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Linking Molecular, Electrical And Anatomical Properties Of Human Epileptic Brain

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LINKING MOLECULAR, ELECTRICAL AND ANATOMICAL PROPERTIES OF HUMAN EPILEPTIC BRAIN

by

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DEDICATION

This work is dedicated to my family: to my Father, who is the greatest inspiration in my life, for giving me strength and encouragement; to my Mother for her unconditional love and affection; to my loving Husband and my best friend for his endless support and understanding; to my doting brother for his friendship; to my son for making me smile even in the toughest times.

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CHAPTER 1

INTRODUCTION

Epilepsy is a chronic neurological disorder characterized by recurrent, unprovoked seizures. Any insult to the brain in the form of stroke, trauma, injury etc can develop into an epileptic focus over time (1). These foci develop abnormal and excessive hyper-synchronous neuronal activity which becomes rhythmic and spreads, and are called seizures. About 60% of seizures are convulsive, of which, two-thirds of the cases are partial idiopathic seizures and begin at the epileptic focus. The other one-third are generalized seizures usually involving larger areas of the brain and often leading to loss of consciousness. The remaining 40% are non-convulsive seizures. Clinical symptoms of epilepsy usually depend on the location of the epileptic focus, but can range from a brief loss of awareness (absence seizure) to as wild as a thrashing movement (tonic-clonic seizure). In rare cases, epilepsy can be fatal due to sustained, generalized convulsions.

Epilepsy affects about 65 million people globally (2) and about 1% of the population by the age of 20 (3). Epilepsy prevails irrespective of the geographic location, race or ethnicity of the patients (4). It is a life-long condition and is often debilitating, preventing the person from carrying out even day-to-day tasks such as driving alone (5).

Despite the severity, prevalence and the fact that this disease has been recognized for over a millennia, it is still one of the least understood disorders, especially with respect to the underlying causes of epilepsy. Inherited epilepsy has been associated mostly with single-gene defects in ion-channels and neurotransmitter receptors (6-8) but these mutations account for a very small percentage of epilepsy cases. In the most common form of epilepsy, seizures originate at an epileptic focus as a result of enhanced neuronal excitability which depolarize rhythmically.

As seizures spread through the brain, they become more generalized. While any brain lesion can develop into an epileptic focus, in many cases, there is no obvious brain pathology and the underlying cause of epilepsy remains unknown (9). The wide-range of causes and lack of distinctive pathology adds to the complexity of the disease and makes it difficult to treat.

Current anti-epileptic drugs were designed using animal models with acute seizures and therefore only target the symptom of seizures, without addressing the underlying abnormality (10). In most patients, seizures persist if the drug is discontinued, so the patients have to be on medication for the rest of their lives. There are several side-effects such as sedation, loss of cognitive function and in-coordination which make things worse for the patient. Even with the side effects, in almost 30% of epilepsy patients, the medication fails to completely reduce seizure frequency (11). The best available treatment for epilepsy is the surgical removal of epileptic brain tissue which suggests a clear malfunction of normal physiological pathways at or near the regions of brain producing seizures. This is the same hypothesis that led to the first surgical approach towards the treatment of epilepsy more than 100 years ago (12). In recent years, with advancement in electrical mapping and imaging techniques, doctors have been able to identify regions of abnormal brain activity more precisely for surgical resection, thus improving the outcomes of epilepsy surgery (13).

For a better understanding of the disease, there is a need for, a.) a clearer understanding of the molecular changes occurring in the regions of human brain that displays abnormal epileptic behavior, b.) newer approaches with minimum or no side effects that could potentially reverse the abnormal molecular changes and treat the disease itself rather than the symptoms alone, and c.) an animal model that mimics the human epileptic condition closely, to test the potential therapeutic targets.

Interictal Spiking in Epilepsy

Seizures are by far the most widely studied electrical abnormalities in epilepsy as observed in electroencephalographs (EEG) and electrocorticographs (ECoG). Seizures are also known as 'ictal discharges'. Careful study of brain electrical recordings have identified small, sharp abnormal wave forms occurring in between seizures referred to as 'interictal' discharges. The interictal discharges occur far more frequently and develop long before the actual seizures, and there has been a growing interest in the electrophysiological and molecular role of interictal spikes in epilepsy and seizures (11).

There has been a lot of controversy in the field of epilepsy over the relationship between ictal and interictal discharges. Several studies on human intracranial EEG recordings have reported high frequency of occurrence of interictal spikes in regions of seizure onset (14-21). It has also been reported that interictal spike frequency increases after a seizure rather than before a seizure as observed in cases of temporal lobe epilepsy (22-25). Some studies that have used interictal spike frequency as a measure to identify and surgically remove epileptic areas have also reported good surgical outcomes (17,26,27). Together these findings suggest an important but poorly understood role of interictal spikes in epilepsy.

Activity dependent molecular changes in epilepsy

An extraordinary feature of human nervous system is its ability to remodel itself in response to environmental stimuli, particularly in early stages of development and childhood. This remarkable property of human brain, referred to as 'plasticity' is critical to its normal development and function. Plasticity aids learning and memory and is likely to play a role in epileptogenesis. Activity-dependent changes in gene expression are the fundamental mechanism

by which synaptic activity is translated into structural changes and connectivity (28). In some systems it is thought to be achieved by the action of neurotransmitter receptors and voltage-gated ion channels by direct activation of synaptic genes (29,30). In others this is achieved by the release of neurotrophic factors which either directly alter synaptic plasticity or indirectly result in changes in gene expression.

Rakhade et al reported that expression level of a common group of genes, such as EGR1, EGR2, c-fos and MKP3 correlated precisely with interictal spike frequency (31). Even though no clear correlation could be established between activity-dependent gene expression and seizure frequency, the local neocortical areas of seizure onset were also identified as regions of highest interictal activity. Interictal spikes in these regions had remarkably higher amplitude than the neighboring 'control' areas. Activity-dependent genes, c-fos, EGR1, EGR2 are known to play important roles in synaptic reorganization in models of learning and memory. Thus, correlation between expression levels of these genes and interictal activity suggests that interictal spiking may be critical in maintaining the aberrant synaptic networks underlying epilepsy. Furthermore, despite the variation between patients such as cause, medication, age, ethnicity etc, we observe similar molecular changes, suggesting a common link between most focal, epileptic disorders.

In continuation with this work, Beaumont et al (2012) reported the underlying molecular changes in areas where seizures originated as compared to neighboring 'silent' regions from high-throughput gene expression arrays on regions of seizure onset. It is important to note that even though our primary experimental design was using seizure onset areas, the same areas also presented high interictal activity. The study identified 137 genes being differentially expressed in seizure onset areas in five patients (32). Ontology analysis of these genes implicated MAPK pathway, followed by CREB activation. About 40% of these genes were targets of activated

CREB, such as the Immediate Early Genes (EGR1, EGR2, BDNF etc) and other transcription factors. Further validation using western blotting for the signaling protein resulted in increased levels of dpERK1/2 and pCREB in epileptic regions. Similar patterns of increased gene expression of EGR1, EGR2, ARC and other CREB targets were observed in epileptic samples. To explore the spatial distribution of MAPK activation, immunohistochemical and in situ hybridization assays were performed immunohistochemical which suggested that MAPK/CREB activation was restricted only to superficial (layers 2/3) layers of the neocortex, whereas unphosphorylated CREB was expressed ubiquitously along all 6-layers of neocortex. Another interesting observation was the presence of distinct boundaries of activated MAPK/CREB which matched the anatomical structure of the cortex. Signal for activated ERK1/2 and CREB as maximal along the gyrus and terminated abruptly at the sulcal boundary. The studies also reported similar anatomical distribution of EGR1 and ARC from in-situ hybridization studies. This was particularly interesting as it suggested a role of intrinsic pattern of the human brain in the MAPK activation, especially at the gyral-sulcal interfaces.

From both these studies, it is evident that activity-dependent targets of CREB are activated in regions associated with both seizures and interictal spiking. This came as no surprise, because epilepsy is mainly a state of disrupted neural activity, and most genes that are differentially regulated in epileptic regions play an important role in synaptic plasticity. Since all the tissue used in these studies was in interictal state, it also suggests a possible role of interictal activity in maintaining high expression levels of these genes. However, one of the major limitations of studying human tissue is that it is almost impossible to infer the cause and effect. For this, we needed to develop an animal model of epilepsy that closely mimics the human epileptic condition, with both interictal activity and seizures.

Long-term monitoring studies in animal models that will eventually develop seizures demonstrate that interictal spikes are the first epileptic changes to appear in the neocortex after a localized insult to the brain (33). Barkmeier et al (2012) reported a successfully developed and characterized model of chronic, focal interictal activity in rats with a long latency period before the development of seizures, using tetanus toxin (34). Tetanus toxin was injected into the left somatosensory cortex of the rat brain, which served as a site of epileptic focus. With a latency period of 4-7 days where we did not find any abnormal brain activity, there was a steady increase in frequency of interictal activity for the next 4-6 weeks. Therefore, this model serves as a perfect parallel to focal epilepsy in humans and for the study of role of interictal spikes in epileptogenesis and seizures.

Electrical recordings over a period of 1-2 weeks in rats injected with tetanus toxin suggested that there is a steady increase in interictal spike frequency, which then spread out and cluster into smaller groups over time. There was no clear association of these interictal spikes with seizures in this animal model. But the most interesting aspect of this study was sustained CREB-activation in layers 2/3 of rat cortex, similar to the observations made in human neocortex with interictal activity (31,32). Persistent interictal activity for one week in tetanus-toxin injected rat was sufficient to induce CREB-activation, as well as induction of previously studied activity-dependent genes such as EGR1, EGR2, NARP, DUSP1 etc. Additionally, it was also reported that using a selective MAPKK (MEK) inhibitor to block MAPK activation, we can significantly reduce the development of interictal activity post tetanus-toxin injection. This finding raised the possibility that blocking MAPK/CREB pathway after an insult to the brain could prevent the synaptic remodeling that leads to interictal activity and therefore seizures. Since the drug used to

inhibit MAPK activation effectively reduced interictal spiking in the rat tetanus toxin model of epilepsy, suggests a more possible role as a post-trauma therapeutic.

Together, these studies suggest a close relationship between interictal activity and underlying molecular changes involving activation of MAPK/CREB pathway and activity-dependent genes. This implies an important role of interictal spiking in synaptic reorganization underlying epilepsy and raises the need for a deeper understanding of their molecular role in epilepsy. This can help us identify biomarkers of epilepsy and also design therapeutic drug targets which can be tested in our animal model.

Project overview

In this project, we aim to link the electrical properties of the human epileptic brain to the underlying molecular changes and the anatomical structure of the brain. We focus on the role of interictal activity in epilepsy and begin by describing the molecular changes underlying regions of high interictal activity in a larger set of patients (n = 20). With the increased number of patients we identify a set of genes with a much higher statistical significance and confidence as compared to previously identified 137 genes from a smaller group of patients. We demonstrate the molecular changes at both 'macroscopic' and 'microscopic' level. Studies carried out on whole tissue are critical in linking the molecular changes to electrical properties of the epileptic tissue. Further studies at microscopic level help us define the spatial relationships between the two. Our data suggests a strong association between the anatomical structure of the human neocortex and the underlying molecular changes. This led to our hypothesis about the role of intrinsic structure of the brain and molecular changes, in the spread of interictal activity. In the last part of the project, we will discuss the relationship between interictal spiking and the

anatomical structure of the brain and how the unique brain structure plays a role in the spread of interictal activity and thereby leads to development of generalized seizures from an epileptic focus.

CHAPTER 2

SPATIAL ORGANIZATION OF THE MAPK SIGNALING NETWORK IN HUMAN EPILEPTIC BRAIN

SUMMARY

Epilepsy is a disease of recurrent seizures; however, the exact mechanisms and networks that produce epileptic activities in the neocortex are not known. Surgical removal of the focal brain regions that display a variety of epileptic activities presents an excellent opportunity to discover the molecular and cellular basis of human epilepsy. We have taken a high-throughput genomics approach and identified a consistent group of differentially expressed genes that correspond to these epileptic regions. Highly represented in regions of high interictal activity are genes that implicate MAPK/CREB signaling, immediate early genes (IEGs), and synaptic plasticity genes. Bioinformatic analyses showed a number of clusters within the MAPK/CREB genes that include both activators and inhibitors of MAPK signaling, raising an important question as to why both activators and inhibitors are both induced. As a means to answer this, we mapped these activators and inhibitors in human epileptic neocortical foci using in situ hybridizations and immunolabeling. DUSP4, one of the most potent inhibitors of MAPK/CREB signaling, was significantly upregulated in high spiking areas. DUSP4 is a member of a family of dual specificity phosphatases and targets both isoforms of ERK1/2. In situ hybridization of serial brain sections shows that DUSP4 is expressed in discrete micro-domains in the superficial neocortical layers and is inversely related to expression of di-phosphoERK, phosphoCREB, EGR1, and DUSP6, suggesting that DUSP4 activation in regions of high interictal activity directly inhibits the spread of electrical activity in these focal regions. To test this, we developed an in vitro model using repeated depolarization of human neuronal Sy5Y cells to determine the mechanism of action of DUSP4. DUSP4 protein was induced within several hours of chronic

depolarization, irrespective of CREB activation. These studies utilizing high throughput genomic studies from human neocortex begin to define both the mechanistic and spatial roles of MAPK signaling in neocortical epilepsy and have the potential to produce novel therapeutics.

INTRODUCTION

Epilepsy is a disabling neurological disorder of recurrent seizures affecting 1% of the world's population (1). Although single-gene defects in ion channels or neurotransmitter receptors have been associated with certain inherited forms of epilepsy, these mutations do not account for the majority of patients with epilepsy (6-8). In most patients with partial epilepsy, seizures start in a focal brain region in response to a variety of insults to the brain (9). Neocortical epileptic foci show a similar electrophysiological pattern of localized, abnormal electrical discharges that can become rhythmic and spread across brain regions to produce clinical seizures. Most pharmacological treatments available for patients with epilepsy were not designed from an in-depth understanding of the disorder because of which there is currently no medication in the market that can effectively treat epilepsy. In the most intractable cases, surgical resection of the brain region where seizures originate is the most effective cure.

Seizures are not the only abnormal electrical discharges observed in the electrical recordings of patients with epilepsy. Between seizures, epileptic focal brain regions generate localized "interictal" discharges, which are small, sharp and far more frequent than seizures. Studies have shown that interictal discharges can be helpful in identifying regions of seizure onset or spread. Additionally, the fact that interictal spikes develop long before actual seizures may represent an important driving force in epileptogenesis.

Little is known of the spatial organization of cortical neurons that produce both interictal and ictal epileptic discharges, due in part to the complex structure and function of the neocortex. Moreover, there are no imaging methods or molecular markers that can reliably detect epileptic neurons. Molecular studies of brain tissue that has been surgically removed after 2-5 days of

EEG monitoring can greatly facilitate our ability to understand and treat human neocortical epilepsy.

Epilepsy surgery is usually a two-stage procedure. In the first stage of this procedure, the neurosurgeon places an evenly spaced grid of electrodes directly on the cortical surface. Electrical recordings are made for 2-5 days to identify regions of seizure onset and high interictal activity, which are then removed in the second stage. One of the most critical decisions in epilepsy surgery is to identify which tissue to remove to prevent the recurrence of seizures without destroying normal brain function. We collect the neocortical tissue that is removed in the surgery after informed consents from patients treated in the Comprehensive Epilepsy Program at the Harper Hospital and Children's Hospital of Michigan. This tissue is then mapped to its underlying electrical activity using intra-operative brain images, with and without the electrode grid. We need extreme precision in mapping the tissue to make sure we have accurate values for interictal activity for the tissue under study. Approximately, 1cm x 1cm piece of tissue is recovered underneath each electrode, which is processed carefully for various molecular studies.

