CHARACTERIZATION OF CHRONIC FOCAL RECURRENT SEIZURES BY IRON CHLORIDE

By

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Submitted in partial fulfillment of the requirements for the degree of

Master of Science

Biomedical Engineering

CASE WESTERN RESERVE UNIVERSITY

May, 2023

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Dedications

"O Lord our God, grant us peace, for You render everything to us" Is 26:12. To God who has always stood by my side and blessed the works of my hands. To my family, whom without I would have never been able to pursue any of my passions or chase any of my dreams. To my friends who have supported and encouraged me in times of difficulties. I will always work to honor your presence in my life.

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List of Abbreviations

AED	Anti-Epileptic Drug
4-AP	4-Aminopyridine
ANT	Anterior Nucleus of the Thalamus
BBB	Blood Brain Barrier
CA3	Cornu Ammonis region superior
CC	Corpus Callosum
DBS	Deep Brain Stimulation
EEG	Electroencephalography
FCE	Focal Cortical Epilepsy
GABA	Gamma Amino Butyric Acid
HC	Hippocampus
IACUC	Institutional Animal Care and Use Committee
IPSP	Inhibitory post-synaptic potential
KA	Kainic Acid
LFS	Low-Frequency Stimulation
MTLE	Mesial Temporal Lobe Epilepsy
sAHP	Slow after-hyperpolarization
TBI	Traumatic Brain Injury
TRE	Traumatic Brain Injury-Related epilepsy
VNS	Vagus Nerve Stimulation

Acknowledgement:

Dr. Dominique Durand for the continuous guidance and support throughout the past years. Thank you for always challenging me and believing in me.

Dr. Chia Chu Chiang for guiding me through the complicated procedures and helping me with the experiments.

Dr. Grant McCallum for his support with the recording and data acquisition system.

Dr. Paul Marasco for his support and insight.

Dr. Chaitali Ghosh for her support and insight.

Dr. Kenneth Laurita for his support and insight.

Muthumeenakshi Subramanian for helping with the procedures.

Nrupen Pakalapati for his help with data analysis and code reviews.

Characterization of Chronic Focal Recurrent Seizures by Iron Chloride

Abstract

By

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Epilepsy is the second most burdensome disease worldwide. Despite multiple advancements, a significant fraction of patients cannot attain freedom from epilepsy through current treatments such as anti-epileptic drugs (AED) or deep brain stimulation (DBS). However, low-frequency stimulation (LFS) has shown promising results *in-vitro* and acute *in-vivo* studies as an alternative treatment modality. One limitation of chronic experiments for LFS is the lack of a well-established focal cortical seizure model. The stability of recurrent focal seizures generated by the Iron Chloride model was examined in this study. We investigated the number of seizures and percent time per day seizing for animals (N=5) after intraoperative intracortical injection of iron chloride for 14 days. Additionally, we investigated the capability of seizures generated to propagate to the contralateral cortex and hippocampus. Recorded seizures in our chronic study had a mean duration of 31±7.9 seconds, a mean peak-to-peak voltage of 2.5±1.5 mV, and an average peak frequency of 22.8±5 Hz (N=3370 seizure events). On average, animals showed a stable baseline of 50 seizures per day. Recording on the 8^{th} day post-injection yielded a stable baseline of seizures for thirteen days. We observed 402 seizures (N=364) propagating to the contralateral cortex and 91 seizures to the hippocampus. The mean delay was 4±8 msec for the contralateral cortical and 1±1 msec for the hippocampal propagation. The Iron Chloride model provides a good alternative to genetically available models for the generation of focal cortical seizures.

Chapter 1
Introduction

1.1. Epilepsy

Epilepsy is a chronic disorder that is characterized by recurrent seizures. Seizures have been classified into two main waveforms: ictal and interictal activity. Ictal activity has been defined by spontaneous spiking with an amplitude twice the background EEG, lasting more than 5 seconds, and composed of frequencies higher than 5 Hz (Nissinen et al. 2000). Interictal activity has been defined as short bursts of low-frequency spikes occurring between ictal activities (Nissinen et al. 2000). Seizures are further subdivided into two focal or generalized based on the size and spread of the epileptogenic zone. In a focal seizure, patients suffer from an abnormal electrical activity that is localized to a single area called the focus. Oftentimes, patients can have multiple foci depending on the nature and cause of the seizure. Patients suffering from focal seizures oftentimes do not have an impaired consciousness, but rather experience symptoms of confusion and present with an "absent stare." Focal seizures can lead to the development of a secondary generalized seizure. A general seizure often presents with loss of consciousness, myotonic or myoclonic convulsions ranging from the extremities to the trunk, and post-ictal confusions prohibiting them from recollecting any aura that preceded the epileptic episode.

A clinical diagnosis for epilepsy requires two seizure events occurring within 24 hours, or one seizure event with an underlying predisposing factor. There are multiple underlying causes that can lead to the development of epilepsy such as genetic variations, trauma, abnormal brain development, metabolic syndromes, medications, and infections.

Epilepsy is identified by the World Health Organization as the second most common burdensome neurological diseases worldwide, with more than 50 million people from around the globe affected ("EPILEPSY A Public Health Imperative International League Against Epilepsy"

2019). In 2015, the estimated incidences of epilepsy were 67.77 per 100,000 persons worldwide (Kirsten M. Fiest et al. 2016). In the United States, more than 3.5 million people reported having active epilepsy accounting for 1.2% of the population (Zack and Kobau 2019).

1.2. Treatments of epilepsy

Though it is the second most common burdensome neurological disease, the treatment gap for epilepsy stands to be one of the greatest barriers to eliminating this disease. Most current treatments are either ineffective or unaffordable for many, particularly low-income patients.

Many modalities, however, are currently used in clinical settings to treat and/or control seizures and epilepsy. The first line of treatment usually involves the use of anticonvulsant drugs (AEDs) such as Levetiracetam, Benzodiazepines, and Carbamazepine. Though AEDs present to be the least invasive control/treatment modality, it also carries a significant burden of adverse effects on patients' quality of life. AEDs commonly cause adverse effects such as fatigue, weakness, vertigo, diplopia, ataxia, tremors, behavioral changes, aggression, and hypersensitivity (Klehm et al. 2014; Sisodiya et al. 2002; Steinhoff et al. 2021). Additionally, not all patients with epilepsy can benefit from AEDs due to the development of drug-resistant seizures. Almost one-third of the cases of epilepsy develop drug resistance due to an unknown mechanism (Aronica et al. 2003; Sisodiya et al. 2002; Sultana et al. 2021; Schmidt and Löscher 2005). Due to the tight regulation of brain-medication transport through the Blood Brain Barrier (BBB), researchers have not yet found a solution within the pharmaceutical realm to address this gap (Achar and Ghosh 2021).

Contrarily, the most invasive approach to the treatment of epilepsy is surgical resection of the epileptogenic zone. In clinical settings, invasive surgeries such as corpus callosectomy or

focal neocortical resection are always considered a last resort due to the inherent risks associated with neurological surgeries, such as infection, paralysis, and even death. Studies have shown that almost a third of patients undergoing surgery do not experience relief in long-term follow-up (Spencer and Huh 2008; Noachtar S and Borggraefe I 2009). In addition, the nature of the epileptogenic zone can pose a challenge to the success and feasibility of surgical intervention. The complexity of the epileptogenic zone and its lack of confinement to a certain network, which can extend beyond a certain lesion, decrease the chances of benefiting from surgery (Noachtar S and Borggraefe I 2009; Choi and Kim 2019). Thus, there is a need for the development of new therapies that can address the disparity in the efficacy of the current treatment of epilepsy.

In the past decade, there has been a transition towards the use of electrical stimulation in the treatment of epilepsy with multiple modalities currently used in clinical settings such as deep brain stimulation (DBS) and Vagus nerve stimulation (VNS) (M. C. H. Li and Cook 2018). DBS constitutes the use of high frequency stimulation (HFS) ranging from 130 Hz to 185 Hz for the stimulation of the anterior nucleus of the thalamus (ANT) and the hippocampus (HC). Stimulation of the ANT as part of the medial limbic system, which has been shown to play a role in seizure propagation, has shown success in 40% (n=110) of patients involved in the Stimulation of the Anterior Nucleus of the Thalamus (SANTE) (Fisher et al. 2010; Velasco et al. 2006; Roberta MORACE et al. 2016). Similarly, the role of HC in the medical limbic circuit was the underlying cause for its consideration as a DBS target (Roberta MORACE et al. 2016; Velasco et al. 2006). Multiple trials have reported a 40% success rate in patients undergoing HC DBS (Roberta MORACE et al. 2016).

Though 40-70% of patients report significant seizure reduction following VNS, AEDs dosage cannot be reduced in patients undergoing VNS, as they are thought to work concurrently to reduce seizure activity, as they are thought to be working concurrently to reduce seizure activity (Jason Lee et al. 2022). The mechanism of VNS is not clearly understood yet, however, it is thought to suppress seizure activity by modulating midbrain and hindbrain structures promoting seizure suppression (Muthiah et al. 2022). Additionally, it is thought that VNS moderates the release of Serotonin and Norepinephrine which are thought to have anti-seizure activity (Muthiah et al. 2022). Therefore, the identification of alternative stimulation targets and frequencies remains a key area of research in the field of epilepsy treatment by electrical stimulation.

1.3. Low Frequency Stimulation

Low-frequency stimulation (LFS) has been investigated for the treatment of multiple movement disorders such as Parkinson's disease but has been gaining attention in the realm of seizure suppression in the past decade (Xie et al. 2015; Kile, Tian, and Durand 2010). LFS, though less widely used, offers multiple advantages over the use of high frequency stimulation. The major advantage of LFS is its ability to spread stimulation to a larger area. This is particularly beneficial when applied to white fiber tracts such as the corpus callosum and the anterior commissural fibers. LFS is defined as the delivery of stimulation with frequencies of 0.1-30 Hz(Durand and Bikson 2001). LFS has shown promising results in seizure suppression in amygdala-kindled seizures (Goodman, Berger, and Tcheng 2005; Weiss et al. 1995). Additionally, LFS has been shown to be successful in the reduction of hippocampal seizures, acute focal cortical seizures, and temporal lobe epilepsy (Couturier and Durand 2018; 2020; Kile, Tian, and Durand 2010; Koubeissi et al. 2013). One of the advantages of LFS is that it

delivers low-power therapy to tissue, which eliminates the risk of tissue and electrode damage in long-term therapy. Consequently, low-power therapy overcomes one of the hurdles encountered in medical device design, battery life. Despite the involvement of multiple other parameters such as amplitude, duty cycle, and waveform, the overall energy expenditure needed to power an implantable device decreases drastically due to the use of low-frequency waves. This is a significant advantage of LFS over high-frequency stimulation.

LFS mechanism has been previously studied in-vitro by our lab (Toprani and Durand 2013). In the study, LFS was applied to the ventral hippocampal commissure (VHC) in an in-vitro preparation containing two hippocampi connected via the VHC. Studies showed more than a 90% reduction in seizure metrics including seizure duration and power (Toprani and Durand 2013). It is believed that the mechanism underlying this seizure reduction is long-lasting hyperpolarization that is mediated through GABA-B inhibitory post-synaptic potentials (IPSPs) and slow after-hyperpolarization (sAHP) (Toprani and Durand 2013). The stimulation of the white matter tract was the underlying basis for the bilateral seizure suppression observed; stimulation of white fiber tract spreads stimulation to areas innervated by its axons and suppresses epileptogenic activity.

