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Factors that Determine Spreading Depolarization Propagation in Brain Slices

Linday Selters
University of New Mexico - Main Campus

Linday Selters

Candidate

Biomedical Engineering

Department

This thesis is approved, and it is acceptable in quality and form for publication:

Approved by the Thesis Committee:

C. William Shuttleworth , Chairperson

Heather Canavan

Russell Morton

FACTORS THAT DETERMINE SPREADING DEPOLARIZATION PROPAGATION IN BRAIN SLICES

by

LINDAY SELTERS

B.S., BIOLOGY, NEW MEXICO STATE UNIVERSITY, 2015

THESIS

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Abstract

In the US alone, more than 750,000 people had a stroke in 2017, more than 1.7 million reported a traumatic brain injury, and more than 9 million suffered from migraines with aura. While all three of these neurological conditions have vastly different causes and possible outcomes, they all have a common phenomenon occurring within the brain. A wave that slowly propagates through grey matter, hitting neurons with a large burst of energy, opening them up to a flood of ions and silencing them for an extended period of time. These waves are known as spreading depolarizations (SD). Within well-nourished tissue, neurons recover from SD. However, in vulnerable tissues, SD events can cause increases in injury. There are currently no approved treatments that specifically target SD, therefore understanding the progression of SD may lead to a better plan for targeting these waves in the future.

Using brain slice preparations from mice I have investigated factors which "prime" SD to propagate more quickly through the brain. I found that a large amount of extracellular potassium ions released into the extracellular space can significantly increase the propagation of the wave in the same direction of the stimulus. Additionally, accumulation of K^+ can prime tissues to be more susceptible to an SD traveling through a region of the brain, and that this can be sufficient to launch an SD event from a remote location following a relatively benign stimulus. The propagation rates, absent a chemical stimulus, were also demonstrated utilizing an induced electrical stimulus highlighting the presence of excitatory factors regardless of initiation within hippocampal mouse slices. These results increase understanding of the factors responsible for propagating SDs, which may lead to the development of new approaches to interrupt these damaging events.

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Introduction

Spreading Depolarizations

Within the brain, there are around 100 billion neuronal cells (Kandel, Schwartz, & Jessell, 2000; Pubmed Health, 2018) and regardless of the type or location of the neuron, its primary function is communication. It waits for a signal and, once received, interprets it and relays the message per the signal's indication. The main way for a neuronal cell to receive a signal is to follow the circuitry of the brain and obtain a stimulus from axon to dendrite via a synapse. The neuron will receive multiple synapses this way at once, but only once enough excitatory neurotransmitters, such as glutamate, have accumulated will an action potential begin down the axon to the next neuron. In this way the brain forms a network of rapid communications. A neuron can also receive a signal from the extracellular fluid when the electrochemical gradient of ions is unbalanced, and cannot be corrected by leaky ion channels. This is generally caused by an upset in the brain, such as oxygen depletion, and can either be instigated by a large extracellular stimulus or a sizeable proximal neuronal depolarization without the need for an axon to dendrite synapse. Once the neuron is pushed past its threshold level, both signaling methods lead to the same outcome, an action potential.

Within well-nourished tissue there is a rapid amount of recovery with repolarization occurring in milliseconds, therefore allowing the neuron to fire action potentials

numerous times per second (Kandel, Schwartz, & Jessell, 2000, pp. 175–298; Lodish et al., 2000). Within certain medical cases, however, the signal is so large that the neuron has a hard time repolarizing. During a migraine with aura, as an example, a large amount of energy, supplied through adenosine triphosphate (ATP), is required to restore the desired ionic gradients after a large wave of depolarization propagates throughout the brain. The needed ATP is supplied through cerebral blood flow delivered with the need for regulation, but the neurons hit by the wave still remain open and depressed for periods that can range from 5 to over 10 minutes (Leao, 1944). The neurons do, however, recover with little remaining damage. During large injuries to the brain, however, such as a blood vessel obstruction during an ischemic stroke, the same wave of depolarization can be seen propagating throughout the brain, initialized by the damaged tissue's perfusion of potassium ions and glutamate. Through damaged parts of the tissue, however, lack of blood perfusion, caused by the clot, prevents summoning of ATP in order to restore ion gradients. Thus, without the necessary ATP, neurons remain depolarized with high levels of sodium and calcium. This unequal distribution of ions recruits water into the cell, further delaying recovering and leading to an overload of calcium, resulting in neuronal death, therefore enlarging the damaged area of tissue (Hartings et al., 2017; Aiba & Shuttleworth, 2012). In this case somehow halting this highly demanding wave of depolarization, means the difference between the recovery of damaged neurons in the brain and the further spread of the injury. These waves, discovered in 1944 by Artistedes Leao, are called spreading depolarizations and have been found as mechanisms of further brain injury in strokes, subarachnoid hemorrhages, and traumatic brain injuries (Hartings et al., 2017; Lauritzen et al., 2011; Leao, 1944; G. G. Somjen, 2001; Strong et al., 2002).

Circuitry

As stated previously, there are billions of neurons within the brain, with each neuron having thousands of connections (Kandel et al., 2000, p. 173; Pubmed Health, 2018). It has been estimated that within every cubic millimeter of the brain there are half of a billion synaptic connections (Changeux & Ricoeur, 2002, p. 78). These connections form the neural network that determines actions, thoughts, and the continuous processes, like breathing, that keep us alive. A common saying is that "neurons that fire together, wire together. Those out of sync, fail to link." This saying originates from psychologist Donald Hebb's ideas on synaptic strength and is now known as the Hebbian theory (Hebb, 2005). It is important because, not only does it simplify how neural networks are formed within the brain, but it also introduces the importance of these connections. Without a strong connection between neurons there is no communication. Therefore, it can be inferred that introducing a factor that throws a neuron out of sync will disconnect communication from further neurons downstream. Consequently, it is important to understand the interactions between spreading depolarization propagation and the circuitry it propagates through.

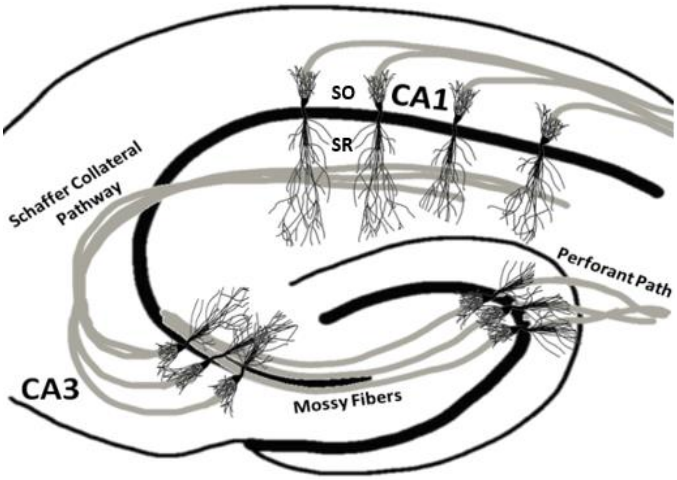

Figure 1: Basic diagram showing the circuitry of the hippocampus. SO: stratum oriens, SR: stratum radiatum

Within the hippocampus, the main path of circuitry is called the trisynaptic circuit (Figure 1). It was first drawn by Ramón y Cajal in intricate detail showing the perforant path extending from the entorhinal cortex to the neurons in the dentate gyrus, then the mossy fibers extending from those granule cells up to CA3 pyramidal neurons, and finally the Schaffer collaterals providing synapses from the CA3 neurons to the CA1 pyramidal neurons (Andersen, 1975). In 1993, it was discovered that spreading depolarization events can propagate throughout different areas of the same cells autonomously, moving forward through dendrites while the remainder of the cell is still catching up (Herreras & Somjen, 1993b). This is important to note, because while Schaffer collaterals initially extend from CA3 pyramidal neurons, the soma of these neurons do not have to be depolarized for the axons of the same neurons to be affected by a spreading depolarization event occurring in the CA1 region; therefore, there is potential for circuit induced priming during a spreading depolarization event.

Glutamate

When a neuron depolarizes, a number of neurotransmitters are released, with glutamate taking part as the main excitatory neurotransmitter within the brain (Kandel et al., 2000; Pubmed Health, 2018). This was first seen by Antoni van Harreveld in the muscles of crustaceans (Harreveld A. Van, 2006) and has been further studied in an attempt to understand the full role of glutamate during an SD. While an axon, such as a Schaffer collateral, does release glutamate in response to a spreading depolarization, (Fabricius, Jensen, & Lauritzen, 1993; A. van Harreveld, 1978) increasing its extracellular amounts to 97.6µm higher than baseline, (Iijima, Shimase, Iwao, & Sankawa, 1998) it does not appear to be in quantities that could induce an SD event singly. Therefore, it may instead contribute to "priming" of neurons downstream from the initiation point of the SD by introducing enough glutamate for a lowering, but not reach, of the threshold. This period of "priming" may explain the heightened neuronal activity, known as prodromal excitation, that has been seen in previous studies briefly after the stimulus but before the SD wave (Aiba & Shuttleworth, 2012; Pietrobon & Moskowitz, 2014).