Our initial studies in the lab were focused on comparing regions where seizures started to the nearby control regions of the epileptic brain. Microarray analysis of five patients, as described by Beaumont et al (2012), resulted in a set of 137 genes that were differentially expressed (32). These genes when analyzed using various gene ontology and pathway analysis tools such as Onto-tools and David, implicated MAPK/CREB signaling pathway and activation of IEGs and other transcription factors. About 40% of the differentially regulated genes were identified as targets of activated CREB from promoter analysis studies and play a role in synapse strengthening and plasticity. Additionally, activation of MAPK/CREB (ERK1/2 and CREB) and downstream CREB target genes (EGR1, ARC etc) was limited to the superficial layers (layers

2/3) of the gray matter only. This expression pattern also correlated with the anatomical structure of the neocortex with lack of signal at the sulcal boundaries. Similar findings were made for the downstream genes as well.

An important point to note here is that even though the above mentioned study was designed to look at molecular changes occurring in seizure onset (SO) areas, most of these SO areas were also identified as regions of high interictal activity. Rakhade *et al* (2007) also suggested a close correlation between activation of IEGs and interictal spike frequency. Similar observations on the role and importance of interictal spikes were also made by Dan Barkmeier in the rat tetanus-toxin model of epilepsy. In order to generate chronic interictal spiking in the animal model, a single injection of tetanus-toxin was given to the rats in their somatosensory cortex followed by long-term video EEG monitoring. We reported that interictal spiking was sufficient to generate similar patterns of MAPK/CREB activation in layers 2/3 and plasticity genes as observed in human epileptic samples. Furthermore, studies on the animal model also implied that inhibiting MAPK (ERK1/2) activation can effectively reduce the frequency of interictal activity (35). Together this data suggests a strong association between interictal activity and the underlying molecular changes as seen in both human high spiking samples and animal model of interictal spiking. Based on these observations, we were highly intrigued to further explore the role of interictal spiking in human epilepsy, both with respect to the underlying molecular changes and the anatomical structure and organization of the human brain.

In this chapter, we extend earlier studies to interictal spiking and their relationship to the underlying molecular changes both at macroscopic level as well as at the level of spatial distribution along the 6-layered neocortex. We confirmed the previous findings that a consistent group of genes implicating MAPK/CREB pathway, IEG activation and synaptic organization,

are induced at human epileptic foci, regardless of the underlying cause. We found activity-dependent expression of CREB target genes and their expression varies precisely with interictal rather than ictal activity. We extended the analysis of activated MAPK/CREB signaling pathway to both activating and inhibiting MAPK/CREB genes in the regions of high interictal activity, which corresponds to differences in their spatial distribution as observed from in situ hybridization (ISH). The most potent inhibitor of MAPK/CREB pathway, DUSP4 (Dual Specificity Phosphatase 4), which acts by inactivating both isoforms of ERK1/2, is significantly upregulated in high spiking areas. ISH studies demonstrated 'micro-domains' of activated DUSP4 in layers 2/3 of neocortex which were inversely related to expression of EGR1, a member of activating MAPK/CREB pathway. This inhibitory function of DUSP4 suggests a naturally occurring mechanism in the brain to block MAPK/CREB activation and a potential role in preventing the spread of interictal activity. This also raises further questions on the role of interictal state of the human neocortex, in particular, how persistent interictal activity is associated with gene expression changes both at macroscopic and microscopic level and how it produces regions of the brain that are prone to generating clinical seizures in patients with epilepsy. From this study, we have identified biomarkers of epilepsy including members of MAPK/CREB signaling cascade and their downstream targets and an important gene candidate for future therapeutic testing.

MATERIALS AND METHODS

Electrocorticography and tissue resection

Human brain tissue was removed as a part of planned surgery for medically intractable epilepsy. Informed consent was obtained through an approved IRB protocol at Wayne State University. Extreme care was observed to make sure our study did not affect the surgical decision-making. Fresh cortical tissue was rapidly collected from the surgeries of 20 patients with neocortical epilepsy. Tissue underlying each electrode represented unique electrical properties and was mapped precisely to the interictal spiking based on long-term electrocorticograms (ECoG) (Figure 1). To measure the state of interictal activity for each electrode, we used the interictal spike frequency value per 10 minutes. Data from three 10 minute ECoG segments for each patient were taken while the patient was awake and atleast six hours before a seizure, preferably from three different days. Our previously published MATLAB algorithm (36) was used to mark all interictal spikes and to calculate the total number of spikes occurring in each of the 10 minute ECoG file. The interictal spike frequency value for each patient was calculated by averaging the number of interictal spikes from all three 10 minute files.

Tissue fixation and RNA isolation

Brain tissue obtained from epilepsy surgery was carefully divided into two halves across the pial surface. One half was fixed in 4% paraformaldehyde for 48 hours followed by treatment in 30% sucrose for 24 hours before embedding into an OCT block, for histological studies. From the other half, only gray matter was isolated and frozen for further RNA and protein isolation.

Alternating strips of full-thickness (layers I-VI) gray matter alone from the neocortex were pooled to produce total RNA and protein for a given recording electrode location. Total

RNA was isolated from multiple high and low interictal spiking areas (used as an internal control) from each patient using the Qiagen RNeasy Lipid Tissue Kit.

Microarrays and Bioinformatics

Microarrays were performed on 20 patients (Table 1) with normal histology as observed from Hematoxylin and Eosin (H&E) staining. In order to have sufficient accuracy of differentially expressed genes we used a quadruplicate, flip-dye experimental design for each pair of samples for a given patient. Amino-allyl aRNAs were generated using the Epicenter protocol. These aRNA samples were labeled with Alexa dyes (Alexa-555 and Alexa-647) for co-hybridization to 60-mer Agilent whole human genome oligonucleotide arrays (catalog #G411A, Agilent) using a two-color dye-swap technique. Microarrays were scanned with an Agilent Technologies Microarray Scanner and the fluorescence signals were detected and calculated using Agilent's Feature Extraction software (V10. 3. 1).

For data analysis, the statistical software, R, was used to normalize the data for dye, slides, and technical replicates biases within a patient and finally across patients, followed by two-step mixed ANOVA. This design yields statistically powerful data for our further study.

For Ontological and Pathway analysis, we used multiple Ontology software, such as Onto-Tools, DAVID and Genomatix, and compared results across them. The key analysis software used was Pathway Express from Onto-Tools available at http://vortex.cs.wayne.edu/ontoexpress to identify biological pathways in the Kyoto Encyclopedia of Genes and Genomes.

Protein extraction and Western Blot

For tissue underlying a single electrode, alternating strips of frozen brain tissue (gray matter only; layers I-VI) were pooled. About 100mg brain tissue was suspended in 1ml homogenization buffer (320 mM Sucrose, 10 mM Tris-HCl, pH7.4, 10 mM $Na_4P_2O_7$, 10 mM NaF, 1 mM PMSF, 2 mM Na_3VO_4) with a cocktail of protease inhibitors (10mg/ml each Antipain, Aprotinin, Leupeptin and Peptistatin, Sigma) and homogenized using the Polytron for 15 seconds. Homogenates were centrifuged at low speed (2300rpm) for 10 minutes at 4°C. Supernatant was carefully collected in a separate mini eppendorf tube and the pellet was suspended in 500µl homogenization buffer without sucrose. The supernatant was centrifuged further at maximum speed for one hour at 4°C to isolate the membrane (pellet) and cytosolic (supernatant) fractions. The membrane fraction was suspended in 500µl of sucrose-free buffer. We used the Coomassie (Bradford) Assay to determine the protein concentrations in each fraction, using Bovine Serum Albumin (BSA) for the standard. Western blotting procedures were followed using a previously established protocol (37). 25µg of protein fraction was loaded onto each lane and subjected to SDS-PAGE, followed by transfer onto a PVDF membrane (Millipore). Membranes were incubated overnight in primary antibodies against diphospho-ERK1/2 (Cell Signaling), ERK1/2 (Cell Signaling), phospho-CREB (Cell Signaling), CREB (Cell Signaling), and alpha-tubulin (Santa Cruz) at 4°C. HRP-conjugated goat anti-mouse IgG or anti-rabbit IgG secondary antibodies were used and the signal was detected using ECL substrate (Thermo Scientific Super Signal West Pico Chemiluminescent Substrate). Since most of these proteins appear around similar molecular weight (42-44kDa), we used β-mercaptoethanol treatment along with 20% SDS and Tris-buffer for 30 minutes at 50°C to strip the membrane after each probing. Metamorph Image Analysis software was used for quantification.

Immunolabeling

For immuno-histochemical staining, paraformaldehyde fixed human neocortical tissue was cryosectioned at -22°C to obtain 20 µm thick sections. The protocol was carried out at room-temperature unless otherwise specified. Sections were equilibrated for 10 mins at room temperature (RT) and permeablized in 0.5% Triton X-100, 1X TBS for 1 hour, followed by 3 washings in 1X TBS for 5 minutes. Sections were then treated with 0.3% Hydrogen Peroxide/50% Methanol/1X TBS for 30 mins. Slides were again washed in 1X TBS and blocked in 0.05% Triton X-100/5% heat inactivated goat serum/1X TBS for 1 hour at RT. Primary antibodies against diphospho-ERK1/2 and phospho-CREB-S133 (Cell Signaling) were diluted in blocking buffer at optimum concentrations and applied overnight at 4°C. Sections were then washed and treated per the Vector Elite ABC kit for Rabbit IgG (Vector Laboratories) and developed with a DAB substrate (Sigma). Finally slides were dehydrated in an ethanol gradient followed by xylene and mounted using Cytoseal XYL. Digital images were captured using a Nikon Eclipse microscope fitted with an 8-bit digital camera.

in situ hybridization

We used 20 µm thick sections to examine spatial expression of genes of interest. Non-radioactive in *situ* hybridization was performed using Digoxigenin (Dig)-labeled RNA probes. Anti-sense and Sense Dig-labeled RNA probes were generated from whole plasmids containing the gene of interest (EGR1, DUSP4 and DUSP6) after restriction digestion with appropriate enzymes based on the construct and gene/site of insert. Linear plasmids obtained after restriction digestion were transcribed *in vitro* (Roche). Probes were hydrolyzed at 60°C using 60mM

Na_2CO_3 and 40 mM $NaHCO_3$, pH 10.2 for exact time as calculated from the below mentioned formula to obtain an optimum length of ~250 bp.

$$T_{\min} = \frac{Length_{initial} - Length_{desired}}{k(Length_{initial} \times Length_{desired})}$$

Hydrolyzed probes were purified on Sephadex G-50 spin columns. Purified probes were washed with 100% and 70% ethanol and then air-dried. Dried-up RNA pellet was dissolved in DEPC and stored at water at -20°C. These probes are good for about one year.

For in situ hybridization, sections were fixed again in 4% paraformaldehyde (PFA) at RT for 10 minutes. After three washes in 1x PBST (1x PBS + 0.1% Triton X-100), 5 minutes each wash, sections were treated with 1 µg/ml proteinase K (Sigma) for 10 minutes at RT. After washing the sections again, and re-fixation in 4% PFA, sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0 for 15 min. After washing the sections, they were incubated in pre-hybridization buffer (10mM Tris-HCl pH 7.5, 600mM NaCl, 1mM 0.5% EDTA, 25% SDS, 1x Dendhardt's solution,50% Formamide and 300µg/ml yeast tRNA in DEPC Water) for 4 hours at 60°C. The sections were then incubated with 2 µg/ml probes in hybridization buffer (20mM Tris-HCl pH 7.5, 0.3M NaCl, 5mM EDTA, 1x Denhardt's solution (Sigma), 50% formamide, 500µg/ml yeast tRNA, 10% Dextran Sulphate, 10 mM $NaPO_4$ in DEPC water) at 60°C overnight. After hybridization, the sections were first washed in 1x SSC containing 50% formamide for 10 minutes at 65°C. To remove excess unhybridized RNA, the sections were treated with 20 µg/ml RNase A (Sigma) in TNE buffer (10 mM Tris-HCl pH 7.5, 0.5 M NaCl, 1 mM EDTA), at 37°C for 30 min followed by sequential washes in 2x, 0.2x and 0.2x SSC washes, 20 minutes each at 65°C. The sections were then processed similar to immunohistochemistry except that here we used Roche Washing/Blocking/Detection kit. Sections are incubated at 4°C in sheep anti-DIG conjugated with alkaline phosphatase (Roche)

overnight and next day the signal is detected using BM Purple (nitroblue-tetrazolium-chloride (NBT)/5- bromo-4-chlor-indolyl-phosphate) as a substrate. Once the signal developed, slides were washed and processed in the same manner as in immunohistochemistry.

RESULTS

Transcriptional profile of high interictal spiking regions of the human epileptic brain

Gene expression microarrays were performed on twenty patients comparing regions of high and low interictal activity (Table 1). For each patient we had a unique quadruplicate design with dye-swaps. Data analysis, has revealed 1280 unique genes that are differentially regulated with a fold-change > 1.4 and FDR 5%. The twenty patient dataset has resulted in a much stronger group of genes with high confidence and statistical power. Pathway analysis of all the 1280 differentially regulated genes using Onto Tools - Pathway Express software implicated MAPK activation in regions of high interictal activity, in addition to other less significant pathways. A large percentage of these genes were targets of activated CREB including Immediate Early Genes (IEGs) and other transcriptional factors playing a role in synaptic strengthening and plasticity. These results was similar to our previous findings (31,38), but with increased number of patients, we have a larger set of differentially expressed genes with the same statistical cut-offs as compared to initial 137 genes identified from five patients.

Analysis of all the differentially regulated genes, using KEGG, identified 199 genes that are direct members of MAPK pathway out of the total of 1280 differentially expressed genes. Several of these are genes that are targets of activated CREB and are downstream of MAPK/CREB pathway, such as the IEGs (EGR1, EGR2 etc). Figure 2 gives an extensive layout of MAPK pathway as obtained from KEGG. The genes that are differentially regulated in the high spiking samples were identified and superimposed onto the existing pathway. The green boxes indicate genes that are members of activated MAPK pathway, whereas the red ones (DUSPs and HSP) are direct inhibitors of MAPK.

MAPK pathway is tightly regulated by coordinate action of kinases and phosphatases. In the high spiking regions of the epileptic brain, we identified both activators and inhibitors of MAPK pathway being upregulated. Dual-specificity phosphatases (DUSPs) comprises of a large family of catalytically active enzymes which have a direct or 'atypical' (complex) relationship with MAPK activation (39). MKPs (MAP kinase phosphatase) represent a distinct subfamily within DUSPs that play a critical role in inactivating MAPK signaling cascade by dephosphorylating both serine/threonine and tyrosine residues on various MAPK (ERK1/2, p38 etc) (40-42). Some of the DUSPs, including DUSP1, DUSP4, DUSP5, and DUSP6 etc. appeared to be highly upregulated in the high spiking samples. DUSP4 was highly and significantly upregulated in the high spiking regions consistently across all twenty patients. DUSP4 is the most potent inhibitor of MAPK (43-45) and acts by inhibiting (de-phosphorylating) both isoforms of ERK1/2. It is previously known that DUSP4 itself is stabilized by ERK1/2 via phosphorylation of its serine residues at C terminus (46). Therefore, when levels of ERK1/2 reach a certain threshold, they phosphorylated and stabilize DUSP4, which in-turn dephosphorylates ERK1/2. When levels of ERK1/2 decrease, DUSP4 is no longer phosphorylated and is prone to degradation, establishing a feed-back loop between the kinase and phosphatase.

