional work beyond these initial studies needs to be done, these results so far imply that this model may be an accurate representation of human epilepsy at the molecular level and that targeted therapeutics developed in this model are likely to translate well into human use.

MAPK-CREB pathway inhibition and drug development

In this study we showed that a MAPK pathway inhibitor can reduce CREB activation and prevent the development of an interictal spiking focus. These results support our conclusions from the human epileptic transcriptome, which implicated MAPK-CREB signaling and present the exciting possibility that entirely novel therapeutics for epilepsy can be developed. It has been shown that constitutive activation of ERK, the kinase blocked in this study, causes epilepsy (156) so blocking its activity may be an effective treatment of epilepsy, or at least prevent its development after injury. A recent study showed that current antiepileptic drugs block seizures, but do not prevent spike activity in vitro (157). Since results from our studies suggest a role for interictal spikes in epilepsy and epileptogenesis, new therapies that target interictal spiking may be more effective at treating epilepsy than current medications only treat seizures.

CHAPTER 5

Behavioral consequences of interictal spiking in a rat model

INTRODUCTION

Epilepsy is a disease of recurrent seizures, with most research attention over the years focusing on the seizures themselves. While seizures are often the major cause of morbidity for patients, patients with epilepsy also suffer from a wide range of comorbid brain disorders, not always linked directly to seizures. For example, estimates of attention-deficit hyperactivity disorder (ADHD) in children with epilepsy range from 20-40%, while the incidence in the general population is only 3-7% (66-72). Patients with epilepsy are also more likely to have depression (68,73-78), anxiety disorders (68,73,75,77-79), and other psychiatric conditions (68,71,78-82). Given that seizures occur infrequently and that most of a patient's life is spent in the interictal state between seizures, it is conceivable that these comorbid conditions are better related to a process going on between seizures, rather than the seizures themselves. Interictal spikes would be a prime candidate for this abnormal process.

Outside of the increased comorbidities in patients with epilepsy, interictal spikes have also been associated with most of these conditions in the absence of an epilepsy diagnosis as well. Interictal discharges have been most strongly linked to children with ADHD, even in the absence of epilepsy (158-163). Several other studies have linked epileptiform discharges to a variety of other psychiatric ailments, including depression, anxiety disorders, and obsessive-compulsive disorder (84-86). With all the evidence linking interictal discharges to behavioral and psychiatric disorders, an animal model of

chronic interictal spiking without seizures may provide insights into these disorders and allow them to be studied in vivo in a way not previously possible.

In Chapter 4 we showed that the tetanus toxin model of interictal spiking can reliably produce spikes without seizures. Additionally, it shows induction of several activity-dependent genes in a similar pattern found in human epilepsy, suggesting that this model may also be a good model to explore comorbidities. In this chapter we demonstrate that our animal model of interictal spiking leads to specific behavioral abnormalities. We also demonstrate for the first time that interictal spiking can be induced by environmental stimuli in animals with an epileptic focus. We show that spiking rats are more active, that the level of their activity correlates with their level of spiking on a day-by-day basis, and that lateralized spikes cause lateralized movements. Suppression of interictal spiking with a MEK inhibitor normalizes these behaviors. These studies not only raise important questions on the significance of interictal spiking in patients with epilepsy, but also provide a novel animal model to develop novel pharmacological treatments for interictal spiking that could also have important effects on behavior.

METHODS

Open-field activity measurements

Rats underwent the same surgical procedure and tetanus toxin injection to initiate neocortical interictal spiking as described previously in Chapter 4. Four tetanus-injected and four sham-operated (electrodes implanted, but no injection into brain) animals were

followed over time to measure the effect of progressive interictal spiking on open-field activity. Immediately after EEG was recorded for one hour, rats were placed in an open-field activity chamber (ENV-515, MedAssociates, Inc., St. Albans, Vermont) for an hour, which uses an array of infrared beams to track movement. The included Activity Monitor software was used to calculate total ambulatory distance over each one-hour recording period.

To examine the effect of focal interictal spiking on behavior in more detail, a group of seven tetanus-injected animals were placed in the open-field activity chamber immediately following an EEG recording session. The ratio of the number of spikes from the left hemisphere to the number of spikes in the right hemisphere as well as total spike power (frequency * amplitude) were compared to the ambulatory distance, resting time, and the ratio of counter-clockwise to clockwise rotations, as calculated by the Activity Monitor software. For these comparisons, the numbers within each rat were converted to Z-scores so that data from multiple rats could be combined together for an overall correlation.

Auditory evoked interictal spikes

To measure the effect of a startle evoked by an auditory stimulus, a total of 11 spiking rats and five vehicle rats were monitored over time for their response on EEG to a repeated stimulus. To induce spikes in a reproducible manner, a digital audio recording of a single clap was made. While EEG was being recorded, this sound was played in the recording chamber three times at intervals of 10 minutes apart, followed 10 minutes later by a train of three repetitions, each one second apart (Figure 11A). Responses were

marked on the EEG files and spike amplitude in each channel was calculated using a Matlab script. The response to the three claps spaced 10 minutes apart were averaged together for each day as a measure of response magnitude at each time point, while the train of spikes was considered separately to measure extinguishment of the response.

Effect of suppressing interictal spikes on behavior

From the results in Chapter 4 that showed that SL327, a selective MEK inhibitor, can prevent the development of a spiking focus, we hypothesized that this suppression could normalize behavior in tetanus-injected rats. Five tetanus-injected rats, four vehicle-injected rats, and three rats who received tetanus toxin followed by SL327 for one week after surgery were followed over time to measure the effect of progressive interictal spiking on open-field activity. While EEG was being recorded for one hour, rats were placed in the open-field activity chamber and monitored for ambulation distance over time.

RESULTS

Interictal spiking increases rat motile behavior

As a first step, we determined the ambulatory behavior of rats with progressive increases in interictal spiking after tetanus toxin injection into somatosensory cortex. Infrared measurements of ambulatory mobility performed daily showed that interictal spiking is associated with a significant increase in rat ambulation, leading to what could be termed a ‘hyperactive’ rat (Figure 10A). The average ambulatory distance of spiking

rats was greater than that of sham controls at all time points measured, and showed the most divergence toward the end of the three week period when tetanus-injected rats were at their maximal spiking (1 week: p=0.082, 7946.5±851.0cm versus 5605.7±759.4cm; 2 weeks: p=0.079, 7808.2±1046.1cm versus 5501.3±571.3cm; 3 weeks: p=0.0005, 8610.0±733.8cm versus 4668.0±504.5cm; distance±SEM, all p values after Bonferroni correction).

Next, we looked in more detail at the relationship between interictal spiking and movement parameters. For seven rats followed either for two weeks (n=3) or three weeks (n=4), spiking was measured for one hour immediately prior to placing the rat in the open field activity chamber for one hour. The ratio between the number of spikes originating in the left hemisphere versus the number originating in the right hemisphere correlated significantly with the ratio between the number of counter-clockwise and clockwise rotations the rats made (Figure 10B; R=0.396, p=0.013 Bonferroni correction). This result means that more interictal spikes on a rat's left caused it to rotate more frequently counter-clockwise to its own left side, while right-sided spikes are associated with clockwise rotations. Total spike power correlated positively with ambulatory distance (R=0.350, p=0.039 Bonferroni correction) and negatively with resting time (R=-0.410, p=0.008 Bonferroni correction). Therefore, not only are interictally-spiking rats hyperactive as a group relative to control animals, but within just spiking rats the level of their activity varies with the level of spiking on that day.

Startle-induced spikes occur in same field as spontaneous spikes

A startling auditory stimulus, such as a clap in this case, consistently evokes an interictal spike in rats that are already spiking. The field of the spike induced is always the same as each rat's individual spontaneous spike field. Figure 11B shows an example rat that has spontaneous spikes originating predominantly under the middle, left electrode (L2) with a small contribution in the contralateral electrode (R2). When this rat is startled, the induced spike occurs with the exact same distribution, although with a slightly higher amplitude. As an illustration of a rat with an entirely opposite field, Figure 11C shows a rat which has spontaneous spikes originating from the right, middle electrode (R2). Predictably, the spikes induced by an auditory stimulus in this rat are restricted to the same electrode, but again at slightly higher amplitude than the average spontaneous spike. Every tetanus-injected rat showed this correspondence between spontaneous and induced spike fields, while vehicle rats did not have a response on EEG when startled.

Not only do induced spikes have an identical field as each rat's spontaneous spikes, but the response changes with time as the spiking focus changes. As the average amplitude of a rat's spontaneous spikes increases over time, so too does the average amplitude of the induced spikes. Figure 11D shows the correlation between average spontaneous and induced spike amplitudes over time, measured at the channel of maximum amplitude in each rat and normalizing amplitudes in each rat so that the maximum value is 100% ($R=0.535$, $p=1.42\text{x}10^{-9}$).

The EEG response of induced spikes seems to depend on the rat being genuinely startled. When the recorded claps are spaced only one second apart instead of 10 minutes apart, the response extinguishes rapidly (Figure 11E). The first response was

significantly larger than the response to the second stimulus (650.2±40.0μV versus 273.1±43.4μV; $p=8.1x10^{-9}$, Bonferroni correction; amplitude±SEM) and the response to the third stimulus (650.2±40.0μV versus 156.2±28.7μV; $p=1.1x10^{-21}$, Bonferroni correction; amplitude±SEM). The second and third responses did not significantly differ after correcting for multiple comparisons (273.1±43.4μV versus 156.2±28.7μV; p=0.079, Bonferroni correction; amplitude±SEM). Anecdotally, a sudden, unexpected touch on the rat's back can also induce an EEG spike when the rat is startled. While this was not possible to repeat in a consistent enough manner to quantify, it suggests that the response observed in this study is not a unique feature of the auditory system, but more likely represents a startled response to any stimulus.

Suppression of interictal spiking normalizes behavior

To show that interictal spiking truly alters rat behavior, tetanus-injected rats were compared to rats who received both tetanus toxin and a selective MEK inhibitor that prevents the development of a spiking focus. Pooling ambulation data for weeks one, two and three after surgery shows that while tetanus-injected rats increase in ambulatory distance over time, rats treated with the MEK inhibitor SL327 for one week after tetanus injection (same as those described in Chapter 4) remain about the same (Figure 12). In contrast to the ambulatory measurements described previously in this chapter, these rats were monitored for ambulation while simultaneously having EEG recorded. Therefore, the presence of the recording tether reduced overall movement distance, accounting for the vastly different scaled between Figure 10A and Figure 12. The two groups do not differ in the first week (p=0.645, t-test, Bonferroni correction; 1144.5±197.3cm and

711.8±293.8cm), but by the second week the spiking animals were moving more than the SL327 treated ones ($p=0.025$, t-test, Bonferroni correction; 1205.4±127.9cm and 627.7±150.5cm). By the third week, the two groups had diverged widely ($p=0.012$, t-test, Bonferroni correction; 1692.6±193.3cm and 759.0±223.1cm). Vehicle animals showed no increase over time and ambulated almost exactly as much as the SL327-treated animals (729.4±93.2cm).

DISCUSSION

Interictal spiking causes hyperactive movement

In this chapter, we showed that spiking rats are hyperactive relative to sham-operated control rats. This result is especially interesting in the context of reports showing that ADHD is frequently associated with epilepsy and that interictal discharges are more common in children with ADHD than normal children, even in the absence of epilepsy. More spiking was also associated with more ambulation and less resting time on a day-by-day basis. Again, these results strengthen the conclusion that interictal spikes directly cause the observed changes in behavior. Further, location of the interictal spiking focus caused asymmetric ambulation in spiking rats. The ratio between left and right hemispheric spikes was correlated with the ratio between counter-clockwise and clockwise rotations, meaning that as the number of spikes originating from the left hemisphere increased, so too did the number of counter-clockwise rotations. Since tetanus toxin was injected into the left somatosensory cortex, it is possible that interictal spikes in this region caused the rat to feel a negative sensation on the right side of its

body, and then turn away from it. Therefore, not only does the presence of interictal spikes affect behavior, but the type of behavior observed appears to depend on their location. These are the first in vivo results showing an effect of neocortical interictal spiking on behavior, and together suggest that the tetanus toxin model of neocortical interictal spikes may be able to offer insights into a range of psychiatric disorders where interictal spikes have been observed.

