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OBSERVED RELATIONSHIP BETWEEN SEIZURES AND SPREADING DEPRESSION IN THE TETANUS TOXIN MODEL OF TEMPORAL LOBE EPILEPSY

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ABSTRACT

Spreading Depression (SD) and Seizure dynamics were assumed in older literature to be strongly associated, and early acute (evoked) seizure investigations were accompanied by the emission of SD waves [12]. There has recently been renewed interest in the link between seizure and SD - including theoretical work implicating that these source from a unified set of instabilities [9], and from the hypothesis that Sudden Unexpected Death in Epilepsy is mediated by SD invasion of brainstem [11].

There have been only rare if any observations of spontaneous seizure-associated SD events. We hypothesize this is primarily due to technical/instrumentation limitations. We first observed SD events associated with spontaneous seizures in a murine model of post-cerebral malaria epilepsy [6] with an in-house constructed digital recording system with DC sensitivity, sufficient dynamic range, and non-polarizing electrodes.

Here we report that spontaneous SD events occur frequently in the tetanus toxin model of temporal lobe epilepsy.

We have extended our system to provide 16 channels of biopotential recording plus head acceleration in rats. We utilized this technology for experimental measurements of cortical activity and hippocampal field potentials.

We find that initiation of SD events is linked to the seizure focus and that SD events potentially mediate seizure severity and underlie seizure clusters. These measurements are the first record of spontaneous seizure-related spreading depolarization in an animal model of temporal lobe epilepsy. They provide a platform for mechanistic investigation of seizure-SD dynamics in chronic epilepsy and cases of sudden unexplained death in epilepsy (SUDEP) that may lead to

new intervention and treatment approaches.

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ACKNOWLEDGEMENTS

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Chapter 1 : Introduction

Section 1.1: Epilepsy

Epilepsy is a neurological disorder that induces repeated seizure events. A seizure is an event where a neurological pathway has excessive excitatory activity. Symptoms of seizures include loss of consciousness and massive muscle contractions. In addition, multiple seizures can eventually cause brain cellular loss, which are known as lesions [1]. The etiology of a seizure stems from a variety of conditions, including genetic disease, stroke, trauma, or infection of the central nervous system. Epilepsy can develop from a single seizure event over time via morphological and biological changes.

Epilepsy is an important area for research because it is a neurological disorder that significantly impacts quality of life. Some of the pathology includes cognitive impairment, loss of consciousness, and cardiorespiratory abnormalities [2]. Although the symptoms are easily definable, the actual neurological pathway that leads to the development of these symptoms are not well understood.

This work was aimed at understanding those underlying pathological pathways that lead to these symptoms. Specifically, this work focuses on spreading depression and their relationship to seizure events.

Section 1.2: Spreading Depression (SD)

Spreading depression (SD) is a depolarization of neuronal bioelectric activity via a massive surge of extracellular potassium [3]. In a typical action potential, an influx of sodium will create a depolarization in neurons, which is approximately 60mV. This sodium channels close and inactivate during the repolarization for a short amount of time while the outflux of potassium continues. A normal action potential will have slight hyperpolarization; however, SD is an extension of this hyperpolarization due to the massive surges of extracellular potassium and intracellular calcium. This SD event propagates throughout the brain, suppressing neural activity.

SD exacerbates cellular damage due to the excess energy required to reestablish ionic equilibrium in tissues that already have cellular damage. This can cause SD to effect blood flow to meet the energy demands of the SD event. This is only amplified by the events that surround the SD event such as seizures. However, many other neurological disorders such as migraines, cerebrovascular diseases, head injury, and transient global amnesia are also associated with SD events.

Section 1.3: Importance of Research

Seizure and SD dynamics have been assumed to be strongly associated in early literature. Specifically, early acute seizures were accompanied by SD waves. This work focuses on the link between seizures and SD, with understanding the goal of understanding whether SD invasion of the brainstem leads to Sudden Unexpected Death in Epilepsy Patients (SUDEP).

SUDEP takes the lives of 0.2% of all epileptic patients per year. While there are effective treatments for many with epilepsy, they require strict adherence and are not without side effects. In addition, certain patients have seizures that are refractory to treatment. Therefore, it is estimated that current treatments only adequately control seizures in about 66% of patients [2]. This leaves many people with few options to combat the diseases, so understanding SUDEP to develop a treatment that prevents this fatal outcome becomes critically important. Currently, the only possible links between seizures and SUDEP that are known are apnea and heart arrhythmias.

Our work explores a spontaneous seizure associated SD events in a tetanus toxin model of the temporal lobe. Previously, this lab has observed SD events associated with spontaneous seizure in a murine model of post-cerebral malaria epilepsy using an in-house constructed digital recording system with DC sensitivity [1]. In addition, peripheral manifestation of seizures can include autonomic effects including cardiac arrhythmia such as seizure-associated tachycardia, bradycardia, and asystole. These ideas have only recently begun to be explored and are expected to be the natural successor to this research.

Section 1.4: DC Sensitive Potential Recording System

The type of recording system is critical for neuroscience to observe freely behaving animals. However, in order to record SD events, we also needed a recording system that was capable of recording EEG potential under 1Hz, which is also known as DC-sensitive potential recording. Typically, commercial recording systems have too much noise under 1Hz, so that data is filtered out to improve quality of the recordings [4]. However, this means that SD events could be occurring more frequently than expected, but they are not visible due to the limited recording tools available.