1.4. Focal Cortical Epilepsy

Focal cortical epilepsy (FCE) is a particularly important subcategory due to its resistance to treatment options. FCE has the highest prevalence for drug resistant epilepsy (Sultana et al. 2021; Sisodiya et al. 2002; Schmidt and Löscher 2005; Achar and Ghosh 2021). This prevents patients from being considered for the use of AEDs for relief of symptoms, even if minute or paired with adverse side effects as mentioned above. Additionally, multiple surgical reviews reported that more than 50 percent of patients with FCD are ineligible for surgical intervention

(Noachtar S and Borggraefe I 2009; Spencer and Huh 2008). A multifactorial decision considering the complexity of focus localization and ease of focus resection leaves most patients ineligible for surgery. Even those who are eligible for surgical intervention have reported seizure recurrence five-year post-op (Noachtar S and Borggraefe I 2009). In some patients, the development of a focal epileptogenic network is paired with the development of a mirror-focus (Wilder 2001; McCarthy, O'Connor, and Sperling 1997). This development requires callosal connections and subcortical polysynaptic connections (Wilder 2001). This phenomenon decreases the feasibility of surgical interventions as the mirror-focus could persist even after the disruption of the callosal connections through corpus callosectomy.

Further studies on the long-term outcome of VNS on patients with generalized epilepsy compared to their counterparts with focal epilepsy showed higher success rates in patients with generalized epilepsy (Muthiah et al. 2022). Nonetheless, patients were taking the same number of AEDs two years post-VNS therapy. Even though LFS can suppress cortical seizures in acute preparations, the long-term outcome is still unknown. Therefore, a chronic model of focal cortical epilepsy will be required to test the efficiency of LFS.

1.5. Corpus Callosum

More specifically, LFS stimulation has been successful at suppressing seizures in in a 4-Aminopyridine (4-AP) acute focal cortical seizure model (Couturier and Durand 2020; 2018). Stimulation of the Corpus Callosum (CC) with LFS resulted in 65% reduction of seizures in the focus and 97% reduction in the mirror focus (Couturier and Durand 2018). The Corpus Callosum, one of the brain's major white fiber tracts, is a structure most of the cortex depends on for the transfer of information from one hemisphere to the other (Kaas 1995). The CC remains to be one of the most important pathways for the spread of epileptogenic activity between

hemispheres(Funncll, Corballis, and Gazzaniga 2000). Callosal fibers stem from pyramidal cells in the cerebral cortex and develop into two dichotomous fibers: fast-conducting fibers and slow-conducting fibers (Eccher 2014). The fast-conducting fibers are the main interhemispheric connection between the somatosensory cortices (Eccher 2014).

Furthermore, the anatomical organization of the CC into anterior and posterior fibers each innervating the various parts of the cortex. Research has shown that the anterior midbody of the CC transfers motor information, the posterior midbody transfers somatosensory information, while the isthmus and the splenium transfer auditory and visual information respectively (Funnell, Corballis, and Gazzaniga 2000). Consequently, targeting pre-determined parts of the CC can, and has been proven to, stimulate the corresponding parts of the motor cortex and decrease epileptogenic activity.

1.6. Seizure Models

There are currently multiple animal models utilized in epilepsy research, each presenting a unique mechanism with advantages and disadvantages. The most common model is the 4-AP seizure model. 4-AP model is widely known convulsant agent that was discovered due to its toxicity to mammals and birds (Schafer, Brunton, and Cunningham 1973). This K^+ channel blocker can penetrate the BBB easily and readily, inducing spontaneous seizure activity. Despite its ability to block multiple voltage-gated channels, 4-AP's epileptogenic effects are mediated through N-methyl-D-Aspartate (NMDA) receptors leading to neurogenic hyperexcitability (Yamaguchi and Rogawski 1992; Cramer et al. 1994). However, the effects of 4-AP are short-lived and are only viable for acute recordings or would require constant dosing to achieve chronic seizures.

Kainic Acid (KA), a chemical analog to L-glutamate, is an antagonist of KA receptors found in the brain. KA was found to cause severe neural depolarization that is followed by cell death (Victor Nadler 1981). This property, along with the characteristic physiological electroencephalography (EEG) features, pushed it to become a useful model in the evaluation of mesial temporal lobe epilepsy (MTLE) (Lévesque and Avoli 2013). The resulting epileptiform was shown to begin as focal seizures that progress to generalized seizures and focal status epilepticus (Ben-Ari et al. 1979).

Another model is the kindling model, in which animals are chronically implanted with stimulation electrodes placed in the limbic system, the stimulation of the limbic structures results in the development of focal seizures (Goddard, McIntyre, and Leech 1969). This model, however, is limited in the sense that it requires daily stimulation to achieve seizures and has been shown to result in secondary generalized seizures as stimulation progresses. (Löscher 2006).

One promising model is the Cortical Iron Chloride Injection model. Injection of iron chloride in the somatosensory motor cortex of rats causes the production of recurrent focal seizures (Willmore, Sypert, and Munson 1978). It is thought that the deposit of reactive oxidative species in the brain leads to lipid peroxidation, consequently leading to demyelination and neural damage (Willmore and Triggs 1991; Triggs and Willmore 1984). Multiple studies have utilized the Iron Chloride model to replicate focal cortical seizures; however, no quantification or analysis of the epileptogenic waveforms produced has been established in the literature (Wu et al. 2015; Zou et al. 2017). It has been established though that the Iron Chloride model produces recurrent focal seizures that can last up to three months post-injection (Zou et al. 2017; Willmore and Triggs 1991; Willmore, Sypert, and Munson 1978). Additionally, it has been hypothesized in the literature that the same peroxidative mechanism utilized by iron chloride is the underlying

cause of the development of seizures secondary to traumatic brain injury (TBI)(Willmore, Sypert, and Munson 1978; Bragin et al. 2016; Willmore and Triggs 1991; Lucke-Wold et al. 2015).

1.7. Traumatic Brain Injury

Traumatic Brain injury is a major contributor to symptomatic epilepsy accounting for 20% of active epilepsy in the general population (Hauser, Annegers, and Kurland 1993). TBI is hypothesized to lead to epileptogenic activity via BBB degradation and other vasculature mechanisms (Tomkins et al. 2011). This mechanism is thought to correlate with the mechanism utilized in the Iron Chloride model (Lucke-Wold et al. 2015; Willmore and Triggs 1991; Pitkänen et al. 2009). Moreover, patients with TBI-related epilepsy (TRE) exhibit FCE as shown through fMRI and EEG recording (Irimia and van Horn 2015; Tomkins et al. 2011; Pitkänen and Immonen 2014).

Patients with TRE exhibit similar characteristics to those with FCE as the pathology is the same, thus they do not benefit from the use of AEDs and are ineligible for surgical intervention. In clinical settings, and in other seizure models, the development of the mirror focus is a characteristic of focal seizures and TRE. In pediatric TBI patients, multichannel recordings revealed a high degree of synchrony across both hemispheres (Nenadovic et al. 2008; Proix et al. 2018). In 4-AP and low Mg models of seizures, 30-40% of seizures have been shown to propagate to the contralateral focus (Sip et al. 2021; Cammarota et al. 2013). However, no research has been completed to investigate the propagation characteristics of the Iron Chloride model in correlation with the clinical findings of patients with TRE.

1.8. Thesis Objectives and Organization

Though there have been various research projects investigating the efficacy of LFS in seizure suppression, the experiments have either addressed the stimulation paradigm in-vitro, acutely in-vivo, or have addressed other sorts of LFS that are not applied to white matter tracts (i.e.CC). Furthermore, the assessment of LFS in CC stimulation in chronic settings requires a robust characterization of the focal cortical model for long-term recording. *It is our hypothesis that the Iron Chloride model can generate chronic focal recurrent seizures in-vivo for the assessment of LFS paradigms in the CC*. This is achieved two-fold through the following objectives:

Objective I: Establish and characterize the Iron Chloride model in rodents for chronic recurrent seizure generation.

Hypothesis: Iron Chloride model can produce a two-week stable baseline of recurrent spontaneous focal cortical seizures within one week following intracerebral injection.

Rationale: Though LFS has been investigated in seizure suppression, experiments have been limited to *in-vitro* preparation or acute *in-vivo* experiments (Couturier and Durand 2020; 2018; Koubeissi et al. 2013). Similarly, the utilization of the Iron Chloride model has been limited or poorly characterized in chronic in-vivo preparations, thus, limiting the utilization of such a model in our current project. Therefore, a stable baseline of recurrent spontaneous focal cortical seizures through a single intracerebral injection is required to test the efficacy of LFS.

Objective II: Examine the spatiotemporal extent of seizure generation/propagation in the Iron Chloride model.

Hypothesis: Iron Chloride injection will generate seizures that will extend beyond the ipsilateral focus.

Rationale: Epileptic neural networks have been shown to have a high degree of interhemispheric synchrony (Nenadovic et al. 2008; Couturier and Durand 2018; 2020; Sip et al. 2021). Thirty to forty percent of seizures generated in well-established models, such as 4-AP and low-Mg, propagate to either the HC or the contralateral focus. Furthermore, LFS has been shown to cause bilateral seizure reduction in *in-vitro* experiments, thus we test to see if the Iron Chloride model can produce seizures that propagate to the contralateral motor cortex for future experiments.

Chapter 2

Establishments and characterization of the Iron Chloride Model

2.1. Introduction

Focal cortical seizures contribute to the highest burden of drug resistant epilepsy leading to a decreased quality of life for those suffering from FCE (Sultana et al. 2021; M. C. H. Li and Cook 2018). More than fifty percent of patients with FCE are ineligible for surgical intervention due to the complexity of resecting the epileptogenic zone or the increased number of involved neural networks in the epileptogenic activity(Noachtar S and Borggraefe I 2009). Furthermore, the eligible cohort of patients reported recurrent seizure activity five-year post-surgery (Spencer and Huh 2008). This disparity in treatment modalities for FCE has led researchers to investigate stimulation paradigms such as DBS and VNS. Despite the apparent benefit of being less invasive than DBS, VNS has received mixed reviews from researchers and clinicians. Some studies have even confirmed the decreased efficacy of VNS in the treatment of FCE in comparison with generalized seizures (Muthiah et al. 2022).

Chronic use of DBS is also linked to tissue-electrode interface damage which leads to decreased therapeutic efficacy. Only 16% of patients were seizure free for six months only as reported in a five-year follow-up (Salanova et al. 2015). The continuous search for a treatment modality that can alleviate FCE and improve the quality of life for patients has led to the investigation of a unique stimulation paradigm utilizing low-frequency stimulation of white-fiber tracts (Koubeissi et al. 2013; Couturier and Durand 2020). Of the many white fiber tracts in the brain, the CC has been the most investigated in FCE and MTLE; the CC is thought to play a role in interhemispheric information shuttling (Eccher 2014). The functional arrangement of the CC aids in the compartmentalization of the information from different cortices in both hemispheres. Furthermore, the existence of GABA (B) interneurons that synapse onto cortical neurons has

been shown to play a critical role in seizure suppression achieved by LFS through sAHP (Couturier and Durand 2018; Toprani and Durand 2013).

