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Development of an Intrahippocampal Kindling Model of Epilepsy

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DEVELOPMENT OF AN INTRAHIPPOCAMPAL KINDLING MODEL OF EPILEPSY

BY

Carter F. Jones

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Development of an Intrahippocampal Kindling Model of Epilepsy

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ABSTRACT

Epilepsy is a neurological condition that affects about 1% of the global population. This debilitating condition is associated with overexcitation and ineffective inhibition of neuronal pathways in the brain causing serious and a diverse set of symptoms, most prominently seizures. While some antiepileptic drug (AED) regimes have been proven to be effective in treating this condition, there are many cases where the drugs do not do enough. The ketogenic diet (KD) has been used for decades as an effective anticonvulsant. Its powerful and natural processes result in some patients becoming seizure-free. Sometimes, these patients remain free of seizures even after returning to a normal diet. Kindling is a modern technique of training the brain to be more susceptible to synchronicity and therefore, seizures over time through the electrical or chemical stimulation of specific brain areas. The objective of this project is to assess whether a KD can prolong the development of major seizures in an electrically kindled rat model. Male Sprague-Dawley rats between 280 and 300 grams were used in this study. A stereotaxic surgery was performed to implant electrodes into the hippocampus and cortical regions. These brain regions were electrically stimulated daily according to an electrophysiological timeline until ten class five seizures were obtained. Although only one animal was successfully taken through the procedure, the results indicate a proof of concept and provide a foundation towards the integration of more animals in the future of the study.
INTRODUCTION

Epilepsy is a disruptive condition that affects millions of people worldwide. More than 50 million people worldwide are considered epileptic, accounting for a significant portion of the worldwide disease burden. Globally, it is estimated that up to 2.4 million people are diagnosed with epilepsy each year ("Epilepsy," 2019). As defined in 2005, epilepsy, in the most general sense, is a disorder in which the brain is more likely to spontaneously experience a seizure. These seizures are recurrent and unpredictable and lead to the interruption of normal brain activity and function (Fisher et al., 2005). This is not an all-encompassing definition however, as epilepsy is usually also accompanied by neurobiological, cognitive, psychological, and/or social changes and deficits (Fisher et al., 2005). It is also important to point out that not all seizures are indicative of an epileptic diagnosis. Seizures can occur in healthy and normal brains, and the diagnosis of epilepsy would be withheld. It is when a measurable alteration in the brain like the increased probability of subsequent seizures that a patient can be diagnosed with epilepsy (Fisher et al., 2005). While the seizure activity experienced by patients with epilepsy does not lead to death, it has been found that people with epilepsy have up to three times higher chance of dying prematurely (Forsgren et al., 2005). It is therefore important to understand the condition and develop treatments and remedies to assist those who suffer from its symptoms.

Seizures or epileptic activity are episodes unusually synchronous neuronal activity and excessive excitation with or without insufficient inhibition of both small and large brain areas (Shorvon, 2010). These episodes are often spontaneous but brief. These episodes of abnormal neuronal activity that define a seizure, however, can result in a myriad of responses including something as small as the loss of awareness for a few seconds to violent, full body convulsions. There are many other physiological, or psychic processes changed or interrupted as a result of
epileptic seizures including motor control, autonomic and sensory functions, and consciousness. Furthermore, epileptic activity is not specific to any part of the brain and can affect different numbers of brain areas between patients (Shorvon, 2010). This property accounts for the diversity of symptoms seen across patients. Abnormal activity in smaller neuronal populations is known as a partial seizure while activity that stretches across most of the brain creates a generalized seizure.

In the most severe cases of epilepsy, these episodes of partial or generalized seizures occur multiple times in a single day, causing serious interruptions to mental and physical life (Shorvon, 2010).

The recognition of epilepsy as a serious abnormal neurological condition has afforded it extensive research to search for effective treatments to reduce or neutralize the behavioral and neurological symptoms.

