Histological Characterization of Inter Ictal Epileptiform Discharges Generating Brain Regions using a Preclinical Model of Focal Cortical Dysplasia

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HISTOLOGICAL CHARACTERIZATION OF INTER ICTAL EPILEPTIFORM DISCHARGES GENERATING BRAIN REGIONS USING A PRECLINICAL MODEL OF FOCAL CORTICAL DYSPLASIA

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

BIOMEDICAL ENGINEERING

by

Abhay Shivaji Deshmukh

2015
To: Interim Dean Ranu Jung  
College Engineering and Computing

This thesis, written by Abhay Shivaji Deshmukh, and entitled Histological Characterization of Inter Ictal Epileptiform Discharges Generating Brain Regions using a Preclinical Model of Focal Cortical Dysplasia, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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Jorge J. Riera, Major Professor

Date of Defense: November 13, 2015

The thesis of Abhay Shivaji Deshmukh is approved.

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Interim Dean Ranu Jung  
College of Engineering and Computing

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Dean Lakshmi N. Reddi  
University Graduate School

Florida International University, 2015
DEDICATION

This thesis is dedicated to my family. Thank you for supporting me through all these years and being a constant source of inspiration.
ACKNOWLEDGMENTS

I would like to thank Dr. Riera for giving me the opportunity to work in his laboratory, supervise my research thesis and having confidence in my abilities. He did not only guide me to complete a degree but also pushed me achieve it with excellence. I would also like to thank Dr. Jung for allowing me to collaborate with her laboratory to perform certain tasks required for this project and for her help during different stages of the project. I am also grateful to Dr. Lin for his time, patience, co-operation and valuable guidance about academic and research aspects.

I also appreciate the co-operation of Yinchen Song, Jihye Bae and my all other lab mates in every step of my thesis.

I have found my coursework throughout the Curriculum and Instruction program to be stimulating and very inter-disciplinary. This provided me with challenges as well as the tools to successfully complete my research project.
Current clinical practice of resective surgery in focal epilepsy involves electroencephalogram (EEG) brain source imaging to localize irritative brain areas from where Inter-ictal epileptiform discharges (IEDs) emerge, useful to localize the seizures-onset zones. Unfortunately, there are no previous systematic studies to characterize the pathophysiological mechanisms and abnormal cellular substrates in these irritative areas since histological data are available only from the final resective zones. To address this issue we applied a combination of EEG brain imaging described by Bae et al. (2015) using cutting-edge technology for high-density scalp EEG in rodents and histological analysis on a chronic rat model of focal cortical dysplasia. Post-mortem brain sections were stained for anatomical, functional and inflammatory biomarkers. Abnormal anatomical structures and increased expression of inflammatory biomarkers were found in the irritative regions. We conclude that IED-based brain source imaging can help to localize abnormal tissues highly prospective for epileptogenesis.
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1. INTRODUCTION

1.1 FOCAL EPILEPSY

Epilepsy is a diverse set of chronic neurological disorders characterized by seizures. There are several types of epilepsies, but those named focal are the ones of special interest for this thesis. Focal or partial epilepsy is characterized by disturbance in electrical activity followed by seizures. Typical symptoms include unconsciousness, muscle rigidity, loss of muscle tone, convulsions, impairment of sensory functions, and automatism. Refractory focal epilepsy is a condition where antiepileptic drugs are ineffective against seizures. Focal epilepsies, also classified as partial epilepsies, are in many cases refractory. There are two major groups of focal epilepsy: temporal lobe epilepsy (TLE) which is more incident in adults and focal cortical dysplasia (FCD) which is more incident in children (Battaglia et al., 2009; Cavalheiro et al., 1995; Curia et al., 2008). FCD which is the one studied in this thesis results from malformations of cortical development that become electrically excitable with time. FCD forms a heterogeneous group of cortical malformations of development (Montenegro et al., 2003) which resemble the following properties.

1) Cortical architecture abnormalities: Columnar disorganization (formation of vertically oriented microcolumns consisting of at least eight neurons) and alterations in 6-layered tangential composition of the cortex (Montenegro et al., 2003).

2) Cytological abnormalities: Hypertrophic neurons outside typical location in layer V, immature neurons, round or oval cells with large nucleus and thin rim of cytoplasm,
absent in matured cortex, dysmorphic neurons with abnormal size and morphology of axons and dendrites, as well as increased accumulation of neurofilament proteins, balloon cells, usually large cells with ill-defined membrane, single or multiple eccentric nuclei and eosinophilic cytoplasm, demonstrating immunohistochemical features of neuronal and glial lineage (Montenegro et al., 2003).

1.2 PRESURGICAL EVALUATION OF FCD PATIENTS

The primary aim of the presurgical evaluation of patients with refractory focal epilepsy is to determine the location of the epileptogenic zone which is a tissue region responsible for seizure generation and that has to be removed to stop the seizures. Although surface electroencephalography (EEG) recordings are less sensitive than intracranial recordings, they still provide the best overview and, therefore, EEG based brain source imaging of interictal epileptiform discharges (IEDs) is the most practical and efficient method to localize and determine the epileptogenic region (Noachtar et al., 2009; Verhellen et al., 2007).

1.3 INTERICTAL EPILEPTIFORM DISCHARGES (IEDS)

The term “spike discharge” describes a pattern of EEG characterized by a spike followed by a wave in epileptic subjects. Spike discharges are observed not only during the ictal phases, when subject is experiencing seizures but also during the interictal phases, characterized by the absence of seizures. The spike discharges during the interictal phase are called as interictal epileptiform discharges. There is no standard definition of the IEDs, but are generally classified in typical sub-types as spikes (20-70
ms in duration, 15-50 Hz) and sharp-waves (70-200 ms in duration, 5-15 Hz) (Tyvaert et al., 2008; Grova et al., 2006; Neymotin et al., 2010).