Potassium

Spreading depolarizations are caused by a large disruption of electrochemical gradients, which can be induced through a variety of methods. The most commonly used for introduction of SD events within hippocampal brain slices is a microinjection of K^+

(George G. Somjen, 2001, p. 1083). It has been found that exceeding a value of 8-10mM of potassium ions in the extracellular spaces is enough to instigate an SD event, which is exceeded with a microinjection of KCl (Heinemann & Dieter Lux, 1977). In 1955 Grafstein made a hypothesis, that has since been supported by additional studies, (Kager, Wadman, & Somjen, 2002; Reid, Marrannes, & Wauquier, 1988) that K^+ not only initiates a spreading depolarization, but it does so because potassium is one of the necessary ingredients to the propagation of SD events. She suggested that if you look at a neuron that has just depolarized within an SD you will likely see a large amount of potassium ions that are released into the extracellular space from the neuron. In this way, it is believed that the accumulation of K^+ within this space not only further depolarizes the same neuron it was released from, but it also begins to depolarize the adjacent cells consequently continuing the propagation (Grafstein, 1956). This accumulation of potassium ions is further influenced by the swelling of the neurons depolarized by an SD. As water is pulled into the cell along with an increase in sodium, chloride and calcium the extracellular space can decrease by 40-70% leading to a consequentially higher concentration of K^+, therefore aggravating the effect (Jing, Aitken, & Somjen, 1994; Pietrobon & Moskowitz, 2014). This self-propagating mechanism allows spreading depolarizations to radiate in multiple directions, including against circuitry components.

Temporal Impedance

As has been stated previously, the amount of potassium ions within the extracellular space of a neuron needed to initiate a spreading depolarization is 8-12 mM (Heinemann

& Dieter Lux, 1977). However with a homeostatic concentration of 2.7-3.5 mM of K^+ extracellularly, (Pietrobon & Moskowitz, 2014) there is constant reuptake of K^+ by sodium potassium channels and astrocytic buffering to maintain the homeostatic concentration. The removal of glutamate through glial and neuronal uptake is also an issue when examining the amount of glutamate being released at the initiation of an SD through Schaffer collateral depolarization. Over time, the amount of extracellular potassium ions and glutamate present in the slice downstream of the wave likely diminishes significantly from the amounts discharged from the axons at the initiation of the wave, over the course of time. Upon stimulation axonal propagation does initiate glutamate release and recordings of prodromal excitation have shown a continuation of the activity until the SD event reaches the recording electrode an average of 4.6 seconds after the initiation of the event (Aiba & Shuttleworth, 2012). What has not been determined is whether or not this excitation is coinciding with the hit of the wave front or if it is preceding the wave and diminishing before the front passes by.

Goal of the Study

The goal of these studies was to determine whether spreading depolarizations are influenced by brain connectivity and the orientation of projecting fibers in relation to an SD stimulus. The support of this hypothesis would introduce the need for methodical circuitry interruption in the attempt to prevent further spread of neuronal injury in deleterious SD inducing pathological conditions.

Specific Aims

Specific Aim 1

How does circuitry contribute to the propagation of spreading depolarizations in brain slices?

These experiments instigated SD events using a concentric bipolar electrode, therefore, removing outside influences of chemical stimuli, within a hippocampal slice model with well-known afferent fiber directionality. The effect of propagation was then monitored using bicuculline, a $GABA_A$ antagonist (Johnston, 2013). While glutamate is the main excitatory neurotransmitter in the brain, GABA is the main inhibitory neurotransmitter (Petroff, 2002). Blocking GABA, therefore, was expected to heighten the effect of glutamate and its effect on SD propagation. These experiments tested the hypothesis that the wave of a spreading depolarization event will travel at an increased velocity in the lateral direction of Schaffer collaterals due to antecedent threshold lowering within neurons due to glutamate and potassium ion excitation provided by the aforementioned depolarized Schaffer collaterals. Thus providing information on the amount of influence circuitry within the brain has on the propagation of SD events in the absence of GABA.

Specific Aim 2

Does K^+ applied extracellularly through ejection pipettes affect propagation velocities?

These studies were designed to compare the effect of directional K^+ diffusion into the hippocampus of mouse brain slices. It was hypothesized that the introduction of extracellular potassium ions will heighten the propagation rate of an SD event and that this effect will depend on the directionality of K^+ diffusion within a slice. These experiments were expected to highlight the challenges of the common hippocampal slice model and confirm the influence of potassium ions on SD propagation in order to further highlight the importance of K^+ and it's "priming" effect on neurons within the wake of an SD event.

Specific Aim 3

Do spreading depolarizations and "priming" mechanisms have a temporal relationship?

These experiments sought to eliminate the possibility of excitatory dispersion, therefore exploring the impact of time on SD event "priming". It was hypothesized that limiting the reuptake of potassium ions into neurons affected by Schaffer collateral depolarization would increase the velocity of wave propagation due to increased excitability. It was expected that these studies would highlight the range that excitatory "priming" can have on propagation and its limitations due to the speed of synapses in contrast to the sluggishness of SD events.

Methods and Procedures

All experimental protocols were approved by the institutional animal care and use committee (IACUC) of the University of New Mexico.

Solutions Preparation

aCSF solutions contained (in mM): NaCl 126, KCl 3, NaH_2PO_4 1.5, $NaHCO_3$ 26, $MgSO_4$ 1, glucose 10, and $CaCl_2$ 2, and was then equilibrated with a carbogen 95% O_2 and 5% CO_2.

Cutting solutions contained (in mM): sucrose 220, KCl 3, NaH_2PO_4 1.5, $NaHCO_3$ 26, $MgSO_4$ 6, glucose 10, and $CaCl_2$ 0.2, and was then equilibrated with a carbogen of 95% O2 and 5% CO2.

Bicuculline was prepared as a 30μM solution with 1.53mL of a 10mg/mL dilution added to 1L of normally prepared aCSF.

Tetrodotoxin was prepared as a 1μM solution with 50μL of a 1mg/0.31321mL dilution added to 500mL of normally prepared aCSF.

Slice Preparation

Adult C57BL/6 mice were used for all experiments. Animals were anesthetized with 0.2mL of a 15% Xylazine and 85% Ketamine solution then decapitated. The brain was then briefly cooled in partially frozen cutting solution that had been bubbled with

carbogen (95% O_2 and 5% CO_2). Two cuts were first made through the bone, laterally, over the cerebellum before a cut anteriorly above the brain, through the bone, to the olfactory bulb was made. The bone covering the brain was then removed gently using forceps before being reemerged into the cutting solution to ensure proper cooling. Using a spatula, the brain was then gently removed from the skull by severing the olfactory bulb, cranial nerves, and spinal cord connection. A second partially frozen beaker of cutting solution, also bubbled with the previous carbogen, containing an addition of 0.2 mL of ketamine, was obtained and the brain was immersed for two minutes. The brain was then cut twice laterally, once severing the hippocampus from the midbrain and once ensuring the olfactory bulb had been fully severed from the brain. A small amount of super glue was then added to a cutting dish and the brain was glued on with the posterior side down. 350um slices were then cut using a vibratome and are hemisected along the midline before being transferred to a warm 35°C artificial cerebral spinal fluid (aCSF) bath bubbling in the same carbogen. The slices were incubated for one hour before being transferred to room temperature aCSF until transfer to the recording chamber.

SD Recording and Imaging

Individual hemisected brain slices were transferred to a submersion recording bath of aCSF bubbling in 95% O_2 and 5% CO_2 and warmed to a temperature of 32°C. A continuous flow of aCSF was added while the slice was in the bath at a rate of 1.9 ml/min. A glass microelectrode, filled with aCSF and a silver wire, was then positioned into the stratum radiatum in order to record the effect of a stimulus-evoked SD.

Recordings were recorded at 10 kHz with an Axon MultiClamp amplifier, and analyzed by using Clampex 9.

Bipolar Stimulation

After the placement of the recording electrode, a concentric bipolar stimulating electrode was placed within the stratum radiatum of the CA1, directly between the Schaffer collaterals and the perforant path, and was connected by a direct current power supply. Following a twenty-minute rest period, spreading depolarization within the CA1 area of the mouse hippocampus was then initialized by 10 pulses, each at 50 Hz with a 1 mA current for 5 ms, repeated every every 20 ms (Figure 2). At these short intervals an SD event is initialized by rising postsynaptic potentials (Zucker & Regehr, 2002).

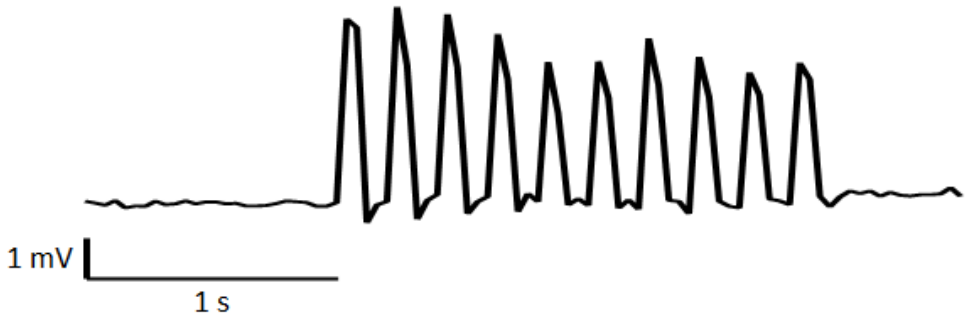

Figure 2: Concentric bipolar pulse

KCl Stimulation

After the placement of the recording electrode into a submerged slice on the rig. A glass microelectrode, filled with 1M KCl, was then positioned within the stratum radiatum of the CA1. The exact placement of the microelectrode changed with the nature of the experiments, but each electrode was placed 30-50 µm below the slice surface. Following a twenty-minute rest period, spreading depolarization within the CA1 area of the mouse hippocampus was then initialized by a brief pressure pulse of 30ms at 60psi released KCl into the slice using a WPI PV830 Pneumatic Picopump.