The highly varied forms of epilepsy result in complex and diverse treatment methods. This results in a high societal and medicinal cost to find the best remedy on a case-to-case basis. Indeed, it was found that brain disorders like epilepsy account for around one-third of all medical costs in Europe alone (Olesen, Gustavsson, Svensson, Wittchen, & Jönsson, 2012). Much of the current treatment plans for this condition center around the use of antiepileptic drugs (AED). These drugs work by modifying neuronal processes and activity to favor inhibition over excitation. While they all work to achieve the same goal of preventing epileptic activity in excitable brain areas, the mechanisms of inhibition vary from one AED to the next. AEDs work by targeting specific neurochemicals, up-regulating or down-regulating them, or by blocking certain receptors in the brain. In general, there are four major targets of AEDs: sodium channels, calcium channels, gamma-aminobutyric acid (GABA), and glutamate (Deckers et al., 2000). Drugs that work on sodium channels often increase the refractory period of neurons by delaying the recovery time after an action potential, effectively decreasing the potential for the sustained and repetitive firing
of neurons during seizures (Deckers et al., 2000). T type calcium channels are another target for AEDs. These channels are blocked by these drugs, preventing calcium from entering the cells and therefore preventing action potential propagation (Deckers et al., 2000). GABA is one of the primary inhibitory neurotransmitters in the brain. By increasing GABA neurotransmission, some AEDs are able to reduce epileptic activity. These mechanisms are determined by the region they act on; however, as they can have the opposite effect of increasing instances of some absence type seizures (Deckers et al., 2000). Glutamate receptors, particularly AMPA and NMDA types, are the last of the major targets of AEDs. These drugs act as antagonists for these glutamate receptors to reduce the chance of activation (Deckers et al., 2000). Finally, it is important to note that most AEDs that are prescribed to patients have multiple mechanisms of actions (Deckers et al., 2000).

Unfortunately, AEDs do not have a 100% effectiveness. Between 30% and 40% of all cases of epilepsy experience no or no significant reduction in epileptic episodes when treated with medication. These patients are diagnosed with refractory epilepsy. This term is given to those who continue to experience severe seizures that disrupt everyday life after the use of at least two or more AEDs either separate or together (Engel, 2014). In these cases, it is necessary to explore other treatment options to stop the epileptic episodes and give the patient a better quality of life. One such alternative method that has been proven effective at treating refractory epilepsy is the ketogenic diet (KD).

*The Ketogenic Diet*

The KD is a low carbohydrate, adequate protein, and high-fat diet. It has been recognized for years as an effective alternative treatment for epilepsy. One of the proposed anti-epileptic mechanisms hypothesizes that by restricting a person’s diet to mostly fat-based, the body is forced
into a fasting-like state due to the low levels of carbohydrates. Researchers in the early 20th century found this state to be effective at reducing epileptic activity. Intermittent starvation was reported to be up to 90% effective at treating epileptic patients back in 1921 by Dr. H.W. Conklin. This idea was later built upon by doctors Wilder and Winter who proposed that a diet higher in fat and lower in carbohydrates would result in similar effects to starvation diets (Bailey, Pfeifer, & Thiele, 2005). Even though the use of the KD to treat epilepsy was known for some time, the invention of AEDs combined with the level of difficulty associated with the maintenance of the KD lead to its reduction in popularity with patients. However, in the case of refractory epilepsy, the KD has been shown to be highly effective (Elamin, Ruskin, Masino, & Sacchetti, 2017).

The body is remarkably adept at switching the metabolomic processes in the body to favor lipids instead of glucose without the side effects that society associates with an increase of fat in a diet. As stated previously, the KD induces a fasting-like state in the body. Under the lack of carbohydrates, the body must find another way to manufacture the adenosine triphosphate (ATP) necessary to function. This process to maintain energy homeostasis is started in the liver where fatty acids and amino acids are converted to ketone bodies such as β-hydroxybutyrate, acetoacetate, and acetone (S.A Masino, Kawamura, Wasser, Pomeroy, & Ruskin, 2009). Organs like the brain have difficulty in utilizing these fatty acids and amino acids for fuel in the production of ATP as well as converting these molecules to ketone bodies (S.A Masino et al., 2009). The liver, therefore, has a crucial role in the production of energy in a body undergoing a Ketogenic Diet. These ketone bodies are then transported around the body, where they are oxidized into acetyl-CoA. At this point, the acetyl-CoA is allowed to enter the Krebs cycle, and the normal function of cellular respiration occurs. Overall these ketone bodies are able to replace glucose as the primary fuel source for cellular respiration in the body. Interestingly, cellular respiration driven
by ketone bodies is more efficient than the alternative, leading to the production of more ATP after
the process is complete (S.A Masino et al., 2009).

There are several benefits for using the KD over AEDs. Unlike AEDs, the KD is relatively
free of side effects. Patients who take AEDs sometimes have trouble with dull or decreased
alertness, memory loss, and other cognitive defects (Yudkoff et al., 2007). These side effects are
absent when patients are put onto the KD. Another major benefit to the KD is the lack of
physiological changes like blood pressure, weight, serum insulin, and blood glucose and lipids
(Yudkoff et al., 2007). The completely non-invasive nature of the diet and the ability to easily
reverse its effects by returning to a more conventional diet are two more powerful beneficial
factors. With a non-invasive treatment, there is no risk for infection or damage that might be an
issue with procedures like surgeries. Furthermore, since the treatment is just a change in a diet, if
there are any side effects found to be too detrimental to the patient, the diet can be simply reverted
to a traditional carbohydrate-rich diet, and the body will switch back to using glucose as the
primary fuel source. Comparatively, stopping an AED regime can lead to a potentially deadly
condition call status epilepticus in which seizure activity persists for more than 30 minutes
(Cherian & Thomas, 2009). Interestingly, there have been documented cases where the KD was
able to “cure” epileptic patients in that after the diet had been ceased, the patients continued to be
seizure free (Elamin et al., 2017). With all of these significant benefits over AEDs, it is curious
as to why the KD is not a more universally used therapy.