Co-expressed MAPK Signaling Genes Network

Upregulation of DUSP4 was particularly interesting because it raised a major question, "Why are both activators and inhibitors induced in high spiking regions of neocortex?" To understand how MAPK genes, in particular, the phosphatase DUSP4 and members of activating MAPK pathway such as EGR1, EGR2, DUSP6 etc, correlate with each other we generated a

molecular map using the normalized fluorescence intensities for each of these genes in high and low spiking samples from 15 patients. The Pearson correlations between all the genes were calculated using MATLAB or R resulting in a correlation matrix. For visual analysis, all the genes which correlated at R>0.80 were plotted in the form of a network layout using Cytoscape software (V 2.8). The network for differentially regulated MAPK genes (R>0.80) resulted in a closer association between a smaller set of MAPK genes as compared to other MAPK members. The biolayout is weighted for the pearson correlation between the genes, therefore, the distance between the genes is directly proportional to the correlation between the two. Closer the genes to each other, more strongly they correlate. While EGR1, EGR2 and some other DUSPs such as DUSP5, DUSP6 clustered together tightly (R>0.95), DUSP4 correlated loosely with these genes at R>0.80 (Figure 3). This suggested that even though DUSP4 and activating MAPK genes are up-regulated at the macroscopic level, there might be some differences in their expression pattern spatially.

Activity-dependent transcription of MAPK/CREB pathway

Previous studies in our lab, as well as my current study using high throughput genomics suggested activation of IEGs and other transcription factors in regions of high interictal activity. Most of these genes are targets of activated CREB, such as the early-growth response 1 (EGR1), EGR2 and some the DUSPs such as DUSP4, 5 and 6. A large subgroup of these genes is transcribed by activated CREB and represent activated MAPK/CREB pathway. A smaller set of genes (mostly phosphatases) are classified as inhibitors of MAPK/CREB pathway because they play a critical role in inactivating various kinases within MAPK/CREB pathway.

We selected key members of MAPK/CREB pathway as activators of the pathway, including EGR1, EGR2, DUSP1, DUSP5 and DUSP6. We examined the level of these genes as compared to DUSP4 in multiple regions of varying interictal activity using real time PCR in eight patients. Gene expression profiles with respect to increasing spike frequency resulted in two distinct group of patients (n = 8, Figure 4A). In five out of eight patients, we observed an initial increase in gene expression with increasing spike frequency with an inflection to lowest gene levels in the highest spiking sample. Three out of the eight patients resulted in an opposite pattern of inflection, where the medium spiking sample had lowest gene level.

The correlation matrix for EGR1, EGR2, DUSP1, 4, 5 and 6 is shown in Figure 4B. The last in the matrix corresponds to correlation between spike frequency and gene expression. All the genes correlate positively with each other as evident from real-time PCR data. The correlations between these genes ranges from R = 0.56 to as high as R = 0.92. On the other hand, neither of the genes shows any correlation with interictal spike frequency, suggesting spike frequency may not be the best measure to study underlying gene expression changes. Together this data suggests that even though EGRs and DUSPs have opposite molecular functions in the regulation of MAPK pathway, they all 'travel' together in brain regions of varying interictal spiking.

Activity-dependent of induction of MAPK/CREB pathway

We extended the gene expression studies to study the levels of activated and inactivated MAPK (ERK1/2) and CREB proteins in regions of varying interictal spiking. Extracellular signal regulated kinases (ERK, isoforms 1 & 2; also known as MAPK) is a MAPK, a key member of the whole signaling pathway and a direct target of DUSP4. Activated ERK1/2

directly and indirectly (via p90-RSK) phosphorylates CREB, which is also implicated in our microarrays results. Figure 5A presents the quantification of western blot data for five patients showing levels of activated and inactivated ERK and CREB. Phosphorylated proteins (diphospho-ERK1/2 and pCREB were normalized to their inactivated counter-proteins (ERK1/2 and CREB).

Western blotting data resulted in extremely high variability in the levels of diphospho-ERK1/2 and phospho-CREB when normalized to their inactivated counterparts (ERK1/2 and CREB respectively). Phosphorylation is a transient state and our data clearly suggests high lability and dynamics of MAPK/CREB signaling pathway. Figure 5B depicts a correlation matrix for activated ERK1/2 and CREB and how they correlate with interictal spike frequency. dpERK1/2 and pCREB correlate positively but weakly (R = 0.58) but do not show any correlation with spike frequency. It would be important to note that spike frequency value is calculated as average number of spikes per ten minutes for approximately 1cm x 1cm region of the epileptic brain. Western blotting may not be the best technique to identify microscopic differences in MAPK/CREB signaling proteins from the analysis of whole gray matter tissue.

Spatial distribution of MAPK/CREB pathway in epileptic neocortex

Human neocortex comprises mainly of gray matter, has a distinct folding pattern of gyri and sulci, and a six-layer laminar structure. Each of the six layers has its unique cell type and neuronal connections.

Previously published studies from our lab indicate that the MAPK/CREB changes occur strictly in the superficial layers (layers 2/3) of the neocortex as seen from histochemical staining for diphospho-ERK and phospho-CREB. Similar pattern of expression was observed for the

IEGs, EGR1 and ARC. In addition to layer-specific induction, the MAPK/CREB and gene-expression changes also corresponded with the anatomical structure of the brain. Maximal expression was observed along the gyrus but the signal for activated ERK and CREB diminished along the sulcus. We confirmed these findings on multiple tissue samples across several patients. There appears to be a direct correlation between the molecular boundaries and anatomical structure of the brain.

To extend these studies, we examined the spatial distribution of the members of MAPK/CREB pathway both at gene and protein level on 20µm thick serial tissue sections fixed in 4% paraformaldehyde. Histochemical analysis of medium/high spiking regions for activated ERK1/2 and CREB resulted in distinct regions in the layers 2/3 of the neocortex where the signal for the activated proteins was diminished, even on the same gyrus (Figure 6). Thus, irrespective of the anatomical location, we discovered spatial differences in expression of MAPK/CREB proteins. These differences can be attributed to the underlying electrical properties of the tissue at higher resolution.

Further, we investigated the gene expression patterns of activators and inhibitors of MAPK including EGR1, DUSP6 and DUSP4 using in-situ hybridization on medium/high spiking tissue. Interestingly, data from ISH depicted an inverse pattern of expression of inhibitor DUSP4 to the MAPK/CREB activating genes, EGR1 and DUSP6 (Figure 7). In six out of eight patients, we found maximal expression of DUSP4 in regions where EGR1 and DUSP6 were completely shut-off. GAPDH was used as a positive control and was ubiquitously expressed along all six-layers of the neocortex. The tissue section in Figure 7B was particularly interesting, because on a single brain section representing two electrodes, we see three regions that show the distinct inverse relationship between DUSP4 and EGR1/DUSP6. This was incredible and

presented a clear inhibitory role of DUSP4 in activating MAPK/CREB pathway in small regions of the neocortex. Some patients also exhibited a subtle inverse relationship between DUSP4 and its direct target diphospho-ERK1/2 (data not shown). We observed lack of signal of diphospho-ERK1/2 around the region where DUSP4 was maximally expressed and vice-versa. It would be imperative to analyze additional tissue sections to confirm this subtle pattern of inverse spatial relationship between DUSP4 and dpERK1/2, which has been observed in three patients so far.

DISCUSSION

High-throughput gene expression studies were performed on a large set of patients (n = 20) comparing regions of high interictal activity to neighboring low activity regions. These near-by regions served as perfect internal 'controls' for each patient. We can confidently rule out any differences in gene expression resulting from genetic or ethnic variability which can be a major concern when using 'control' tissue from a non-epileptic patient undergoing brain surgery or from an autopsy. The patients have a general clinical diagnosis including various pathological conditions, but the tissue samples used in the project are histologically normal as verified from hematoxylin and eosin staining. Thus, the common underlying pathology for all the patient samples in this study is epilepsy.

Consistent with our previous findings, we reported induction of MAPK/CREB pathway in regions mapped to high interictal spiking from ECoG. Since all the MAPK genes identified in the initial seizure onset studies are also implicated from the recent interictal spiking studies, it is safe to conclude that MAPK/CREB changes are more loosely associated with interictal activity rather than seizures. Common underlying molecular changes in seizures and spiking are suggestive of an important role of interictal activity in the pathogenesis in epilepsy.

With a statistically strong dataset generated from the high-throughput studies on twenty patients, we identified 199 genes within the MAPK pathway as differentially regulated in epileptic brain out of a total of ~450 MAPK members. This was a significantly high number of genes and strongly implicated role of MAPK/CREB changes in tissue pathology. Within the MAPK/CREB pathway we identified activation of both activators and inhibitors, specifically DUSP4 which functions as the most potent inhibitor of MAPK (ERK1/2). The fact that MAPK is

persistently induced in epileptic brain and that DUSP4 functions as a direct inhibitor of ERK1/2, makes DUSP4 a great target for therapeutic testing in the animal model of epilepsy.

Careful study of epileptic tissue at both macroscopic and microscopic levels provided insight on the underlying molecular mechanisms and how they relate to the electrical and anatomical properties of the neocortex. Microarrays and real-time PCR studies suggested a positive correlation between DUSP4 and activating MAPK genes such as EGR1, EGR2, DUSP1, DUSP5 and DUSP6 at different spike frequencies despite their opposing functional properties. Further analysis of the activators and inhibitors of MAPK/CREB signaling pathway at microscopic level suggested a distinct inverse spatial relationship between the two. In-situ hybridization studies to analyze the gene-expression patterns along the 6-layered neocortex resulted in maximal signal of DUSP4 in regions where there was lack of EGR1 and DUSP6 (n = 6). Comparative studies between histochemistry and in-situ implicated subtle differences in the spatial distribution of DUSP4 and activated ERK1/2. Signal for activated ERK/2 diminished around the region of activated DUSP4 supporting the fact that DUSP4 directly inhibits ERK1/2 activation.

Our current model of molecular mechanisms underlying epilepsy describes presence of 'micro-domains' of activated DUSP4 along the superficial layers (layers 2/3) of the neocortex. These micro-domains act like 'zones of inhibition' and prevent activation of MAPK/CREB members within them. This could possibly be how DUSP4 also prevents the spread of MAPK/CREB changes and thereby interictal activity beyond their 'inhibitory pockets' (Figure 8).

As a next step, it would be crucial to study the mechanism of DUSP4 as an inhibitory molecule in the spread of molecular changes and thereby interictal activity. Repeated

depolarization of semi-neuronal Sy5Y cells with KCl produces a rapid activation of the MAPK, ERK, CREB, and EGR1 and closely mimics the patterns we see in high-spiking human neocortex. The Sy5Y cells serve as great model for the study of underlying mechanisms in seizures and epilepsy, particularly because we can study the effects of over-expression and knock-down of genes of interest in a system that most closely represents a model of sustained-activity like the epileptic neocortex in human cells.

From our high-throughput and molecular studies of epileptic brain samples, DUSP4 remains to be of key interest as a therapeutic target in the treatment of epilepsy. Therefore, we started off with a series of experiments to study the role of DUSP4 in epileptic brain's natural defense mechanism. Some of the preliminary findings from the in vitro studies implicate DUSP4 activation only in the Sy5Y cells repeatedly depolarized with KCl, and not in the control cells (serum-containing media change only), even though diphospho-ERK1/2 was induced in cells depolarized with KCl as well as cells undergoing serum containing media change. Additionally, we have some preliminary data on the effects of over-expression of DUSP4 on depolarization. Interestingly our data suggests a decreased diphospho-ERK1/2 induction in cells over-expressed with DUSP4 as compared to the control cells. These preliminary experiments also indicate a high therapeutic potential of DUSP4 and extended studies on the in vitro model would be highly crucial towards the understanding and mechanism of DUSP4 as an inhibitory agent in MAPK activation and spread of interictal activity.

Taken together, our extensive molecular studies present DUSP4 as one of the most important molecules in epilepsy with a potential therapeutic function. According to our hypothesis, DUSP4 plays an essential role in inhibiting the spread of interictal activity along the neocortex by increased expression in specific domains in the superficial cortical layers. The rat

tetanus-toxin model of interictal spiking and epilepsy closely parallels the layer-specific molecular (MAPK) changes observed in humans. The model is progressive over both time and space and the frequency of interictal activity is stable enough to study both reversal of established interictal spiking and prevention of epileptogenesis after injury (34,35). This serves as a perfect model to study the therapeutic role of DUSP4 in inhibiting MAPK as a means to prevent or modify epilepsy. An exciting approach would be to over-express DUSP4 using lentiviral vectors (47-49) in focal brain regions of the tetanus-toxin injected rats and study the effects on MAPK activation and epileptogenesis.

Even though gene therapy is still in its infancy in epilepsy, having a strong biomarker candidate with great therapeutic potential can be advantageous. Several preclinical studies of viral gene therapy for various kinds of epilepsy have been conducted over the past decade with promising results (50-54). DUSP4 is a strong and consistent biomarker of neocortical epilepsy and holds great potential for future gene therapy testing for region-specific inhibition of neuronal and circuit excitability.

CHAPTER 3

DISTINCT PATTERNS AND VELOCITIES OF HUMAN INTERICTAL SPIKE PROPAGATION THROUGH CORTICAL AND SUB-CORTICAL PATHWAYS

SUMMARY

Interictal spiking occurs far more frequently than seizures, however, the mechanism of spike generation and spread as well as its relationship to seizures remains poorly understood. Here, we have developed a software to quantify both the timing and spatial spread of intracranial spikes on long-term subdural electrographical recordings. We found that spikes occur in multiple channels of the subdural electrode grid within a hundred milliseconds and used these events to study the spatial and temporal patterns of interictal spike spread across cortical sites. Using a previously established interictal spike detection algorithm, we identified spikes occurring within 100 ms of each other on all channels of the electrode grid. Three different patterns of spike propagation were observed including 1) spread to electrodes on the same gyrus, 2) spread to neighboring electrodes but separated by a sulcus, and 3) Spread to distant electrodes (separated by multiple sulci). Interestingly, we found that the velocity of spread to distant electrodes was much higher compared to spikes that spread to adjacent electrodes, often on the same gyrus. This suggests at least two distinct conduction velocities through grey matter (slow) and white matter (fast). Using the datasets from five patients, we developed an equation to predict the pathway of spike propagation that incorporates both components of gray matter and white matter conduction for a given spike. These studies help our understanding of neocortical epilepsy and warrant further studies to determine whether patterns of interictal spike propagation could possibly play a role in surgical decision-making and improve epilepsy surgery outcomes.

INTRODUCTION

Synchronization among neurons is critical for normal brain function and information processing (55). It is becoming clear that both spatial and temporal components of neuronal firing are equally critical for normal physiological network functions (56). Disruption of normal synchrony between the neurons is visible in the electrical recordings in the form of seizures, interictal discharges or any other abnormal waveforms at both spatial and temporal level.

Interictal spikes are small sharp discharges that occur in between seizures, hence given the name 'interical'. They are non-repetitive discharges, comprised of brief synchronous firing of a population of neurons. Although the key feature of an epileptic brain is its ability to seize, studies have reported an equally characteristic role of interictal spikes (57,58). Interictal spikes can be described as the electrographic hallmark of epilepsy. They occur far more frequently than seizures and their relationship to seizures is highly controversial (33). Seizure onset zones are often the areas of highest interictal activity, but not always. Some studies on scalp EEG of epilepsy patients have suggested occurrence of spikes in regions with high probability for spontaneous seizures in neocortical epilepsy (59,60). Others report no spiking in scalp EEGs of human subjects with increased risk for provoked seizures (seizures evoked by stimulation), for instance in cases of drug overdose of alcoholism or in patients with complex febrile seizures (61,62). Even though interictal spikes develop long before seizures (33), some studies have reported an increase in interictal spike frequency after a seizure rather than before a seizure (63). Epileptologists believe that apart from seizures, a better understanding of interictal spikes can also provide crucial information on disease physiology and pathology (64).

