# CAN SEIZURES BE FORGOTTEN? RAPAMYCIN AND ISOFLURANE DO NOT DISRUPT EPILEPTOGENESIS IN A MOUSE MODEL OF EPILEPSY

**CARLOS JOEL HOWEY**
Bachelor of Science, University of Lethbridge, 2022

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

**MASTER OF SCIENCE**

in

**NEUROSCIENCE**

Department of Neuroscience
University of Lethbridge
LETHBRIDGE, ALBERTA, CANADA

CAN SEIZURES BE FORGOTTEN? RAPAMYCIN AND ISOFLURANE DO NOT DISRUPT EPILEPTOGENESIS IN A MOUSE MODEL OF EPILEPSY

CARLOS JOEL HOWEY

Date of Defence: December 10, 2024

| | | |
|---|---|---|
| Dr. Artur Luczak<br>Thesis Supervisor | Professor | PhD |
| Dr. Ian Whishaw<br>Thesis Examination Committee Member | Professor | PhD |
| Dr. Robbin Gibb<br>Thesis Examination Committee Member | Professor | PhD |
| Dr. David Euston<br>Chair, Thesis Examination Committee | Associate Professor | PhD |

**Dedication**

*For Skailer and Lexus*

**Abstract**

Epilepsy is a neurological disorder, characterized by recurring seizures, that affects approximately 50 to 70 million people worldwide. Understanding how neurons generate abnormal electrical activity is central to the search for an effective treatment. It was hypothesized that the same biochemical processes that support normal synaptic plasticity may play a role in epileptogenesis. To explore this, a kindling model of epilepsy was used. Seizures were induced in mice through electrical stimulation of the amygdala. After the mice displayed seizures of intermediate intensity, they received a treatment of rapamycin (40 kg/mg, i.p.), a compound implicated in affecting synaptic plasticity. Following rapamycin treatment, the mice were also treated with isoflurane (2.5 % in oxygen), an anesthetic that reduces neuronal activity. It was found that rapamycin and isoflurane did not reduce seizures. It is possible that the treatments had no effect, since they had commenced after the animals displayed intermediate seizures; treating the animals at earlier stages could be more likely to disrupt the progression of seizures, however this question will be addressed in further studies. Following the treatments, the mice were then subjected to a Morris water maze task to test the effects of kindling on spatial memory. It was found that the mice with advanced seizures performed the task as well as the mice that were not kindled.

## Preface

The work presented in this thesis, as part of the master of science program, took two and a half years to complete between September 2022 to December 2024. The first year and a half was spent developing the experimental apparatus and the necessary skills (e.g. surgery) to complete the project. Experiments occurred in the second half of the second year, followed by analysis for the remainder of the program.

**Acknowledgements**

I joined the Luczak lab in the latter half of my undergraduate program as an independent study student; after which I joined as a master's student. I would like to express my gratitude to Dr. Artur Luczak for taking me on in the lab and for his mentorship and guidance throughout. The opportunities that Dr. Luczak has provided to me are ones that I never imagined having and has enabled me to pursue a career in research and science with a PhD program. I will be forever grateful to Dr. Luczak, as his mentorship and guidance serves as the foundation for my interest in research and science.

I would also like to acknowledge my master's thesis committee, Drs. Ian Whishaw and Robbin Gibb, for the advice and comments they provided to me which helped to improve the quality of this project. I will always consider their advice in my future pursuits in research and science.

The other Luczak lab members, Ritwik, Yoshi, Sean and Maryam, all served an important role during my master's project, as their diverse backgrounds in academia helped to enhance this project. This environment created by the Luczak lab members provided me with an experience during my time in the lab that was holistic. I am also grateful to the undergraduate lab members, Jacob, Nyah, Brooklyn, Vidisha, Matthew, and to Tiana and Autumn, both recipients of the Alberta innovates summer research studentship. Your support enabled me to be ambitious with the project. I wish you all the best in your future endeavours.

I also need to thank my friends and family, Matthew, Shayla, Dominik, Shannon, Philip, Casey, Maleha, Autumn, my cat Shaundi (R.I.P), mother Susan, sister Erin, and brother Jeremie for making my time in Lethbridge as best as possible.

This project was also supported by the CIHR Project and NSERC DG grants awarded to Dr. Artur Luczak, and the Alberta graduate excellence scholarship (AGES) to Carlos Howey.

**Table of Contents**

**List of Figures**

**List of Abbreviations**

| | |
|---|---|
| 4EBP1 | 4E-binding protein 1 |
| AD | Afterdischarge |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazole |
| ANOVA | Analysis of Variance |
| EEG | Electroencephalogram |
| E-LTP | Early Long-Term Potentiation |
| fEPSP | Field Excitatory Postsynaptic Potential |
| FKBP12 | FK506-binding Protein |
| GABA | ɣ-aminobutyric Acid |
| ILAE | International League Against Epilepsy |
| LFP | Long-Term Potentiation |
| LTD | Long-Term Depression |
| LTP | Local Field Potential |
| L-LTP | Late Long-Term Potentiation |
| mTOR | The Mammalian Target of Rapamycin |
| MWT | Morris Water Maze Task |
| p70s6K | p70-kDa ibosomal s6 kinase |
| PKCζ | Protein Kinase C ζ |
| PKMζ | Protein Kinase M ζ |
| SIU | Stimulus Isolator Unit |
| TSC | Tuberous Sclerosis (or Tuberous Sclerosis Complex) |
| TSC1 | Tuberous Sclerosis 1 (Hamartin) |
| TSC2 | Tuberous Sclerosis 2 (Tuberin) |
| ZIP | ζ-pseudosubstrate Inhibitory Peptide |

## Chapter 1 Introduction

> The general idea is an old one, that any two cells or systems of cells that are repeatedly active at the same time will tend to become "associated" so that activity in one facilitates activity in the other (Hebb, 1949, p. 70).[1]

### 1.1 Memory

> There has been one striking and totally unexpected behavioural result: a grave loss of recent memory in those cases in which the medial temporal-lobe resection was so extensive as to involve the major portion of the hippocampal complex bilaterally. (Scoville & Milner, 1957, pp. 13-14)[2].

In the late summer of 1940, near the village of Montignac in the Dordogne region of occupied France, Marcel Ravidat, while on a walk with his dog Robot, had stumbled upon a cave lined with over 600 paintings, determined later to be created by palaeolithic humans living in the region approximately 17 000 years ago (Leroi-Gourhan, 1982). Following the war, the French government seized the cave to protect it and opened it to the public as the Lascaux cave (Leroi-Gourhan, 1982). The depictions of the paintings varied, most of which were of the animals living in the region at the time, some extant (e.g. bison, horses, etc.) and extinct (e.g. woolly rhinoceros) (Leroi-Gourhan, 1982). While the significance of these paintings are under speculation, some suggesting that they served as a guide for the palaeolithic humans, depicting the animals that can be hunted, or as cautionary depictions of dangerous animals; although, it is known for certain that the habitat of these animals were not in caves (e.g. the preferred habitat for the European bison are the grasslands) (Kuemmerle et al., 2010). This discovery, although obvious, demonstrates the ability of memory in early humans, and illustrates its importance in aspects of human life, such as survival and art.

---

[1] This was later summarized by Carla Schatz as “cells that fire together, wire together”.
[2] Case H.M

Throughout human history, researchers and scholars have made speculations and theories in regards to memory. For example, Aristotle (384 - 322 B.C.) suggested that memories exist physically as residues of sense-perception associations (Lautner, 2012). However, it wasn't until the latter part of the 20th century, following the emergence of cognitive and systems neuroscience that the current definition of memory was defined. In his book, Memory and Brain, Larry R. Squire (1987, p. 3) described learning as "the process of acquiring new information" and memory as "the persistence of learning in a state that can be revealed at a later time" stating that "Memory is the usual consequence of learning." While these definitions are elegant, they do however pose several implications that researchers have been working to address within the past decades. First, that engrams of memories exist and are represented by network activity of neurons. Second, that learning and memory are two unique processes that are required for information processing that can be described as acquisition and consolidation respectively. The first implication is also suggested by previous research, that was made possible with development of single-unit multi electrodes, that illustrated the correlations between cognitive processes (e.g. perception) and the activity of individual neurons (Milner et al., 1998). These early findings also supported the postulation made by Donald Hebb in 1949, that the synaptic strength between neurons increases when they engage in repeated coactivity.

The selective strengthening and weakening of the synapses between neurons is referred to as synaptic plasticity, and is considered to be a physiological substrate of several neurological processes, including learning and memory. Cellular models of synaptic plasticity include long term potentiation (LTP), a lasting increase in synaptic

strength, and long-term depression (LTD), a lasting decrease of synaptic strength (Okuda et al., 2021). The former was first demonstrated by Bliss and Lømo in 1973 when they showed that high frequency stimulation (tetanic stimulation) applied to the perforant path resulted in potentiated responses, measured by slope of field excitatory postsynaptic potentials (fEPSP), in populations of interneurons in the dentate area (Bliss & Lømo, 1973). From their results, Bliss and Lømo (1973) suggested that the mechanisms for learning and memory might include the mechanism required for LTP; however, this suggestion was made at a time when the knowledge of the hippocampus was limited and implicated in learning and memory. Additionally, tetanic stimulation does not occur naturally in the brain, suggesting that LTP is an artificial response that cannot accurately model the synaptic plasticity required for information storage. These limitations would lead Bliss and Lømo (1973) to acknowledge in their conclusion that the elucidation of the mechanisms for information storage is still required. However, their work provided the initial framework to model the synaptic plasticity required for learning and memory, and the significance of their discovery is demonstrated by the thousands of LTP studies that followed. In most of these studies, tetanic stimulation is analogous to the learning tasks that are typical for most learning and memory experiments, which allows researchers to investigate in more depth the mechanisms (e.g. molecular) required for learning and memory.

It was later found that LTP can be distinguished as early-LTP (E-LTP), induced by weak tetanization and lasts one to three hours, or late-LTP (L-LTP), induced by strong tetanization and lasts for at least 10 hours (Frey et al., 1993). Following the induction of L-LTP, an increase in α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) type glutamate

receptors at the postsynaptic terminal is observed in addition to the activation of dopamine receptors near the postsynaptic terminal; however, only the increase of the AMPA type glutamate receptors, although transient, is observed following the induction of E-LTP (Frey et al., 1993). These findings lead Frey and Morris (1997; 1998) to hypothesize that LTP is sufficient for the protein-independent synthesis of a 'synaptic tag' on the postsynaptic terminals that capture the proteins, synthesized via the modulation of dopaminergic receptors to establish the persistence of LTP. They had demonstrated evidence for this hypothesis when they showed that L-LTP can be induced in a weakly tetanized pathway if a strong tetanus was applied to another local pathway (Frey and Morris, 1997; 1998). It was suggested that this is due to the abundance of the plasticity-related proteins that are synthesized following a strong tetanus that are shared by both of the 'tagged' pathways to establish L-LTP (Frey and Morris, 1997, 1998). This work consequently led researchers to identify candidate proteins that are required for learning and memory. In a particularly striking example, Sajikumar et al (2005) demonstrated that the protein kinase C (PKCζ) isoform, protein kinase Mzeta (PKMζ), the independent catalytic domain of PKCζ, is necessary for the persistence of L-LTP. This was done by applying a strong tetanization to synaptic inputs located in stratum radiatum of the CA1 region in hippocampal slices, followed by bath applications of the myristoylated ζ-pseudosubstrate inhibitory peptide (ZIP), a constituent of the regulatory domain of PKCζ, to inhibit PKMζ one hour following the induction of L-LTP (Sajikumar et al., 2005). It was found that the slope of the evoked fEPSPs increased following tetanization, then returned to baseline responses 75 minutes following the bath

application of ZIP; suggesting that PKMζ is necessary for the persistence of L-LTP (Sajikumar et al., 2005).

Once a molecule is implicated to be necessary for LTP, it is then important to determine its role in learning and memory. Following the work that determined the role PKMζ for LTP, Pastalkova et al (2006) conducted a study to determine if the protein is also necessary for the persistence of spatial memory. In their experiment, the animals were trained on a place avoidance task, a hippocampal dependent task, that required the animals to learn the location of a shock zone in the apparatus (Pastalkova et al., 2006). Twenty-two hours following training, the rats were injected with either ZIP or saline into both hippocampi, and two hours later, they were placed back into the apparatus for a test session with the shock zone turned off (Pastalkova et al., 2006). Retention of spatial memory was measured by the amount of time between the placement of the animal in the apparatus and the initial entry in the shock zone (Pastalkova et al., 2006). The saline treated animals demonstrated typical spatial memory retention, while the animals treated with ZIP did not (Pastalkova et al., 2006). While these results suggest that PKMζ is necessary for the persistence of spatial memory, results from other studies have provided contradictory evidence. For example, Volk et al (2013) conducted a similar study as Pastalkova et al (2006), that subjected mice with the PKCζ and PKMζ genes knocked out to a Morris water maze task (MWT). They found no significant difference between the average latencies to find the platform for the knockout and wildtype animals (Volk et al., 2013). Additionally, they found that L-LTP was maintained in the knockout mice following strong tetanization, in addition to its reversal with ZIP (Volk et al., 2013). Volk et al (2013) concluded that PKMζ is not required for the induction of LTP and memory

storage, and that ZIP has nonspecific effects, or that its activity is independent of PKMζ. However, it has been suggested that the results from Volk et al (2013) could be explained by the compensation of PKMζ with other related proteins (Tsokas et al., 2016).

By the 1990s, memory research had reached a boiling point; following the discoveries of LTP by Bliss and Lomo in 1973 and hippocampal place cells by O'Keefe and Dostrovosky in 1971, an overwhelming amount of evidence was generated that implicated that synaptic plasticity formed the basis of learning in the brain and prompted researchers to search for the mnemonic operations represented by the physiological changes in the synapses of neurons. It was established that neuronal activity in the hippocampus changes following a given experience, for example, Pavlides and Winson (1989) found that the firing rate of hippocampal pyramidal cells increased during the sleeping states that followed exposure to the corresponding place field, relative to pyramidal cells with a non-overlapping place field.

In 1993, Wilson and McNaughton found that neuronal activity in the hippocampus develops into a robust ensemble of activity as an animal is exposed to a novel space. This was demonstrated by parallel recordings of single neurons in the hippocampus that were subsequently used to accurately predict the animals' location in a familiar environment (Wilson & McNaughton, 1993). When the animals' were exposed to a novel environment, it was found that the error of predictions were greater than the error in the familiar environment, and that the error decreased in the latter half of the period where the animals were exposed to the novel environment (Wilson & McNaughton, 1993). Additionally, it was found that while the animals were exposed to the familiar environment, that the activity of inhibitory interneurons was suppressed, and

would return to normal when the animals returned to the familiar environment. From their results, Wilson and McNaughton (1993) suggested that the development of the neuronal activity in a novel environment does not interfere with established activity representative of a familiar environment, and that the suppression of the inhibitory interneurons allows for synaptic enhancement. This provided the necessary foundation for the interpretation of neuronal activity that is thought to be representative of a particular experience in the absence of that experience (e.g. sleep; Pavlides & Winson, 1989).

In 1994, Wilson and Mcnaughton were the first to demonstrate that neuronal network activity, representative of a particular experience, is replayed during subsequent sleep, by recording the activity of single neurons in large populations in the hippocampus, followed by the computation of spike-train cross-correlations for all principle neuron pairs. They found an increase in the coactivity of neuron pairs with overlapping place fields, relative to pairs with non-overlapping place fields during exploration and in a subsequent sleep, compared to a sleep prior to exploration (Wilson & McNaughton, 1994). It was previously proposed that memory consolidation occurs during periods of rest and sleep (Pavlides & Winson, 1989), in particular, during sharp-wave (SPW) sleep when neurons display a burst-suppression like activity (ripples). It had been previously found that synaptic modification is suppressed during SPW sleep (Leonard et al., 1987), which has been proposed to be necessary for the transfer of information from the hippocampus to the cortex for long-term storage.

### 1.2 Epilepsy and Seizures

> Case D.R. *Illusion of memory* followed by nausea. This patient stated that when he was talking to people he sometimes said something that he had said before, or

> heard something that he had said before, or heard something that he had heard before. When he had that sudden feeling of familiarity or remembrance, he usually went away, being afraid of having an attack. At such times he was apt to be nauseated. Attacks seen in hospital were characterized objectively by his sitting up in bed, then by unconsiousness with mastication, grunting, and facial twitching, followed by generalized seizures (Penfield, 1954, pp. 442-443).

Seizures have been observed throughout a majority of human history and were originally defined by the behavioural manifestations that a patient exhibits, ranging from loss of consciousness to limb and full body convulsions. Early advances in technology, particularly in electroencephalogram (EEG), suggested that a cellular correlate of seizures was the hypersynchronous neuronal activity resulting from an imbalance of neuronal inhibition and excitability (Bower et al., 2012; Truccolo et al., 2011). However, further advances in technology, particularly in single-unit microelectrodes, suggest the contrary, that neuronal activity in an epileptic network is highly heterogeneous (Bower et al., 2012; Neumann et al., 2017; Truccolo et al., 2011). Early research suggested that y-aminobutyric acid-ergic (GABA)-ergic inhibitory neurons play a critical role in countering membrane depolarization and decreases the frequency of action potentials during a seizure, as it has been previously found, and well documented, that regions of inhibition adjacent to regions of hypersynchronous activity exist, and were proposed to have a restraining role on the spread of seizure activity (Prince & Wilder, 1967; Shevon et al., 2012). This, in addition to other lines of evidence, such as the loss of GABAergic inhibitory neurons in clinical (de Lanerolle et al., 1989) and experimental (Sloviter, 1987) models of epilepsy, suggest that seizures occur due to the dysfunction of GABA mediated inhibition (Cossart et al., 2005). However, while this theory is intuitive, it is simple and does not account for the heterogeneity of the morpho- physiological features observed across different GABAergic neurons (Cossart et al., 2005). For example, spared

GABAergic interneurons in the hippocampus were found to participate in both interictal and ictal spiking activity, suggesting that GABAergic interneurons also contribute to the formation of epileptic discharges (Muldoon et al., 2015; Neumann et al., 2017).

Additionally, while most patients experience a seizure in the absence of an external cue (spontaneous seizures), approximately 5 % experience reflex seizures in which a seizure is induced by some external cue in the environment (e.g. intense flashing lights). Moreover, in some cases of reflex seizures, it has been reported that a patient's seizure can be induced by thinking about the same seizure inducing stimuli (Navarro et al. 2006). Thus, it can be suggested that spontaneous seizures are a form of reflex epilepsy, where instead of an external cue, an internal one is inducing the seizure.

Epileptogenesis is the process by which a normal neuronal network is physiologically altered, resulting in an epileptic network that increases the susceptibility and intensity of recurrent seizures (Kadam et al., 2010; Neumann et al., 2017; Stover et al., 2017). It was originally theorized that epileptogenesis only occurs in the period of time between the precipitating factor of an epileptic condition (e.g. trauma) and the first unprovoked seizure (Pitkänen et al., 2015). However, a considerable amount of evidence has revealed that the intensity and frequency of seizures continue to increase following the first seizure (Pitkänen et al., 2015). To account for these results, the international league against epilepsy (ILAE) revised the definition to describe the physiological changes that develop in to an epileptic condition, in addition the changes that progress the severity and frequency of seizures after the condition has been established (Pitkänen et al., 2015).

The identification of a universal mechanism responsible for epileptogenesis would be critical for the development of treatments, especially for individuals at risk for developing an epileptic condition. With advances in molecular biology, the search for this mechanism has been under investigation by many research groups for several decades (Pitkänen et al., 2015). However, it is unsurprising due to the wide variety of epilepsies that inconsistent mechanisms have been identified, proving this task to be challenging (Pitkänen et al., 2015). For example, the protein kinase, referred to as the mammalian target of rapamycin (mTOR), has been implicated to have a strong role in driving epileptogenesis, particularly in epilepsies associated with the tuberous sclerosis (Curatolo et al., 2008; Krueger et al., 2013; Lam et al., 2010). However, the results from studies that investigate mTOR signalling in an electrical stimulation model of epilepsy are controversial (Drion et al., 2016).