Although the KD has many benefits for the treatment of epilepsy, there are some challenges
as well. Primarily, the diet is difficult to maintain and not particularly palatable. Some long-term
studies of the diet show that patients have a hard time sticking to its constraints and often drop the
diet all together before the prescribed length of the treatment (Henderson, Filloux, Alder, Lyon,&
Furthermore, the diet is not 100% effective in all cases, and it is not possible to predict its successfulness (S.A Masino et al., 2009). The shortcomings in success could be related to the specific mechanisms that define the activity of the KD. Understanding these mechanisms in the body and central nervous system (CNS) is an important part of any research study involving the KD.

By lowering the intake of carbohydrates, the body can switch the primary fuel type. While this part of the mechanism is well documented by neuroscientists and biochemists alike, the exact way that the KD becomes an effective treatment for epilepsy is still unknown to researchers. The reduced excitability of the neurons could be caused by the increase in ketone bodies and/or the decrease in glucose or a result of another mechanism entirely (Ruskin & Masino, 2012). There are multiple hypotheses as to the mechanism that explains the KD’s profound antiepileptic properties. There is likely a complex relationship between different networks of biochemical and physiological systems, resulting in a combination of more than one mechanism that would provide the answers to the neuroprotective effects of the diet. While it is likely that the explanation of how the KD works so well is not due to just one proposed hypothesis, it is the adenosine hypothesis that is the focus of this study.

**Adenosine Hypothesis**

The adenosine hypothesis focuses on the inhibitory effects of the neurotransmitter. Studies have found that increased activation of the adenosine A₁ receptors inhibits neuronal excitability. Since overexcitability is a primary cause of epileptic activity, adenosine has been identified as a strong, endogenous, seizure reducing neuroprotective molecule (S. A. Masino, Kawamura, Ruskin, Geiger, & Boison, 2012). Mechanistically, adenosine can work presynaptically by closing
calcium channels, reducing neurotransmitter release (Ribeiro, 1979), or postsynaptically by opening potassium channels which hyperpolarizes the neurons, preventing the creation of action potentials (Haas & Greene, 1984). From these studies, it would seem that the upregulation of adenosine would be an effective treatment to reduce hyper-synchronicity of neurons and susceptibility to seizures.

A study performed by Masino et al. (2011) found that in an *in vivo* model, the KD acts on the adenosine levels in the brain. Specifically, the KD results in a greater production of adenosine in the brain. These elevated levels of adenosine, in turn, increase the activation of A<sub>1</sub> receptors. This resulted in a significant seizure reduction in the animals on the KD. Furthermore, adenosine A<sub>1</sub> knockout mice saw no beneficial effects from the KD on seizure reduction, indicating that the A<sub>1</sub> receptors play an important role in the suppression of seizures (Susan A. Masino et al., 2011). The mechanism of the interaction between the KD and adenosine was theorized in a later publication claiming that when the levels of intracellular ATP in pyramidal cells in the CA3 region of the hippocampus reach a certain point due to the increased production under ketone body-fueled cellular respiration, pannexin-1 channels open and release intracellular ATP. These, now extracellular, ATP molecules are quickly transformed to adenosine by nucleotidases. The elevated levels of extracellular adenosine activate adenosine A<sub>1</sub> receptors which, in turn, open ATP-dependent potassium channels. Finally, by opening these potassium channels, the neurons become hyperpolarize, reducing the chance of firing (Kawamura, Ruskin, & Masino, 2016).

Relating to adenosine expression, the study in 2011 also found that KD reduced adenosine kinase (ADK), the enzyme that breaks down adenosine. This results in further increases in extracellular levels of adenosine as the molecule is broken down less often. The lower levels of ADK and resulting higher density of extracellular adenosine are associated with a reduction of
epileptic seizures (Susan A. Masino et al., 2011). These results suggest that there is a close relationship between the KD and adenosine receptors that could explain the neuroprotective effects of the KD.

