In-Silico analysis, characterization and quantification of EEG alterations in a mouse model of temporal lobe epilepsy

THESIS

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To my father
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<tr>
<td>ILAE</td>
<td>International League Against Epilepsy</td>
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<td>SRS</td>
<td>Spontaneous recurrent seizures</td>
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<tr>
<td>HSV-1</td>
<td>Herpes Simplex Virus Type 1</td>
</tr>
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<td>HHV-6</td>
<td>Human Herpes Virus Type 6</td>
</tr>
<tr>
<td>AEDs</td>
<td>Anti-epileptic drugs</td>
</tr>
<tr>
<td>ASDs</td>
<td>Anti-seizure drugs</td>
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<td>SE</td>
<td>Status epilepticus</td>
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<tr>
<td>TMEV</td>
<td>Theiler's murine encephalomyelitis virus</td>
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<tr>
<td>DA</td>
<td>Daniels strain</td>
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<tr>
<td>SJL / J</td>
<td>Swiss Jim Lambert / Jackson laboratories</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<td>IEDs</td>
<td>Interictal epileptiform discharges</td>
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<td>HFOs</td>
<td>High frequency oscillations</td>
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<td>ECoG</td>
<td>Electrocorticography</td>
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<td>EEG</td>
<td>Electroencephalography</td>
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<td>ASD</td>
<td>Autism spectrum disorder</td>
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<td>LFP</td>
<td>Local field potentials</td>
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<td>IFCN</td>
<td>International Federation of Clinical Neurophysiology</td>
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<tr>
<td>ADHD</td>
<td>Attention-deficit / hyperactivity disorder</td>
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<tr>
<td>EZ</td>
<td>Epileptogenic zone</td>
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<td>SEEG</td>
<td>Stereotaxic electroencephalography</td>
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<tr>
<td>PET</td>
<td>Positron emission tomography</td>
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<tr>
<td>fMRI</td>
<td>Frontal magnetic resonance imaging</td>
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<tr>
<td>SPECT</td>
<td>Single-photon emission computed tomography</td>
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<tr>
<td>MRS</td>
<td>Magnetic resonance spectroscopy</td>
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<tr>
<td>EMG</td>
<td>Electromyography</td>
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<tr>
<td>TLE</td>
<td>Temporal lobe epilepsy</td>
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<tr>
<td>QEEG</td>
<td>Quantitative EEG</td>
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<tr>
<td>CNS</td>
<td>Central Nervous System</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>SNR</td>
<td>Signal-to-noise ratio</td>
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<td>ANN</td>
<td>Artificial neural networks</td>
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<td>DM</td>
<td>Data mining</td>
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<td>LFPs</td>
<td>Local field potentials</td>
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<td>MUA</td>
<td>Multiple unit spiking activity</td>
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<tr>
<td>vEEG</td>
<td>Video EEG monitoring</td>
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<tr>
<td>DSI</td>
<td>Data Science International</td>
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<tr>
<td>ROC</td>
<td>Receiver operating characteristic</td>
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<tr>
<td>B6</td>
<td>C57Bl/6</td>
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<tr>
<td>dpi</td>
<td>Days post infection</td>
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<tr>
<td>pi</td>
<td>Post infection</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>FFT</td>
<td>Fast Fourier transformation</td>
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<tr>
<td>RMS</td>
<td>Root Mean Square</td>
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<tr>
<td>TLE</td>
<td>Temporal lobe epilepsy</td>
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<tr>
<td>DG</td>
<td>Dentate gyrus</td>
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<tr>
<td>CBZ</td>
<td>Carbamazepine</td>
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<td>VPA</td>
<td>Valproic acid</td>
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1. General Introduction

1.1. Epilepsy

1.1.1. Definition of seizures and epilepsy

Epilepsy is a brain disorder typically manifested by sudden brief periods of altered and diminished consciousness, involuntary movements, or convulsions due to abnormal electrical activity in the brain (Merriam-Webster, 2018; World Health Organization, 2018). Globally, epilepsy is one of the most common and significant diseases of the central nervous system (Löscher et al., 2013), affecting approximately 50-70 million people (Ngugi et al., 2010; World Health Organization, 2018). Approximately 4.6 million people develop epilepsy every year (Fiest et al., 2017). According to the International League Against Epilepsy (ILAE), someone is presumed epileptic when he or she has an epileptic seizure and “demonstrates a pathologic and enduring tendency to have recurrent seizures”. (Fisher et al., 2014a). Conceptually, an epileptic seizure is “a brief occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain” (Fisher et al., 2005). The imbalance between excitatory and inhibitory neuronal activity, overshooting and synchronous electrical discharges by groups of neurons in the brain, gives rise to epileptic seizures (Wu et al., 2015). The abnormal neuronal activity in neural pathways involved in seizure development, known as epileptic networks, may lead to inter-ictal and ictal epileptic activity (Luo et al., 2014; McCormick & Contreras, 2001). Although, the hallmark for epilepsy is the presence of epileptic seizures, it is important that seizures and epilepsies are considered and understood separately (Fisher et al., 2014a; World Health Organization, 2018).

1.1.2. Diagnosis of epilepsy

Traditionally, the onset of two spontaneous seizures at intervals of at least 24 hours has been necessary for the diagnosis of epilepsy (Beretta et al., 2017; Fisher et al., 2005). However, epilepsy can be diagnosed after a single unprovoked seizure if sufficient supporting diagnosing evidence from, but not limited to, inter-ictal EEG and brain imaging is available (Beretta et al., 2017). Such a diagnosis also requires knowledge of recurrence risks, in particular clinical circumstances (Fisher, 2015). According to the ILAEs “new operational definition”, epilepsy is diagnosed clinically, when someone has i) at least two unprovoked or reflex seizures > 24 hours apart ii) one unprovoked or reflex seizure and a risk of at least 60% to have another within the following 10 years or iii) an epilepsy syndrome (Fisher, 2015;
Fisher et al., 2014a). A recent retrospective study (PRO-LONG), where patients were diagnosed with one unprovoked seizure along with potential epileptogenic abnormality evidences, has supported the practicality of this new epilepsy definition in clinical practice with a recurrence rate of 83.6% at 10 years (Beretta et al., 2017). However, a person does not necessarily become epileptic after having experienced one seizure, as at least 10% of people worldwide encounter one seizure during their lifetime (World Health Organization, 2018).

In epilepsy, generally, we talk about unprovoked or spontaneous recurrent seizures (SRS). In epilepsy research unprovoked seizures (which are also referred to as late or spontaneous recurrent seizures (SRS)) need to be distinguished from early (also referred to as provoked or symptomatic) seizures and their underlying diseases, which are a risk factor for development of epilepsy (Löschner & Brandt, 2010; Pitkanen et al., 2016). Early seizures are caused by transient factors, e.g., acute nervous system insults such as stroke, trauma, toxicity or infections (Beghi et al., 2010; Rizvi et al., 2017), high grade fever, concussions, or alcohol withdrawal (Scheffer et al., 2016), and are not a symptom of epilepsy, as they temporarily lower the seizure threshold of an otherwise normal brain (Fisher et al., 2014a).

The burden of comorbidities in epileptic patients is often high (Keezer et al., 2016; Löschner et al., 2013; Tellez-Zenteno et al., 2007), as epilepsies are often associated with psychological, neurobiological, and cognitive disorders with social impact affecting quality of life (Fisher et al., 2005; Jacoby et al., 2009; Rudzinski & Meador, 2013). Along with humans, animals such as dogs (Berendt et al., 2015; Frank et al., 2018) cats (Pákozdy et al., 2010) and horses (Lacombe et al., 2012) also suffer from this disease.

1.1.3. Classification of seizures

Epilepsies can be classified based upon the type of seizures and their underlying causes (Falco-Walter et al., 2018). Recently there have been revisions and updates in the classification of different seizure types (Fisher et al., 2017a; Fisher et al., 2017b) and etiologies of epilepsies (Scheffer et al., 2017). These classifications serve as a key clinical tool in evaluating individuals presented with spontaneous seizures and selection of corresponding therapies with antiepileptic drugs (Scheffer et al., 2017).

Seizures can be defined by their onset as: focal, generalized, and unknown (or unclassifiable) (Falco-Walter et al., 2018; Fisher et al., 2017b). Based on their key signs and symptoms seizures can be of known (focal or generalized) or unknown origin (Fisher et al., 2017b; Scheffer et al., 2017). In order to make a decision whether a seizure onset is focal or
generalized, a confidence level of 80% is assumed, otherwise onset is unknown (Fisher et al., 2017a). The presumed seizure origin (focus or network) can be located in different brain areas (Fisher et al., 2014a). Focal-onset seizures originate from neuronal networks limited to one hemisphere of the brain, whereas generalized-onset seizures arise and propagate rapidly involving neural networks in both cerebral hemispheres (Falco-Walter et al., 2018; Fisher et al., 2017b). Unknown-onset seizures are not a category as such, but rather a suitable placeholder for our unawareness about their origin due to limited information available at the time of evaluation (Fisher et al., 2017b).

Focal seizures can be further classified by the state of awareness, which can be either retained (“focal aware seizures”) or impaired (“focal impaired seizures”). Awareness is defined as a knowledge of self and environment (Fisher et al., 2017b). Furthermore, the first sign or symptoms seen during a seizure, marking the seizure focus or network (Falco-Walter et al., 2018), can be used to further characterize focal seizures, which could be of both motor (e.g., clonic, tonic, hyperkinetic) and nonmotor-onset (e.g., cognitive, sensory). An epileptic seizure that has a focal origin and then propagates to both cerebral hemispheres is referred to as a "focal to bilateral tonic-clonic seizure", was previously described by term “secondarily generalized tonic–clonic seizure”, and is placed in a special category due to it common occurrence and importance (Fisher et al., 2017b).

Generalized-onset seizures (usually associated with impaired awareness) can also be broadly divided into motor or non-motor seizures. Non-motor seizures describe absences entailed by a sudden behavioral arrests followed by amnesia, particularly in younger age groups (Meeren et al., 2002). Muscle tone and severity of muscle contractions provide the basis for categorizing motor seizures into various types e.g., atonic, tonic, tonic-clonic, clonic or myoclonic (Fisher et al., 2017a). This classification applies to seizures in adults as well as children, whereas, there is a separate classification for neonatal seizures (Falco-Walter et al., 2018). The frequency of seizure occurrence is highly unpredictable and ranges from many seizures per day to once in a year or even longer (World Health Organization, 2018) with variable severities ranging from brief losses of attention, muscle twitching, and sensory malfunctions to prolonged life threatening seizures (Scheffer et al., 2016). The results of neuroimaging studies, electroencephalography and additional investigations exploring underlying causes of epilepsy are taken into account to classify both seizure and epilepsy type (Scheffer et al., 2017).
1.1.4. Classification of epilepsies

The classification of epilepsies is broader in scope than classifying seizures. However, the seizure type provides a starting point for this classification (Scheffer et al., 2017). Additionally, the overall clinical picture, hereditary tendencies, diagnostic test outcomes and comorbidities are taken into account. Epilepsies can be classified into i) focal, ii) generalized, iii) combined generalized & focal, and iv) unknown (Falco-Walter et al., 2018; Scheffer et al., 2017).

The information collected from clinical observations, patient’s age, family history, types of existing sensory and motor seizures, and EEG findings aids in diagnosing patients with a specific epilepsy type (Falco-Walter et al., 2018). The inter-ictal EEG analysis generally shows the presence of typical generalized spike-wave activity (generalized epilepsy), focal epileptiform discharges (focal epilepsies), and generalized spike-wave and focal epileptiform discharges (combined generalized & focal epilepsies) at the time of diagnosis. In cases when a clinician is sure of a patient having epilepsy, but does not have enough clinical evidence to support the decision as well as a normal EEG, an unknown epilepsy is identified (Scheffer et al., 2017).