Environment can influence interictal spiking

Next, we demonstrated that interictal spikes can be induced in spontaneously-spiking rats by a startling sound. Further, these induced spikes always occur in the same field distribution as each rat's spontaneous spikes, implying that the same population of hypersynchronized neurons is likely being activated in both cases and that these neurons are more easily brought to threshold than normal neurons. This is perhaps the first observation that a common environmental stimulus such as sound can alter interictal activity in vivo.

Suppression of interictal spiking normalizes behavior

Finally and perhaps most importantly, we showed that treatment with a MEK inhibitor during the first week following tetanus injection prevented the hyperactivity seen in the spiking rats. While it is true that the drug itself could reduce rat movement by negatively impacting their health, this effect would be expected to cease once treatment stopped in the first week. By the third week any residual drug effects are gone and rats have returned to their normal health state, yet SL327-treated rats show no increase in

ambulation at this time. Instead, their total ambulation remained constant throughout the monitoring period and was essentially identical to that of the non-spiking vehicle rats. Together, these results imply that interictal spiking itself is directly responsible for the increased activity and that this effect can be mitigated with a selective molecular inhibitor. While this study has only explored the prevention of a spiking focus with a MAPK pathway inhibitor, MAPK inhibition should be explored as an entirely new therapeutic strategy for a variety of psychiatric disorders with epileptiform abnormalities.

Chapter 6

Discussion

Quantitation of interictal spikes to improve surgical outcome and advance research

In Chapter 2 we demonstrated that professional electroencephalography (ECoG) reviewers varied widely in their detections of interictal spikes. Such variation could lead to subjective clinical decisions and make it difficult to compare results between research studies on interictal spikes. This may explain why there have been mixed results in the use of interictal spikes to identify the seizure onset zone and improve surgical outcome (16-23). Due to the tremendous variation in spiking rates over time within patients(17) and the frequency of spikes on ECoG, manual marking of interictal spikes is simply not feasible as regular, reliable method of quantifying ECoG. While many other algorithms for spike detection have been published (for review see: (122)), only a few have been verified on intracranial recordings (29,121,123,124) and these have either only examined single channels or verified spikes already detected by humans. Here, we showed that our method can be run on full ECoG recordings and still preserve differences in activity between channels. Additional benefits to this method are that it requires no changing of parameters for each patient, runs very quickly, and is fairly simple to implement.

We subsequently used this spike detection algorithm to see if other parameters besides spike frequency could be used to identify regions of seizure onset. We determined that brain areas which either generate or participate in seizures cannot be distinguished from each other based on these interictal spike parameters, but that these epileptic regions differ from control regions. In this group of non-lesional patients, the

parameters examined did not significantly improve upon spike frequency for the identification of seizure onset zones, but several characteristics of spikes did perform equally well. In the future, it might be possible to show that different parameters do a better job with different patients. For example, spike morphology might be affected by disease duration, initiating etiology, antiepileptic medications, or functional area of the brain in which it occurs. With a larger sample of patients, it may be possible to stratify patients into groups and find which parameters of spike morphology best fit each subpopulation.

The use of ECoG in tumor surgery patients provided new and interesting results, since this method is rarely used in tumor surgeries. Here we found that the spiking parameters which best identify seizure onset zones in lesional patients are different than those found in non-lesional patients. In this group of tumor patients, frequency was not the best measure of seizure onset areas; duration of the second halfwave was a much better marker. More surprisingly, we found that seizure onset areas regularly lie outside of the immediate tumor area, and that increasing spike frequency was actually correlated with increasing distance away from the tumor. These results suggest that the use of ECoG in tumor patients with comorbid epilepsy may give valuable additional information to the surgeon. An expanded group of patients followed after surgery for post-operative outcomes will eventually answer whether there is a true clinical advantage to using ECoG data in these tumor resections and for which types of patients it is most helpful.

One problem in the world of spike detection is that techniques are developed and papers are published, but then no one but the authors use the algorithm that they

developed. Widespread adoption of this algorithm could be facilitated by integrating it into a broader set of tools. Now that we have developed and verified an automated method of spike detection, a whole host of research questions can be asked about the relationship between interictal spikes and epilepsy which would have been impossible to do in an objective, efficient manner previously. The next steps in development could be to integrate it into ECoG recording software so that detections can be made in real time, and then include new quantitation and visualization tools. We are currently working with Dr. Jing Hua in the Computer Science department to be able to display spike detection results on a three-dimensional image of a patient's brain in real time. This would allow clinicians and researchers to visualize how groups of electrodes are linked to each other, whether spikes in one area lead and perhaps drive spikes in another area, and determine how spiking patterns change with time or patient activity.

Interictal spikes in epileptogenesis

In Chapter 3, we described the development and characterization of an animal model of chronic, neocortical interictal spiking. In this model, a single injection of tetanus toxin is followed by a latent period of several days before spikes are first detected. Spikes then increase in frequency, amplitude, electrode involvement and clustering with time, even after the initial insult (tetanus toxin) is gone. This pattern mirrors the development of acquired epilepsy in humans, although on a shorter time scale. The latent period before seizure development in humans can range from months to years after the initial insult (1,55,56). The absence of an initiating seizure in this model allows the effects of in vivo interictal spiking to be isolated, and the increase with time

shows that they are not benign. In fact, the progressive clustering of spikes may represent the process that takes place during the latent period before clinical seizures in human epilepsy. More frequent and longer clusters of spikes indicated that the brain is changing with time in a way that allows faster recovery from a massive depolarization and the ability to depolarize again more quickly. As a focal seizure is often arbitrarily defined as lasting for 10 seconds (164), a sufficiently long train of interictal spikes would be indistinguishable from a focal seizure, and likely represents a transition from single interictal spikes to electrographic seizures. Recent work in the kainic acid model of hippocampal epilepsy supports the role of interictal spikes in epileptogenesis. White, et. al. demonstrated that after kainic acid-induced status epilepticus, interictal spikes appear before spontaneous seizures (149). Further, increasing spike frequency and increasing spike clustering were predictive of which animals would develop convulsive seizures in the future. Our work adds to this study and shows a similar pattern of spike progression, but without any initiating seizure and in an entirely different animal model.

Data from our laboratory's work in human epilepsy showed CREB phosphorylation and activity-dependent gene expression predominantly in layers II/III of the neocortex (10,11). Initial work in the tetanus toxin model of neocortical interictal spiking agrees with these findings, which both supports the validity of our human findings, as well as the use of the tetanus toxin model as a molecularly-accurate model. It is important to note, however, that only 57% of canonical cyclic AMP response elements (CREs; sequence TGACGTCA) are conserved between human and rat, so the two species may exhibit somewhat different transcriptional profiles (165). Further characterization therefore needs to be done in order to be certain that this animal model sufficiently

replicates the human disease. The significance of layers II and III is that these are the layers primarily responsible for lateral, associative communication with other cells of the cortex (166). It is conceivable therefore that that the spiking focus expands through these lateral connections. Under this model, an initially small focus of hyperexcitability is created by some initial lesion (human patients) or tetanus toxin's cleavage of synaptobrevin (167) (rat model). The affected neurons would signal excessively on the 'neighbors' they are connected to, which would induce activity-dependent pathways in these neurons and cause them to become synchronized with the original group (see Figure 13 for schematic of MAPK pathway's proposed role in this model). Continued spiking could expand the focus in a similar matter until a large population of neurons in the superficial cortical layers has become excessively synchronized, and spikes can be detected as field potentials. Further alterations in synaptic efficacy and recovery times could lead to the generation of clustered spikes and eventually seizures.

Further studies would be required to prove this hypothesis. First, the results from *in situ* hybridizations presented in Chapter 3 showed changes in gene expression only in a subset of animals which were undergoing increases in spike frequency and amplitude at the time of sacrifice, while most animals showed no lateralized changes, even if spikes were frequent and well-restricted to one hemisphere. These data lead us to the hypothesis that activity-dependent gene expression in this model can only be detected while neurons are actively undergoing synaptic plasticity and long-term potentiation. To confirm this hypothesis, instead of deciding to sacrifice rats at an array of time points as was done here, rats should be continually monitored for spiking parameters and sacrificed during periods of rapid increase. The initial work on the tetanus toxin model of neocortical

interictal spiking presented here demonstrates that individual rats vary widely in the rate of progression of their spiking focus, so that it will be more logical to group rats by observed spiking parameters than to attempt to group them by arbitrary time points. This method may more consistently reveal the link between activity-dependent gene expression and interictal spikes in this model.

Finally, one of the most important studies to next be carried out in this model will be to examine the progression from interictal spikes to seizures. We recently installed equipment for continuous EEG monitoring of rats, so we can now follow rats constantly and determine when seizures first develop in this model. This will hopefully allow us to determine which variables, such as spike clustering, are most important for the progression from interictal spikes to seizures. We would also be able to sacrifice rats that have just developed seizures and ask how they differ at a molecular level from rats who do not have seizures. In fact, it may be prudent to categorize rats into non-spiking, individual spiking, clustered spiking, and seizures to see if different signaling and transcriptional profiles are activated in each. For example, blocking Phospholipase C in a hippocampal slice culture model is able to prevent the initiation of interictal spiking, but not the continuation of spiking once it has been established (168). Protein Kinase C (PKC) activation, on the other hand, is able to convert single interictal discharges to clustered bursts of spikes in guinea pig hippocampal slices (169).

Behavioral changes of the interictal state

In Chapter 5, we showed that interictally-spiking rats exhibit several behavioral changes. These observations are important in light of the numerous conditions comorbid

with epilepsy (66-82), and the observations of spikes in a broad array of psychiatric disorders as described previously (158-163). We first observed that interictal spikes could be induced by a simple, external stimulus such as a clap. The field of the induced spike was identical to each rat's spontaneous spikes and its amplitude varied with spontaneous spikes amplitude as well, strongly suggesting that the same group of neurons is involved in both cases. Given the old adage among epileptologists that "seizures beget seizures" (170,171), it may also be true that spikes beget spikes, in which case this study could have implications for how epileptic patients should be treated. To test this hypothesis, groups of rats could be set up in parallel, where one group is subjected to random, startling sounds while the other group is not, and spontaneous spike frequency could be monitored over time. Given the innate variability in this model, however, a large number of rats would need to be used in order to detect differences in spiking with sufficient power.

The next major result is that rats with interictal spiking are hyperactive relative to non-spiking controls. This is a very significant observation, as interictal spikes are still often considered symptomatically benign, despite the associative studies in psychiatric literature. Neocortical interictal spikes without seizures have never been previously shown to have behavioral effects in an animal model. The only other studies showing an effect of interictal spikes on behavior or cognition in animal models were just published this year. One that showed hippocampal spikes impaired memory retrieval and response time to tasks (172) and another showed that hippocampal spikes during neonatal development impaired reference memory and long-term potentiation in these rats as adults (173). Finally, we observed that spiking rats turned counterclockwise more often

than clockwise in proportion to the number of spikes originating from the left versus right hemispheres on the day of recording. Therefore, the location of the interictal spike actually influences the behavior displayed.

Taken together, these results argue for a potential role of interictal spikes in the comorbidities of epilepsy or psychiatric disorders. It may therefore be beneficial to treat not only seizures, but also interictal spikes in epilepsy patients experiencing comorbid conditions, and to look for the presence of interictal spiking more often in psychiatric patients. The tetanus toxin model described here provides a new animal model in which hypotheses and eventually treatments for these human ailments can be tested.