In our lab, we have developed a low cost, highly efficient, and high-fidelity epilepsy monitoring unit that can bypass this issue. It is composed of a custom cage that synchronizes data and video for long-term continuous recording. In addition, it is capable of DC-sensitive recording because rather than using nichrome wire bundles in typical recording systems, the lab has developed electrodeposited iridium oxide (EIROF) wires that have more accurate recordings. These were developed by comparing the nichrome wire with the EIROF electrode using an experimental chamber that emitted a specific current. The EIROF electrodes in the chamber yielded a sensitivity at 10mHz, which is capable of recording SD events, unlike nichrome wires. It should be noted that silver chloride pellet also had good results, but these are more expensive and difficult to implant into an animal.

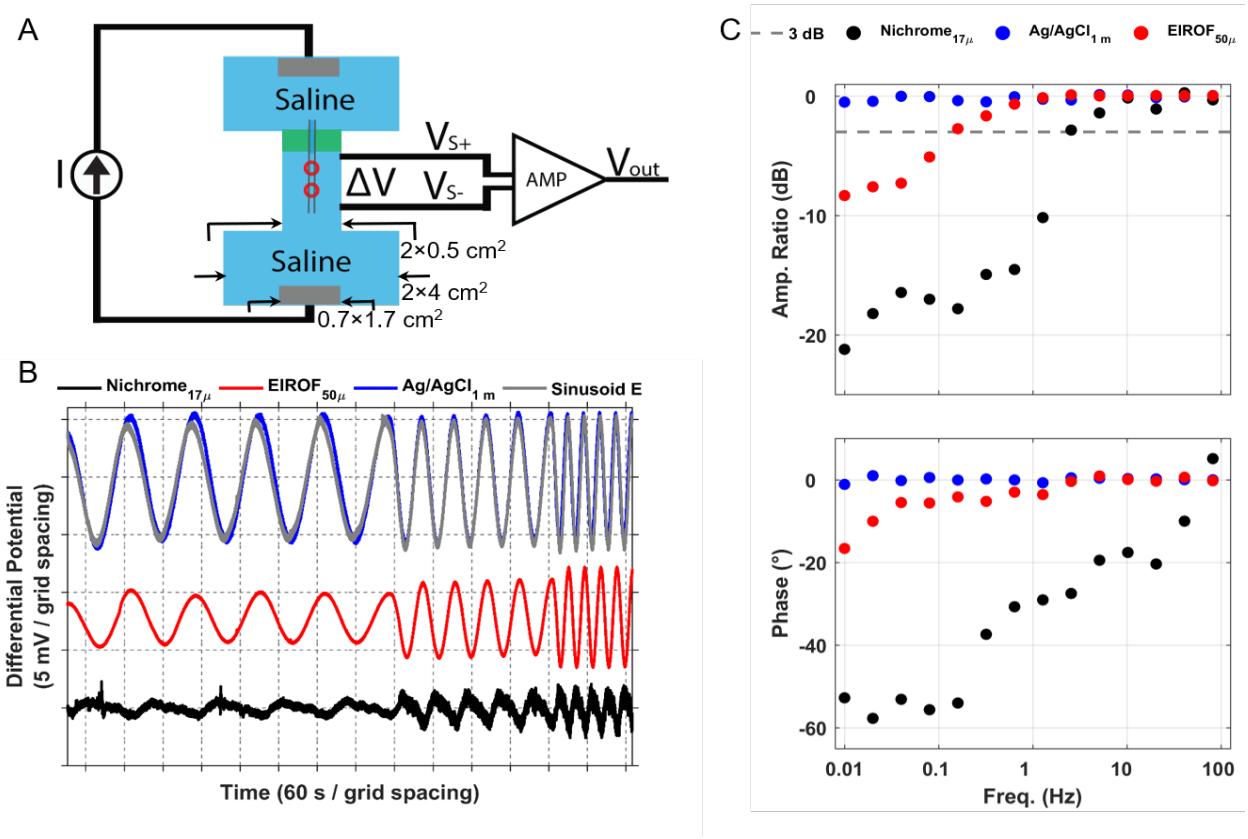


Figure 1: DC-sensitive Potential Recording Validation.

(A) The schematic of the experimental chamber. The system had two chambers that were filled with saline (blue) and separated by clay (green). A reference and ground electrode (red circles) were placed in the center and the electric field was induced by platinum electrodes (silver). (B) The sinusoidal signal (grey) is the signal that the experimental chamber is emitting. The silver (Ag/AgCl) pellet (blue) followed the amplitude and phase of the sinusoidal signal at all frequencies (0.01,0.02,0.04Hz). Similarly, the EIROF electrodes (red) also followed the sinusoidal signal as well; however, the amplitude was smaller. Compared to the commercial nichrome wire (black), which had a distorted signal that would not be capable to record SD events, the EIROF electrodes were the best electrode to use in terms of accuracy and cost. (C) This showed the low frequency sensitivity, where a similar trend was found as before. Overall, the EIROF wire was able to follow the range of frequencies consistently with only minor issues.

Chapter 2 : Methods

All of this research was done under PSU IACUC oversight. All of the animal work was approved by and performed under the oversight of the Institutional Animal Care and Use Committee at the Pennsylvania State University.

Section 2.1: Recording System

These low-cost chronic recording systems were based on a previous system that was built for chronically recording electroencephalogram (EEG) data from cerebral malaria mice [1]. The recording system began as a normal rat box with food and water. The roof was then adjusted with two separate recording systems. The first system was a camera that connected to a single board computer. This camera continuously filmed the animal.