1.1.5. Etiologies of epilepsy

Finding the underlying cause of epilepsies on primary patient contact is desired for appropriate treatment options (Falco-Walter et al., 2018). Modern diagnostic methods have tremendously improved the possibility of precisely diagnosing the underlying disease causes and epilepsies can be divided into different groups based on their etiology (Scheffer et al., 2017). For better clinical application and comprehension of disease, these classification groups have been subdivided in to (i) structural, (ii) genetic, (iii) infectious, (iv) metabolic (v) immunological and (vi) unknown groups in recent updates by the ILEA (Falco-Walter et al., 2018; Scheffer et al., 2017). A broad spectrum of epilepsy inducing factors is largely covered in aforementioned groups explained below & there is a possibility that there are multiple underlying known or unknown causes to the development epilepsy disease (Scheffer et al., 2017; Scheffer et al., 2016).

i) **Structural etiology:** Structural epilepsies are due to abnormalities of brain structure, observed during neuroimaging studies such as MRI, leading to a reasonable inference that imaging abnormality being the probable cause of seizures. These abnormalities can be acquired (e.g. trauma, stroke & infection) or
innate (e.g. cortical malformation). One common finding is the presence of hippocampal sclerosis associated with mesial temporal lobe epilepsy (Scheffer et al., 2017).

ii) Genetic etiology: Epilepsies which have a known or presumed underlying genetic abnormality are included in this group. These could be acquired de-novo mutations, e.g. SCN1A (sodium channel gene) mutations (Claes et al., 2001), or hereditary autosomal dominant disorders, e.g. Benign Familial Neonatal Epilepsy (Grinton et al., 2015). Although they can be familial or acquired, involvement of the environmental factors is not ruled out (Scheffer et al., 2017).

iii) Metabolic etiology: Metabolic causes related to biochemical changes in the body such as porphyria, uremia, and aminoacidopathies associated with seizures fall in this group. They also most likely have genetic bases, but could be acquired such as cerebral folate deficiency (Scheffer et al., 2017).

iv) Immune etiology: When an immune disorder has seizures as a primary symptom of the underlying immune illness, it is denoted to be an immune epilepsy (Scheffer et al., 2017). Many of these have been recently characterized in both adults and children. Autoimmune mediated CNS inflammation may provide an indication of immune system mediated epilepsies (Vezzani et al., 2016).

v) Infectious etiology: Epilepsies caused by infectious etiologies are the most common worldwide (Vezzani et al., 2016). Infectious pathogens such as viruses (e.g. herpes viruses, West Nile viruses), parasites (e.g. Plasmodium falciparum), bacteria (e.g. mycobacteria) and fungi (e.g. Cryptococcus and Candida albicans) can cause epilepsies (Vezzani et al., 2016). The acute seizures caused by insults such as meningitis or encephalitis do not represent the disease, which is characterized by the presence of late seizures (Scheffer et al., 2017).

vi) Unknown etiology: when the underlying cause of epilepsy is not known it is referred to as “unknown etiology”. This diagnosis can improve with time upon availability of further information on subsequent examinations (Scheffer et al., 2017).

During this PhD work, the focus has been on an animal model of epilepsy representing viral infectious etiology, which will be described later.
1.2. **Infections and epilepsy**

Worldwide, infections are one of the significant risk factors for developing provoked seizures and later epilepsy (Ngugi et al., 2013; Singhi, 2011; Vezzani et al., 2016). Patients suffering from cerebral infections or infestations by pathogens such as viruses, bacteria, parasites and fungi (Vezzani et al., 2016) frequently suffer from infection associated early (acute, provoked) seizures and subsequently develop late seizures (spontaneous recurrent seizures, SRS) (Vezzani et al., 2016). Within the first one to two weeks post CNS infection, up to 30% of patients exhibit early seizures as a symptom of the underlying inflammation (Beghi et al., 2010; Singhi, 2011; Vezzani et al., 2016). Although, early or provoked seizures are a risk factor for the later development of epilepsy (Christensen, 2012), their absence is not a safeguard against the development of late seizures and vice versa (Lowenstein, 2009). It might take from months to years that late seizures, characterizing epilepsy, appear after the resolution of CNS infections (Vezzani et al., 2016). Early seizures are often insult-associated and mechanistically different form subsequent late or epileptic seizures (Shorvon & Guerrini, 2010). Factors governing the risks for developing epilepsies after infections are variable and only partly understood (Michael & Solomon, 2012; Vezzani et al., 2016). The risk of suffering from CNS infections is often high in poor income countries and probably the most common preventable risk factor for epilepsy development (Sander & Perucca, 2003).

1.3. **Viral-infection and epilepsy**

Viral infections of the CNS are amongst the prominent causes of developing epilepsy (Getts et al., 2008; Libbey & Fujinami, 2011; Misra et al., 2008; Theodore, 2014). The chances of developing epilepsy in patients surviving viral encephalitis range from 4 to 20% (Getts et al., 2008). There are 100 or more neurotropic viruses showing affinity to CNS and causing encephalitis (Getts et al., 2008; Libbey & Fujinami, 2011; Misra et al., 2008; Vezzani et al., 2016). Many of these have been associated with the development of post-infectious epilepsy (Vezzani et al., 2016). Especially herpesviruses (Herpes Simplex Virus Type 1 [HSV-1], Human Herpes Virus Type 6 [HHV-6]) and various arboviruses (arthropod borne virus, insect-transmitting viruses, e.g., Japan Encephalitis Virus, West Nile Virus) are important epilepsy-causing viruses (Bonello et al., 2015; Libbey & Fujinami, 2011; Vezzani et al., 2016).
1.4.  Epileptogenesis

The phenomenon of developing epilepsy is described by the term “epileptogenesis”, which is a combination of the two ancient Greek words epilēpsis = attack, invasion and genesis = genesis (Lösch & Brandt, 2010). About 40% of all epilepsies have an identifiable cause, i.e., they develop after an initial brain insult (Banerjee et al., 2009; Lösch & Brandt, 2010). These initial brain assaults, e.g. trauma, stroke, brain tumors, status epilepticus or infections, often accompanied by early seizures, play a role in transforming an otherwise healthy brain into an epileptic brain (Lösch & Brandt, 2010; Ravizza et al., 2011). After an initial brain insult, the failure of intrinsic repair mechanisms, followed by a second hit, gene sensitivity, or comorbidities provides a favoring milieu to develop late seizures (Lösch & Brandt, 2010).

Various underlying mechanisms (c.f. Fig. 1) such as inflammation, blood brain barrier changes, neurodegeneration, gliosis, neuronal hyper-excitability, alterations of receptor and ion channel expression are involved in this transformation (Lösch & Brandt, 2010; Vezzani et al., 2016). The seizure free period, also known as “latency” or “latent period”, between the initial insult and the occurrence of the 1st unprovoked epileptic seizure, ranges from months to years in humans and results in the manifestation of chronic epilepsy (Jozwiak et al., 2017; White & Loscher, 2014). A schematic representation of epileptogenesis and potential intervention strategies is given below (Fig. 1).

**Figure 1:** Steps of the development and progression of temporal lobe epilepsy and possible therapeutic interventions: modified form (Lösch & Brandt, 2010).
1.5. Pharmacotherapy, pharmacoresistance & epilepsy prevention

There are currently more than 20 therapeutic agents available for the management of epilepsy (Lösch et al., 2013; Pitkanen et al., 2016). Fifteen, belonging to the 3rd generation, have been added in the past three decades, which have provided clinicians more chances of attaining successful therapeutic goals (Lösch & Schmidt, 2011). Although these drugs are primarily known as “anti-epileptic drugs” (AEDs) they do not cure epilepsy itself, but rather provide symptomatic relief from seizures and are thus more appropriately called “anti-seizure or anticonvulsant” drugs (Kaminski et al., 2014). However, only 70-80% of new onset epilepsy patients become seizure free when treated with current AEDs, while 20-30% of patients fail to respond to these treatments (Brodie et al., 2012; Lösch et al., 2013; Sillanpaa & Schmidt, 2006).

Patients, who do not remain adequately seizure free for a prolonged period of time or do not respond to any of two or more well tolerated anti-epileptic drugs, are said to be pharmacoresistant (Kwan et al., 2010). The underlying mechanisms of this pharmacoresistance has not been understood completely (Kwan & Brodie, 2006). Hence, the quality of life of these patients is compromised with psychological and social consequences (Weaver & Pohlmann-Eden, 2013). There are many drugs that are currently in clinical trials or under development having novel mechanisms of action for pharmacoresistant epilepsy (Younus & Reddy, 2018). The final resort for these patients is surgical resection of brain regions involved in seizure formation or transmission (Schmidt et al., 2004). However, the risks and adverse effects of these neurosurgical procedures limit the usefulness of such measures in patients with pharmacoresistant epilepsy (West et al., 2016) and only 50% of such patients are able to achieve long term seizure freedom. In order to control the seizures, many patients continue to receive the drug therapy after surgery (Loscher & Schmidt, 2006).

Another extensively studied strategy in animal models of epilepsy and in clinical trials, termed as “anti-epileptogenesis”, is to stop the phenomenon of “epileptogenesis” before the appearance of spontaneous seizures (Lösch, 2016; Lösch & Brandt, 2010; White & Lösch, 2014). This strategy could be more appropriate in preventing the development of epilepsy in people at risk after trauma or disease. Finding the biomarkers or changes that would indicate pathological conditions of patients after initial brain injuries marks another important area in ongoing research (Pitkanen et al., 2016).
1.6. Animal models of epilepsy

The intricacy of the nervous system advocates for the use of various animal models to understand the pathobiological aspects of chronic neurological diseases such as epilepsy. (Lösch, 2002; Lösch, 2016; Lösch & Brandt, 2010). Laboratory rodents (rats and mice), like in many other biomedical fields (Halliwell, 2018), seem to be the animals of choice in a number of models of seizures and epilepsy proposed for preclinical research (Lösch, 1999; Lösch, 2016; Lösch & Brandt, 2010). In order to develop anti-seizure drugs (ASDs) (Lösch et al., 2013; Simonato et al., 2014; Simonato et al., 2012), and to address the clinical questions effectively, the selection of authenticated and predictive animal models is necessary (Lösch, 2016).

In order to render animals epileptic, they are treated with electrical or chemical stimulus to induce acquired epilepsy, with late seizures (Lösch, 1997, 2016). However, animal models representing genetic epilepsy with late seizures without any further treatment are also available (Lösch, 2016; Pitkänen & Engel, 2014). Transgenic animal models with inherent epilepsy can be used for the evaluation of anticonvulsant drug potency, while pure seizure models cannot be used as model of chronic epilepsy (Lösch, 2011). Epilepsy models are characterized by the presence of late seizures. Normal animals where seizures are induced electrically cannot be used as epilepsy model (Lösch, 2011).

An example of the electrical stimulation model is the well-established Kindling model, where different brain regions of the animals especially rat, can be used as targets via a depth electrode e.g. amygdala or hippocampus. Animals are given electrical stimuli in a repetitive manner from day to weeks to induce focal and later generalized seizures. The length and severity of these seizures increases over time with continued stimulation (Gorter et al., 2016; Lösch, 2016). This model causes persistent brain alterations very similar to temporal lobe epilepsy in humans (Sato et al., 1990) and can reasonably forecast the clinical usefulness of many ASDs against focal seizures in epilepsy patients (Lösch, 2011).

In animal models of chemically induced epilepsies, pro-convulsant substances are administered either systemically (pilocarpine model) or locally (intra-hippocampal kainic acid model), which induces status epilepticus (SE) (Lévesque et al., 2016), a continuous seizure lasting for more than 5 minutes (Cherian & Thomas, 2009), causing a severe epileptogenic insult. This is followed by a seizure free period, termed as “latent period” after which we see the occurrence of late seizures. The length of the latent period may range from
days to weeks or even months (Maguire, 2016) and it shows the presence of epileptiform activity although not the motor seizures (Gorter et al., 2001). Although a large number of seizure and epilepsy models is available to date (Löscher, 1999), the selection of appropriate predictive models to answer the pertinent clinical questions is necessary in order to improve the translational value of preclinical work and avoid potential failure of costly clinical trials (Löscher, 2016; Simonato et al., 2014).