*Epigenetics Related to Adenosine*

In recent years the field of epigenetics has begun to increase in popularity. Simply put, epigenetics studies examine changes in gene expression that does not involve changes to the underlying DNA sequence. The two major types of changes identified at this point include DNA methylation, the “on” switch, and DNA acetylation, the “off” switch of epigenetics. While there are many possible avenues in which epigenetics could be applied, the work in epilepsy-related cases has given a new view on how the KD’s mechanism provides antiepileptic properties. Studies have shown an epigenetic foundation to epileptogenesis. These properties could help explain the permanent changes seen in epilepsy that lead to overexcitation of neurons affected (Williams-Karnesky et al., 2013). Finding the causes of these changes to neuronal DNA and identifying ways to modify them to the benefit of the patient could lead to an extremely effective treatment not only for those with refractory epilepsy but for all who live with the disorder. It is the interest of researchers in this field and this lab to identify and study pathways and neurochemicals whose genetic code can be manipulated to achieve this goal.

To induce DNA methylation, there are reactions that need to occur that are equilibrium-dependent. First, a methyl group must be donated from S-adenosylmethionine (SAM). This methyl group is, in turn, given to the DNA in question resulting in increased expression of that gene. The enzyme, DNA methyltransferase (DNMT), facilitates this reaction (Williams-Karnesky et al., 2013). In addition to the methylation of DNA, DNMT produces S-adenosylhomocysteine
(SAH), the demethylated SAM product. For DNMT to continue to react with SAM and methylate more DNA, SAH must be transformed through another reaction to keep the equilibrium favoring the products. Facilitated by the enzyme SAH hydrolase, this reaction transforms SAH to adenosine and homocysteine (HCY) (Williams-Karnesky et al., 2013). The SAH hydrolase reaction favors SAH; therefore it is necessary for the two products, adenosine, and HCY, to be cleared out or broken down to maintain an equilibrium that would favor the products (Williams-Karnesky et al., 2013).

Adenosine has already been identified as a strong candidate in seizure inhibition. It is no surprise then that it has been targeted in epigenetic research. Williams-Karnesky et. al. (2013) were able to show that the hippocampus had a large instance of DNA methylation in animal models of epilepsy. Using this, the research group was able to show that the addition of adenosine to the hippocampus was effective at reducing the amount of DNA methylation in the area. Additionally, the adenosine treatments were able to stop the progression to more severe cases of epilepsy under a kindling model. Even after the treatments were stopped, the neuroprotective effects were seen for at least three months (Williams-Karnesky et al., 2013). These data are promising foundations for the continued study into changes in DNA methylation homeostasis as a treatment for epilepsy.

Relating what has been found by Williams-Karnesky et. al. to what is known about the KD’s effect on adenosine and ADK, more can be added to the adenosine hypothesis. As stated before, the KD has been shown to increase extracellular levels of adenosine and reduce those of ADK. Based on the epigenetic capabilities of high levels of adenosine, there could be an epigenetic effect facilitated by the diet through the prevention of SAH breakdown and therefore DNA methylation. Due to this relationship, there is increased interest in determining the epigenetic
effects of the diet. Understanding this could give valuable insight to the neuroprotective effects of the KD or new targets for epigenetic therapies.

**Kindling**

In studying epileptic models, there are multiple ways for researchers to observe epileptogenesis and epileptic seizures. One very popular model over the past 30 years has been kindling. An effective tool that has been used for the discovery for AEDs, kindling involves repeated focal stimulation that leads to lasting change in brain function and to increased neuronal sensitivity to hyper-synchronicity (Bertram, 2007). The major advantages of the kindling model for epilepsy research are four-fold: a precise activation of desired brain area, a reliable timeline for epileptogenesis, easily monitored epileptic activity, and excellent control of when an animal experiences an epileptic episode (Kumar, Sharma, & Bhardwaj, 2016). Kindled seizures can and have been used successfully in a number of species, including rats and mice. A kindling protocol can induce these seizures either electrically or chemically. For the purpose of this study, an electrical kindling procedure was chosen because the lab was better equipped to initiate it.

The repetitive stimulations that are associated with kindling gradually reduce the threshold needed to induce a seizure or epileptic behaviors and train the neurons to fire synchronously, eventually culminating in tonic-clonic seizures after enough subsequent stimulations. The development of epileptogenesis is believed to be a consequence of excessive activation of the excitatory pathways in the brain. These mechanisms include glutamate transmission and a developed imbalance between neuronal excitation and inhibition. It has been documented that overactive glutamate receptors are a key factor in the initiation and propagation of epileptic activity.
after kindling (Szyndler et al., 2012). These properties lead to an accurate and controllable model of epilepsy that is optimal for electrophysiological studies.