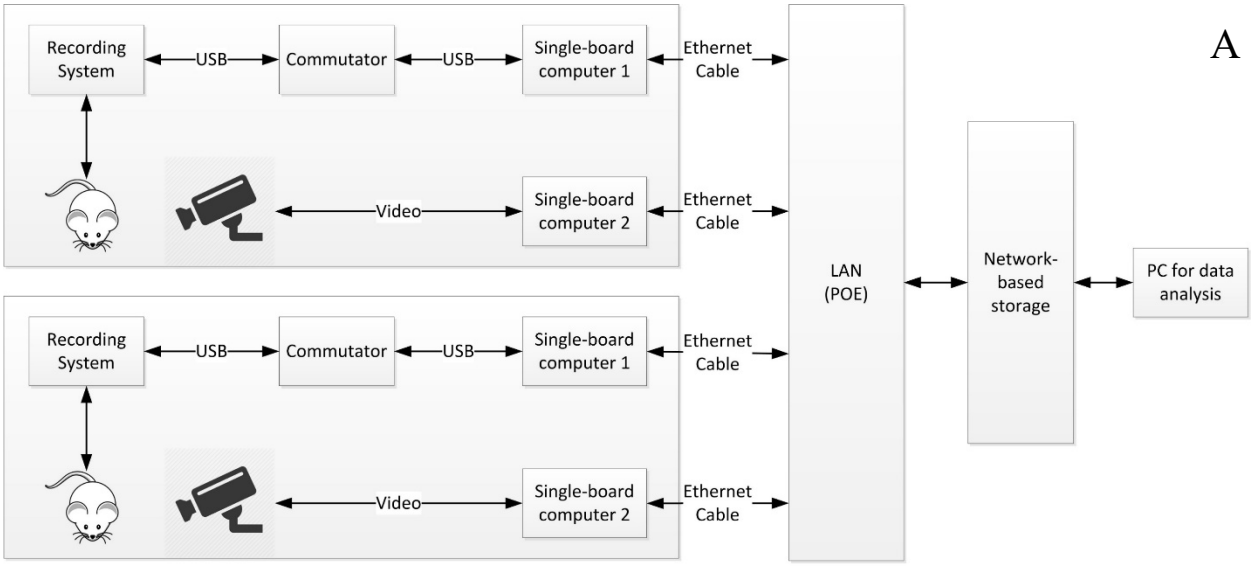
The corresponding recording system was the EEG recording system. This recording system comprised of a small EEG board (1”x1”x0.25”) with 16 open channels for EEG, field potential, EMG, and ECG recording known as the Electronic Interface Board (EIB) [5]. It was DC sensitive and had an input dynamic range of [-2.5V,2.5V]. The frontal circuit on the EIB even had a three electrode potentiostat that was capable of recording PO2, temperature, and pressure. In the current experiments, only the EEG signaling was used; however, the goal is to eventually be able to implement all of these systems simultaneously in an epileptic animal.

The EIB was held within a 3D printed box so the rat could not damage the recording system. The 3D printed box itself had a small opening on the top and no floor. The box sat on a platter that is mounted and secured onto the rat’s head. It was then secured onto the platter via

screws to allow for easy access to the EIB whenever it was necessary. Finally, the opening on the top allowed the EIB to be connected to a wire which would connect it to a single board computer. This wire would have extra insulation to prevent the rat from chewing the wire and interrupting the recording.

Both single board computers, which were Raspberry Pi Model 3 computers, were connected to a LAN storage system and sent to the network-based storage for analysis. The overall system can be seen in Figure 2.

A



B

Figure 2: Low-cost, highly efficient and high-fidelity epilepsy monitoring units (EMU).

(A) Schematic of two recording systems recording simultaneously. These systems are capable of recording both video and EEG data 24/7 without any input from the user. (B) An example of the actual recording systems that were being used for recording. The door on the top is used for easy access to replace the wire, food, or water. These cages were cleaned once a week and checked on daily.

Section 2.2: Electrodes

Animals were implanted with electrodes to monitor hippocampal and cortical activity for at least two weeks using procedures described in previous research [6]. Six stainless steel screw electrodes (#000 self-tapping, Morris Co.) were placed in the cortex. Four of these electrodes monitored cortical activity while the other two acted as the reference and ground electrodes for the cortical activity.

The hippocampal depth electrodes were fabricated from 50μm gold-plated 316L stainless steel wire insulated with polyimide (California Fine Wire). The ends of these electrodes were placed in a micro-reaction chamber (μRC) to provide low impedance (typically <3 kΩ) and DC sensitivity. The stainless steel was etched out on the end to form a chamber. This chamber was then coated with gold and iridium oxide via electro depositing. This provided a larger surface area that could pass large amounts of charge without increasing the geometric surface of the electrode.

Some other electrodes that were used in various experiments include ECG electrodes, EMG electrodes, and oxygen sensors. The only electrodes that have been used currently from this list is the ECG electrode, which was used in a few animals in this experiment and previous experiments. These electrodes were comprised of three separate wires comprised of gold that were insulated everywhere except for the ends. These were placed directly under the skin over the heart.

Section 2.3: Targeting

In order to be able to record from specific regions of the brain, an atlas had to be used to calculate the coordinates of specific targets. The brain has been mapped and categorized into different regions, making it possible to record from specific regions in the brain. In these surgeries we did not adjust the angle of the electrode, which meant that the electrode simply needed to be placed over its target. The areas that were targeted were the cortex and the dorsal and ventral hippocampus. The ground and reference electrodes were placed in the cortex alongside four cortical electrodes.