Tuberous sclerosis is a genetic disease caused by inactivation mutations of the Tsc1 and Tsc2 genes that encode for the hamartin and tuberin proteins respectively (Curatolo et al., 2008; Drion et al., 2016; Krueger et al., 2013; Lam et al., 2010). The hamartin and tuberin complex has been found to have an inhibitory role against mTOR; thus, the disease is characterized by hyperactive mTOR signalling that leads to the formation of benign tumours that can present in any organ, including the brain, and is often associated with other neurological comorbidities, such as epilepsy and autism (Curatolo et al., 2008; Drion et al., 2016; Krueger et al., 2013; Lam et al., 2010). This finding has lead to the approval from the American food and drug administration for the use of mTOR inhibitors (e.g. rapamycin) to treat TSC in the clinic (Saffari et al., 2019), in addition to the development of several animal models of TSC by knock out of the

TSC1 and TSC2 genes, that also present with the common neurological comorbidities that are found in clinical cases (Saffari et al., 2019; Uhlmann et al., 2002; Wang et al., 2007). In both clinical and experimental research, regression of a TSC patient's tumours, and in some cases necrosis, was observed in addition to the reversal of the neurological comorbidities following a treatment of mTOR inhibition (Franz et al., 2006; Lam et al., 2010; Silva et al., 2008). While it is intuitive to theorize that the comorbidity of epileptogenesis in TSC arises due to the presence of the cortical tumours (Saffari et al., 2019; Wu et al., 2010), the validity of this hypothesis is under question when considering cases that have reported the persistence of epilepsy in TSC patients following cortical resectioning (Jansen et al., 2007), and experiments that utilize a kinate mouse model of temporal lobe epilepsy, in which no tumours are present, demonstrate that mTOR inhibition prevents epileptogenesis (Zeng et al., 2009).

### 1.3 Theory and Hypothesis

While there is a plethora of literature that discusses both memory and epilepsy, the topics discussed in this chapter were selected to illustrate the similarities of these two phenomena. Indeed epileptogenesis resembles the synaptic modification processes that are required for information storage and consolidation. Understanding the abnormal processes that generate seizures in epilepsy, in addition to the normal memory consolidation processes, is fundamental to investigate this link and provide vital information when identifying novel anti-seizure medications.

It has been previously hypothesized that epileptogenesis is utilizing the same mechanisms that are required for information storage and consolidation (Beenhakker & Huguenard, 2009). Neumann et al. (2017) has provided more evidence to support this

hypothesis, as they found that the neuronal activity during periods of rest in between seizures, resemble the neuronal activity during seizures. Thus, there has been emerging evidence that the neuronal activity during a seizure recruits networks of neurons that are representative of experiences to create a larger epileptic network that is 'trapped in attractor-like dynamics[3].'

The purpose of the study presented here is to investigate the link between epilepsy and memory by disrupting the memory reconsolidation processes that occur during the recall of an association between a conditioned stimulus paired with a seizure.

*Hypothesis: Protein inhibition therapy for epilepsy.* Drugs that have been identified as plasticity related protein inhibitors such as rapamycin or anisomycin are capable of reducing seizures in epileptics. To test this hypothesis, seizures will be induced in mice with electrical stimulation (kindling). Epileptogenesis occurs with this form of kindling, as with repeated stimulation the seizures propagate out from the seizure initiation site across the brain (focal to generalized). For example, when a seizure propagates to the motor cortices, behavioural seizures that include limb convulsions are generated (Teskey, 2020). Additionally, with repeated stimulation, the minimum current required to induce a seizure is lowered, and seizure intensity (duration and behavioural manifestation) increases (Botterill et al., 2014; Neumann et al., 2017; Teskey, 2020). The drugs selected for the following experiments are rapamycin (40 mg/kg, i.p.) and isoflurane (2.0 % in oxygen).

Rapamycin was originally discovered from a soil sample taken from Easter Island, as an antifungal metabolite (Li et al., 2014). Rapamycin readily passes through

[3] This was communicated personally to Artur Luczak by Bruce McNaughton (n.d).

the blood brain barrier due to its hydrophobic properties (Zambrotta & Houlé, 2015) and forms a protein complex with the FK506-binding protein (FKBP12) that inhibits the mammalian target of rapamycin (mTOR) protein through allosteric regulation (Li et al., 2014). mTOR regulates mRNA translation in neurons through the phosphorylation of the p70-kDa ribosomal s6 kinase (p70s6K) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1); in a variety of fear learning paradigms, neurons exhibit an increase in phosphorylated p70s6K (Mac Callum et al., 2014). Parsons et al. (2006) was the first to demonstrate that mTOR signalling is required for the induction and maintenance of activity dependant synaptic plasticity in learning and memory, as they had found that rats trained in a fear conditioning paradigm that also received a post-training treatment of rapamycin displayed attenuated fear responses and phosphorylated p70s6K. Additionally, rapamycin has been shown to be an effective treatment for epilepsy in cases of TSC (see section 1.2). Isoflurane was selected due to its properties that potentiate GABAergic activity (Hall et al., 1994).

In a classical conditioning paradigm, an unconditioned stimulus (US) that elicits an unconditioned response (UR) is paired with a conditioned stimulus (CS). Through repeated pairings, the CS then elicits a conditioned response (CR) that is the same as the UR in the absence of the US. In this study, the convulsive stimulus (US) is paired with visual and auditory sensory stimuli (CS) to create a model of reflex epilepsy; however, this was unsuccessful as the animals did not display a seizure (CR) in response to the sensory stimuli in the absence of the convulsive stimulus. Generating reflex seizures in an electrical stimulation model of epilepsy is a challenging task as a large amount of kindling sessions (> 300 kindling sessions) are required for seizures to occur

spontaneously; even when spontaneous seizures are generated, it is difficult to attribute them to the morpho-physiological changes induced by epileptogenesis, as with a large amount of kindling sessions tissue is damaged at the initiation site, which has also been reported to have an epileptogenic effect (Teskey, 2020).

## Chapter 2. Materials and Methods

### 2.1. Subjects

18 C57BL/6J mice aged 3-6 months, obtained from the Jackson Laboratories, Bar Harbour, Maine were utilized for the three experiments described in this chapter. The mice were housed within the vivarium at the University of Lethbridge on a 12h light/12h dark cycle (700 h - 1900 h) and had access to food and water ad libitum. All procedures were conducted in accordance with the Canadian Council of Animal Care and were approved by the University of Lethbridge Animal Care and Use Committee.

The rapamycin experiment consisted of a rapamycin-treated group (n=6) and a saline-treated group (n=6); both groups contained 3 males and 3 females.

The isoflurane experiment consisted of an isoflurane-treated group (n=4) and a non-treated group (n=6); the isoflurane-treated group contained 2 males and 2 females, whereas the non-treated group contained 3 males and 3 females.

The animals used in the rapamycin and isoflurane experiments underwent the same stereotaxic amygdala electrode implantation surgery.

The Morris water maze task (MWT) was used to assess learning and memory in a kindled (n=6) and naive non-kindled group (n=6) of animals; the kindled group contained 5 females and 1 male, whereas the non-kindled group contained 4 males and 2 females. The kindled group can be further divided into a treated (rapamycin and isoflurane) (n=3) and non-treated group (n=3); the treated group contained only females, whereas the non-treated group contained 2 females and 1 male.

**2.2 Surgery**

The animals received an analgesic of buprenorphine (0.075 mg/kg), administered subcutaneously 30 minutes pre-operatively. After the 30 minutes, the animals were anaesthetised with a maximum of 2.5 % isoflurane in oxygen and placed in the stereotaxic frame followed by an injection of the local anesthetic, lidocaine (7 mg/kg), administered subcutaneously at the incision site. Throughout surgery, breath rate and internal temperature were monitored and recorded every 15 minutes; the percent of isoflurane in oxygen was adjusted as needed, and internal temperature was maintained at 37 °C with a regulated heating pad.

The skulls were then exposed and bregma was marked for reference. Two holes were drilled, one for the amygdalar electrode, located -2.8 mm lateral and -1.3 mm anterior from bregma, and the other for the ground electrode located ~1 mm anterior from lambda. The bipolar electrodes were loaded into a stereotaxic arm and positioned over the hole drilled for the amygdala with the electrode tip touching the surface of the brain. The arm was then lowered -4.6 mm ventrally. A unipolar ground electrode with an uninsulated tip was inserted through the hole posterior to lambda and positioned in between the surface of the cerebellum and the skull.

With the electrodes in place, they were fixed in place with a drop of Krazy Glue followed by a drop of dental acrylic. Once dried, they were removed from the stereotaxic arm. A layer of metabond was then applied over the surface of the skull which served as the base for the pedestal. The pins soldered to the electrodes were then carefully loaded into a 6-channel electrode pedestal (plastics1) (only 3 channels were utilized). The pedestal was then positioned by placing the exposed parts of the pins on the surface of the

dried metabond. The head cap was then built by layering on dental acrylic, surrounding the pedestal.

Surgery was completed once the dental acrylic had dried. The animals were taken off anesthetic and recovery began. The animals were given seven days to recover and received post-operative care for a minimum of three days, in which the animals were monitored twice a day, once in the morning and again in the evening. During the morning check, the animals were weighed and received a combined treatment of an analgesic (Metacam: 7.5 mg/kg, s.c) and antibiotic (Baytril: 5 mg/kg, s.c.). The animals were only weighed during the evening checks. After three days, if the animals displayed no complications due to surgery, they were then moved out of post-operative care and placed back into their home cages where they continued recovery.

**2.3 Seizure Induction Apparatus**

The apparatus consisted of a translucent chamber equipped with two LEDs mounted inside and on opposite walls (visual stimulus), and two speakers that were placed outside facing the chamber (auditory stimulus). The roof of the chamber was opaque, equipped with the RasPicamera mounted at the center, and a tether that connects to the animal's head caps was fed through a hole near the center. The tether served two purposes: it had carried the LFP signal and delivered the electrical stimulus.

The LFP was recorded with a Digital Lynx SX acquisition system, in addition to videos of the behavioural seizures recorded with a Raspberry Pi model 4 B (DigiKey: 2648-SC0194(9)-ND). The Raspberry Pi and Digital Lynx SX were synchronized by programming the Raspberry Pi to output a pulse corresponding to each frame to the Digital Lynx SX, in addition to a pulse corresponding to each stimulus. See figure 2.1.

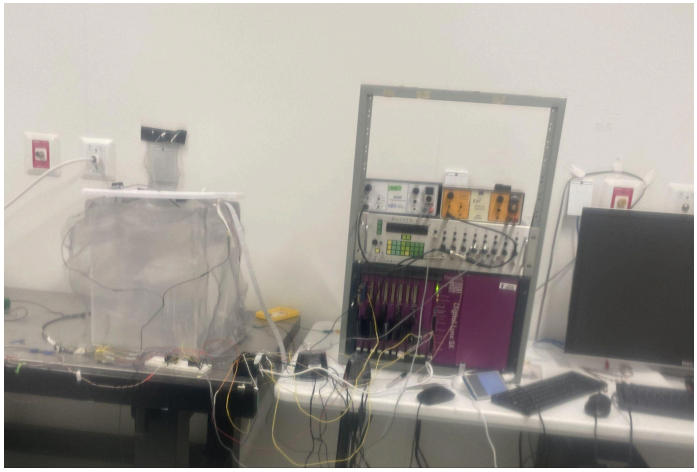

**Figure 2.1** Image of the apparatus.

### 2.3.1 Electrode Fabrication

The bipolar electrodes were fabricated by soldering the ends of a stainless steel wire (A-M systems, Inc.; 791400) with a diameter of 0.008 in to female connector pins with a diameter of 0.05 in (A-M systems, Inc.; 520100). The soldered pins were held together, and a pair of hemostats clamped to the center of the wire were suspended and spun, to twist the wire together. The twisted wire was cut close to the hemostats at an angle of 45 to create a tip separation of ~0.05 mm, while ensuring that the electrode was more than 5 mm in length.

### 2.3.2 Recording

The LFP from the amygdala was carried through the bipolar electrodes, tether, the open contacts of the relay (DigiKey: PR21-5V-400-2C), and a HS-18mm headstage pre-amplifier (Neuralynx; 31-0601-0110) directly connected to Digital Lynx SX. The LFP was sampled at 1kHz. The LFP and the timestamps corresponding to the video frames were saved in separate channels as a .ncs (continuously sampled) file. The

timestamps corresponding to the sensory and electrical stimuli were saved as a .nev (events) file.

The RasPicamera was positioned to capture the entire base of the chamber to ensure that the animals could not move out of the camera's field of view when the seizures were generated. The videos were sampled at 30 frames per second and saved as a .h264 file by the Raspberry Pi.

To ensure that the pulse corresponding to the first video frame was present in the LFP data, a session would begin by starting the recording on the Digital Lynx SX before the recording on the Raspberry Pi.

### 2.3.3 Stimulation

A one second 50 Hz biphasic train of square wave pulses delivered directly to the amygdala was used to generate seizures. The stimulus was generated by programming a Master-8 pulse generator (A.M.P.I.) to output a train of 50 one msec square wave pulses every 20 msec for one second, with each pulse triggering an additional square wave pulse that was outputted 2 msec following the initial pulse (Drion et al., 2016). The pulse was outputted by the Master-8 to the stimulus isolator unit A365 (SIU) (WPI: SYS-A365D) that isolated it from ground, converted to a constant current, and made biphasic by reversing the polarity of the triggered pulse. See figure 2.2. The pulse was carried through the closed contacts of the relay that was triggered to open, through the tether, and the bipolar electrodes to be delivered to the amygdala.

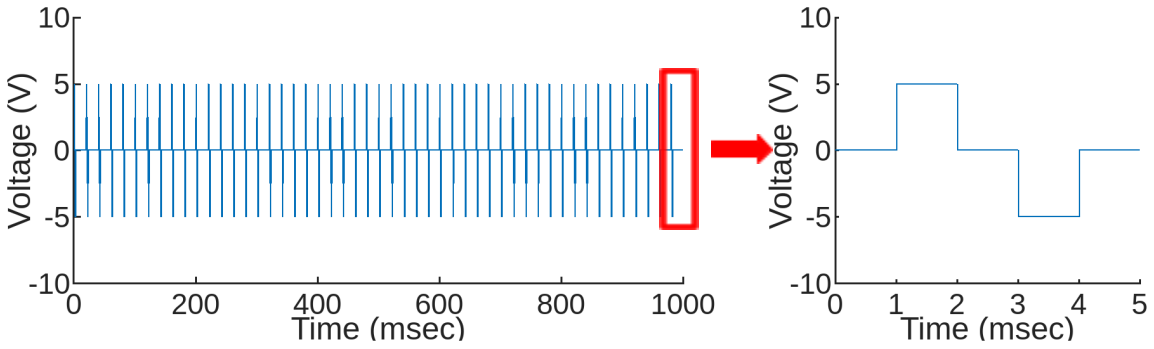


**Figure 2.2** 50 Hz biphasic square wave train of pulses. The figure on the left illustrates the train of pulses. The figure on the right illustrates a single pulse from the train.

In addition to recording the animal's behaviour, the Raspberry Pi controlled the delivery of the stimuli, to ensure that the order and timing of the delivery were consistent across all sessions and animals. The Raspberry Pi was programmed to initiate the stimulus protocol with a single keystroke when an experimenter determined that enough baseline data had been collected. The protocol for stimulation is as follows: one second auditory tone (110-130 dB; 12 kHz), one second pause, one second LED flash (white), four second pause, the relay opens, one second pause, electrical stimulation, one second pause, the relay closes. Unfortunately, during the two second period when the closed contact on the relay were opened, the connection between the amygdala and the digital Lynx Sx was broken, preventing the LFP from being acquired. See figure 2.3.

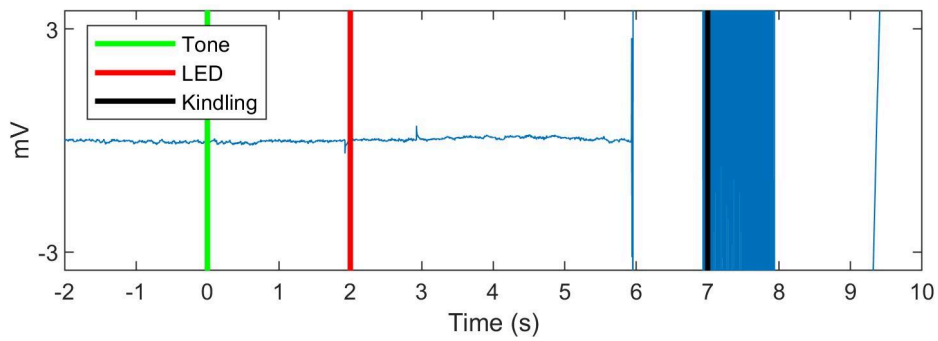


**Figure 2.3** Local field potential recording with stimuli timestamps.

## 2.4 Experimental Design

The rapamycin and isoflurane experiments were divided into 3 main phases: thresholding, kindling, and treatment. The experimental and control groups would undergo sessions on alternating days with the order in which the animals were experimented maintained across all sessions. Following the kindling experiments, 6 kindled (3 treated, and 3 non-treated) and 6 naive non-kindled animals were subjected to a MWT.

The purpose of the rapamycin experiment was to test the effects of mTOR inhibition on epileptogenesis, since mTOR has been implicated to play an important role in a variety of genetic (TSC) and acquired (kainic acid) models of epilepsy. Additionally, this experiment was also a test of rapamycin on the reconsolidation of long-term fear memories, as an association between the auditory tone and seizure is learned in the sessions prior to the treatments, and with at least 48 hours in between each session this association becomes stable in long-term memory. During the treatment sessions, the fear memory is recalled, and treatments occur while the memory is a labile state. See figure 2.3 for the rapamycin experimental timeline.

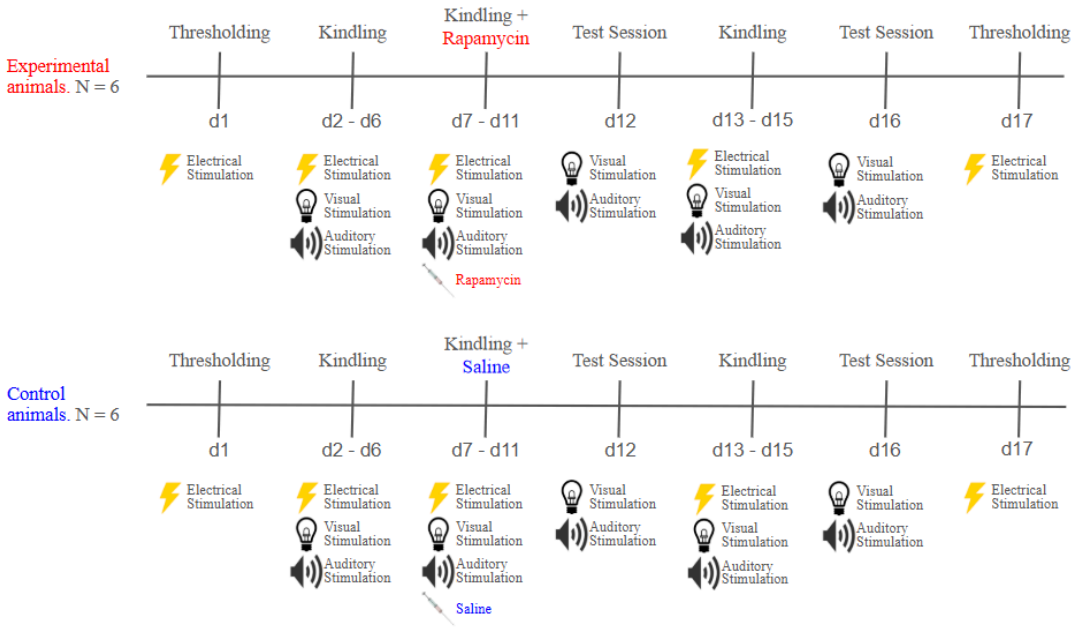


**Figure 2.4** Timeline for rapamycin experiment. The timeline displays the order and the approximate day for each experimental phase.

The purpose of the isoflurane experiment was to test the effects of isoflurane on epileptogenesis, since it has been known to inhibit glutamatergic transmission and excites GABA transmission, which has been implicated to have an antiepileptogenic effect (Bar-Klein et al., 2016). Like the rapamycin experiment, the isoflurane experiment tested the effects of isoflurane on the reconsolidation of long-term fear memories. However, the isoflurane treatments were administered following a majority of all the kindling sessions, thus epileptogenesis had developed the seizures to be fully generalized before the treatments occurred, and with each kindling session, the fear memory association was reinforced. See figure 2.4 for the isoflurane experimental timeline.