In our lab, we have been extremely interested in both molecular and anatomical role of interictal spikes in epilepsy. Molecular studies in our lab on human brain samples exhibiting high

interictal activity implicate a strong association between spiking and gene-expression changes specifically in the gray matter region of the neocortex. Similar molecular changes were also observed in regions of 'seizure onset' as reported by Beaumont et al (2012), and in the rat tetanus-toxin model of interictal spiking and epilepsy (35). Together, our previous and current studies suggest common molecular changes, i.e. activation of MAPK/CREB pathway and induction of IEGs and other transcription factors playing a role in synaptic strengthening and plasticity in regions of high interictal activity. Barkmeier et al (2012) have also reported that by blocking MAPK/CREB pathway, we can effectively prevent development of interictal spiking in the rats after tetanus toxin injections. This data also advocates a direct relationship between MAPK/CREB pathway and interictal spikes. Despite such in-depth understanding of molecular changes associated with interictal spiking, we are still unclear of the relationship between spiking and the anatomical structure and organization of the epileptic brain.

Epileptologists have been using interictal spikes as a means to diagnose epilepsy for decades now, but interictal spikes are still not considered as a reliable marker in locating seizure focus. This lack of reliability of interictal spikes as a strong diagnostic feature can be attributed to their tendency of propagation from their initial site of origin. Several studies reported from both in vivo intracortical recordings in rabbits, and in vitro slice recordings in guinea pigs and rats have identified distinct pathways for generation and propagation of spikes in the animal models of epilepsy (65-67), but the spatial-temporal properties of interictal spikes in human neocortical epilepsy still remain unknown.

Our lab has focused on the role of seizures and interictal discharges and various parameters associated with them in the initiation and progression of epilepsy in human brain for several years now. From careful review of the electrical recordings over past several years,

interictal spike spread has been one of the most common features we have observed consistently across several patients. Preliminary observations such as high interictal activity in distant regions of the epileptic brain intrigued us about the possible spread of interictal activity in these regions through cortical or sub cortical networks. According to our hypothesis, the spread of interictal spikes is largely associated with the anatomical structure and organization of the human brain. Interictal spikes possibly play an important role, not only in generating seizures but also in their spread, by creating network connections where a small neuronal population repeatedly communicates with several other neuronal populations in different parts of the brain, eventually making them hyper-excitable and creating new epileptic foci. In this chapter, we will explore the spatio-temporal relationships between interictal spikes. We will start by addressing the spatial spread of interictal spikes along the neocortex and move on to their temporal properties with respect to their anatomical locations.

MATERIALS AND METHODS

Patient selection

A total of eight patients with neocortical epilepsy who underwent cortical resection for treatment of uncontrolled seizures in Children's Hospital of Michigan or Harper University Hospital were selected for this study. Three of these eight patients were excluded because they did not present enough spike propagation events. We had a mix of both children (age 2-7 years) and adults (age 23 and 56) as well as both males and females. All these patients underwent scalp video-EEG monitoring, MRI and several other medical tests as a part of their pre-surgical evaluation. Based on this, prolonged intracranial ECoG monitoring with subdural electrodes was carried out for two to five days as the first step of epilepsy surgery.

In this first stage of the procedure, multiple electrode grids with 10mm inter-contact distance between the electrodes of each grid were surgically implanted directly on the cortical surface. Total number of electrodes placed on each patient ranged from 64 to 128. Electrode grids were placed covering mostly frontal parietal region of the cortex.

ECoG recording and analysis

Intracranial recordings were obtained with a 128-channel Stellate Harmonie digital recorder (Stellate Inc., Montreal, PQ, Canada) sampled at 1000Hz. For each patient, three 10-minute ECoG segments were selected from periods of quiet wakefulness at least six hours away from a seizure. Using a previously established and validated algorithm all three ECoG recordings for each patient were marked to identify interictal spikes (36).

To study spike propagation, smaller, couple of seconds long, segments of the ECoG that showed patterns of spike propagation, i.e. spikes in more than four channels within 100

milliseconds, were isolated from the whole 10-minute recordings. These were then analyzed for a.) order of electrodes in which the spikes occur, and b.) time between occurrence of spikes using a custom MATLAB algorithm. Almost 200 segments (< 1 second long) consisting of obvious patterns of interictal spike spread were selected from two and a half hours of ECoG recordings across five patients (30 minute recordings each). Each individual spike propagation pattern included between five to fifteen electrodes within less than a hundred milliseconds. Based on this observation, we set the time-limit for the spike propagation as 100 milliseconds. We successfully validated later that by choosing 100 milliseconds as our time-limit, we did not lose any spikes within a given set of spike propagation.

From the 200 spike propagation events, using a MATLAB script, we identified a total of 1988 unique spikes that were propagating from one particular electrode to the other. The MATLAB code also provided data on the number of times we observed spike propagation between the two unique electrodes. Only electrodes that had three or more spike propagation events within the 30-minute ECoG were used for further analysis. The same analysis was carried out using propagations between three unique electrodes.

3D brain rendering and mapping

A 3D rendering software developed by our collaborators, Dr Jing Hua, Dr Darshan Pai and Hae Kim in Computer Science Department, Wayne State University was used to generate patient-specific 3D brain renderings mapped with intracranial electrodes. Cortical surfaces were generated from preoperative volumetric T1- weighted MRI images using the automated cortical surface reconstruction software (BrainSuite09, http://neuroimage.usc.edu/neuro/BrainSuite). Post-implantation skull x-rays and/or CT scans were co-registered with the pre-implantation

MRI to localize precisely the intracranial electrodes on the patient's 3D-brain reconstruction. In all cases, accuracy of electrode localization was manually confirmed by matching gyral patterns between intraoperative photographs and 3D-reconstructions.

To calculate the geodesic distance, a smooth representation of the brain was generated by using a roller-ball algorithm, which removes all the sulcal folds that are present. The smooth surface is re-meshed to a very high resolution, ensuring that the geodesic distance calculation results in a line that is not aliased. To calculate the geodesic line between any two points on the surface we use Dijkstra's shortest path algorithm. The algorithm treats the mesh as a graph and selects the set of edges that result in the direct shortest path between the two surface points. This is used as the geodesic distance between the two electrodes.

From time and geodesic distance values, velocity of spike propagation was calculated. Velocity of propagation (mm/msec) was plotted against the geodesic distance (mm) for each spike propagation.

RESULTS

Distinct patterns of interictal spike propagation across the neocortex

We identified segments of electrocorticographic recordings from subdural electrodes arrays on human epileptic neocortex which exhibit interictal epileptic discharges occurring across multiple electrodes within hundred milliseconds. Figure 1A shows an example of one such event. These were then analyzed for their spatial spread across the electrode grid using the MATLAB algorithm. Finally, the order in which spikes occur, and the direction of propagation were mapped onto the patient-specific 3D brain rendering for visual analysis (Figure 1B).

We discovered different patterns of interictal spike spread including, a.) spread to adjacent electrodes located on the same gyrus, b.) adjacent electrodes separated by a sulcus and c.) distant electrodes separated by several sulci as shown by different colors in Figure 1b.

Identifying a true sulcus based on cortical thickness

The 3D brain rendering obtained from MRI images generates a shading pattern on a gray scale in the regions of sulcus. The thickness and shading depends upon the depth of the sulcus and helps us identify a sulcus which can otherwise be challenging from 2D intra-operative images alone. However, in some cases, 3D brain renderings may not have high enough resolution especially in the parietal region based on the quality of MRI, making it hard to identify a true sulcus from 3D brain rendering alone. It is well established that the normal cortical thickness along the gyrus is higher as compared to the thickness along the sulcus. Using this information, we generated a plot representing how cortical thickness changes between two electrodes using MRI data to calculate gray matter thickness perpendicular to the point of interest along the surface (Figure 1D). Thinning of the cortex between two electrodes was used to

identify presence of a true sulcus between them and also to distinguish the presence of a sulcus from regular folding of the brain.

Interictal spikes favor a certain direction of propagation more than the other

Further study of patterns of spike spread resulted in events where there appeared to be a cross-talk between a smaller set of electrodes. Interictal spikes seem to propagate in multiple directions between a smaller set of electrodes within a few milliseconds. We isolated all the spike propagation events occurring between a unique set of three electrodes. Figure 2 represents an example of spike propagation occurring between Electrodes A, B and C. Top panel is mapped with spikes initiating at Electrode A, spreading to B or C, middle panel spikes propagating from Electrode C and bottom panel shows spikes that first appear at Electrode B. The arrows indicate the direction of propagation and thickness of the line is representative of the number of times spikes propagate in that direction. Clearly, there are more spikes that originate at Electrode A and spread to B and C, as compared to spikes spreading from Electrodes B or C. This data suggests that interictal spikes favor one particular order of propagation more than the other.

Spikes propagate faster to distant electrodes

Spatial distribution and spread of interictal spikes resulted in distinct patterns of interictal spike spread between adjacent and distant electrodes. To extend these observations, we examined the temporal properties of interictal spike spread. We extracted the information on time of occurrence of spikes from the Stellate Harmonie software using a custom-written MATLAB code to calculate time differences between the spikes as they propagate. With the help of our collaborators, Dr Jing Hua, Darshan Pai and Hae Kim, geodesic distance and shortest

linear distance between any two electrodes was calculated from the MRI renderings. The velocities of interictal spike propagation were calculated from both geodesic distance and linear distance and time. The graph between velocity and distance were plotted to analyze the rate of spread of interictal activity between different patterns of propagation. Figure 3 depicts plots of velocity against geodesic distance for three different patients. It was evident from this data that the velocity of spike propagation was much higher for distant electrodes than adjacent electrodes, suggesting a faster mode of propagation, possibly through the white matter. The same pattern of increase in rate of propagation with increase in distance was observed for all five patients.

Additionally, the group of spikes propagating between adjacent electrodes separated by a sulcus could be divided into two distinct subgroups. One subgroup of spikes perfectly overlaps with the spike propagation events occurring between electrodes on same gyrus, whereas the other subgroup overlaps with spikes propagating to distant electrodes. This observation also implies two distinct modes of propagation.

DISCUSSION

In the course of this project, we analyzed more than 2000 spike propagation events from five patients, including three distinct patterns of propagation, i.e. spikes propagating between adjacent electrodes on the same or different gyri and spikes propagating to distant electrodes. The spikes tend to spread to distant parts of the brain as commonly as they spread to neighboring areas, suggesting a cross-talk between the neuronal populations in different regions. The neuronal connectivity between distant regions of the brain may be crucial to the development of multiple epileptic foci that eventually produce seizures. From the patterns of spike spread on ECoG alone, it can be hard to determine whether a single spike is spreading from one electrode to the other, or a single electrode is generating multiple time-lagged spikes which spread to multiple electrodes within the selected time interval. Another possibility could be a third unknown source spreading out spikes to multiple electrodes at the same time. The fact that interictal spikes spread multiple times between any given set of electrodes in the 30-minute recording suggests that it is more than random chance that we observe that pattern of spike propagation. For our analysis, we excluded any events that occurred only twice or less within the 30-minute EEG.

Our current model of spike propagation (Figure 4) suggests a faster white matter propagation of interictal spikes to distant electrodes (separated by several sulci) as compared to a slower gray matter propagation occurring between adjacent electrodes mostly on the same gyrus. Together with these findings, we came up with an equation presenting two major components of spike propagation, i.e. propagation through gray matter and propagation through white matter. For any given spike, total time of propagation can be divided into time it travels through gray matter plus the time travelled through white matter. According to our hypothesis, spikes that

spread to neighboring electrodes on same gyrus, most likely have only the gray matter component of propagation due to their proximity, whereas, spikes traveling to distant electrodes will have a major component of white matter propagation due to their speed and distance. Average velocity for spikes spreading to adjacent electrodes on same gyrus was noted to be around 7 m/sec from our analysis, whereas velocity distribution for distant electrodes ranges from 15m/sec to as high as 40 m/sec, thereby suggesting an almost two- to three-fold increase in propagation speed for distant regions.

It is well established that axonal diameters play an important role in the dynamics of neural connections mainly by regulating the conduction speed of impulses (68-70). Non-invasive brain imaging techniques such as diffusion MRI tract tracing (DTT) have been widely used to study axonal lengths, diameters etc for the understanding of white matter conduction times and velocity. The conduction time between the left and right hemisphere ranges from 30ms through myelinated callosal fibres to 150-300ms through unmyelinated fibers (71). While these numbers depict conduction times between the two hemispheres, they are similar to the time delays observed in interictal spikes occurring on brain regions almost 40 to 150 mm apart geodesically on the same hemisphere. In future, it would be interesting to study the actual conduction pathways of interictal spike spread through gray vs. white matter using the DTI images for the same patients.

As the next steps, it would be highly informative to study the relationship between interictal spike spread and seizure onset zones. Again quoting our hypothesis, we would expect the most common regions of interictal spike spread to be same as the regions that have eventually developed into epileptic foci over period of time. The underlying phenomenon that could possibly cause interictal spike spread to develop into an epileptic focus can be similar to

what we see in 'kindling'. Kindling can be describes as an increase in the likelihood of having more seizures from a single seizure (72). It is commonly used to study the seizure initiation and spread circuitry. Most intractable focal epilepsies have been associated with multiple epileptic foci and much of the clinical support for kindling of the second site have come through observations made on the differences in localization of interictal spikes over a period of several years where initially the EEG recorded spikes only in one primary focus (72). Another common observation in focal epilepsy is occurrence of a whole new type of seizure after removal of the first reported seizure onset areas in the epilepsy surgery. Thus, while there are still a lot of unanswered questions, our data on the patterns and velocities of interictal spike spread provides great insights on the possibilities of what might be going on in the cortical and subcortical parts of human epileptic brain.

CHAPTER 4

CONCLUSIONS AND DISCUSSION

Epilepsy is a debilitating neurological disorder of recurrent unprovoked seizures. About 1% (65 million people) people worldwide have epilepsy (2). About 80% of epilepsy cases are reported in developed countries (73). Even with drug treatment, more than 30% of patients with epilepsy continue to have seizures (74-76). Tremendous developments have been made in the field of medicine over the past more than hundred years since this disease was first recognized. We have great insights into the cellular and molecular structure of the human brain and how it functions, but the pathological mechanisms leading to, and underlying epilepsy still remain largely unknown. Several studies have linked single gene mutations that modulate neuronal excitability, such as neuronal receptors and ion-channels to some rare forms of epilepsy; but in most epilepsy cases, no real genetic cause can be identified. One of the most common forms of epilepsy is medically intractable epilepsy with a focal onset. In this form of epilepsy, there is enhanced excitability in a focal region or a neural network, which rapidly depolarizes and spreads rhythmically leading to generalized convulsions. In focal epilepsies, seizures generally remain limited to a single hemisphere.

Most of the anti-epileptic medications work as anti-convulsants and might help in reducing the frequency of seizures, but do not cure the disease itself. To date, the best available cure for this debilitating disease is the surgical resection of regions of the brain where seizures originate and spread. While seizures are the most important characteristic of epilepsy, there has been a growing interest in interictal epileptic discharges due to their high frequency of occurrence. In the preceding chapters we have made efforts in understanding the role of interictal

spikes in epilepsy, with respect to the underlying molecular changes in regions of high interictal activity and their relationship to the anatomical structure and organization of the brain.