### 1.7. TMEV-induced animal model of epilepsy

To study the mechanisms of virus-induced epilepsy, rare possibilities existed even until few years ago (Vezzani et al., 2016), since appropriate animal models of epilepsy representing infectious etiology were not available. High mortality rates after viral infections (Libbey & Fujinami, 2011) in laboratory rodents was the primary reason that made it difficult to investigate the complex pathobiological phenomenon spanning over several weeks to months in epilepsy to deduce results (Libbey & Fujinami, 2011). However, a group at the University of Utah in Salt Lake City described a novel model of viral infection associated epilepsy in mice following Theiler's murine encephalomyelitis virus (TMEV) inoculation, where animals developed infection-induced early seizures and later spontaneous seizures representing epilepsy (Libbey et al., 2008; Stewart et al., 2010a).

Theiler's murine encephalomyelitis virus (TMEV) is a naturally occurring enteric pathogen of the mouse (Theiler, 1937; Theiler & Gard, 1940). It is a non-enveloped, positive-sense, single stranded RNA virus belonging to Picornaviridae family and Cardiovirus genus (Daniels et al., 1952; Tsunoda & Fujinami, 2010). Different TMEV strains have been used for research on neuroinflammation, whereby the Daniels (DA) and BeAn 8386 (BeAn) strain are most commonly used. The ability to initiate early seizures by different TMEV strains post infection varies considerably (Libbey & Fujinami, 2011). Although the seizure prevalence was different among virus strains, all of the tested virus strains (DA, BeAn, GDVII, WW, DapBL2M, H101) were able to generate early seizures in C57BL/6 mice. About 40% of the BeAn-infected mice developed early seizures as compared to 60% DA-infected animals (Libbey & Fujinami, 2011). After early experiments, the standard virus strain at the University of Utah for developing seizures is the DA strain.

Intracerebral viral infection with TMEV leads to an inflammation of CNS (Carpentier et al., 2008). For many decades, this virus has been used in susceptible mouse species (SJL/J) for studying multiple sclerosis (MS), a chronic demyelinating disease of the CNS mediated by
immune system (Lipton & Dal Canto, 1976; Procaccini et al., 2015; Tsunoda & Fujinami, 2010). Another mouse strain, C57BL/6 or short B6, shows a different disease manifestation: There is acute encephalitis but secondary demyelinating disease does not develop due to absence of virus persistence (Libbey & Fujinami, 2011; Tsunoda & Fujinami, 1996). The group of scientists from the Institutes of Pathology and Pharmacology, Salt Lake City, University of Utah, USA, Robert Fujinami and Steve White observed seizures in B6 mice during the encephalitis period. After this coincidental observation, further monitoring was performed that ensued in description of the “1st model for virus-induced seizures” (Libbey et al., 2008) and subsequently the “1st model for virus-induced epilepsy” (Stewart et al., 2010a).

The occurrence of seizures in B6 mice was mostly during first 3-7 days after infection, with the maximal seizure frequency on 6dpi. Visual observations confirmed 50% of animals suffering from early seizures (Libbey et al., 2008) that went up to 75% upon 24/7 video recording (Stewart et al., 2010a). Since the recordings were only from surface electrodes, seizure frequencies might have been misreported especially for focal seizures, as it is difficult to record focal seizures from surface electrodes (Stewart et al., 2010a). After the acute phase of infection and encephalitis, in the absence of clinical signs of disease and seizures for some time (latent period), a significant proportion of mice developed late seizures or epileptic seizures.

1.8. Epilepsy biomarkers

A biomarker could be defined as an objectively quantifiable trait helping in evaluating the physiological or pathological state of an individual e.g. serum glucose level in case of diabetes. (Engel et al., 2013). Biomarkers in epilepsy could be

- Blood or tissue metabolites
- Alterations in gene expressions
- Imaging biomarkers and
- Electroencephalography parameters

Electrophysiological or EEG biomarkers include seizure (ictal) patterns, interictal spikes or interictal epileptiform discharges (IEDs), High frequency oscillations (HFOs). EEG biomarkers are better identified from depth electrodes or cortical surface electroencephalography (ECoG). Our target in the current study was the identification of biomarkers in electroencephalography from an animal model of epilepsy.
1.9. Electroencephalography (EEG) – An introduction

According to the International Federation of Clinical Neurophysiology, electroencephalography is “the science related to the electrical activity of the brain” encompassing “the practice of recording and interpreting encephalograms” (Kane et al., 2017). The history of electroencephalography (EEG) is more than a century old (Beres, 2017; Tudor et al., 2005). English physician Richard Caton presented his findings on EEG from open brains of monkeys and rabbits for the 1st time in 1875. He entailed first sensory evoked response and observed ‘continuous spontaneous electrical activity’ from the brain surface (Goldensohn, 1998). However, the discovery of EEG recordings form the human scalp is credited to German neuropsychiatrist Hans Berger (Berger, 1929; Haas, 2003; Zeidman et al., 2014). The electroencephalogram (device to record EEG) was also invented by Berger, described "as one of the most surprising, remarkable, and momentous developments in the history of clinical neurology" (Millet, 2002). These novel findings paved the way for advanced research and utilization of EEG in different physiological and pathological conditions (Beres, 2017).

Currently EEG is being used in different fields to monitor the brain activity, e.g. for diagnosis of brain death (Chen et al., 2008; Lee et al., 2017), assessment of head trauma (Ianof & Anghinah, 2017; Nuwer et al., 2005; Rapp et al., 2015), diagnosis of concussions (Arciniegas, 2011), stroke (Wijaya et al., 2015; Wolf et al., 2017), Alzheimer’s disease (Tsolaki et al., 2014), epileptic activity (Noachtar & Rémi, 2009; Okanishi, 2018), Sleep disorders (Abad & Guilleminault, 2003; Tan et al., 2012), Parkinson’s disease (Johnsen et al., 2014) or autism spectrum disorder (ASD) (Bhat et al., 2014). It can also be useful for investigating various cognitive functions, such as memory and attention (Bell & Cuevas, 2012) and in language and clinical research, for example in studies investigating the EEG pattern in individuals with aphasia (Riley & McFarland, 2017).

1.10. Neural source of EEG generation

There has been a general consensus on the generation of the EEG from the cerebral cortex since the early days of clinical EEG. This rhytmical synaptic activity recorded from the scalp originates from populations of cortical neurons (pyramidal neurons arranged in column format in cortical regions) (Avitan et al., 2009; Jackson & Bolger, 2014). Early recordings from subcortically located electrodes (i.e. depth electrodes) confirmed the typical EEG source from the grey matter instead of white matter and higher amplitude electrical potentials were recorded from the exposed cortex or fractured bones (Avitan et al., 2009).
Neurons are excitable cells with intrinsic electrical properties (Llinas, 1990) and the intercommunication between approximately 86 billion neurons in an average human brain is the major brain activity (Herculano-Houzel, 2009). The interplay between neurons results in the generation of magnetic as well as electrical fields (Buzsaki et al., 2012). The recording of these fields is possible as (i) local field potentials (LFP) or local EEG, when electrodes are placed in the proximity of sources, (ii) electrocorticogram (ECoG), from electrodes placed on the brain cortex or (iii) electroencephalogram (EEG, most commonly used) from scalp electrodes placed at larger distances from source (Buzsaki et al., 2012; Da Silva, 2009). The summed electrical activity of cortical neuronal cells or pyramidal cells, recorded in a graphical manner over time with the help of recording electrodes is termed as electroencephalogram or EEG (Beres, 2017).

Pyramidal neurons are a common class of neurons found in the cerebral cortex and other subcortical regions of the brains, e.g. amygdala and hippocampus, of almost all mammals. They consist of a cell body (soma), longer apical dendrites and shorter basal dendrites (Bekkers, 2011). The separation of negative and positive charges across a small distance in pyramidal cells results in the formation of a dipole – just like in little batteries. The positive regions are termed as source, while the negative as sink (Jackson & Bolger, 2014). The net negative or positive charge in the extracellular space at the top of the pyramidal cell depends on two factors; the input stimulus (excitatory or inhibitory) and the location of the synapse in reference to the cell body (proximal or distal). For example, in case of an excitatory signal (Fig. 2A) near the distal end of a dendrite (close to the cortical surface, away from the cell body), causes an Na+ influx into the cell which will leave the outer extracellular space more negative and due to the dipole nature of the pyramidal cells, as described above, the extracellular space near the proximal end of the dendrite will be more positive. If a large number of pyramidal cells in close proximity act similarly, the EEG surface electrode will record a negative extracellular potential. This situation will be reversed in case of an inhibitory stimulus (Fig. 2B) or a change of stimulus location. Hence the algebraic sum of inhibitory or excitatory post synaptic potentials is recorded and represented by the EEG (Da Silva, 2009; Jackson & Bolger, 2014). It is important to note that the measured EEG cannot determine if the activity is excitatory or inhibitory (Fig. 2B), as it would result in a positive deflection in the EEG.

The neuronal activity can be of two types: action potentials and postsynaptic potentials. Neuronal depolarization, upon reaching a threshold due to the alteration of membrane
permeability to Na+ and K+ ions, results in the firing of action potentials from the cell body to the end of axons lasting for very brief periods of about 1 ms. These action potentials, being very brief and rapid with a fixed propagation rate in axons, cannot be detected by scalp EEG electrodes. On the other hand postsynaptic potentials, mediated by the binding of neurotransmitters on membranes of postsynaptic cells, usually produce slower changes in membrane potentials, causing a charge alteration in extracellular space, described above in as an example of pyramidal cells, lasting for about 200 ms. This extracellular electrical charge, negative or positive, is measured by electrodes on the scalp (EEG) or cortex (ECoG) (Da Silva, 2009; Jackson & Bolger, 2014).

**Figure 2:** Schematic representation of a pyramidal cell – as a dipole, possible stimulation conditions and resultant scalp EEG recording (A&B). Resultant EEG deflections due to dipole position (C&D). Modified from (Da Silva, 2009)

### 1.11. Electroencephalography (EEG) and epilepsy

Nowadays, the use of EEG has become a routine practice in epilepsy diagnostics (Noachtar & Rémi, 2009). EEG is highly reliable laboratory investigation tool that has successfully been used over decades for classifying seizures and epilepsies based on use of functional marker: interictal and ictal epileptiform discharges (ILEA Commission, 1989; Koutroumanidis et al., 2017). EEG can be used to answer questions such as presence of epilepsy (Smith, 2005),
localization of possible epileptic foci (Vulliemoz et al., 2010), identify epileptic zone for surgical resection in intractable epilepsies e.g. lobectomy in temporal lobe epilepsy (Burkholder et al., 2014), prognosis of epileptic surgery (Abou-Khalil, 2012; Hildebrandt et al., 2005), evaluating the effects of drug therapy (Sato et al., 1990), estimating the side effects of standard AEDs e.g. phenobarbital and others on CNS (Bauer, 1982), assessing drug safety and toxicity (Authier et al., 2016) alone or in combination with other diagnostic techniques (De Ciantis & Lemieux, 2013).

1.12. EEG – A biomarker of epilepsy and other neurological diseases

Presence, frequency, and distribution characteristics (So, 2010) of interictal epileptiform discharges (IEDs) in the EEG is a key diagnostic tool for distinguishing epileptic from nonepileptic paroxysmal events (Mohan et al., 2016; Noachtar & Rémi, 2009). The EEG patterns which are considered as epileptiform discharges are spikes, sharp waves, spike-wave complexes, slow spike-wave complexes, 3-Hz spike-wave complexes polyspikes, hypsarrhythmia, seizure patterns, status patterns and benign epileptiform discharges of childhood (Noachtar & Rémi, 2009).