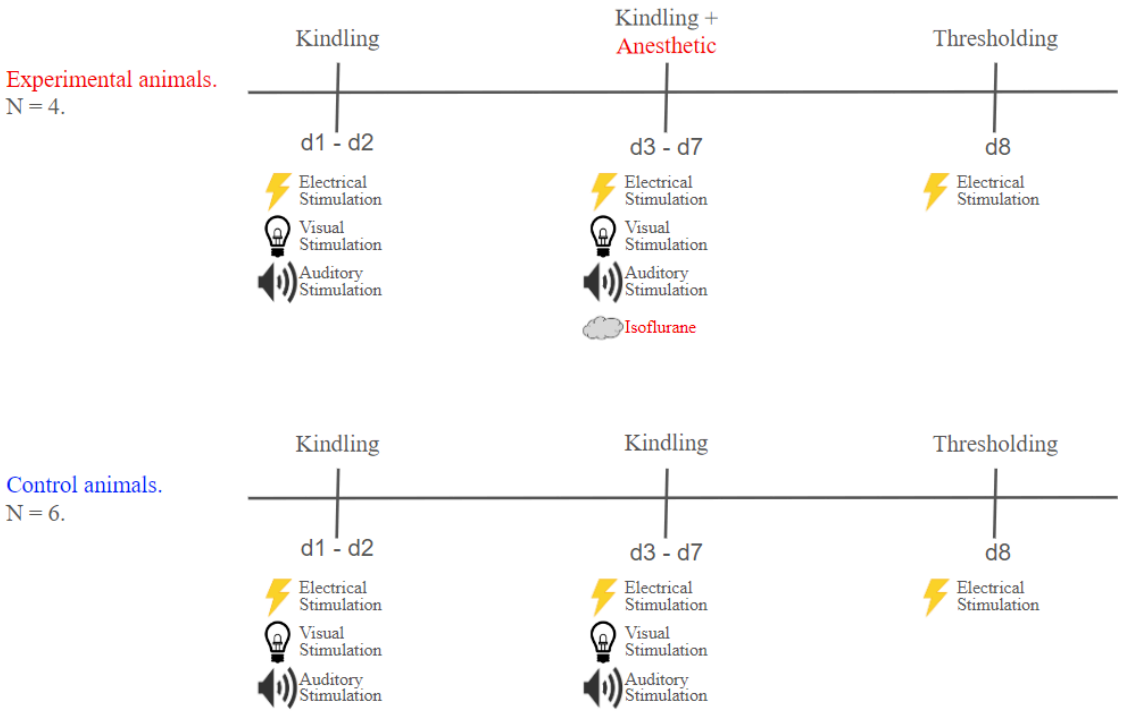


**Figure 2.5** Timeline for isoflurane experiment. The timeline displays the order and the approximate day for each experimental phase.

### 2.4.1 Thresholding

Before kindling, the minimum current to induce an afterdischarge (AD) (see figure 2.2) was determined for each animal. The sensory stimuli were absent for all thresholding sessions, and every animal received electrical stimulation at a starting current at 50 uA. Following stimulation, the EEG was monitored for an AD. If no AD was present, the current was increased by 50 uA and reapplied in intervals of 5 min until one was elicited. Once present, the suprathreshold was determined by adding 100 µA to the current that elicited the AD (Teskey, 2020). Follow up thresholding sessions were conducted, to determine any effects on sensitization to the electrical stimulation.

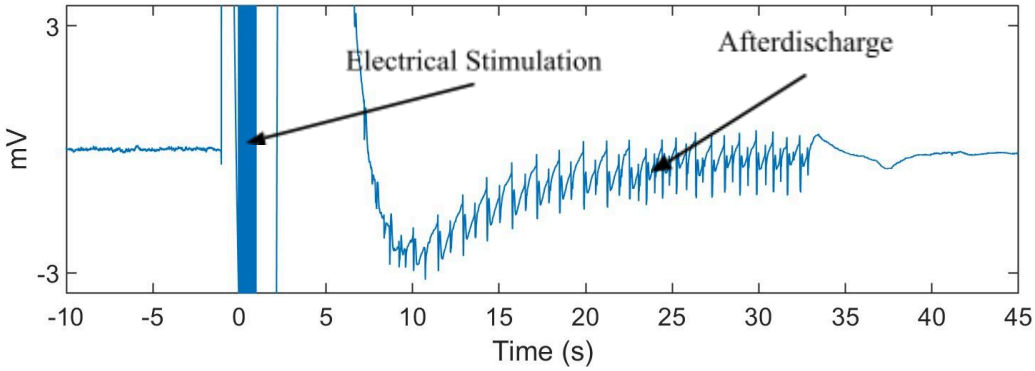


**Figure 2.6** Local field potential with afterdischarge present following electrical stimulation.

### 2.4.2 Kindling

During the kindling phase, each animal was presented with the sensory stimuli first, followed by the electrical stimulation at a current equal to the suprathreshold for each animal (see figure 2.2, the green, red and black vertical lines). Baseline activity was recorded for at least three minutes, followed by the stimulation protocol, and an additional seven minutes of recording following stimulation. Because repeated kindling progressively amplifies the strength and duration of a seizure (Das et al., 2024; Neumann et al., 2017; Stover et al., 2017; Teskey, 2020), treatments were started before the animal's seizures had become fully generalized when a stage three on the Racine scale (see section 2.5.3 Racine Scale) was observed. In addition to generating sufficient seizures, this phase was also an attempt to create a model of reflex seizures. Following the rapamycin treatment, the animals were subjected to three additional kindling sessions, followed by an additional test session, to determine if the effects of the rapamycin were persistent.

### 2.4.3 Treatment

The treatment phase consisted of five kindling sessions that were followed with an intraperitoneal injection of either rapamycin (40 mg/kg) or Saline. The rapamycin was

dissolved in a stock solution of 5 % ethanol, 4 % PEG 400, and 4% Tween 80 in distilled water that was no older than one week (Mac Callum et al., 2014). The control animals had received an injection of saline with volume equal to that if it were rapamycin. 48 hours following the last injection, the animals were exposed to the sensory stimuli in the absence of the electrical stimulus to determine if a model of reflex epilepsy had been established.

### 2.4.4 Isoflurane experiment

Following the rapamycin experiment, the animals that did not die from their seizures were subjected to an additional experiment to test the effects of isoflurane on seizures. With new thresholds determined at the end of the rapamycin experiment, the animals underwent two kindling sessions followed by five sessions in which the experimental animals were placed under 2.5 % isoflurane in oxygen before being returned to their home cage. Following this experiment, the animals were thresholded again (see figure 2.6).

### 2.4.5 Morris Water Maze Task

The MWT consisted of a 154cm diameter pool filled with water ~15 cm from the top, that was kept between 20-22°C and made opaque by mixing in non-toxic white tempera paint. The animals were required to locate a platform with a diameter of 11 cm that was placed in the northeast quadrant of the pool and was submerged ~1 cm under the surface of the water. The pool was located at the center of the testing room which contained distal cues facing the pool in each cardinal direction (Jafari et al., 2019; Morris, 1981).

The animals were trained over seven consecutive days, with each day containing four trials starting at one of the four cardinal locations around the perimeter of the pool, which was randomized. Each animal was placed in the pool facing the perimeter and was allowed 60 s to locate the platform. If the animals were unable to locate it, the experimenters placed them on the platform for 10 s without obscuring their view of the distal cues. On the 8th day (probe), the platform was removed from the pool and each animal was allowed to swim freely for 60 s. Data was recorded with HVS, an automated tracking software that had virtually divided the pool into 4 quadrants (northeast, northwest, southwest, and southeast) and calculated swim time (s), swim speed (m/s), and swim distance (m) which was used for analysis. The time spent swimming in the target quadrant on the probe day was calculated.

**2.5. Analysis**

The LFP and video data was organized into 8 phases: thresholding 1, kindling 1, test 1, kindling 2, test 2, thresholding 2, kindling 3, thresholding 3. Phases 1-5 correspond to the rapamycin experiment and phases 6-8 correspond to the isoflurane experiment. Data recorded during the maze task were imported into an excel spreadsheet and analyzed in MATLAB. Videos were converted to a .AVI format and trimmed to 3 minutes, 1 minute before and 2 minutes following the presentation of the auditory stimulus, by using the raw LFP files as they contained timestamps corresponding to each stimuli and each video frame in separate channels that were generated by the raspberry pi 4. The LFP data was also trimmed to 3 minutes, 1 minute before and 2 minutes following the presentation of the auditory stimuli, and saved as a .MAT file along with other useful information for each session (e.g. sample rate, current) and animal.

### 2.5.1 LFP Analysis

LFP data collected by the Neuralynx Sx was imported into MATLAB for analysis. When calculating seizure duration, only the abnormal signal generated by the seizure was of interest, thus a high pass filter was used to remove any artefacts or deflections in the signal that was generated by the relay switching from stimulation to recording. The baseline consisted of the LFP signal recorded 1 minute before the onset of the electrical stimulus, a threshold was then set by calculating the standard deviation of the baseline activity and multiplying it by 5. Using the find peaks function in MATLAB, the seizure activity could then be detected by disregarding the peaks that fell below the set threshold; the final peak above the threshold was considered as the seizure offset. The time in which the electrical stimulus was presented was stored by the Neuralynx Sx and was considered as the seizure onset. Seizure duration was then calculated by taking the difference between the seizure offset and onset.

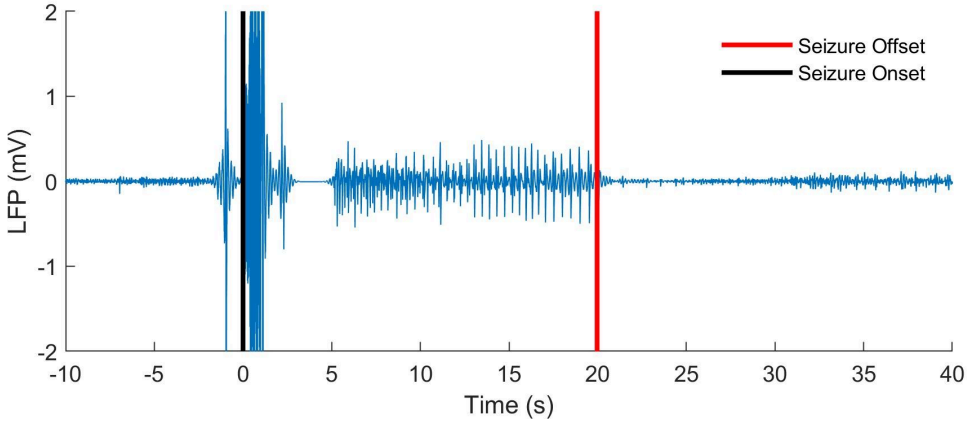


**Figure 2.7** Sample LFP recording from the amygdala, filtered by passing through a 10 Hz highpass filter. Red and Black bars indicate seizure offset and onset respectively.

### 2.5.2 Frame Differencing

Post-cue freezing was determined by calculating the percentage of time that an animal does not move during the 7 second period in between the presentation of the

auditory and electrical stimuli. Frame difference was conducted in MATLAB. Each video had a frame rate of 30 frames per second, 600 frames before and after the presentation of the auditory stimulus were extracted (1200 frames = 20 seconds), and downsampled to 10 frames per second and binarized. Each frame was subtracted from the following frame and the absolute value of the sum of negative numbers were used to quantify the movement between frames (see figure 2.8). These sums were then convolved with a gaussian filter where $\mu = 3$ and $\sigma = 5$. A threshold was determined by calculating the mean of the rescaled values that correspond to frames before the presentation of the auditory stimulus. The threshold was then applied to the seven second period in between the presentation of the two stimuli; the percentage of time spent freezing in this period was determined by dividing the number of values that fell below the threshold by the total number of values in this period.

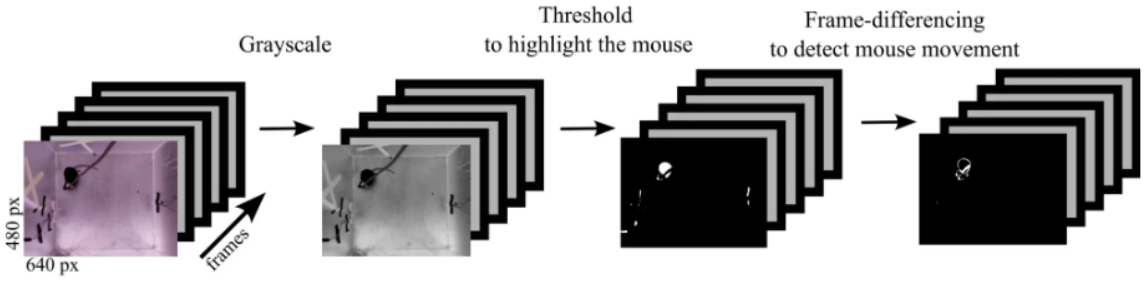


**Figure 2.8** Frame processing in MATLAB. This figure was adapted from Das et al. 2024.

### 2.5.3 Racine Scale

The Racine scale was utilized to quantify the animal's behavioural seizures during the kindling experiments. The scale is progressive, with scores 1-5, where the animal's seizure progresses from anterior to posterior, as it progresses from a focal to a fully generalized seizure, with characteristic behaviours at each stage (e.g. bilateral clonus and rearing at stage 4). The animal's seizures were captured by video, which allowed for each

behavioural seizure to be analyzed by several individuals, and the average of scores was utilized for analysis (Racine, 1972).

### 2.5.4 Statistics

An equal number of sessions were analyzed for each animal in all experiments; however, because it was ensured that the animals started treatment at roughly stage 3 of the Racine scale, several animals particularly in the control group, required additional kindling sessions, likely due higher tolerance to the convulsive stimulus.

To determine any significant effects of the treatments and sessions on seizure duration, behavioural seizure, and post-cue freezing, a two-way analysis of variance (ANOVA) was conducted for both the rapamycin and isoflurane experiments for each dependent variable. To determine if the means of the dependent variables between both groups on a particular session were significantly different, a t-test was utilized.

To determine any significant effects of the treatments, kindling, and session on the swim latencies during training of the MWT, a three-way ANOVA was conducted. A two-way ANOVA was utilized to determine if kindling and/or the treatments had a significant effect on spatial memory retention during the MWT probe. $p < 0.05$ was considered statistically significant.

## Chapter 3. Results

The analysis was conducted on four sessions before the injections of rapamycin until the final isoflurane session. The session where sufficient seizures were observed is the session before treatment began during the rapamycin experiment (session 0, figure 1 top left).

The two parameters used to determine the intensity of the animal's seizures were the electrographic seizure duration in seconds and behavioural seizure, quantified with the Racine scale (see section 2.5.3 Racine Scale). Similarly to a fear conditioning paradigm, post-auditory cue freezing was quantified as a percentage of time not moving during the seven second period in between the presentation of the auditory and electrical stimuli (see section 2.5.2 Frame Differencing). Results from the thresholding sessions for both groups were analyzed to determine if there were any effects of the treatment on sensitization to the convulsive stimulus (see section 2.4.1 Thresholding).

### 3.1 Rapamycin results

During the rapamycin experiment, the animals were exposed to two sensory stimuli, auditory and visual, followed by the electrical stimulus to generate a seizure (see section 2.3.3). Once sufficient behavioural seizures were observed (session 0 figure 3.1), the animals received a treatment of rapamycin or saline approximately seven minutes after being electrically stimulated over 5 sessions (sessions 1 through 5, figure 3.1).

Figure 3.1 illustrates the results of the rapamycin experiment. The seizure duration (A), behavioural seizure (B), and post-cue freezing duration (C) were determined for every animal for each session, and the minimum currents required to induce an electrographic seizure were determined at the thresholding phases before and

after the rapamycin experiment (D). A two-way ANOVA was used to determine if a significant effect of treatment and session on the dependent variables existed, and a t-test was used to determine any significant differences between the groups each session.

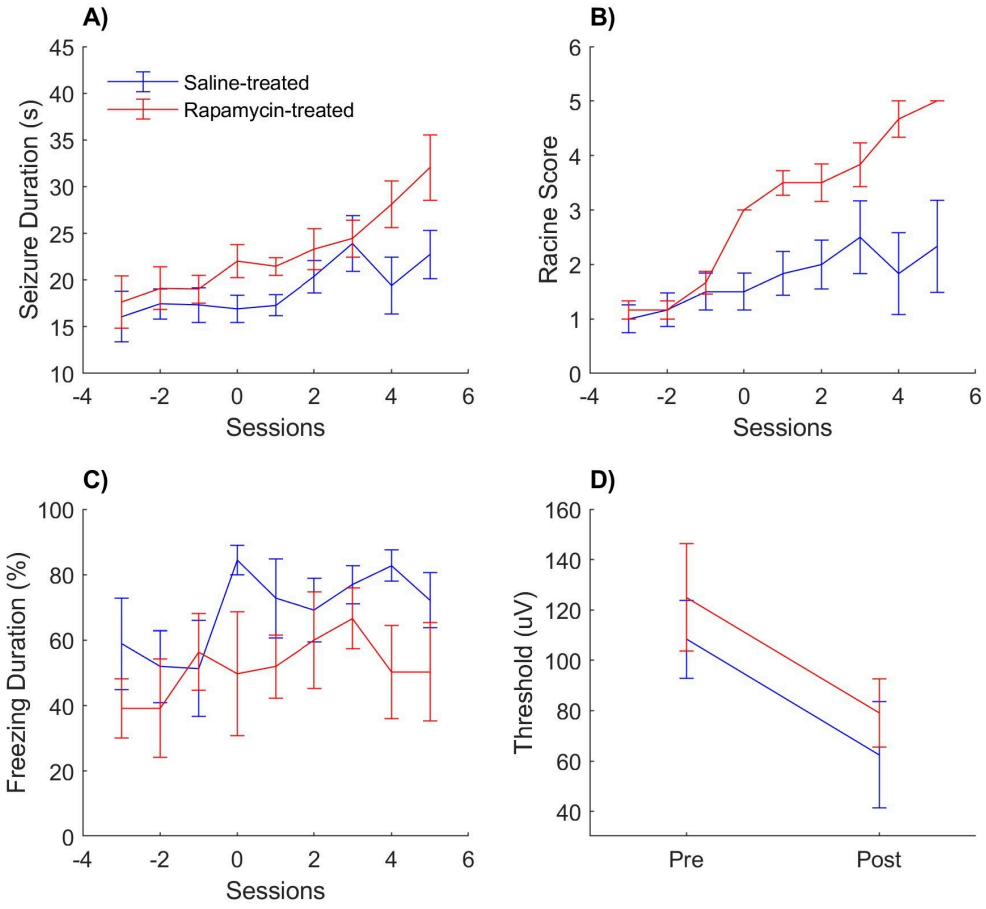


**Figure 3.1.** Results of the rapamycin experiment. **(A)** Seizure duration for both control and experimental animals. Rapamycin had no effect on seizure duration. **(B)** Racine scores for both control and experimental animals. Saline-treated animals scored lower on the Racine scale. **(C)** Racine scores for both control and experimental animals. Rapamycin-treatment impairs fear memory consolidation. **(D)** Minimum thresholds for both control and experimental animals. Rapamycin had no effect on sensitization to the convulsive stimulus. Legend in the A applies to all subfigures. Session 0 in A, B, and C represents the session where sufficient behavioural seizures were first observed. N = 6 for both groups. Data reported as mean ± S.E.M.

### 3.1.1 Seizure Duration (Rapamycin)

A two-way ANOVA revealed a significant effect of session and treatment on seizure duration; on average, the saline-treated animals had shorter seizure durations [figure 3.1 A: main effect of the session, $F(8, 90) = 4.81$, $p < 0.05$; main effect of the treatment, $F(1, 90) = 14.05$, $p < 0.05$; effect of the interaction, $F(8, 90) = 1.01$, $p > 0.05$]. These results suggest a potential effect of the rapamycin increasing the duration of seizures; however, this can not be confirmed as the saline-treated animals had on average, seizures of less duration even before the treatments had begun and required extra kindling sessions to generate sufficient seizures, suggesting that the rapamycin-treated group had longer seizure durations regardless of treatment [figure 3.1 A: session 0, $t(10) = -2.24$, $p < 0.05$].

### 3.1.2 Behavioural Seizure (Rapamycin)

A two-way ANOVA revealed a significant effect of session and treatment on behavioural seizures; on average, the saline-treated animals scored lower on the racine scale [figure 3.1 B: main effect of the session, $F(8, 90) = 10.52$, $p < 0.05$; main effect of the treatment, $F(1, 90) = 46.42$, $p < 0.05$; effect of the interaction, $F(8, 90) = 3.24$, $p < 0.05$]. These results suggest that there is a potential effect of rapamycin causing the animals to have seizures that were more generalized than the saline-treated animals; however, this can not be confirmed as the saline-treated animals had on average, less generalized seizures even before the treatments had begun and required extra kindling sessions to generate sufficient seizures [figure 3.1 B: session 0, $t(10) = -4.39$, $p < 0.05$].