Using high-throughput genomics, we have identified 1280 genes that are differentially regulated in regions of high interictal spiking when compared to the neighboring low spiking areas (control) from twenty patients. Ontology studies of these genes implicated activation of MAPK/CREB pathway and downstream target genes such as the IEGs and other transcription factors playing a role in synaptic strengthening and plasticity. Within the MAPK/CREB signaling cascade, we found induction of both activators (downstream CREB targets such as EGR1, EGR2) and inhibitors (DUSPs) of the pathway in regions of high spiking. DUSPs are a class of phosphatases that function by inhibiting various members of MAPK/CREB pathway. One of the most potent inhibitors of MAPK (ERK1/2) is DUSP4 and is highly up-regulated in high spiking regions consistently across all the patients. Further, we demonstrated an inverse spatial relationship between DUSP4 and EGR1/DUSP6, both of which occur downstream of MAPK/CREB pathway and represent activating MAPK/CREB signaling. Most molecular changes were limited to superficial layers (layers 2/3) of the neocortex and within these molecular patterns of MAPK/CREB activation, we observed 'micro-domains' of activated DUSP4 in the regions where EGR1 and DUSP6 were not expressed. Previous studies in our lab reported that by blocking MAPK we can effectively prevent spiking in the tetanus toxin injected rats (35). Therefore, DUSP4 was one of the key molecules of interest in our studies because of its high therapeutic potential in lieu of its inhibitory role in the activation of MAPK (EKR1/2).

In the second part of these studies, we identified distinct patterns of interictal spike propagation across the human epileptic brain. We made efforts towards investigating the role of the anatomical structure and organization of human brain in the spread of electrical activity. Our

data indicate two distinct modes of propagation of interictal spikes through grey matter (slow) and through white matter (faster).

Taken together, these findings shed great insight on the molecular role of interictal spikes in epilepsy, as well their relationship to the cortical and sub-cortical organization of the human brain. We have identified biomarkers of epilepsy associated with high interictal spiking regions. Our studies provide new targets for diagnostics and novel therapeutics and raise exciting questions about the role of interictal activity and its spread in epilepsy.

Interictal spikes in epilepsy

Interictal spikes are perhaps the first recognized signatures of cortical assemblies (77,78). They can be described as electrographic hallmarks of epilepsy. Despite their high frequency of occurrence interictal spikes cannot be used as a biomarker to identify regions of seizure onset or for evaluation of surgical resection of intractable epileptic foci. This is because of high variance and the tendency of interictal spikes to spread across the brain (20,79). Data from previous studies in our lab as well as from recent work described in this thesis collectively suggests a strong association of interictal spikes to disease physiology.

In Chapter 2, we described the molecular pathways and gene expression changes occurring in regions of high interictal spiking in the human epileptic brain. Our studies were primarily focused on comparing regions of high and low interictal activity. We demonstrated MAPK/CREB activation and gene expression of EGR1, EGR2 etc predominantly in layers 2/3 of the neocortex. The pyramidal cells in layers 2/3 exhibit extensive arborization within the same layers leading to monosynaptic recurrent connections between pyramidal cells in these layers (80). These intralaminar connections possibly mediate lateral excitatory connections. These

layers also receive feed-forward excitatory inputs from deeper layers and thalamus and are responsible for amplifying these inputs by local feedback excitation (81). This could possibly be the mechanism by which recurrent interictal activity leads to sustained MAPK/CREB activation and activity-dependent gene expression in these superficial layers.

This data suggests an important role of interictal spiking in the underlying molecular changes in epilepsy. However, in the present study, we have investigated all the molecular changes in relationship to the average interictal spike frequency per ten minutes. In future projects it would be fundamental to look at additional interictal spike parameters to better characterize the molecular changes. The published algorithm by Dan Barkmeier (36) to study interictal spikes has given us numerous other parameters associated with spiking, such as the slope, duration and amplitude of the whole spike as well as first-half wave and second half-wave. Another important characteristic of interictal spikes as observed from ECoG is the difference in their shape and their tendency to spread. Future studies to understand the relationship between these additional parameters and underlying molecular changes could provide greater insight in the role of interictal spikes in epilepsy.

Epileptic brain's defense mechanism

The role of MAPK pathway and ERK1/2 activation has been well established in epilepsy in both humans (82,83) and animal models (84-87). Nateri et al (2007) have demonstrated that ERK activation alone is sufficient to cause epilepsy via NMDA receptor stimulation in the animal model (88). We observe up-regulation of both activators and inhibitors of MAPK/CREB pathway in the high spiking regions of human epileptic brain from our high-throughput studies. A deeper look into the expression patterns of these positive and negative regulators of MAPK

suggested a strong inverse spatial relationship between DUSP4 and members of activating MAPK/CREB pathway, EGR1 and DUSP6. It was striking that we observed micro-domains of DUSP4 activation where there was lack of expression of EGR1/DUSP6 suggesting inhibition of MAPK/CREB and its downstream targets in regions of DUSP4 activation. One possible hypothesis generated from these findings is that DUSP4 prevents the spread of interictal activity along the neocortex by creating these 'zones of inhibition' where excessive DUSP4 activation turns off activation of MAPK/CREB and its downstream targets. This can be viewed as a defense mechanism adapted by human epileptic brain in response to abnormal electrical activity.

Similar observations were reported by John Huguenard's group in Stanford, where they observed increased levels of Neuropeptide Y (NPY), an endogenous anti-convulsant, in thalamus and hippocampus of rats in response to valproic acid (89). These regions of human brain are associated with petit mal and temporal lobe epilepsies and valproic acid has been long used in the treatment of epilepsy. Valproate is largely used in the treatment of absence and other seizures and can effectively treat status epilepticus. Therefore, increase in levels of NPY after valproic acid treatment suggests an inherent property of human brain to prevent seizures.

DUSP4: Therapeutic target

Mitogen activated protein kinase pathway constitutes a series of highly conserved kinase proteins that relay information from extracellular signals to intracellular effectors mediating diverse cellular processes such as proliferation, differentiation, migration and apoptosis (90-94). The activation of MAPK is tightly regulated by coordinate action of MAPKK and DUSPs. Dual specificity phosphatases (DUSPs) play a critical role in the inactivation of MAPK signaling

cascade by dephosphorylating both serine/threonine and tyrosine residues (40-42). Additionally DUSPs also function to anchor or shuttle MAPK between cytoplasm and nucleus (95,96).

Several DUSPs that play a direct or indirect role in regulation of MAPK signaling pathway have been implicated in initiation and development of human cancer for over a decade (97,98). DUSPs have been associated with both tumor progression and tumor suppression activity in different cancers. Functional studies on DUSP4 along with some other DUSPs such as DUSP6, DUSP7 etc have implicated their roles as tumor suppressors (99,100). DUSP4 (also known as MKP-2), which is primarily localized in the nucleus and functions by dephosphorylating (inactivating) both isoforms of ERK (ERK1 and ERK2) (44,45,101), has been widely implicated in many cancers including glioblastomas (GBM). While DUSP4 is observed to be upregulated in liver, breast and rectal carcinomas (102-105), it has also been reported to be down-regulated in ovarian, lung, breast carcinomas and GBMs (106-110). Most DUSPs including DUSP4 can be regulated at multiple levels, such as gene transcription, protein stability and phosphatase activity. In most cases, DUSP4 is observed to be transcriptionally regulated through DNA methylation, especially in the gliomas as demonstrated by genome-wide DNA methylation analysis (110,111). Waha et al (2010) also reported that over-expression of DUSP4 in GBM cell lines significantly reduces cellular proliferation and colony formation by blocking MAPK signaling cascade, suggesting DUSP4 as a novel tumor suppressor gene candidate.

Taken together, the studies in GBMs and our recent data on molecular changes in high interictal spiking regions demonstrate a highly robust role of DUSP4 in inhibiting MAPK/CREB pathway and suggest high therapeutic potential of DUSP4, warranting further investigation. The next step would be to examine the spatial localization of MAPK/CREB activators and inhibitors (particularly, DUSP4) in a well-characterized animal model of interictal spiking, which closely

mimics the human physiology in epilepsy. It would be crucial to determine the effect of over-expression and knock-out of DUSP4 on epileptic spikes as a means of therapeutic development in the animal model. Kullmann et al (2014) have extensively discussed need and possibilities of gene therapy in both focal epilepsies (112,113) and status epilepticus (114). The same group has also described a highly effective gene therapy approach in the rat tetanus toxin model of epilepsy using lentivirus (52). Using a similar design, an exciting approach would be to over-express DUSP4 in the superficial layers of neocortex as a 'natural' mechanism to round-off excessive MAPK signaling and possibly development or progression of epilepsy.

Cortical and sub-cortical connectivity in epilepsy

Our data so far, suggested an important role of the intrinsic structure of the human neocortex in the MAPK activation in the epileptic regions. This led to our hypothesis about a possible role of anatomical boundaries of the neocortex and the gyral-sulcal interfaces in blocking the spread of molecular changes and therefore preventing the spread of interictal activity beyond these anatomical boundaries.

To address this, in Chapter 3 we discussed the relationship between interictal spikes and the structure and organization of the human brain. The molecular studies in our lab have been performed using average spike frequency as a way to measure the interictal activity of the tissue. While there are several other properties exhibited by interictal spikes, the one property that intrigued us was their tendency to propagate. Spike propagation is one of the most common phenomena observed in electrical recordings in epilepsy and one of the primary reasons why interictal spikes are not accurate measures in the pre-surgical evaluations in epilepsy.

Our data demonstrates distinct patterns of interictal spike propagation, including propagation to a.) adjacent electrodes on same gyrus, b.) adjacent electrodes separated by a sulcus, and c.) distant electrodes separated by multiple sulci. Based on the time delays in occurrence of spikes in different regions, we speculate slower gray matter propagation between neighboring regions on the same gyrus, as opposed to faster white matter propagation between distant areas of the brain. This suggests an important role of interictal spikes in the initiation and progression of epilepsy.

The most common type of epilepsy is characterized by partial seizures initiating at a single epileptic focus. This focal brain region exhibits heightened electrical excitability capable of producing repeated depolarizations that can spread rapidly through the brain and result in generalized convulsions. Even though the relationship between interictal spikes and seizures is highly controversial, our recent data on the spread of interictal spikes suggests a possible mechanism by which electrical activity spreads through the epileptic brain. In some patients undergoing epilepsy surgery, multiple regions of the brain are resected to obtain adequate surgical outcomes. Information on how interictal spikes spread and their possible role in the spread of seizures can provide great insight on the evaluation of surgical resection. As a future direction, it would be imperative to study the spike propagation after surgical resection. If the resected tissue happens to be a region of the brain where we observe maximum spike initiation, it would be useful to examine occurrence and frequency of interictal spikes post epilepsy surgery. Based on our recent findings, we would expect no interictal activity in the remaining region of the brain where we observed spike spread before surgical resection.

It would also be crucial to compare the regions of spike initiation and spread to regions of seizure initiation and spread. This can provide essential information on the relationship between interictal spikes and seizures in epilepsy.

APPENDIX A

TABLE AND FIGURES

Table 1.

Patient No.	Age/Sex	Spike Frequency (High:Low)	Patient Diagnosis
1	15/F	6:0	Polymicrogyria
2	10/F	116:1	Diffuse gliosis, acute inflammation but normal laminar pattern
3	33/M	576:157	Normal temporal lobe
4	11/F	5:0	Heterotopia
5	7/F	425:0	Mild gliosis.
6	27/M	27:2	White matter gliosis
7	1/M	85:0	Mild gliosis
8	3/F	141:66	White matter gliosis, superficial heterotopia
9	3/F	212:56	Mild gliosis
10	7/F	215:25	Cortical dysplasia
11	6/F	124:26	Mild gliosis
12	8/M	172:3	Mild gliosis
13	56/F	299:51	Hippocampal sclerosis
14	16/M	176:44	Diffuse gliosis
15	11/F	66:2	Cortical dysplasia
16	1/F	80:1	Gliosis, Focal dysplastic hamartomas
17	23/M	137:90	Mild gliosis
18	2/M	80:8	Left craniotomy, Mild gliosis
19	17/F	44:42	Mild gliosis
20	7/M	150:25	Diffused white matter, Gliosis

Table 1. Lists clinical information of patients used for highthroughput study. The table gives the list of patients that were used for microarrays analysis, along with their age and sex. Column 3 presents the interictal spike frequency per ten minutes, averaged over three ten-minute ECoG samples for high and low interictal spiking tissue. Column 4 gives the patient's general clinical diagnosis.

FIGURE 1

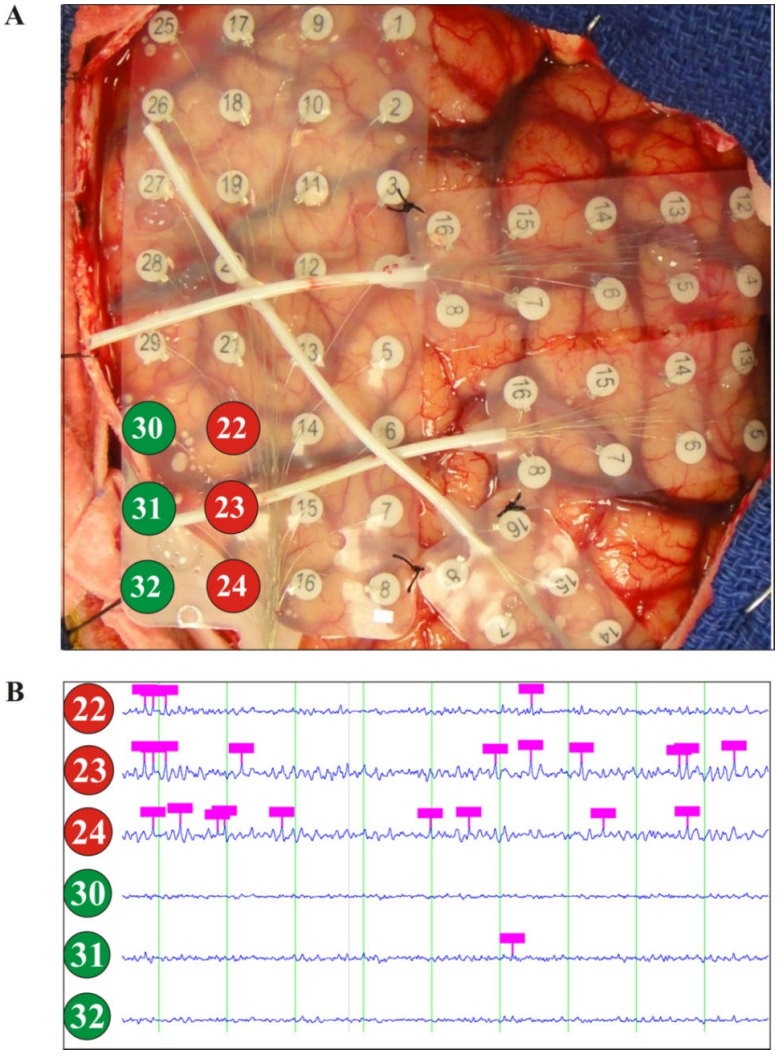


Figure 1. Identification of regions of High and Low Interictal Activity (A) Electrical recording grids with 1 cm evenly-spaced electrodes placed on the neocortical surface in the first stage of a two-step epilepsy surgical procedure. Cortical regions of high interictal activity and relatively low-spiking "control" tissue identified in the electrocorticogram are marked. High spiking electrodes are colored red and low spiking electrodes are colored green (B) Electrocorticogram depicting high (red) and low (green) spiking regions of selective electrodes from (A). Part of the brain tissue underneath these electrodes was removed during the second surgical stage.