The ground and references electrodes were found at (AP -6.5, ML ±4) while the other four cortical electrodes had the coordinates (AP 129 +1.5, ML ±4 mm) and (AP -2, ML ±3 mm). Meanwhile, the EIROF electrodes that targeted the dorsal and ventral hippocampus had the coordinates (AP -2.5, ML ±2.0, DV -3.2 mm) and (AP -3.9, ML ±2.2, DV -3.1 132 mm).

The tetanus toxin was injected at the coordinates (AP -6.0, ML 5.0, DV -5.5 mm) and a depth electrode was also placed there. All of these targets can be seen in Figure 3, which shows where holes were made in the skull to reach these targets.

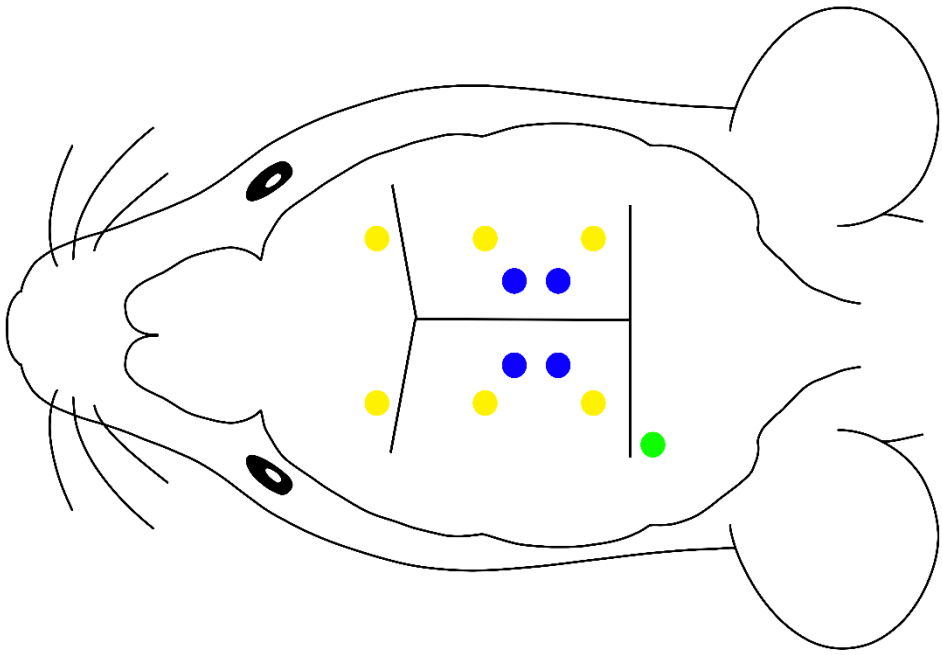


Figure 3: Cortical and Hippocampal Brain Targetting Diagram.

(A) Transverse cut of the brain to show the relative position of the targets. The lines on the brain are the sutures on the skull that are used as reference for targeting specific regions in the brain. The different electrodes pictured in this image are the cortical electrodes (yellow), EIROF electrodes (blue), and the tetanus injection site (green).

Section 2.4: Animal Handling & Surgery

The rats had to be under minimal stress to survive the surgery. As a result, the surgeon began interacting with the animal every day for two weeks prior to the surgery. It began with simply putting a hand in the cage and allow it to explore the surgeon's presence at its own pace. Once it became familiar with the hand, the surgeon approached the rat slowly with its hand and made physical contact the animal. This process was continued until the rat had no reaction to the hand. After that, the rat was held by supporting it close to the surgeon's chest. This provided comfort to the animal, which allowed it to remain calm as it entered the surgical room.

The day before the surgery, all of the surgical tools, gauze, and containers were autoclaved overnight to prepare for the surgery. On the day of the surgery, the animal was brought into the surgical room and placed into the induction chamber that used liquid isoflurane. While the animal was being placed under anesthesia, a ketamine and xylazine mixture was being withdrawn. The amount of this mixture that was administered depends on the weight of the animal, which was typically within 250-350g. Once the animal was anesthetized, the ketamine and xylazine mixture was administered in two doses to the femoral arteries. The animal was then given a specific amount of buprenorphine depending on the rat's weight as general anesthetic.

Once the animal has been properly anesthetized, the areas where the incision was made were shaved and cleaned quickly. After that, the animal was intubated with a custom intubation device that goes down to the larynx. Unlike most intubation devices which only go to the trachea, this device allowed more oxygen to reach the lungs. A general anesthetic was sprayed on the intubation device to relax the muscles of the pharynx as it was installed. After the intubation device was secured in the rat's mouth, it was attached to a high flow vaporizer, which provided oxygen to the animal. It was also capable of providing isoflurane if necessary. After,

the rat's head is secured into the stereotaxic frame and a local anesthetic known as bupivacaine is administered subdermal on the skull to prepare for the incision.

After the rat completely prepared, the incision was made and the skull was cleaned. The head was leveled using the stereotax and bregma, the point where the sagittal and coronal sutures joined together, was marked for reference. Once the reference was set, a drill was used to make the holes for each of the electrodes at specific locations. However, the drills only went partially through the skull and never went completely through the skull to prevent any cellular damage. The drill was stopped once the final layer of skull was fragmented and the remaining fragments of skull are manually removed.