### 3.1.3 Post-cue Freezing (Rapamycin)

A two-way ANOVA revealed no significant effect of session, and a significant effect of treatment on post-cue freezing behaviour; on average, the rapamycin-treated animals had lower freezing durations [figure 3.1 C: main effect of the session, $F(8, 90) = 1.11$, $p > 0.05$; main effect of the treatment, $F(1, 90) = 9.97$, $p < 0.05$; effect of the interaction, $F(8, 90) = 0.53$, $p > 0.05$]. The post-cue freezing results reveal that the saline-treated animals had developed a greater fear response to the sensory stimuli than the rapamycin-treated animals, despite having displayed on average, less intense electrographic and behavioural seizures, indicating a potential effect of rapamycin decreasing post-cue freezing; however, a significant difference was observed in only one of the treatment sessions [figure 3.1 C: session 4, $t(10) = 2.17$, $p < 0.05$].

### 3.1.4 Thresholding (Rapamycin)

A two-way ANOVA revealed a significant effect of session, but no significant effect of treatment on the minimum threshold required to induce an electrographic seizure [figure 3.1 D: main effect of the session, $F(1, 20) = 6.34$, $p < 0.05$; main effect of the treatment, $F(1, 20) = 0.84$, $p > 0.05$; effect of the interaction, $F(1, 20) = 0$, $p > 0.05$]. The thresholding results indicate that there was no effect of treatment on sensitization to the convulsive stimulus; this can be further confirmed when considering the average differences in minimum current between the thresholding sessions for each group. Both groups had an average difference of 45.8 uA, obviously, a t-test revealed no significant difference between the two groups [figure 3.1 D: $t(10) = 0$, $p > 0.05$].

### 3.1.5 Test Session (Rapamycin)

Following the rapamycin experiment, the animals were subjected to a test session where they were exposed to the sensory stimuli in the absence of the electrical stimulus (see section 2.4.3 Treatment). The test session revealed that a model of reflex epilepsy was not established as no animal regardless of treatment, displayed an electrographic or behavioural seizure; however, it should be noted that several animals in the rapamycin-treated group had displayed spontaneous seizures in the absence of the sensory and electrical stimuli on several occasions in different environmental contexts (e.g. home cage in the housing room, perfusion lab, ect.). Because this experiment was similar to a fear conditioning paradigm, post-cue freezing was analyzed during the test session (figure 3.2 left).

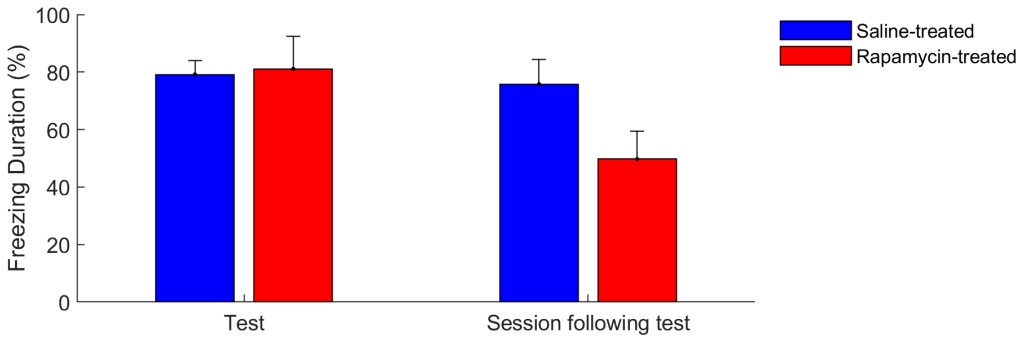


**Figure 3.2** Freezing durations during the test session, and the session following. The experimental animals displayed less post-cue freezing following the test session. N = 6 for both groups. Data reported as mean ± S.E.M.

A t-test revealed no significant difference of post-cue freezing during the test session between the experimental and control animals; however, an additional t-test on the first session following the test, where the electrical stimulation was reintroduced, revealed a significant difference between the two groups [figure 3.2: test session, $t(10) = -0.16$, $p > 0.05$; session following the test, $t(10) = 2.02$, $p < 0.05$]. These results taken

with the results obtained from the post-cue freezing analysis, indicate a potential effect of the rapamycin-treatment on post-cue freezing behaviour. However, these results need to be investigated in more depth before any conclusions can be drawn.

### 3.2 Rapamycin Correlation

The initial analysis revealed that the experimental animals had on average seizures that were longer in duration (figure 3.1 A) and more generalized (figure 3.1B), however, they also had on average shorter post-cue freezing durations (figure 3.1 C). To determine the relationships between these results, Pearson's *r* was calculated for seizure duration and behavioural seizure (figure 3.3 A), seizure duration and post-cue freezing (figure 3.3 B), and behavioural seizure and post-cue freezing (figure 3.3 C).

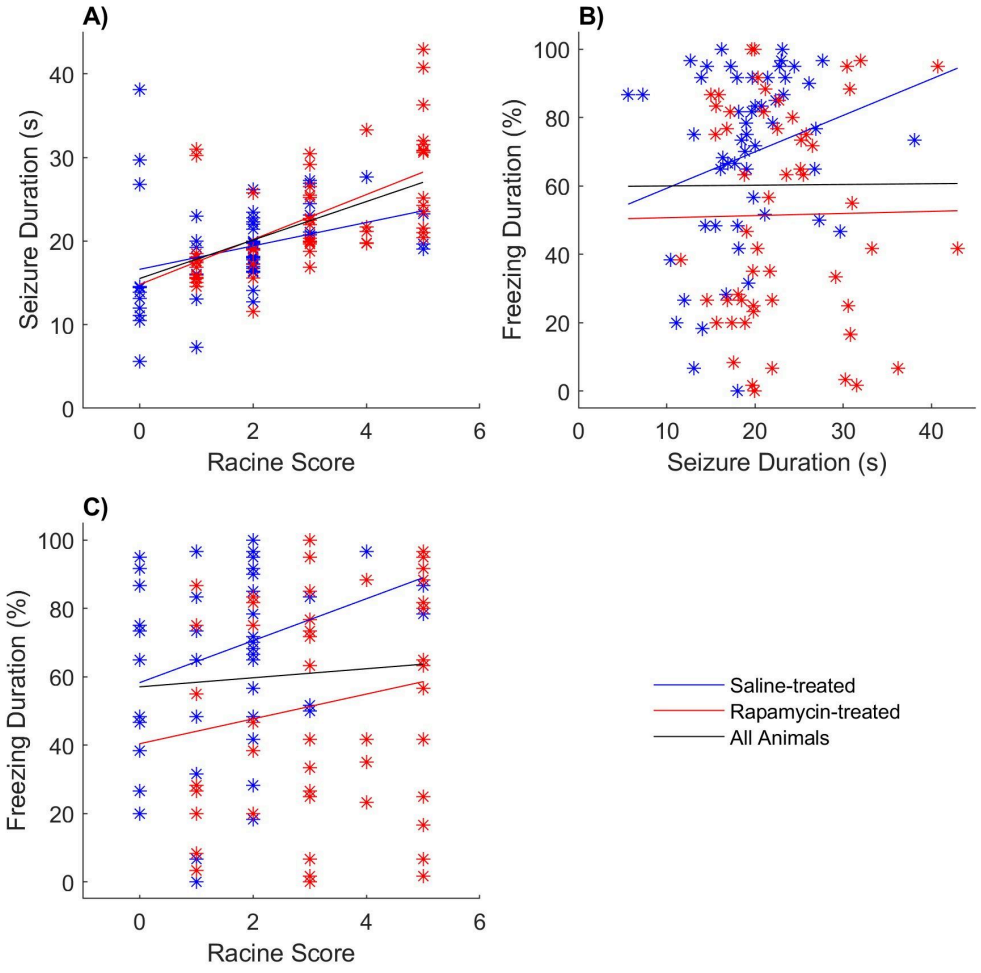


**Figure 3.3** Correlation between seizure duration, behavioural seizure, and freezing behaviour during the rapamycin experiment. **(A)** Behavioural seizure and seizure duration are the most positively correlated in both groups. **(B)** Seizure duration and post-cue freezing have a correlation close to 0 for the rapamycin-treated animals. The control animals have an insignificant positive correlation **(C)** Behavioural seizure and post-cue freezing have a positive correlation. Only the correlation for the rapamycin-treated animals is significant. Regression lines are included for each group for each correlation. The legend in the bottom right applies to all subplots.

### 3.2.1 Behavioural Seizure and Seizure Duration (Rapamycin)

Pearson's *r* revealed a positive correlation between the behavioural seizures and seizure durations for both the saline- and rapamycin- treated animals. Regardless of treatment, a positive correlation was also found [figure 3.3 A: control animals, $r(52)$ =

0.32., $p < 0.05$; experimental animals, $r(52) = 0.59$, $p < 0.05$; all animals, $r(106) = 0.54$, $p < 0.05$]. These results reveal that seizure duration and behavioural seizure are the most correlated dependent variables, as the *r* values obtained for both groups are greater than the *r* values for seizure duration and post-cue freezing, and behavioural seizure and post-cue freezing.

### 3.2.2 Seizure Duration and Post-cue Freezing (Rapamycin)

Pearson's *r* between seizure duration and post-cue freezing revealed an insignificant positive correlation for the saline-treated animals, and an insignificant correlation close to 0 for the rapamycin-treated animals; regardless of treatment, an insignificant correlation close to 0 was found [figure 3.3 B: control animals, $r(52) = 0.23$, $p > 0.05$; experimental animals, $r(52) = 0.01$, $p > 0.05$; all animals, $r(106) = 0.004$, $p > 0.05$]. The results presented here and in section 3.1.1, suggest that post-cue freezing is only partially dependent on the duration of the seizures, as the saline-treated animals displayed high post-cue freezing, unlike the rapamycin-treated animals that had less post-cue freezing and greater seizure durations. The *r* statistics for the saline-treated animals provides more evidence for this, as the correlation between these two dependent variables is higher than the *r* statistic for the rapamycin-treated animals, indicating that longer seizure durations are not required for high post-cue freezing.

### 3.2.3 Behavioural Seizure and Post-cue Freezing (Rapamycin)

Pearson's *r* revealed a positive correlation between behavioural seizure and post-cue freezing for the saline-treated animals, and an insignificant positive correlation for the rapamycin-treated animals. Regardless of treatment, an insignificant correlation close to 0 was found [figure 3.3 C: control animals, $r(52) = 0.31$, $p < 0.05$; experimental

animals, $r(52) = 0.17$, $p > 0.05$; all animals, $r(106) = 0.07$, $p > 0.05$]. The results presented here and in section 3.1.1, suggest that post-cue freezing is only partially dependant on the generalization of seizures, as the saline-treated animals displayed high post-cue freezing, unlike the rapamycin-treated animals that had less post-cue freezing and greater behavioural seizures. The *r* statistic for the saline-treated animals provides more evidence for this, as the correlation between these two dependent variables is higher than the *r* statistic for the rapamycin-treated animals, indicating that highly generalized seizures are not required for high post-cue freezing. These results are consistent with the *r* values for seizure duration and post-cue freezing, reinforcing the correlation between seizure duration and behavioural seizure.

**3.3 Isoflurane Results**

During the isoflurane experiment, like in the rapamycin experiment, the animals were exposed to two sensory stimuli, an auditory tone and white LED, followed by electrical stimulation to generate seizures (see section 2.3.3 Stimulation). Because seizures were already induced in the previous experiment, there were only two kindling sessions (sessions -1 and 0 in figure 3.4) before the treatment had begun. During the treatment sessions, the isoflurane-treated animals were placed under isoflurane (2.5 % of isoflurane in oxygen) for five minutes, approximately seven minutes after being electrically stimulated, for a maximum of five sessions. The non-treated animals were placed back into their home cages approximately seven minutes after being electrically stimulated (sessions 1 through 5 in figure 3.4).

The same animals and experimental groups were maintained for both experiments, however, before the isoflurane experiment began, two of the treatment

animals died during kindling, thus only four animals could be analyzed instead of six for the treatment group during the isoflurane experiment.

Figure 3.4 illustrates the results of the isoflurane experiment. The seizure duration (figure 3.4 A), behavioural seizure (figure 3.4 B), and freezing duration (figure 3.4 C) were determined for every animal each session. The minimum currents required to induce an electrographic seizure, were determined at the thresholding phases before and after the isoflurane experiment (figure 3.4 D). A two-way ANOVA was used to determine if a significant effect of treatment and session on the dependent variables existed, and a t-test was used to determine any significant differences between the groups each session.

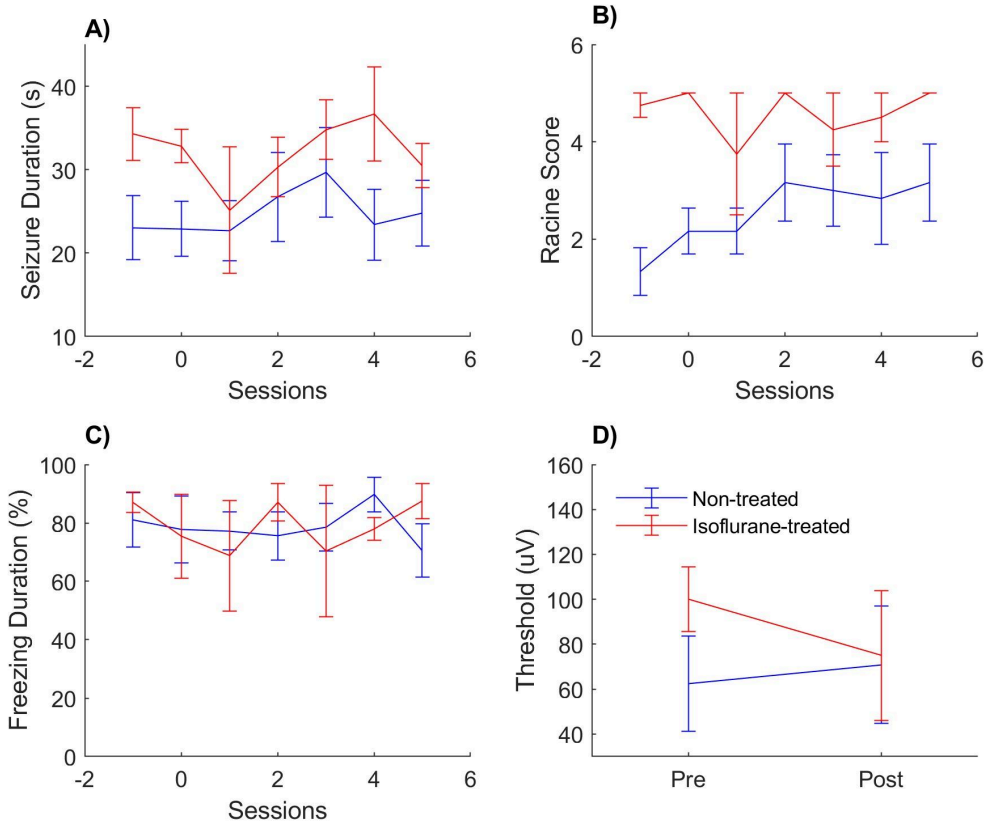


**Figure 3.4** Results of isoflurane experiment. **(A)** Isoflurane-treated animals had longer seizure durations than the non-treated animals. **(B)** Isoflurane-treated animals had seizures that were more generalized than the non-treated animals. **(C)** There was no effect of isoflurane on post-cue freezing. **(D)** Isoflurane had no effect on sensitization to the convulsive stimulus. Legend in C applies to all subfigures. Session 0 in A, B, and C represents the session where sufficient behavioural seizures were first observed. N = 6 for both groups. Data reported as mean ± S.E.M.

### 3.3.1 Seizure Duration (Isoflurane)

A two-way ANOVA revealed an insignificant effect of session and a significant effect of treatment on seizure durations; on average, the non-treated animals had shorter seizure durations [figure 3.4 A: main effect of the session, $F(6, 56) = 0.63$, $p > 0.05$; main effect of the treatment, $F(1, 56) = 9.19$, $p > 0.05$; effect of the interaction, $F(6, 56) = 0.42$, $p > 0.05$]. An effect of isoflurane cannot be determined as the isoflurane-treated animals

had significantly longer seizures than the non-treated animals before the isoflurane treatment began, as determined by a t-test [figure 3.4 A: session -1, $t(8) = -2.09$, $p < 0.05$; session 0, $t(8) = -2.27$, $p < 0.05$].

The effects of isoflurane are consistent with the effects of rapamycin (figure 3.1 top left), in that on average, the treated animals had significantly longer seizure durations than the control animals. However, the effects of session during the isoflurane experiment are not consistent with the effects of session during the rapamycin experiment, in that the average seizure durations for both experimental groups in the isoflurane experiment did not progress across sessions, whereas they did during the rapamycin experiment. The differences between the average seizure durations at the start of each experiment is much greater than the differences between the average seizure durations at the end of the rapamycin experiment and the start of the isoflurane experiment, indicating that seizures had progressed before the isoflurane experiment began for both groups.

### 3.3.2 Behavioural Seizure (Isoflurane)

A two-way ANOVA revealed an insignificant effect of sessions and a significant effect of treatment on behavioural seizures; on average, the non-treated animals scored lower on the Racine scale [figure 3.4 B: main effect of the session, $F(6, 56) = 0.82$, $p > 0.05$; main effect of the treatment, $F(1, 56) = 30.52$, $p < 0.05$; effect of the interaction, $F(1, 56) = 0.61$, $p > 0.05$]. The effects of isoflurane on behavioural seizures cannot be determined as the isoflurane-treated animals scored significantly higher on the racine scale than the non-treated animals before the treatments began, as indicated by a t-test [figure 3.4 B: session -1, $t(8) = -5.27$, $p < 0.05$; session 0, $t(8) = -4.75$, $p < 0.05$]. These results are consistent with the results obtained for seizure durations during the isoflurane

experiment (figure 3.4 A), in that both revealed a significant effect of the treatments, but not sessions.

Only the effect of treatment on behavioural seizures during the isoflurane experiment is consistent with the effects of treatment on behavioural seizures during the rapamycin experiment (figure 3.1 A), in that the treated animals scored significantly higher on the Racine scale. The effects of session on behavioural seizure during the isoflurane experiment are not consistent with the effects of session during the rapamycin experiment, in that the average Racine scores for both groups during the isoflurane experiment did not progress across sessions, while they had during the rapamycin experiment. Additionally, the differences between the average Racine score at the start of each experiment is much greater than the difference between the average Racine score at the end of the rapamycin experiment and at the start of the isoflurane experiment, indicating that seizures had progressed before the isoflurane experiment began.

### 3.3.3 Post-cue Freezing (Isoflurane)

A two-way ANOVA revealed an insignificant effect of session and treatment on post-cue freezing during the isoflurane experiment [figure 3.4 C: main effect of the session, $F(6, 56) = 0.35$, $p = 0.90$; main effect of the treatment, $F(1, 56) = 0.01$, $p > 0.05$; effect of the interaction, $F(1, 56) = 0.56$, $p > 0.05$]. These results indicate that there is no main effect of the isoflurane or sessions on post-cue freezing and no interaction, which provides more evidence for a potential effect of the rapamycin that disrupts the consolidation of fear memories.

The effect of treatment on post-cue freezing during the isoflurane experiment is not consistent with the effect of treatment during the rapamycin experiment, as there is no

significant difference between the groups during the isoflurane experiment, while a significant difference was found during the rapamycin experiment, where the rapamycin-treated animals displayed less post-cue freezing. The effect of session for the isoflurane experiment is also not consistent with the effect of session for the rapamycin experiment, in that for both groups, the average freezing durations during the isoflurane experiment did not increase across sessions, while they did for the rapamycin experiment. Additionally, the difference between the average freezing durations at the end of the rapamycin experiment and at the start of the isoflurane experiment is greater for the treated animals, indicating that post-cue freezing increased for the treatment group following the rapamycin experiment, providing even more evidence that suggests a potential effect of rapamycin that disrupts the consolidation of fear memories.

### 3.3.4 Thresholding (Isoflurane)

A two-way ANOVA revealed an insignificant effect of the sessions and isoflurane on the minimum current to induce an electrographic seizure [figure 3.4 D: main effect of the session, $F(1, 14) = 0.03$, $p > 0.05$; main effect of the treatment, $F(1, 14) = 0.31$, $p > 0.05$; effect of the interaction, $F(1, 14) = 0.10$, $p > 0.05$]. These results indicate that there was no effect of the isoflurane that increases tolerance to the convulsive stimulus; however, a t-test revealed a significant difference between the difference in current for both groups [figure 3.4 D: final minus initial current, $t(8) = -2.16$, $p < 0.05$]. The average difference for the non-treated group is 8.33 uA, and -25.0 uA for the isoflurane-treated group.