1.13. Interictal epileptiform discharges (IEDs) in EEG – Biomarkers of epilepsy

Interictal epileptiform discharges (IEDs), epileptiform activity or epileptiform pattern describes the transients typically, but neither solely nor always, found in the interictal EEG of epilepsy patients. These transients are clearly separable from background activity with a distinctive morphology (Kane et al., 2017). According to the guidelines recently updated by the International Federation of Clinical Neurophysiology (IFCN) 4 out of the 6 criteria mentioned below have to be met do classify a transient as an epileptiform pattern (Kane et al., 2017):

1. A sharp spikey shape (i.e. pointed peak) with di or tri phasic wave
2. Shorter or longer wave-duration than current background activity
3. Asymmetrical waveform: sharp ascending, slow decaying descending component or vice versa
4. An associated slow after-wave follows the transient
5. Disrupted background activity (around IEDs) due to epileptiform activity presence
6. Negative and positive potentials distribution on scalp based on source in brain (dipole)
High frequency oscillations (HFOs) and interictal epileptiform discharges (IEDs) are considered as important biomarkers of epilepsy (Worrell & Gotman, 2011). Presence, frequency, and distribution characteristics (So, 2010) of IEDs in EEG are a key diagnostic tool in order to distinguish between epileptic and nonepileptic paroxysmal events (Mohan et al., 2016; Noachtar & Rémi, 2009). The EEG patterns (see definitions below) which are considered as epileptiform discharges are spikes, sharp waves (Krakow et al., 1999), spike-wave complexes (Seneviratne et al., 2012), slow spike-wave complexes (Crespel, 2000), 3 Hz spike-wave complexes (Kakisaka et al., 2011), polyspikes (Seneviratne et al., 2017), hypsarrhythmia (Khreisat, 2011), seizure pattern (Karoly et al., 2016), status pattern (Amzica, 2015) and Benign epileptiform discharges of childhood or rolandic spikes (Liu et al., 2017). However, there are also normal sharp transients in EEG like wickets (4-7 Hz frequency), small sharp spikes (Single, frontally localized), and 14- and 6- Hz positive spikes, which need to be distinguished from epileptiform discharges in order to avoid misinterpretation and overinterpretation leading to an incorrect epilepsy diagnosis (Noachtar & Rémi, 2009).

Characteristics and definitions of few commonly identified IEDs and normal transients are as under (Kane et al., 2017):

- **Spike**: Transient with pointed peak, 20-70 ms duration, amplitude varies but > 50uV typically, main component generally negative
- **Sharp wave**: A varying amplitude transient, clearly distinguishable from background activity, 70-200 ms duration, generally negative main component, may be followed by a slow wave of same polarity
- **Sharp-and-slow-wave complex**: Sharp wave with an associated following slow wave, single or multiple
- **Spike-and-slow-wave complex**: Spike with an associated following slow wave, single or multiple
- **Polyspike and-slow-wave complex**: Two or more spikes associated with one or more slow waves
- **Small sharp spikes (SSS)**: Normal transient, very short in duration (<50 ms), low in amplitude (<50 µV), often followed by a small theta wave.

There is inter-individual variability in the frequency of IEDs among epilepsy patients, linked with certain clinical aspects such as disease duration (Selvitelli et al., 2010) or time to last
seizure (Sundaram et al., 1990); effects of seizure frequency and use of antiepileptic drugs are under discussions (Gotman & Koffler, 1989; Janszky et al., 2005).

1.13.1. **Interictal epileptiform discharges (IEDs) in EEG of non-epileptic people**

On the contrary, rarely IEDs can be identified from young children to adults, without any previous seizure history (Sam & So, 2001). However the inferences shall be drawn keeping in view the age and health status of the individuals, and spontaneous IEDs must be segregated from induced IEDs by hyperventilation or photic stimulation (So, 2010). EEG has been routinely used in screening aircrew members in civilian and military setups, but this evaluation has decreased over time (So, 2010). In a study of over 13000 aircrew members 0.5% showed IEDs, out of these individuals 58% showed IEDs only upon photic stimulation. Only one person out of 43 individuals monitored for 29 years developed epilepsy, leading to a conclusion by researchers that there are 2 – 3% chances of developing epilepsy in healthy individuals (Gregory et al., 1993).

1.13.2. **Interictal epileptiform discharges (IEDs) in brain disorders other than epilepsy**

Although 50 – 70 % patients with neurobehavioral and psychiatric disorders show abnormalities in EEG recordings they are not epileptiform discharges. There is slowing of background that could be due to effects of medications being used, drowsiness or simultaneously occurring other non-epileptic cerebral disorders (Shelley et al., 2008). IEDs were detected in 24 hour digitally recorded ambulatory EEGs of approximately 60% patients suffering from autism spectrum disorder with no previous history of abnormal EEG or seizures. 55% of the identified IEDs were temporally located. When these patients were treated with Valproic acid 47% showed a normalized EEG, whereas another 17% showed EEG improvement (Chez et al., 2006). 30 %EEGs recorded from children suffering from Attention-deficit / hyperactivity disorder (ADHD), without history of prior seizures, have been identified with IEDs (Hughes et al., 2000). ADHD in children has been reported to be a risk factor for unprovoked seizures and epilepsy (Hesdorffer et al., 2004). Interictal epileptiform discharges are often associated with cognitive impairment (Aldenkamp & Arends, 2004). A causal relationship between IEDs and cognitive function impairment relating to poor psychosocial functions has been discussed by (Jaseja, 2007) and suppression
of IEDs with medication in terms of incidence, frequency or duration has resulted in improving behavioural and cognitive problems (Pressler et al., 2005).

1.13.3. Interictal epileptiform discharges (IEDs) and epileptogenic zone (EZ) identification

Approximately 30% of focal epilepsy patients with identifiable paroxysmal discharges in local brain tissue are medically refractory (Pedersen et al., 2017). Patients requiring resective epilepsy surgery (Noachtar et al., 2003), after failing to respond to a combination of two antiepileptic drug treatments (Kwan et al., 2010), need precise localization of epileptiform discharges to identify the epileptogenic zone (EZ) for successful surgical outcomes (Yang et al., 2018). Nowadays, non-invasive clinical evaluation methods like functional brain imaging (PET, fMRI, ictal SPECT, MRS or EMG), long term video/EEG monitoring and neuropsychological test evaluation are considered sufficient before surgical procedures to identify EZs (Hupalo et al., 2017). However, invasive EEG recordings using stereotaxic electroencephalography (SEEG) or intracerebral EEG recording using stereotactically implanted electrodes (epidural, subdural or depth electrodes, Fig. 3B) are still considered the “Gold standard” (Cossu et al., 2005), when non-invasive procedures mentioned above fail to localize the EZ (Noachtar, 2003). Prejudiced or uncertain visual human analysis to identify IEDs for localization of EZ is the limitation of SEEG (Harvey et al., 2008) and attempts have been made in recent years to quantify the interneuron networking by computational modelling as changes in brain network are thought to be associated with epileptogenesis (Panzica et al., 2013). IEDs identified in EEGs from the anterior temporal lobe have long been associated with temporal lobe epilepsy (TLE) (Gibbs et al., 1948), often a medically refractory epilepsy form requiring surgery (Salanova et al., 2002). Seizure freedom in 80.5% patients is achieved in case of rare IEDs as compared to 28.6% with frequent IEDs (Krendl et al., 2008).

1.13.4. Electroencephalography and evaluation of therapeutic efficacy

The use of EEG in diagnosing brain disorders such as epilepsy is well established (Koutroumanidis et al., 2017), whereas, the utilization of EEGs features as a biomarker i.e. Quantitative EEG (QEEG) to evaluate effects of AED treatment are being studied (Ouyang et al., 2018). Traditionally, EEGs are used to record seizure frequency (both electrographic and electroclinical) to observe the effectiveness of AED treatment in research and practice (Abend et al., 2013). Practically a decrease in seizure frequency over time is considered as a success of AED therapy, despite of the unpredictability of seizure occurrence and recording in clinical settings (Ouyang et al., 2018). Prior studies have demonstrated that there is a
positive co-relation between IEDs frequencies and number of seizures in some, but not all, patients with seizures (Duncan, 1987). Many researchers have reported a greater probability of detecting IEDs or IED frequency with higher clinical seizure frequency but the degree of association is not clear (Drury & Beydoun, 1998; Janszky et al., 2005).

Treatment with standard AEDs such as carbamazepine, phenobarbital, valproate and phenytoin may exert non-specific CNS side effects (Bauer, 1982). These drugs could slow down the dominant rhythm and increase the slow activity e.g. carbamazepine particularly appears to deteriorate background activity, though improves the clinical outcome (Ebersole & Pedley, 2003). Fast activity is enhanced by Benzodiazepines and barbiturates while intravenous phenytoin and benzodiazepines result in both IEDs suppression and early seizure control (Duncan, 1987).

1.13.5. Electroencephalography with invasive electrodes

Recording of local field potentials from invasively implanted cranial electrodes (Fig. 3B) have several advantages (Noachtar & Rémi, 2009):

- Better signal-to-noise ratio (SNR) than scalp EEG
- Detection of focal, subclinical or electrographic seizures which may not be detected with surface EEG
- Less artifacts in invasive EEG due to a better localization near epileptic foci
- Reduction of muscle activity artefacts, very common in surface EEG whereas they are much less in ictal invasive recordings

![Figure 3](modified from Noachtar, 2009)

**Figure 3:** (A) Continuum between various states in epilepsy patients

(B) Schematic diagram of different invasive electrode types

(modified from Noachtar, 2009)
1.14. **EEG analysis by experts or electroencephalographers**

Despite advances in computational power and the development of a lot of computer assisted algorithms and programs, visual analysis by human EEG experts is still considered as “gold standard” for identification, annotation and assessment of different alteration in EEGs, such as IEDs and seizures (Moyer Jason et al., 2017). While performing such analysis there are always inter-rater variability and a consensus value is usually attained after consultation (Stroink et al., 2006).

1.15. **Automatic detection algorithms for interictal spikes detection**

Long term EEG recordings and monitoring, to document and describe IEDs (such as interictal spikes), may be required for diagnosis and treatment of epilepsy (El-Gohary et al., 2008). However, the visual analysis by human experts is time consuming, requires a certain training time, and is subject to inter-observer variability and laborious (Tzallas et al., 2009; Wang et al., 2017). Therefore, a solution to this obstacle is the use of automatic spike detection methods (Puspita et al., 2017; Tadanori et al., 2018). Many researchers are developing and using automated methods to detect and quantify the interictal spikes and other EEG alterations (Orosco et al., 2013; Quang et al., 2016). Several methods have been proposed for this purpose, which could be divided in six categories based on detection rules (El-Gohary et al., 2008).

i) **Feature-based detection or mimetic techniques:** Certain features such as Spike slope, height, duration, and sharpness are compared against EEG expert threshold values (Gotman et al., 1979; Ktonas et al., 1984).

ii) **Template matching algorithms:** Spikes are marked by human experts as templates and new events are found based on this template. Wavelet detection is often used in this method (Kalayci & Ozdamar, 1995; Le Douget et al., 2017).

iii) **Parametric approaches:** Traditional data processing techniques are used where spikes are detected based on non-stationary behaviour against stationary of background activity (Soriano et al., 2017; Tzallas et al., 2006).

iv) **Artificial neural networks (ANNs):** ANNs recognise patterns similar to data learned from raw sources during training phases. Spikes, seizures, and sleeping behaviours have been detected successfully using these methods (Nguyen et al., 2018; Tzallas et al., 2004).
v) **Data mining techniques (DM):** These techniques have been used to develop automatic detection models and they do not require a priori spike morphology definitions (Puspita et al., 2017; Valenti et al., 2006).

vi) **Knowledge-based rules:** This technique uses rules from knowledge of experts to make a final decision after extracting the basic features in 1st step (Park et al., 1998; Truong et al., 2017).