Once the holes were prepared, Dr. Gluckman prepared the tetanus toxin in a Hamilton syringe by withdrawing a specific amount of the tetanus toxin and saline mixture depending once again on the size. The tetanus toxin was then placed in the temporal lobe and it sat there to reduce potential swelling before administration. The tetanus toxin was then completely administered and all of the tools used to administer the tetanus toxin are immediately autoclaved. There were also a set of chronic animals that were not injected with the tetanus toxin to use as the control for the experiment.

After the toxin was administered, the cortical electrodes, which were measurement of electrocorticogram (ECoG), were placed into the cortex by screwing it in a specified amount of turns to control the depth of these electrodes. As for the depth electrodes, they were implanted into the hippocampus to measure hippocampal field potentials and observe SD events. The electrodes were placed above their targets and were slowly lowered into their target to ensure that the electrode does not bend from the dura mater. If the electrode bent because it was not able

to pierce the dura mater, the targeting process had to be reset for that electrode. After the electrode was placed, dental cement was used to keep the electrode in place.

Once all of the electrodes were placed, the platter was set on the skull via dental cement onto the rat's head. The electrodes were attached to open channels on the EIB and a chart is filled out to know what location the channel is recording from. The animal was then given atipamezole to wake up and it was monitored into an incubator until it can walk. It is then monitored daily for a week until the rat resumed normal behavior.

Section 2.5: Data Analysis

The data was recorded and analyzed with a MATLAB program using LabVIEW as an interface. The program allowed for filtering, spectral analysis, and annotation. For seizure scoring, the raw ECoG and hippocampal local field potentials were filtered at 1-55 Hz and 1-125 Hz to observe major SD and seizure events.

Section 2.6: Histology

After the data was collected from the rat, they were euthanized and the researcher examined the brain's histology to ensure the targeting was accurate. This was done by placing the brain in formaldehyde (4% PFA/30% Sucrose) and allowing it to sit in the solution until it sinks. Once the brain sunk, the brain was removed carefully and a notch was made on one side to orient the brain slices. The ends of the brain were cut and placed on the microtome via dry ice. The brain slices were placed in individual well plates until all the targets have been obtained.

After the brain was sliced, they were manually mounted onto slides via a paintbrush. Once they were mounted, they were dried for at least a day to ensure they are secured onto the slide. After they were secured onto the slide, they are stained with a nissl stain, which stains the nucleic acids and is a common nervous tissue stain.

Once the slides are stained, they were imaged with an electronic microscope to see if the targeting was accurate, which can be seen in Figure 4. While the brain slice appeared identical on either side, the notch on the right side allowed the researcher to orient the brain and know exactly where the target is based on the structures and orientation.

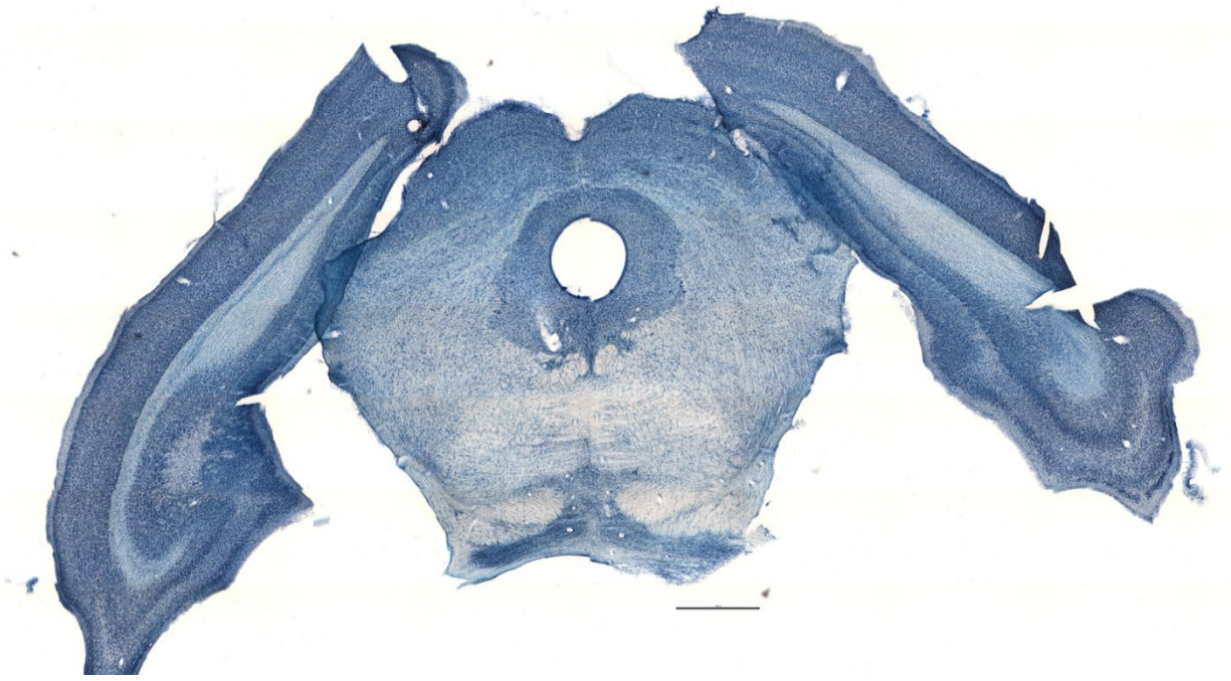


Figure 4: Electrode Path Confirmation via Histology.

A nissl stained brain slice. The electrode track can be seen on the left side (black arrows point to it). The target was confirmed by referencing a brain atlas to determine if the target was reached. If the target was not reached, then any data from that target is rendered null.