1.4 ANIMAL MODEL FOR FCD

Historically, animal models of epilepsy have been mainly developed in rodents. It is because the rat is already the lowest appropriate mammalian preparation to study epilepsy. In particular, it has been very difficult to obtain an appropriate animal model for focal cortical dysplasia (FCD), as methylazoxymethanol acetate and pilocarpine (MAM-PILO) double hit model recently developed (Colciaghi et al., 2011) and proposed for our study using Wistar rats (details given in section 2.1). Rat model for FCD created by prenatal treatment of methylazoxymethanol acetate (MAM) has a potential to mimic the pathological and clinical features of focal epilepsy thus MAM-PILO model is an important model for FCD. Improvements in the strategies to create chronic focal epilepsy in rats and the recent development of cutting-edge technology for high-density scalp EEG in small animals make this particular preclinical model attractive.

1.5 IED-BASED BRAIN SOURCE IMAGING (BSI)

In this study, 32-channel EEG recordings were collected and analysed from chronic rat models of focal cortical dysplasia. In order to determine the candidate tissue to be resected in these animal models, IEDs from EEG were selected using their characteristic features reported in literature (Bortel et al., 2010) and the IEDs sources (irritative zones) were estimated by solving the EEG inverse problem (Kai boriboon et
The inverse problem consists of localizing in the 3D brain space the current sources that produce the IEDs. To that end, it is necessary to make some assumptions about the nature of these current sources. Standardized low-resolution brain electromagnetic tomography (sLORETA) (Pascual-Marqui, et al., 2002) is one such model which considers two sources of variation: the variation of the actual sources, and if present, variation due to noisy measurements and is based on the assumption that multiple sources can be simultaneously active across many locations at a given time. With context to our study, sLORETA underlies the concept that neighbouring neuronal populations are more likely to undergo synchronous depolarization during spontaneous discharge or an evoked response than are non-neighbouring neurons (Verhellen et al., 2007; Kaiboriboon et al., 2012). However, in practice, a critical issue with the IEDs is the inconsistencies in the localization of the epileptic zone in an individual case where solutions of inverse problem give the multiple localized regions for a single IED. The reasons underlying the inconsistencies in the localization of the epileptogenic zones could be due to the errors in the proper classification of IEDs and identification of relevant irritative area by using only information in the IED waveform. To avoid such inconsistencies we used a specific criterion for source determination, based on frequency of sources obtained from localization of each IED subtype.

The existence of multiple IED-generating sources could also be due to improper IED classification. We applied widely recommended methods that classify IED subtypes using both spatial and temporal profiles based on a multi-way principal component analysis known as parallel factor analysis (PARAFAC) (Fumikazu
Miwakeichi et.al, 2004) which is combined with a cluster analysis (Pedreira C, et al., 2004). This method allows us to decompose the topographic maps and temporal courses of IEDs as combination of spatial and temporal atoms to reduce the uncertainly in IED classification.

1.6 HISTOLOGICAL ANALYSIS

As mentioned earlier, localization of the individual IEDs subtypes was performed through the use of the sLORETA (Pascual-Marqui, et al, 2002). In order to analyse this methodology, we used histological biomarkers for anatomical, functional, and inflammatory indicators known to be affected in epilepsy. At the irritative zones identified from localized IEDs, we compared these histological indexes with the contralateral cortical region. From histological analysis it was possible to discuss major molecular and cellular bases that better correlate with the IEDs associated regions.

In the past most of the studies have been focused on determining histological biomarkers associated with the focal epileptic lesion. In this study we aim to find correlates between electrically excitable areas, as determined by applied brain source imaging and IEDs, and focal epilepsy specific histological biomarkers.

1.7 STATEMENT OF RESEARCH

In spite of recent advances in neuroimaging techniques, current clinical practice of resective surgery in focal epilepsy involves EEG brain source imaging to localize brain areas named irritatives, from where interictal epileptogenic discharges (IEDs) emerge to localize the seizures-onset zones (Noachtar et al., 2009; Verhellen et al.,
2007). Unfortunately, there are no previous systematic studies available to characterize the pathophysiological mechanisms and abnormal cellular substrates in these irritative areas since histological data are available only from the final resective zones. To address this issue we combined EEG brain source imaging with histological analysis from the irritative areas which would be only possible using preclinical animal models.

1.8 OBJECTIVE AND HYPOTHESIS

The objective of the project was to characterize the pathophysiological mechanisms and abnormal cellular substrates of the irritative areas using a preclinical animal model by combining: a) an animal model of FCD, b) a method for EEG brain imaging of IED generators, and c) histological data. The following hypothesis was tested to meet the objective.

H1: Chronic focal epilepsy induces abnormal pathophysiological changes in the irritative areas; hence these irritative zones are of clinical relevance for pre-surgical evaluation of patients with refractory focal epilepsy.

1.9 SPECIFIC AIMS

Listed below are the specific aims to test the above mentioned hypothesis. Please refer to the chapter: materials and methods for details.

Aim 1: To develop a preclinical model of focal cortical dysplasia for Wistar rats.

Aim 2: To develop an accurate methodology for performing EEG brain source analysis on IEDs.
Aim 3: To develop a particular protocol to perform histological analysis on the target irritative tissue.
2. MATERIALS AND METHODS

All experimental procedures were approved by and carried out in full compliance with the Institutional Animal Care and Use Committee at Florida International University (Approval No. 13-004).

2.1 ANIMAL MODEL PREPARATION

All male Wistar rats used to create models with focal cortical dysplasia were received from Charles River Laboratories. The pharmaco-resistant FCD preclinical model was established with male Wistar rats following the protocol described previously by Colciaghi et al. In brief, this protocol includes two major steps: first, the cortical malformations of this preclinical model were induced prenatally on Embryonic Day 15 with two doses of methylazoxymethanol (MAM) injections (15mg/kg maternal body weight) 12 hours apart; second, these MAM-treated rats were intra-peritoneally (i.p.) injected with pilocarpine (PILO, 300 mg/kg) on post-natal day 28 to induce status epilepticus (SE). All MAM-PILO rats were monitored by four investigators (YS, RT, AD and JJR) independently to identify the SE marked by occurrence of continuous motor seizure activity according to the Racine scale (Racine et.al, 1972). Rats experiencing SE received intra-peritoneal phenobarbital (20 mg/kg) 90 min after SE onset to reduce mortality, then were hydrated subcutaneously with Lactate Ringer’s solution and fed by the investigators to improve the survival rate. Rats not experiencing SE were euthanized. After two weeks from onset of SE, rats were observed for occurrence of seizures for one week to confirm the chronic epileptic models. Eventually, thirteen MAM-PILO rats survived from this procedure and developed
chronic seizures. From this point on, these MAM-PILO rats will be referred to as epileptic rats. All epileptic rats were housed in standardized cages at a 12h-12h light-dark cycle with food and water ad libitum.