**Current Study**

This study aims to combine all of the aforementioned information into an experiment designed to determine the neuroprotective and epigenetic effects of a ketogenic diet in an epileptic brain. This experiment will span multiple years, this being the beginning stage. Consequently, the work done in this study, thus far, has been to develop and set up an intrahippocampal electrical kindling model of epilepsy that will be used to conduct the experiment. It is the belief of this lab that the KD will show a neuroprotective effect against a kindling model of epilepsy. It is hypothesized that this will manifest in both a resistance to epileptogenesis under the kindling protocol as well as epigenetic differences in neuronal DNA as compared to a control diet group.

The two research questions will be assessed fully in future experiments. To achieve this, a rodent model will be used. Using a stereotaxic surgery, stimulating and recording electrodes will be into the hippocampus, an area commonly used in kindling studies, and the surface of the cortex. Rodents will be separated into two diet groups, control diet (CD) and KD. By stimulating the hippocampus multiple times in a day for subsequent days according to a predetermined timeline, tonic-clonic seizures will eventually be achieved. The passage of time to maximum behavioral response will be compared across diet type, and significance will be determined. This will provide an answer to the first research question on the protective effects against an electrical kindling model. Successful tests have been conducted in lab, and one rat on the KD has successfully made it through the protocol, indicating that a kindling model of epilepsy has been implemented.
The second phase of this project is to determine the epigenetic effects of the KD. While this has yet to occur in lab, it will involve extracting the hippocampus from designated rats at differing points during the kindling procedure, sending the tissue samples to a collaborative lab at the University of North Dakota for examination, and receiving the results for analysis. The DNA methylation will be compared across diet types and within diet types at varying times to assess for significant changes. It is the hope of this lab to be able to ultimately identify specific genes of interest that could help explain the KD’s incredible anti-epileptic properties.

METHODS

*Animals and Housing*

All experimental protocols were performed under the US Public Health Service’s Guide for the Care and Use of Laboratory Animals and were approved by the Trinity College Institutional Animal Care and Use Committee (IACUC). Sprague-Dawley breeders obtained from Charles River Laboratories (Wilmington, MA) were used to produce all experimental animals. Only males were used for this study in order to prevent the estrous cycle, present in female rats, from affecting the procedure and results, thereby reducing the acting variables on the study. Rats were socially housed in ventilated polycarbonate cages in a temperature (20-23 ºC) and pressure (1 atm) controlled room with 20-40% humidity. A 12-hour light/dark cycle (lights on at 7 AM) was used to maintain circadian rhythm. Standard rodent chow and tap water were provided *ad libitum*. Housing and food access were unchanged until after surgical implantation.
**Animal Preparation and Stereotaxic Surgery**

All rat subjects were handled at least three times during the week before surgery for five or more minutes. The rat was placed against the experimenter’s chest, rat’s mid-section enveloped by the hand of the experimenter. Handling was performed until the rat ceased moving while in the hold or until no vocalized resistance. The purpose of handling was to assure the rats became comfortable in the hand of an operator. This was an important learned behavior for the electrical kindling procedure as inserting the stimulation wire for kindling required holding the animal.

All surgical procedures were performed between 7:00 and 19:00 to maintain the animals’ circadian rhythm in the 12-hour light-dark cycle. All surgical instruments were sterilized using a water bath with Decon Labs Dri-Clean labware detergent for at least two hours before putting the instruments into a Steri-Dent dry heat sterilizer for ten minutes immediately before surgery. All surfaces making up the surgical station were prepared for surgery by laying down a sterile surgical drape under the stereotaxic frame and wrapping more sterile drapes around the plexiglass platform where the rat would be resting during the surgery. All other surgical instruments were wiped down with 70% isopropyl alcohol before surgery. Male rats between 280-300g were prepared for surgical implantation of a bipolar twisted pair electrode (model #: E363-2-2TW-SPC) into the CA3 region of the hippocampus, two screw electrodes (model #: E363-2-2TW-SPC) onto the anterior and posterior cortical regions, and one screw (model #: 0-80 X 1/16) for cap stability. All electrodes and screws were provided by PlasticsOne (Roanoke, VA).