MAPK pathway inhibition as a new therapeutic for epilepsy

The role of the MAPK pathway in epilepsy, especially ERK, has been implicated by both human (174,175) and animal studies (176-180). In fact, conditional expression of a constitutively active form of ERK in rats was shown to cause epilepsy, demonstrating that ERK activation alone is sufficient to cause the disease, at least in an animal model (156). Transcriptional analysis by microarrays in our laboratory has provided the strongest evidence to date that MAPK activation is central to human epilepsy (10,11). Components of the MAPK pathway were greatly enriched in the epileptic transcriptome, and a transcription factor binding site search revealed that the downstream target, CREB, was enriched over the next factor (serum response factor) by several orders of magnitude. Therefore, when creating an animal model of epilepsy, we expected to find evidence of MAPK-CREB activation and then to subsequently test inhibitors of this pathway as possible treatments. Frustratingly, this was difficult to

demonstrate consistently in spiking rats either by western blot or immunohistochemistry. This difficulty may be due to the labile nature of MAPK activation after a stimulus in neurons (181,182). Regardless, CREB phosphorylation was greater in layers II and III of the spiking hemisphere in most animals sacrificed one week after tetanus injection, implying that at least initially it corresponds well to the location of spike activity.

More conclusive, however, was the use of SL327, a compound which selectively inhibits the phosphorylation of ERK by MEK (133,134). SL327 administered to rats in the first week following tetanus toxin injection prevented the development of a spiking focus, providing strong evidence that ERK activation is required for initiation of this process. This inhibitor was also able to prevent the increase in ambulation associated with the development of interictal spikes, even weeks after it was discontinued. The next step will be to see whether treatment with this drug once a spiking focus has already been established will be able to reduce or abolish interictal spiking, or more importantly, to prevent the development of seizures. If effective, this would represent an entirely new avenue of therapy for epilepsy, one which could hopefully correct the underlying brain abnormality itself, rather than simply reduce the symptom of seizures.

Other therapeutic targets

The MAPK pathway inhibitor used here was chosen because it could be given simply through an intraperitoneal injection yet still penetrated the brain, and had been shown to be effective at knocking down ERK and CREB phosphorylation in rat brain in vivo (133,134). It is certainly not the only option for targeting this pathway though, and perhaps administration of an inhibitor that does not cross the blood-brain barrier through

an intracranial cannula would allow higher concentrations to be used without the side effects of systemic MAPK inhibition. Recent unpublished data from our laboratory (Bagla S, Brown E, Barkmeier D) suggest that phosphorylated p90-Ribosomal S6 Kinase is a more reliable marker of epileptic brain areas than either dpERK or pCREB, so it may actually make a better target for inhibition.

Outside of the MAPK pathway, other signaling cascades may also prove to be effective therapeutic targets. Serum Response Factor, for example, plays a role in activity-dependent plasticity (183-185) and was the second most enriched transcription factor in our human epileptic transcriptome after CREB. The activation states of other pathways classically associated with activity-dependent plasticity such as Protein Kinase A, PKC and Calcium-calmodulin dependent Protein Kinase II (186-189) should be assessed as well. While they did not appear as top candidates on our human epileptic microarrays, they may play roles at different stages of the disease, such as the ability of PKC to convert isolated spikes to spike clusters in vitro (169). The drawback of all these inhibitors, however, would be that they are not targeted specifically to the abnormal areas of brain, but would act generally. As with many medications, the doses could likely be titrated to find an effective level that causes minimal side effects, but the ideal solution would be to target drugs directly to the epileptic areas. The ability to target drugs to neurons based on their activity would solve this problem, but that technology may be decades away. Instead, a more immediate solution may be to convert spiking neurons from inducing activity-dependent, long-term potentiation (LTP) to inducing activity-dependent, long-term depression (LTD). In normal brain, the balance between LTP and LTD depends on the discharge timing of the two connected neurons, but application of an

alpha$_1$-agonist such as methoxamine can force LTD as the only plasticity option (190-192). In the presence of methoxamine, any stimulus would induce LTD in an activity-dependent manner. It may therefore be possible to use the hyperactive firing of the epileptic focus to preferentially induce LTD in that neuronal network, with less of an effect on normal neurons.

APPENDIX A
TABLES

Table 1 – Reviewer agreement on detecting individual spikes

	Percent of spikes marked by:	
	>=2 Reviewers	All 3 Reviewers
Patient 1	12.7	0.0
Patient 2	30.9	4.3
Patient 3	26.5	4.9
Patient 4	17.8	0.5
Patient 5	39.2	0.7
Patient 6	18.1	1.3
Patient 7	35.7	5.9
Patient 8	16.0	2.9
Patient 9	32.3	7.4
Patient 10	8.1	-
Mean	23.7%	3.1%

Table 2 – Spike detection reviewer variability and algorithm performance

A. HUMAN REVIEWER AGREEMENT WITH OTHER REVIEWERS

	Reviewer 1		Reviewer 2		Reviewer 3	
	Sensitivity	**Precision**	**Sensitivity**	**Precision**	**Sensitivity**	**Precision**
Patient 1	13.1	81.0	0.1	100.0	80.8	13.1
Patient 2	55.1	39.9	15.3	73.6	26.5	53.9
Patient 3	70.1	29.6	19.5	93.9	13.7	56.1
Patient 4	21.2	32.5	4.6	58.7	33.2	26.0
Patient 5	19.4	49.5	31.9	51.8	48.8	64.2
Patient 6	17.0	33.1	7.8	56.6	35.4	25.5
Patient 7	45.7	49.8	14.5	75.8	44.9	53.2
Patient 8	33.7	19.2	11.5	75.0	12.6	31.2
Patient 9	34.8	61.1	17.7	61.5	45.7	41.2
Patient 10	9.7	32.7	-	-	32.7	9.7
Mean	**32.0**	**42.8**	**13.6**	**71.9**	**37.4**	**37.4**

B. RANKING OF CHANNEL ACTIVITY

	Kendall's	Mean Spearman Rank Coefficient*				
	W	**Reviewer 1**	**Reviewer 2**	**Reviewer 3**	**Custom script**	**Commercial**
Patient 1	0.653	0.56	0.284	0.529	0.85	0.799
Patient 2	0.795	0.705	0.708	0.758	0.493	0.585
Patient 3	0.811	0.747	0.747	0.68	0.551	0.199
Patient 4	0.802	0.652	0.687	0.755	0.949	0.801
Patient 5	0.882	0.796	0.822	0.849	0.901	0.817
Patient 6	0.767	0.561	0.667	0.706	0.834	0.794
Patient 7	0.838	0.712	0.744	0.823	0.915	0.725
Patient 8	0.622	0.416	0.448	0.46	0.583	0.352
Patient 9	0.858	0.821	0.806	0.737	0.697	0.579
Patient 10	0.49	0.478	-	0.478	0.726	0.09
Mean	**0.752**	**0.645**	**0.657**	**0.678**	**0.750**	**0.574**

**For human reviewers, this is the average Spearman's Rho from comparison to the other two reviewers. For the computer scripts, it is Spearman's Rho when compared to the average channel rank determined by the three human reviewers*

C. CUSTOM SCRIPT'S ABILITY TO IDENTIFY SPIKES MARKED BY:

	At least 1 Reviewer		At least 2 Reviewers		All 3 Reviewers	
	Sensitivity	**Precision**	**Sensitivity**	**Precision**	**Sensitivity**	**Precision**
Patient 1	47.1	58.6	83.1	13.2	100.0	0.1
Patient 2	42.4	80.6	65.1	38.3	79.0	6.4
Patient 3	8.3	48.2	24.7	38.0	46.2	13.1
Patient 4	49.0	60.1	85.8	18.7	87.0	0.6
Patient 5	33.8	98.9	51.1	58.5	36.0	0.7
Patient 6	20.8	73.4	51.9	33.0	40.5	1.8
Patient 7	44.5	86.8	74.5	51.9	78.0	9.0
Patient 8	30.3	46.0	71.8	17.4	85.7	3.7
Patient 9	67.8	30.5	82.7	12.0	83.6	2.8
Patient 10	58.2	19.3	90.7	2.4	-	-
Mean	**40.2**	**60.2**	**68.1**	**28.3**	**70.7**	**4.3**

D. EXISTING COMMERCIAL SCRIPT'S ABILITY TO IDENTIFY SPIKES MARKED BY:

	At least 1 Reviewer		At least 2 Reviewers		All 3 Reviewers	
	Sensitivity	**Precision**	**Sensitivity**	**Precision**	**Sensitivity**	**Precision**
Patient 1	20.8	52.1	55.7	17.8	100.0	0.1
Patient 2	45.6	45.3	55.7	17.1	39.5	1.7
Patient 3	0.0	0.0	0.0	0.0	0.0	0.0
Patient 4	35.3	92.3	65.1	30.3	46.3	0.7
Patient 5	29.6	95.9	37.3	47.3	18.7	0.4
Patient 6	29.8	82.3	59.7	29.7	48.6	1.7
Patient 7	48.6	100.0	60.0	44.1	16.6	2.0
Patient 8	23.8	14.4	28.2	2.7	14.3	0.2
Patient 9	28.3	26.0	27.8	8.3	6.8	0.5
Patient 10	0.1	100.0	0.8	50.0	-	-
Mean	**26.2**	**60.8**	**39.0**	**24.7**	**32.3**	**0.8**

Table 3 - Spikes marked only by script, but later verified by reviewers (Out of 10 per patient)

	Reviewer 1	Reviewer 2	Reviewer 3
Patient 1	7	0	1
Patient 2	9	1	4
Patient 3	10	4	7
Patient 4	6	1	0
Patient 5	8	0	5
Patient 6	6	0	0
Patient 7	6	0	2
Patient 8	4	0	0
Patient 9	0	0	0
Patient 10	9	0	4
Mean	**6.5**	**0.6**	**2.3**

Table 4 – Lesional patient data

Patient	Tumor type	Age	Sex
1	Oligodendroglioma with astrocytic component - Grade 2	54	M
2	Oligoastrocytoma – Grade 3	40	M
3	Oligodendroglioma infiltrating	35	M
4	Galngliоglioma	22	M
5	DNET	47	F
6	Mixed high-grade tumor: Oligoastrocytoma with a nodule of sarcoma – Grade 3	42	F
7	Anaplastic mixed glioma - Grade 3	36	F
8	Oligoastrtocytoma - Grades 2 and 3 mixed	44	M
9	Dermoid cyst	38	F
10	Epidermoid	50	M
11	Meningioma (transitional type)	40	F

Table 5 – Correlations with interictal spike parameters

	Non-lesional	Lesional	
	Seizure Category	**Seizure Category**	**Distance from tumor**
Frequency	0.111	0.126	0.254
Amplitude 1	0.133	-	-0.134
Duration 1	-	-	0.144
Slope 1	0.124	-	-0.217
Amplitude 2	0.121	-	-0.213
Duration 2	-	0.220	-
Slope 2	-	-	-0.205
Power	0.116	0.144	0.245
Tumor distance	-	-0.183	-

All p<0.01 after correction for multiple comparisons

Table 6 – Interictal spike parameters identify seizure onset zone

	Non-lesional patients		
	Significant Kruskal-Wallis	**Number of patients with max value at onset electrode**	**Number of patients with min value at onset electrode**
Frequency	Yes	4	0
Amplitude 1	Yes	4	2
Duration 1	No	1	0
Slope 1	Yes	4	0
Amplitude 2	No	4	2
Duration 2	No	2	2
Slope 2	No	5	2
Power	Yes	4	0

	Lesional patients		
	Significant Kruskal-Wallis	**Number of patients with max value at onset electrode**	**Number of patients with min value at onset electrode**
Frequency	Yes	2	0
Amplitude 1	No	4	2
Duration 1	No	0	2
Slope 1	No	5	0
Amplitude 2	No	3	0
Duration 2	Yes	4	0
Slope 2	No	5	3
Power	Yes	3	0
Tumor Distance	Yes	2	1

APPENDIX B

FIGURES

Figure 1 - Spike detection using filtering and block scaling balances differences in human reviewers