**Figure-(1): Methodology Overview.** An overview of the methodology illustrating steps involved in brain source imaging and rat brain scaling for further histological analysis.
An overview of the methodology is described in figure-1 with epileptic rat with EEG-mini cap placed on scalp for EEG recording. A red marking in EEG trace show an IED subtype to be selected followed by illustration of IED subtype clustered from 32-channel scalp EEG. sLORETA localized sources are shown based on selected IEDs. The coordinates of these localized brain sources were scaled with respect to experimental brain using Paxinos and Watson atlas as shown in figure-1.

2.2 EEG RECORDING

There is a difficulty for EEG recording in small animals because of small surface area of scalp compared to human scalp. To solve this problem our group has a patented technology which provides EEG mini cap with 32 spatial electrodes suitable for rodent EEG recording (Riera J, et. al., 2011) as shown in figure-1. Weight of the rat was measured before initial anesthesia with isoflurane (5% for initial induction and then 1-2%, 1 L/min O₂, 14 PSI) during all the manipulations. This was followed by shaving of rat scalp and fixing rat head in a stereotaxic frame. The scalp was rubbed with ethanol (90%) for delipidation and a saline swab was kept on the scalp to keep it wet until the cap was fixed. After fixing the cap on the rat scalp an impedance check was
carried out to ascertain the performance of each electrode (Riera J, et al., 2011). With this preparation, EEG ongoing activity was recorded (PZ3, Tucker-Davis Technology) for a period of thirty minutes. The rat body temperature was maintained using a water-circulating heating pad (TPZ-0510EA, Texas Scientific Instruments, LLC) with a pump (TP700, Texas Scientific Instruments, LLC). The rat body temperature (~37°C), heart rate (200-300 beats per minute) and respiration rate (<50 breaths per minute) were monitored continuously by PowerLab 8/35 data acquisition device and LabChart software (AD Instruments) to assure that level of anesthesia remained stable throughout the entire experimental procedures. EEG signals were amplified, filtered (0.5 Hz high-pass, and 250 Hz low-pass), and digitized (5 kHz sampling rate, 0.5 μV resolution).

2.3 EEG PROCESSING, PARAFAC AND CLUSTER ANALYSIS

IEDs that included temporal patterns which are spikes (20-70 ms in duration, 15-50 Hz) and sharp-waves (70-200 ms in duration, 5-15 Hz), were selected by a combination of multiple band-pass/notch filters and an auto-thresholding method from the averaged signals of the 32-channel scalp EEG. The unique multi-linear decompositions of multi-way arrays of data (more than two dimensions) are possible under certain conditions according to the principle of Parallel Factor Analysis (PARAFAC) (Sidiropoulos and Bro, 2000). In PARAFAC, the data is decomposed as a sum of components (space/frequency/time) for three-way arrays. The important improvement in PARAFAC as compared to traditional techniques such as PCA or ICA is that the decomposition of multi-way data is unique even without additional
orthogonality or independence constraints (Fumikazu Miwakeichi et al., 2004). Thus we employed PARAFAC analysis decomposing the EEG data into space/time/sample components along with super paramagnetic clustering (fig. 2), a clustering method from statistical mechanics (Pedreira C, et al., 2014). This combination allows identifying small but consistent differences in the analysed signals. The IED detection method is similar to those reported in human studies (A. Salek-Haddadi et al, 2006; E. Kobayashi et al., 2006), as well as animal studies (R. Rathakrishnan et al., 2010; F. Pittau et al., 2013).
Figure-(2): IED Classification. (A) PARAFAC 3D (space/time/sample) clustering based on k-mean values. (B) Raster plots representing observed clusters with respect to time. (Colors show respective clusters).
Subsequently, spikes and sharp-waves were separated into multiple sub-types (fig 3), using a series of feature extraction and classification methods described previously (R. Rathakrishnan et al., 2010; F. Pittau et al., 2013). Each sub-type of IEDs was considered as a unique event. Event-related 32-channel EEG potentials were created by averaging the EEG data time-locked to the IED onsets within corresponding window size (80-ms for spikes and 200-ms for sharp-waves).

2.4 sLORETA INVERSE SOLUTION

To define a volume conductor model, the open source software, Brainstorm (Tadel F, et al, 2011), was used with the MRI atlas for Wistar rats (Valdez-Hernandez P, et al, 2011). However, individual rat’s MRI can also be used to generate the volume conductor model if available. The input of MRI surface and brain surface were given to the software and head surface was generated with default setting. For lead field computation scalp and inner/outer skull surfaces based on MRI were generated (Tadel F, et al, 2011). The orientation and location of each surface were co-registered with respect to the MRI using the visualization option (Tadel F, et al, 2011). Using the rat head picture with three reference points acquired immediately after EEG recording, their positions were co-registered in the MRI. The same references were used to generate the electrode positions as the electrode positions are fixed with respect to reference points. Electrode position matrix \((N \times 3)\) based on the three reference points was generated. Here, \(N\) is the number of channels \((N = 32)\) and the column represents the corresponding \(x, y,\) and \(z\) coordinate values. Using the generated channel file, the
location of all electrodes was displayed and confirmed. Any misplaced electrodes were modified manually (Tadel F, et al, 2011). It was made sure that the final coordinate system for the electrode positions coincides with the coordinate system used for the MRI and brain surfaces. The conductivity values which satisfy the ratio of skin, skull, and brain as 1 : 1/80 : 1 were specified to obtain the lead field matrix based on the volume conductor model and the electrode positions already created. sLORETA solution (Pascual-Marqui, R. D. 2001) was obtained based on the computed lead field matrix and the input EEG signals. By selecting the source estimation method option, the inverse solution was obtained and estimated sources were plotted (Tadel F, et al, 2011). sLORETA distributed inverse solution was estimated from the event-related potentials for each IED sub-type using Brainstorm (fig. 3) (Y. Lu et al., 2004). For each epileptic rat, surgically accessible location for craniotomy was determined based on the source locations at the initial phases of each IED sub-type. These source locations were believed to be associated with irritative zones. A T2-weighted MRI atlas for Wistar rats was used to calculate the electric lead fields (J. Riera et al, 2004). This atlas is defined on the Paxinos and Watson’s coordinate system (Y. Zheng et al, 2002).
Figure-3: Time series and EEG topography for each IED subtype. Analysis performed at a specific time instance represented by a red line in time series.
2.5 HISTOLOGY AND sLORETA CO-REGISTRATION