### 3.4 Isoflurane Correlation

The initial analysis for the isoflurane experiment revealed that the isoflurane-treated animals had, on average, seizures that were longer in duration (figure 3.4 A) and more generalized (figure 3.4 B) than the non-treated animals, like the rapamycin experiment; however, no significant difference between post-cue freezing between the groups was found (figure 3.4 C).

To determine the relationships between these results, and how they had changed from the rapamycin experiment, Pearson's *r* was calculated for seizure duration and behavioural seizure (figure 3.5 A), seizure duration and post-cue freezing (figure 3.5 B), and behavioural seizure and post-cue freezing (figure 3.5 C).

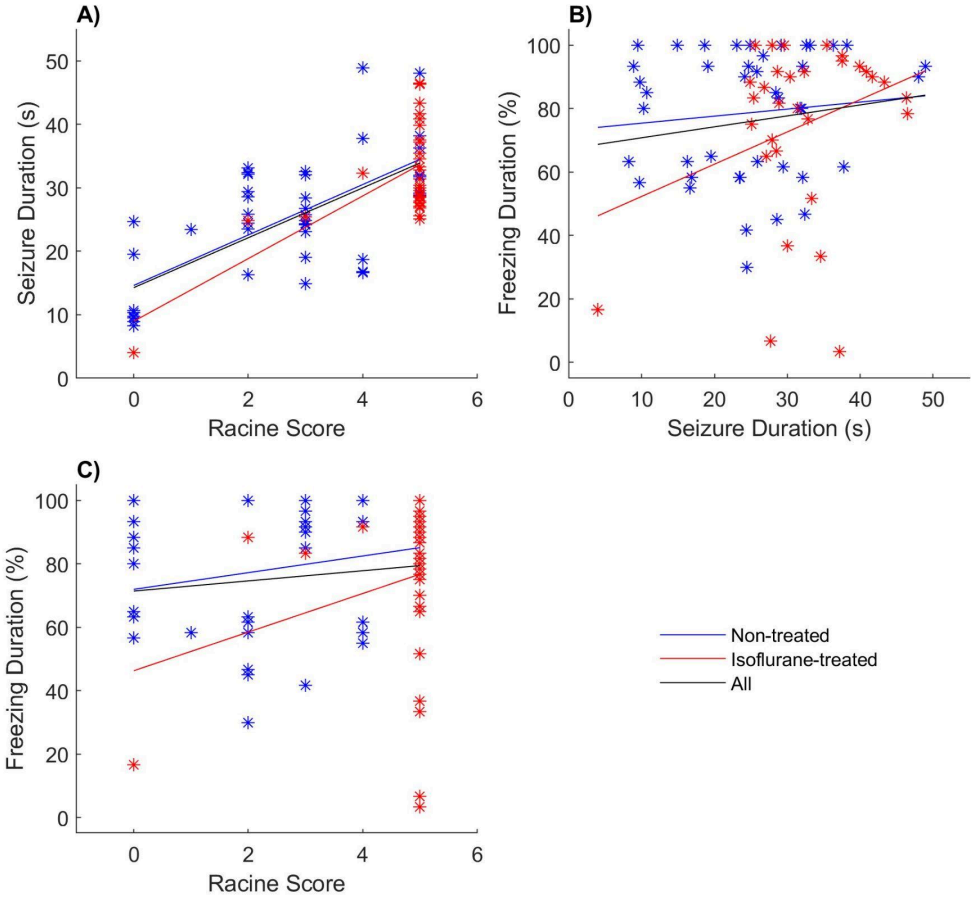


**Figure 3.5** Correlation between seizure duration, behavioural seizure, and freezing behaviour during the isoflurane experiment. **(A)** Behavioural seizure and seizure duration are the most positively correlated in both groups. **(B)** Seizure duration and post-cue freezing have an insignificant positive correlation for both groups. **(C)** Behavioural seizure and post-cue freezing have an insignificant positive correlation for both groups. Regression lines are included for each group for each correlation. The legend in the bottom right applies to all subplots.

### 3.4.1 Behavioural Seizure and Seizure Duration (Isoflurane)

Pearson's *r* revealed a positive correlation between behavioural seizure and seizure duration for the non- and isoflurane- treated animals. Regardless of treatment, a positive correlation was found [figure 3.5 A: non-treated animals, $r(40) = 0.07$, $p < 0.05$; isoflurane-treated animals, $r(26) = 0.67$, $p < 0.05$; all animals, $r(68) = 0.72$, $p < 0.05$].

These results, like the rapamycin experiment, reveal that seizure duration and behavioural seizure are the most correlated dependant variables, as the *r* values obtained for both groups and all animals are greater than the *r* values obtained for seizure duration and post-cue freezing, and behavioural seizure and post-cue freezing.

### 3.4.2 Seizure Duration and Post-cue Freezing (Isoflurane)

Pearson's *r* revealed an insignificant positive correlation between seizure duration and post-cue freezing for the non- and isoflurane- treated animals; regardless of treatment, an insignificant positive correlation was found [figure 3.5 B: non-treated animals, $r(40) = 0.11$, $p > 0.05$; isoflurane-treated animals, $r(26) = 0.30$, $p > 0.05$; all animals, $r(68) = 0.14$, $p > 0.05$]. The results presented here and in section 3.3.3, suggest that post-cue freezing is only partially dependant on the duration of the seizures, as the non-treated animals displayed high post-cue freezing, like the isoflurane-treated animals, but displayed lower seizure durations; the *r* statistics for the non-treated animals provides more evidence for this, as the correlation between these two dependant variables is low, which indicates that longer seizure durations are not required for high post-cue freezing. Compared to the rapamycin experiment, the *r* value for the isoflurane-treated animals has increased, although still insignificant, and is now greater than the *r* value for the non-treated animals, which has decreased and is now insignificant.

### 3.4.3 Behavioural Seizure and Post-cue Freezing (Isoflurane)

Pearson's *r* revealed an insignificant positive correlation between behavioural seizure and post-cue freezing for the non- and isoflurane- treated animals. Regardless of treatment, an insignificant positive correlation was also found [figure 3.5 C: non-treated animals, $r(40) = 0.22$, $p > 0.05$; isoflurane-treated animals, $r(26) = 0.24$, $p > 0.05$; all

animals, $r(68) = 0.12$, $p > 0.05$]. The results presented here and in section 3.3.3, suggest that post-cue freezing is only partially dependant on the generalization of the seizures, as the non-treated animals displayed on average, high post-cue freezing, like the isoflurane-treated animals, but scored lower on the Racine scale; the *r* statistics for both groups and all animals provides more evidence for this, since the correlation between the two dependent variables is low, which indicates that longer seizure durations are not required for high post-cue freezing. Compared to the rapamycin experiment, the *r* value for the isoflurane-treated animals increased, although still insignificant, and is now closer to the *r* value for the non-treated animals, which has decreased and is now insignificant.

**3.5 Rapamycin and Isoflurane Results**

Considering the results in sections 3.1 and 3.3, consistent (e.g. the effect of treatment on seizure duration) and inconsistent (e.g. the effect of treatment on freezing duration) results are identified and become particularly interesting. To investigate these results further, and because the experimental groups were maintained across both experiments, analysis of all the kindling sessions was necessary.

The following analysis was conducted on all kindling sessions, from four sessions before the first injection of rapamycin through to the final isoflurane session, excluding the test sessions. This analysis included the three kindling sessions occurring in between the rapamycin and isoflurane experiments. 12 animals were included, six treated and six non-treated animals, however, two of treated animals died as a result of their seizures; thus, six animals were in the non-treated group for all sessions, six in the treated group sessions one through 12, five in the treated group sessions 13 through 15, and four animals in the treated group sessions 16 through 19.

Figure 3.6 illustrates the results from all kindling sessions. The average seizure duration (A), post-cue freezing duration (B), and behavioural seizure (C) across all sessions were determined. Treatments of rapamycin and saline occurred on sessions five through nine, while the treatment of isoflurane occurred on sessions 15 through 19. A two-way ANOVA was used to determine if a significant effect of treatment and session on the dependent variables existed, and a t-test was used to determine any significant differences between the groups each session.

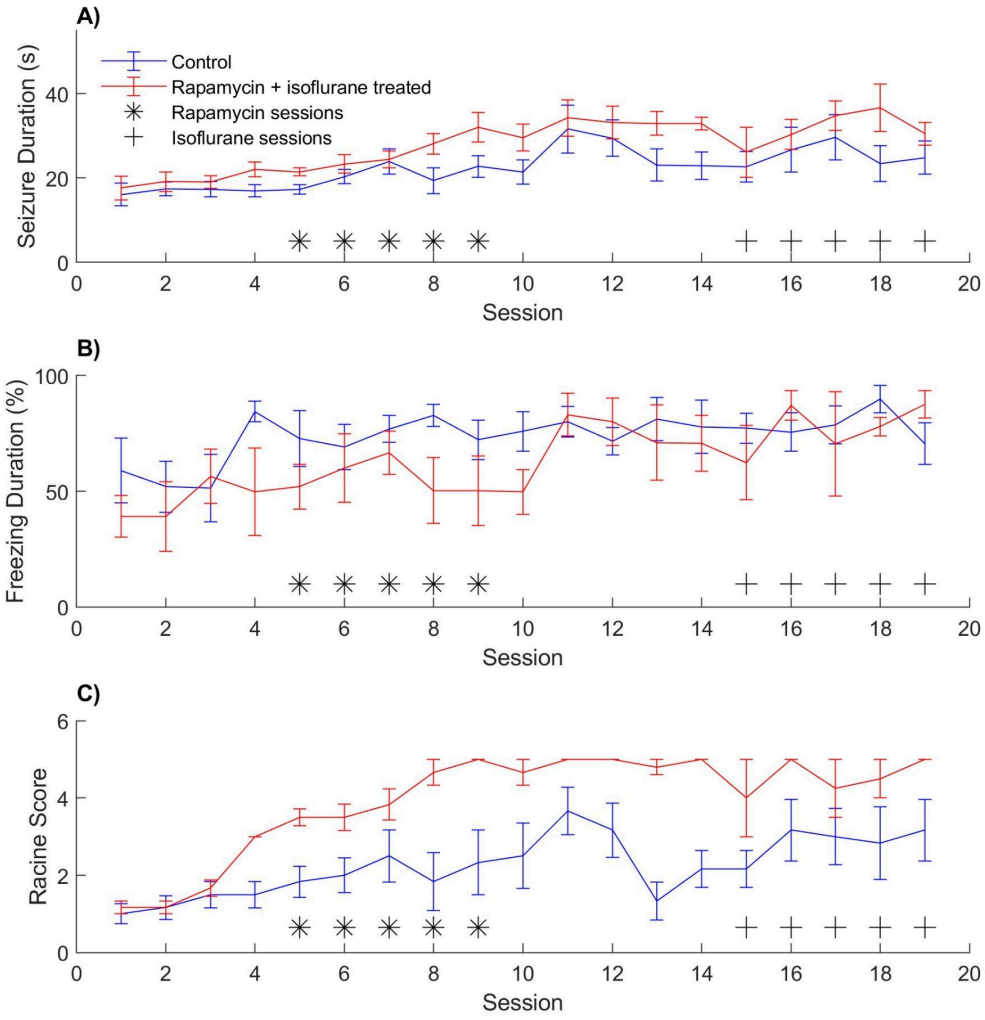


**Figure 3.6** Seizure duration, post-cue freezing and behavioural seizure results for all sessions. **(A)** Seizure duration increased across all sessions for both groups. The treated animals had significantly longer seizures. **(B)** Post-cue freezing increased across sessions for both groups. The control animals froze for significantly longer durations. **(C)** Behavioural seizures increase across sessions for both groups. The treated animals had significantly greater seizures. Rapamycin treatments occurred on sessions 5 through 9; isoflurane treatments occurred on sessions 15 through 19. Legend in A applies to all subfigures. Data reported as mean ± S.E.M.

### 3.5.1 Seizure Duration (Rapamycin and Isoflurane)

A two-way ANOVA revealed a significant effect of the sessions and treatments on seizure duration [figure 3.6 A: main effect of the session, $F(18, 179) = 4.58$, $p < 0.05$; main effect of the treatment, $F(1, 179) = 23.9$, $p < 0.05$; effect of the interaction, $F(18, 179) = 35.26$, $p > 0.05$]. On average, the treated animals had significantly longer seizures.

The effect of the treatments suggests that the rapamycin and isoflurane increase the duration of seizures; however, this conclusion can not be made since the control animals displayed seizures of less duration both consistently and before the treatments began. Instead it is more likely that the control animals were having seizures of less duration regardless of treatment. These results are consistent with the results obtained for each experiment individually (sections 3.1.1 and 3.2.1).

The effects of session from this analysis suggests that seizure duration progressed across all sessions in both groups; however, the results obtained for each experiment (sections 3.1.1 and 3.2.1) reveal that the animal's seizures had developed to a maximum duration before the isoflurane experiment had commenced.

### 3.5.2 Post-cue Freezing (Rapamycin and Isoflurane)

A two-way ANOVA revealed a significant effect of sessions, for both groups, and a significant effect of treatments on post-cue freezing [figure 3.6 B: main effect of the session, $F(18, 179) = 1.91$, $p < 0.05$; main effect of the treatment, $F(1, 179) = 8.05$, $p < 0.05$; effect of the interaction, $F(18, 179) = 0.82$, $p > 0.05$]. On average, the control animals displayed greater freezing responses.

The effects of treatment suggest instead that only the rapamycin treatment decreases post-cue freezing. The treated animals displayed significantly lower post-cue

freezing during the rapamycin experiment. No significant difference was first observed on session 11 (figure 3.6), the second kindling session after the rapamycin experiment, that also took place in between the two experiments, which indicated that the treated animals did not have typical fear memory consolidation until after the rapamycin experiment

The effects of the sessions suggest that post-cue freezing is not dependent on the intensity of the seizures, but is dependant on the fact that the animals are having seizures, as the non-treated animals displayed post-cue freezing close to 100 % in earlier sessions, despite having seizures of less intensity relative to the treated animals across both experiments.

The effects of sessions and treatment obtained here and the rapamycin experiment are consistent (section 3.1.3), however, they are not consistent with the isoflurane experiment. The effects of treatment across all sessions suggest a potential effect of the rapamycin and isoflurane treatments that decrease post-cue freezing duration; however, the treated animals displayed on average, significantly lower post-cue freezing durations than the non-treated animals during the rapamycin experiment only; there was no significant difference between the two groups during the isoflurane experiment.

### 3.5.3 Behavioural Seizure (Rapamycin and Isoflurane)

A two-way ANOVA revealed a significant effect of sessions and treatment on behavioural seizures [figure 3.6 C: main effect of the session, $F(18, 179) = 7.16$, $p < 0.05$; main effect of the treatment, $F(1, 179) = 98.3$, $p < 0.05$; effect of the interaction, $F(18, 179) = 1.59$, $p > 0.05$]. On average, the saline-treated animals scored lower on the Racine scale.

The effects of treatment suggest a potential effect of both the rapamycin and isoflurane treatments that increases the generalization of the seizures, however the control animals displayed seizures lower on the Racine scale both consistently and before the treatments began, thus this conclusion can not be made. Instead it can be suggested that the control animals had seizures that were less generalized, regardless of treatment.

Considering the effects of session obtained from the rapamycin (section 3.1.2) and isoflurane experiment (section 3.3.2) individually, they suggest that the animal's seizures had developed to a maximum generalization before the isoflurane experiment had commenced.

**3.6 Rapamycin and Isoflurane Correlation**

The analysis of all kindling sessions revealed a significant difference of seizure duration and behavioural seizure between the two groups, as the treated group had on average, seizures that were significantly longer (figure 3.6 A) and more generalizied (figure 3.6 C). These results are consistent with the seizure duration and behavioural seizure results presented in sections 3.1 and 3.3. This analysis also revealed a significant difference of post-cue freezing between the two groups; however, when considering the results for post-cue freezing in sections 3.1.3 and 3.3.3, the effects of treatment on post-cue freezing are not consistent, as the treated animals had significantly shorter post-cue freezing durations during the rapamycin experiment (section 3.1.3 and figure 3.1 C) than the control animals, but not during the isoflurane experiment (section 3.3.3 and figure 3.4 C).

To determine the relationships between these dependant variables, Pearson's *r* was calculated for seizure duration and behavioural seizure (figure 3.7, A), seizure

duration and post-cue freezing (figure 3.7 B), and for behavioural seizure and post-cue freezing (figure 3.7 C).

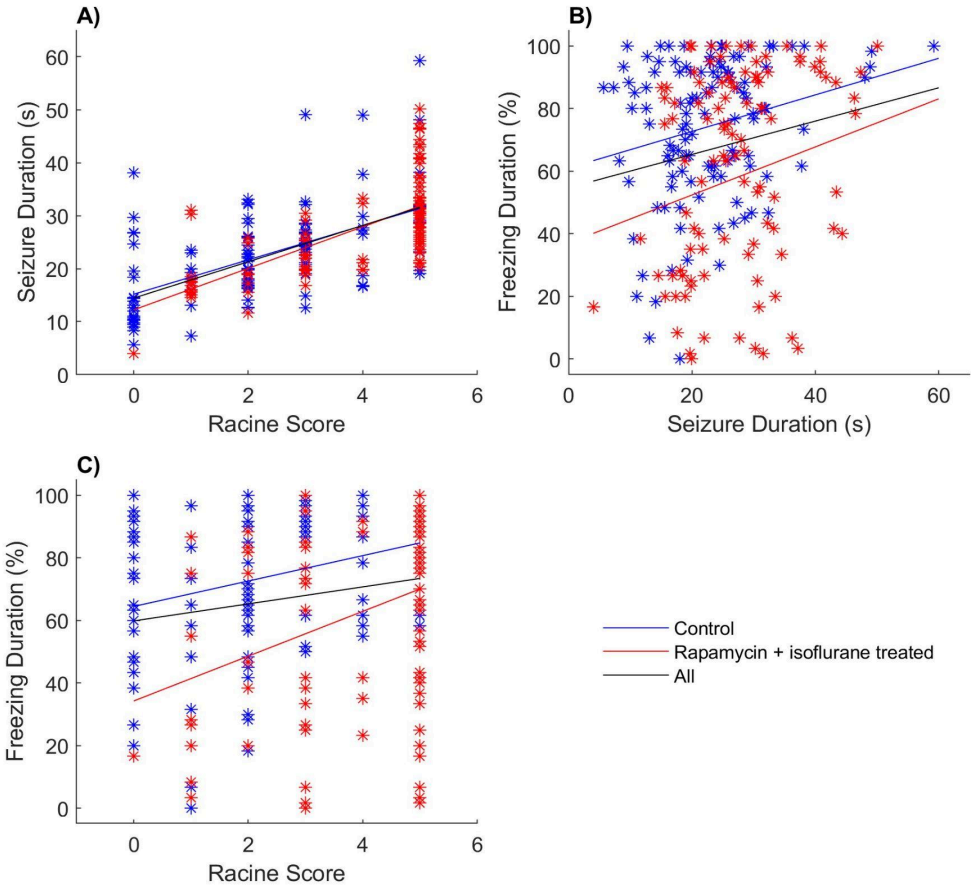


**Figure 3.7** Correlation between seizure duration, behavioural seizure, and freezing behaviour across all kindling sessions. **(A)** Behavioural seizure and seizure duration are the most positively correlated in both groups. **(B)** Seizure duration and post-cue freezing have a significant positive correlation for both groups. **(C)** Behavioural seizure and post-cue freezing have a significant positive correlation for both groups.

### 3.6.1 Behavioural Seizure and Seizure Duration (Rapamycin and Isoflurane)

Pearson's *r* revealed a positive correlation between behavioural seizure and seizure duration for the non-treated animals and isoflurane-treated animals; regardless of treatment, there was also a positive correlation [figure 3.7 A: non-treated animals, $r(112)$

= 0.57, $p < 0.05$; rapamycin and isoflurane treated animals, $r(101) = 0.66$, $p < 0.05$; all animals, $r(215) = 0.65$, $p < 0.05$].

These results reveal that seizure duration and behavioural seizure are the most correlated dependent variables, as the *r* values obtained for both groups and all animals were greater than the *r* values obtained for seizure duration and post-cue freezing, and behavioural seizure and post-cue freezing. These results are also consistent with the *r* values obtained for seizure duration from the rapamycin (section 3.2.1 and figure 3.3 A) and isoflurane experiments (section 3.4.1 and figure 3.5 A).