Passive Potassium Ion Release

Once the recording electrode is placed into a submerged slice on the rig, one glass microelectrode tip is intentially broken and filled with 1MKCl. An additional KCl filled microelectrode is also made (fully intact) and the latter is placed 30-50 µm into the surface of the stratum radiatum of the CA1, on the edge of the slice facing, either left or right unidirectionally dependent on the nature of the experiment. The standard twenty-minute rest period is foremost, followed by placement of the broken KCl filled electrode. This electrode is also placed unidirectionally on the edge of the slice, 30-50 µm under the surface, but on opposite end of where the initial electrode was placed. The slice is then monitored for 5 minutes to ensure that there is a steady leak of KCl into the slice without initiation of an SD event. A spreading depolarization is then initialized within the CA1

area of the mouse hippocampus by a brief pressure pulse, inducted only in the intact pipette, for 30 ms at 60psi.

Slice Visualization and Capturing

Slices were visualized using trans-illumination with visible light and using a 5x or 10x objective. Images of the SD events were captured with a cooled CCD camera and were analyzed using TillVision software. The slow, second phase of increased light transmission can not only be seen easily but can be analyzed to determine the velocity of the wave.

Analysis of SD Wave Propagation Velocity

All data points were taken at 0.5mm and 1.0 mm away from the stimulus of the wave. The distance formula, $Distance = \sqrt{(x_2 - x_1)^2 + (y_2 - y_1)^2}$, was applied by taking the x,y coordinates at the beginning of the 0.5mm frame and taking the second x,y coordinates three frames later; so, that frames 1 and 4 were used, with the 0.5mm distance from the stimulus captured during those frames. The distance was then converted to mm/min by dividing by the time captured between the four frames, which was determined by three multiplied by the frame rate, then dividing by the converted distance of the slice to switch from pixels to millimeters, and finally by multiplying by 60,000 milliseconds. This process was then repeated over four frames containing the 1.0mm point of distance from the stimulus. All data is reported as mean ± SEM.

Statistical analyses run were one-way analysis of variance (ANOVA), paired and unpaired t-tests and were calculated using GraphPad Prism (7.03, La Jolla, CA). Statistical significance was determined by P values of < 0.05, with Bonferroni correction during ANOVA tests.

Materials

The vibratome used for all mouse brain cuts was the Pelco 102 Vibratome (Ted Pella, Inc., Redding, CA). The slice support for use during recording was produced by Warner Instruments (RC-27, Hamden, CT), as was the inline heater assembly (TC-344B). The concentric bipolar was produced by FHC (CBCEG75, Bowdoin, ME). The Axon MultiClamp and Clampex were obtained from Molecular Devices (Sunnyvale, CA). The microscope was an Olympus EXS1W1with both 5x (0.15 W) and 10x (0.30 W) objectives. All images were captured using a Sensicam 12 bit CCD camera from PCO Imaging (382KL2014, Kelheim, Germany) and were analyzed using Till Vision software, version 4.01. All glucose and salts for aCSF were obtained from Sigma-Aldrich Corporation (St. Louis MO). All glass micropipette tips were obtained from Sutter Instruments and were made of borosilicate glass (BF150-86-10, Novato, CA). 50 mg of 1(S),9(R)-(-)-Bicuculline methiodide was obtained from Sigma-Aldrich (CAS# 40709-69-1, St. Louis, MO). The concentric bipolar was obtained from FHC (Bowdoin, ME) and the power supply was an A.M.P.I. (Jerusalem, Israel). 1mg of Tetrodotoxin citrate was obtained from Tocris biosciences (CAS# 18660-81-6, Minneapolis, MN). The KCl

pressure pulse issued by the picopump was obtained from World Precision Instruments (Sarasota, FL).

Chapter 1: Circuitry's Contribution to the Propagation of Spreading Depolarization in Brain Slices

Introduction:

As formerly mentioned, the brain is incredibly vast, having half of a billion synaptic connections within just a cubic millimeter of the brain (Changeux & Ricoeur, 2002, p. 78). The connection between circuitry and spreading depolarizations, however, has not yet been explored. It is known that 1) spreading depolarizations only travel through grey matter, therefore neuronal transmission is crucial (George G. Somjen, 2001) 2) a physical cut through the cortex will halt the wave, (A. V. Harreveld, Terres, & Dernburg, 1955) and 3) loss of neurons, here due to asphyxiation, will do the same, showing SD's dependence on functioning circuitry (Hull & Harreveld, 1964). What is not known is how circuitry affects SD events when the circuitry is aligned along or opposed to SD propagation. It is known that action potentials causes glutamate to be released from presynaptic terminals, and that the cell's attempt at repolarization prompts the release of K^+ through sodium potassium channels. Then, both potassium ions and glutamate are seen in normal concentrations within the extracellular fluid and have the potential to influence further propagation, while being prevented reverse propagation due to neuronal refraction (Kandel et al., 2000b). During a spreading depolarization, however, this release of potassium ions and glutamate is not seen at normal levels, instead, it is a flood, with glutamate rising over 90μm above baseline (Iijima et al., 1998) and potassium ions

following suit increasing from a mere 2.7 – 3.5 mM to as high as 60mM (Pietrobon & Moskowitz, 2014). This overwhelming amount not only initiates depolarization of axons in the circuitry driven direction, but also introduces enough glutamate and potassium ions into the extracellular fluid to initiate reverse propagation as well. The difference between these two directions, one fueled by extracellular concentrations alone and one fueled with both ionic concentrations as well as axonal depolarizations is not yet known. Consequently, this has lead to the hypothesis that a spreading depolarization event will travel at an increased velocity in the lateral direction of Schaffer collaterals due to antecedent threshold lowering within neurons due to glutamate and potassium ion excitation provided by the aforementioned depolarized Schaffer collaterals.

Results:

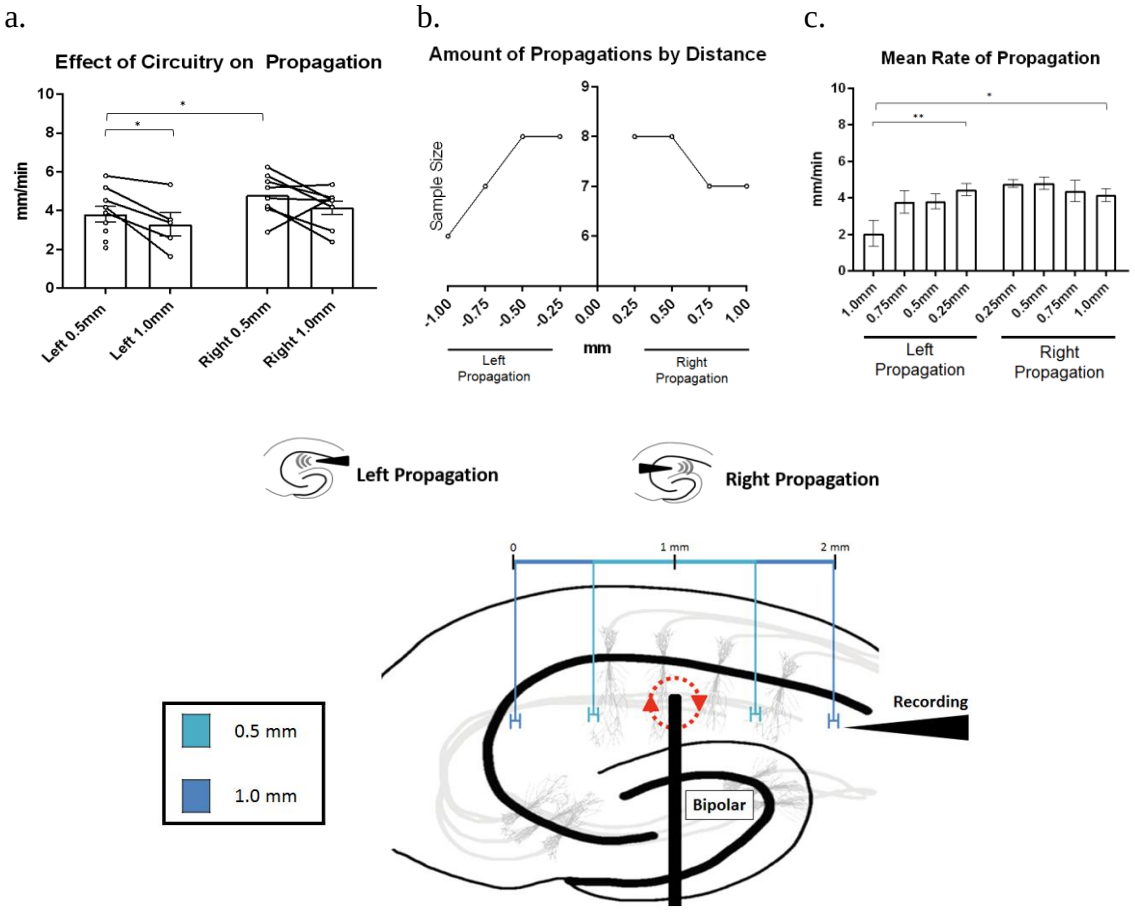

Figure 3: (a) Rates were taken on both left and right sides of the stimulus at distances of 0.5mms and 1.0mm away from the edge of the electrode. All rates were taken over a time period of three frames (750ms in total) and each n (0.5 mm n=9, 1.0mm n=5,8) were paired with the same SD event. There was a significant difference seen between the propagation rates at 0.5mms away from the stimulus ($p=0.027$, paired t-test), and between the distances away from the stimulus in left propagating waves ($p=0.0143$, paired t-test). (b) In all propagating events waves traveled to a distance of 0.5mm away from the stimulus. Increasing the distance from the stimulus decreased the likelihood of further propagation, mainly in left traveling events. Slices were removed from this analysis if propagation was halted due to damaged tissue or blockage. (c) In both directions, SD events did not mainstay a constant velocity throughout the hippocampus, instead decreasing in speed as distance from the stimulus increased. This decrease was mainly seen in the distances 0.5mm and 1.0mm (Left propagation $p=0019$) and between left and right propagations ($p=0.0275$).

To test the hypothesis that the wave of a spreading depolarization event will travel at an increased velocity in the lateral direction of Schaffer collaterals due to

antecedent threshold lowering within neurons due to glutamate and potassium ion excitation provided by the aforementioned depolarized Schaffer collaterals we used electrical stimulation to induce SD events and optically measured their propagation rates. Figure 3a shows the rates of propagation through the hippocampus after an electrically initiated spreading depolarization. With a 50 Hz train of 1mA, the concentric bipolar depolarized the conducting neurons producing an SD event that was self-propagating. While the crest of the wave heading contiguously with the trisynaptic circuitry traveled at an average velocity of 4.8 mm/min, the opposite crest propagating contrary to the known pathways had a significantly slower velocity with mean of 3.8 mm/min. In addition, what is generally thought of to be a self-regenerating phenomenon, the SD events do not retain their velocity, slowing down as they move further away from the stimulus (Figure 3c). This effect is amplified in left propagating SD waves as not only are all waves seen slowing their velocity as distance increases from the point of stimulation, waves do not consistently continue propagation to a distance of 1mm away from the stimulus (Figure 3a,b).

During the depolarization of neurons due to a concentric bipolar it is indicated in Figure 3 that the resulting SD, while not reliant on neural networks, is affected by the cumulative consequence of threshold lowering ahead of the crest of the wave. This suggests that the extracellular potassium ions and glutamate from depolarized axons ahead of the SD initiate the depolarization of neurons, therefore, “priming” through prodromal excitation. On the contrary, Figure 3 indicates the wave heading in opposition to the circuitry is bolstered by its own accumulation of potassium ions and glutamate.

While this is a slower event, it is shown that the copious amount of propellant that is released by the primarily depolarized neurons is enough to also depolarize the proximal neurons in a self-propagating fashion.

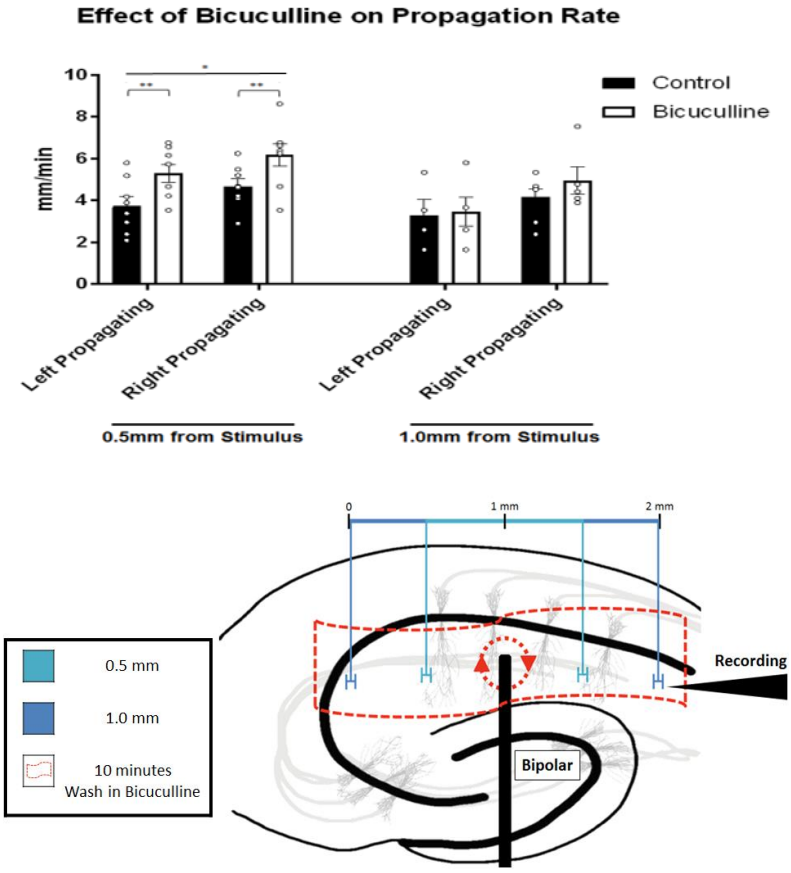

Figure 4: Rates were taken on both the left and right sides of the bipolar at distances of 0.5mm and 1.0mm away from the edge of the stimulus. All rates were taken over a time period of three frames (750ms in total) and while treated vs. untreated slices were not paired, distance, and left vs. right propagating values were paired within individual treated or untreated slices (treated 0.5mm n=8, 1.0mm n=5) (untreated 0.5mm n=8, 1.0mm n=4,7). There was a significant difference seen between both directionality of propagation (p=0.0492, Bonferroni Anova Test) as well as treated vs. untreated slices (p=0.0020, Bonferroni Anova Test). Though not significant, there was also a familiar decrease of propagation velocity 1.0mm from the stimulus following the trend with bicuculline treated slices showing higher propagation rates in accompaniment with higher propagation rates in right propagating waves.

While it has been shown that an SD initiated by electrical excitation is propelled through the hippocampus using the fuel of extracellular K^+ and large amounts of released

glutamate, the full effect of excitatory glutamate is generally dampened by the inhibitory neurotransmitter GABA (Petroff, 2002). A 30μM solution of bicuculline aCSF was introduced to each slice for a wash on period of 10 minutes before an SD was initiated by the same period of stimulation as the previous experiment viewed in Figure 1 (50 Hz, 1mA). Bicuculline is a $GABA_A$ receptor antagonist and as such reduces the amount of GABAergic synaptic transmission by preventing the binding of an agonist to said receptor (Capaday, van Vreeswijk, Ethier, Ferkinghoff-Borg, & Weber, 2011; Johnston, 2013); this, in turn, removes the hyperpolarization induced by GABA and could bring the neurons closer to SD threshold. It was found that 0.5mm away from the concentric bipolar the increase in propagation rate (a mean increase of 1.5 mm/min) in bicuculline perfused slices is significantly faster in both propagation directions (Figure 4). Intrinsically, the velocity of the SD event is significantly faster in both the control and bicuculline waves when paired with the additional "priming" of trisynaptic circuitry, increasing from an average of 5.3 mm/min in left propagating treated slices to an average of 6.2mm/min. That being said, the surge, once again, does not maintain its velocity, slowing down in all instances as it reaches a distance of 1mm away from the point of initiation.

These results strengthen the awareness of glutamate's importance on SD propagation velocity showing that without the inhibition of GABA, SD waves reach threshold easier and propagate faster. Not only is this occurring with glutamatergic priming, provided by the depolarization of axons on neurons downstream, it is also

occurring consistently with the propagation of the wave as individual neurons depolarize providing neighboring neurons with their fuel to follow suit.

Conclusions:

In 1959 Dr. Antonie van Harreveld showed the importance of glutamate during SD events, (A. V. Harreveld, 1959) but even now the degree of its importance is still being studied and discovered. As shown in these results, SD events may not be as self-regenerative as previously thought; (Pietrobon & Moskowitz, 2014) however, glutamate plays an essential role in providing stimulation, ensuring that they propagate faster when relying on only focal neuronal depolarization (Figures 3, 4). Glutamate's effect is further heightened when facilitated by neuronal circuitry showing how the release of glutamate from currently depolarizing neurons can expedite the rate of depolarization of the neurons that will be affected in the future downstream direction. Additionally, while it has been shown that $GABA_AR$ are highly activated at the beginning of an SD event, (Aiba & Shuttleworth, 2012) GABA is not responsible for the fluctuations in velocity. Though GABAergic interneurons project in both directions therefore providing inhibition both downstream, through feed forward inhibition, as well as upstream, through feedback inhibition, hindering $GABA_AR$ does not remove the difference in SD propagation velocity.