FIGURE 2

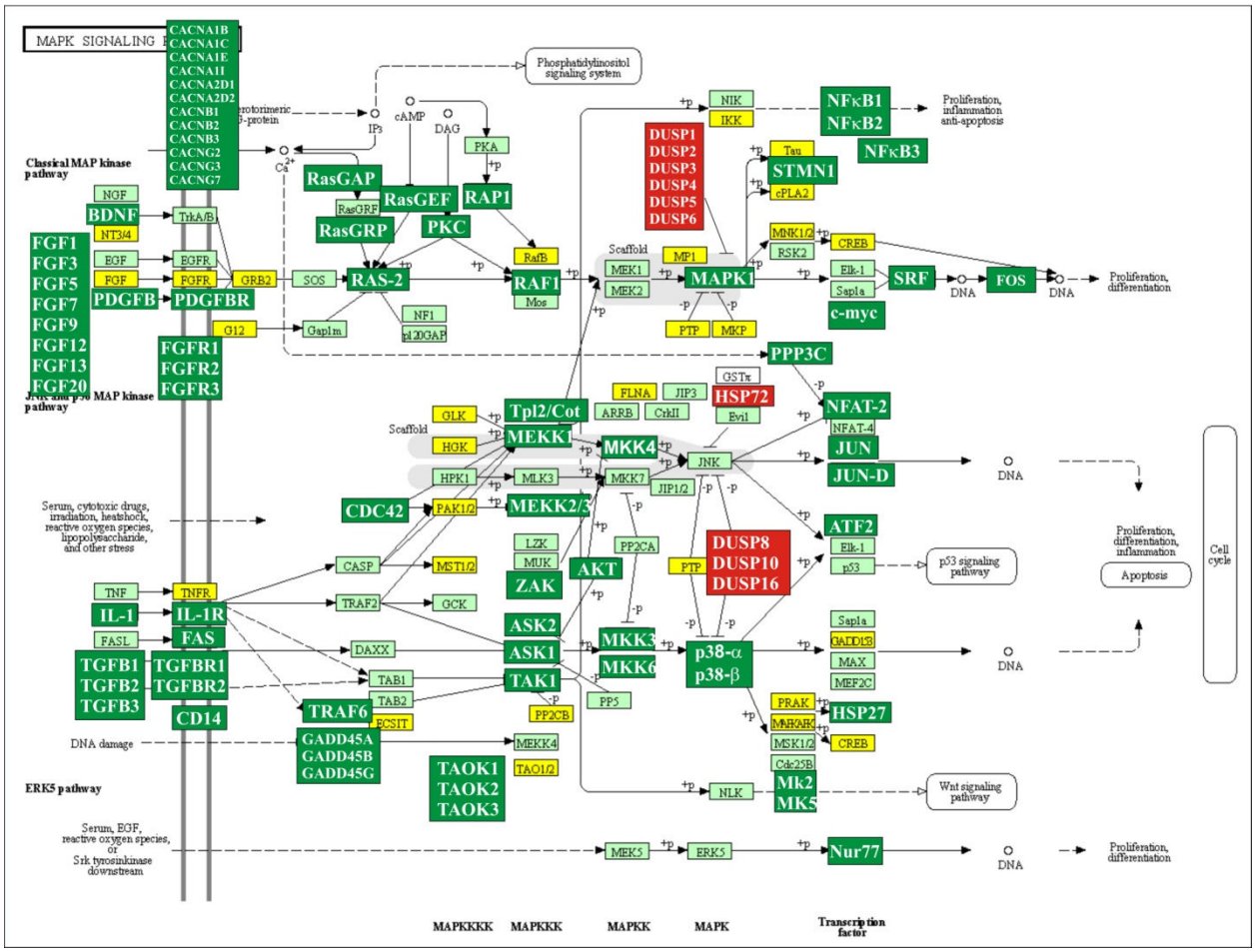


Figure 2. Summary of MAPK pathway implicated in high spiking regions of epileptic brain. Sketch of well-established MAPK pathway was taken from Kyoto Encyclopedia of Genes and Genomes (KEGG). Key components of MAPK pathway that were differentially regulated (fold-change > 1.4; FDR 5%) in epileptic regions of the brain are presented on the schematic. We find both activators and inhibitors of MAPK pathway induced in high spiking areas. Activators are marked in green boxes and inhibitors are in red boxes.

FIGURE 3

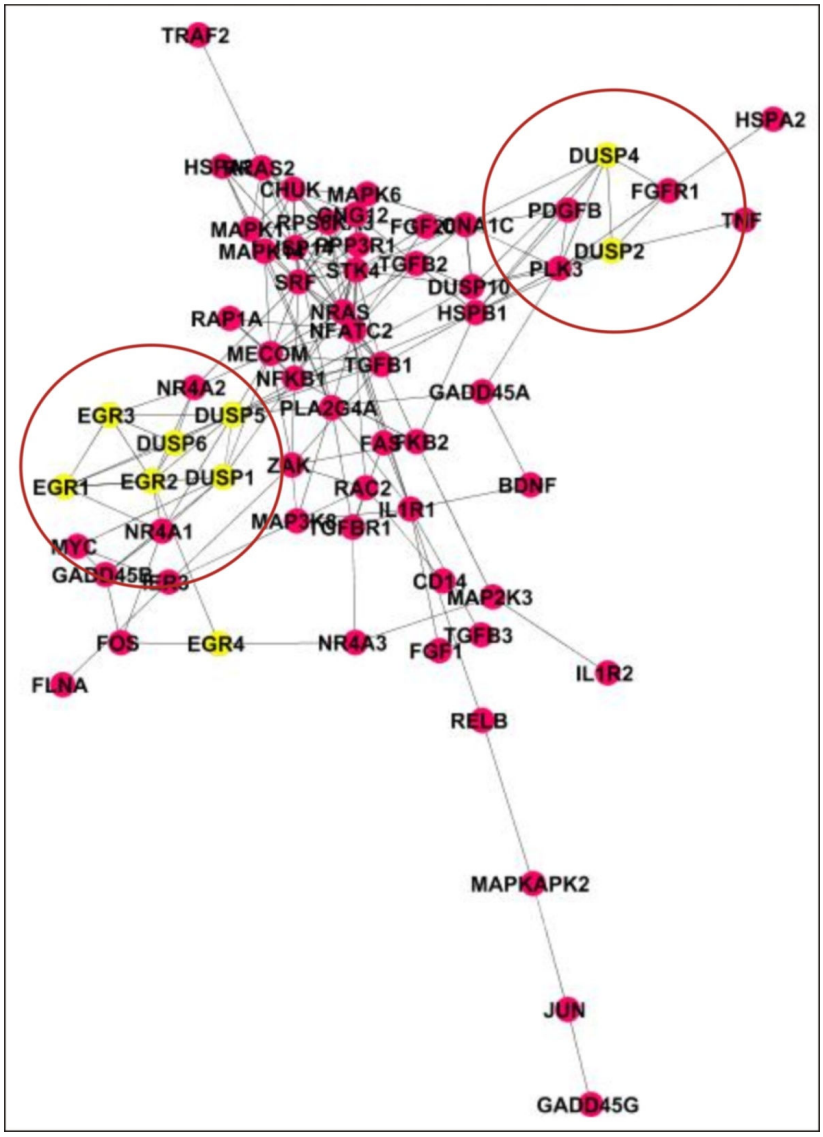
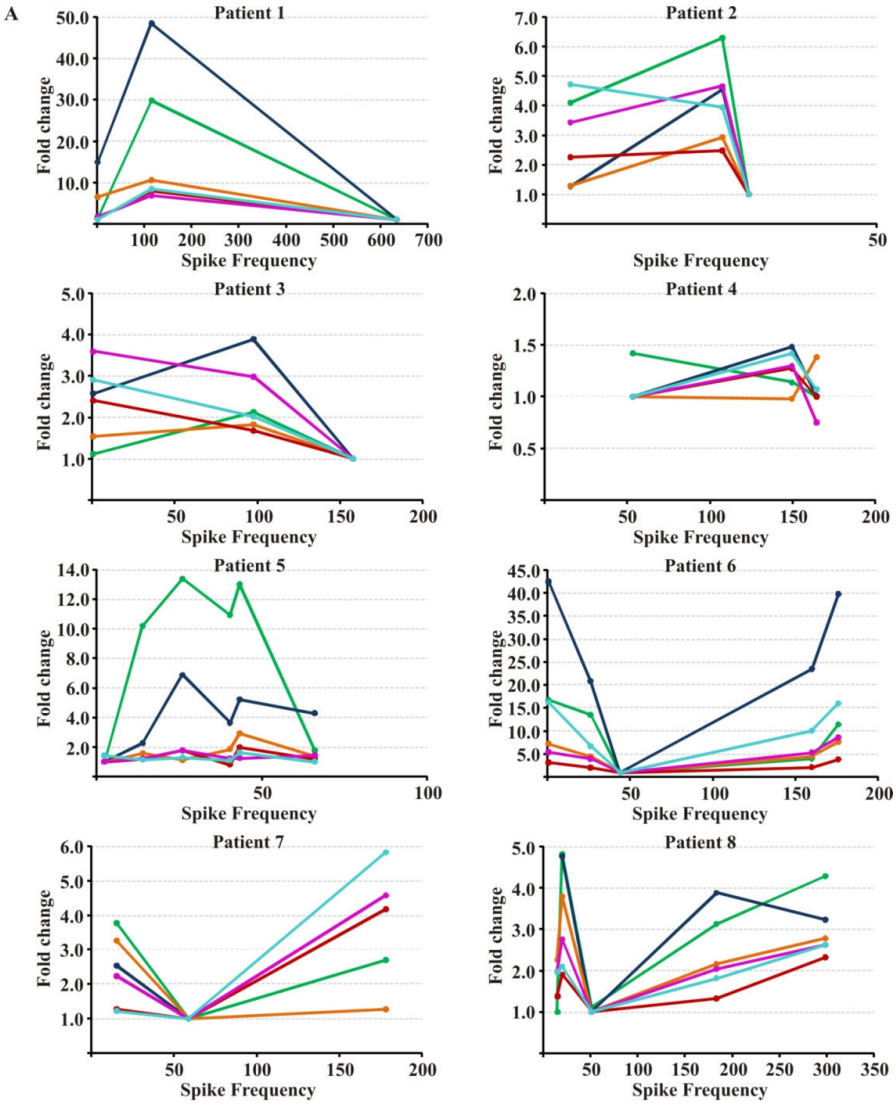


Figure 3. Co-expressed MAPK Signaling Genes Network. The network represents correlation between all MAPK/CREB genes that are differentially regulated (fold-change>1.4; FDR 5%) in epileptic regions of the neocortex at R > 0.80. Raw fluorescence intensities of these genes in high and low spiking samples in microarrays of 15 patients were used. Within MAPK/CREB pathway we found that EGR1, EGR2 and other DUSPs clustered together tightly (R > 0.95), whereas DUSP4 did not correlate with MAPK/CREB activating genes at higher correlation.

FIGURE 4



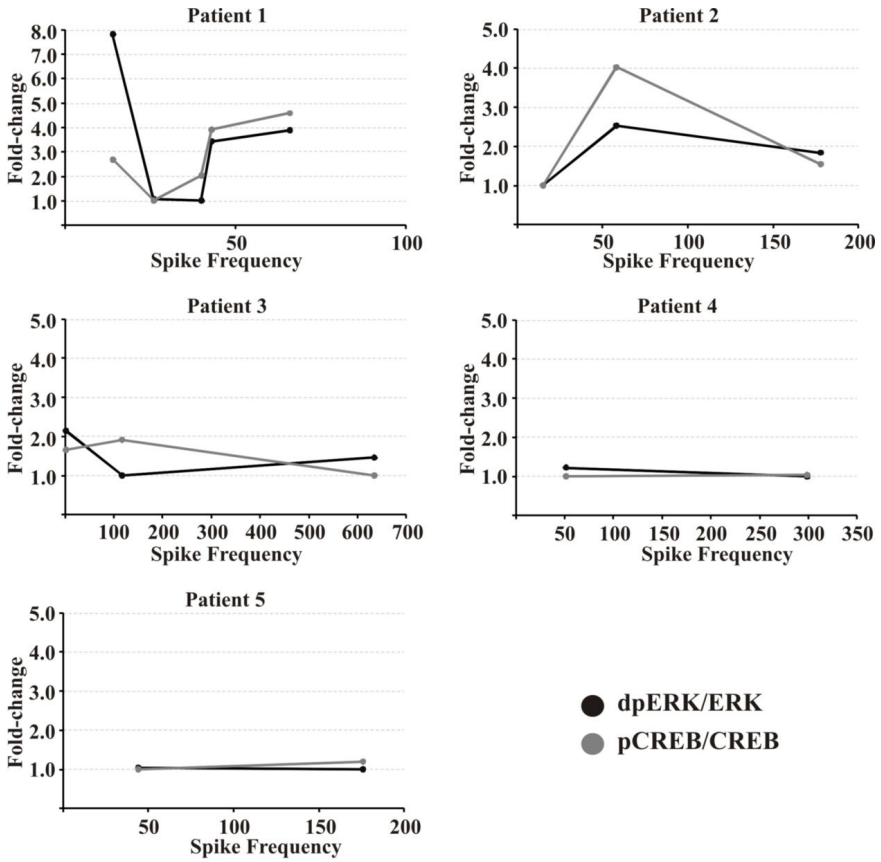
	EGR1	EGR2	DUSP1	DUSP4	DUSP5	DUSP6
EGR1	1.00					
EGR2	0.73	1.00				
DUSP1	0.73	0.92	1.00			
DUSP4	0.80	0.79	0.81	1.00		
DUSP5	0.59	0.83	0.80	0.80	1.00	
DUSP6	0.56	0.87	0.74	0.62	0.89	1.00
Spike Frequency	-0.13	-0.04	-0.06	0.01	-0.02	0.00

Figure 4. Activity-dependent changes in gene expression. (A) Real-time qPCR data representing gene expression changes for the genes EGR1, EGR2, DUSP1, DUSP4, DUSP5 and DUSP6, comparing high, medium and low spiking regions of the epileptic brain in eight patients.

All genes are normalized to the housekeeping gene, GAPDH. All these genes are downstream targets of activated CREB. We see two distinct patterns of changes in gene expression. For patients 1 through 5, data suggests an initial increase in gene expression with increasing spike frequency. Gene expression is maximum in the medium spiking sample and minimum in the highest spiking samples. On the other hand, patients 6, 7 and 8 depict an inverse pattern where the medium spiking sample has lowest gene expression. **(B)** Correlation matrix for all the genes and spike frequency. All the MAPK/CREB genes correlate positively with each other, including the inhibitor DUSP4 which correlates with EGRs at $R > 0.80$. Correlation analysis between MAPK/CREB genes and interictal spiking did not show any relationship between the two.

FIGURE 5

A



B

	dpERK/ERK	pCREB/CREB
dpERK/ERK	1.00	0.58
pCREB/CREB	0.58	1.00
Spike Frequency	-0.24	-0.30

Figure 5. Levels of MAPK/CREB in regions of different interictal spiking in five patients. **(A)** Western blot data was quantified using Metamorph software for the levels of phosphorylated MAPK (ERK1/2) and CREB in regions of different interictal spiking for five patients. Phosphorylated ERK1/2 and CREB were normalized to their unphosphorylated counter-proteins. The graphs depict high variability in levels of dpERK1/2 and pCREB with changes in spike

frequency within a patient, suggesting high lability and dynamics of phosphorylation state of these proteins. **(B)** Activated ERK1/2 and CREB correlate weakly with each other (R = 0.58) as shown in the correlation matrix. Furthermore, ERK1/2 and CREB activation does not correlate with interictal spike frequency, similar to what we observed with gene expression changes.