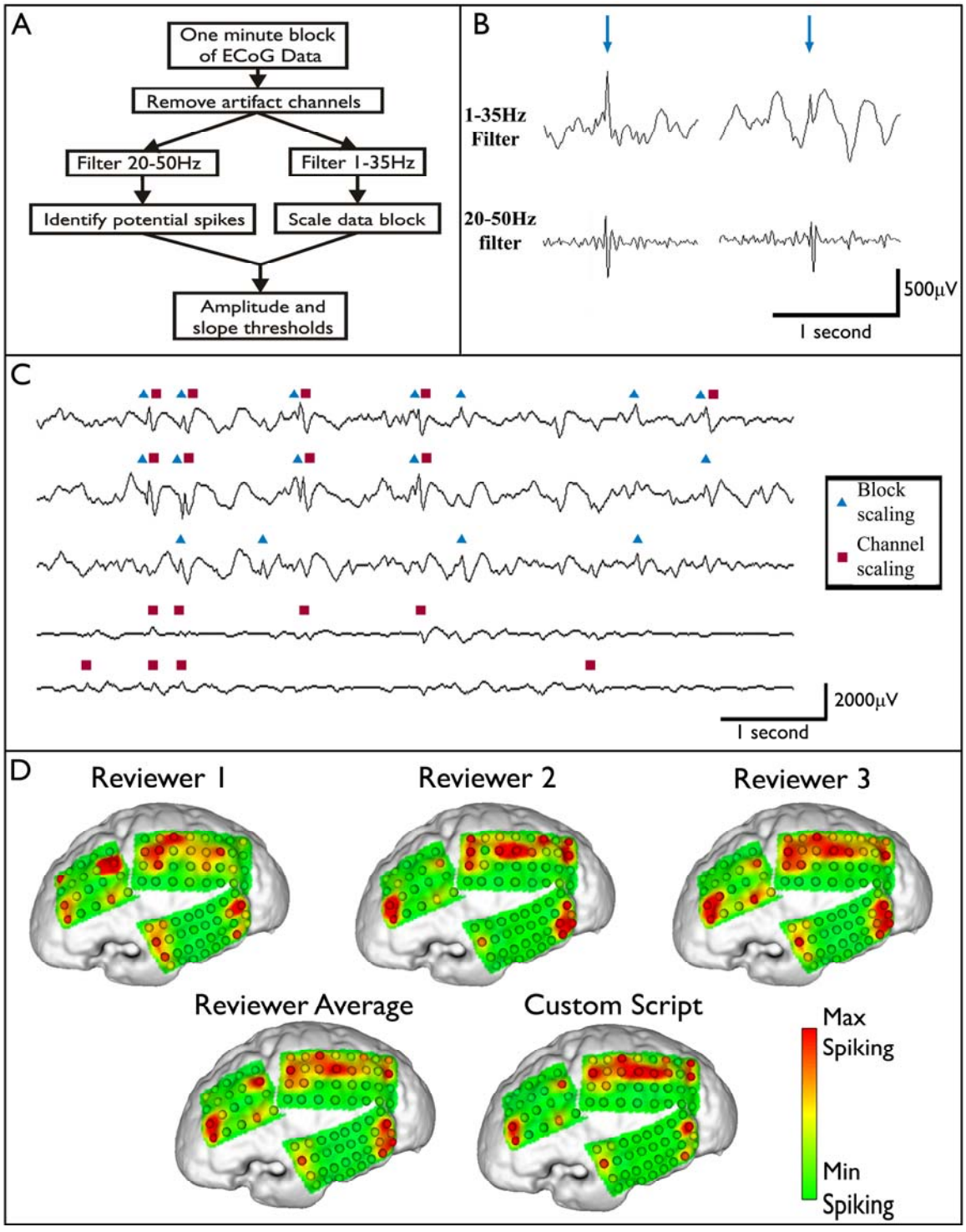


Figure 2 – Frequency and slope of the first halfwave correlate best with seizure category in non-lesional patients

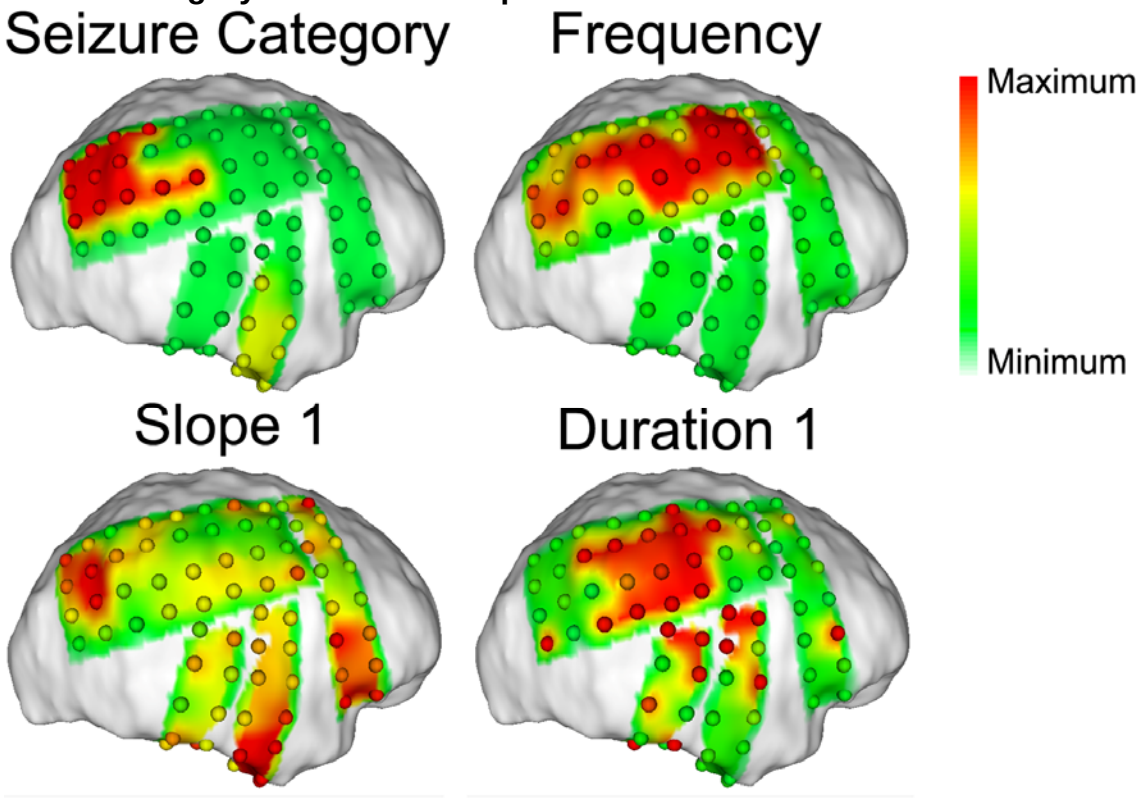


Figure 3 – Seizure category often lies outside the tumor area, but is identified by higher frequency and duration of interictal spiking