However, to our knowledge, the efficacy of LFS has been limited to *in-vitro* and acute *in-vivo* studies due to the lack of availability of a well-established animal model for cortical epilepsy. Available models, such as KA, often lack the precision in localization of seizures or lead to secondary generalized epilepsy. Other models, such as the kindling model, require daily stimulation as it is the mechanism by which cellular physiology is altered leading to hyperexcitability. Despite the availability of a new genetic focal seizure model that is still under investigation, the cost and labor surpass that of many research organizations deeming it unfeasible. One model that has been shown to produce focal cortical recurrent seizure is the Iron Chloride model (Willmore, Sypert, and Munson 1978).

The Iron Chloride model acts to induce seizures through a process called ferroptosis. Ferroptosis has been linked to multiple neurological diseases such as neurodegenerative diseases (Thirupathi and Chang 2019). Ferroptosis is linked to the development of reactive oxidative species (ROS) leading to lipid peroxidation. The same mechanism is linked to patients suffering from TBI-related epilepsy (TRE) where the released iron from the blood leads to lipid peroxidation.

This model has been utilized by multiple studies to investigate other stimulation paradigms (e.g., Transcranial Stimulation) but has not been characterized in literature through standard seizure metrics. We are going to develop a stable recurrent seizure in a chronic model and advance the evaluation of white fiber tract stimulation through LFS in chronic settings.

2.2. Methods

2.2.1. Electrode Preparation

Prior to the surgical procedure, electrodes were prepared into a “head cap” that is cemented on the animal’s head. A 12-pin Micro-360 Solder Cup SS connector (0.27 in, Omnetics Connector Corporation, MN, USA) was used to solder five stainless-steel electrodes (diameter 0.125 mm, Plastics One Inc., Roanoke, VA, U.S.A.) via 40-gauge DFT wire (Fort Wayne Metals, IN, USA). Electrodes were connected to the connector using the schematic shown below in Figure 1. Each electrode was paired to a reference recording to differentially record from each electrode. All the reference electrodes (A-, B-, and C-) were connected to a stainless-steel screw that is referred to as “common reference”. The GND electrode was connected to a separate stainless-steel screw to serve as the ground.

The head-cap was then immersed in Epoxy to avoid any shorting between wires and/or soldering cups. Electrodes were tested in a 0.9% saline bath, a function generator, and the Data Acquisition system (RHD200, Intan Technologies, CA, USA) to confirm the integrity of each induvial connection prior to implantation.

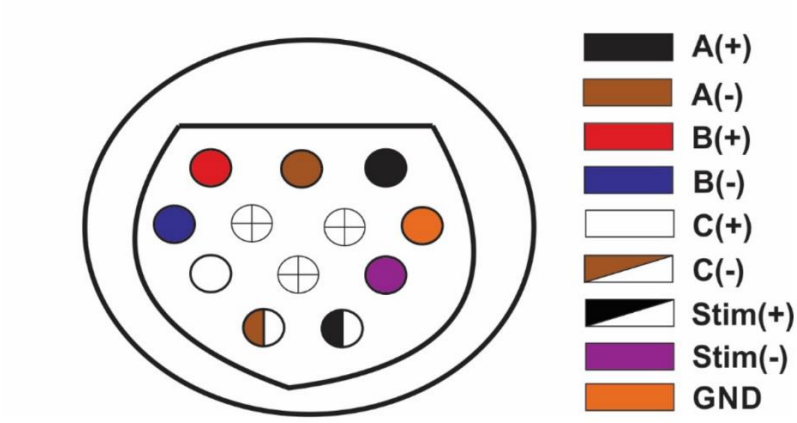


Figure 1: 12 Pin Omnetic Connector Electrode Schematic.

2.2.2. Surgical Procedure

Eight-week-old Sprague Dawley rats (N=5) were used in this study. Rats were housed in a controlled environment prior to the surgical procedure with free access to food and water. One additional animal was used as a sham as described below. All animal procedures were conducted in accordance with guidelines, reviewed, and approved by the Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University.

Animals were given 10 ml of Lactated Ringer's fluid prior to the surgery to ensure sufficient hydration and blood volume. Animals were then anesthetized using Isoflurane gas in an anesthesia chamber. The head was shaved and scrubbed with betadine and alcohol prior to the surgery. The animal was then secured in the stereotactic apparatus. A small incision was made along the rostrocaudal axis to expose the skull. Seven burr holes were made: five for the electrodes and injection, and two for the reference and ground screws.

5 µL of 0.1 M $FeCl_3$ was injected in the M1 cortex using a 10 µL 700-series Hamilton Syringe (Hamilton Company, NV, USA) (1.00 mm posterior to the bregma, 2.00 mm lateral to the bregma, and 1.80 mm ventral to the surface of the brain). Injections were performed at a rate of 0.2 µL/5 mins to avoid any osmotic damage to the motor cortex. For the sham animal, 5 µL of 0.9% saline was injected in the M1 cortex using the same coordinates.

Electrodes were placed in the coordinates shown below in Table 1 according to a stereotactic atlas (Paxinos and Watson 2006) . Three recording electrodes were placed in the injection site as the focal point, the contralateral M1 cortex (1.00 mm posterior to the bregma, 2.00 mm lateral to the bregma opposite the injection site, and 1.80 ventral to the surface of the brain), and the ipsilateral CA3 region of the HC (3.14 mm posterior to the bregma, 3.00 mm ipsilateral to the injection lateral to the bregma, and 3.75 mm ventral to the surface of the

brain) respectively. Additionally, two stimulation electrodes (bipolar configuration placed parallel to the longitudinal axis of the callosal axons) were placed equidistant from the midline, 1.00 mm posterior to the bregma, 0.60 mm laterally on both sides of the bregma, and 3.4 mm ventral to the surface of the brain. These coordinates correspond to the anterior fibers of the CC that are of interest in this stimulation paradigm as explained previously.

Table 1:Electrode and injection Coordinates for the Iron Chloride Model

Position	Coordinate Axis (mm)		
	AP	Lateral	Depth
Injection	-1.00	2.00	-1.80
Focal Cortex Recording	-1.00	2.00	-1.80
Corpus Callosum stimulation + and -	-1.00	+/- 0.60	-3.40
Ipsilateral CA3 Recording	-3.14	3.00	-3.75
Contralateral Cortex Recording	-1.00	-2.00	-1.80

These coordinates have been established through the aforementioned acute LFS experiments in accordance with the Rat Brain Atlas as shown in Figure 2 (Paxinos and Watson 2006; Couturier and Durand 2018; 2020).

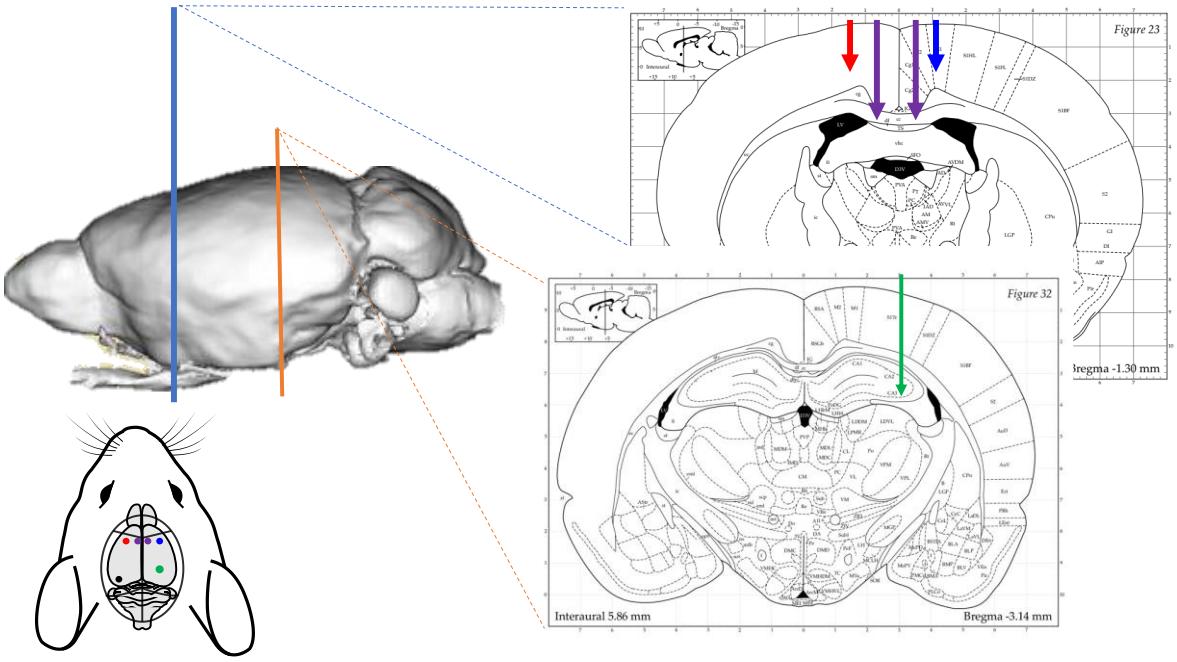


Figure 2: Figures from Rat Brain Atlas indicating the coordinates used in the surgical electrode implantation along with superior view indicating the placement of electrodes. From left to right: Contralateral recording (red), Bipolar Stimulation Electrodes (Purple), Focal Cortex Recording (Blue), ipsilateral CA3 HC Recording (Green), and Common Ground (Black) seen only on the skull.

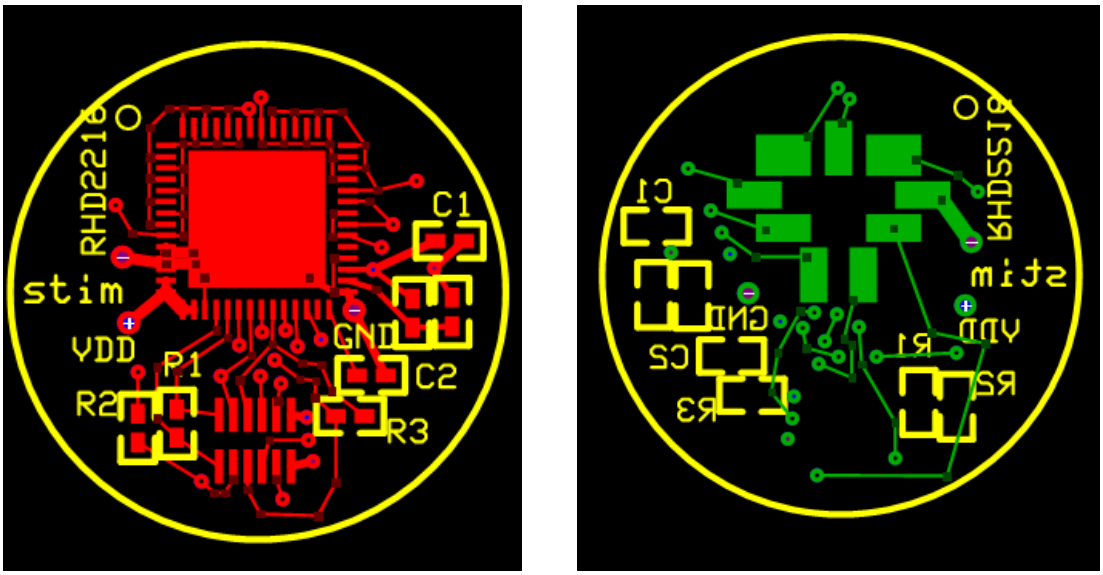
Both the common reference and the ground screws were placed on the posterior aspect of the skull. Three more screws were placed around the skull to act as an anchor point for the dental acrylic. A two-part cold cure Dental Cement (A-M Systems, WA, USA) was used to fixate the head-cap, electrodes, and screws to the skull.