Each rat was anesthetized using a Kent Scientific (Torrington, CT) VetFlo isoflurane vaporizer (model #: 1210S). The initial dose of isoflurane was 5% in an oxygen-rich airflow until the rat was completely anesthetized. The rat was then immobilized in a stereotaxic frame using ear bars and a ventilation nose cone connected to the VetFlo vaporizer. The isoflurane content
was reduced to 2% for the remainder of the surgery. Body temperature was maintained at 38 °C using a Kent Scientific PhysioSuite (model #: PS-02) module and heat pad. The analgesic Bupivacaine was administered at this point as a local pain killer. A local pain killer like Bupivacaine was chosen because of the potential neurological effects of general, anti-inflammatory analgesics. An incision was made with a scalpel down the middle of the top of the skull from behind the eyes to in front of the ears in order to expose the surface of the skull. Using the bregma and lambda as references, the head was adjusted to assure a flat skull surface. Precise burr holes (1mm in diameter) were made into regions of the skull corresponding to desired brain structures using an electrically operated dental drill. The bipolar, twisted pair electrode was lowered into the hippocampus (AP: -5.0 mm; ML: -5.0 mm; relative to the bregma) the DV coordinate was determined by lowering the tip of the electrode onto the surface of the skull at the midline equal distant from the bregma and lambda (DV: -7.5 mm relative to DV measurement of the midline). The two screw electrodes were inserted and screwed into their corresponding burr holes (AP: +4.0 mm & -10.3 mm; ML: +2.0 & -2.0 mm relative to bregma), positions of all insert electrodes can be visualized using Figure 1. Finally, a screw was inserted and fixed into an arbitrability drilled burr hole, leaving space between the surface of the skull and the bottom of the screw so the dental cement used for the cap could seep into that space and increase the stability of the cap. Stereotaxic positions of all inserted electrodes and screws are documented in Table 1.
Table 1. Surgical coordinates of electrode placement based on the position of the bregma. Coordinates are in millimeters AP (Anterior/Posterior) and ML (Medial/Lateral) from the bregma and DV (Dorsal/Ventral) from the midpoint between the bregma and lambda.

Once all electrodes were positioned, an initial layer of dental cement was used to stabilize the electrodes in their places, especially the free-floating twisted pair electrode. After the base layer had completely dried, a PlasticsOne pedestal was utilized to standardize the location of the female ends of the implanted electrodes, as depicted in Figure 2, and allow for secure connections with the male counterparts during kindling. Once the electrodes were inserted, and the pedestal lowered to the surface of the skull, subsequent layers of dental cement were added to the base layer until the pedestal was fixed in place forming the cap on the dorsal side of the skull. The incision was sutured using violet microfilament polydioxanone synthetic absorbable sterile suture, and the incision site was sufficiently covered in antibacterial cream. The rat was removed from the
stereotaxic frame and placed into a clean polycarbonate cage with *ad libitum* access to standard rodent chow and water. The cage was kept under a heat lamp for 24 hours to assure the maintenance of rodent body temperature. After 24 hours another dose of Bupivacaine was administered, and all rats were returned to the surgical recovery room for one week to recover from the surgery.

![Diagram of electrode positions](image)

**Figure 2.** Top-down view of the positions of electrodes in the pedestal. The posterior screw electrode was placed into the bottom left opening and the anterior screw electrode into the top right.

**Kindling Protocol**

All kindling procedures were performed between 7:00 and 19:00 to maintain animal circadian rhythm. After one week, rats were removed from the surgical recovery room and placed into the recording room. Rats were allowed to acclimate to the room for at least 30 minutes before kindling was initiated. The metal wire tops were removed from the cages and replace with filter tops to allow for more space in the cages and reduce the chance for headset damage. Each rat undergoing kindling on a given day was connected to the male end of a metal spring coiled wire, the ends of which led outside of the cage for connection to the stimulation wire. Using LabChart software (v8.1.13) and the PowerLab 4/35 (model #: PL3504) from ADInstruments (Sydney, AUS) along with the A-M Systems’ (Sequim, WA) Model 1800 2 Channel Microelectrode AC
Amplifier (model #: 700000), the EEG signals from the hippocampus and cortex were acquired and recorded throughout the kindling process. Upon stimulation, a Grass S-88 stimulator was used to deliver a 10-second train of 200 μA biphasic pulses at 50 Hz. Full electrophysiological stimulation settings are depicted in Table 2.

After stimulation, the resulting electrophysiological response was recorded by LabChart to assess for afterdischarge (AD) presence. Along with the EEG data, behavioral responses were observed to determine the severity of the epileptic response. A number between 0 and 5, the behavioral response as defined by Table 3, was assigned to each stimulation.

- wet dog shake = 0
- arrest/absence = 1
- chewing, headbobbing, eye twitch = 2
- forelimb clonus = 3
- forelimb clonus + rearing = 4
- forelimb clonus + rearing + falling = 5

During a kindling session, six stimuli were administered to each rat undergoing the procedure. The stimuli were 30 minutes apart. Each rat was put through the kindling session every day until a specific number of behavioral or electrophysiological responses was achieved. The first was the presence of five afterdischarges on the EEG. Upon obtaining this, each rat was assigned into either the control diet (CD) or ketogenic diet (KD) group. The rats were given two weeks between stimulations and had access to their designated diet ad libitum. Once returning to the kindling procedure, each rat was stimulated until three class five behavioral responses were obtained. At this point, the KD group was placed back on standard rodent chow, and all animals were given a
week of rest. When kindling was again resumed, the animals were stimulated until ten class five
responses were achieved, at which point the animal had completed the kindling procedure.