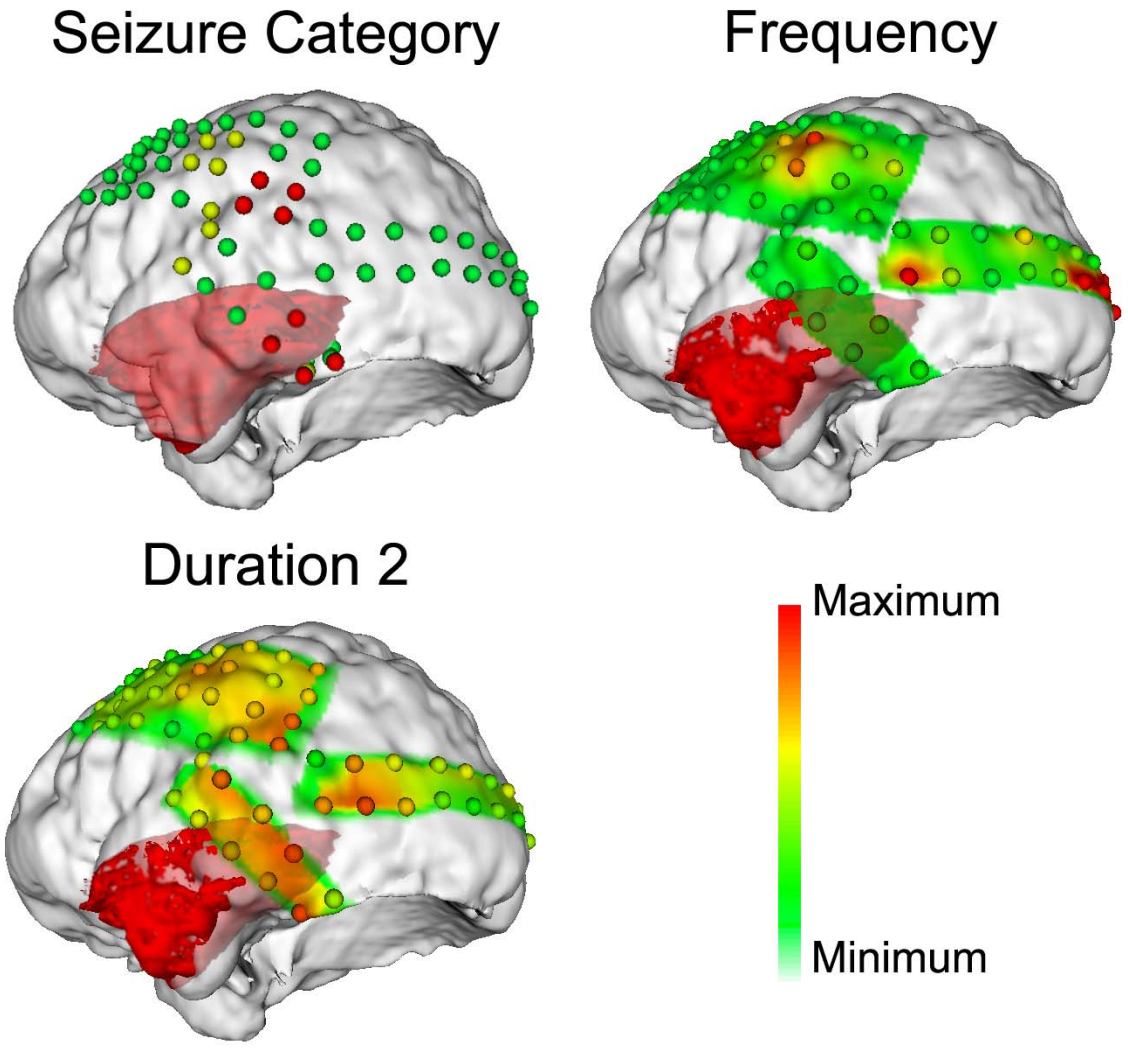


Figure 4 – Setup of animal model of neocortical interictal spiking

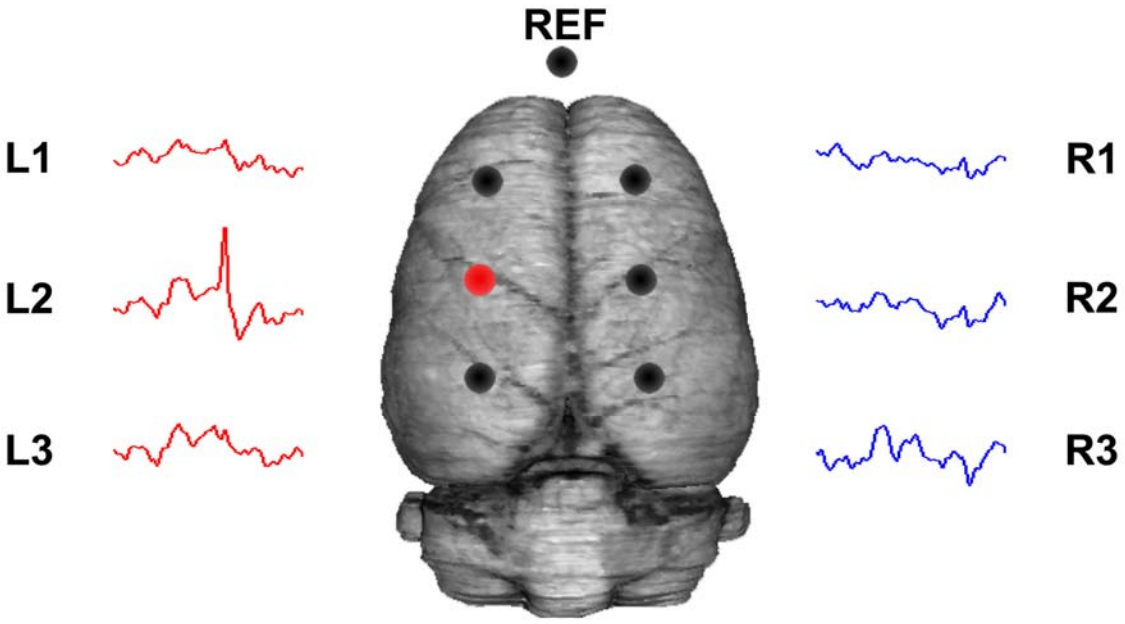


Figure 5 – Interictal spiking focus progresses over time

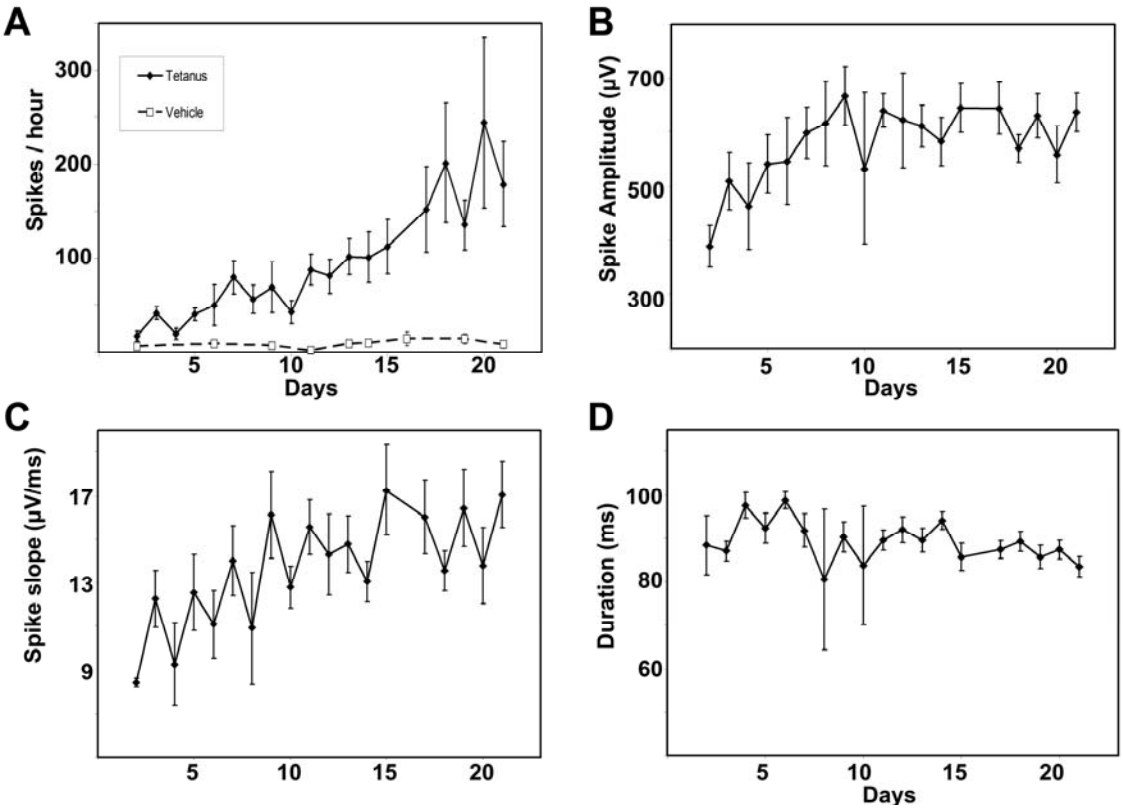


Figure 6 – Spikes progressively cluster together with time