### 3.6.2 Seizure Duration and Post-cue Freezing (Rapamycin and Isoflurane)

Pearson's *r* revealed a positive correlation between seizure duration and post-cue freezing for the non-treated and treated animals; regardless of treatment, there was a positive correlation for all animals [figure 3.7 B: non-treated animals, $r(112) = 0.24$, $p = 0$; rapamycin and isoflurane treated animals, $r(101) = 0.26$, $p < 0.05$; all animals, $r(215) = 0.18$, $p < 0.05$]. The correlation for the treated animals were the greatest in this analysis and in the analysis of the isoflurane experiment, during the rapamycin experiment the correlation for the treated animals was close to zero. These results indicate that the treated animals displayed

### 3.6.3 Behavioural Seizure and Post-cue Freezing (Rapamycin and Isoflurane)

Pearson's *r* revealed a positive correlation between behavioural seizure and post-cue freezing for the non-treated and treated animals; regardless of treatment, there was also a positive correlation [figure 3.7 C: non-treated animals, $r(112) = 0.28$, $p < 0.05$; rapamycin and isoflurane treated animals, $r(101) = 0.34$, $p < 0.05$; all animals, $r(215) = 0.17$, $p < 0.05$].

### 3.7 MWT latencies

Following the isoflurane experiment, the animals were subjected to a MWT to test the effects of kindling on spatial learning. Each animal was placed in a pool of water and were required to locate a submerged platform within 60 seconds, by utilizing the distal cues that were facing the pool in each cardinal direction. The animals were trained on this task over seven sessions with each one consisting of four rounds of trials, where in each the animals were placed in the pool, starting at a different cardinal direction. The swim latencies were determined by calculating the average time to find the platform from the four trials. The eighth session was the probe trial, in which the submerged platform was removed from the pool and the animals were given 60 seconds to swim freely before being removed; the percentage of time spent in the target quadrant was determined. The MWT utilized three animals from the treated and non-treated groups that had displayed the greatest behavioural seizures, in addition to six naive animals from the same cohort.

Figure 3.8 displays the results of the MWT. The average swim latencies (figure 3.8 A) during training, and the average percentage of time spent in the target quadrant (figure 3.8 B) during the probe were determined for all groups. A two-way ANOVA was used to determine the effects of kindling and session on swim latency, and a t-test was used to determine a significant difference in the percentage of time spent in the target quadrant between both groups.

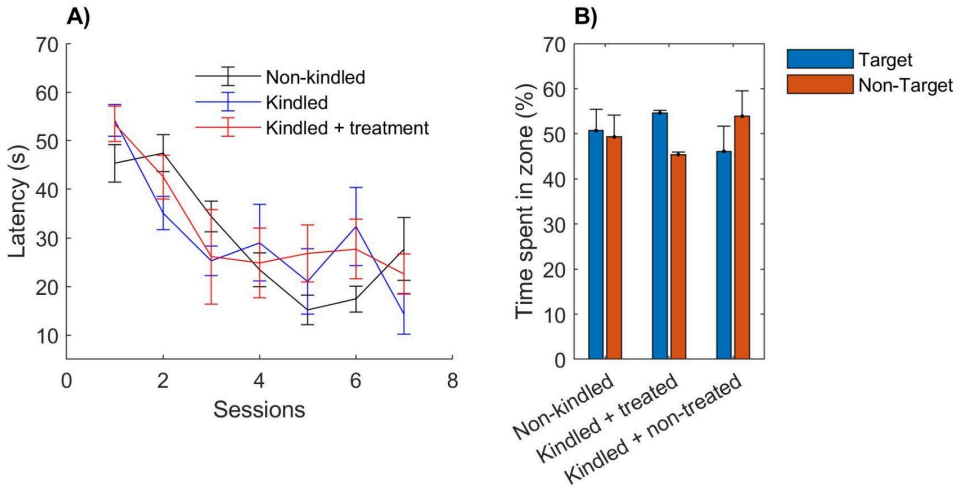


**Figure 3.8** Results of Morris water maze task. **(A)** The average swim latencies for kindled vs treated kindled vs non-treated kindled animals. **(B)** The average time spent in the target quadrant vs all other quadrants for all groups during the probe session. Data reported as mean ± S.E.M.

### 3.7.1 Training

A three-way ANOVA revealed a significant effect of the sessions in all groups, and no effects of the kindling or treatments on the latency to locate the platform [figure 3.8 A: main effect of the session, $F(6, 75) = 14.3$, $p < 0.05$; main effect of the kindling, $F(1, 75) = 0$, $p > 0.05$; effect of the treatments, $F(1, 75) = 0.33$, $p > 0.05$]. The effect of the session indicated that the animals were able to learn the task as the average latency for all groups decreased across sessions. The effects of the kindling indicate that the physiological and morphological changes induced by epileptogenesis does not disrupt spatial memory. The effects of the treatment indicate that the rapamycin and isoflurane treatments that occurred prior to the MWT does not disrupt spatial learning.

### 3.7.2 Probe

A two-way ANOVA revealed no significant effect of kindling or the treatments on the time spent in the target quadrant [figure 3.8 B: main effect of kindling, $F(1, 9) = 0.43$,

$p > 0.05$; main effect of the treatments, $F(1, 9) = 1.11$, $p > 0.05$]. The effects of kindling indicate that the physiological and morphological changes induced by epileptogenesis, does not disrupt spatial memory retention. The effects of the treatments indicate that the rapamycin and isoflurane treatments that occurred prior to MWT does not disrupt spatial memory retention.

## Chapter 4. Discussion

The purpose of this study is to determine if rapamycin or isoflurane could be used as potential treatment for epilepsy by disrupting the normal progression of seizures. Unfortunately, the results in chapter three illustrate several limitations that prevent any reliable conclusions from being made. In this chapter, the major limitations will be discussed before a discussion of the results reported in chapter three, as this discussion will ultimately assume the effects of the treatments.

### 4.1 Limitations

The analysis in chapter three suggests that the rapamycin and isoflurane treatments increase the intensity of seizures, as the treated animals have seizures that are longer in duration and more generalized; however, these effects are observed in the sessions prior to both treatments, suggesting that this is attributed to a methodological error and not the treatments. This is likely due to the lack of randomization when the animals were assigned to a group, where the first three males and females that displayed a stage three seizure on the Racine scale were assigned to the experimental group, when they should have been randomly assigned prior to the first kindling session. An effect of rapamycin on the development of seizures can not be reliably concluded, as the two groups can not be compared.

The effects of session on seizure duration and generalization across both the rapamycin and isoflurane experiments illustrate another important limitation that needs to be addressed. While the model of kindling that these experiments utilize is a reliable tool to study the progression of seizures, the effects however are highly persistent, meaning that the neuronal changes that initiate the seizures are relatively permanent; thus, an

animal that displays a stage five seizure will continue to display a stage five in the seizures that follow, even if there is a large interval of time in between each session. The rapamycin experiment reveals that both seizure duration and generalization increase from low to high across sessions, while the isoflurane experiment reveals that they are consistently high. Taken together, these results suggest that seizure intensity progresses from low to high until a maximum intensity is reached (stage five on the Racine scale) that is maintained in the seizures that follow throughout the isoflurane experiment. The intention of both the rapamycin and isoflurane experiments is to determine if these treatments are capable of disrupting the progression of seizures in epilepsy; thus, the treatments needed to be administered before the seizures are fully generalized. While this is the case for the rapamycin experiment, this can not be the case for the isoflurane experiment, since the experimental animals had stage five seizures that were established prior to the start of the experiment.

The post-cue freezing durations reveal a significant difference in the fear responses between the experimental and control groups during the rapamycin experiment, but not during the isoflurane experiment. While these results suggest that rapamycin disrupts fear memory consolidation, it should be mentioned that the technique used to calculate the percentage of time that an animal freezes for, as described in section 2.5.2, is sensitive to modification. While small modifications, such as having the threshold be equal to the mean of the baseline plus one or two standard deviations, will reveal the same effects, other modifications, such as, setting the threshold to constant value, across all animals and sessions, will reveal no significant difference between both groups across both experiments. To determine if the technique used is reliable, it must be applied to a

similar dataset, where the percentage of time an object freezes or moves is already known, and must replicate those results. The validity of these results requires the effects found to be replicated with another reliable technique.

**4.2 The effects of rapamycin**

Despite the significant differences between the experimental and control animals, the results do suggest that rapamycin does not disrupt the progression of seizures, as the experimental animals' had seizures that progressed from partial to fully generalized over the treatment sessions. The average number of kindling sessions that the experimental animals required to generate a stage five seizure is 11, which is consistent with the literature, where rodents typically require 10 to 15 sessions (Stover et al., 2017; Teskey, 2020). Previous studies, particularly ones that investigate TSC, have implicated that mTOR signalling is required for epileptogenesis (Curatolo et al., 2008; Krueger et al., 2013; Lam et al., 2010); however when also considering the results obtained here, it can be suggested that mTOR signalling is not a universal mechanism that is required for epileptogenesis in all epileptic conditions, but is required for some conditions (e.g. TSC).

The effects of rapamycin on the reconsolidation of a seizure fear memory was assessed, as it has been previously demonstrated that rapamycin inhibits mTOR, which is known to play a critical role in the synaptic plasticity required for fear memory consolidation and reconsolidation (Blundell et al., 2014; Parsons et al., 2006). The US in this case is unfortunately not obvious, since the seizures, electrical stimulation (shock/pain) and after effects of the electrical stimulation (confusion) are all variables that are possibly responsible for the animals freezing. The results of the rapamycin experiment however, suggests that intraperitoneal injections of rapamycin following

recall will disrupt the reconsolidation of fear memories, since freezing was attenuated during the rapamycin experiment only. However, attenuation should be observed following the first injection. While the treated animals exhibit fear memory attenuation, this effect is also observed in several sessions prior to the treatments. Thus, while the rapamycin potentially attenuates fear memory, it is clear that another independent variable is causing attenuation in the sessions prior to the treatments.

These results suggest that it is the intensity of the seizures that is attenuating the fear responses in the sessions prior to the treatment, and that the extent of this impairment is dependent on the generalization of the seizures. It has been previously found that when kindling proceeds fear conditioning, the normal acquisition of the fear memories are disrupted (Botterill et al., 2014; Mao et al.. 2009). Additionally, it has also been found that seizure prone rats, relative to seizure resistant rats, display greater disruptions to both working and reference memory when kindled prior to training (McIntyre et al., 2004). Here, the results from the experimental animals suggest that when the physiological changes that occur following kindling are sufficient, the seizures disrupt the normal acquisition of fear memories. Additionally, the results from the control animals suggest that when the physiological changes are insufficient, the seizures do not disrupt the normal acquisition of fear memories, or at least not to the same extent that sufficient physiological changes do. A significant difference in fear response, seizure generalization, and seizure duration are all first observed the session where sufficient behavioural seizures were determined, indicating that sufficient physiological changes in the experimental animals had been reached following kindling in the session prior, that

allowed for their seizures to be more generalized and disrupt learning of the fear memory in the sessions that followed.

**4.3 The effects of isoflurane**

Isoflurane was selected as a treatment, since it has been shown to suppress neuronal activity (Ranft et al., 2004), thus, it was hypothesized that isoflurane disrupts the normal consolidation and reconsolidation mechanisms that would slow/reverse the progression of seizures. Because the treated animals were kindled to fully generalized seizures prior to the isoflurane experiment, sufficient physiological changes had occurred that resulted in an increased sensitization to convulsive stimulus; thus, the effects of isoflurane on the persistence of fully generalized seizures was tested. The experiment revealed no effects on the persistence of the seizures, as the experimental animals displayed stage five throughout all sessions.

In addition to suppressing normal neuronal activity (Ranft et al., 2004), isoflurane has been shown to prevent both spontaneous (Bar-Klein et al., 2016) and electrically induced seizures, when administered during seizures. Thus, it can be hypothesized instead, that isoflurane disrupts the physiological changes that increase sensitization to the convulsive stimulus, when administered during a seizure. This can be tested with an experiment that would require an experimental and control group, that are treated with either 2.5 % of isoflurane dissolved in oxygen or pure oxygen respectively, during seizures that are induced with electrical stimulation

Unlike the rapamycin experiment, no effects of isoflurane on the reconsolidation of fear memories can be suggested, since the treated animals displayed a fear response similar to the non-treated animals at the start of and throughout the experiment.

### 4.4 Future directions

Future studies that test these interventions at earlier stages would clarify the mechanisms and their therapeutic implications for epileptogenesis. There are also other limitations of our study. For example, using recordings from single neurons (Schjetnan et al., 2011) instead of LFP could provide more direct information about the effect of treatment on neuronal reactivation patterns which are associated with memory consolidation (Wilson & McNaughton, 1994). Using histological analyses, like measuring cell density (Faraji et al. 2013) or neuronal branching patterns (Luczak, 2010) could also provide more details of the effects of rapamycin on epilepsy. Moreover, using machine learning techniques for analyses of animal behavior (Ryait et al., 2019; Torabi et al., 2021; Tanabe et al., 2024) could reveal more nuanced changed in behavioral seizures which we could have missed with the standard Racine scale measure (Racine, 1972). Nevertheless, the results presented here provide new insights into differences between epileptogenesis and memory processes, which could help researchers and clinicians in designing novel treatments for epilepsy.

### 4.5. Associating sensory cues with incoming seizures: developing an animal model of auras.

Ritwik Das, Carlos Howey, Autumn McFetridge, Valérie Lapointe & Artur Luczak[4] [5]

Abstract:

> For patients with epilepsy, one of the biggest problems is the unpredictability of the time when the next seizure will occur. Interestingly, some epileptic patients experience a sensory sensation preceding seizures, called aura, which helps them

[4] R.D. and A.L. designed the experiment. R.D., A.M. and C.H. conducted the experiments and collected the data. R.D. and C.H. performed the surgeries. V.L., A.M., and C.H. performed the histology for the brain sections. R.D. and A.L. analyzed the data and wrote the manuscript, which all authors helped to revised [*sic*].

[5] Permission to include this published paper in this thesis was obtained from all authors.

move to safety before a seizure. Here, we describe the development of the first animal model of auras, which could allow for a more detailed study of this phenomenon. Specifically, in mice, we presented sensory stimuli (sound and light cues) a few seconds before kindling an animal to induce seizures. Animals were kindled by electrical stimulation in the basolateral amygdalar nucleus. Over the course of stimulation sessions, animals started showing progressively stronger freezing behavior to sensory cues preceding kindling. Interestingly, seizures are known to cause retrograde amnesia, thus it was surprising that the association between seizures and preceding sensory cues developed in all experimental animals. In summary, our experiments show that similarly to auras, a sensory sensation can be associated with incoming generalized seizures and is not erased by retrograde amnesia (Das et al., 2024, Abstract).

## Chapter 5. Conclusion

While it was found that rapamycin (40 mg/kg, i.p.) does not prevent or slow the progression of epileptogenesis, it can be determined that mTOR is not required for epileptogenesis in an electrical stimulation model of epilepsy. Interestingly, mTOR signalling has been implicated to play a role in epileptogenesis in cases of TSC; taken with the results obtained here, it is indicated that mTOR signalling is not a universal process that is required for epileptogenesis in all epileptic disorders. These results also suggest that rapamycin inhibits memory reconsolidation of a fear memory association, where a seizure is the unconditioned response; however, more testing is required to determine if this effect is solely due to fully generalized seizures.

It was found that isoflurane (2.5 % in oxygen) has no effect that reverses epileptogenesis. However, it was observed that isoflurane prevents seizures from being generated in a mouse that displayed stage five seizures (see appendix B, figure B.5). Thus, it can be hypothesized instead, that isoflurane will prevent/slow epileptogenesis when administered during early seizures, as it potentiates GABAergic activity, which would prevent neuronal networks from being recruited into an epileptic network.

The MWT revealed that the kindled animals performed the task as well as the non-kindled animals, suggesting that an established epileptic condition does not disrupt spatial learning. However, while the effects of kindling are typically persistent (Teskey, 2020), a considerable amount of time had passed between the isoflurane experiment and the MWT (approximately two weeks). Additionally, only a subset of the kindled animals had displayed seizures in the absence of the electrical stimulation. Perhaps, a better

experiment would be one that tests the effects of kindling prior to a session of training, to determine if the processes required for epileptogenesis disrupts spatial learning.

These findings emphasize the complexity of the mechanisms required for both memory consolidation and epileptogenesis; however, they have the potential to provide insight into both phenomena, which could help researchers and clinicians in selecting novel treatments for a variety of neurological disorders, not exclusive to epilepsy.

**References**


Banerjee, P. N., Filippi, D., & Allen Hauser, W. (2009). The descriptive epidemiology of epilepsy—A review. *Epilepsy Research, 85*(1), 31-45. https://doi.org/10.1016/j.eplepsyres.2009.03.003

Bar‐Klein, G., Klee, R., Brandt, C., Bankstahl, M., Bascuñana, P., Töllner, K., ... & Löscher, W. (2016). Isoflurane prevents acquired epilepsy in rat models of temporal lobe epilepsy. *Annals of Neurology, 80*(6), 896-908.

Beenhakker, M. P., & Huguenard, J. R. (2009). Neurons that fire together also conspire together: is normal sleep circuitry hijacked to generate epilepsy?. *Neuron, 62*(5), 612-632.

Bliss, T. V. P., & Lømo, T. (1973). Long-lasting potentiation of synaptic transmission in the dentate area of the anaesthetized rabbit following stimulation of the perforant path. *The Journal of Physiology, 232*(2), 331-356. https://doi.org/10.1113/jphysiol.1973.sp010273

Blundell, J., Kouser, M., & Powell, C. M. (2008). Systemic inhibition of mammalian target of rapamycin inhibits fear memory reconsolidation. *Neurobiology of learning and memory, 90*(1), 28-35.

Botterill, J. J., Fournier, N. M., Guskjolen, A. J., Lussier, A. L., Marks, W. N., & Kalynchuk, L. E. (2014). Amygdala kindling disrupts trace and delay fear conditioning with parallel changes in fos protein expression throughout the limbic brain. *Neuroscience, 265*, 158-171. https://doi.org/10.1016/j.neuroscience.2014.01.040

Bower, M. R., Stead, M., Meyer, F. B., Marsh, W. R., & Worrell, G. A. (2012). Spatiotemporal neuronal correlates of seizure generation in focal epilepsy. *Epilepsia, 53*(5), 807-816.

Cossart, R., Bernard, C., & Ben-Ari, Y. (2005). Multiple facets of GABAergic neurons and synapses: Multiple fates of GABA signalling in epilepsies. *Trends in Neurosciences (Regular Ed.), 28*(2), 108-115. https://doi.org/10.1016/j.tins.2004.11.011

Curatolo, P., Bombardieri, R., & Jozwiak, S. (2008). Tuberous sclerosis. *The Lancet, 372*(9639), 657-68. doi:https://doi.org/10.1016/S0140-6736(08)61279-9

Das, R., Howey, C., McFetridge, A., Lapointe, V., & Luczak, A. (2024). Associating sensory cues with incoming seizures: developing an animal model of auras. *Scientific Reports, 14*(1), 20881. https://doi.org/10.1038/s41598-024-71885-3

de Lanerolle, N. C., Kim, J. H., Robbins, R. J., & Spencer, D. D. (1989). Hippocampal interneuron loss and plasticity in human temporal lobe epilepsy. *Brain Research, 495*(2), 387. https://doi.org/10.1016/0006-8993(89)90234-5

Drion, C. M., Borm, L. E., Kooijman, L., Aronica, E., Wadman, W. J., Hartog, A. F., ... & Gorter, J. A. (2016). Effects of rapamycin and curcumin treatment on the development of epilepsy after electrically induced status epilepticus in rats. *Epilepsia, 57*(5), 688-697.

Franz, D. N., Leonard, J., Tudor, C., Chuck, G., Care, M., Sethuraman, G., Dinopoulos, A., Thomas, G., & Crone, K. R. (2006). Rapamycin causes regression of astrocytomas in tuberous sclerosis complex. *Annals of Neurology, 59*(3), 490-498. https://doi.org/10.1002/ana.20784

Frey, U., Huang, Y. -., & Kandel, E. R. (1993). Effects of cAMP simulate a late stage of LTP in hippocampal CA1 neurons. *Science (American Association for the Advancement of Science), 260*(5114), 1661-1664. https://doi.org/10.1126/science.8389057

Frey, U., & Morris, R. G. (1997). Synaptic tagging and long-term potentiation. *Nature, 385*(6616), 533-536.

Frey, U., & Morris, R. G. (1998). Synaptic tagging: implications for late maintenance of hippocampal long-term potentiation. *Trends in neurosciences, 21*(5), 181-188.