Chapter 3 : Results

Section 3.1: SD events are paired with spontaneous seizures

Frequent instances of spontaneous seizures occurred with SD events in five epileptic rats used for this experiment. These seizures were clearly induced by the rat tetanus toxin that was injected into the temporal lobe because the three control animals never had any spontaneous seizures occur despite undergoing the same surgery and being housed in the same cages.

Continuous recordings totaling 124 days were taken from these tetanus toxin rats. Meanwhile, the non-epileptic rats were recorded for 228 days continuously. In total, 425 of 1256 seizures had SD events, which leads to an estimated rate of 33±1%. This can be seen in Figure 5, which shows SD events occur alongside seizure events. While there are seizures that can occur without SD events, the opposite is not true. These convulsive seizures occurred 8-12 days post injection and always originating from the focal injection point.

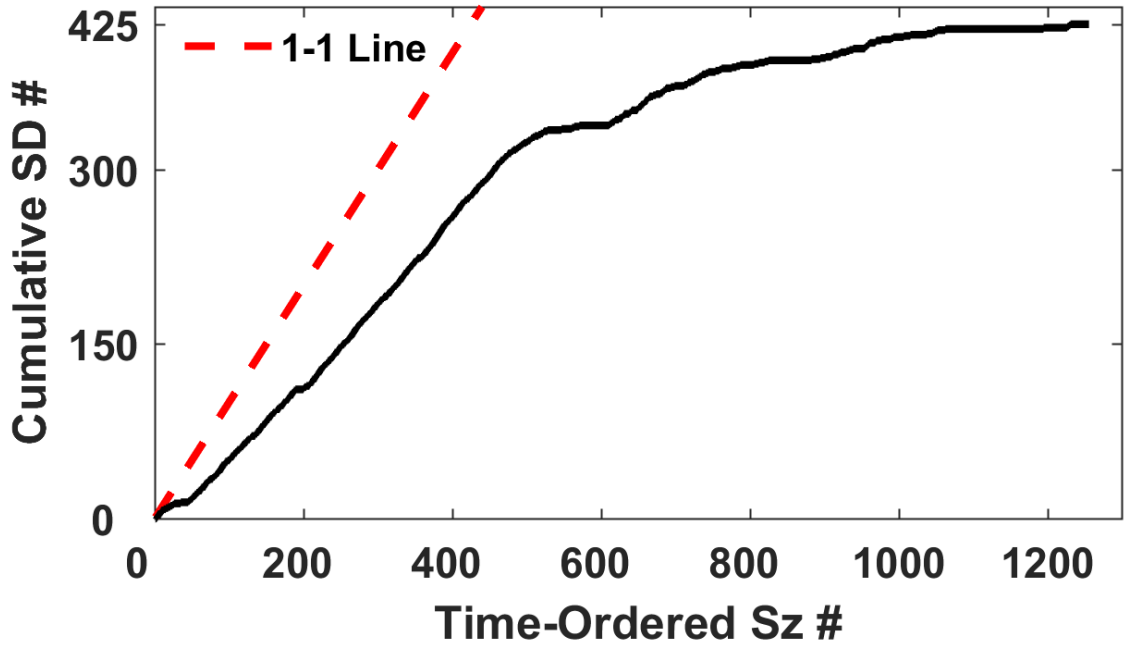


Figure 5: Frequency of SD events occurring alongside Seizure events.

425 of 1256 seizures recorded had SD events alongside them from 5 epileptic rats (124 continuous days recording). This is an estimated rate of 33±1%. There were no SD events detected from 228 days recording from 3 non-epileptic rats.

Section 3.2: SD events increase depression of activity

SD events are typically 10-30mV depressions in amplitude which last for at least 30 seconds [1]. These SD events typically began during seizure events and spread from the initial electrode that observed the SD event to neighboring regions that other electrodes were recording from.

During this time, neurological activity in that area seemed to be suppressed, but return to normal after a short amount of time. Sometimes, this stabilization leads to another bout of hyperexcitability, which can present itself as random spiking or as another seizure event. This can be seen in Figure 6, where a SD event spreads across the hippocampus.