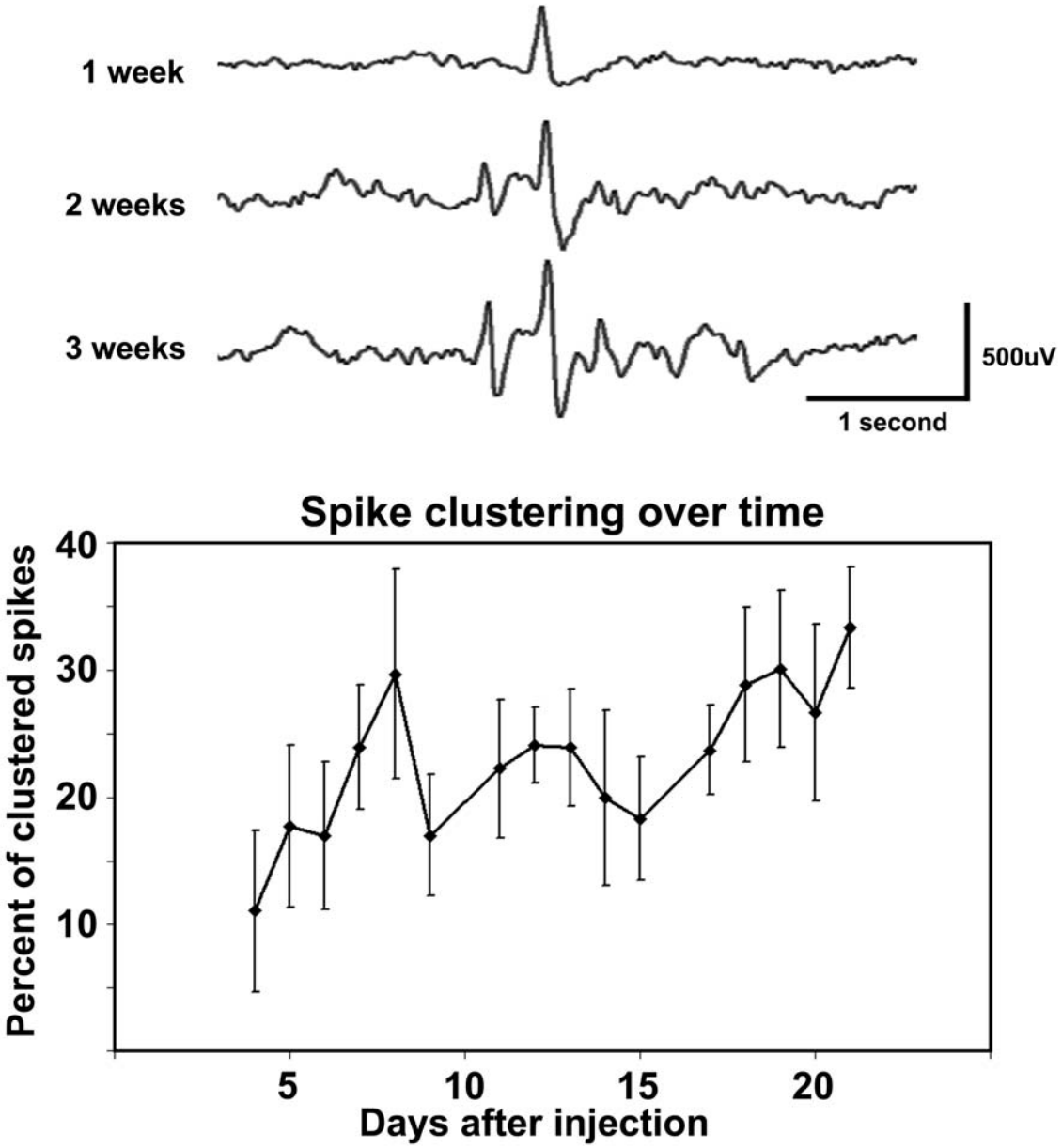


Figure 7 – Expansion of spike field over time

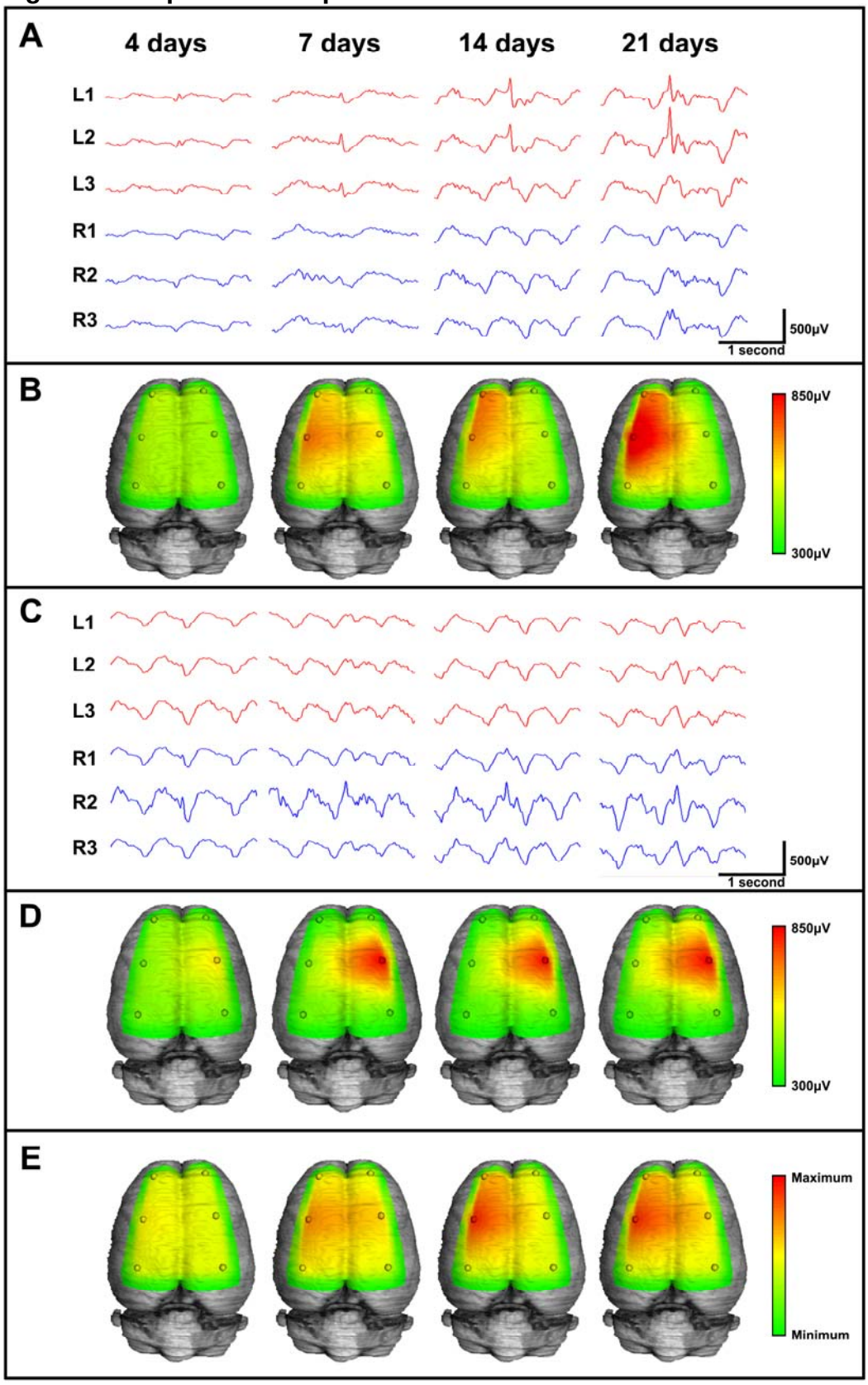


Figure 8 – Interictal spiking causes activity-dependent gene expression

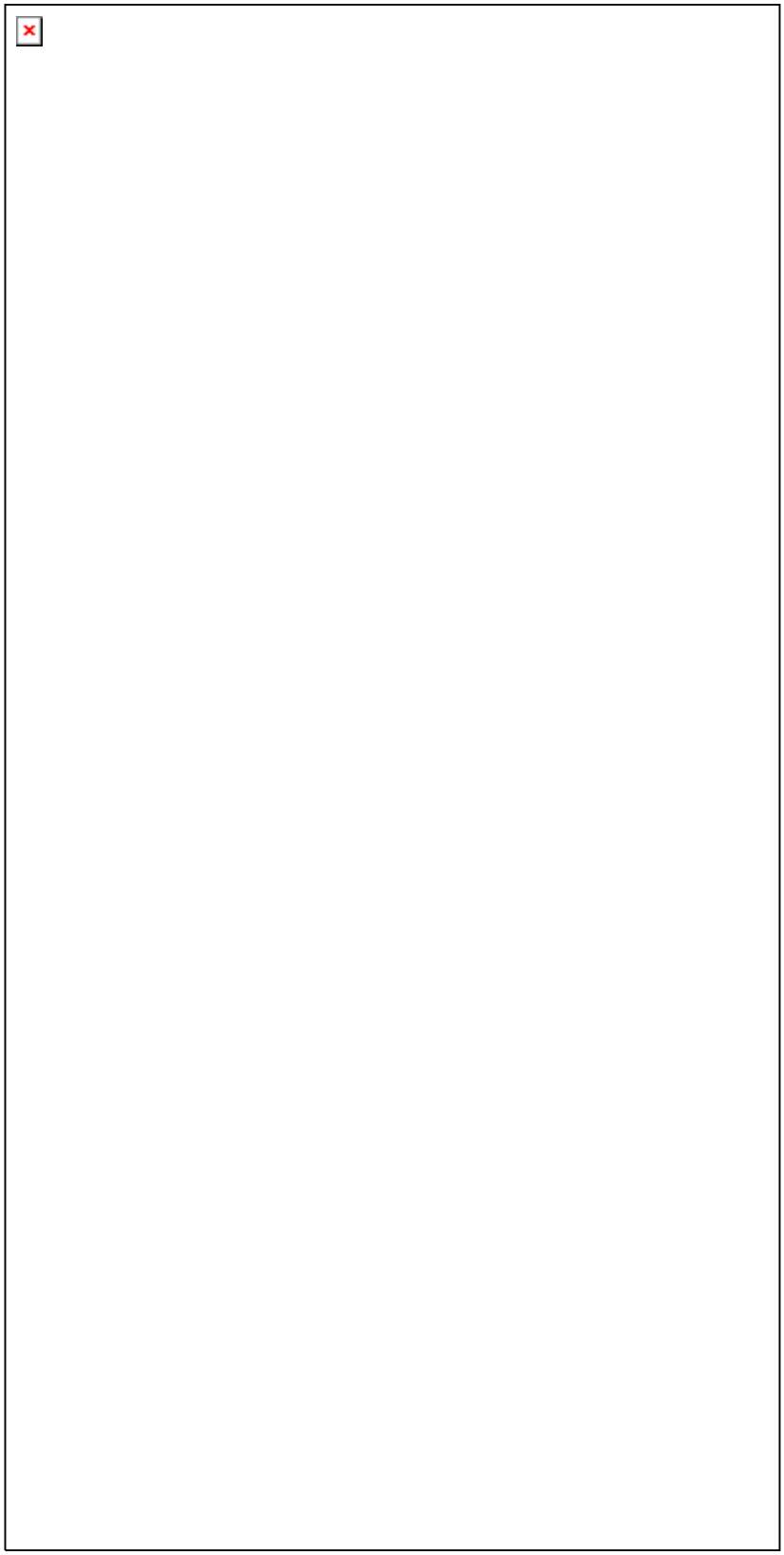


Figure 9 – MAPK-CREB inhibition can block the development of an interictal spiking focus