Animals were monitored for seven days post-op, with daily 0.1 mg/100g meloxicam injections for the first three days. Animals were housed in their original cage individually to prevent surgical site infections.

2.2.3. Data Acquisition System

Data were collected using a recording system composed of two parts. The first part consisted of a custom-made PCB board utilizing the RHD 2216- amplifier chip (Intan Technologies, CA, USA). Briefly, the RHD 2216 is a 16-channel amplifier chip with differential

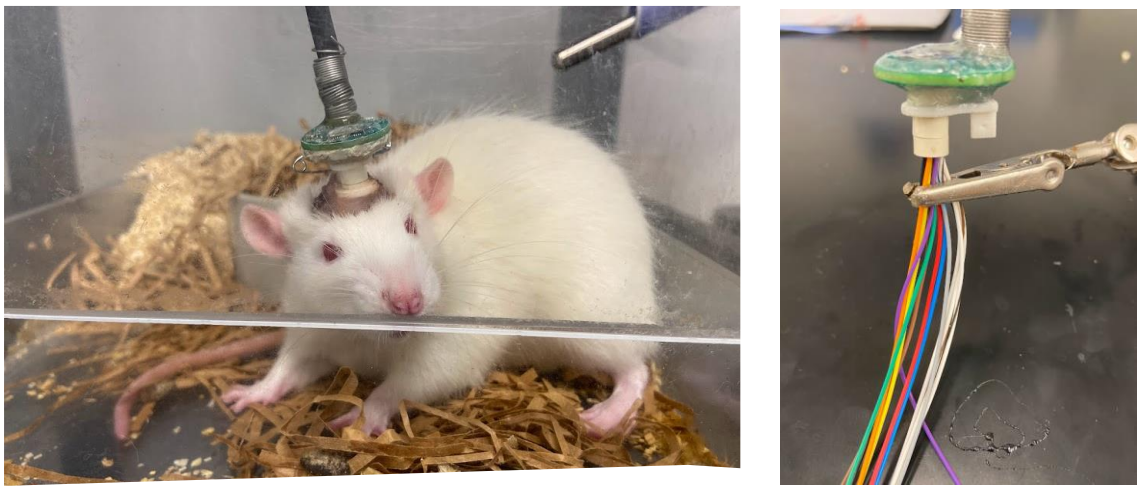
inputs (0.1Hz-20kHz) recording capabilities. Since we are only interested in three recording sites, we utilized the extra channels in performing signal averaging to decrease stochastic noise. Thus, the signal from the focus was connected to six channels, the contralateral signal was connected to four channels, and the CA3 signal was connected to two channels. A schematic of the PCB channels can be seen below in Figure 3.



A B

Figure 3: Front (A) and Back (B) of Custom-made PCB board utilizing a 16-channel bipolar amplifier (Intan Technologies). PCB Board utilized 12 channels through redundant use of multiple channels for the same electrode to allow for signal averaging.

The PCB board was connected to a female-12-pin connector matching the one used in the head cap, the final connection between the rat, head cap, and the Intan chip can be seen below in Figure 4.



A B

Figure 4: Final connection to the animal, with headcap on top, connected to the Intan chip-PCB board (A). Sample connection demonstrating the Intan-chip to headcap connection without the animal.

The second component of the recording system is the custom interface board, located outside the recording cage connected to a computer. The animal was then placed in the recording cage, where an RHD standard SPI cable (2.9mm PZN-12 polarized nano, Intan Technologies, CA, USA) is connected to the headcap and then connected to a breakout board fixed commutator. The commutator is a two-part disc that is fixated on top of the recording cage and acts to relieve the torsion on the SPI cable as the animal moves during the recording period. A second SPI cable is then used to connect the commutator to the interface board.

A digital stimulator (DS8000, WPI, FL, USA) was connected to a DS300 current isolator (WPI, FL, USA). The current isolator output was connected to the aforementioned breakout board, and the output current was fed into the electrodes through the head cap shown in Figure

4A. Additionally, the breakout board was connected to a metal grounding plate to decrease 60Hz noise in the recording.

All signals were acquired at a 2000 Hz sampling rate, a high-pass filter 0.1 Hz, and a low-pass filter 500Hz. The commutator and shielded wires allowed the animals to move freely while being chronically recorded for 24 hrs./14 days. Data were stored in an external hard drive and then processed using MATLAB. Animals had free access to food and water throughout the duration of the experiment with new cage change once a week.

2.2.4. Signal Processing and Seizure Detection

Data were imported into MATLAB where the above-mentioned signal averaging was performed based on the channel assignments. A digital 300 Hz low-pass filter was applied, and EEG signals were down-sampled by a factor of two to streamline the process of EEG analysis. The data were then imported into a custom-made MATLAB script that plotted the EEG in the time domain and allowed the user to visualize the power spectrum and amplitude of individual windows. An electroencephalographic seizure was defined as a segment of EEG with high-frequency power (>5Hz), high amplitude (>2x the baseline), and a duration of at least five seconds (Nissinen et al. 2000). For seizure duration measurements, the first point of increased power and amplitude was considered t=0, while the final point where the power and amplitude meet the requirements is considered $t=t_{end}$. Seizure events less than five seconds apart were considered to be separate seizures.

Epileptogenic activity in the contralateral motor cortex and the HC were both analyzed using the same methodology discussed in 2.2.4. A cross-correlation was performed on each seizure. A segment consisting of five seconds of baseline, the EEG seizure activity, and another

five seconds of baseline in that order was cross correlated to the contralateral motor cortex and the HC EEG signals.

2.2.5. Statistical analysis

The stability of both seizure metrics was evaluated using the gradient test. In short, a linear regression was performed then a regression analysis was utilized to determine if the regression is statistically significant (alpha=0.05).

In order to determine whether the seizures were synchronized with delay or perfectly simultaneous, student's t-test was performed (alpha=0.05) on the mean lag time between the focal seizures and the contralateral seizures and HC seizures, respectively. All statistical analyses were performed using JMP software.

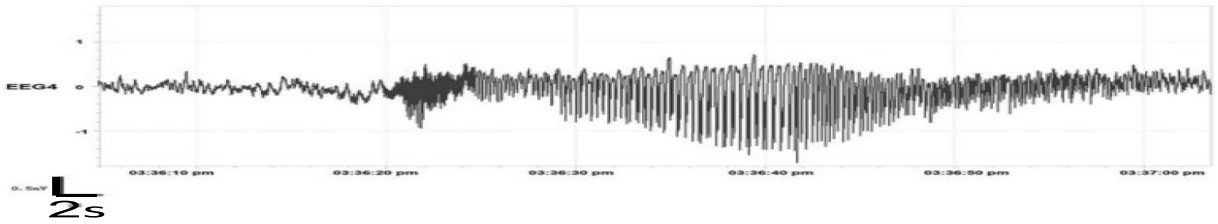
2.3. Results

2.3.1. Seizure Characteristics

The saline-injected animal did not show any seizure activity over the span of the entire recording period (14-days).

The iron chloride injected animals, however, were epileptogenic as expected. An example of the recorded electroencephalographic seizures is shown in Figure 5B. The recorded seizure in our chronic study had a mean duration of 31±7.9 seconds, a mean peak-to-peak voltage of 2.5±1.5 mV, and an average peak frequency of 22.8±5 Hz (N=3370 seizure events). Seizures obtained matched those reported in other acute experiments in the literature or short-term studies (three days) that have been published (Figure 5A). It is worth noting that all seizure events were initiated by a positive baseline shift event which was useful for the detection and counting those events.

A)



B)

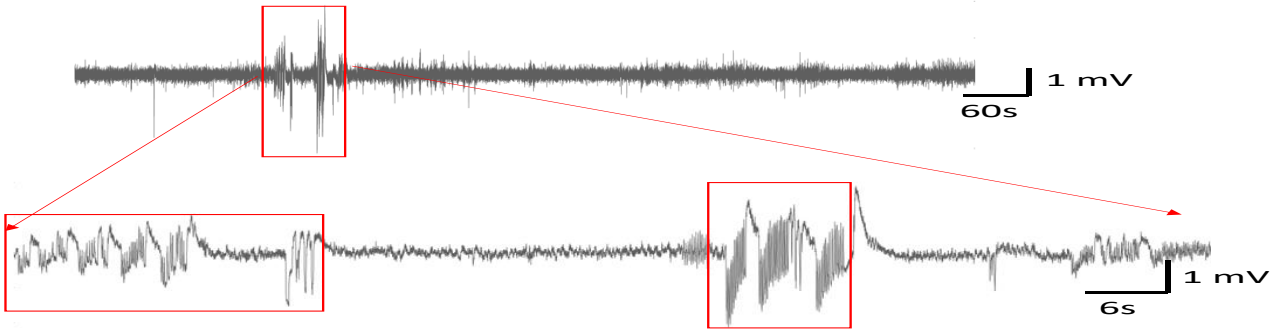


Figure 5: An EEG segment showing an Iron Chloride seizure from a previous acute study (A) compared to an EEG segment with multiple seizure events (red box) from our chronic recording (B).

2.3.2. Seizure metrics

Seizures were counted and compiled for every animal during the 14-day recording period. As seen in Figure 6, there is an established minimum floor of approximately 50 seizures per day. The iron chloride animal model also showed as high variability among the animals, specifically on the first day of recording (day eight post-injection) give numbers. Additionally, the time spent seizing per day, reported as percent of day in Figure 7, shows similar variability.

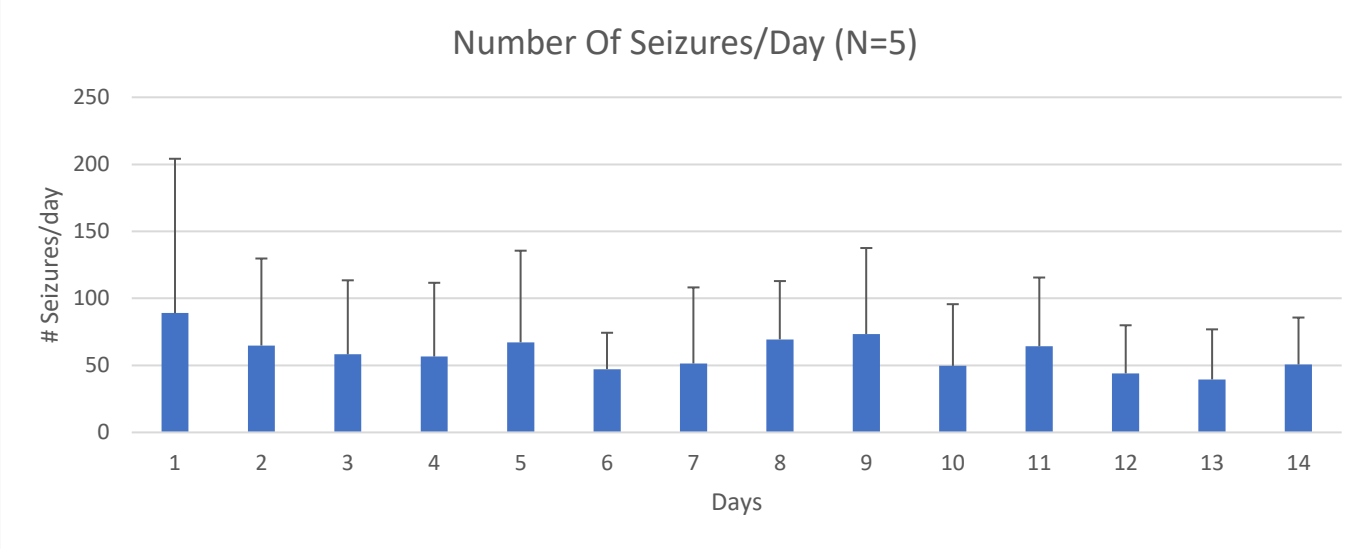


Figure 6: Number of seizures per day (N=5) during the span of 14-day chronic recording of Iron Chloride Injected Animals