Once estimated sources based on the sLORETA inverse solution were visualized in the 3D brain region of the pre-clinical model, the coordinates of the identified target irritative regions were scaled using Paxinos and Watson’s Atlas (G. Paxinos and C. Watson, 2007) with respect to the rat brains to identify the tissue blocks to perform immunohistochemistry. For scaling, known coordinates (0.0) of bregma (B1) and a length of a point (L1) (-9.3 mm from B1) from the anterior end were used as reference from Paxinos and Watson’s atlas. In the rat brains the length of the point (L2) from the anterior end was measured (all length measurements were considered in the anterior to the posterior direction). This information was used to determine coordinates of bregma in rat brains (B2) and to calculate scaling factor (~0.74 for each rat). Product of scaling factor and known distance of the target irritative regions from the bregma (B1) obtained from source localization gave us real-time coordinates of target regions in the rat brains. Tissue blocks of 3mm thickness around the target region were determined for immunohistochemistry.

Immunohistochemistry of tissues is a powerful tool used to demonstrate the presence or absence of an antigen. The immunocytochemical staining of brain tissue can be compromised by factors such as time of tissue dissection, type of fixation and storage time in fixative. Sometimes an animal has to go through different research studies which make it difficult to obtain fresh brain tissue, or brain tissue that has been in fixative for a short time for scientific research purposes and may not be available until after several months of fixation. If fixed for longer times, the antigens are masked by
crosslinking of proteins by aldehyde over fixation. Proteolytic enzyme predigestion is often used to unmask the antigens (e.g., Battiflora and Kopinski, 1986) but the results are not satisfactory with over fixed tissue. Shi et al. (1991) described a method of antigen retrieval from formalin-fixed, paraffin-embedded human brain sections based on microwave heating in metal solutions. However no single methodology is available to immunostain all the antigens in rat brains that are fixed for long times. Here we demonstrate a procedure to process and immunostain whole brains of focal epileptic rat models that have been fixed for few months to a year and to identify three categories of focal epilepsy specific biomarkers namely, anatomical, inflammatory and functional with fluorescence immunohistochemistry.

2.5.1 FIXATION AND TISSUE PROCESSING

Whole animal perfusion was performed on an epileptic Wistar rat after EEG recordings (Gage, G, et al, 2012). The brain was dissected and preserved in fixative (4% paraformaldehyde) for few months till brain sources were identified by applying EEG source analysis as mentioned before. Prior to immunohistochemical staining brain was removed from fixative and washed with distilled water for 3-4 hrs. Following washing, rat brain was incubated in a 4% aluminum chloride solution at room temperature for about 12 hours before heating in microwave for 10 minutes with 1 minute interval after 5 minutes. Dimensions of brain were measured before and after heating to calculate scaling factor to compensate for shrinking of brain upon heating. Accurate position of Bregma was calculated from the scaling factor. Brain tissue was kept in 30% sucrose
solution at 4°C for cryoprotection prior to the sectioning. After about 3 days when tissue sinks in sucrose solution, it was taken out and placed on the solid mold with the coronal matrix with slots positioned 1 millimeter (mm) apart. With this mold a 3 mm coronal segment of interest of rat brain was cut and positioned at the centre of a cubical plastic mold filled with tissue freezing medium which supports the tissue during cryostat sectioning because of its highly viscous and water soluble properties. Tissue was then snap frozen in liquid nitrogen, plastic mold peeled off and tissue block was loaded on cryostat head with the help of specimen disc. Tissue block was allowed to equilibrate at -22°C for 1 hr.

2.5.2 SECTIONING WITH CRYOSTAT

The frozen tissue block was mounted on the cryostat head and its orientation was kept in the proper x-y plane with reference to the blade. A well oriented tissue block will first meet the blade at the centre of the block. After mounting the chuck with frozen tissue embedded in tissue freezing medium, first step was the trimming. The face of tissue block needs to be trimmed till a desired depth in the tissue is reached. Each clockwise rotation of the hand wheel advanced the mounted tissue block in the coronal direction as per the set thickness (30μm). In case of focal epileptic rats, sectioning should be precise to the target area as determined from the BOLD signal evoked by IEDs. The target area was scaled to real-time coronal coordinates using Paxinos and Watson’s Atlas (G. Paxinos and C. Watson, 2007). While sectioning, the coronal
coordinates at which serial sections were taken were tracked by thickness and the number of section from anterior-to-posterior direction.

2.5.3 IMMUNOHISTOCHEMICAL STAINING

Sections on glass slides were rehydrated with 0.01M phosphate buffered saline (PBS). The endogenous peroxidase activity was suppressed by incubating the sections in 3% hydrogen peroxide and 0.2% Triton X-100 in PBS for 30 minutes. Sections were washed and incubated in 3% bovine serum albumin (BSA) in PBS for 1 hour to block non specific antibody binding to the tissue before incubation in primary antibodies followed by secondary antibodies for 1 hr each. Both the primary and secondary antibodies were diluted in PBS.

Serial tissue sections of 30μm thickness were double immunostained using a principle of sequential immunostaining method as described below.

1. Permeabilization and endogenous peroxidase blocking step: Apply 0.2% Triton X-100 with 3% hydrogen peroxide in 0.01M PBS for 30 minutes.

2. Wash the sections with PBS for 10 mins minutes. Remove the excess PBS with a dropper or pipette. Repeat three times.

3. Blocking step: Incubate the sections in 3% Bovine Serum Albumin (BSA) in 0.01M PBS for 60 minutes to block unspecific binding of the antibodies.