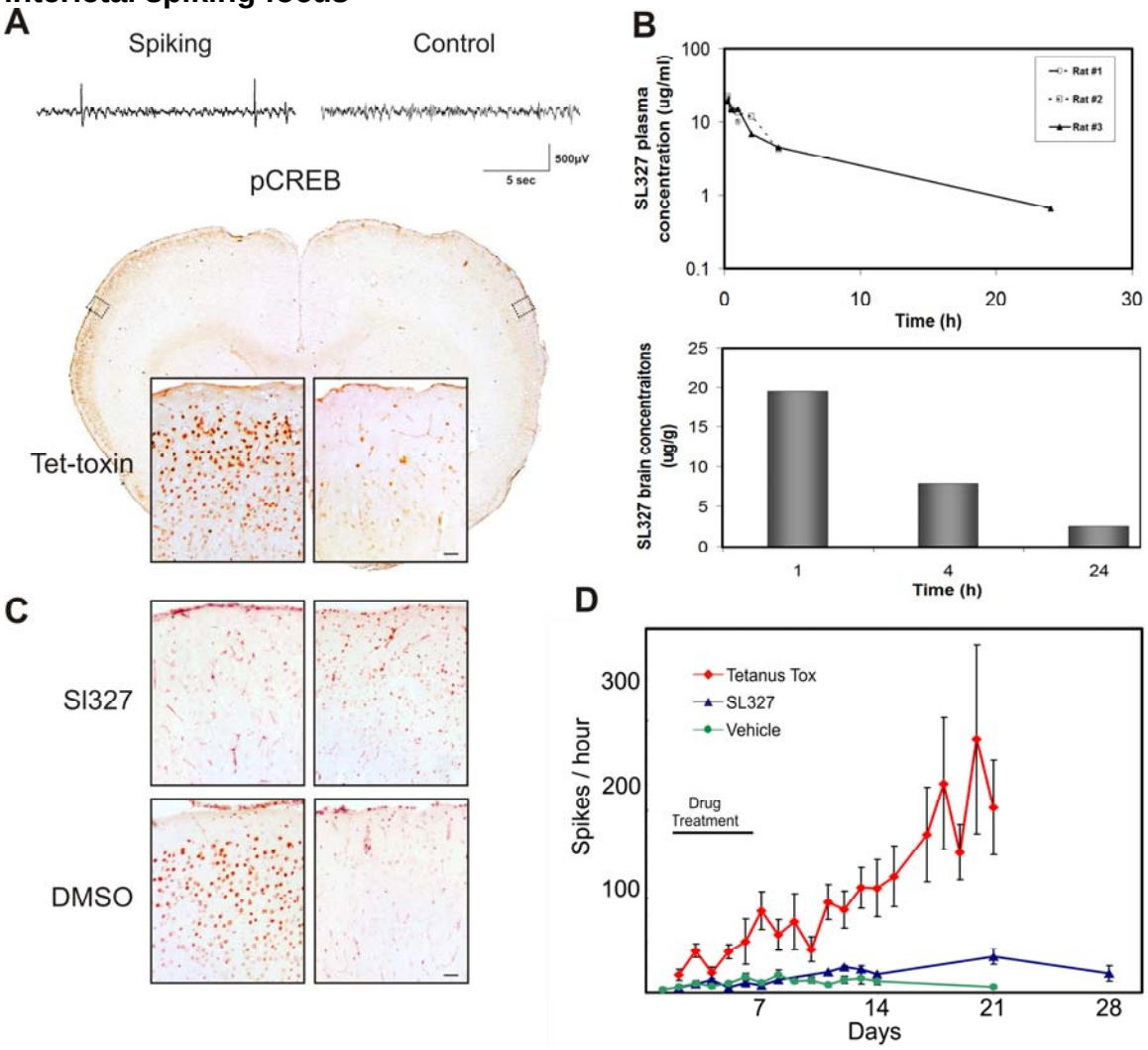


Figure 10 – Interictal spikes alter rat motility behavior

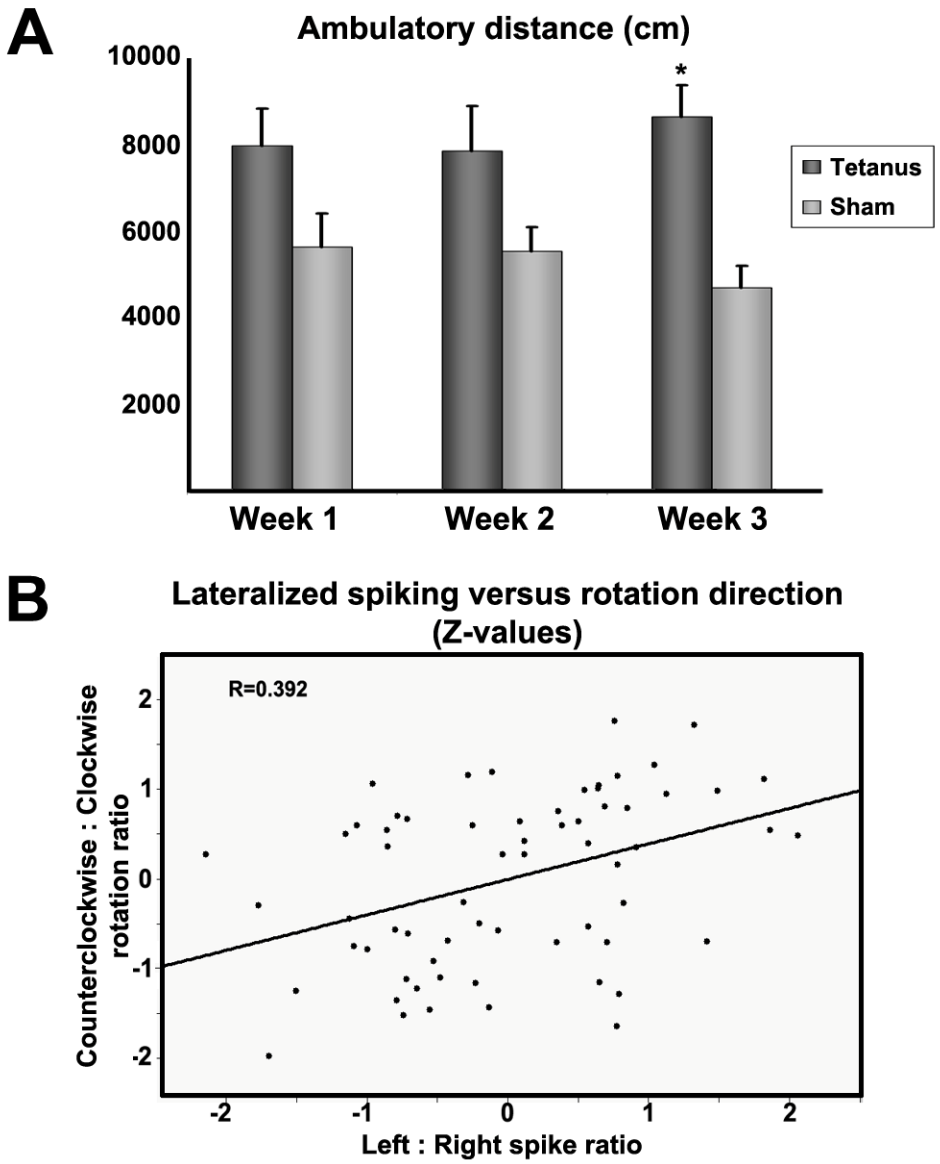


Figure 11 – Interictal spikes can be induced by environmental stimuli

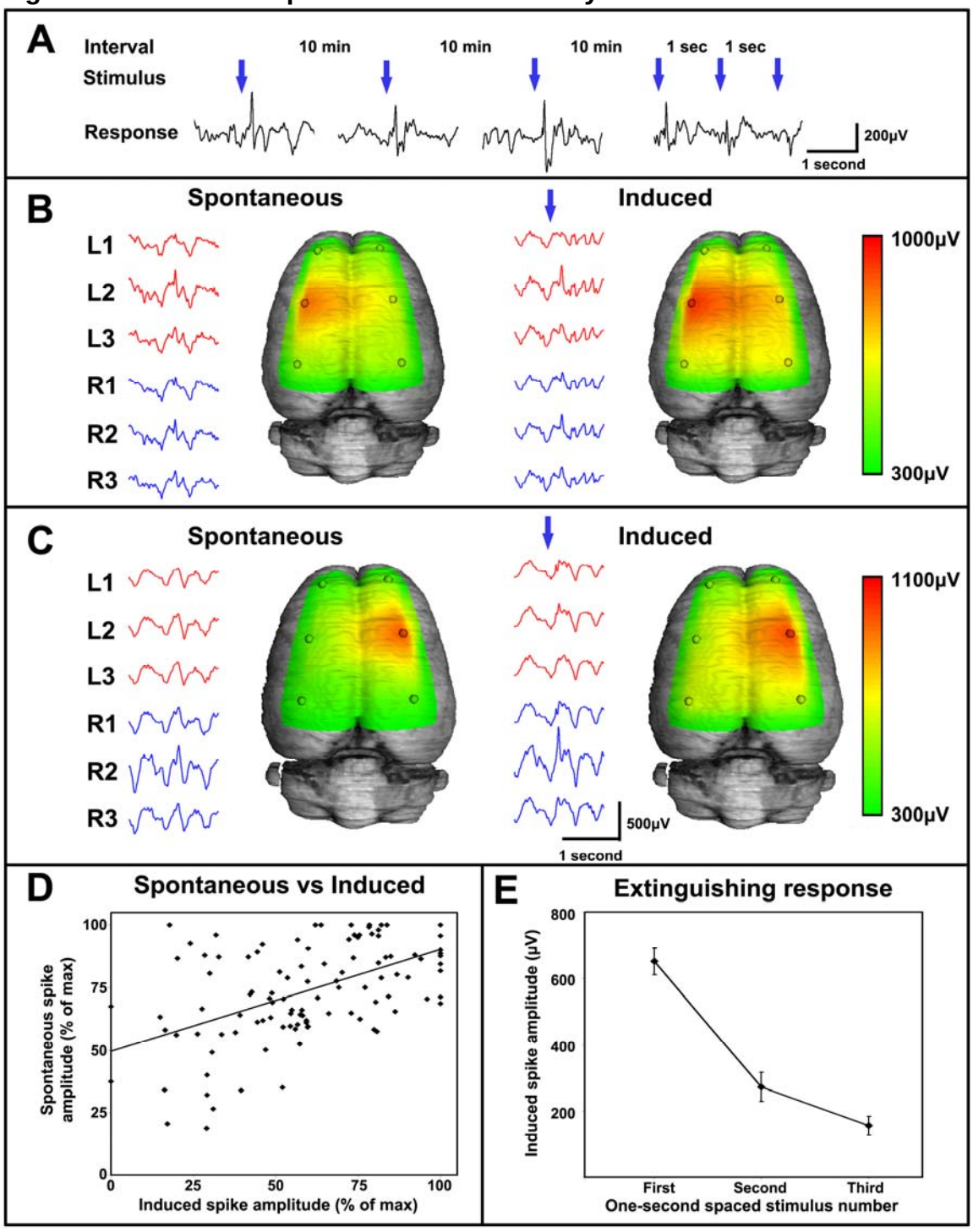


Figure 12 – Suppression of interictal spiking with a MEK inhibitor normalizes behavior

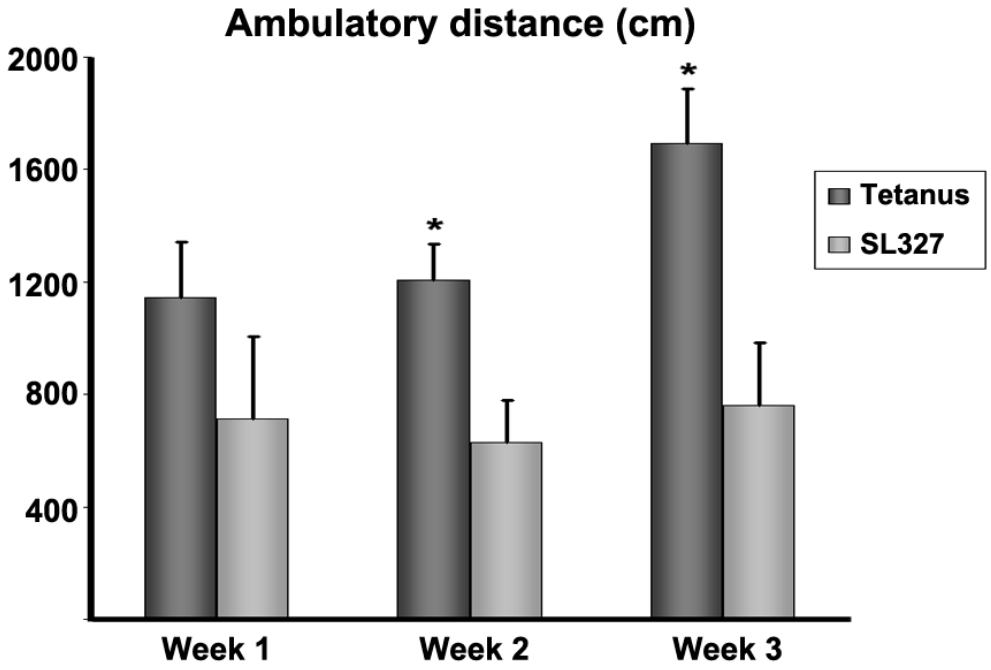
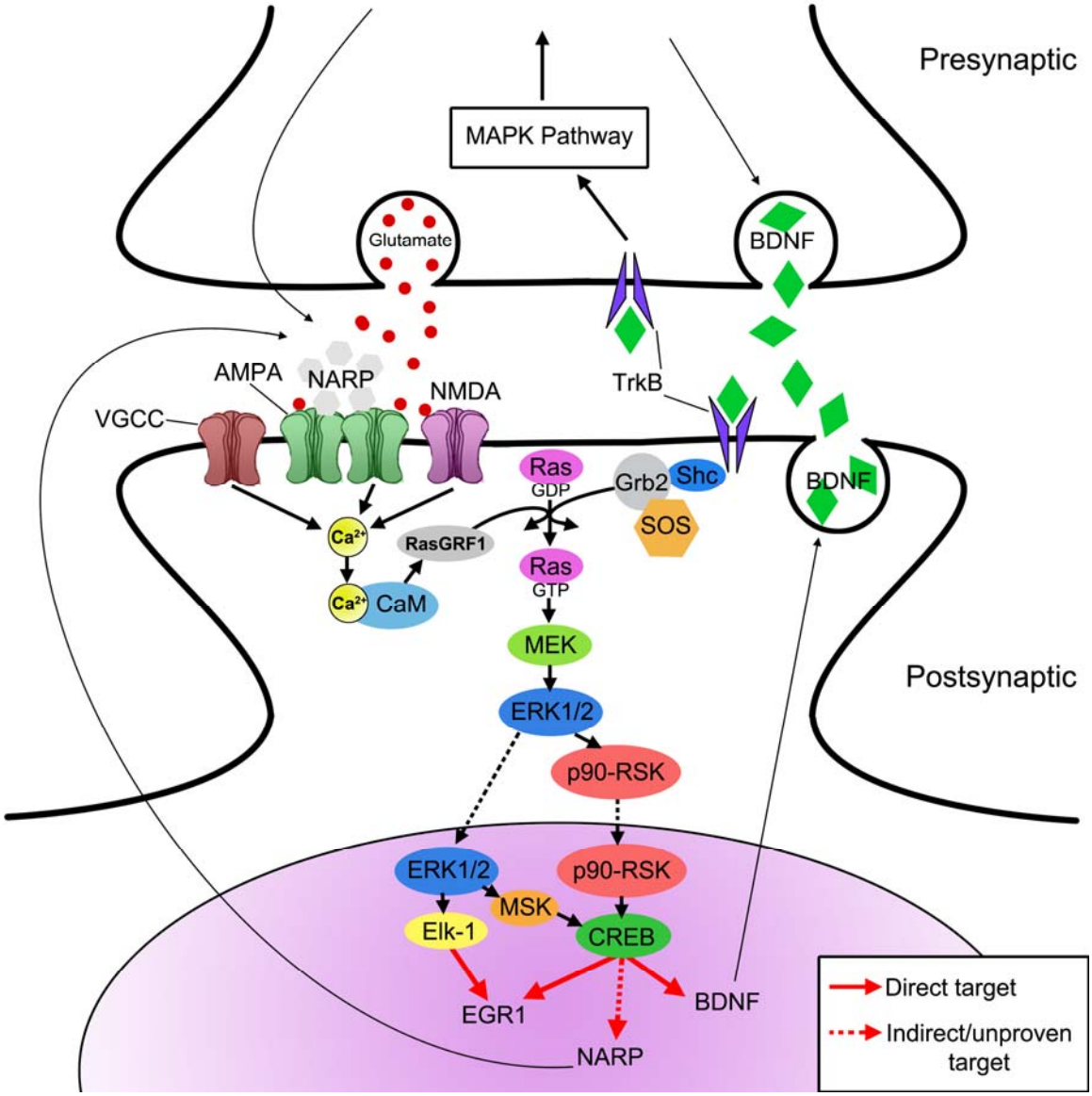


Figure 13 – Proposed role of MAPK pathway signaling in this animal model



APPENDIX C
FIGURE LEGENDS

Figure 1. Spike detection using filtering and block scaling balances differences in human reviewers. **(A)** Flowchart overview of the detection algorithm. **(B)** The algorithm's main initial screening step involves bandpass filtering the data from 20-50Hz (bottom), which makes interictal spikes (blue arrows) stand out from background compared to more standard viewing filters (top). This method accentuates both large, obvious spikes (left) as well as those that may have otherwise been lost in larger slow wave background activity (right). **(C)** Scaling all channels together as a block preserves differences in spike detection (blue triangles) between more and less active channels. Typical methods of scaling each individual channel leads to undermarking of active channels and overmarking of less active channels (red squares). **(D)** Heatmaps of interictal spike frequency superimposed on a patient's 3-dimensional brain rendering show that the three human reviewers rank channels similarly, but not identically (top). The spike detection algorithm, however, balances reviewer discrepancies and produces a similar pattern to the average of all three human reviewers (bottom).

Figure 2 – Frequency and slope of the first halfwave correlate best with seizure category in non-lesional patients. Cortical areas of seizure onset (red) and seizure spread (yellow) can best be distinguished from normal areas of brain (green) in non-lesional patients by higher interictal spike frequency and slope of the first halfwave, as demonstrated in this example patient. Other parameters, such as duration of the first halfwave, are not as reliably associated with seizure-generating areas. (Colored scale bar

shows that for each parameters, high values are colored red, intermediate values are yellow and low values are green.)

Figure 3 – Seizure category often lies outside the tumor area, but is identified by higher frequency and duration of interictal spiking. In most lesional patients (n=7/11), areas of seizure onset (red) lie outside of the immediate tumor area and may not be resected as part of a normal tumor resection, as shown in this example patient. The tumor is highlighted as a large, red mass within the temporal lobe. In contrast to non-lesional patients, interictal spike frequency is not the best marker for tumor area. Instead, duration of the second halfwave is much more reliable. Spike frequency is inversely correlated with distance from tumor, suggesting that low spike frequencies may aide in identifying tumor margins. (Colored scale bar shows that for each parameters, high values are colored red, intermediate values are yellow and low values are green.)

Figure 4 – Setup of animal model of neocortical interictal spiking. Three electrodes are placed over each hemisphere, and one over the nasal sinus as a reference (circles). Tetanus toxin is injected under the left, middle electrode (red circle) into the somatosensory cortex. Spikes are initially detected at the injection site in the left, middle electrode (L2), as shown by a small example segment of EEG recorded at each of the six channels.