Hebb, D. O. (1949). The organization of behavior; a neuropsychological theory. *Wiley.*

Jansen, F. E., Van Huffelen, A. C., Algra, †., & Van Nieuwenhuizen, O. (2007). Epilepsy surgery in tuberous sclerosis: A systematic review. *Epilepsia (Copenhagen), 48*(8), 1477-1484. https://doi.org/10.1111/j.1528-1167.2007.01117.x

Jafari, Z., Mehla, J., Kolb, B. E., & Mohajerani, M. H. (2019). Gestational stress augments postpartum β-amyloid pathology and cognitive decline in a mouse model of Alzheimer's disease. *Cerebral Cortex, 29*(9), 3712-3724.

Jansen, F. E., Van Huffelen, A. C., Bourez‐Swart, M., & Van Nieuwenhuizen, O. (2005). Consistent localization of interictal epileptiform activity on EEGs of patients with tuberous sclerosis complex. *Epilepsia (Copenhagen), 46*(3), 415-419. https://doi.org/10.1111/j.0013-9580.2005.31704.x

Kadam, S. D., White, A. M., Staley, K. J., & Dudek, F. E. (2010). Continuous electroencephalographic monitoring with radio-telemetry in a rat model of perinatal hypoxia–ischemia reveals progressive post-stroke epilepsy. *Journal of Neuroscience, 30*(1), 404-415.

Kalilani, L., Sun, X., Pelgrims, B., Noack‐Rink, M., & Villanueva, V. (2018). The epidemiology of drug‐resistant epilepsy: a systematic review and meta‐analysis. *Epilepsia, 59*(12), 2179-2193.

Koike-Kumagai, M., Fujimoto, M., & Wataya-Kaneda, M. (2022). Sirolimus relieves seizures and neuropsychiatric symptoms via changes of microglial polarity in tuberous sclerosis complex model mice. *Neuropharmacology, 218*, 109203-109203. https://doi.org/10.1016/j.neuropharm.2022.109203

Krueger, D. A., Northrup, H., Roberds, S., Smith, K., Sampson, J., Korf, B., ... & Frost, M. D. (2013). Tuberous sclerosis complex surveillance and management: recommendations of the 2012 International Tuberous *Sclerosis Complex Consensus Conference. Pediatric neurology, 49*(4), 255-265.

Kuemmerle, T., Perzanowski, K., Chaskovskyy, O., Ostapowicz, K., Halada, L., Bashta, A., Kruhlov, I., Hostert, P., Waller, D. M., & Radeloff, V. C. (2010). European bison habitat in the carpathian mountains. *Biological Conservation, 143*(4), 908-916. https://doi.org/10.1016/j.biocon.2009.12.038

Lam, C., Bouffet, E., Tabori, U., Mabbott, D., Taylor, M., & Bartels, U. (2010). Rapamycin (sirolimus) in tuberous sclerosis associated pediatric central nervous system tumors. *Pediatric Blood & Cancer, 54*(3), 476-479. https://doi.org/10.1002/pbc.22298

Lautner, P. (2012). Aristotle and plotinus on memory. *The Classical World, 105*(4), 569-570. https://login.uleth.idm.oclc.org/login?url=https://www.proquest.com/scholarly-journals/aristotle-plotinus-on-memory/docview/1321936375/se-2

Leroi-Gourhan, A. (1982). The archaeology of lascaux cave: Forty years' work has revealed much about how the great paleolithic paintings of lascaux cave were created. it has also focused attention on hundreds of engravings that rival the paintings in their import. *Scientific American, 246*(6), 104. https://doi.org/10.1038/scientificamerican0682-104

Leonard, B. J., McNaughton, B. L., & Barnes, C. A. (1987). Suppression of hippocampal synaptic plasticity during slow-wave sleep. *Brain research, 425*(1), 174-177.

Li, J., Kim, S. G., & Blenis, J. (2014). Rapamycin: one drug, many effects. *Cell metabolism, 19*(3), 373-379.

Mac Callum, P. E., Hebert, M., Adamec, R. E., & Blundell, J. (2014). Systemic inhibition of mTOR kinase via rapamycin disrupts consolidation and reconsolidation of auditory fear memory. *Neurobiology of learning and memory, 112*, 176-185.

Mao, R., Tian, M., Yang, Y., Zhou, Q., Xu, L., & Cao, J. (2009). Effects of pentylenetetrazol-induced brief convulsive seizures on spatial memory and fear memory. *Epilepsy & Behavior, 15*(4), 441-444. https://doi.org/10.1016/j.yebeh.2009.05.015

Meng, X., Yu, J., Song, J., Chi, S., & Tan, L. (2013). Role of the mTOR signaling pathway in epilepsy. *Journal of the Neurological Sciences, 332*(1), 4-15. https://doi.org/10.1016/j.jns.2013.05.029

McIntyre, D. C. , McLeod, W. S. & Anisman, H. (2004). Working and Reference Memory in Seizure-Prone and Seizure-Resistant Rats. *Behavioral Neuroscience, 118*(2), 314-323.

Milner, B., Squire, L. R., & Kandel, E. R. (1998). Cognitive neuroscience and the study of memory. *Neuron, 20*(3), 445-468.

Morris, R. G. (1981). Spatial localization does not require the presence of local cues. *Learning and motivation, 12*(2), 239-260.

Muldoon, S. F., Villette, V., Tressard, T., Malvache, A., Reichinnek, S., Bartolomei, F., & Cossart, R. (2015). GABAergic inhibition shapes interictal dynamics in awake epileptic mice. *Brain (London, England : 1878), 138*(Pt 10), 2875-2890. https://doi.org/10.1093/brain/awv227

Neumann, A. R., Raedt, R., Steenland, H. W., Sprengers, M., Bzymek, K., Navratilova, Z., Mesina, L., Xie, J., Lapointe, V., Kloosterman, F., Vonck, K., Boon, Paul A. J. M., Soltesz, I., McNaughton, B. L., & Luczak, A. (2017). Involvement of fast-spiking cells in ictal sequences during spontaneous seizures in rats with chronic temporal lobe epilepsy. *Brain (London, England : 1878), 140*(9), 2355-2369. https://doi.org/10.1093/brain/awx179

O'Keefe, J., & Dostrovsky, J. (1971). The hippocampus as a spatial map: preliminary evidence from unit activity in the freely-moving rat. *Brain research.*

Okuda, K., Højgaard, K., Privitera, L., Bayraktar, G., & Takeuchi, T. (2021). Initial memory consolidation and the synaptic tagging and capture hypothesis. *The European Journal of Neuroscience, 54*(8), 6826-6849. https://doi.org/10.1111/ejn.14902

Pavlides, C., & Winson, J. (1989). Influences of hippocampal place cell firing in the awake state on the activity of these cells during subsequent sleep episodes. *Journal of neuroscience, 9*(8), 2907-2918.

Parsons, R. G., Gafford, G. M., & Helmstetter, F. J. (2006). Translational control via the mammalian target of rapamycin pathway is critical for the formation and stability

of long-term fear memory in amygdala neurons. *Journal of Neuroscience, 26*(50), 12977-12983.https://doi.org/10.1523/JNEUROSCI.4209-06.2006

Pastalkova, E., Serrano, P., Pinkhasova, D., Wallace, E., Fenton, A. A., & Sacktor, T. C. (2006). Storage of spatial information by the maintenance mechanism of LTP. *Science (American Association for the Advancement of Science), 313*(5790), 1141-1144. https://doi.org/10.1126/science.1128657

Penfield, W., & Jasper, H. H. (1954). Epilepsy and the functional anatomy of the human brain (1st ed.). *Little, Brown.*

Pitkänen, A., & Lukasiuk, K. (2011). Mechanisms of epileptogenesis and potential treatment targets. *The Lancet Neurology, 10*(2), 173-86. Retrieved from https://login.uleth.idm.oclc.org/login?url=https://www.proquest.com/scholarly-journals/mechanisms-epileptogenesis-potential-treatment/docview/856800667/se-2

Pitkänen, A., Lukasiuk, K., Dudek, F. E., & Staley, K. J. (2015). Epileptogenesis. *Cold Spring Harbor perspectives in medicine, 5*(10), a022822. https://doi.org/10.1101/cshperspect.a022822

Prince, D. A., & Wilder, B. J. (1967). Control mechanisms in cortical epileptogenic foci*: surround inhibition. *Archives of neurology, 16*(2), 194-202.

Racine, R. J. (1972). Modification of seizure activity by electrical stimulation: II. Motor seizure. *Electroencephalography and clinical neurophysiology, 32*(3), 281-294.

Ranft, A., Kurz, J., Deuringer, M., Haseneder, R., Dodt, H., Zieglgänsberger, W., Kochs, E., Eder, M., & Hapfelmeier, G. (2004). Isoflurane modulates glutamatergic and GABAergic neurotransmission in the amygdala. *The European Journal of Neuroscience, 20*(5), 1276-1280. https://doi.org/10.1111/j.1460-9568.2004.03603.x

Saffari, A., Brösse, I., Wiemer-Kruel, A., Wilken, B., Kreuzaler, P., Hahn, A., Bernhard, M. K., van Tilburg, C. M., Hoffmann, G. F., Gorenflo, M., Hethey, S., Kaiser, O., Kölker, S., Wagner, R., Witt, O., Merkenschlager, A., Möckel, A., Roser, T., Schlump, J., . . . Syrbe, S. (2019). Safety and efficacy of mTOR inhibitor treatment in patients with tuberous sclerosis complex under 2 years of age - a multicenter retrospective study. *Orphanet Journal of Rare Diseases, 14*(1), 96-96. https://doi.org/10.1186/s13023-019-1077-6

Sajikumar, S., Navakkode, S., Sacktor, T. C., & Frey, J. U. (2005). Synaptic tagging and cross-tagging: The role of protein kinase M{zeta} in maintaining long-term potentiation but not long-term depression. The Journal of Neuroscience, 25(24), 5750-5756. https://doi.org/10.1523/JNEUROSCI.1104-05.2005

Schevon, C. A., Weiss, S. A., McKhann, J., Guy, Goodman, R. R., Yuste, R., Emerson, R. G., & Trevelyan, A. J. (2012). Evidence of an inhibitory restraint of seizure activity in humans. *Nature Communications, 3*(1), 1060-1060. https://doi.org/10.1038/ncomms2056

Scoville, W. B., & Milner, B. (1957). Loss of recent memory after bilateral hippocampal lesions. *Journal of neurology, neurosurgery, and psychiatry, 20*(1), 11.

Silva, A. J., Ehninger, D., Han, S., Shilyansky, C., Zhou, Y., Li, W., Kwiatkowski, D. J., & Ramesh, V. (2008). Reversal of learning deficits in a Tsc2 +/− mouse model of tuberous sclerosis. *Nature Medicine, 14*(8), 843-848. https://doi.org/10.1038/nm1788

Sloviter, R. S. (1987). Decreased hippocampal inhibition and a selective loss of interneurons in experimental epilepsy. *Science (American Association for the Advancement of Science), 235*(4784), 73-76. https://doi.org/10.1126/science.2879352

Stover, K. R., Lim, S., Zhou, T. L., Stafford, P. M., Chow, J., Li, H., Sivanenthiran, N., Mylvaganam, S., Wu, C., Weaver, D. F., Eubanks, J., & Zhang, L. (2017). Susceptibility to hippocampal kindling seizures is increased in aging C57 black mice. *IBRO reports, 3*, 33–44. https://doi.org/10.1016/j.ibror.2017.08.001

Squire, L. R., & MyiLibrary. (1987). Memory and brain (1st ed.). *Oxford University Press.*

Teskey, G. (2020, August 27). Kindling. *Oxford Research Encyclopedia of Psychology.* Retrieved 15 Sep. 2024, from https://oxfordre.com/psychology/view/10.1093/acrefore/9780190236557.001.0001/acrefore-9780190236557-e-790.

Thiele, E. A. (2004). Managing epilepsy in tuberous sclerosis complex. *Journal of Child Neurology, 19*(9), 680-686. https://doi.org/10.1177/08830738040190090801

Thijs, R. D., Surges, R., O'Brien, T.,J., & Sander, J. W. (2019). Epilepsy in adults. *The Lancet, 393*(10172), 689-701. doi:https://doi.org/10.1016/S0140-6736(18)32596-0

Tsokas, P., Hsieh, C., Yao, Y., Lesburguères, E., Wallace, E. J. C., Tcherepanov, A., Jothianandan, D., Hartley, B. R., Pan, L., Rivard, B., Farese, R. V., Sajan, M. P., Bergold, P. J., Hernández, A. I., Cottrell, J. E., Shouval, H. Z., Fenton, A. A., & Sacktor, T. C. (2016). Compensation for PKMζ in long-term potentiation and spatial long-term memory in mutant mice. *Elife, 5* https://doi.org/10.7554/eLife.14846

Truccolo, W., Donoghue, J., Hochberg, L. et al. Single-neuron dynamics in human focal epilepsy. *Nat Neurosci 14,* 635–641 (2011). https://doi-org.uleth.idm.oclc.org/10.1038/nn.2782

Uhlmann, E. J., Wong, M., Baldwin, R. L., Bajenaru, M. L., Onda, H., Kwiatkowski, D. J., ... & Gutmann, D. H. (2002). Astrocyte‐specific TSC1 conditional knockout mice exhibit abnormal neuronal organization and seizures. *Annals of neurology, 52*(3), 285-296.

Volk, L. J., Bachman, J. L., Johnson, R., Yu, Y., & Huganir, R. L. (2013). PKM-ζ is not required for hippocampal synaptic plasticity, learning and memory. *Nature (London), 493*(7432), 420-423. https://doi.org/10.1038/nature11802

Wang, Y., Greenwood, J. S., Calcagnotto, M. E., Kirsch, H. E., Barbaro, N. M., & Baraban, S. C. (2007). Neocortical hyperexcitability in a human case of tuberous sclerosis complex and mice lacking neuronal expression of TSC1. *Annals of Neurology: Official Journal of the American Neurological Association and the Child Neurology Society, 61*(2), 139-152.

Wilson, M. A., & McNaughton, B. L. (1993). Dynamics of the hippocampal ensemble code for space. *Science, 261*(5124), 1055-1058.

Wilson, M. A., & McNaughton, B. L. (1994). Reactivation of hippocampal ensemble memories during sleep. *Science, 265*(5172), 676-679.

Wu, J. Y., Salamon, N., Kirsch, H. E., Mantle, M. M., Nagarajan, S. S., Kurelowech, L., Aung, M. H., Sankar, R., Shields, W. D., & Mathern, G. W. (2010). Noninvasive testing, early surgery, and seizure freedom in tuberous sclerosis complex. *Neurology, 74*(5), 392. https://doi.org/10.1212/WNL.0b013e3181ce5d9e

Zambrotta, M. E. & Houlé, J. D. (2015). Chapter 29 - MicroRNA Regulation of mTOR Function. In C. K. Sen (Ed.) *MicroRNA in regenerative medicine* (pp. 757-790) Academic Press. https://login.uleth.idm.oclc.org/login?url=https://www.proquest.com/other-sources/microrna-regenerative-medicine/docview/1680244952/se-2?accountid=12063

Zeng, L., Rensing, N. R., & Wong, M. (2009). The mammalian target of rapamycin signaling pathway mediates epileptogenesis in a model of temporal lobe epilepsy. *The Journal of Neuroscience, 29*(21), 6964-6972. https://doi.org/10.1523/JNEUROSCI.0066-09.2009

Zhang, B., McDaniel, S. S., Rensing, N. R., & Wong, M. (2013). Vigabatrin inhibits seizures and mTOR pathway activation in a mouse model of tuberous sclerosis complex. *PloS one, 8*(2), e57445. https://doi.org/10.1371/journal.pone.0057445

## Appendix A Histology

Following the MWT task, all animals were euthanized, perfused and had their brains extracted for histology. Before euthanization, all animals with amydalar implants were electrolytically lesioned to allow for the confirmation of electrode implantation in the amygdala. The animals were euthanized with 0.05mL of euthansol (i.p.) and immediately perfused with 1x phosphate buffered saline (PBS) followed by 4 % paraformaldehyde (PFA) to fixate the samples before extraction. Their brains were collected and suspended in tubes of PFA for at least 24 h in the fridge before being transferred into tubes of 30 % sucrose + sodium azide and placed back into the fridge.

The brains had to be kept frozen with dry ice while they were coronally sectioned into 40 um slices with the blockface apparatus; images of each slice were taken with the mounted camera. Slices were stored in containers with 4 x 6 wells filled with 1x PBS + 0.02 % sodium azide in the fridge until they were to be mounted for staining. Each container stored a maximum of 72 slices.

Slices were mounted onto Superfrost Plus microscope slides and dried overnight in the oven before they were stained. Dried slides were placed in a rack and bathed in several solutions for a specified amount of time. See figure 2.X.X for the staining protocol. Images of the stained slides were then taken with a Nanozoomer microscope (Nanozoomer 2.0-RS, HAMAMATSU, JAPAN) at 40x resolution.

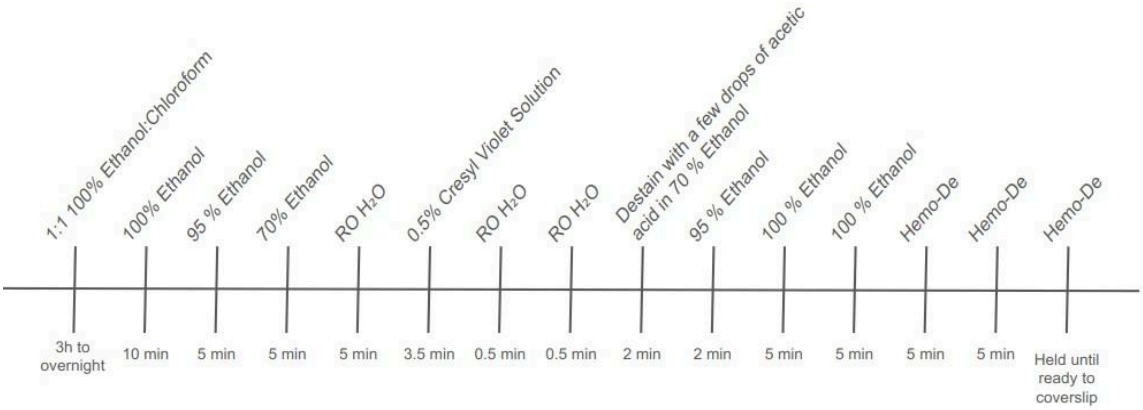


**Figure A.1** Cresyl violet staining protocol.

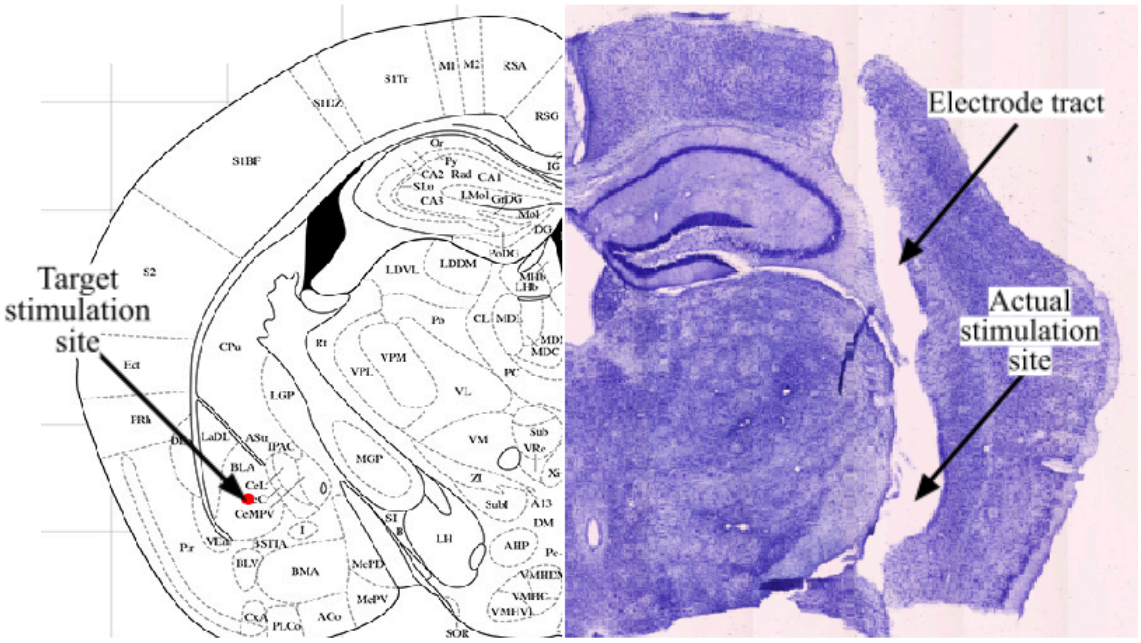


**Figure A.2** Stained coronal section with electrode tract, and actual and target stimulation sites.

## Appendix B Power Spectrum Density

Prior to the isoflurane experiment, a pilot experiment was conducted with an animal kindled to stage five that was used for testing and troubleshooting the apparatus. The animal was anaesthetised with isoflurane in oxygen at concentrations of 1.0 %, 2.0 %, and 2.5 % (figures 3.4 to 3.7). An additional recording under 2.5 % of isoflurane was performed in which the animal was electrically stimulated (figure 3.8).