4. Incubate sections with the first primary antibody in 2% BSA in 0.01M PBS in for 1 hour at room temperature.
5. Decant the first primary antibody solution and wash the cells three times in PBS, 10 minutes each wash.

6. Incubate cells with first secondary antibody (labelled with Fluorochrome-1) in 2% BSA in 0.01M PBS for 1 hour at room temperature in dark.

7. Decant the first secondary antibody solution and wash three times with PBS for 10 minutes each in dark.

8. Incubate cells with the second primary antibody in 2% BSA in 0.01M PBS in the dark for 1 hour at room temperature or overnight.

9. Decant the second primary antibody solution and wash sections three times with PBS for 10 minutes each in dark.

10. Incubate cells with second secondary antibody (labelled with Fluorochrome-2) in 2% BSA for 1 hr at room temperature in dark.

11. Decant the second secondary antibody solution and wash three times with PBS for 10 minutes each in dark.

12. Remove excess of the PBS from the sides of the slide and cover slip sections with mounting medium (ProLong® Diamond Antifade Mountant).

   For biomarker staining experiments following biomarker staining protocols were applied on serial tissue sections (30μm).

1. Neurofilament (SMI311) staining.
2. TNF-α and GFAP sequential double immunostaining.

3. HMGB1 and GFAP sequential double immunostaining.

4. IL-1β and GFAP sequential double immunostaining.

5. GABAARα6 and Neurofilament (SMI311) sequential double immunostaining.

6. mGluR5 and GFAP sequential double immunostaining.

7. NMDAR2B and Neurofilament (SMI311) sequential double immunostaining.

8. Nissl immunostaining.

For data analysis biomarkers were grouped in three categories as anatomical, inflammatory and functional biomarkers and details about reagents used are given following sections.

2.5.3.1 ANATOMICAL BIOMARKER STAINING

The sections were incubated for 1 hour at room temperature with one of the following primary antibody mouse anti-neurofilament (SMI 311, 1:2000). Sections were washed and incubated with respective secondary antibody: Bovine anti-mouse IgG (sc-362246, 1:400). For Nissl staining sections were incubated for an hour with Neurotrace stain (N-21480, 1:300) after permeabilization (0.2% Triton X-100) step.

2.5.3.2 INFLAMMATORY BIOMARKER STAINING

The sections were incubated for 1 hour at room temperature with one of the
following primary antibodies: rabbit anti-IL-1β (sc-7884, 1:300), goat anti-TNF-α (sc-1350, 1:300), rabbit anti-HMGB1 (ab79823, 1:500). Sections were washed and incubated with respective secondary antibodies: donkey anti-rabbit IgG (Alexa Fluor 680, 1:1000), donkey anti-goat IgG (sc-362255, 1:300), donkey anti-rabbit IgG (Alexa Fluor 680, 1:1000). For GFAP staining sections were incubated for an hour with primary monoclonal mouse Anti-Glial Fibrillary Acidic Protein-Cya3 (Sigma C9205, 1:500).

2.5.3.3 FUNCTIONAL BIOMARKER STAINING

The sections were incubated for 1 hour at room temperature with one of the following primary antibodies: rabbit anti-NMDAR2B (AB1557P, 1:500), goat anti-mGluR-5 (sc-47147, 1:300), rabbit anti-GABAA Receptor alpha6 (AB 5610, 1:300). Sections were washed and incubated with respective secondary antibodies: donkey anti-rabbit IgG (Alexa Fluor 680, 1:1000), donkey anti-goat IgG (sc-362255, 1:300), donkey anti-rabbit IgG (Alexa Fluor 680, 1:1000).

2.5.3.4 DATA COLLECTION AND ANALYSIS

To evaluate the IED-generating irritative regions, localized by using sLORETA inverse solution, coronal sections from corresponding cortical region in seven FCD rat brains were photographed with a Delta Vision Elite System. Raw images were processed using background subtraction feature in Image-J open source software. Number of Nissl bodies, perivascular cuffing and number of neuronal processes were
counted using cell counter plug-in for Image-J software. Perivascular cuffing structure was counted as continuous vasculature visible in photographs. Expression of all other epilepsy specific biomarkers were computed as stained area as number of pixels in an image after applying frequency threshold to avoid detecting background signal using ImageJ software. For each rat, average of six 20x images spanning cortical thickness for each biomarker from target region were considered for analysis. Similarly control data were collected and analysed from contra-lateral cortex. In this way target and control data was collected from seven chronic epileptic rat brains. To determine the distribution of the data, Q-Q plot (probability plot) a graphical method was employed where linearity of data suggests normal distribution. Based on the distribution of the data, statistical analyses were performed using Paired t-test where data were normally distributed and Wilcoxon’s Signed Rank test where data were not normally distributed.
3. RESULTS

3.1 SOURCE LOCALIZATION

Once all procedures are properly applied, estimated sources can be visualized in 3D brain region of the pre-clinical model. Figure 1 (A) show step wise methodology applied on preclinical rat models of FCD to determine the estimated source from localization of spikes and sharp-waves from IEDs. In addition, Figure-2 and 3 display classification of subtype spikes and sharp-waves from IEDs. This methodology was successfully applied to seven preclinical rat models and a target irritative region was decided for each rat based on the criteria as given in Table-1. For each rat, number of clearly classified averaged spikes and sharp-waves were localized separately (IEDs in Table-1). Each spike and sharp-wave gave certain localized regions represented by assigned numbers (localized structures in Table-1, where structures listed as most frequent to least frequent) according to the Paxinos–Watson atlas and the averaged MRI. From this data, for each rat most frequently localized region (most frequent structure in Table-1) was considered as target irritative region for further histological analysis. These results support the capability of the applied methodologies to record high-resolution EEG on rats with focal epilepsy and to conduct source analysis using the recorded EEG.
Table-1: Criteria for source determination

<table>
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<tr>
<th>Rat</th>
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<th>Localized Structures</th>
<th>Most Frequent Structure</th>
<th>Target area</th>
<th>Seizure During Recording</th>
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3.2 ANATOMICAL BIOMARKERS

We used Nissl staining with 0.2% triton X-100 and NeuroTrace stain (N21478, Invitrogen) method to detect the Nissl bodies which are large granular endoplasmic reticulum with rosettes of free ribosomes in the neurons. Nissl bodies show changes under various pathological conditions.