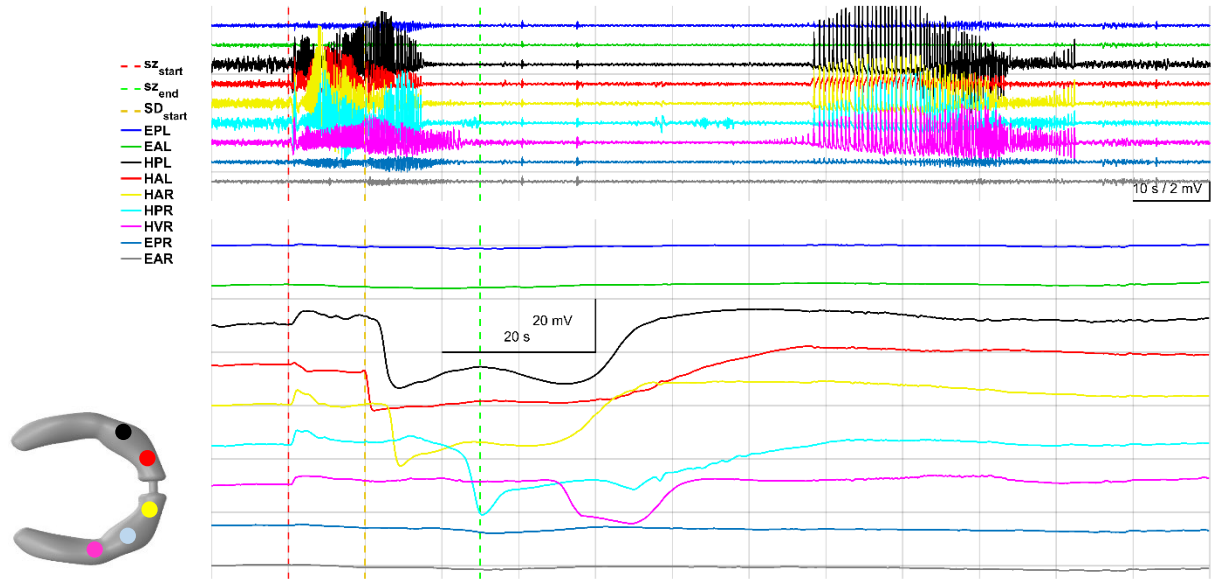


Figure 6: Hippocampal SD events dissociate along Hippocampus.

The first seizure initiates a SD event as usual. However, shortly after the SD event ends, another SD event propagates and initiates another seizure. This second seizure also produces another SD event.

Section 3.3: SD events lack of behavioral changes

Seizures tend to have rapid convulsions of motion, which tend to paralyze the rat, so it is reasonable to hypothesis that the depolarization of the neurons during SD events should also affect the rat's behavior similar to the seizure event. However, there was no significant change in behavior during SD events. This is determined by using accelerometer electrodes to observe the movements of the head alongside the continuous video that was recorded with the EEG data.

Section 3.4: SD may increase susceptibility to future seizures

As mentioned before, SD events were observed in multiple seizure clusters. In 21 separate instances, two or more seizures were within a 10-minute interval. The SD event always occurred after the first seizure and significantly shortened the time before the next seizure.

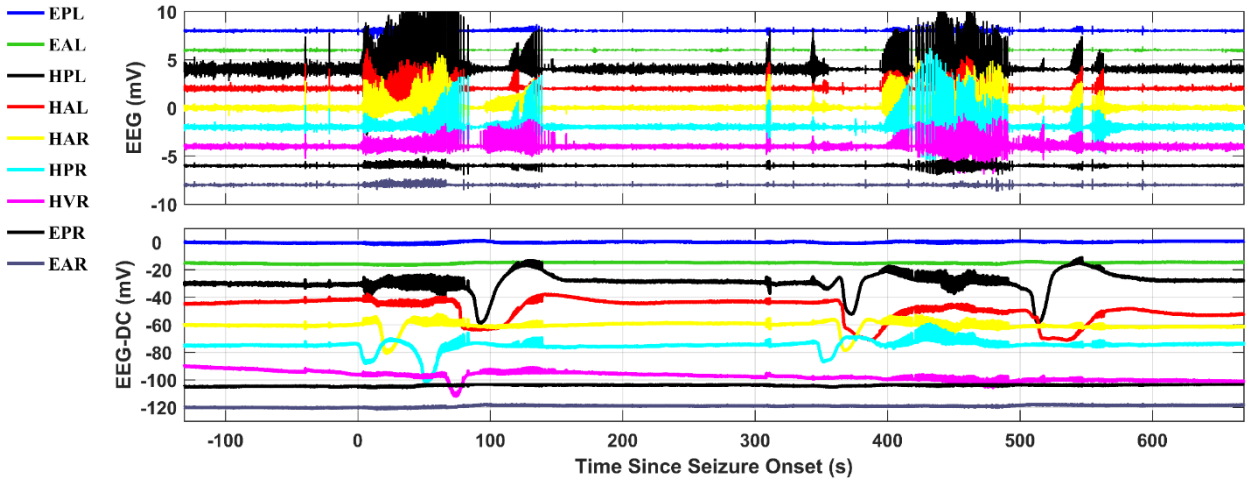


Figure 7: Seizure Clusters.

The first seizure initiates a SD event as usual. However, shortly after the SD event ends, another SD event propagates and initiates another seizure. This second seizure also produces another SD event.

In addition, SD events shorted the time between seizure events within a cluster, which suggests that SD events could have a direct impact on when the next seizure will occur. This can be seen in Figure 7, where a SD event occur right before another seizure.

Chapter 4 : Discussion

Section 4.1: SD relationships

Spontaneous seizure-associated SD events were recorded in a tetanus toxin animal model for weeks to months per animal. This data was compared to a control animal model that went underwent the same procedures as the tetanus model rats. The system that was used allowed observation of SD events while not impairing their behavior. Overall, this experiment has shown that SD events are associated with more than a third of all seizure and appear to increase the frequency of seizure clusters dramatically.

SD events in humans, which can be measured with an EEG, have been observed in clinical settings from patients that have low mobility in intensive care units. These SD events are a propagating depolarization of EEG potential which is followed by a gradual recovery. These SD events last for 1.5-3 minutes and decrease 1-3mV in amplitude for humans [7]. Even though these SD events have been observed in humans, there are no attempts for long term EEG recordings in epileptic patients for SD events. This could be a potential path to explore on the explanation for the propagation of seizures in humans.

Furthermore, the SD events we had observed created seizure clusters, which suggests that SD events could also be a major factor in how epilepsy develops. Seizures are a hyperexcitability of neural activity while SD events are a suppression of neural activity. This means that epilepsy could be a neural disease where the neuronal tissue cannot regulate neural activity sometimes, which leads to these extreme events.