Visualization of the procedure timeline is depicted in Figure 3.

![Figure 3](image)

Figure 3. Procedural timeline post-surgical implantation of the electrodes. AD refers to
afterdischarges, and Class V refers to a behavioral response to the stimulation that would be
categorized as a 5

RESULTS

The objective of this experiment was to implement and begin a kindling study at Trinity
assessing the neuroprotective effects of the KD. The extent of the protective measures would be
determined by the time and number of stimulations taken for a rat to respond to an intense
stimulating train in the behavioral way defined as most severe (class V). The two diet groups
would be compared for time to class V response, and the significant difference would be assessed
based on time to ten class V responses. All data to date resulted from one animal. Consequently,
there can be no significance drawn from these data. However, these results provide a proof of
concept and a foundation to which the study can grow as more animals are added to the protocol.
This particular animal was given the KD during the kindling protocol.
EEG Recordings

**Figure 4.** Baseline EEG recorded from Hippocampus (red) and anterior cortex (blue)

**Figure 5.** Ten-second stimulation train administration. Increases in amplitude and forced synchronicity recorded in both the hippocampus (red) and anterior cortex (blue)
Figure 6. Electrophysiological response to a stimulation train. Increased amplitude, synchronicity, and repeating EEG pattern seen in both the hippocampus (red) and anterior cortex (blue). This electrophysiological response was paired with a one behavioral score (absence/arrest).

Figure 7. Electrophysiological response to a stimulation train. Increased amplitude, synchronicity, and repeating EEG pattern seen in both the hippocampus (red) and anterior cortex (blue). This electrophysiological response was paired with a five behavioral score (rearing, forelimb clonus, and falling).

The EEG recordings shown in Figures 4-7 suggest a successful application of the stimulating train and recording from the two electrode locations. The synchronicity, repeated
patterns, and increased amplitude in post-stimulation recordings seen in Figure 6 and 7 as compared to the baseline EEG in Figure 4 indicate successful initiation of epileptic activity in the brain. These data are promising to the future of the study.

*Behavioral Responses*

![Behavioral Score to Electrical Kindles](image)

**Figure 8.** Seizure progression of the animal throughout the kindling protocol. A break in the graph indicates a checkpoint in the kindling timeline has been reached (5 ADs, 3 class V responses, and 10 class V responses) and a rest period from kindling as indicated by the dates of stimulation. The numbers correspond to the behavioral responses seen in the animal.

Data gathered from the behavioral responses, depicted in Figure 8, show a clear progression in the severity of behavioral responses to the ten-second kindling stimulations. These data also show a tendency to maintain high-level responses towards the end of the Kindling protocol. These results indicate that kindling has successfully been implemented in the lab. The results shown by
the beginning of the study provide encouraging outlooks to effectively begin introducing more animals to the protocol.

**DISCUSSION**

The objective at this stage of this multi-year study was to successfully set up a kindling model of epilepsy that could be taught and effectively used in future experiments. To this effect, the efforts thus far have achieved this goal; however, there is still much work to be done in producing results. The goal of this study is to eventually assess the neuroprotective effects against epileptogenesis induced by an electrical kindling model of epilepsy and the epigenetic changes on neuronal DNA by the KD. While there are no data gathered at this stage to discuss these possible effects of the KD, the work that has been done in the lab thus far has provided a proof of concept for the study as well as a starting point for the inclusion of more animals and expansion of the protocol.

*Lack of Results*

The biggest setback for this study was the lack of results found. Only one animal was tested through the entire kindling protocol. This is an issue as there cannot be any significance determined. It is the hope that this section will provide insight into the factors that led to the sparsity of data.

The novel nature of the study required a large amount of training and learning to become sufficiently skilled in all parts of the procedure. A stereotaxic surgery was familiar to the lab; however, the addition of new techniques and equipment provided a new challenge. The capping method was novel to the lab which was necessary to master in order to create identical and effective
electrode connections to the stimulator. Furthermore, the anesthetic for the surgery was changed from previous surgical procedures from an injectable ketamine cocktail to a ventilation rig that administered isoflurane continuously throughout the surgery. While this change in anesthetic may not seem to affect the techniques during surgery, there was even a learning curve here relating to fixing the head of the animals securely into the frame through the use of ear bars while maintaining good airflow to the animal through a nose attachment. Another novel skill was determining the correct level of isoflurane administration as to prevent an overdose or the animals from regaining consciousness during surgery. The first semester of this study focused around achieving a high level of skill in the surgical procedure so multiple animals could be introduced into the study with accurate electrode placement and cap manufacturing. This was achieved by the end of the semester.