![Nissl & Neurofilament (SMI-311) staining](image)

Figure-(4): Anatomical Biomarkers. Nissl & Neurofilament (SMI-311) staining. Scale bars: 50μm.

We observed that cortical neurons in the control group showed organized orientation compared to Nissl staining observed in target cortex as shown in figure-4. We also reported neuronal loss as indicated by number of Nissl bodies (figure-5) compared in control and target area (p-value< 0.01).
Overexpression of SMI311 positive neurofilaments is another important feature in FCD. These neurofilaments targeted with monoclonal anti-SM311 antibody enable to identify the presence of abnormally large pyramidal neurons often known as cytomegalic neurons, frequently grouped in cell clusters, with increased expression of SMI311 positive neurofilaments (figure-4), particularly in the apical dendrites. We analysed over expression of SMI311 positive neurofilaments in target area by counting the neuronal processes (p-value< 0.01) and by measuring the total area of neuronal processes (p-value< 0.01) in comparison with control cortex as presented in figure-5.
3.3.1 INFLAMMATORY BIOMARKERS

Expression of Glial Fibrillary Acidic Protein (GFAP) is associated with FCD. GFAP is a useful marker which was a target for monoclonal anti-GFAP antibody (Sigma C9205) to demonstrate the increased glial activity and presence of astrocytes which are abnormal in their size and orientation (figure-8) in the pathology of FCD. The GFAP expression was quantified by computing total stained area of glial processes (p-value< 0.01) as shown in figure-10. Neuroinflammation in FCD can be identified by release and presence of specific inflammatory agents such as Interleukin 1 beta (IL-1β), tumor necrosis factor alpha (TNF-α) in inflamed cortical tissue which activates and triggers further immune response.

Fig-(6): Inflammatory Biomarkers. IL-1β, HMGB1 & TNF-α staining. Scale bars: 50μm.
Significantly increased expression of IL1β and TNFα in target area was demonstrated by comparing stained area with control cortex (p-value< 0.05, p-value< 0.01 respectively) (figures-6 and 7). Also, IL-1β was found to be colocalized with GFAP at perivascular cuffing (figure-8). High mobility group protein 1 (HMGB1) secreted by immune cells and inflamed cells as an indicator of cell death during neuroinflammatory process. Presence of HMGB1 was confirmed by HMGB1: rabbit polyclonal antibody (Pharmigen, 1:100, Maroso et al., 2010). HMGB1 expression was found to be significantly increased in target area (p-value< 0.01) (figures-6 and 7).
3.3.2 PERIVASCULAR CUFFING

We report numerous diffuse and patchy areas of infiltration of the cortex and white matter by inflammatory cells, predominantly glia defined as perivascular cuffing (figures-9). Sections from target area showed intensive perivascular cuffing (p-value<0.05) (figure-10) underlined by presence of glial cells around inflamed vasculature. The astrocytes were hypertrophied and increased in number.

Figure-(8): IL-1β and GFAP Co-registration. Scale bars: 50μm.
Figure-(9): Perivascular Cuffing. GFAP staining. Scale bars: 50μm.

Figure-(10): Statistical representation of Perivascular Cuffing. Bars represent standard error of mean (SEM). **P<0.01.
3.4 FUNCTIONAL BIOMARKERS

Functional markers are associated with specific post-translational changes in the pathology of FCD. Increased glutamate function which is associated with hyperexcitability in epileptogenic cortex was analysed with amplified expression of the NR2B subunit of the N-methyl-D-aspartate glutamate receptor (NMDR) (p-value< 0.05) (figures-11 and 12) and expression of group I metabotropic glutamate receptor 5 (mGluR5) in target area was identified (p-value< 0.001) (figures-11 and 12). NR2B was found to be intensively co-localized with neurofilaments in target area (figures-13) whereas mGluR5 was poorly co-registered with GFAP (figures-14). The inhibitory function of gamma-aminobutyric acid (GABA)ergic system was observed by analysing the expression of the GABAA-receptor α-6 subunit by directing anti-GABAAr α-6 rabbit polyclonal antibody against it. We found no significant difference between localized and control cortex (figures-11 and 12).
Figure-(11): Functional Biomarkers. NR2B, GABA_A-α6 & mGluR5 staining. Scale bars: 50μm.

Figure-(12): Statistical representation of Functional biomarkers. Bars represent standard error of mean (SEM). *P<0.05; ***P<0.001.
Figure-(13): Co-registration of NR2B and SMI-311. Scale bars: 50μm.

Figure-(14): Co-registration of mGluR5 and GFAP. Scale bars: 50μm.
4. DISCUSSION

The PI has developed several experimental and analytical tools in the past to study the Wistar rats. This constitutes the main reason for modifying the (Colciaghi et al., 2011) model, which was originally developed using Sprague–Dawley, for Wistar rats. Rat model for FCD created by prenatal treatment of methylazoxymethanol acetate (MAM) has a potential to mimic the pathologic and clinical features of FCD. We have provided extensive evidence for the validity of this chronic epileptic model using EEG data (Bae et al., 2015), IED-evoked fMRI response (Song et al., 2015), and intracranial recordings (Song et al. 2016). This model gave us insights about the cellular and molecular mechanisms of epileptogenesis which are evident with similar markers observed human FCD subjects. Unfortunately, this model does not show presence of balloon cells (abnormally large neurons with abundant cytoplasm) which are observed in human FCD subjects.