Figure 5 – Interictal spiking focus progresses over time. (A) A single injection of tetanus toxin causes interictal spikes to develop after a delay of three to five days, which

then increase in frequency with time (n=11 animals). Vehicle animals (n=5) show very little spiking and do not change with time. Tetanus-injected animals have higher spike frequency than vehicles by the first week (p=0.029, one-tailed t-test, Bonferroni correction; 79.1±18.1 and 9.4±2.9 spikes/ hour, ± sem), which persists at two weeks (p=0.020, one-tailed t-test, Bonferroni correction; 101.3±19.5 and 9.5±2.9 spikes/ hour, ± sem) and three weeks (p=0.040, one-tailed t-test, Bonferroni correction; 178.33±45.3 and 8.6±4.2 spikes/hour±sem) **(B)** Tetanus-injected animals also initially increase in amplitude, which then plateaus by 10 days (Best fit model: $y=a*x^b+c$; $R^2=0.667$). **(C)** Spike slope increases with time ($R^2=0.530$; Linear regression slope=0.307μV/day, p=0.0004), while duration does not change much ($R^2=0.148$; Linear regression slope=-0.299ms/day, p=0.104). **(D)**. Error bars are standard error of the mean.

Figure 6 – Spikes progressively cluster together with time. Example EEG samples from the same rat show that spikes initially appear as single, isolated discharges in the first week. By the second week, 'doublets' become increasingly common, and by three weeks longer runs of three to six spikes occur. Quantifying this increase by calculating the percent of spikes in a recording that occur clustered with other spikes shows a progressive increase in clustering with time. (n=11; $R^2=0.492$; Linear regression slope=0.75%/day, p=0.0025)

Figure 7 – Expansion of spike field over time. (A) Sample EEG traces from a rat which develops spikes predominantly in the left middle (L2) and anterior (L1) channels. **(B)** Heatmap plots of the average amplitudes of spikes in each recording channel over

time show the progression in both amplitude and distribution over time. Similarly, example EEG traces **(C)** and heatmaps **(D)** show one of the rats which developed right-sided spikes, despite injection of tetanus toxin on the left. Averaging the field distribution of 11 rats over time **(E)** shows that spikes begin initially at the injection site, but soon spread anteriorly and sometimes contralaterally over time. No rats showed spread to posterior channels.

Figure 8 – Interictal spiking causes activity-dependent gene expression. Coronal brain sections near the injection site show changing patterns of activity-dependent gene expression with time via ^{35}S *in situ* hybridization. An animal sacrificed after one week shows an interesting pattern of increased expression at the injection site, with an annulus of suppression surrounding it. While Egr1 shows this most robustly, the same pattern can be seen in superficial layer II with both Narp and Bdnf. Gapdh shows even expression as a non-activity-dependent control. By two weeks, animals that were sacrificed while the spiking focus was still increasing in frequency and amplitude show increased gene expression in layers II/III of the spiking hemisphere relative to the contralateral hemisphere, while Gapdh expression is even. By three weeks, the spiking focus of most rats has generally stabilized and no longer shows lateralized expression differences, despite having focal, left-hemispheric spiking. Surgery-naïve animals showed even expression and antisense probes showed no detectable signal.

Figure 9 – MAPK-CREB inhibition can block the development of an interictal spiking focus. (A) By one week after injection of tetanus toxin, most spiking animals

(n=5/7) show Creb phosphorylation in layer II/III of the spiking hemisphere relative to the contralateral hemisphere. To attempt to block this activation, the pharmacokinetics of a selective MAPK inhibitor were first determined by measuring drug concentrations in plasma **(B, top)** and brain **(B, bottom)** at multiple time points after intraperitoneal injection. The results of this study were used to determine a dosing regimen of the inhibitor which, when given to rats in the week following tetanus toxin injection, reduces Creb phosphorylation by immunohistochemistry relative to animals treated with the vehicle, DMSO **(C)**. Rats treated with intraperitoneal injections of 25 mg/kg SL327 dissolved at 25 mg/kg in DMSO twice daily for one week after tetanus toxin injection and then monitored for interictal spiking for four weeks showed significantly decreased spike frequency at one week ($p=0.005$, one-tailed t-test, Bonferroni correction; 79.1 ± 18.2 and 7.0 ± 3.6 spikes/ hour, $\pm$ sem), 2 weeks ($p=0.044$, one-tailed t-test, Bonferroni correction; 100.5 ± 27.3 and 18.0 ± 9.0 spikes/ hour, $\pm$ sem) and 3 weeks ($p=0.013$, one-tailed t-test, Bonferroni correction; 178.3 ± 45.3 and 30.7 ± 7.6 spikes/ hour, $\pm$ sem), suggesting that MAPK is necessary for the development of interictal discharges **(D)**. Scale bars = 10μm**(A,C).**

Figure 10 – Interictal spikes alter rat motility behavior (A) Monitoring open field activity immediately after recording EEG shows that tetanus treated spiking rats are consistently hyperactive relative to sham-operated controls (n=4 tetanus, 4 sham; 1 week $p=0.082$, 2 weeks $p=0.079$, 3 weeks $p=0.0005$, Bonferroni correction). **(B)** Left versus right spike frequency ratio correlates with the ratio of counter-clockwise to clockwise rotations rats make ($R=0.396$, $p=0.013$). Similarly, total spike power correlated

positively with ambulatory distance (R=0.350, p=0.039 Bonferroni correction) and negatively with resting time (R=-0.410, p=0.008 Bonferroni correction). Values within each rat were normalized to Z-scores before being compiled for the final correlation.

Figure 11 – Interictal spikes can be induced by environmental stimuli. (A) Study overview: Each day of EEG recording, an audio file of a clap is played repeatedly. The first three sounds are spaced 10 minutes apart and are then followed by a train of three spaced one second apart. **(B)** A rat with spontaneous left-sided spiking has induced spikes that appear in the same pattern on the left. EEG traces show individual examples, while the three-dimensional heatmaps show the average of all spontaneous or induced spikes for a particular day. **(C)** A rat with spontaneous right-sided spiking has induced spikes on the right. All rats showed induced spikes in the same field distribution as the rat's spontaneous spikes. **(D)** The amplitude of induced spikes increased over time as each rat's spontaneous spikes increased in amplitude. The scatter plot shows the amplitude of induced spikes versus the amplitude of spontaneous spikes, normalizing the values within each rat so that the highest value is 100%. ($R=0.535$, $p=1.42\times10^{-9}$). **(E)** When a train of sounds only one second apart is played, EEG response to the stimulus extinguishes rapidly, likely because rats no longer appear startled by the sound.

Figure 12 – Suppression of interictal spiking with a MEK inhibitor normalizes behavior. Monitoring open field activity while simultaneously recording EEG shows that tetanus treated rats increase in ambulation over time as spiking increases, but treatment with a selective inhibitor of a component of the MAPK pathway, SL327,

during the first week after tetanus injection prevents this increase. Rats were administered intraperitoneal injections of 25 mg/kg SL327 dissolved at 25 mg/kg in DMSO twice daily for one week following surgery. By two weeks, rats given tetanus alone ambulate significantly more than rats given tetanus and SL327 ($p=0.025$) and this difference widens by week 3 ($p=0.012$). SL327 animals were similar to animals injected with a vehicle solution (0.01M sodium phosphate) instead of tetanus toxin (729.4±93.2cm; not shown).

Figure 13 – Proposed role of MAPK pathway signaling in this animal model. The MAPK pathway can be activated both by calcium influx into the cell and by binding of BDNF to its receptor, TrkB. Intracellular calcium can be increased by the binding of glutamate to ionotropic glutamate receptors (AMPA and NMDA types) or by activation of voltage-gated calcium channels (VGCC) (193). The resulting increase in intracellular calcium (Ca^{2+}) binds to and activates calmodulin, which in turn binds to Ras protein-specific guanine nucleotide-releasing factor 1 (Ras-GRF1) (194,195). Ras-GRF1 acts as a guanine nucleotide exchange factor (GEF) which causes Ras to release guanosine diphosphate (GDP) and instead bind guanosine triphosphate (GTP), thus activating Ras. From the TrkB receptor side, binding of BDNF to a TrkB homodimer causes autophosphorylation of tyrosine residues in the intracellular kinase domain of the receptor (196). Phosphorylation of a tyrosine at position 515 in the juxtamembrane region of TrkB recruits Shc adaptor molecules through their phosphotyrosine-binding (PTB) domains (197). The recruitment and phosphorylation of Shc adaptors leads to the binding of growth factor receptor-bound protein 2 (GRB2) complexed with son of sevenless

(SOS) (198) , which acts as a GEF to activate Ras to the GTP-bound form (199). Active Ras then phosphorylates mitogen-activated protein kinase (MEK), which in turn phosphorylates extracellular signal-regulated kinases 1 and 2 (200). ERK can both translocate to the nucleus and phosphorylate cytosolic ribosomal S6 kinase (p90-RSK) which itself translocates to the nucleus (201-203). Once inside the nucleus, p90-RSK phosphorylates the transcription factor, cyclic AMP response element binding protein (CREB)(202). Nuclear ERK can also lead to CREB phosphorylation through mitogen- and stress-activated protein kinase (MSK)(202). ERK phosphorylates a variety of additional targets, including ets-like gene1 (Elk1) shown here (133). Genes whose transcripts were found upregulated in this project include early growth response 1 (EGR1), neuronal activity-regulated pentraxin (NARP) and brain-derived neurotrophic factor (BDNF). EGR1 is an immediate early gene (IEG) rapidly induced after long-term potentiation(LTP)-inducing stimuli (204). EGR1 is a direct target for pCREB, since the EGR1 promoter contains binding sites for both Elk-1 and CREB, and both these proteins were found to be phosphorylated coincident with the onset of EGR1 expression after glutaminergic stimulation in vivo (150-152). Additionally, inhibition of the MAPK pathway prevents phosphorylation of both these proteins, prevented EGR1 induction and resulted in rapidly decaying LTP (133). EGR1 causes transcription of a variety of other mRNAs, many of which are involved in synaptic plasticity (138). The control of NARP transcription has not been well studied, but the NARP promoter contains a CREB binding site, and NARP is an IEG upregulated in neurons by physiologic synaptic activity and by BDNF-induced LTP (154,155). It is a pentameric protein secreted at synapses where it clusters AMPA receptors together and mediates synaptic refinement (205-207). BDNF

has been shown to have a functional CRE (153) and has long been known to play a role in synaptic plasticity and LTP (137,208-211). BDNF is secreted in an activity-dependent manner from both pre- and post-synaptic terminals and can bind to TrkB receptors on both surfaces, leading to further MAPK pathway activation and causing modulation of neurotransmitter signaling (212-216).

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ABSTRACT

THE INTERICTAL STATE IN EPILEPSY AND BEHAVIOR

by

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August 2010

Advisor: Dr. Jeffrey A. Loeb, M.D., Ph.D.

Major: Molecular Biology and Genetics

Degree: Doctor of Philosophy

Epilepsy is one of the most common neurological diseases, affecting up to 1% of the world population. Epilepsy remains poorly understood and there are currently no medications to cure it. Patients with epilepsy have both seizures as well as another type of abnormal activity between seizures, known as interictal spikes. Interictal spikes have thus far been poorly researched, yet growing evidence supports an important role for them in epilepsy. In this project, we first show the high variability between reviewers in marking interictal spikes on intracranial EEG, and then develop and test an automated detection method to solve this problem. Next, we use this automated detection algorithm to identify spikes on intracranial EEG in both tumor and non-tumor patients in order to determine the best spiking parameters to identify the seizure onset zone in each group. We then develop and characterize an animal model of chronic, neocortical interictal spiking to test our observations previously made in human epilepsy and to have a molecularly-accurate model on which to test new therapeutics. Finally, we show that interictal spikes are associated with behavioral changes in this animal model and that a

targeted inhibitor can both prevent the development of a spiking focus and normalize behavior.

AUTOBIOGRAPHICAL STATEMENT

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EDUCATION

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AWARDS/HONORS

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2008	Best Oral Presentation, Graduate Student Research Day, Wayne State University
2008	Epilepsy Foundation Pre-doctoral Fellowship
2007	Best Poster, Center for Molecular Medicine and Genetics Department Retreat, Wayne State University
2007	Best Oral Presentation, Medical Student Research Symposium, Wayne State University
2006	MD/PhD Preclinical Excellence Award, Wayne State University