These findings support the trajectories in the Ullah et al. model [8]. In this model, it suggests that the brain is typically in steady state (SS), but can move to different states depending on the oxygen level and the extracellular potassium levels. These are important because extracellular potassium directly impacts neuronal potential while oxygen is essential for the neuron's survival. As such, there is way to directly map these different states, as seen in Figure 7. It can move to either a seizure (SZ) event or a spreading depression (SD) event depending on the levels of the two factors stated previously. This can even explain why SD events sometimes induce other seizure events as the brain tries to return to steady state, only to induce another seizure.

All of this data is limited to the hippocampus, so SD events in other areas of the brain may vary; however, this data shows prolonged suppression of hippocampal activity. It shows that SD might be a potential route to move forward in the future.

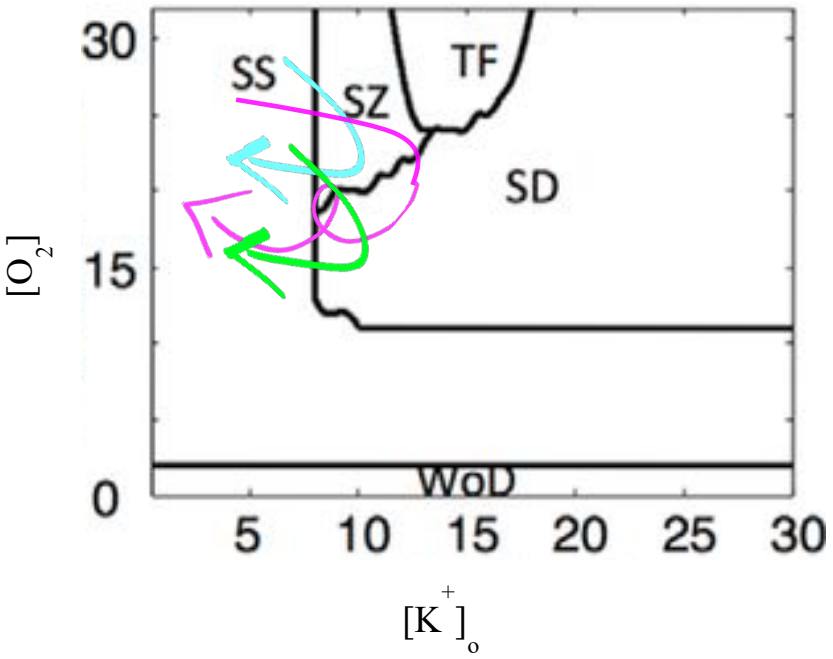


Figure 8: Seizure/SD Unification Theory

This space state model that is based on extracellular oxygen and potassium concentrations is adapted from Wei et. al. 2014 [9]. It separates neural brain activity into different regions, which are steady state (SS), seizure (SZ), spreading depression (SD), and tonic firing (TF), depending on the different concentrations. It is a potential explanation on how seizures can occur with (green arrow) and without SD events (blue arrow). In addition, it explains how SD events can initiate another seizure event via the pink arrow. However, this theory still has not been explicitly proven.

Section 4.2: SUDEP relationships

SUDEP affects 1 in 1000 adults with epilepsy every year approximately [10]. Research from Aiba et. al. has suggested that brainstem SD may be responsible for SUDEP [11]. This suggests that SD could potentially be used as a biomarker for SUDEP clinically. In the rats that we had tested, there were no instances of SUDEP, which suggests that more testing may be

necessary to test whether this relationship exists. However, any path towards a way to prevent SUDEP is a major step into a treatment.

Another potential theory that has only begun to be explored is the relationship of cardiac activity and SUDEP. In some preliminary data, there were entire heartbeats that were missed during both seizure and SD events. These arrythmias could be another route to explore as a potential treatment for SD.

The combination of previous models along with the data collected in this experiment suggest that SD is an important path to explore for future experiments.

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ACADEMIC VITA

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Education: The Pennsylvania State University
Major(s) and Minor(s): Biology - Neuroscience
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Thesis Title: OBSERVED RELATIONSHIP BETWEEN SEIZURES AND SPREADING DEPRESSION IN THE TETANUS TOXIN MODEL OF TEMPORAL LOBE EPILEPSY
Thesis Supervisor: Bruce Gluckman

Work Experience: VA Hospital
Date: May 2018-June 2018
Title: Observer
Description: Participated in Infectious Disease consult rounds led by Dr. Leonard Sacks.
Institution/Company: VA (Washington D.C.)
Supervisor's Name: Dr. Leonard Sacks

Work Experience: University of Iowa General Hospital
Date: May 2019-August 2019
Title: Observer
Description: Observed the treatment of neurological patients in the Parkinson's Disease and Dementia field respectfully. In addition, I was able to shadow in the emergency room with multiple doctors and observe a variety of illnesses.
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Presentations: Neuronal Basis of Sleep Regulation – Model Development using Long – Term Recordings from a Single Neuron in Rats (REU Summer Symposium 2017), Spontaneous Seizure-Associated Spreading Depolarization in a Chronic Rodent Model of

Temporal Lobe Epilepsy (SfN 2018), Spontaneous Seizure – Associated Spreading Depression Relationship with Heart Rate Fluctuations in a Chronic Rodent Model of Temporal Lobe Epilepsy (College of Engineering Research Symposium 2019), & The Effect of the Absence of Serotonergic Neurotransmission on Spreading Depolarization in Mice (University of Iowa REU Symposium 2019)

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