4.1 SOURCE LOCALIZATION

A methodology to non-invasively record multichannel EEG in a preclinical animal model of chronic focal epilepsy is described. The details for the recording and analysis procedures are provided. There were key factors to consider achieving successful results. First, obtaining high quality signals is essential during EEG recordings. There is a lot of evidence about the negative effect of different type of anesthesia on the genesis of IEDs which was confirmed by our preliminary experiments where EEG recording was performed under isoflurane (2%). These negative effects were significantly absent in EEG recordings performed under sedative (0.25mg dexdomitor). For this reason we
employed a protocol where anesthesia induction was done with 5% isoflurane during animal manipulation phase (i.e. fixing an animal to stereotaxic frame, scalp shaving) the reducing isoflurane slowly to 0.5% and obtaining EEG recording under sedative. Second, for brain source imaging, generating proper volume conductor model is crucial. Each surface of the model should be co-registered. Even though we have applied source analysis procedures using Brainstorm (Tadel F, et al, 2011), other open softwares (Delorme, A, et al, 2004; Oostenveld R, et al, 2011) and commercial products (Koessler L, et al, 2010; Manganotti P, et al, 1998) can be used. Also, besides sLORETA, other inverse solutions such as multiple dipole models and Beamformer can be applied (Baillet S, et al, 2001). Our results support the importance for a precise classification of IED markers from EEG recordings to determine the IED-generating irritative regions in a rat with focal epilepsy and further to evaluate their relationship with the underlying mechanisms for seizure initiation (Song Y, et al, 2014). In addition, it has been shown that EEG source localization for such specific IEDs showed a good correspondence with the respective BOLD activation and deactivation regions (Bae J, et al, 2015).

We believe that the use of this methodology will produce similar results when applied to patients with FCD, in this regard preparing IRB protocols for the evaluation of this and other aspects of the methodology in human dataset will be a near future prospect. Moreover, the use of preclinical models will help us understand the capabilities and limitations of EEG source localization in epilepsy (Birot G, et al, 2014). Accurate estimation of brain sources underling epileptogenesis is crucial for therapeutic strategies and resective surgical planning. Also, having a standard platform for EEG
recording in rats will be useful for the evaluation of the effectiveness of several anti-
epileptic drugs in preclinical trials. The entire methodology presented in this study is 
extendable to other experimental conditions and brain disorders. This methodology has 
been developed for Wistar rats because of the existence of an MRI atlas for this 
particular rat strain. However, it can be applied to other rodent types with respective 
standard format of atlas including mouse (Hawrylycz M, et al, 2014), Sprague-Dawley 
rats (Schweinhardt P, et al, 2003), and Paxinos and Watson rats (Schwarz A, et al, 
2006). We have provided extensive evidence for the validity of the used MAM-PILO 
chronic epileptic model using EEG data (Bae et al., 2015), IED-evoked fMRI response 
(Song et al., 2015), and intracranial recordings (Song et al. 2016).

4.2 ANATOMICAL BIOMARKERS

Hyperexcitability in a neuronal tissue is a pathological condition leading to 
epileptic seizures underlines the focally injured tissue in FCD (Badawy RA, et al, 2012) 
characterized by an abnormal neuronal activity in surrounding cortical tissue 
analyzed morphology and protein expressions of key biomarkers in IED-generating 
irritative areas in FCD with seizures, to investigate the hypothesis that chronic epilepsy 
induces pathological changes in the irritative areas. We found that the irritative area of 
focal epileptic rats was characterized by the abnormal neurons over expressing 
neurofilaments, as well as reduced neuronal mass demonstrated by Nissl staining. 
Consistent results were reported previously in dysplastic compared to non-dysplastic 
areas in type IIA and IIB FCD, (Thom M, et. al, 2005).
4.3 INFLAMMATORY BIOMARKERS

Recent evidence strongly suggests that glia mediated inflammation plays a role in the pathogenesis of seizures and epilepsy (Orrin D, et al, 2013). We found extensive reactive gliosis (with altered astrocyte morphology) in localized irritative region which corroborates finding that longer chronic epilepsy is associated with more intensive reactive gliosis and astrocyte alterations in epileptogenic region, further evidence that epilepsy is a progressive pathology (Finardi A, et al, 2013). Astrocytes undergo changes in morphology, molecular composition, and proliferation in epileptogenic region. The activation of astrocytes includes changes that vary with the severity and chronicity of seizure pathology (Sofroniew, M.V, 2009). Reactive astrocytes has been reported in animal models of epilepsy and in neural tissue from human patients with mesial temporal sclerosis (MTS), focal cortical dysplasia (FCD), tuberous sclerosis complex (TSC), Rasmussen’s encephalitis (RE), and glioneuronal tumors (Jabs R, et al. 2008; Bauer J, et al, 2007; Binder D, et al, 2006). In case of Rasmussen’s encephalitis, epilepsy with chronic brain inflammation, astrocytes are a specific target of immune system, particularly cytotoxic T cells (Bauer J, et al, 2007). MTS, the most common pathology associated with temporal lobe epilepsy (TLE), is characterized by astroglial and microglial activation and proliferation (Wetherington J, et al, 2008), with increased complexity of astroglial processes, often approaching glial scar-like formations in late stage MTS (Martinian L, et al, 2009).

Astrocytes are closely related to the microvasculature and with their endfeet ensheathing blood vessels (figure-8 and 9) known as perivascular cuffing contribute to
blood-brain-barrier (BBB) function by releasing chemical signals that help to form and maintain tight junctions between endothelial cells, thus regulating the exchange of water and molecules between the blood and brain tissue (Orrin D, et al, 2013). We observed perivascular cuffings and inflammatory glial nodules in epileptic rats which were more intense in target irritative areas. These structures have been attributed in the past to tissue breakdown associated with frequent seizures (Theodore Rasmussen, et al, 1958).

The brain microvasculature undergoes several structural, molecular, and functional changes in epilepsy. Vessel proliferation in TLE positively correlates with seizure frequency (Rigau V, et al, 2007) and is associated with compromised BBB permeability (Rigau V, et al, 2007; Marcon J, et al, 2009). Proinflammatory chemokines and cytokines released by inflamed epileptogenic tissue can interact with their respective receptors over expressed by brain microvasculature in epilepsy, thus compromising BBB permeability at multiple levels [e.g., by disrupting tight junction proteins (Morin-Brureau M, et al. 2011), increased infiltration of leukocytes and viral particles through the BBB into the brain parenchyma]. In response to inflammatory cytokines, excessive leukocyte trafficking by interacting with adhesion molecules on endothelial cells may alter BBB permeability to serum proteins and circulating molecules (Fabene P, et al, 2008; Kim J, et al, 2009).