In this study we used basic feature based detection, mentioned in the 1st category, by estimating values regarding spike width, amplitude, slope and entropy (power).

### 1.16. Electroencephalography in animal models of epilepsy

For the past several decades, direct recording of neuronal electrical activity has served as an important tool for the identification, diagnosis, management and research of neurological diseases such as epilepsy (Niedermeyer & da Silva, 2005). Principally, inhibitory and excitatory neuronal electrical activity from animal skulls can be amplified and recorded between slightly apart recording and reference electrodes, using wired or wireless (telemetry) electroencephalographic equipment (Martín del Campo et al., 2009). Complex synchronous brain communication among different brain areas determines appropriate behaviours, however during seizures, failure of such mechanisms among brain networks might lead to paroxysmal activity (Gibbs et al., 2002).

Furthermore, failure of intercommunicating neuronal networks may also affect the interictal periods that could be associated with behavioural disturbances (Kramer & Cash, 2012). Hence EEG recording and analysis over time during different disease development states, could prove helpful to understand the neural functioning and complexity of epileptogenesis, instead of only characterizing single events i.e. seizures (Cambiaghi et al., 2015). One model of epilepsy development is the TMEV-induced animal model of epilepsy, in which the presence of epileptiform activities have been shown as spikes, spike clusters and seizures (Stewart et al., 2010a). There is a need to analysis and characterize the EEG parameters in TMEV-induced animal model of epilepsy. Identification of suitable electroencephalographic biomarkers, such as spikes, spike cluster and seizures is required. This will help to understand course of disease development and provide a window of opportunity for possible therapeutic interventions.
1.16.1. EEG spikes in rodent models of epilepsy

Interictal EEG spikes have long been considered as a biomarker in diagnosing epilepsy in human patients (Staley et al., 2011). Development of such biomarkers has been identified as one of the key research targets in order to predict epilepsy after an acute brain insult (Kelley et al., 2009). Biomarkers could provide valuable clinical information during latent period. They can also help in accelerating research to understand the mechanisms underlying epileptogenesis as well as developing potential anti-epileptogenic therapies. In some experimental animal models of epilepsy such as kainate-induced epilepsy model, there is a progressive increase in seizures frequency from a very low number over a period of time (Williams et al., 2009). In order to record these low number of seizures, very long and intensive continuous (24/7) vEEG recordings are needed. These long term recordings are becoming a rate limiting step in the field of epilepsy research that could be efficiently replaced by accurate biomarkers such as interictal EEG spikes (White et al., 2010).

Interictal spikes from temporal lobe regions in rodent models have been recorded using depth EEG electrodes (Suárez et al., 2012; White et al., 2010; White et al., 2006). Different quantification aspects of EEG transients have been entailed previously such as spike counting (Spencer et al., 2008), automatic detection (Gotman, 1999), shape features (Wadman et al., 1983), and localization of source (Michel et al., 2004). These interictal spikes have been frequently observed in studies conducted in both patients and animal models during chronic phase of epilepsy describing spike features with reference to mature epileptogenic networks (Schwartzkroin & Wheal, 1984).

In few other studies in animal models of epilepsy it has been reported that these spikes could be observed as early as during epileptogenesis and latent period before chronic phase of epilepsy (Avoli et al., 2006; Staley & Dudek, 2006). However, in rodent models of epilepsy, long term extensively standardized quantitative and qualitative studies, describing EEG spike parameters like spike frequency, type and morphology has only been reported rarely (Chauviere et al., 2012; Clément et al., 2013; White et al., 2010). Moreover most of the studies conducted in rodent model of epilepsy are customized according to the research question and standardized parameters in terms of EEG recording settings, analysis and interpretation have not been laid (Moyer Jason et al., 2017). However a series of guideline papers have recently been published by various ILEA established task force working groups in order to provide harmonization in preclinical epilepsy studies (Harte-Hargrove et al., 2017; Hernan et al., 2017; Kadam et al., 2017; Raimondo et al., 2017).
1.16.2. EEG spikes frequency and morphology in rodent models of epilepsy

White et al. (2010) reported that there is a correlation between seizure frequency and occurrences rate of interictal spike before the first late seizure. This study was conducted in rats, which were injected with kainic acid systemically. Another finding regarding the spike frequency was reported, where the animal that did not develop epilepsy had low number of interictal spikes as compared to the ones which had developed late seizures. For spike detection they used a common definition employed to define human EEG spikes. A computerized algorithm was developed based on spike slope to identify spikes against normal EEG background (White et al., 2010). Spike width was chosen between 20-800 ms. They performed analysis for 24-48 hours per week. They also observed and counted the spike clusters (frequency range 0.15-0.7 Hz).

EEG spikes in control rats were also detected by algorithm, which they attributed to physiological dentate gyrus spikes (Bragin et al., 1995), increased external noise detected as spikes due to prolonged EEG recording (White et al., 2006; Williams et al., 2006), and electrode induced local injury spikes. However absence of seizures in control animals, consistent spike frequency over time and similarity of waveforms with other acute studies (Bragin et al., 1995) suggests that electrode injury is less likely source of these EEG alterations. White et al. (2010) did not try to localize the anatomical source of EEG alterations, however they recorded both from depth bipolar and two epidural electrodes from both hemispheres and were able to detect spikes in all three channels suggesting that spike frequency is not affected by location, but morphology could be affected over time due to gliosis around electrodes and electrode position shift because of brain injury and skull growth (Williams et al., 2009). However that change in spike morphology was not documented. For typical spike shapes observed by White et al. (2010) see Fig. 4(a).

Chauvière et al. (2012) described the spike morphology in an extensive detail during latent period in kainic acid treated rat model of epilepsy. The spikes were categorized in two shapes, type 1 (spikes followed by a long lasting wave) and type 2 spikes (spike without a wave) [see Fig 4(b)]. They showed that type 1 spike frequency, duration and amplitude decreased progressively over time before the 1st SRS was observed, whereas frequency of type 2 spikes increased. They tried to explain that type 1 spikes are generated due to neuronal activity in large number of excitatory (spike) and inhibitory (wave) cells, whereas the type 2 spikes are generated due to more locally occurring excitatory cells activity.
The increase in type 2 spike frequency and a decrease in type 1 spike frequency were associated to a continuous change in build-up of excitatory epileptic networks and a decline in inhibitory intracellular circuits during epileptogenesis.

Clément et al. (2013) have tried to count single epileptic spikes (ESs) from CA1 region of intrahippocampal kainate injected mouse model of temporal lobe epilepsy. These ESs are described by a “spike-wave complex” having an initial sharp spike component and a slow wave component followed by spike. They described the shape changes in ES spike and wave components over time and observed that there is a progressive increase in both amplitude spike and surface area of wave component in identified ESs [c.f Fig. 4(c)]. They proposed a novel ratio (WA/Sa) based on wave area (WA) and spike amplitude (Sa) as a novel epilepsy-predicting biomarker after testing results in different inbread strains of mice such as C57BL/6J, DBA/2J, 129/SvTer, FVB/N, BR/Orl, and CBA/H. They augmented their findings by in vitro and in silico computational modeling describing the role of excitatory and inhibitory input on pyramidal interneuron functioning and their correlation with spike and wave components of ESs. A decrease in the inhibitory GABAergic input progressively resulted in large wave components along with increased excitatory output shown as high Sa.

**Fig. 4:** (a) Typical spikes observed during epileptogenesis (White et al., 2010) (b) Type 1 (A) and type 2 (B) spikes during latent phase in long term vEEG recordings (Chauviere et al., 2012) (c) progression of spike and wave morphology over time (Clément et al., 2013).
1.16.3. **Local field potentials (LFPs)**

Extracellular electrical signals recorded from animal brains can be divided into two frequency-bands.

(i) **Multiple unit spiking activity (MUA):** Above ~ 200-500Hz, representing neuron action potentials around electrodes in 140 – 300 μm radius (Buzsaki, 2006; Henze et al., 2000)

(ii) **Local field potentials (LFPs):** below ~ 200 Hz, synaptic transmembrane current flows in neurons around electrode in 200 μm – few mm radius (Cambiaghi et al., 2015; Kajikawa & Schroeder, 2011).

LFPs are an approximation of synaptic signals of neural populations around electrode tips, which may provide information regarding cognitive and sensory processes. LFPs recorded from the cortex or deep brain structures combined with behavioural analysis using a simultaneous video recording (video – EEG monitoring) can be used as an investigative tool to detect seizures or other electrical anomalies such as interictal spiking to evaluate the effects of certain pharmacological compounds in animal models (Cambiaghi et al., 2015).

1.16.4. **Data acquisition and analysis softwares in animal models of epilepsy**

In humans, EEG recording montage, signal acquisition, signal interpretation, and equipment design has been standardized over time by clinicians (American Clinical Neurophysiology Society, 2006). In animal models of epilepsy however, parameters like placement of electrodes, electrode arrangements, data acquisition, and analysing algorithms differ considerably between research laboratories (Moyer Jason et al., 2017), and epilepsy researchers around the globe have developed customized methods and algorithms, using different software for data collection and processing, meeting their individual needs to answer specific questions (Moyer Jason et al., 2017). Recently a co-ordinated attempt has been made by the ILEA to review the current systems being used in collection, processing, storage, and in-silico analysis of neural data, and to develop general guidelines to enhance the validity of collected data and increase effective conversion of preclinical data into clinical use (Moyer Jason et al., 2017). Enormous volumes of data can be processed nowadays due to advances in experimental methods and computational powers using advanced mathematical processing. However, in-silico analysis using different software can, on one hand, handle huge data volumes but, on the other hand, distort results naively due to improper usage (Moyer Jason et al., 2017).
1.16.4.1. Data acquisition systems

Examples of the highly refined data collection systems available as complete packages, including recording systems (hardware), electrode implantation, to analysis software, being used in epilepsy and other highly sophisticated studies are Blackrock microsystems (Schevon et al., 2010), Plexon (Zheng et al., 2017), Neuralynx (Kremen et al., 2017), Tucker-Davis (Kros et al., 2017). These systems can record up to 512 channels of EEG. Many of these systems have spike detection and sorting capabilities (Moyer Jason et al., 2017), but are highly expensive and offer only limited customization capabilities as compared to systems assembled from simple components. Another frequently used complete data acquisition system from Data Science International, USA (DSI) ®, with mini implants and telemetric recording capabilities, which can record from up to 4 animals (Chemaly et al., 2018; Moyer Jason et al., 2017) is bundled with NeuroScore™ software. Another commonly used system is PowerLab® from ADInstruments, Ltd., Sydney, Australia. In this study we have used PowerLab® 8/30 ML870 (ADInstruments, Ltd., Sydney, Australia), an 8 channel bio-amplifier analogue-digital converter, along with LabChart® v 6.0 or higher software (ADInstruments) for windows to record data (Bröer et al., 2016).