FIGURE 6

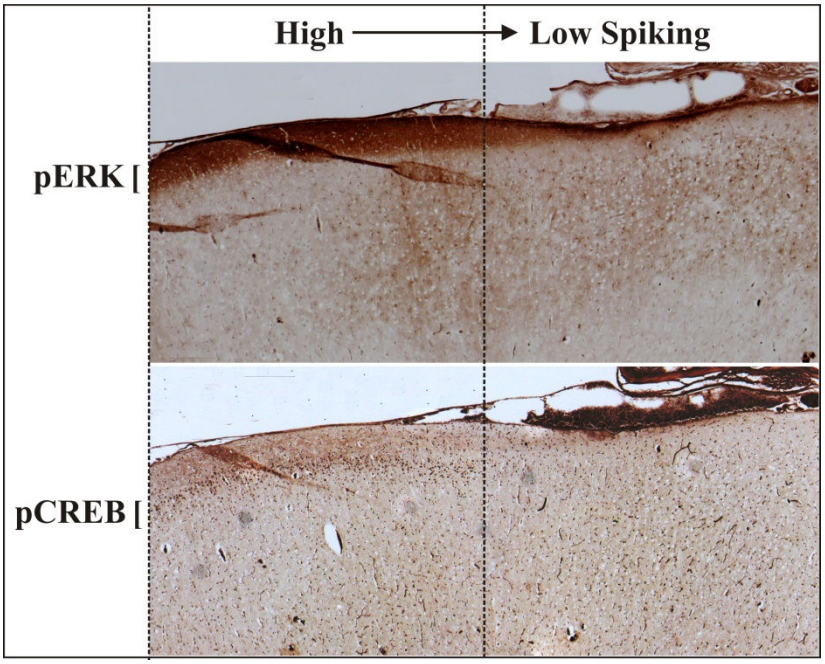


Figure 6. Spatial pattern of expression of dpERK1/2 and pCREB. Immuno histochemical staining of regions of high and low interictal spiking on the same gyrus. Signal for activated ERK1/2 and CREB is present in the region of high spiking and diminishes in the low spiking area. The black arrow depicts the molecular borders of dpERK1/2 and pCREB.

Figure 7

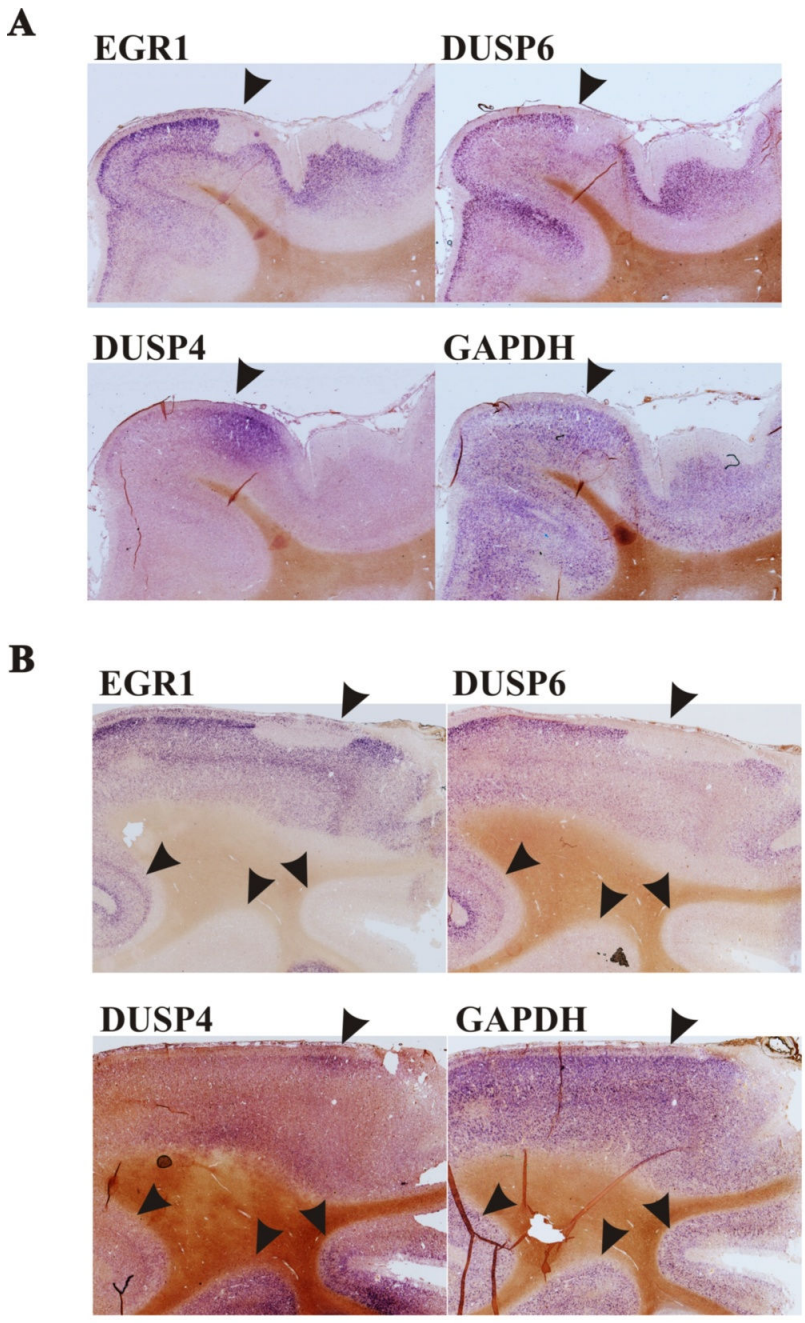


Figure 7. Inverse spatial relationship between DUSP4 and EGR1/DUSP6. DIG-labeled in-situ hybridization showing an inverse spatial relationship between the expression of MAPK inhibitor DUSP4 and downstream MAPK/CREB targets, EGR1 and DUSP6. Region of DUSP4

expression coincides with the region where there is lack of signal for EGR1 and DUSP6. Serial sections were used to analyse the spatial distribution of the genes for two different patients, (A) and (B) on a medium spiking tissue. GAPDH was ubiquitously expressed along the 6-layered neocortex and was used as a positive control.

Figure 8

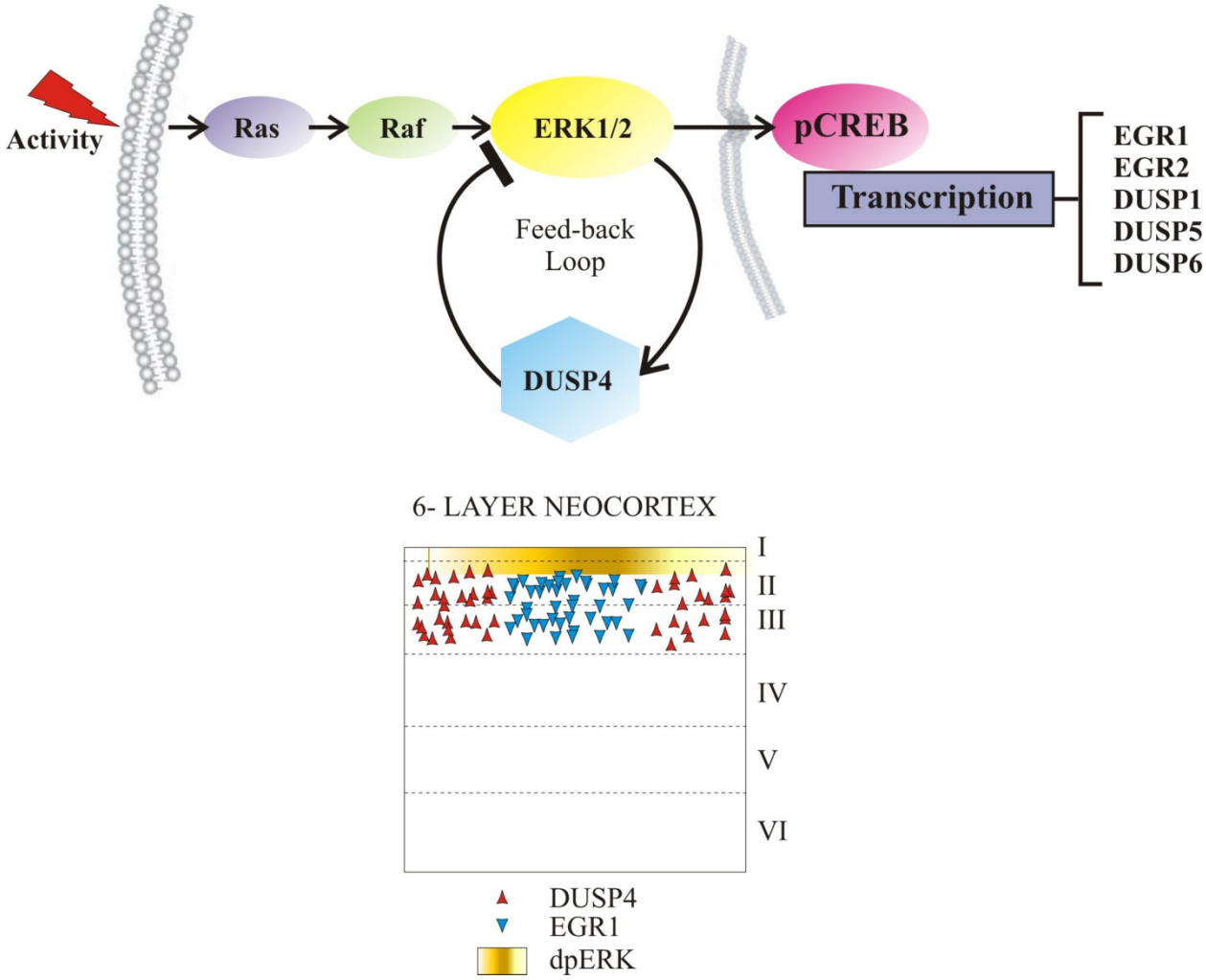


Figure 8. Proposed model for MAPK/CREB pathway and mechanism of DUSP4 in epileptic neocortex. (A) Schematic representation of key members of MAPK pathway that are implicated in the regions of high interictal activity, followed by CREB activation and transcription of downstream target genes, including EGR1, EGR2, DUSP1, DUSP5 and DUSP6. The model also presents well-established feed-back loop between DUSP4 and ERK1/2. **(B)** A proposed role of DUSP4 along the 6-layered epileptic neocortex. We hypothesize that micro-domains of activated DUSP4 act as inhibitory pockets for MAPK/CREB activation and prevent

the spread of molecular changes beyond themselves. This could possibly be the self-defense mechanism adapted by epileptic brain to prevent the spread of interictal activity.

Figure 9

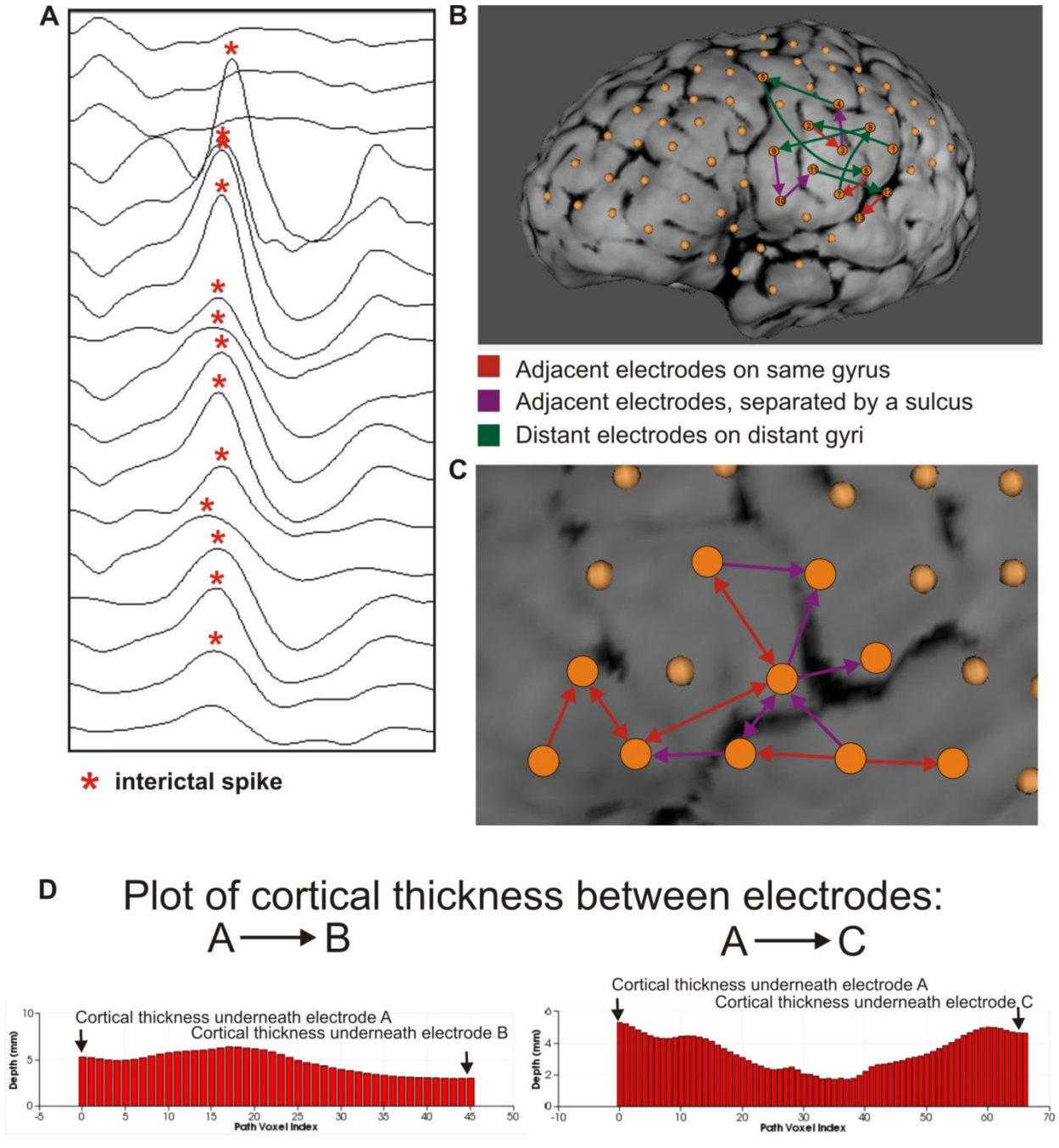


Figure 9. Distinct patterns of interictal spike propagation. (A) A segment of the electrocorticogram of an epileptic patients undergoing surgery. We observe occurrence of interictal spikes in multiple channels within a few milliseconds, as identified from the spike

detection algorithm and validated by a trained neurologist. Each interictal spike is marked with the pink asterisk sign. **(B)** Pattern of interictal spike propagation as depicted in (A) is mapped onto the 3D brain rendering for the patient for visual analysis. Interictal spikes present three distinct patterns of propagation as depicted. Spikes propagating to adjacent electrodes on the same gyrus are marked with orange-colored arrows; spikes propagating to adjacent electrodes separated by a sulcus are colored with pink arrows and spikes spreading to distant electrodes are marked with green colored arrows. **(C)** Zooming into the epileptic brain for a better visual presentation of different patterns of spike propagation. **(D)** Graph showing how cortical thickness changes between two electrodes on the same gyrus as compared to the electrodes separated by a sulcus. There is thinning of the cortex in the sulcul region of the brain as visible in the graph on the right. The plots of cortical thickness were used to validate presence of a sulcus between two electrodes where it couldn't be confirmed from the 3D rendering due to poor resolution.