Glial cells play a critical role by maintaining balance between proinflammatory and anti-inflammatory pathways. Activated glia release cytokines inducing transcriptional as well as, post-transcriptional signalling in the astrocytes and other immune cells. Immune system and glia including astrocytes release IL-1β and high-
mobility group box 1 protein which activate nuclear factor kappa B (NF-kB), an important regulator of proinflammatory gene expression which has been reported to be upregulated in MTS and TSC tissue (Crespel A, et al, 2002; Maldonado M, et al, 2003). In case of IL-1β and HMGB1 release, signaling occurs through activation of the pro-inflammatory IL-1 receptor/Toll-like receptor (IL1R/TLR) system. This system is activated in epilepsy models and in human MTS, TSC, and FCD tissue (Vezzani, A. et al. 2013; Maroso, M. et al. 2010; Zurolo, E. et al. 2011). Astrocytes help to limit the immune response by controlling microglial activation. It has been demonstrated in in-vitro experimental settings that astrocytes can reduce the production of proinflammatory and neurotoxic TNF-α, nitric oxide and reactive oxygen species from microglia to inhibit microglial phagocytosis (Tichauer, J. et al. 2007). However, our results showed increased TNF-α staining in target area with lack of colocalization with GFAP indicating presence of proinflammatory immune response with neuronal source of TNFα.

In in-vivo seizure models, astroglial cells are also key sources of anti-inflammatory molecules such as the IL-1 receptor antagonist (IL-1ra), an endogenous competitive IL-1 receptor blocker that controls IL-1β-mediated inflammation. IL-1ra has powerful anticonvulsant effects in experimental seizure models (Vezzani, A. et al. 2000; Auvin, S. et al. 2010; Marchi, N. et al. 2009) and mice over expressing IL-1ra in astrocytes were reported to be intrinsically resistant to seizures (Vezzani, A. et al. 2000). However, in MTS and experimental epilepsies, astrocyte expression of IL-1ra is significantly lower than that of IL-1β (De Simoni, M.G. et al. 2000; Ravizza, T. et al.
2006), which may explain poor anti-inflammatory response due to the dominance of proinflammatory pathways over anti-inflammatory pathways which we demonstrate to be consistent with our finding of IL-1β over expression in epileptogenic region. Also, proinflammatory IL-1β was found to be colocalized with GFAP at perivascular cuffing (figure-8) indicating active inflammation thus supporting previous study that report astrocyte derived interleukin-1 beta (IL-1β) can compromise BBB integrity during seizures (Librizzi, L. et al. 2012).

4.4 FUNCTIONAL BIOMARKERS

So far, it is clear that cortical injuries are associated with abnormal excitability of the adjacent neuronal networks focal epilepsy. It has been assumed that the development of this abnormal neuronal activity is the result of an imbalance between excitation and inhibition, where changes in inhibition are believed to play the most important role (Imbrosci B, et al, 2011). An impaired inhibitory transmission has been reported in several animal models of both traumatic and ischemic brain injury (Buchkremer-Ratzmann I, et al, 1996; Mittmann T, et al, 1994). Functionally, the impaired GABAergic transmission has been attributed to reduction in release of GABA from presynaptic terminals (Li H, et al, 2002) and changes in the expression of GABA receptors (Schiene K, et al, 1996). In an attempt to assess the inhibitory function at localized epileptogenic tissue, we found no significant reduction (figure-12) in GABAA-receptor-α6 subunit of GABA receptor family. To reach a proper conclusion in this regard we need to analyse the expression profiles of entire GABA receptor family and also need to take into account the previous studies (Schiene K, et al, 1996) reporting differential GABAergic transmission in and around epileptic foci in epileptogenic cortex.

Activation and high expression of group I metabotropic glutamate receptors (mGluRs) may contribute to neuronal hyperexcitation, as suggested by the convulsant action of group I agonists, which are responsible for seizure discharge and epileptogenesis in different experimental models (Chapman AG, 1998; Wong R. et al, 1999). Neuronal mGluRs are known to have important roles in synaptogenesis and synaptic plasticity and in pathophysiological processes (Chapman AG, 1998; Merlin L,
et al, 1997). In our study we observed elevated expression of mGluR5 (p-value < 0.001) in irritative region (figure-11 and 12) with the dominant source being neuronal mass as evident from lack of co-localization of mGluR5 with GFAP (figure-14). Since consistent findings were reported in similar study involving glioneuronal tumours (GG) and dysembryoplastic neuroepithelial tumours from clinical patients associated with chronic intractable epilepsy which are highly epileptogenic (Aronica E, et al, 2001), we can speculate that chronic seizure activity could contribute to the strong expression of mGluR5 in the neuronal component of the tumours although the role of group I metabotropic glutamate receptors (mGluRs) is not completely understood (Bruno V, et al, 1999; Herrero I, et al, 1998).
5. CONCLUSION

In this study, we applied a standard non-invasive, multiple electrode scalp EEG source imaging methodology in preclinical ‘double-hit’ rat models of focal epilepsy. We were able to apply this methodology to evaluate preliminarily the underlying brain sources of two types of IEDs. Proper classification of EIDs by using only information in the IED waveform was performed and sLORETA inverse solution was obtained to localize specifically classified IED subtypes.

In order to evaluate this methodology using histological biomarkers as gold standards, we demonstrated a procedure to process and perform immunohistochemistry on brains of chronic focal epileptic rats that have been long time under fixation (for a few months to a year). We were successful in immunostaining three categories of focal epilepsy specific biomarkers with fluorescence immunohistochemistry. The results showed the existence of abnormal neuronal expression as well as glioreactivity, vascular cuffing substantial inflammatory processes in irritative brain areas, independent whether they were the seizure-onset zones or not. We conclude that chronic focal epilepsy induces abnormal pathophysiological changes in the irritative areas; hence these irritative zones are of clinical relevance for pre-surgical evaluation of patients with refractory focal epilepsy and IED-based brain source imaging helps localize these abnormal tissues (irritatives) highly prospective for epileptogenesis.
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