Chapter 2: The Effect of Exogenous Potassium Ion Introduction on Spreading Depolarization

Introduction:

Experiments in chapter 1 showed that circuitry affects the velocity of SD propagation due to additional release of potassium ions and glutamate through axonal depolarization. The introduction of a spreading depolarization *in vitro*, however, is more commonly introduced with a microinjection of K^+, which disrupts extracellular gradients, than with the concentric bipolar. This not only introduces additional potassium ions to the extracellular fluid, potentially increasing excitability of neurons influenced by the heightened gradient but also presents an overlooked variable, microinjection directionality. Therefore this study examines the hypothesis that the introduction of extracellular K^+ will heighten the propagation rate of an SD event and that this effect will depend on the directionality of K^+ diffusion within a slice.

Results:

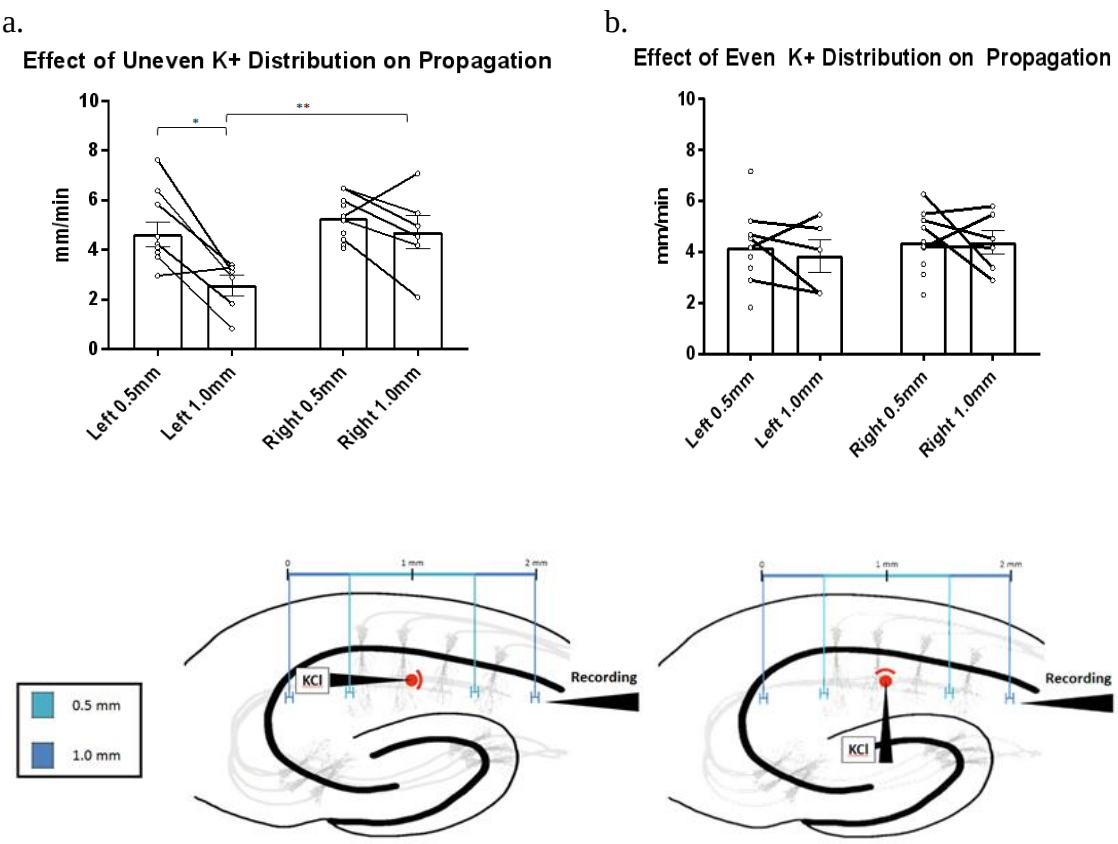

Figure 5: (a) Rates were taken on both left and right sides of the stimulus at distances of 0.5mms and 1.0mm away from the edge of the pipette. All rates were taken over a time period of three frames (750ms in total) and each n (0.5mm n=10, 1.0mm n=6) were paired from the same SD event. There was a significant difference seen between the propagation rates in both distances from the stimulus in left propagating slices, (p=0.0116, paired t-test) but also in directionality between 1.0mm propagations. (p=0.0056, paired t-test) (b) Analysis was repeated in the same way as in (a) with the exception of the directionality of the microinjection facing parallel with the neurons in the CA1 region of the hippocampus. Sample sizes (0.5mm n=10, 1.0mm n=5) were paired and no significance was found in the rates of propagation in comparison to the significance found with the unidirectional microinjection.

As mentioned previously, the most common way to introduce a spreading depolarization into a slice is by injecting a small amount of K^+ into the CA1 region of the hippocampus (George G. Somjen, 2001, p. 1083). This way K^+ is diffused into a small area of extracellular space where neurons rapidly respond to its upset of the

extracellular concentrations and depolarize, blooming into an SD event. What is not usually taken into account, however, is the effect of both circuitry within a slice as well as the directionality of the microinjection itself. When a K^+ stimulus is introduced into a slice it is commonly done through a pipette of discernable resistance and driven by a small puff of air. In this way it is projected into the slice in a discernable direction, potentially causing an uneven diffusion of K^+ into the extracellular space. As can be seen in Figure 5a, when the K^+ microinjection is faced unidirectional with the circuitry of the slice the rates of propagation are higher in right directional waves. Whereas in left propagational waves not only do the waves significantly slow the further away from the stimulus they are they are also significantly slower at a distance of 1.0mm away from the stimulus than their counterparts are with their aid of both circuitry and partisan stimuli. In contrast (Figure 5b) when an even microinjection of KCl is introduced into the slice, by positioning the electrode in parallel with the neurons, instead of in the standard perpendicular fashion, a consistent rate average of 3.9-4.4 mm/min is seen and the large variance in rates is removed between leftward and rightward directionalities.

In comparing Figure 5a to 5b, it is important to note that while this bidirectional stimulus did not appear to significantly affect the rate of propagation at 0.5mm away from the stimulus, its effect on the propagation of the wave 1.0mms away from the stimulus, removing the significant decreases in rate, is crucial. This shows the importance of extracellular potassium ions during SD events. When looking at left propagating waves, close to the stimulus, the average velocity of propagation is around 4.6 mm/min which compares to the faster velocity average of 5.3 mm/min in right propagation waves.

Yet, this difference increases drastically with the right propagating waves barely slowing down to around 4.7 mm/min and left propagating waves dropping down to a mean of 2.6 mm/min. Even when looking at the concentric bipolar induced SD (see previous chapter), which included no introduction of supplementary chemicals, the average rate of propagation 1.0mm from the stimulus was around 3.8mm/min. Allowing the K^+ to distribute and diffuse evenly gives the neurons the ions they need to continue the SD event. Therefore neurons starved of potassium ions have slower propagation rates and SD may abate sooner than neurons able to share the K^+ introduced through extracellular means.

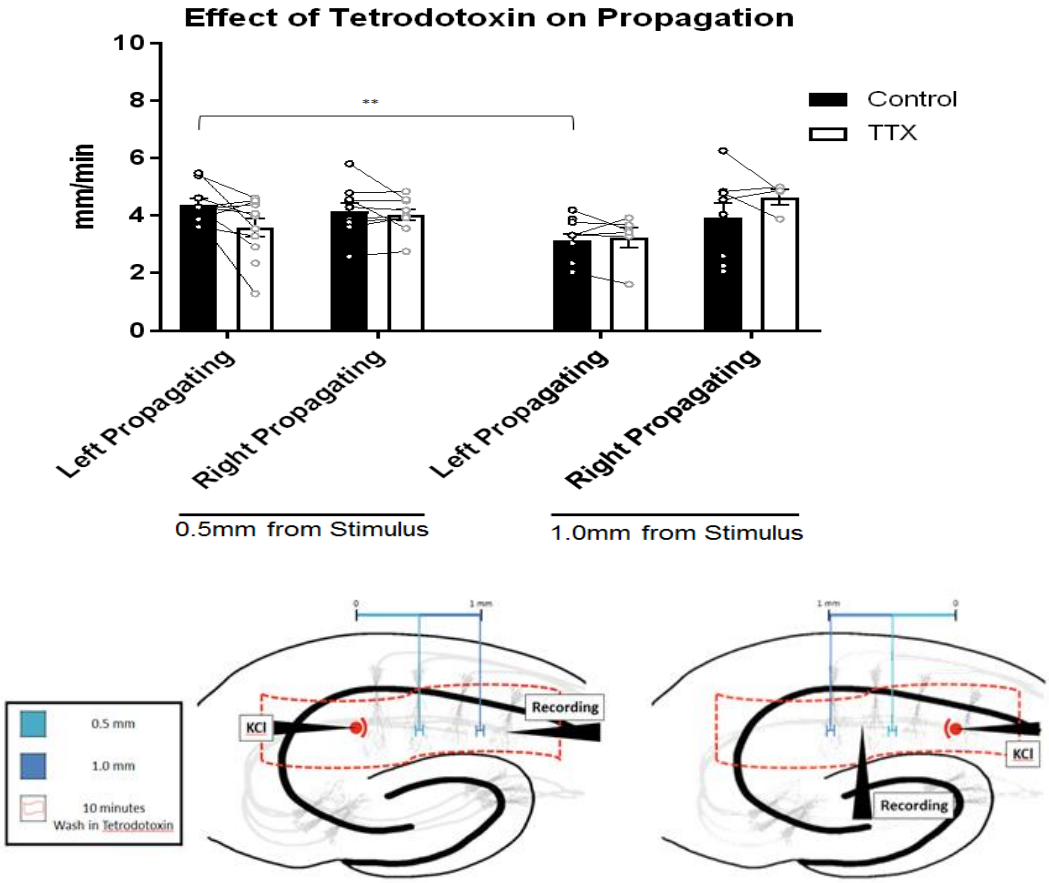

Figure 6: SD events were stimulated using either a left or right directional microinjection at the edge of the hippocampus and were measured at distances of 0.5mms and 1.0mm away from the tip of the electrode. All rates were taken over a time period of three frames (1350ms in total) and each n was paired through distance and controls vs. treated, but not through directionality (Untreated 0.5mm n=10, 1.0mm n=8,10) (Treated 0.5mm n=10,11, 1.0mm n=4,6). As seen in similar experiments left propagating untreated waves decreased significantly as they grew further away from the stimulus (p=0.0037, paired t-test). Directionality statistics were performed through unpaired t-tests and no significance was found.