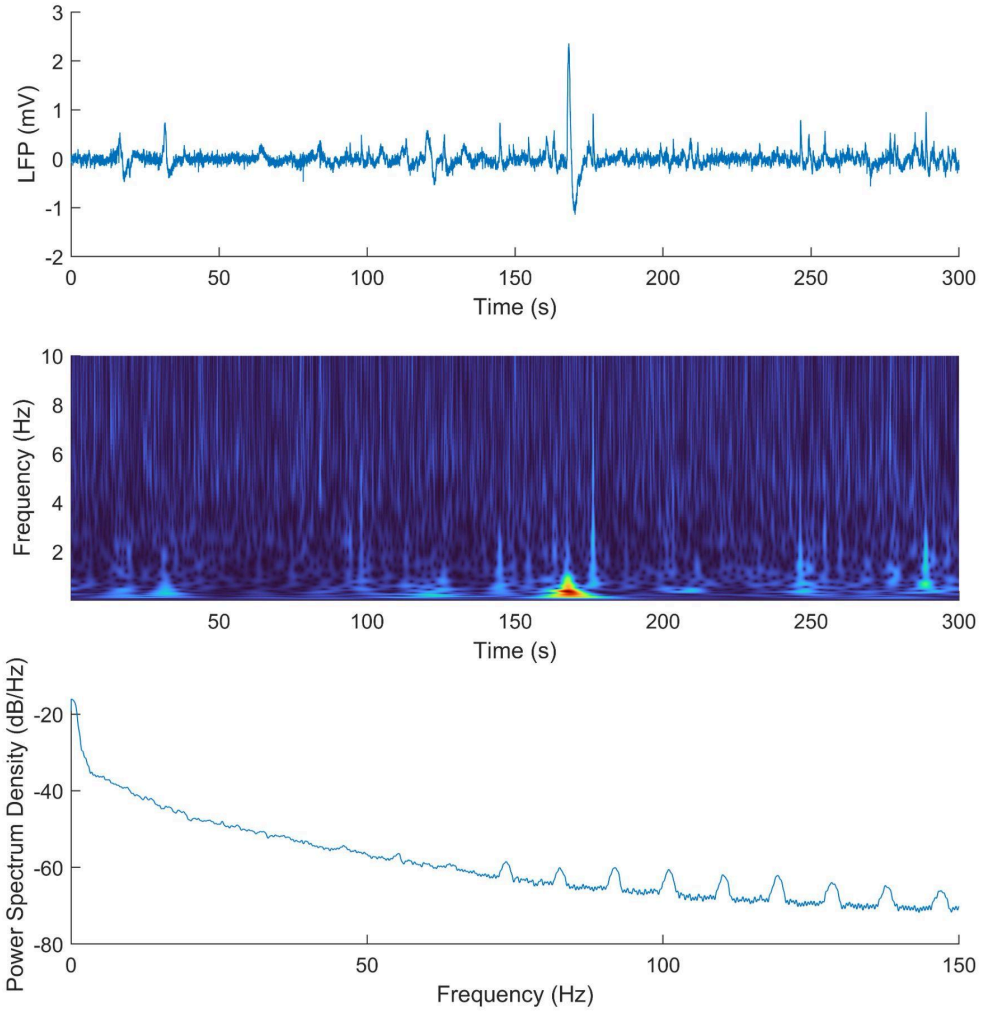


**Figure B.1** Five minute recording of amygdala LFP under 1.0 % of isoflurane in oxygen.

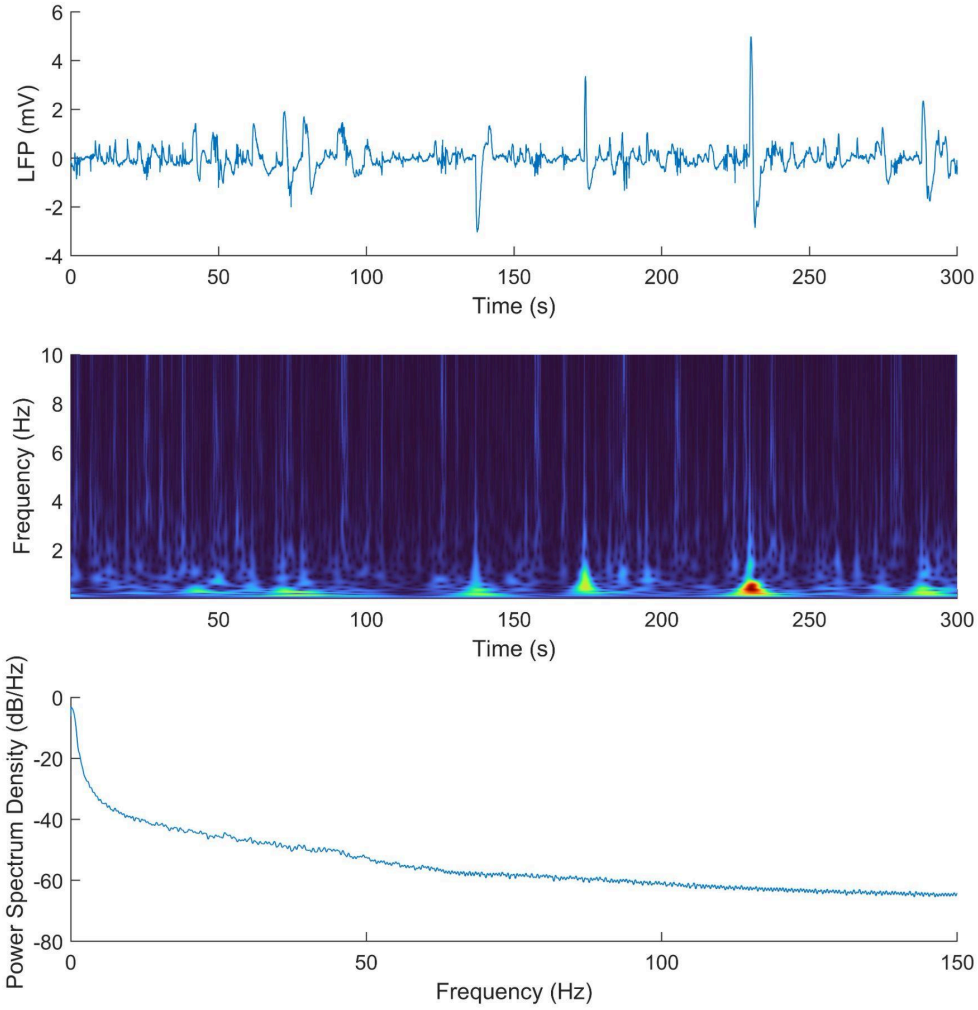


**Figure B.2** Five minute recording of amygdala LFP under 1.5 % of isoflurane in oxygen.

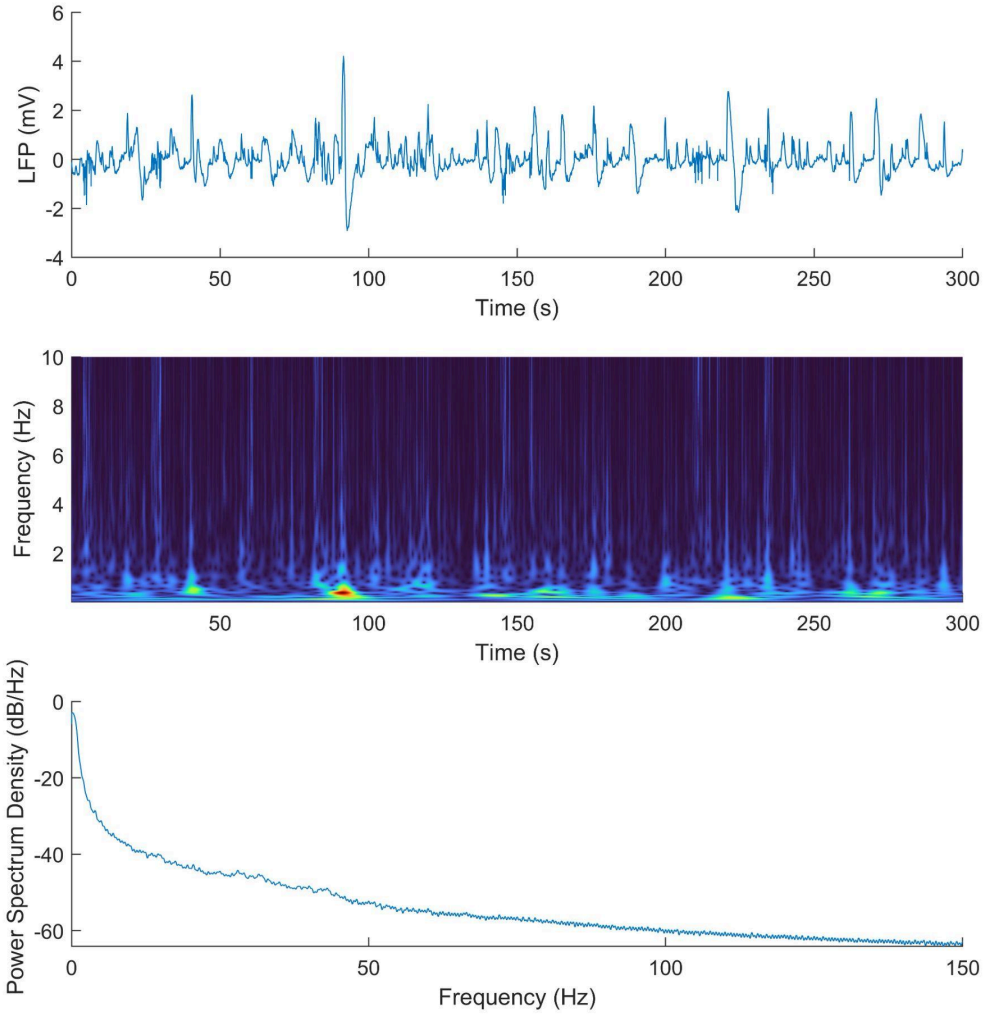


**Figure B.3** Five minute recording of amygdala LFP under 2.0 % of isoflurane in oxygen.

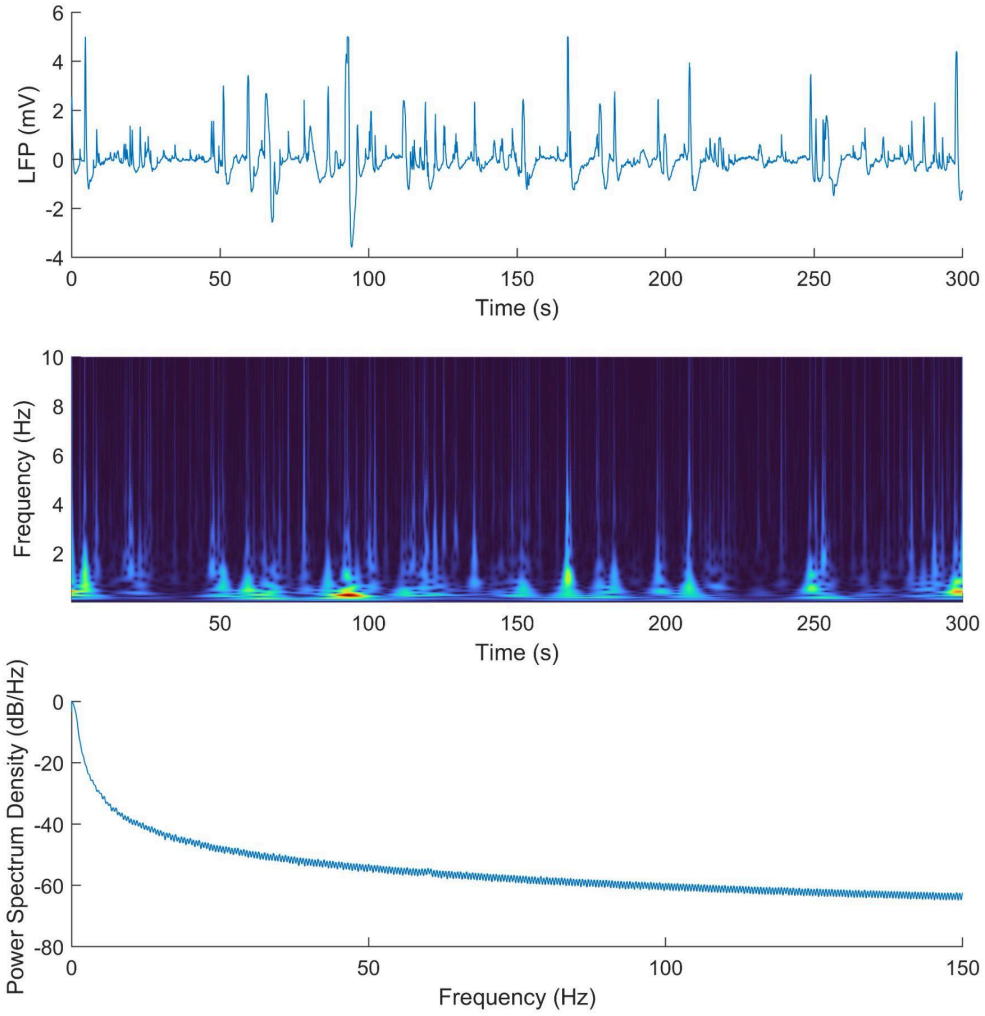


**Figure B.4** Five minute recording of amygdala LFP under 2.5 % of isoflurane in oxygen.

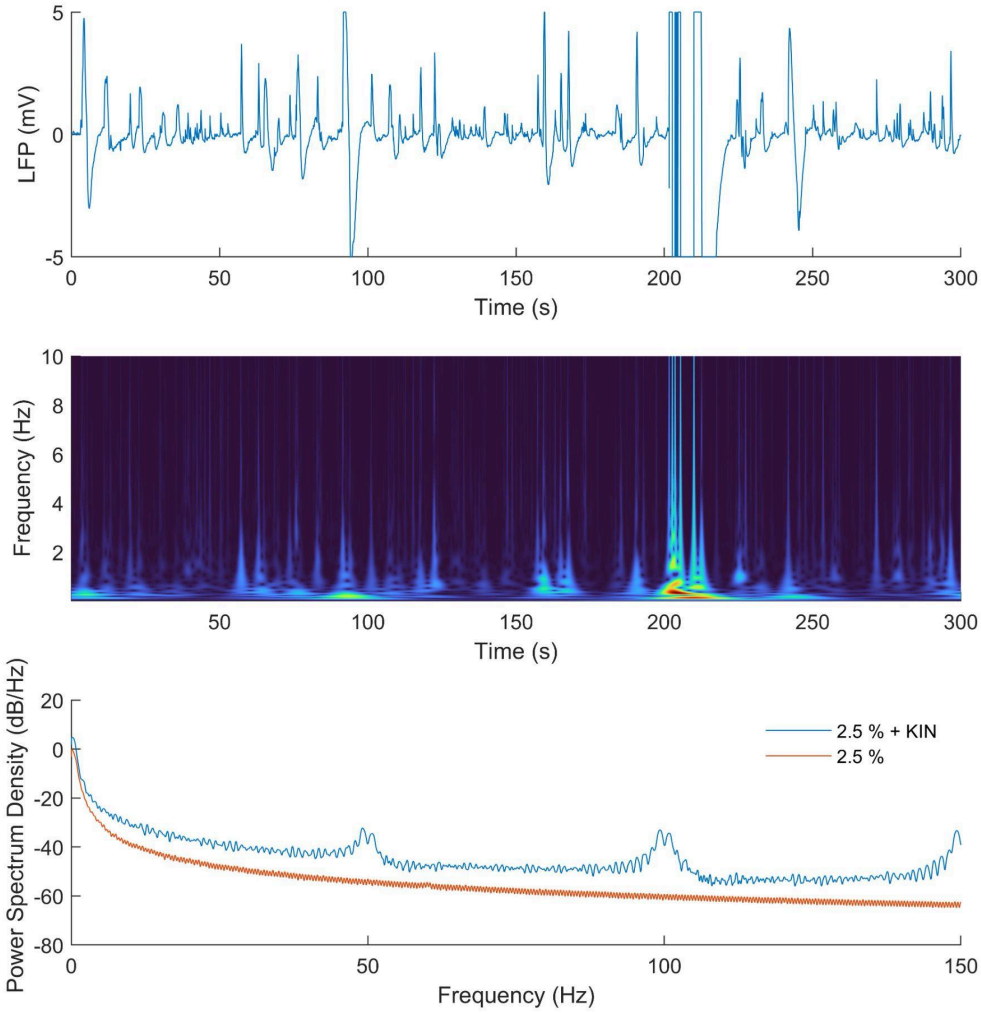


**Figure B.5** Five minute recording of amygdala LFP under 2.5 % of isoflurane in oxygen with electrical stimulation.

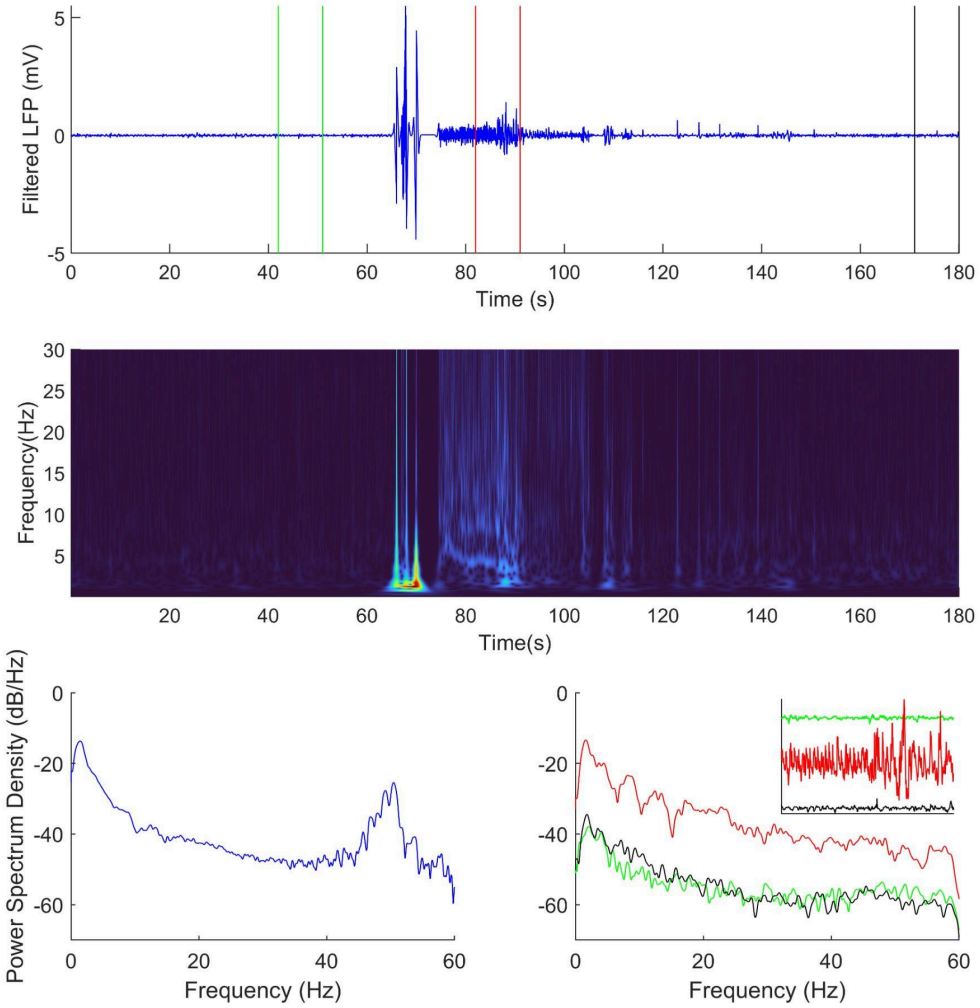


**Figure B.6** LFP of a kindling session with frequency spectrogram and power spectrum density of 10s of samples before, during and after a seizure.