1.16.4.2. Data analysis softwares

Analysis software can be used alone or in combination for processing data recorded by different data acquiring systems (hardware). Such software packages are shipped with built-in tools to perform analyses or they can be programmed by users to match their requirements. Software can be differentiated by ways of functions offered or user license (free or commercial) (Moyer Jason et al., 2017). Some examples of freely available software are polymen, EEG.Rev, NeuroScope, EDFviewer, or the web based iEEG.org etc., which can be used as standalone applications for basic analysis of EEG, such as viewing and commenting. On the other hand commercially available programs such as spikes2 or NeuroExplorer are specialized for neurophysiological analysis. These software packages are well tested for specified purposes and come with very good user support. Among the most popular solutions is the utilization of powerful computational platforms, e.g. MATLAB, Python, LabVIEW or R. There are various free to use readily available scripts and toolboxes, which can be used with these programs for EEG analysis and viewing (PyEEG, EEGLab, eegkig etc.). Although these programs are more flexible, they require certain levels of expertise and training before proper utilization (Moyer Jason et al., 2017).
For this study we tested NeuroScore™ (DSI®, USA) and LabChart® (ADInstrument, Australia), the latter being used as a standard software for recording and analysing EEG data routinely in our lab. The use of LabChart® over NeuroScore™ was preferred after testing as

- It was difficult to program NeuroScore™
- Automatic artefact rejection was not possible
- Data had to be converted to another compatible format before analysis
- Calculations from multiple data channels were not possible
- More processing and verification time for performing data analysis

Although, I had to learn the programing with LabChart as well, it was preferred because of following reasons,

- Default software being used in our lab
- Familiarity with basic interface by colleagues
- Ease of programming
- No need to convert data to other formats
- Multi-channel calculations possible using simple arithmetic formulas
- Excellent software technical support (ADInstrument, Europe support)

1.16.5. Typical composition of EEG signal recording in rodents

An EEG signal can consist of multiple components, such as

(i) **Background EEG activity** – Rodents have dominant broader theta rhythms recorded with depth hippocampal electrodes unlike human posterior-dominant rhythm (PDR) in alpha frequency ranges (Leuchter et al., 2017). This can be affected by the state of the animal, i.e. sleep vs awake, and changes are visible as a change in amplitude and relative spectral power of the signal (White et al., 2006).

(ii) **Epileptiform activity**: Can be ranked from normal to severe in terms of spiking (grade 1- 4) (Griffey et al., 2006).

(iii) **Ictal activity** – in terms of seizures usually > 10s in duration, electrographic seizures (present only in EEG without behavioural correlates) and electroclinical seizures (both in EEG with motor correlates – verifiable in video) (Löscher, 2017).

(iv) **Artefacts** - with either identifiable triggers (physical activity, chewing, drinking, grooming, scratching, severe head movement, removal from cage, electrical artefacts or unknown source) or non-identifiable sources, termed as noise (White et al., 2006).
1.17. Working hypothesis and study objectives

The TMEV-induced animal model of epilepsy, as described above, has opened new horizons to understand the pathobiological mechanisms involved in the induction of epilepsy and ultimately new avenues for studies on disease prevention (anti-epileptogenesis) and disease modification (anti-seizures) (Stewart et al., 2010a) in infection induced epilepsies. The presence of early seizures during the early phase (0-7dpi) of infection, was observed visually and confirmed with 24/7 video EEG (vEEG) recording (Stewart et al., 2010a). However, the frequency of behavioural seizures per week during the chronic disease phase (2-7 months pi) was very low and continuously decreased over time. This renders seizure frequency, generally used as measure of disease progression and therapeutic efficacy in various animal models of epilepsy (Twele et al., 2016), a non-suitable biomarker to evaluate the effects of drug treatments in this model. However, all the infected animals showed epileptiform activity (IEDs) in terms of spikes (Stewart et al., 2010a), providing a window of opportunity to characterize and quantify these as a potential biomarker for epilepsy development and later therapeutic disease modification (Engel et al., 2013).

Hypothesis:

Interictal spiking activity can be used as a potential biomarker to differentiate between epileptic and non-epileptic mice

Most of the studies conducted by the University of Utah group are on early seizure development, focusing on underlying mechanisms and the role of the immune system, with a relatively short period of visual observations for seizure occurrence (2 hours per day at same time every day) or vEEG recordings. Thus there was a need for extensive long term (up to 90 days post infection) simultaneous 24/7 video EEG recordings to answer the following questions, (1) What are the general characteristics of EEG recorded in TMEV model of epilepsy? (2) Is it possible to develop an algorithm to characterize and analyze EEG in-silico? (3) What is the length of latent period, if any? (4) How seizure pattern evolve over time? (5) What is the frequency and intensity of early and chronic seizures (6) Is it possible to find out a potential EEG biomarker such as spikes and spike clusters in EEG recordings for distinguishing between epileptic / non-epileptic mice? (7) Can we use observed spikes and spike clusters as a quantifiable biomarker for therapeutic targeting?
Brain inflammation, neurodegeneration and seizure development following picornavirus infection markedly differ among virus and mouse strains and substrains

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Personal contributions

SB designed the study and performed the experiments, analyzed the data and wrote the manuscript. CK performed the experiments, analyzed the data and wrote the manuscript. VH designed the study and performed the experiments. LL performed the experiments. IG analyzed the data and wrote the manuscript, MA learned the model, performed the experiments, analyzed EEGs, MB helped in analysing the data. WB supervised and analyzed the data and wrote the manuscript. WL supervised and analyzed the data and wrote the manuscript. All authors revised the manuscript.
Abstract

Infections, particularly those caused by viruses, are among the main causes of acquired epilepsy, but the mechanisms causing epileptogenesis are only poorly understood. As a consequence, no treatment exists for preventing epilepsy in patients at risk. Animal models are useful to study epileptogenesis after virus-induced encephalitis and how to interfere with this process, but most viruses that cause encephalitis in rodents are associated with high mortality, so that the processes leading to epilepsy cannot be investigated. Recently, intracerebral infection with Theiler's murine encephalomyelitis virus (TMEV) in C57BL/6 (B6) mice was reported to induce early seizures and epilepsy and it was proposed that the TMEV mouse model represents the first virus infection-driven animal model of epilepsy. In the present study, we characterized this model in two B6 substrains and seizure resistant SJL/J mice by using three TMEV (sub) strains (BeAn-1, BeAn-2, DA). The idea behind this approach was to study what is and what is not necessary for development of acute and late seizures after brain infection in mice. Receiver operating characteristic (ROC) curve analysis was used to determine which virus-induced brain alterations are associated with seizure development. In B6 mice infected with different TMEV virus (sub)strains, the severity of hippocampal neurodegeneration, amount of MAC3-positive microglia/macrophages, and expression of the interferon-inducible antiviral effector ISG15 were almost perfect at discriminating seizing from non-seizing B6 mice, whereas T-lymphocyte brain infiltration was not found to be a crucial factor. However, intense microglia/macrophage activation and some hippocampal damage were also observed in SJL/J mice. Overall, the TMEV model provides a unique platform to study virus and host factors in ictogenesis and epileptogenesis.
Automated quantification of EEG spikes and spike clusters as a new read out in Theiler’s virus mouse model of encephalitis-induced epilepsy

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Personal contributions

SMMA Designed and performed the experiments, conceived, designed, executed and wrote algorithm, analyzed the data and wrote manuscript. CK performed experiment, edited manuscript, RH analyzed algorithm technically, wrote manuscript, IW performed experiments, SB designed and performed experiments, and WL supervised and analyzed the data and wrote the manuscript.
Abstract

Intracerebral infection of C57BL/6 mice with Theiler's murine encephalomyelitis virus (TMEV) replicates many features of viral encephalitis-induced epilepsy in humans, including neuroinflammation, early (insult-associated) and late (spontaneous) seizures, neurodegeneration in the hippocampus, and cognitive and behavioral alterations. Thus, this model may be ideally suited to study mechanisms involved in encephalitis-induced epilepsy as potential targets for epilepsy prevention. However, spontaneous recurrent seizures (SRS) occur too infrequently to be useful as a biomarker of epilepsy, e.g., for drug studies. This prompted us to evaluate whether epileptiform spikes or spike clusters in the cortical electroencephalogram (EEG) may be a useful surrogate of epilepsy in this model. For this purpose, we developed an algorithm that allows efficient and large-scale EEG analysis of early and late seizures, spikes, and spike clusters in the EEG. While 77% of the infected mice exhibited early seizures, late seizures were only observed in 33% of the animals. The clinical characteristics of early and late seizures did not differ except that late generalized convulsive (stage 5) seizures were significantly longer than early stage 5 seizures. Furthermore, the frequency of SRS was much lower than the frequency of early seizures. Continuous (24/7) video-EEG monitoring over several months following infection indicated that the latent period to onset of SRS was 61 (range 16–91) days. Spike and spike clusters were significantly more frequent in infected mice with late seizures than in infected mice without seizures or in mock-infected sham controls. Based on the results of this study, increases in EEG spikes and spike clusters in groups of infected mice may be used as a new readout for studies on antiepileptogenic or disease-modifying drug effects in this model, because the significant increase in average spike counts in mice with late seizures obviously indicates a proepileptogenic alteration.
4. General discussion

4.1. TMEV-induced epilepsy model

4.1.1. Model establishment & EEG characterization

An infection-induced animal model of epilepsy after intracerebral infection in C57BL/6 mice with DA strain of Theiler’s virus has recently been reported (Stewart et al., 2010a). Discovery of this unique model has paved the way to understand the underlying mechanisms of infection, inflammation, immune system activation and their therapeutic modulation in seizure development (Cusick et al., 2013; DePaula-Silva et al., 2018; Waltl et al., 2018). In order to understand the pathophysiology of disease development, the model was reproduced in our lab and important differences in both virus and mouse strains were observed (Bröer et al., 2017; Bröer et al., 2016). In this model mice develop early (insult-associated) and late (spontaneous) seizures as a result of virus-induced encephalitis, which resembles human epilepsy manifestations (DePaula-Silva et al., 2018; Singhi, 2011; Stewart et al., 2010a). Moreover, histopathological analysis of brain sections of seizing animals has shown a progressive neurodegeneration in hippocampal CA1 & CA2 regions, likely due to inflammatory reactions & immune cell involvement, resembling brain lesions of epileptic patients with temporal lobe epilepsy (TLE) (Bröer et al., 2016; Kirkman et al., 2010; Loewen et al., 2016).

The recorded EEG from cortical electrodes matched in terms of background and interictal activity similar to (Stewart et al., 2010a) i.e. EEG spikes, spike clusters and seizures (Bröer et al., 2016). We could observe spiking activity in all animals soon after plugging the animals into recording system, though it is higher in infected animals when compared with controls. Majority of the spiking activity was observed when animals were immobile and it was confirmed visually by analyzing the simultaneously recorded videos. Appearance of spike clusters and seizures in EEG was less frequent as compared to spikes. We could also observe a change in EEG background activity based on state (awake and sleep) of the animals observed by others as well (White et al., 2006).

4.1.2. Early Seizures

4.1.2.1. Visual observations

While performing the experiments in order to understand the role of the immune system or its modulation in development of early seizures (Cusick et al., 2013; Howe et al., 2012), animals
were observed visually relatively for a short period of time (2h/day) for 7 – 14 days after infection to confirm the presence of seizures and evaluate the effects of therapeutic modulation on early seizure occurrence (Libbey et al., 2008; Waltl et al., 2018). During visual observations, we noticed early behavioural seizures in 60% of infected animals (Bröer et al., 2016), which is in line with initially reported 50 – 55% animals with early seizures (Libbey et al., 2008; Stewart et al., 2010b). The seizures could see seizures 1 dpi after infection, although they occurred mostly between 3dpi to 7dpi with maximum seizure activity occurring on day 6 post infection (Libbey et al., 2008). This narrow time period of 3-7dpi could be crucial in terms of underlying inflammatory and virus associated pathological responses (Stewart et al., 2010a). During this period, a significant upregulation of chemokines, cytokines (e.g. IL-6 and TNF- α), adhesion molecules in infected C57BL/6 mice has been observed, causing a damage to the blood brain barrier and enhancing the CNS infiltration of peripheral leukocytes (de Vries et al., 1996). We have shown recently in another study that the incidence of early seizures in TMEV-infected mice could be as high as 84% (Waltl et al., 2018). The incidence of early seizures in human patients with encephalitis after CNS infection is between 2% – 67% (Singhi, 2011), while the chances of developing epilepsy are maximum within the 1st 5 years after encephalitis, and might occur up to 20 years of life (Singhi, 2011).