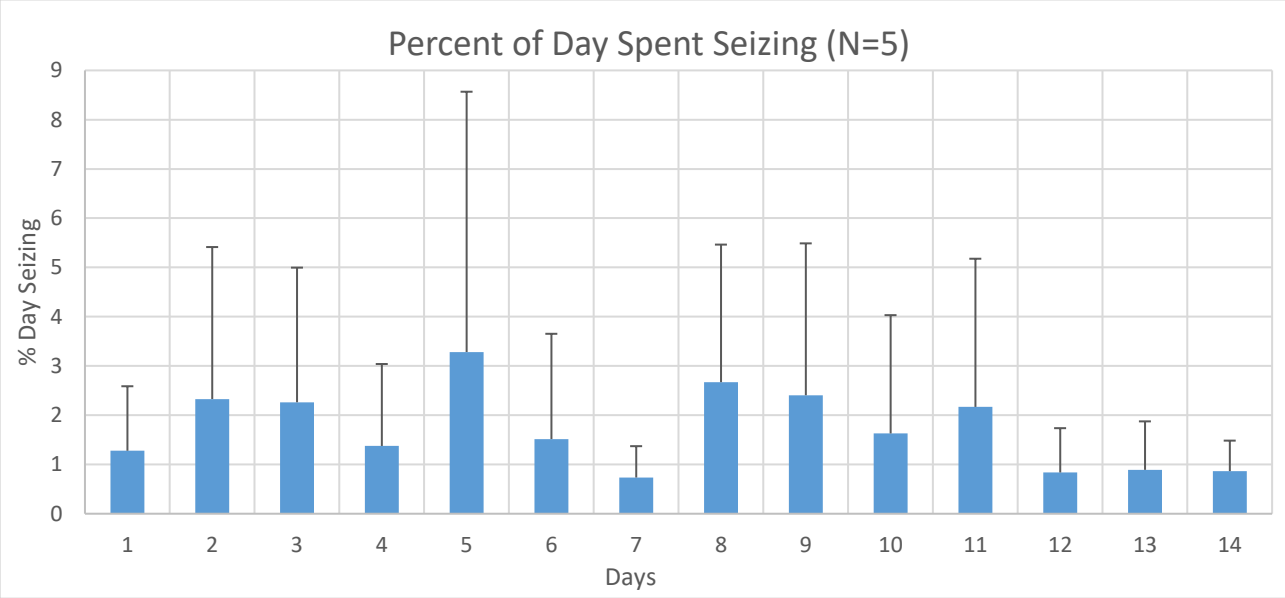


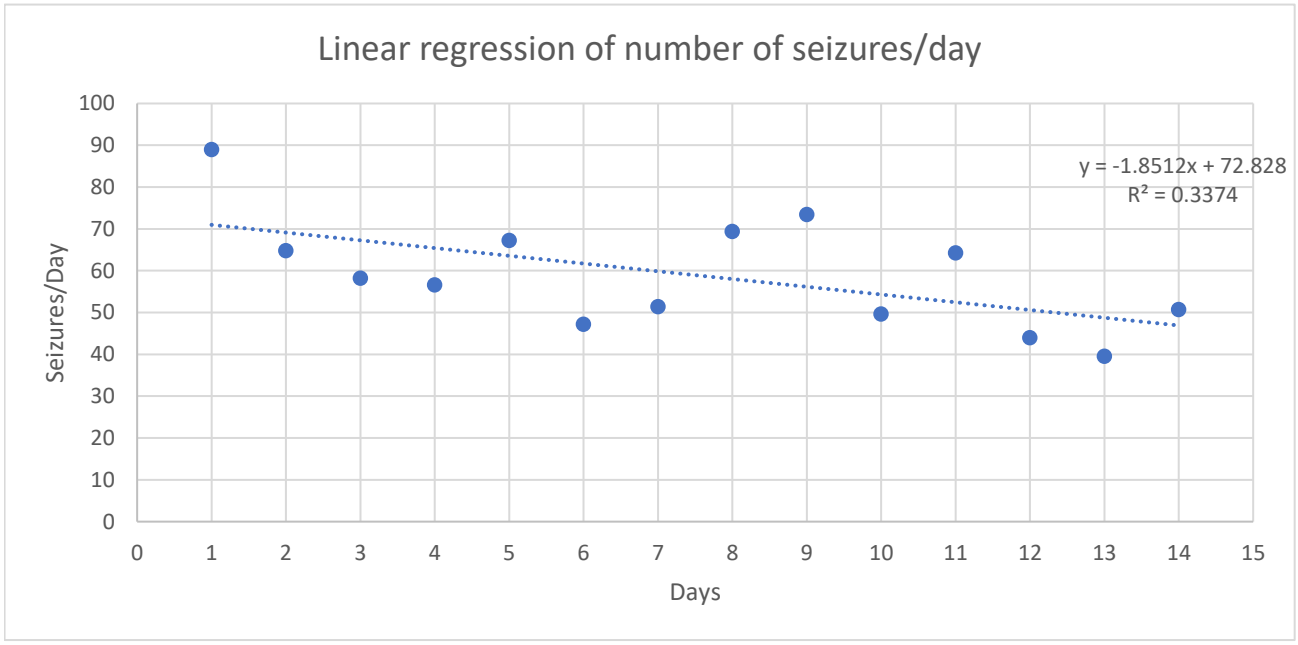
Figure 7: Percent of day spent seizing (N=5) reported for 14-days of chronic recording for Iron Chloride injected Animals.

2.3.3.Stability of the Baseline Seizure Activity

In order to determine the stability of the number of seizures per day, the hypothesis that the slope of the regression line for the amount of seizures/day over time was not significantly different from zero was tested (Figure 8). A first linear regression including all fourteen days of recording and a second regression line excludes the first day of data due to its high variability were performed. An ANOVA performed on all fourteen days of recording, Figure 8 panel A, was statistically significant ($p=0.02$). However, excluding the first day of recording (day eight post-implantation), Figure 8 panel B, was statistically insignificant ($p=0.15$). Examining the data seen in Figure 6, the variance between animals is highest at the first day of recording reaching 115 seizures per day compared to the rest of the recording period where the variance is between 24-65 seizures per day.

An ANOVA performed on the linear regression for the percent time spent seizing was statistically insignificant ($p=0.19$), Figure 9, throughout the 14-day chronic recording period. No exclusions or adjustments needed to be made. Taken together the results indicate that seizures are stable over a 13-day period.

A)



B)

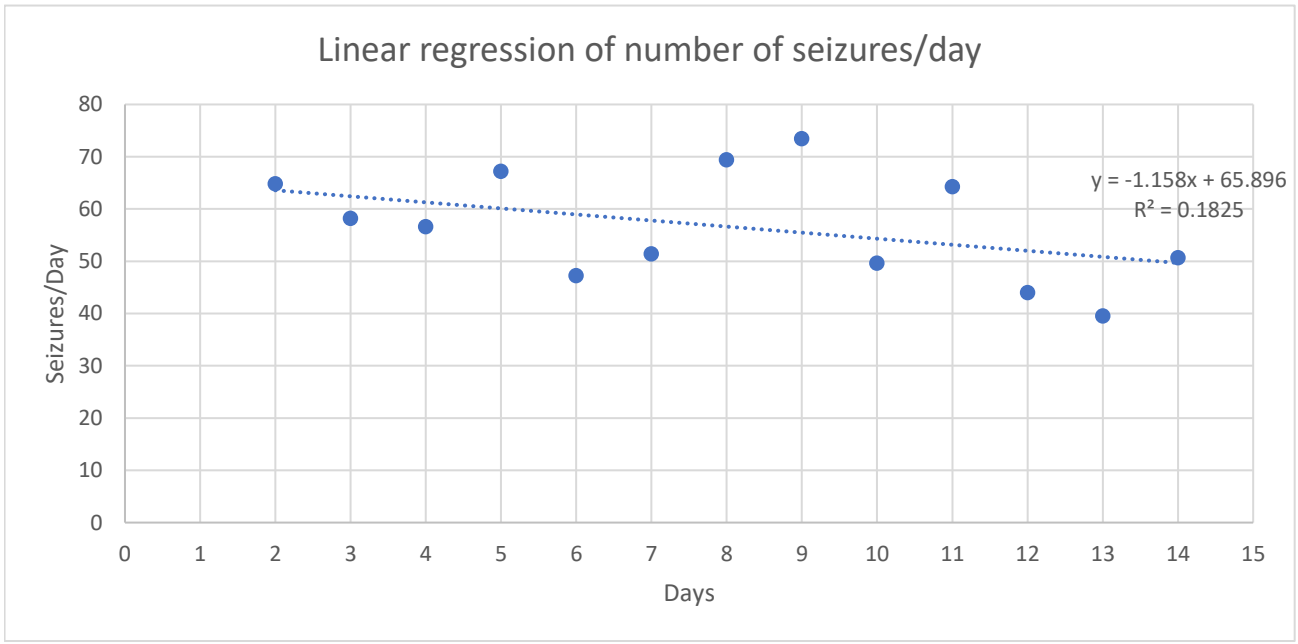


Figure 8: Linear regression of number of seizures per day for all 14-days of chronic recording (p=0.02) (A) compared to linear regression of number of seizures per day excluding the first day of recording (p=0.015) (B).

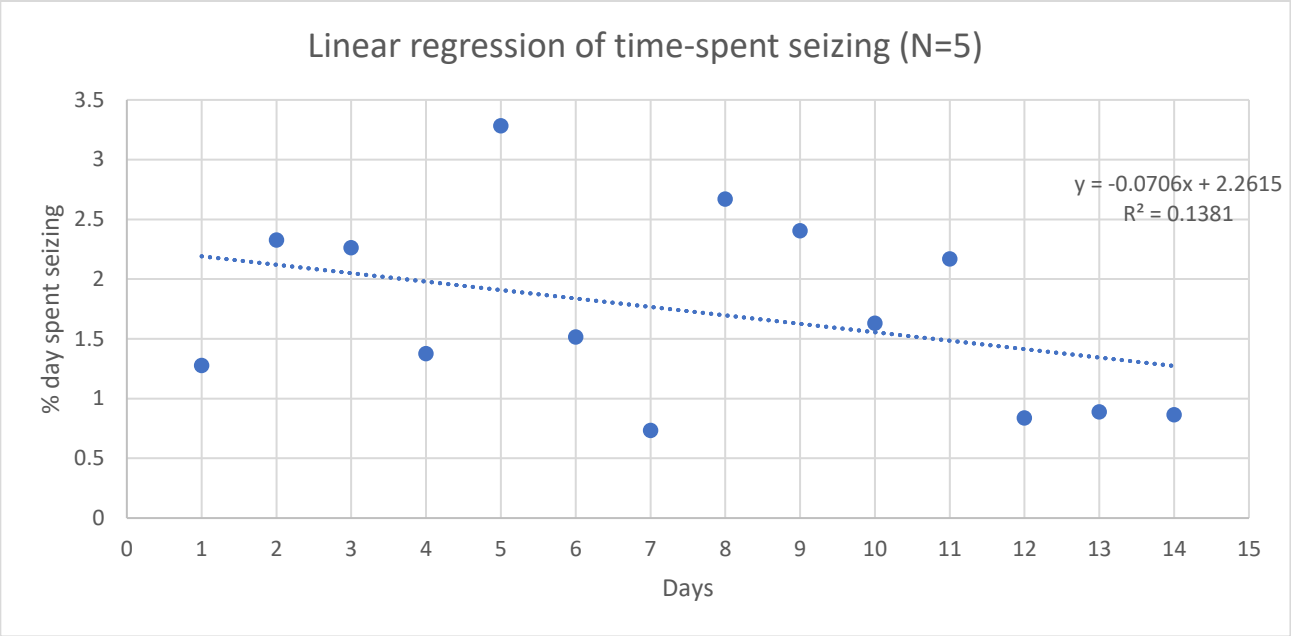


Figure 9:Regression analysis of the percent time spent seizing for 14-day recording (N=5) (p=0.19)

2.3.4. Propagating Seizures

The hypothesis that seizures from the Iron Chloride model could propagate to other areas of the brain was then investigated. Seizures were observed to propagate from the focus to the contralateral focus and hippocampus, with a high degree of synchrony. An example of such propagation is shown in figure 10.