To test the hypothesis that the activation of action potentials through sodium channels is a main contributor to the release of glutamate during a spreading depolarization, Figure 6 examines a combination of a KCl stimulus with retardation of glutamate release via circuitry. Tetrodotoxin (TTX), a well-known neurotoxin found in pufferfish (Lago, Rodríguez, Blanco, Vieites, & Cabado, 2015) blocks sodium channels in neurons therefore inhibiting action potentials, and release of neurotransmitters, such as

glutamate. During an SD event, however, it was found that glutamate is still released from depolarized neurons due to activation of NMDAR, however this is likely only occurring to adjacent cells, not through synaptic activity (Zhou et al., 2013). For that reason, this experimental set examines the effect of KCl diffusion and glutamate release void of circuitry. As can be seen 0.5mm away from the stimulus both left and right propagating stimuli are fairly similar. Control SD propagations average out to around 4 mm/min, (Left propagating mean=4.4mm/min, Right propagating mean=4.2mm/min) and decrease to a little less than 4 mm/min with the addition of TTX. (Left propagating mean=3.6mm/min, Right propagating mean=4.0mm/min)This is comparable to the rates seen when SD was introduced with electrical stimulus. In comparison, 1.0mm away from the KCl stimulus left propagating waves average at 3.2 mm/min, increasing from the control (mean=3.1mm/min) slightly with the addition of TTX. Right propagating SD events within the same slice are found to have a mean of 3.9 mm/min increasing to 4.6mm/min with the addition of TTX.

While 0.5mm away from the stimulus TTX introduces a familiar effect decreasing the rate of propagation in both directionalities as neurons struggle to release glutamate in a nonregulated way, the effect was not what was expected. With the removal of circuitry induced glutamate “priming” and the reliance on self-generated propagation, the decrease of right propagation rates was expected to be significantly lower in treated SD events than in control SD events, an example of expected results being Figure 3a. As an added factor SD wave propagation rates increased in both directionalities as the waves reached 1.0mm away from the stimulus. A phenomenon that had not yet been seen within this set

of experiments. Therefore it can be deduced that there is either a factor affected by the opening of sodium channels which provides an inhibitory protective effect on neurons or an excitatory component activated along with the NMDA receptor activation of glutamate release that may be introduced with the removal of circuitry induced "priming". A possible inhibitory factor that has been removed may be suggested by revisiting the effect of bicuculline on a slice. Introduction of bicuculline inhibits the opening of $GABA_A$ channels, but so does TTX by inhibiting action potentials. So not only is glutamate prevented from being released, so is GABA. This may explain the increase of propagation rate as the wave expands outwards, much like removing the brakes of a train on a downhill slope. However, it has also been found that activation of NMDA channels alone can stimulate the release of GABA (Chen, Xiong, & Shepherd, 2000). Therefore there is likely another explanation of this phenomenon which could contribute to further explanation of the mechanisms of SD propagation.

Conclusions:

When using a common model for scientific practice, such as a unidirectional microinjection of KCl into a mouse hippocampal slice, it is important to understand how the normal standard may be an influenced result. This has been examined (Figures 5a, 5b) and it was found that an even introduction of KCl into the extracellular space of a slice increases the amount of propagation against the neuronal circuitry of the slice. Not only does this provide more information about the standard KCl induction of an SD within a slice it also provides a look at the importance of extracellular potassium ions during an

SD. Without the supplemental K^+ the wave slowed down substantially while the opposite side saw both additional potassium ions as well as preceding neuronal excitation from circuitry. This was then challenged by TTX treated SD events (Figure 5) which showed that while TTX should have removed circuitry leaving only self-regenerating waves using the introduced K^+, they instead thrived in the TTX induced environment. This effect, while unprecedented should be examined more in future studies.

Chapter 3: Examination of the Temporal Interaction between "Priming" Mechanisms and Spreading Depolarization

Introduction:

In chapter 1 circuitry was examined, and it was seen that spreading depolarization events traveling in the downstream direction, with the direction of the Schaffer collaterals, tend to travel at a faster velocity than waves traveling in the upstream direction. In chapter 2 the effect of K^+ on a mouse hippocampal slice was examined and while it was seen that the propagation velocities followed the same trend, preferring a downstream direction, it was also seen that the directionality of the initial stimulus of K^+ could influence the propagation of an event as well.

This deceleration of the wave's velocities, seen in both chapter 1 and 2 indicate that this excitation may be diminishing, signifying a proceeding decrease in the potassium ions and glutamate once serving as fuel. This study explores the prospect that both potassium ion and glutamate levels decrease over time due to dissipation and cellular uptake. Therefore the deceleration of SD events may be a factor of time caused by diminishing ionic concentrations. The experiments in this chapter seek to eliminate the possibility of excitatory dispersion, therefore exploring the impact of time on SD event "priming". It is hypothesized that limiting the reuptake of K^+ into neurons affected by

Schaffer collateral depolarization will increase the velocity of wave propagation due to increased excitability.

Results:

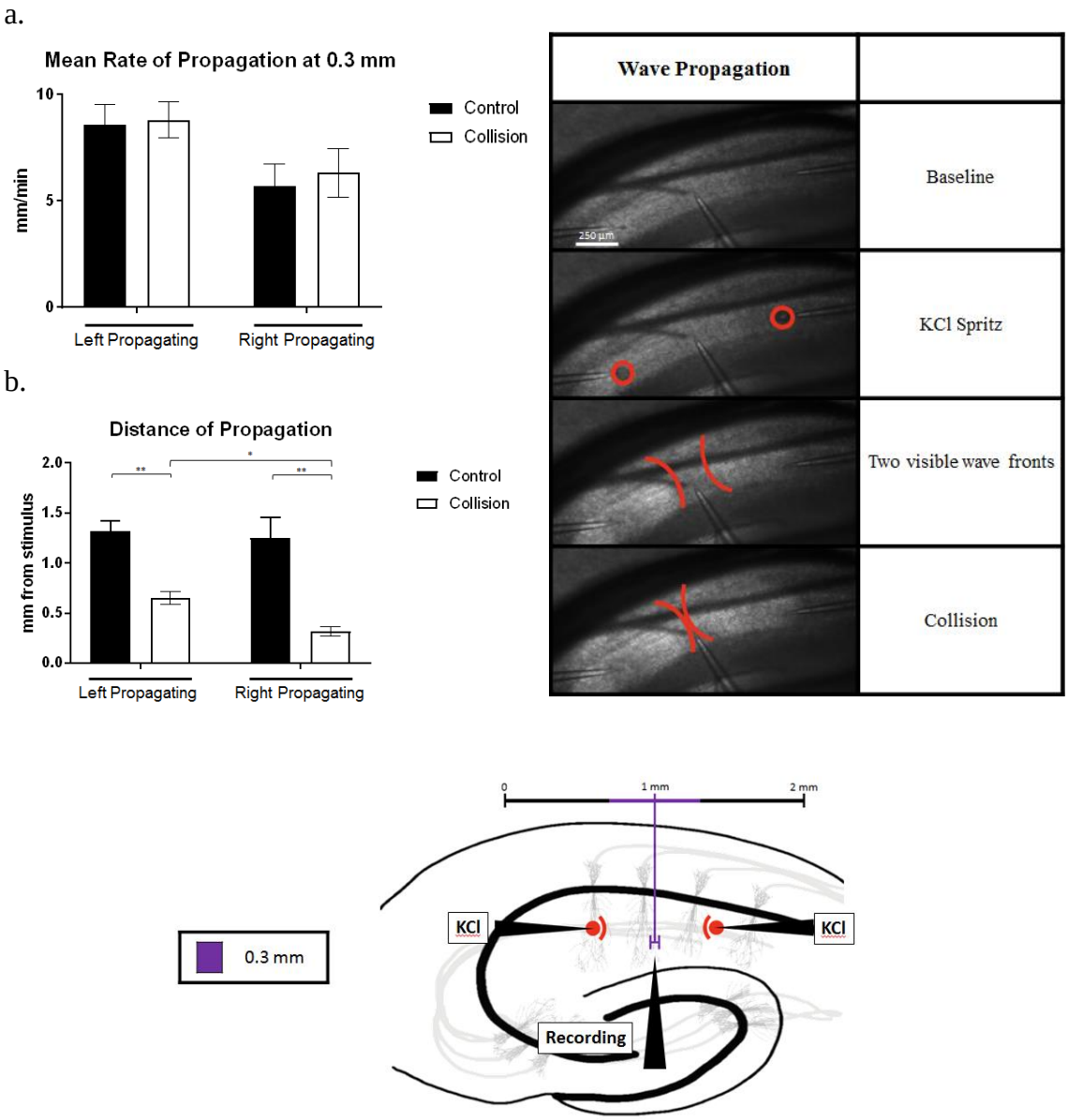

Figure 7: (a) Spreading depolarizations were initialized at opposite ends of the hippocampal slice initiating two events propagating towards the other. Rates were taken on both left and right sides of the stimulus at 0.3mm from the tip of the stimulus. All rates were taken over a time period of three frames (750ms in total) and each n (n=6) and were paired within the same slice. (b) Instigating two events colliding with each other significantly decreased the amount of space allowed for analysis within the slice therefore making it necessary to perform analysis at 0.3mm away from the stimulus instead of the previous 0.5 and 1.0mm distances.