4.1.2.2. Characterization of EEG recording during early time point

Owing to the short period of visual observation it was hypothesized that probably many early seizures could have been missed, advocating for a continuous vEEG recording (Stewart et al., 2010a). Thus 24/7 vEEG recording with cortical screws revealed that seizure incidence in recorded animals was 77% which is in line with the previously described 75% (Stewart et al., 2010a). Keeping in view the hippocampal sclerosis, the generation of hippocampal focal seizures could not be ruled out, which are difficult to record with cortical screws, so deep electrodes implanted in the dentate gyrus of the dorsal hippocampus showed 68% seizure incidence (Patel et al., 2017). The incidence and frequency of early seizures per mouse during acute phase after infection are higher (14.5 ± 10.5, mean ± SD) as compared to late seizures, 4 months post infection (0.75 ± 0, mean ± SD), (Stewart et al., 2010a). Whereas, in our study the average seizure frequency/week during early phase recording was (5.5 ± 1.7, mean ± SD) significantly higher as compared to seizures (0.998 ± 0.39, mean ± SD) during late phase (16-98 dpi) of recording.
We could record early seizures in vEEG as early as on 0 dpi few hours after infection, although the maximum seizure frequency (43%) occurred on 4-5 dpi, similar to previous findings (Stewart et al., 2010a). This suggests that possible pharmacological interventions to counter epileptogenesis shall be initiated as early as possible after the acute insult, since early seizures itself are a risk factor for epilepsy development (Wilson et al., 2018). As expected, a considerable proportion (54.7%) of seizures were focal or partial (Racine scale, I-III), while (45.3%) were generalized seizures (Racine scale, IV-V), with vEEG recording during early phase of recoding (0-7dpi). We could record focal seizures with cortical electrodes, although it is considered difficult, especially when brain regions like hippocampus is believed to be involved in generating seizures (DePaula-Silva et al., 2017; Stewart et al., 2010a). The average length of the focal seizures was much shorter than generalized seizures. Moreover, we observed animals pausing or becoming immobile during video analysis of focal seizures, suggesting that these were different from electrographic seizures where usually no behavioural correlates are observed in videos in another animal model of epilepsy (Twele et al., 2017). However, the depth hippocampal electrode recordings from dentate gyrus (DG) region (Patel et al., 2017), showed that electrographic activity was recorded before appearance of behavioural components in many generalized seizures and continued even after the behavioural seizures subsided. Patel et al., (2017) could not correlate any specific observable behaviours with recorded electrographic activity and classified them as focal (electrographic) seizures. The recording for such focal seizures from depth hippocampal electrodes is suggestive of the fact that there is extensive epileptiform activity and neuronal hyperexcitability taking place in hippocampus post TMEV infection (Patel et al., 2017).

The average duration of seizures also increased as the seizure stage changed, from type – I, 16.41 ± 9.84 sec (mean ±SD) to type-V, 35.88 ± 18.02 sec. The average seizure length recorded was 25.67 ± 15.83 sec, whereas the average seizure length recorded during previously reported studies for focal seizures was 24 ± 13 sec and 50 ± 17 sec for stage IV-V seizures (Stewart et al., 2010a). There has been contradicting results after treatment with anti-seizure drugs during acute phase after infection in this model (Barker-Haliski et al., 2017). Valproic acid (VPA) administration resulted in a decrease in severity and frequency of early seizures, whereas treatment with Carbamazepine (CBZ) worsened the disease with increased severity and incidence of early seizures (Barker-Haliski et al., 2015). Testing of a number of antiepileptic drugs with varying results suggests the robustness of this model to screen for seizure-reduction effects of pharmacological compounds (Barker-Haliski et al., 2017). However there are no acute phase studies with vEEG recording extensively discussing the
quantitative and qualitative aspects of EEG tracing after drug administrations. The last seizure recorded in vEEG was on 8dpi by us as compared to 7dpi by (Stewart et al., 2010a) with cortical electrodes. However, with depth electrode recordings, focal seizures could be recorded in many animals after 8dpi, while the generalized seizures were largely subsided. In our recorded data after 8dpi the animals did not show any seizures for several days before the start of late seizures or epilepsy. In between the early and late seizures occurrence, there is a so called asymptomatic seizure free latent period (Monteyne et al., 1997) of unknown duration, requiring a long term continuous vEEG recording for its determination. Long term vEEG recordings from weeks to months, though laborious, are recommended for rigorous epilepsy studies (Gu & Daltone, 2017).

4.1.3. Latent period

Traditionally, patients suffering from acquired epilepsies, due to known initial insults or injuries, often have a seizure free period between the initial insult and the 1st spontaneous seizures depicting epilepsy (Sloviter, 2008). This concept has been challenged on the basis of new evidences both from human studies and animal models of epilepsy (Löschler et al., 2015). It has been suggested that a large number of molecular, cellular & functional changes take place in glial and neuronal networks during this period, establishing its simultaneous role in epileptogenesis (Pitkänen & Lukasiuk, 2011). Epileptogenesis, the process of epilepsy development, does not stop to evolve after the appearance of the 1st late seizure, rather it is a progressive phenomenon (Löschler et al., 2015; Maguire, 2016). In this virus-induced epilepsy model, Libbey et al., (2010a) recorded animals only for 14 days post infection with continuous vEEG monitoring, and they did not record any generalized seizures from 8 to 14 dpi. Although recent studies with depth electrode recordings from DG region of hippocampus demonstrate the presence of focal seizure in the second week pi (Patel et al., 2017). We were able to continuously record animals for several days post infection to estimate and report that 1st late seizure in an epileptic animal appeared on 16dpi, then late seizures started appearing in many animals on different days between 16-91dpi and the calculated average was 61dpi. However vEEG recordings with depth hippocampal electrodes after infection has revealed the presence of focal (electrographic) seizures up to 15 dpi and no information is provided for the appearance of 1st late clinical seizure after infection. This estimation could prove critical in providing a window for studying epileptogenesis and pharmacological interventions (Löschler, 2012).
4.1.4. Late seizures

4.1.4.1. Visual observations

We observed a set of infected animals visually during chronic phase after infection (2-13 weeks pi) following the same protocol used for early seizures with modifications i.e. 2hrs/day once weekly (Bröer et al., 2016). We could only observe late seizures in 2/8 (40%) infected animals between 9-10 weeks pi. However the owing to the fact that observation period was too short, a continuous 24/7 vEEG monitoring was also performed for the animals for a week after 13 weeks pi (91-97dpi), but we could not record any late seizure during that phase in any of the animals, including those two where late seizures were observed visually, although epileptiform activities in terms of spikes and spike clusters were recorded (Bröer et al., 2016). No such visual observations during chronic phase of epilepsy were performed by others (Stewart et al., 2010a). This observation further advocated for a continuous 24/7 vEEG recording for longer periods of time as late seizures might have been missed (Gu & Daltone, 2017).

4.1.4.2. Characterization EEG recording during late time point

In order to confirm the presence of late seizures, a time-locked vEEG recording was performed (Bröer et al., 2016; Stewart et al., 2010a). We recorded vEEG till 3 months pi with minimum interruptions, while Stewart et al., (2010a) recorded vEEG for 1 week at 2 months pi and for 4 weeks at 4 and 7 months pi. They showed a gradual decrease in the percentage of seizing animals from 64% to 40% after 7 months pi, either due to loss of headsets or unexplained reasons, whereas we could report an epilepsy incidence of 33% as compared to the previously reported 38% (Bröer et al., 2016). One explanation for the difference between the two research group findings could be the fact that we included all animals in chronic studies post infection, without considering whether they exhibited early seizures or not, whereas Stewart et al., (2010a) recruited only animals that showed at least one seizure during the acute phase. Another point worth mentioning here is that we could record late seizures in some animals, which did not show any early seizures, emphasizing the need to record all the infected animals, with or without early seizure in acute phase after infection. Despite of the long recording duration, the frequency of late seizures was very low in contrast to early seizures and decreased over time from 2.1(2 months pi) to 0.37, (7 months pi) per mouse per week (Stewart et al., 2010a). The average seizure frequency was 1.4 ±1.6 per animal /week (Stewart et al., 2010a), as compared to 0.998 ± 0.39 calculated in this study. The intensity and
duration of the late seizures was higher as compared to early seizures with type I – III seizures comprising 46% and type IV – V seizures 54%, with an average duration of 66.2 ± 27.0 sec (Stewart et al., 2010a) and in our case 54.41 ± 11.97 sec, suggesting an intensification and progression of the seizure activity (Rakhade et al., 2011). This low frequency of late seizures and low number of epileptic animals along with a long latent period is similar to post traumatic epilepsy TBI models (Pitkänen et al., 2009). Therefore, it is difficult to use seizures as readout for epilepsy development for the disease modification studies, although long term behavioural outcomes after therapeutic administrations have been evaluated (Barker-Haliski et al., 2016).

All of our studies were performed using cortical electrodes, but we have shown that large proportion of seizures are only focal in nature, where the animals showed brief pauses, indifferent from normal physical activity. Seizure activity recoded with hippocampal depth electrodes in EEGs of human and animal models of epilepsy can occur without behavioural co-relates or during immobility (Bragin et al., 1999; Riban et al., 2002), difficult to separate from normal pauses requiring vEEG recording (Fisher et al., 2014b). So it is highly likely that with deep intra-hippocampal depth electrodes, one could record more events, thus increasing the seizure frequency during both acute and chronic phases of epilepsy.

4.1.5. Analyzed spikes vs interictal epileptiform discharges (IEDs)

We could easily visualize transients other than seizures, i.e. spikes and spike clusters, in recorded EEGs of animals during the experiments. These events were not only observed and recorded in the EEG of all the infected animals like previous studies (Bröer et al., 2016; Stewart et al., 2010a, 2010b), but also in sham controls as well. A quick visual scan indicated that probably these events were more frequent in infected animals as compared to controls. Nevertheless, the frequency of these events was too high to count them manually, requiring a computer-assisted algorithm. The ultimate objective was to analyze the EEGs and find out whether these events could serve as potential discriminators between epileptic and nonepileptic animals acting as biomarkers.

We used a common definition based on spike amplitude (2 times background activity), having a “rise and fall” with “pointy shape” and width (<200ms). This definition was obtained by combining the “spike” (30-70ms) and “Spike-wave” (70-200ms) width definitions, found in human EEGs to identify, mark and quantify events electronically (Gloor, 1975). We did not know whether these spike and clusters were epileptiform or interictal
epileptiform discharges (IEDs). The term IEDs or epileptiform patterns in EEG is considered to be linked with high probability for having seizures (Fisher et al., 2014b). In order to manually mark an EEG event as epileptic, certain pre-defined criteria have to be met as described by International Federation of Clinical Neurophysiology (IFCN, 2018; Kane et al., 2017). Manual EEG analysis is still considered as the “gold standard” for identification and marking of spikes and seizures in humans (Moyer Jason et al., 2017). In order to identify, mark and report IEDs, electroencephalographers require a certain level of training, but, sometimes even experienced electroencephalographers disagree on a consensus identification of IEDs, resulting in high inter-observer variability and poor reliability (Grant et al., 2014; Williams et al., 1985). A solution to this problem could be in-silico analysis of such events, minimizing the human input and automatizing the spike detection in EEGs (Webber & Lesser, 2017).