However, not all seizures propagated to the contralateral cortex and the HC (see example in Figure 11). Out of 1600 seizures analyzed, 402 (25%) were observed to be in synchrony with seizures in motor cortices. Only 45 seizures in the focus (2.8%) were observed to be synchronized with seizures in the hippocampus.

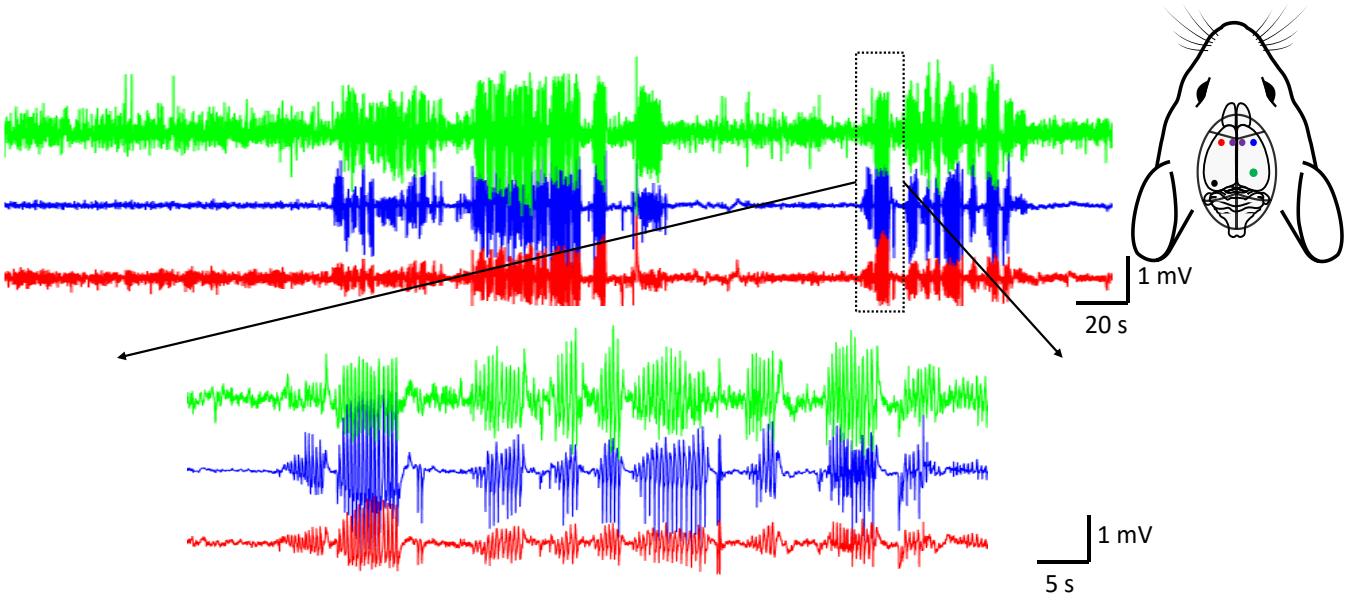


Figure 10: Seizures initiating in the focus (blue trace) are seen to propagate to both the contralateral motor cortex (red trace) and the HC (green trace) (top). A magnified EEG trace of multiple seizure events showing high degree of synchrony amongst the focus (blue), contralateral motor cortex (red), and the HC (green) (bottom)

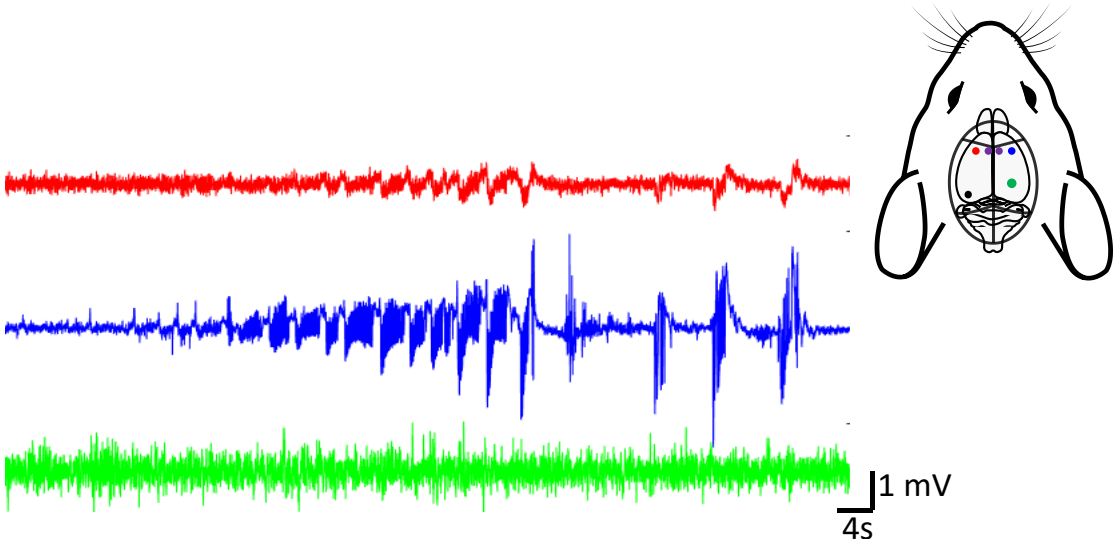


Figure 11: An example of a non-propagating seizure initiated in the focus (blue trace) but not detected in the contralateral motor cortex (red trace) nor in the HC (green trace)

Although the seizures observed in two different locations appear to be simultaneous, a closer examination of the seizure initiation event reveals otherwise. In Figure 12, a closer look at one of the seizure events reveals a minute delay between the onset of the seizure event in the focus compared to that in the mirror focus. The small difference of 2 ms between both depolarization events suggests that there is a propagation delay between secondary foci and original focus.

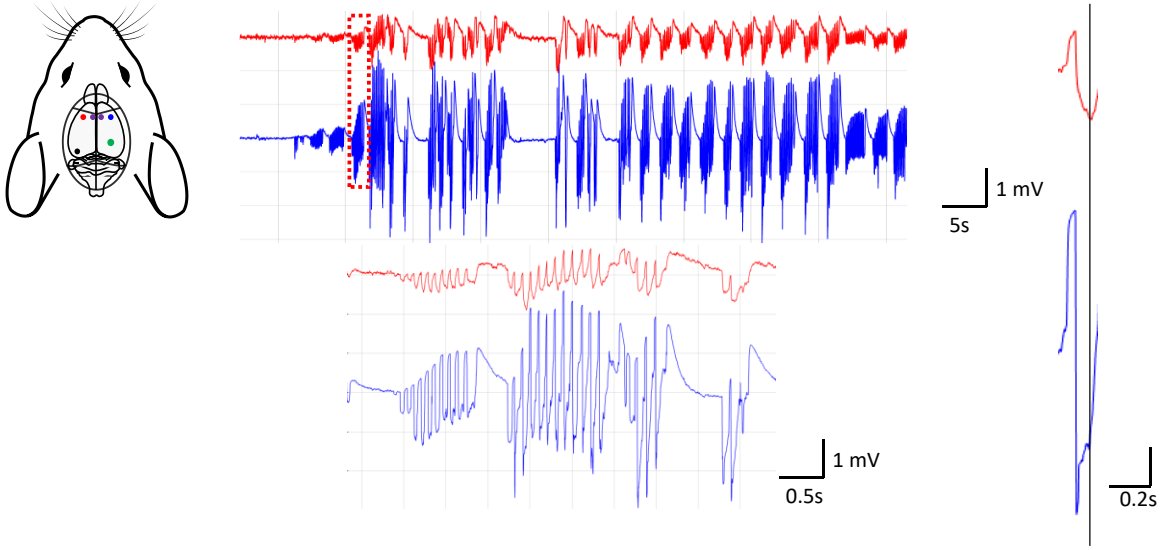


Figure 12: A closer evaluation of the onset of a seizure event between the focus (blue) and the mirror focus(red) (top). An enlarged version of the seizure event (dotted red box) (bottom) reveals a slight delay between the onset of the seizure event in the focus and the mirror focus (right) demonstrated by the difference of the initial depolarization event.

Multiple events (N=63) occurred simultaneously between the ipsi- and contra-lateral motor cortices. The cross-correlation analysis shows a high degree of correlation with a mean R-value of 0.79±0.07 (N=402) between contralateral events. The delay time from the correlation analysis was broadly distributed with a mean value of 4±8 msec. The delays values could be further divided into positive and negative values with, a negative phase shift indicating as focus-leading seizures while a positive phase-shift was defined as mirror-focus-leading seizures. The majority (86%, N= 402) of seizures initiated within the focus; however, a few seizures (14%, N=402) events were observed to start in the mirror-focus.