Figure 10

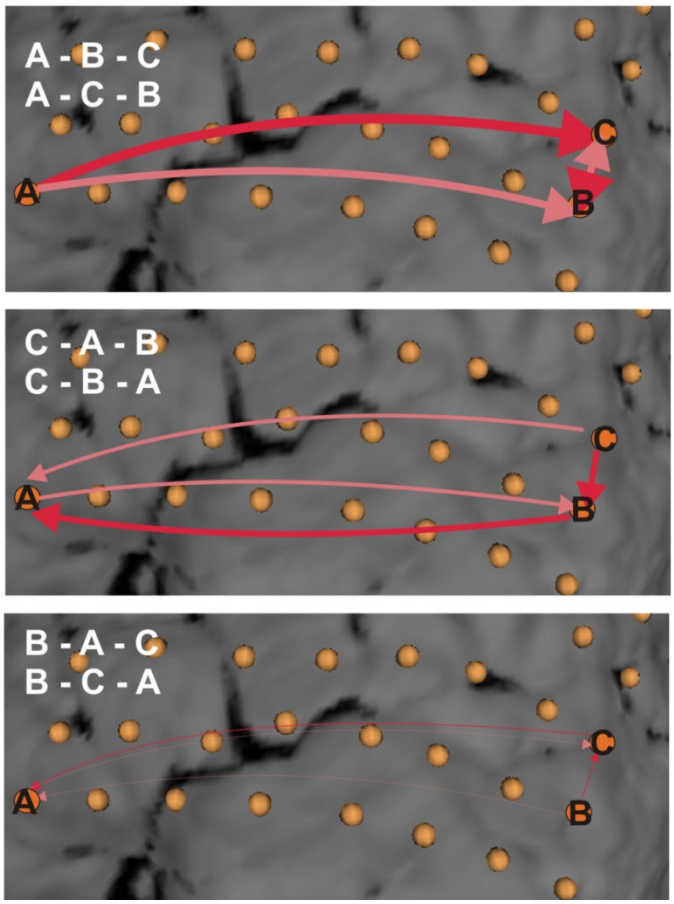


Figure 10. Spikes prefer a certain direction of propagation more than the other possible paths. This figure depicts six possible directions a spike could propagate in, two in each panel. Different panels consist of spikes initiating at Electrode A (top), Electrode C (middle) and Electrode B (bottom). The arrows indicate direction of propagation and thickness of the arrow is representative of the frequency of spike propagation in that orientation. Clearly, there are more spikes that first appear on Electrode A and propagate to B/C as compares to spikes initiating at and propagating from Electrodes B or C. Therefore, it appears that spikes follow a particular direction more than the others.

Figure 11

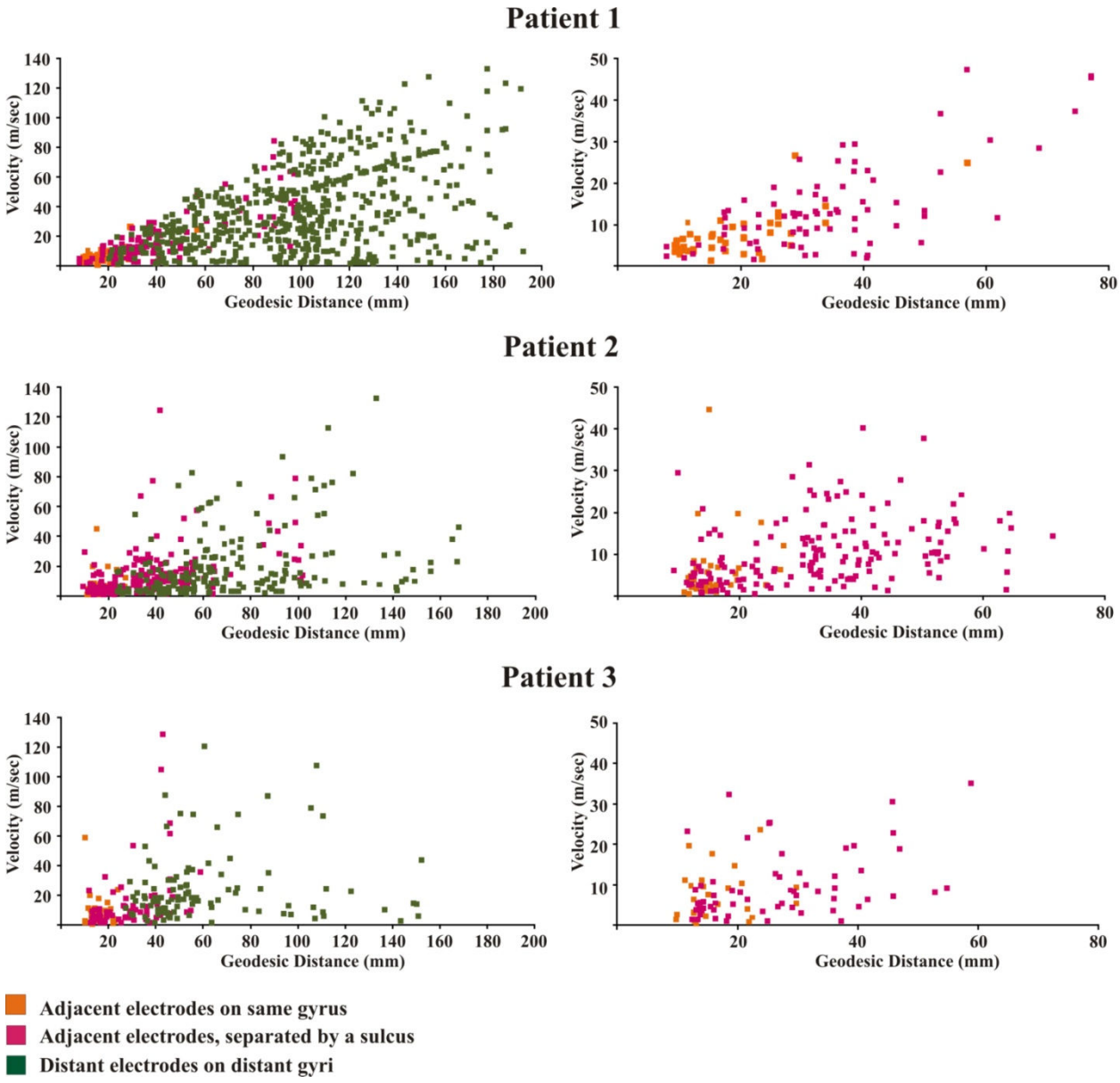
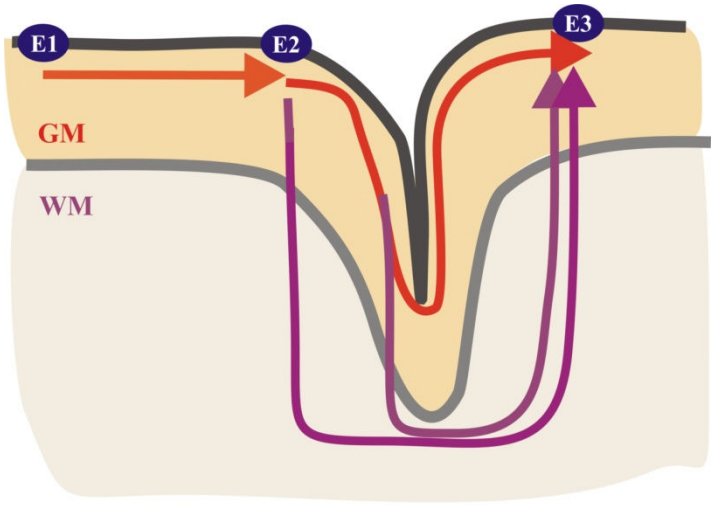


Figure 11. Higher velocities of spike propagation to distant electrodes as compared to adjacent electrodes. Graphs depicting velocity of spike propagation as a function of distance between the electrodes for three patients. Data clearly suggests an increase in velocity of spike propagation as distance increases. For each patient, the graph of the left depicts data for all the spike propagation events, and graph on the right present spike propagations between adjacent electrodes only to a better visualization. Spike propagation between adjacent electrodes on the same gyrus as marked in orange series; spikes propagating between adjacent electrodes separated by a sulcus are colored by pink data points; and distant spike propagations are colored green**.**

Figure 12



$$\text{Time}_{\text{Total}} = \text{Time}_{\text{GM}} + \text{Time}_{\text{WM}}$$

$$= \frac{\text{Distance}_{\text{GM}}}{\text{Velocity}_{\text{GM}}} + \frac{\text{Distance}_{\text{WM}}}{\text{Velocity}_{\text{WM}}}$$

APPENDIX B

HIC APPROVAL

WAYNE STATE UNIVERSITY

IRB Administration Office
87 East Canfield, Second Floor
Detroit, Michigan 48201
Phone: (313) 577-1628
FAX: (313) 993-7122
http://irb.wayne.edu

NOTICE OF FULL BOARD CONTINUATION APPROVAL

To: Jeffrey Loeb
Neurology
3122 Elliman Clinical Research

From: James Chinarian, M.D. or designee
Chairperson, Medical/Pediatric Institutional Review Board (MP2)

Date: June 14, 2012

RE: IRB #: 086000MP2F(5R)
Protocol Title: Molecular Analysis of Human Epileptic Tissue
Sponsor: ° NATIONAL INSTITUTES OF HEALTH
Protocol #: 1105009778

Expiration Date: June 13, 2013

Risk Level / Category: Pediatric: 45 CFR 46.404 - Research not involving greater than minimal risk
Adult: Research not involving greater than minimal risk

Continuation for the above-referenced protocol and items listed below (if applicable) were **APPROVED** following Full Board review by the Wayne State University Institutional Review Board (MP2) for the period of 06/14/2012 through 06/13/2013. This approval does not replace any departmental or other approvals that may be required.

- Medical Adult Research Informed Consent Form with HIPAA Authorization (revision dated 7/27/2011)
- Parental Permission/Research Informed Consent Form with HIPAA Authorization (revision dated 7/27/2011)
- Documentation of Adolescen Assent Form Ages 13-17 (dated 6/29/2011)

° Federal regulations require that all research be reviewed at least annually. You *may* receive a *"Continuation Renewal Reminder"* approximately two months prior to the expiration date; however, it is the Principal Investigator's responsibility to obtain review and continued approval ***before*** the expiration date. Data collected during a period of lapsed approval is unapproved research and can *never* be reported or published as research data.

° All changes or amendments to the above-referenced protocol require review and approval by the IRB **BEFORE** implementation.

° Adverse Reactions/Unexpected Events (AR/UE) must be submitted on the appropriate form within the timeframe specified in the IRB Administration Office Policy (http://www.irb.wayne.edu/policies-human-research.php).

NOTE:

1. Upon notification of an impending regulatory site visit, hold notification, and/or external audit the IRB Administration Office must be contacted immediately.
2. Forms should be downloaded from the IRB website at **each** use.

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ABSTRACT

LINKING THE MOLECULAR, ELECTRICAL AND ANATOMICAL PROPERTIES OF HUMAN EPILEPTIC BRAIN

by

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Epilepsy is a common neurological disorder of recurrent unprovoked seizures. It affects almost 1% of the world population. Although there is a wide range of anti-epileptic drugs (AEDs) available, they only treat the seizure symptoms and do not cure the disease itself. The poor role of AEDs can be attributed to the lack of knowledge of exact mechanisms and networks that produce epileptic activities in the neocortex. At present, the best cure for epilepsy is surgical removal of electrically localized epileptic brain tissue. Surgically removed brain tissue presents an excellent opportunity to discover the molecular and cellular basis of human epilepsy.

Patients with epilepsy have both seizures as well as another type of abnormal electrical wave form occurring in between seizures, and referred to as 'interictal' spikes. Interictal spikes occur far more frequently than seizures and in most epilepsy cases, cannot be associated with a clinical symptom. There is growing evidence supporting the role of interictal spikes in seizures and epilepsy. We have taken a high-throughput genomics approach and identified a consistent group of differentially expressed genes implicating MAPK/CREB signaling, immediate early genes (IEGs), and synaptic plasticity genes in regions of high spiking. Bioinformatic analyses showed a number of clusters within the MAPK/CREB genes that include both activators and

inhibitors of MAPK signaling. DUSP4, a member of a family of dual specificity phosphatases function by inhibiting both isoforms of ERK1/2, is one of the most potent inhibitors of MAPK/CREB signaling. DUSP4 was significantly upregulated in high spiking areas, raising an important question as to why both activators and inhibitors are induced in high spiking areas. In situ hybridization of serial brain sections shows that DUSP4 is expressed in discrete micro-domains in the superficial neocortical layers and is inversely related to expression of di-phosphoERK, phosphoCREB, EGR1, and DUSP6, suggesting that DUSP4 activation in regions of high interictal activity directly inhibits the spread of electrical activity in these focal regions. These studies utilizing high throughput genomic studies from human neocortex provide great insight on the role and importance of interictal spiking and associated molecular changes and begin to define both mechanistic and spatial roles of MAPK signaling in neocortical epilepsy and have the potential to produce novel therapeutics.

Furthermore, we have developed a software to quantify both the timing and spatial spread of intracranial spikes on long-term subdural electrographical recordings as a means to understand the relationship between spike spread and anatomical structure of the anatomical brain. We found that spikes occur in multiple channels of the subdural electrode grid within a hundred milliseconds and used these events to study the spatial and temporal patterns of interictal spike spread across cortical sites. Three different patterns of spike propagation were observed including 1) spread to electrodes on the same gyrus, 2) spread to neighboring electrodes but separated by a sulcus, and 3) Spread to distant electrodes (separated by multiple sulci). Interestingly, we found that the velocity of spread to distant electrodes was much higher compared to spikes that spread to adjacent electrodes, often on the same gyrus. This suggests at least two distinct conduction velocities through grey matter (slow) and white matter (fast). Using

the datasets from five patients, we developed an equation to predict the pathway of spike propagation that incorporates both components of gray matter and white matter conduction for a given spike. These studies help our understanding of neocortical epilepsy and warrant further studies to determine whether patterns of interictal spike propagation could possibly play a role in surgical decision-making and improve epilepsy surgery outcomes.

AUTOBIOGRAPHICAL STATEMENT

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2002 – 2006 B.E. – Biotechnology
University Institute of Engineering and Technology
Panjab University, Chandigarh, INDIA

AWARDS & HONORS

2013 First prize in poster presentation at Graduate Exhibition, Wayne State University, Detroit

2012 Thomas Rumble Fellowship awarded by Center for Molecular Medicine and Genetics, Wayne State University, Detroit

2009 First prize in poster presentation at Systems Biology Symposium, University of Michigan, Ann Arbor

PUBLICATIONS

- **Bagla S**, Dachet F, Loeb JA. Spatial Organization of MAPK Signaling Interactome in Human Epileptic Brain *(In preparation)*
- **Bagla S**, Pai D, Kim H, Hua J, Loeb JA. Distinct Patterns and Velocities of Human Interictal Spike Propagation through Cortical and Sub-cortical Pathways *(In preparation)*
- Dachet F, **Bagla S**, Keren-Aviram G, Morton A, Balan K, Kupsky W, Song F, Dratz E, Loeb JA. Predicting Novel Histopathological Lesions in Human Epileptic Brain through Transcriptional Clustering *(Submitted)*
- Keren-Aviram G, **Bagla S**, Dachet F, Balan K, Loeb JA, Dratz E Proteomic Analysis of Human Epileptic Neocortex predicts Vascular and Glial changes in regions of Epileptic Spiking *(Submitted)*
- Lipovich L, Dachet F, Cai J, **Bagla S**, Balan K, Jia H, Loeb JA. Activity-dependent human brain coding/noncoding gene regulatory networks. *Genetics*, November 2012
- Malik S, **Bagla S**, Chaurasia P, Duan Z, Bhaumik SR. Elongating RNA Polymerase II is disassembled through specific degradation of its largest but not other subunits in response to DNA damage in vivo. *Journal of Biological Chemistry* June 2008