In humans, this epileptiform activity is described by events which are clearly distinguishable form background activity with a specific spike morphology (Kane et al., 2017). These events are typically found in interictal EEG of epilepsy patients but they are not always present and neither limited to epilepsy patients (Kane et al., 2017). Based on age and disease progression status, 60-90% of patients with epilepsy show IEDs (Bourien et al., 2005; Marsan & Zivin, 1970; Schaul, 1998). We could note in our study that spike frequency increased over time in infected mice, whereas the frequency remained more or less the same during recording period in controls, which might be indicative of a disease progression in this TLE model (Staley et al., 2011). The EEG may appear normal in up to 50% of epileptic patients upon the 1st examination (Goodin & Aminoff, 1984; van Donselaar et al., 1992), but falls dramatically below 10% on subsequent EEG recording and analysis (Binnie & Prior, 1994; Salinsky et al., 1987). High frequency oscillations (HFOs) and interictal epileptiform discharges (IEDs) are considered as important biomarkers of epilepsy (Worrell & Gotman, 2011). Presence, frequency, and distribution characteristics (So, 2010) of IEDs in EEG are a key diagnostic tool in order to distinguish between epileptic and nonepileptic paroxysmal events (Mohan et al., 2016; Noachtar & Rémi, 2009). The presence of spikes in control animals adds to ambiguity, however it has been shown that spikes are present, though in a very small percentage, both in sham controls in other animal models of epilepsy (Twele et al., 2017) and normal humans without history of seizures (Staley & Dudek, 2006). Prevalence of spontaneous IEDs is 0 – 5.6% in healthy volunteer children (Sam & So, 2001) as compared to 0 – 6.6% in healthy adult volunteers (Jabbari et al., 2000). Non-seizing patients submitted to EEG analysis based on suspicion of neurological disease showed a higher prevalence of
spontaneous IEDs from 2 – 12% (Bridgers, 1987), out of these patients three quarters suffered from acute or progressive brain disorders (Sam & So, 2001). IEDs are also present in EEGs of patients suffering from brain disorders other than epilepsy and without prior history of seizures (Shelley et al., 2008) such as autism spectrum disorder (ASD) (Chez et al., 2006), Attention-deficit / hyperactivity disorder (ADHD) (Hughes et al., 2000), cognitive impairment disorders (Jaseja, 2007). As discussed already, lesions in rodents associated with prolonged electrode implantations and surgical procedures could cause brain alteration which could initiate phenomena contributing to epileptogenesis, e.g. lowering seizure threshold in local regions, blood brain barrier damage, inflammation and depth electrode implantations in the hippocampus (Groothuis et al., 2014; Löscher et al., 1995; McConnell et al., 2009; Polikov et al., 2005).

We could identify many spike shapes that could be categorized in two major groups “Type 1” (Chauvière et al., 2012; Wendling et al., 2012) and “Type 2” spikes (Chauvière et al., 2012), based on similarities in morphology from rodent EEGs recorded with depth bipolar electrodes from dorsal hippocampus (Chauvière et al., 2012; Wendling et al., 2012). The majority of the spikes observed in this study were “Type 2” (generally monophasic with −Ve or +Ve polarity), with varying shapes, amplitude and width (<100ms), where as we could also find a few typical epileptiform “Type 1” spikes from one epileptic animal before the onset of 1st late seizures (width > 100ms). These epileptiform spikes are often found in EEGs of both human and animal models of epilepsies (Schwartzkroin & Wheal, 1984). These spikes could appear during epileptogenesis and latent period in animal models (Avoli et al., 2006; Staley & Dudek, 2006). A typical morphological description of “Type 1” spikes has been described by (Clément et al., 2013), where an initial sharp component referred as “spike” is preceded by slow “wave” of opposite polarity. These typical epileptiform spikes could easily be identified in a commonly used mouse model of TLE (Riban et al., 2002) and human EEGs (Kane et al., 2017). Clement et al., (2013) has quantified the frequency and described changes in shape features of these typical “Type 1” spikes over time. They proposed a computational model describing the role of phasic changes in GABAergic inhibition, causing a progressive change in spike morphology (Clément et al., 2013). However data from these studies were from dorsal hippocampal depth electrodes, where as we have recorded from cortical electrodes. For clinical purposes variability among morphology of different IEDs are less important than the certainty with which they can be separated from non-specific or physiologic sharp transients as well as artefacts. (Kane et al., 2017):
General discussion

We could demonstrate that, in a small group of epileptic animals analyzed over 2 months pi, the average spike frequency per hour increased gradually until the animals experienced the late seizures. Following the seizures there was a sharp decline in spiking activity, whereas the average frequency in mock infected animal remained almost unchanged. On the other hand, despite the high average number of spikes in epileptic animals as a group, in few epileptic animals the average number of spikes was quite low and in one epileptic animal with lots of seizures no progression in spike number was recorded as reported above. These conflicting findings coincide with the inconsistent points of views regarding the relationship between interictal spikes and seizure occurrence (Avoli et al., 2006; Gotman, 1991; Karoly et al., 2016). According to one point of view, interictal spike frequency increases over time due to increased neuronal activity, ultimately leading to seizure generation (De Curtis & Avanzini, 2001). Conversely, people have reported that spike rate does not change over time or even decreases before proceeding to ictal phase (Gotman & Marciani, 1985; Librizzi & de Curtis, 2003).

In this study we have shown that spike and spike cluster frequency in infected seizing (early and/or late) mice are significantly higher than in controls and can be used to discriminate among groups of seizing, non-seizing and mock infected animals. But the presence of spikes with similar morphology in control animals in a similar average range renders them a poor identifier for individual animals solely. However, our proposed spike count along with body weight and other surrogate markers such as cognitive impairment, seizure threshold, anxiety-like behaviour and changes in motor functions, identified previously (Stewart et al., 2010b; Umpierre et al., 2014), can be used to evaluate the progression of epilepsy development in this model (Stewart et al., 2010b).

4.2. Spike detection algorithm

The custom build algorithm using in house resources enabled us to identify, mark, quantify and subsequently report the EEG alterations in terms of spikes, spike clusters, and seizures. With above 86 – 98 % sensitivity and up to 98% specificity rate, we were able to detect spikes in all groups of animals, using very commonly used definitions for characterizing spikes, i.e. spike height (relative to baseline) and spike width. Two other easily implementable spike identifying parameters, spike slope and Teager Kaiser Energy operator were also incorporated to improve the algorithm’s specificity. The algorithm initially detected spikes that were verified by human experts to rule out the detection of artefacts, which were
not removed by the algorithm automatically. A spike detection algorithm using similar detection parameters in rats (model: i.p. administered kainate) has been described by (White et al., 2006), but no sensitivity or specificity values for spike detections has been given. This algorithm used spike slope (12 times the upslope of EEG background) and spike width (<200ms) parameters for spike detection. Spikes detected by the algorithm were then further employed to automatically detect spike clusters and seizures in animal EEGs. Range autocorrelation method, most efficient of the 4 methods used, to automatically detect seizures, resulted in 95 % positive predictive value, and 100 % sensitivity and specificity.

A more recent publication has described an algorithm using total signal variation and an advanced wavelet transform technique to find spikes, seizures and other abnormal EEG activity (Bergstrom et al., 2013). The algorithm was established on epileptic mice (intrapiriform kainic acid model) and the signal was subdivided in to normal, spikes, seizures and abnormal EEG data. The authors claimed to provide an alternative in place of long used “Racine scale” for behavioural analysis (Bergstrom et al., 2013). The algorithm used an automatic approach to remove the artifacts by employing a 2nd empty channel to record extra cerebral input and then subsequently remove them from mouse EEG. Furthermore, the algorithm was verified on data collected with a multi-channel EEG recording system from a model of absence seizure epilepsy in γ-butyrolactone treated mice (Bergstrom et al., 2013). The identification of different events was with 99% specificity and 91% sensitivity.

The major challenge in the spike or seizure detection algorithms is to prevent the detection of noise or artefact data which closely resembles interictal spikes and seizures (White et al., 2006). We were able to correctly detect and mark 76 – 84 % of spikes, while 16 – 24 % were marked as artefacts. Among marked artefacts, 76 – 83 % were removed automatically by the algorithm, while the remaining had to be excluded manually. Animal studies yield huge volumes of experimental data during pre-clinical studies. To analyze this large data volume we need to use computationally efficient algorithms. Highly sophisticated, time consuming softwares and algorithms used for human EEG analysis cannot be used on large data sets due to impracticality (White et al., 2006). The solution is developing time saving, simple to execute and computationally efficient algorithms like White et al., (2006) and ours. With our simple, though efficient algorithm, we were able to reduce the analysis time by up to 80%. Furthermore, our algorithm can be customized using various data processing techniques according to the individual requirements, examine bulk EEGs in short time, detect events of interest using common parameters, like amplitude, duration, spike frequency and power etc.
5. Summary

**IN-SILICO ANALYSIS, CHARACTERIZATION AND QUANTIFICATION OF EEG ALTERATIONS IN A MOUSE MODELS OF TEMPORAL LOBE EPILEPSY.**

Syed Muhammad Muneeb Anjum

Epilepsy is one of the most common chronic neurologic disorders that affects approximately 1% of the general population. Brain insults such as viral encephalitis may initiate the process of epilepsy development known as epileptogenesis. The Theiler’s murine encephalomyelitis virus (TMEV) animal model of viral encephalitis-induced epilepsy, first described by Libbey et. al. (2008), is the first proposed infection driven animal model of epilepsy. This model was reproduced in our lab and we have recorded vEEG (Video-EEG) using cortical electrodes. This vEEG recording is a primary tool used to characterize the electrophysiological parameters in animal models of epilepsy. Interictal epileptiform discharges have been associated with epilepsy, and EEG alterations such as spikes have widely been accepted diagnostically as sign of epilepsy in humans.

While characterizing the EEG of this animal model of epilepsy, we could show that animals suffer from early seizures after infection. These early seizures recorded in EEGs were either focal or generalized. We have reported that the percentage of animals suffering from seizures after acute infection is about 77%, verifying the findings of others. With visual observations this percentage values is usually lower. An average latent period of 61 days has been recorded and reported using long term continuous (24/7) vEEG recordings with cortical electrodes. Late seizure frequency during chronic phase of the disease was low in this model: only 38% of infected animals developed epilepsy during this study. In late EEG recording of the animals, we could, just like acute phase after infection, record both focal and generalized seizures. The low frequency of late seizures as well as epileptic animals prompted us to look for alternative read outs as a biomarker for developing epilepsy. We could verify the presence of inter-ictal spikes and spike clusters in all the infected animals. Quantification of spikes in humans and experimental models of epilepsy is often done manually by visual inspection, which requires intensive training, is very laborious, subjective, and error-prone.

However, the number of spikes and spike cluster was too high for visual analysis and hence a computer assisted algorithm was needed to objectively quantify them. We have developed a novel method to reliably and objectively quantify EEG spikes and spike clusters automatically. The idea of this detection technique is to analyse bulk EEG data recorded over
an extended time period and comparing the results with the standardized visual inspection of specialists.

A multitude of constantly updated algorithms over a period of time using EEG recording software LabChart® (AD Instruments) helped us develop a novel arithmetic & macro based signal processing method to quantify and characterize the inter-ictal spikes with high precision (90-99%). Although spikes and spike clusters have been seen in all the animals (mock & infected) during the early (0-7 days post infection) and late phase (91-97 days post infection) following infection, it is found that they are more frequent in TMEV-infected animals with seizures (early or late). The average number of spikes and spike clusters during the acute and chronic phase of epilepsy development was significantly higher in infected animals compared to controls. A comprehensive analysis of EEGs recorded in the late phase after infection showed that infected mice without early or late seizures were indifferent in spike frequency from controls. However, mice with any type of seizure, early or late, exhibited significantly increased spike and spike cluster frequencies in the late phase. On the contrary, few of the epileptic animals showed low number of spikes and spike clusters, with in the range of control animals. This suggested that spike and spike cluster frequency alone cannot predict individual animals to be epileptic or not. However, this can be used along with other previously identified surrogate markers to access the disease development such as seizure threshold, behaviour and cognitive studies.

Our results suggest that we can use increased spike and spike cluster frequencies in groups of infected animals as a new readout for disease modification or antiepileptogenesis studies to evaluate the effects of pharmacological compounds.
6. Zusammenfassung

IN-SILICO-ANALYSE, CHARAKTERISIERUNG UND QUANTIFIZIERUNG VON EEG-
ÄNDERUNGEN IN EINER MAUSMODELLE DER TEMPORALLAPPEN-EPILEPSIE.

Syed Muhammad Muneeb Anjum


manuell durch visuelle Inspektion, was ein intensives Training erfordert, sehr mühsam, subjektiv und fehleranfällig ist.


References


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Pakistan Zindabad!

Ich liebe Deutschland und du wirst vermisst warden!