Figure 7 shows the rates of SD events 0.3mm away from their initiating stimulus set on a collision course in the attempt to determine the amount of "priming" preceding a slice and, consequently the influence of SD events on each other. What is seen (Figure 7a) is a negligible amount of difference between control spreading depolarizations and colliding SD waves. This was possibly caused by the restrictions of space within the slice. While other velocity comparisons were done 0.5mm and 1.0mm away from the stimulus due to the differences seen in Figure 3c, the majority of waves in this experiment collided 0.3-0.5mm away from the stimulus (Figure 7b), preventing further analysis. This may or may not be the reason for the lack of difference between control and treated slices, but is likely the reason for such high propagation amounts seen in left propagating waves. The average rate of propagation in Figure 3c never exceeded 6 mm/min, thus this average is still fairly high in left propagating events compared to past studies. Consequently, the interactions between SD waves within the slice in reference to proceeding excitation should be further explored in a more applicable model. In this regard it has now been shown that the collision of two spreading depolarization events can occur within a slice and in doing so prevent further propagation of both sides. This and multiple other SD interactions have been shown optically within the gyrencephalic brain of a pig (Santos et al., 2014), including the most commonly seen semi-planar pattern, which has also been recreated in intact mouse and rat brains (Kaufmann et al., 2017). However, this does not give conclusive information on the influence of "priming" on coinciding SD events.

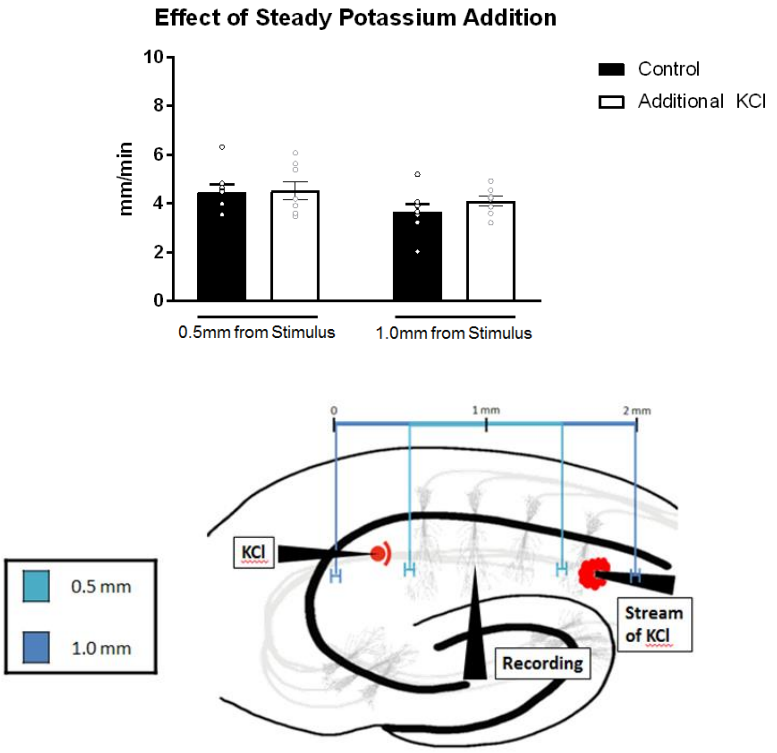

Figure 8: For a period of five minutes a pipette steadily expelling KCl was inserted into the edge of the hippocampus. Following the five minute period, SD events were stimulated towards the primarily added pipette using a right directional injection of KCl at the opposing edge of the hippocampus and were measured at 0.5mm and 1.0mm distances away from the stimulus. All rates were taken over a period of three frames (1350ms in total) and each n was paired in slice (n=8). Like similar experiments waves decelerated over distance. In comparison, treated waves, while still slowing over distance, did not slow as much as control waves.

Figure 8 displays the rate of SD events when provided a steady release of K^+ in an area of the slice opposite to the stimulus. What is seen upon initiation of the SD event is that treated waves had faster propagation rates 1.0mm out from the stimulus compared to control SD events. While both experimental designs still showed slowing propagation, the amount of deceleration in treated SDs was an average of 0.4mm/min less. This suggests that the velocity of SD events may not be able to keep up with the rapid reuptake of the potassium ions and glutamate released through axonal transmission

relying instead on lower levels of excitation as the wave propagates further from the stimulus.

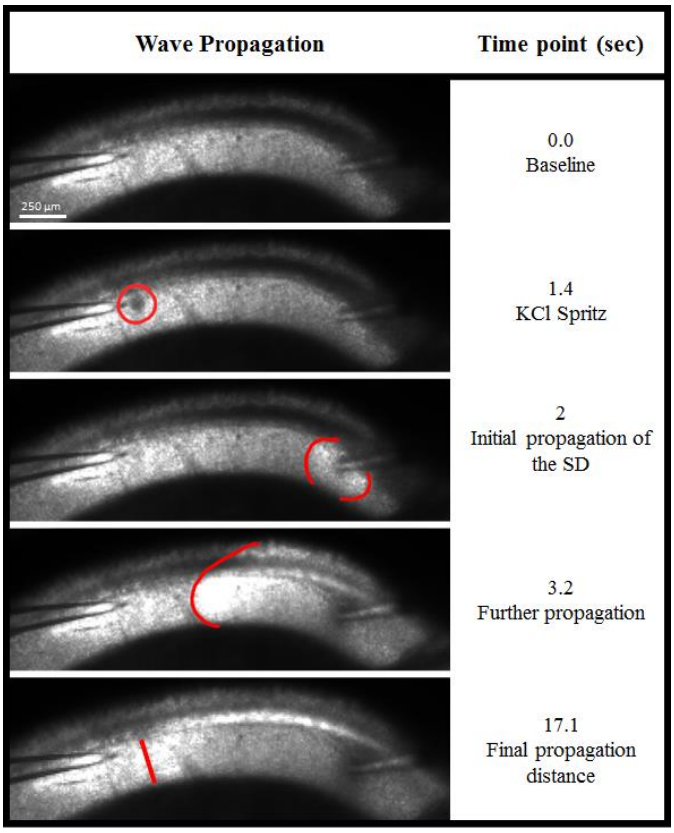

Figure 9: This SD event was stimulated using a right directional microinjection, and initiated using a pool of K^+ introduced over a time period of 5 minutes. As can be seen descending through the figure, 1.4 seconds after the baseline image, the active stimulus is introduced into the slice. 0.6 seconds later a wave can be clearly seen propagating from the pool of K^+ which continues propagating for over 15 seconds.

Figure 9 shows an SD event initiated not from the point of stimulation, but instead from 0.9mm away, propagating towards the stimulus. As in the previous experiment, a pipette providing a steady stream of KCl into the slice was placed opposite the stimulus and after 5 minutes the stimulus provided a microinjection of KCL. However, unlike the previous experiments, the reserve of potassium ions was verging on the precipitous

threshold of 8-10mM, therefore the axonal transmission was enough to initiate an event therefore eliminating the need for priming and the influence of time controlled reuptake and dissipation.

Conclusions:

In the first set of experiments the steady addition of KCl at the forefront of the wave increased the velocity of SD events over time and distance compared to untreated experiments. In the second experiment this was further tested and the important interaction of extracellular potassium ions and circuitry within the brain during SD events was demonstrated.

In healthy neuronal events potassium ions and glutamate are excreted and taken up quickly decreasing the amount of excitation "priming" an SD event. However in these experiments, it was shown that increasing the amount of chemical excitation to an insurmountable quantity not only increased the rate of propagation it also increased the prospect that an event would be initiated through axonal transmission alone. Therefore this demonstrated an event that relied not only on K^+ priming and the amount of priming occurring at the time, but also synaptic excitation introduced through the circuitry of the slice. This shows the important effect of both maintained excitatory threshold levels, influenced by potassium ions and glutamate, and neural circuitry within the brain on SD events.

Summary and Future Outlook

The primary goal of these studies was to examine factors that reduce threshold within neurons, therefore "priming" SD events to propagate more quickly throughout the brain. The trisynaptic circuit, K^+ stimulus directionality, and reduction of priming over the course of time, was examined in the scope of this goal.