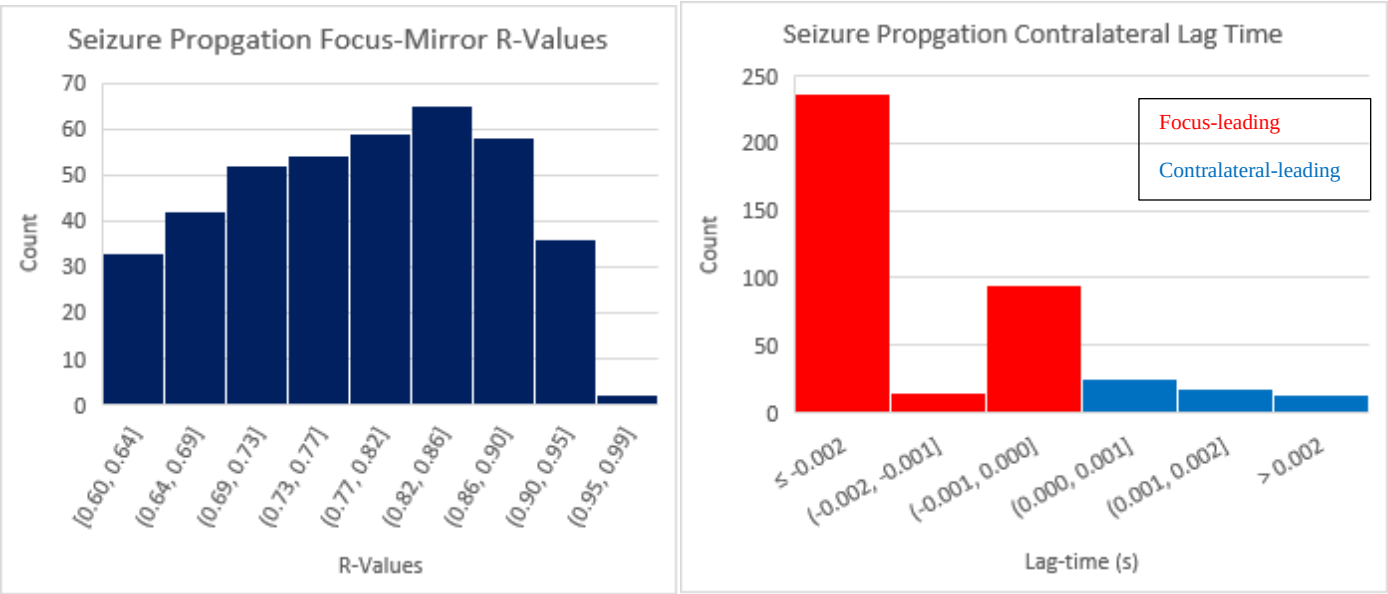


Figure 13: Cross-correlation results showing the R-values of (left) and Phase-shift in second (right) between seizure events in the focus and contralateral cortex. A negative phase-shift is defined as seizures initiated in the focus, while a positive phase shift is defined as seizures initiated in the contralateral cortex.

Similar patterns were observed in the evaluation of the HC (Figure 14). However, as mentioned before, fewer seizures were observed to propagate. Seizures that did propagate had a mean R-value of 0.71±0.072 and a mean delay-time of 1±1 msec.

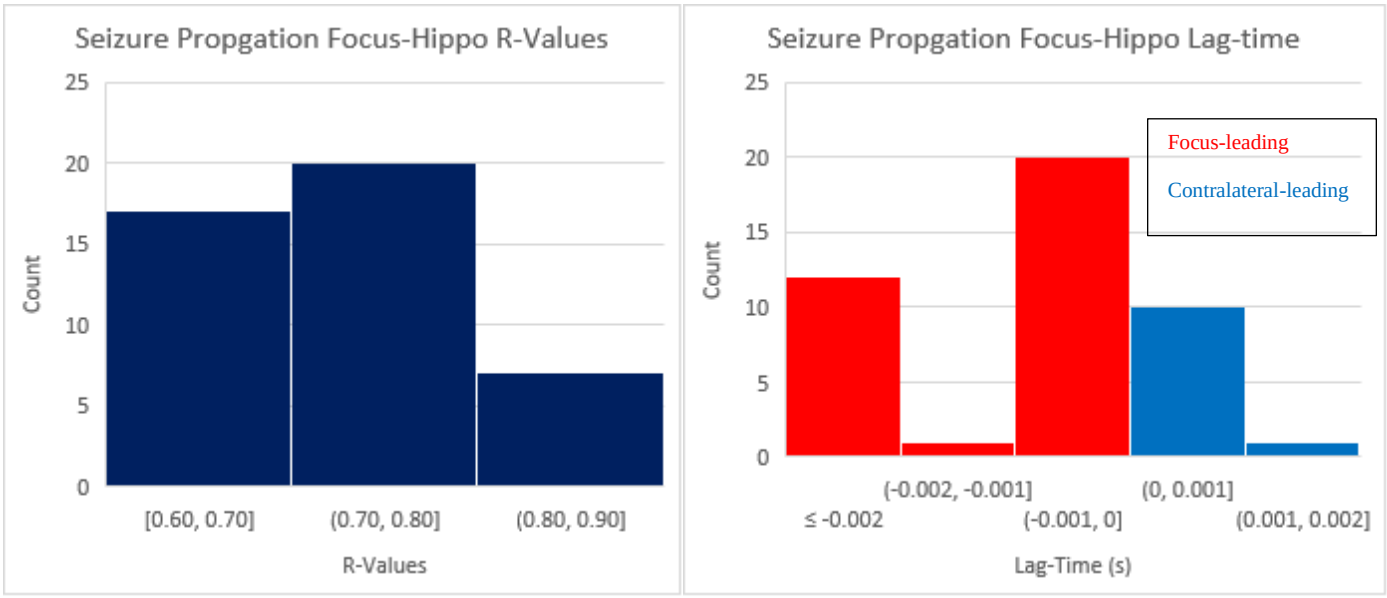


Figure 14:Cross-correlation results showing the R-values of (left) and Phase-shift in second (right) between seizure events in the focus and the HC. A negative phase-shift is defined as seizures initiated in the focus, while a positive phase shift is defined as a seizure initiated in the HC.

A two tailed t-test showed that the mean values for both two delays are significantly different ($\mu \neq 0$), $p<0.001$ and $p=0.019$ respectively.

2.4. Discussion:

The Iron Chloride model has been widely used in various experiments and research, but its stability for long-term experiments has not been well characterized. The main objective of this part of the project was to investigate whether the seizures induced by iron chloride are stable in chronic settings, and if so, to characterize the nature of the seizure events. The ultimate purpose of this aim is to establish a stable baseline of seizures over a two-week period (14 days) to investigate the efficacy of white fiber tract LFS in chronic settings.

Although the seizures produced by the Iron Chloride model may appear different from those seen in other models such as the 4-AP or KA model, they fit one of the most established electroencephalographic definitions of seizure activities by Nissinen et al. The seizures are clearly

distinguishable from the baseline, as demonstrated in Figure 5B, as they often exhibit relatively higher frequency components and are often higher than twice the amplitude of the baseline. Although the seizure events were often short, lasting 31±7.9 seconds, some seizures would last up to a minute. Overall, the seizures produced by this model are generally mild to moderate across all animals over the span of two weeks. Morphologically, the seizures observed match those previously reported in the literature, as shown in Figure 5 (Willmore and Triggs 1991; Das, Singh, and Sharma 2017; Willmore, Sypert, and Munson 1978). No statistics were found in the literature regarding the voltage, duration, or frequency content of those seizures, which highlights the need for this study to characterize these seizures.

The injection of Iron Chloride in the M1 cortex was capable of producing recurrent seizures that lasted at least two weeks. Figure 6 shows that the baseline number of seizures stabilizes at approximately 50 seizures per day across all animals (N=5). Moreover, linear regression performed on the data, excluding the first day, shows that the number of seizures per day is statistically stable ($p=0.15$), along with the percentage of time spent seizing ($p=0.19$).

The iron chloride model produces more seizures than other models that have been studied previously. For example, one study reported that the 4-AP model produced an average of 12 seizures per hour declining over the span of two hours to less than six seizures per hour (Osborne et al. 2019). Previous studies have also reported an average of 15 seizures per day for the Kindling model over the span of seven days (Couturier 2014). However, the seizures are more stable and recurrent without the need for further intervention past the initial injection. It is possible to produce more seizures through the injection of more $FeCl_3$, however the severity of the seizures could possibly increase, leading to a generalization of seizures as observed in an acute study that utilized different concentrations of $FeCl_3$ (Q. Li et al. 2019).

One additional characteristic of interest for the iron chloride model is the is the spatiotemporal extent of the seizures produced through a single injection. We observed bilateral seizure propagation that was synchronous but not simultaneous. Comparing the focal motor cortex to the contralateral motor cortex, we observed highly correlated and synchronized EEG activity that resulted in a quarter of the seizures propagating from the injected motor cortex to the contralateral hemisphere. Although these seizures seem simultaneous, they are observed to propagate to the contralateral hemisphere. Some seizures were observed to propagate in the opposite. Similar results were obtained on the hippocampus whereby propagation into the hippocampus was also observed. A majority of the seizures were initiated in the injected focal cortex and propagated into the HC (75%, N=44) with a few initiating in the HC (25%, N=44) and propagated into the cortical focus. Taken together, these results indicate that the iron chloride model generates seizures that can generalize and propagated into the contralateral cortex and into the hippocampus. They also indicate that the model can generate temporary secondary generated seizures that can propagate back into the cortical focal site.

TRE contributes significantly to the incidence and prevalence of FCE (Hauser, Annegers, and Kurland 1993). TRE has been shown to have a localized epileptic effect that was confirmed through electroencephalographic studies and fMRI imaging (Irimia and van Horn 2015; Tomkins et al. 2011; Pitkänen and Immonen 2014). Furthermore, these patients develop secondary epileptic foci (Nenadovic et al. 2008; Proix et al. 2018). The development of secondary foci is not quite understood but has been confirmed and studied for multiple years (Wilder 2001; McCarthy, O'Connor, and Sperling 1997). The secondary focus, often called the mirror focus, is synchronized to the epileptogenic focus to various degrees in clinical and research settings (Proix et al. 2018; Cammarota et al. 2013). In laboratory settings, 4-AP results in a mirror focus that

mirror around 40% of all epileptogenic waveforms (Couturier and Durand 2020; Osborne et al. 2019; Sip et al. 2021). These mirror foci often resolve with surgical interventions such as corpus-callosectomy or focal neocortical resection. This suggests that the corpus callosum plays a role in the propagation or synchronization of those foci as it is responsible for the interhemispheric transportation of motor and sensory information (Wong et al. 2006; Funnell, Corballis, and Gazzaniga 2000).

Though the concept of synchrony is not well-understood, it has been established in other models and clinical scenarios. One common explanation for the concept of spontaneous seizures observed is the concept of coupled oscillators. The idea has been heavily researched in mechanical, electrical and biological systems and is known as “Huygen’s clock.” If we are to analogize the primary focus to a secondary focus of the two clocks used in Huygen’s experiment, the CC would become analogous to the bar connecting both clocks. As aforementioned, the CC plays a critical role in the transfer of information and is highly compartmentalized. This nature of the CC could be playing a role in the propagation of these seizures from one focus to the other. However, if we are to reach this conclusion, we are to also hope that by the same principles in which the CC synchronizes, that those two individual neural networks would apply to the spreading of LFS to both primary and secondary epileptogenic zones.

Chapter 3

Conclusions and future work

3.1. Conclusion

Iron Chloride model can generate chronic focal recurrent seizures in-vivo. We demonstrated the ability to produce stable and focal recurrent cortical seizures through a single injection of Iron Chloride ($p=0.15$ for gradient test). We also demonstrated the capability of generating a stable baseline of focal recurrent cortical seizures through an intraoperative injection of ferric chloride into the M1 cortex. Mild-moderate seizures were recorded encephalographically over a 14-day period with no further intervention needed to trigger those events. The stability of both seizure metrics, number of seizures per day, and percentage of the day spent seizing demonstrates the capability of performing a chronic experiment using this model to evaluate the therapeutic efficacy of LFS in rat models.

Iron Chloride injection will generate seizures that will extend beyond the ipsilateral focus. We demonstrated that seizure generation extended beyond the ipsilateral focus. We demonstrated the development of a secondary epileptogenic zone in the contralateral motor cortex as well as the ipsilateral CA3 region of the hippocampus. This was done through quantification and correlation of electroencephalographic seizure events in both hippocampus and contralateral motor cortex. Seizures were more likely to propagate to the contralateral motor cortex than to the ipsilateral hippocampus, 26% compared to 2.5% respectively. Seizures propagated with a mean delay of 4ms to the contralateral motor cortex while propagating to the ipsilateral CA3 region with a mean delay of 1ms.