Possibly the largest factor relating to the lack of results was the timing of the collection of necessary equipment. Even though many electrophysiological studies have been successfully initiated and finished in this lab, practically all electrophysiological equipment used in this study had to be purchased or manufactured. The only piece of equipment that was salvaged from previous experiments was the Grass S-88 stimulator. The acquisition of these tools did not begin until the second semester of this study and continued even until April. By nature of acquiring new equipment, there was an extended learning and troubleshooting process that need to take place before any tests could be administered. Once all of the necessary tools were gathered, a new wire had to be assembled to connect the stimulator and amplifier and record from two separate locations of the brain simultaneously through the same wire. This had never been done in this lab resulting in a long troubleshooting process with animals that had gone through the surgical procedure. Consequently, these animals could not be used for the study as their kindling was not identical to
those after the stimulation had been setup and finalized. During the troubleshooting process, it was even found that a wire supplied by A-M systems, necessary for recording signals from the brain, was faulty and needed to be returned and replaced before any kindling could occur.

The last of the troubleshooting occurred at the end of March. At this time there was one rat ready to go through the protocol. The results from this rat are the sole data reported for this study. Unfortunately, while more rats needed to enter the protocol for significant results to be found, there were no rats near the specific and small range of weights to undergo the surgical procedure. This resulted in a waiting period where no progress could be made on the study besides progressing the eligible rat through the kindling timeline seen in Figure 3. By the time more rats could be added to the study, there was not enough time to complete the kindling protocol. During the kindling protocol, there are four weeks of mandatory rest periods depending on certain checkpoints (Figure 3). Based on this timeline, the new rats would not reach the terminal point of the procedure before the end of the semester. Furthermore, new wires were obtained to create an opportunity to stimulate more than one rat at a time during the kindling protocol. This cause more manufacturing and modification of old wires. Based on this change in electrophysiological equipment and the timing issue, these rats were omitted from the results as they would not be consistent with the rat that made it through the procedure.

**Future of the Study**

This study will be continued after this year. Building off of the work that has been completed, the amplification of animal subjects will be initiated. By increasing the number of animal subjects, the first hypothesis will be successfully tested. Based upon the stimulation number and time needed to achieve the requisite number of class V behavioral responses in both
groups, the KD’s neuroprotective effect during feeding and after the return to a control, standard chow, diet will be determined. It is the belief of this lab that the KD will result in an extension in the timeline both in getting to three class V behavioral responses as well as ten class V responses. This hypothesis is depicted in Figure 3 in the KD group’s proposed timeline.

By introducing more animals into this study, the lab will be able to begin looking into the possible epigenetic effects of the KD proposed before. This will be performed by sacrificing animals during multiple points during the procedure and taking hippocampus tissue samples. These samples will be sent to a collaborative lab at the University of North Dakota. The methylation levels of DNA sequences in the hippocampus will be determined and the results sent back for analysis. Every instance of hippocampus collection is shown in Figure 9.

**Figure 9.** Procedural timeline post-surgical implantation with hippocampus collections indicated by asterisks.

The collections coincide with many of the major checkpoints in the kindling protocol. The first collection is designed to provide a baseline for each feeding group. Collecting tissue samples after five ADs aims to find initial methylation results after the brain has been primed to react more readily to electrical stimulations. The third tissue collection after three class V behavioral
responses are achieved will look for the methylation changes between the two different diets. Finally, the last collection will assess for any lasting methylation changes that the KD provides as possible neuroprotective measures. The tissue collection results will be compared both across diets and within diets to find significant data.

By identifying certain DNA sequences that are promoted or turned off as a result of the KD, the mechanistic properties of the diet can be identified. Furthermore, the results from this study could provide the foundation for studies to target particular genes with epigenetic measures for the treatment of epilepsy. This could provide an effective, non-invasive, and easily administered treatment for cases of drug-resistant epilepsy.

CONCLUSION

An intrahippocampal electrical kindling model was successfully set up for the continuity of this study to research the neuroprotective effects of the KD. Both the protection from epileptogenesis and any epigenetic effects will be assessed in the future. It is the hope of this lab to identify any particular DNA sequences that can be targeted for seizure suppression. These DNA sequences may be targeted for the development of new and effective treatments for epilepsy.

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LITERATURE CITED