The trisynaptic circuit was examined in chapter 1 to test the hypothesis that the wave of a spreading depolarization travels faster from the CA1 region of the hippocampus to the subiculum than from the CA1 region to the CA2 region because of the additional stimulation provided by the Schaffer collateral pathway. These studies revealed that SD events do in fact propagate more rapidly in the downstream direction, in line with the Schaffer collateral pathway and with the addition of bicuculline it was suggested that this was likely due to "priming" caused by the release of excitatory glutamate on downstream non-depolarized neurons. Within these studies there has been no evidence of prodromal excitation as it has been defined in the literature (Aiba & Shuttleworth, 2012). However, the evidence of "priming" shown throughout these experiments highly indicate the likelihood of present prodromal excitation. Further studies regarding this correlation should be performed with voltage-clamp recordings in order to distinguish between self-propagation and proceeding excitation during SD events.

In chapter 2, the effect of potassium ion "priming" was observed in a challenging view of standard hippocampal mouse slice stimulations to test the hypothesis that the effect of K^+ diffusion from a primary KCl stimulus is enough to increase the rate of propagation of an SD event. These studies revealed that in lieu of proper circuitry to promote SD propagation, diffusion of introduced K^+ is enough to increase the velocity of an SD event upstream through a slice. It was also seen that with the introduction of tetrodotoxin, thus removing action potentials, and in theory the circuitry of the slice, KCl induced events not only propagated, as seen in previous studies, self-regenerative priming alone increased the propagation rate of the event in the downstream direction. This indicates that there may be additional effects of either removal of the typical GABA detainment during an SD event or undiscovered heightening of SD events through differential pathways such as NMDA activation of glutamate release.

The dependency of priming on time in the slice was challenged in chapter 3 and was found as the reason for decreases in velocity correlating with distance from the stimulus. The hypothesis that an introduced pool of K^+ within the slice would propel the propagation of waves in an increased velocity towards the "priming" pool was both shown and heightened by the induction of an SD event, not from the stimulus, but from the benign pool of K^+. The unanticipated reverse propagation of the SD event invigorated the potential indication of K^+ priming introduced through hippocampal circuitry.

It is significant to note that there are multiple hypotheses on the role of potassium ions during SD events. But even these hypotheses agree that K^+ likely has a downstream

"priming" effect on the neurons. How this effect arrives downstream, however, has been a matter of debate. It was hypothesized that K^+ is not the extracellular factor that aids SD through the slice as it has been shown that outer K^+ concentrations are not always seen to increase when the initial depolarization shift occurs but only soon after the SD peak is seen (Herreras & Somjen, 1993a, pp. 287, 290). This indicates that while K^+ in the extracellular space contributes to the full development of the spreading depolarization, by not only promoting the release of glutamate, but also preventing neuronal recovery, this extracellular K^+ may play less of a role in the actual propagation of the event. Since this discovery in 1993, the role of K^+ in SD events has been deliberated, however, multiple studies have discussed the possibility that K^+ is still significantly involved through chemical synapses introduced through glial to neuronal cell gap junctions (Herreras, Largo, Ibarz, Somjen, & Rio, 1994; Mori, Miller, & Tomita, 1976). It has long been known that astrocytes, a type of glial cell, are highly permeable to potassium ions, and in being so, protect the nearby neuronal cells from excess K^+ in the extracellular space (Gardner-Medwin, 1981; Kandel et al., 2000). What is still being discovered, however, is the amount of influence astrocytes have on neurons and their signals. What is known, is that astrocytes form their own networks throughout the brain sharing extracellular ATP and glutamate and that astrocytes and neurons share chemical synapses through gap junctions (Cornell-Bell, Finkbeiner, Cooper, & Smith, 1990; Fields & Stevens-Graham, 2002, p. 3). In 2008, a study additionally showed that astrocytes released glutamate, activating NMDA receptors within neurons of the hippocampus, all through non-synaptic communication (Navarrete & Araque, 2008). It has been suggested that once SD has begun its propagation through tissue astrocytes then communicate their abundance of K^+

to neurons though this non-synaptic gap junction communication hence "priming" neurons ahead of the wave (Herreras et al., 1994; George G. Somjen, 2001, p. 1082). However, while these studies are important to note, the experiments explored throughout this study have shown that K^+ does play a highly influential role on both the initiation of SD events but also, even more so, on the propagation of these waves.

Limitations

Animal models

There is debate within the scientific community over the efficacy of animal models as there is often a large disconnect between the models and the clinical trials they seek to recreate as well as general researcher bias and error leading to very few treatment effects being seen in animal's human counterparts. This, in turn, extends to the use of animal models within SD studies, especially in the comparison between lissencephalic and gyrencephalic animal models. In fact, prior to 2002, it was highly debated whether or not spreading depolarizations occurred in humans at all, leading to a loss of advancement in the field (Strong, 2002). Now evidence of SD events during varying neurological injuries in humans is well established, (Dohmen et al., 2008; Dreier et al., 2006, 2009; Fabricius et al., 2006) and it has been observed that the smooth brains of rats are able to show the same patterns of propagation as a complex brain with gyri and sulci, (Kaufmann et al., 2017; Santos, Sánchez-Porras, Sakowitz, Dreier, & Dahlem, 2017) while exhibiting very similar characteristics as those found in the brains of humans. Therefore the studies of spreading depolarizations within mouse brain slices should contribute large amounts of information about the phenomenon of SD that is translatable to clinical settings.

Acute Hippocampal Slice Preparations

A large confound of using 350 μm mouse hippocampal slices is the lack of vasculature throughout the slice, therefore removing blood perfusion, and the deficiency of space within the slice to do further analysis of changes in velocity over distance. The ability, however, to confine SD events within an area of known synaptic circuitry and control variables such as temperature, perfusion rate and known neuronal density makes the mouse hippocampal model ideal for these studies. The hippocampal region is also known to have much fewer glial cells than in most areas of the brain, limiting the amount of reuptake of "priming" elements (G. G. Somjen, 2001). In future studies it would be useful to examine the contribution of circuitry to propagation rate within an *in vivo* whole brain model.

Neurological Pathology

Another confound in this group of experiments is the lack of damaged brain slices. While SD events have been found in multiple neurological conditions such as stroke, traumatic brain injury, migraine and hemorrhaging. The slice model here represents tissue that is well perfused with oxygen and glucose, therefore only indicative of a non-pathological model. Within chapter 3, a model is shown that may be similar to what could occur to a stroke patient that has an SD inducing stimulus introduced outside of the ischemic penumbra, but even still the neurons within the K^+ perfused tissue are likely not similar concentrations as would be seen within a clinical setting. Future studies

regarding the mechanisms of SD propagation should be studied with a more detailed model of damaged tissue as would be seen during most SD events. The suggested model for this study was used here (Reinhart & Shuttleworth, 2018). In a past study the difference between normoxic and hypoxic optical SD propagation rates saw an increase of up to 2mm/min in hypoxic slices (Aitken, Tombaugh, Turner, & Somjen, 1998). Thus, it would be expected that a slice with limited perfusion would see similar rate increases followed by limited, or no, recovery due to inability to repolarize. If so, this comparison between increasing propagation velocities and damaged slices may be a correlation worth exploring within prevention means. It has been shown that spreading depolarization can be blocked with the addition of ketamine, an NMDAR antagonist, in both animal and human models, (Hernández-Cáceres, Macias-González, Brožek, & Bureš, 1987; Sakowitz et al., 2009) but only at very high dosages. Instead the application of ketamine with the purpose of slowing the propagation may be beneficial to future studies in order to examine the correlation of damage to wave velocity. In conjunction with this, ciliary neurotrophic factor (CNTF) has been shown to activate astrocytes increasing neuronal survival at central nervous system focal injuries, (Albrecht, Dahl, Stoltzfus, Levenson, & Levison, 2002) and to increase the threshold of SD resulting in a decrease in SD incidence (Seidel Jessica L. et al., 2014). This indicates that the controlled application of CNTF, may serve to slow, if not eliminate, the propagation of spreading depolarizations. Both ketamine and CNTF applications need further exploration regarding clinical consequences, however, they are both promising applications for the exploration of SD propagation velocities and neuronal damage.

Conclusion

Overall the experiments in this study highlight the importance of potassium ions and glutamatergic excitation during an SD which is already well regarded in the field, however, this study also introduced the steep importance of neural networks to the propagation and excitation of SD events. In neurological conditions, such as stroke, a large pool of potassium ions and glutamate, present as a peri-infarct core, may simply be waiting for upstream activation of neurons in order to initiate an SD event. In 2015, a study found that SDs could be initiated from focal ischemic lesions with the introduction of a somatosensory stimulus, in this case, the stimulation of whiskers on a mouse (von Bornstädt et al., 2015). It was hypothesized that this was a result of increased oxygen demand brought about by neuronal stimulation, but the experiments in my study show that it may also be that the depolarization of neurons introduce a synapse to the damaged penumbra sending it over threshold and thereby initiating an event. This suggests that in a clinical setting, heightened stimulation of any population of neurons upstream of damaged tissue may be enough to introduce an SD, in turn spreading the amount of damage and increasing the likelihood of further SDs. This possibility needs to be considered and studied further in both *in-vivo* and clinical settings.

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