3.2. Future work

Our research has shown that the ferric chloride model of epilepsy can be a cost-effective and efficient method for inducing focal chronic recurrent seizures without the need for genetic engineering or daily kindling stimulation. By intraoperative injection of ferric chloride in the M1 cortex, we were able to develop stable and recurrent seizures that mimic the symptoms of

epilepsy in human patients. Furthermore, we assessed the extent of seizure generation past the primary focus, providing valuable insights into the underlying mechanisms of epileptogenesis.

These findings have important implications for the development of new treatments for epilepsy, particularly the potential for the treatment of focal cortical seizure and seizure arising from traumatic brain injury. Future work should investigate the nature and extent of the epileptogenicity of iron chloride through histological studies. This study provides a basis for evaluating the efficacy of LFS stimulation of white-fiber tracts that innervate the focal zone and therefore further research is needed to assess the stimulation paradigm and in vivo seizure reduction in chronic animal models of iron chloride induced epilepsy.

If the efficacy of LFS is demonstrated in animal models, it could serve as a steppingstone for the integration of LFS in focal epilepsy patients and its integration into medical devices. Clinical trials would then be needed to evaluate the effectiveness of LFS in focal epilepsy treatment, quantifying and qualifying its benefits as a new treatment modality.

The results of our work provide a framework for the integration of LFS into chronic studies, which can ultimately lead to improved treatment options for patients suffering from epilepsy. By utilizing the ferric chloride model of epilepsy and exploring new treatment strategies like LFS, we can gain a better understanding of the mechanisms underlying epileptogenesis and develop more effective and personalized treatments for patients.

Appendix

Chronic Cage Re-design:

An indirect result of this work was the re-evaluation of our chronic recording set-up. The set-up explained in 2.2.3 proved to be incompetent and unhelpful to the long-term aims of this project. A chronic recording is a meticulous yet important aspect of the evaluation of new therapies in the realm of DBS, however, multiple experiments failed due to the mechanical restrictions imposed by a wired signal acquisition system. Multiple animals suffered from premature disconnection of the recording system, requiring the early termination of four experiments and costly time delays, which restricted our ability to acquire recordings for longer than 14-days. Though a telemetry system could be the ultimate solution, the development, verification, and validation of such a system extend beyond the goals of this thesis.

However, in order to achieve the current aims, we aimed to re-model the current chronic recording cages available at CWRU. An enhanced system, inspired by previously published research, was implemented for the continuation of this project (Medlej et al. 2019).

Though there was a previously implemented torsion-relief system through the aforementioned commutator, another system was needed to alleviate the tension in the SPI cable. We implemented a swivel-balance cage system. Here we provide a brief methodology for this modification. A 3-D model of the modification can be seen below in Figure 15. A standard cage is utilized with an additional top board. The top board is fixed to the cage through a hinge (not shown) on the back of the cage. The board contains a hole that accommodates the commutator at the center of the cage. Additionally, the board contain a screw in the back that carries weight. The weight acts to counteract the strain on the SPI cable via swiveling in countermotion with the tension applied to the cable. The weight is adjusted so that at rest the board is leveled with the

cage. This has allowed us to increase the recording time to a month without interruptions or early terminations in two animals.

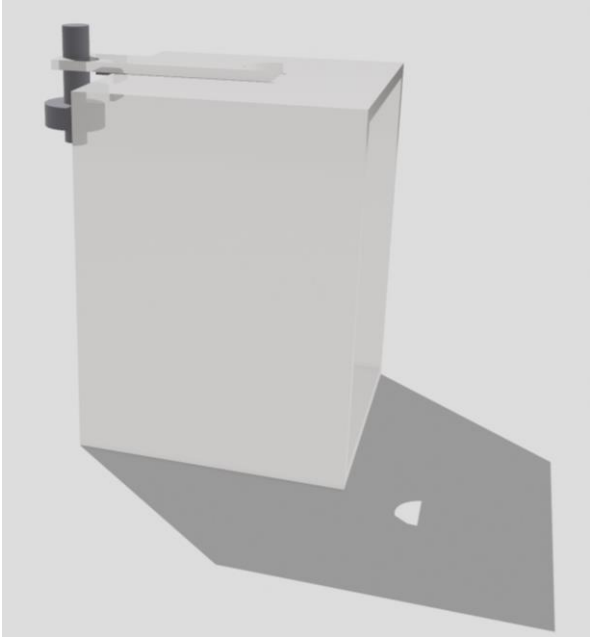


Figure 15: Swivel-balance modification for chronic recording cage implemented to aid in chronic recording for future LFS experiments.

Early LFS Data

The continuation of the main aims of this work naturally lead to the evaluation of LFS as a treatment modality for seizure suppression. Despite the presence of multiple factors to be considered in the design of the stimulation paradigm in chronic experiments, we present preliminary results on the efficacy of LFS in seizure suppression.

Stimulation Paradigm

Acute studies of LFS have shown 20Hz stimulation to be effective at suppressing 4-AP seizures. The amplitude was determined as 80% of the maximum evoked potential as seen in Figure 16C. Based on this the stimulation was applied in the form of 2-mA biphasic current for

these experiments (N=2) with a current pulse of 100-microsecond (total biphasic pulse width of 200 μS). Pulses were delivered continuously for six hours followed by six hours rest for seven days. The stimulation was delivered after the baseline was established, 21 days post-implantation (seven days of recovery followed by 14 days of baseline recording).

Data Acquisition and Artifact Removal

During the stimulation period, all signals were acquired at 20kHz to avoid any aliasing of the stimulation artifact. Removal of stimulation artifacts was done through the utilization of the Hampel filter.

Results

Animals that underwent stimulation (N=2) showed a decreased number of seizures per day over the span of the seven-day period. Statistical analysis comprised of performing a paired student t-test to compare the means for pre-stimulation and post-stimulation. All seven days of stimulation showed a statistically significant reduction in the number of seizures and time spent

seizing (Figure 16D and Figure 16E). Animals (N=2) had an average of one seizure per day with a mean time of seizing of 47.5±40 seconds per day.

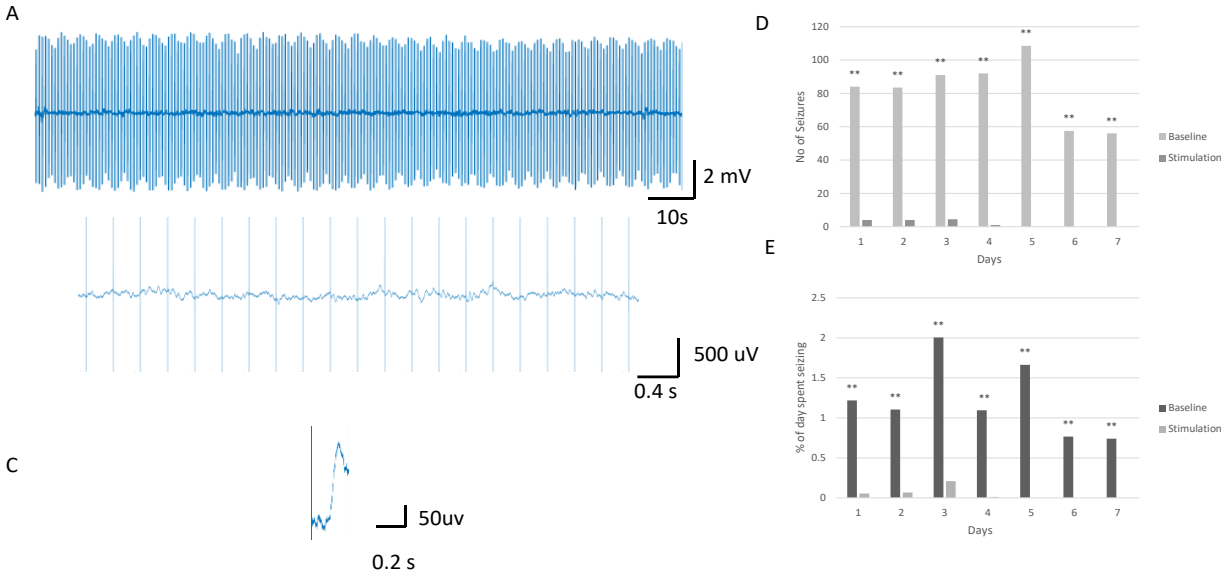


Figure 16: Preliminary LFS data. Sample recorded EEG strip with 20Hz stimulation (A) with an enlarged portion shown below (B). An example of an evoked potential recorded during stimulation of animals (C). Results of stimulation on number of seizures (D) and time spent seizing (E) during a 7-day period of stimulation.

Tables

Table A 1: Iron Chloride Experiment 1 Rat 1. Animals were denoted differently signifying new rats being used in the experiment. Animals were recorded for 14 days post-implantation.

Day	No. of Seizures	**Duration**
1	148	1942
2	144	1733
3	151	3264
4	150	1653
5	181	2555
6	66	679
7	66	679
8	89	958
9	94	699
10	90	812
11	88	744
12	87	732
13	90	802
14	91	850

Table A 2:Iron Chloride Experiment 1 Rat 2. Animals were denoted differently signifying new rats being used in the experiment. Animals were recorded for 14 days post-implantation.

Day	No. of Seizures	Duration
1	20	164
2	23	175
3	31	201
4	34	240
5	36	318
6	49	645
7	46	599
8	56	688
9	59	803
10	62	1195
11	50	645
12	38	300
13	31	256
14	28	234

Table A 3:Iron Chloride Experiment 1 Rat 3. Animals were denoted differently signifying new rats being used in the experiment. Animals were recorded for 14 days post-implantation.

Day	No. of Seizures	Seizure Duration
1	265	2629
2	127	1318
3	67	534
4	57	491
5	45	474
6	62	651
7	140	1500
8	132	3165
9	173	6660
10	94	5005
11	119	6106
12	51	1862
13	37	2020
14	33	1157

Table A 4:Iron Chloride Experiment 1 Rat 4. Animals were denoted differently signifying new rats being used in the experiment. Animals were recorded for 14 days post-implantation.

Day	No. of Seizures	Seizure Duration
1	2	711
2	12	6622
3	24	5512
4	34	3507
5	72	10806
6	59	4573
7	5	396
8	54	6182
9	33	2117
10	0	0
11	0	0
12	0	0
13	0	0
14	0	0

Table A 5:Iron Chloride Experiment 1 Rat 5. Animals were denoted differently signifying new rats being used in the experiment. Animals were recorded for 14 days post-implantation.

Day	No. of Seizures	**Duration**
1	10	76
2	18	210
3	18	264
4	8	54
5	2	30
6	0	0
7	0	0
8	16	546
9	8	112
10	2	30
11	0	0
12	0	0
13	0	0
14	0	0

Table A 6:Iron Chloride Experiment 3 animal 1. Note this is the same as animal 1 in experiment 1. Animals were stimulated for 7 days.

Day	No. of Seizures	Duration
1	2	15
2	3	16
3	3	18
4	0	0
5	0	0
6	0	0
7	0	0

Table A 7:Iron Chloride Experiment 3 animal 2. Note this is the same as animal 2 in experiment 1. Animals were stimulated for 7 days.

Day	No. of Seizures	Duration
1	6	80
2	5	100
3	6	346
4	2	20
5	0	0
6	0	0
7	0	0

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