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The Effect of pH on Synaptic Transmission at the Neuromuscular Junction in *Drosophila melanogaster*

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Dr. David Weisrock, Director of Graduate Studies

The Effect of pH on Synaptic Transmission at the Neuromuscular Junction in *Drosophila melanogaster*

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Arts and Sciences
at the University of Kentucky

By

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Lexington, Kentucky

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ABSTRACT OF THESIS

The Effect of pH on Synaptic Transmission at the Neuromuscular Junction in *Drosophila melanogaster*

Synaptic transmission is the main avenue of neuronal communication and can be affected by a multitude of factors, both intracellularly and extracellularly. The effects of pH changes on synaptic transmission have been studied for many years across many different models. Intracellular acidification at the presynaptic terminal is known to occur with increased neuronal activity and can also occur in pathological conditions. The effects of these pH alterations are therefore an important area of study. Here, intracellular acidification using either propionic acid or the ammonium chloride pre-pulse technique was examined for the effects on both spontaneous and evoked synaptic transmission at the neuromuscular junction in larval *Drosophila*. The fly NMJ is glutamatergic and is used as a model of graded synaptic transmission. Propionic acid is shown to increase spontaneous quantal event frequency while also attenuating evoked transmission and depolarizing the cell. Ammonium chloride withdrawal has more diverse and complex effects that are shown to be dose- and condition-dependent. These studies are significant in further developing a model of the effects of intracellular acidification on evoked and non-evoked synaptic transmission.

KEYWORDS: pH, Propionic Acid, Synaptic Transmission, Ammonium Chloride, *Drosophila*, Electrophysiology

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CHAPTER 1. BACKGROUND

1.1 Synaptic Transmission and Vesicular Dynamics

One of the major avenues of communication between cells relies on synaptic transmission. This is the mechanism by which cells interact through chemical messengers that are released via synaptic vesicle fusion from a presynaptic terminal across the synaptic cleft to have some effect on postsynaptic receptors. Synaptic vesicles are filled with neurotransmitter in the cycle of vesicle recycling involving neurotransmitter release, endocytosis, and refilling. It has been shown that vesicular packaging of glutamate is driven by VGLUTs which exchange glutamate for protons inside the vesicle (Juge et al., 2010). The gradient is maintained by H^+-ATPase pumps that keep the interior of vesicles acidic to allow for greater neurotransmitter packaging capacity (Figure 1.1).

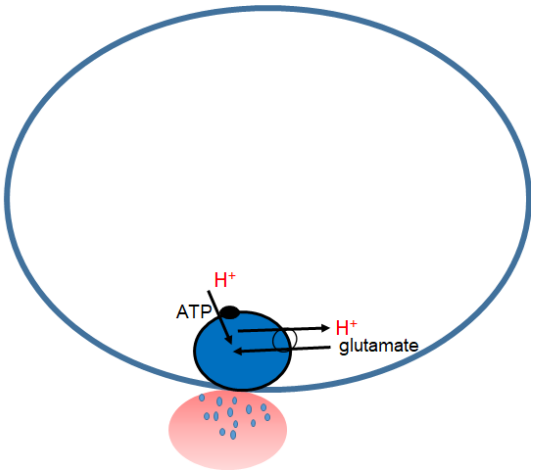

Figure 1.1 Vesicular packaging

A vesicle (in solid blue) docked at the presynaptic membrane. A H^+-ATPase pump allows for the accumulation of protons inside the vesicle, and this gradient permits packaging of glutamate into the vesicle using the VGLUT transporter that exchanges protons for glutamate.

Interestingly, it has been shown in *Drosophila* that VGLUT on the vesicular membrane also plays a role in acid efflux in response to acidification of the presynaptic terminal (Rossano et al., 2017). When located at the plasma membrane during exocytosis acid efflux is enhanced, and inhibition of vesicle fusion reduces the rate of acid efflux. This proposes a dual purpose of VGLUT as both a vesicular neurotransmitter packager as well as a regulator of intracellular pH (pHi).

Docking of synaptic vesicles at the presynaptic terminal involves a variety of proteins, including the SNARE proteins v-SNARE (vesicular) and t-SNARE (target membrane). The v-SNARE proteins on the vesicle bind t-SNAREs on the presynaptic membrane, forming a complex that holds the vesicle in place at the membrane. However, fusion of the vesicular and plasma membrane is inhibited by the complexin protein (McMahon et al., 1995). During an action potential or graded membrane depolarization in a neuron, depolarization arriving at the presynaptic terminal allows for the opening of voltage-gated calcium channels, causing an influx of calcium. Synaptotagmin is a calcium sensor associated with the SNARE complex, and upon sensing the influx of calcium synaptotagmin removes the inhibition from complexin (Schiavo et al., 1997). This allows the membranes to fuse and results in exocytosis of the packaged neurotransmitters. These proteins along with many others in this complex process likely function best at optimal pH levels. In rats, it has been shown that the voltage-gated calcium channels that mediate calcium influx are affected by intracellular pH (pHi) alterations and produce differential calcium flux in alkaline or acidic environments (Tombaugh & Somjen, 1997). Thus, pH within a cell must be tightly regulated to allow for the process of synaptic transmission to function effectively.

In the process of vesicle recycling, the three-pool model has been widely accepted (Rizzoli & Betz, 2005). This model suggests that there are three different pools or groups of vesicles that are differentially utilized in the process of synaptic transmission. The first pool is classified as the readily releasable pool (RRP), and the vesicles in this pool undergo synaptic vesicle fusion and neurotransmitter release the most rapidly following stimulation. The second pool, the recycling pool, is suggested to consist of vesicles that recycle during intermediate levels of stimulation such that the pool is constantly being replenished with newly filled vesicles in order to maintain neurotransmitter release in response to stimulation. The last pool is called the reserve pool; this group of vesicles consists of those that only fuse to the plasma membrane and release neurotransmitter during strong and prolonged bouts of stimulation.

Each vesicle pool consists of different numbers of vesicles. The RRP is the smallest, consisting of only 1-2% of the total vesicles at the presynaptic terminal. These vesicles are thought to be located in the active zone of the presynaptic membrane. At the active zone, these vesicles remain docked and ready to fuse following stimulation in order to act upon the postsynaptic membrane which is being targeted during synaptic transmission. The recycling pool is generally larger, approximately 5-20% of vesicles. In *Drosophila*, it has been shown that this recycling pool is distinct from the reserve pool and is largely located in the peripheral sections of the terminal bouton (Kuromi & Kidokoro, 1998). The reserve pool is thought to be the largest of the vesicle pools, consisting of up to 90% of all vesicles in the terminal. It has further been shown in *Drosophila* that these vesicle pools undergo preferential replenishment, in which vesicles that are retrieved from the plasma membrane

first refill the recycling pool and, once it is full, then refill the reserve pool of vesicles (Kuromi & Kidokoro, 1998).

Drosophila have been estimated to have approximately 84,000 vesicles at the neuromuscular junction (NMJ) according to depletion studies using tetanic stimulation (Delgado et al., 2000). Using cytochasalin D, an inhibitor of reserve pool vesicles, it was estimated that there are around 12,000-16,000 vesicles in the *Drosophila* recycling pool. The RRP is very small in comparison; approximately 300 vesicles comprise this pool. The remainder of vesicles make up the reserve pool, approximately 68,000-72,000 vesicles. The majority of *Drosophila* synaptic transmission is noted to be comprised of the processes of exo- and endocytosis following fusion of the vesicle to the presynaptic membrane. However, knockout of a protein required for endocytosis still allows for synaptic transmission following vesicle depletion, suggesting that some small percentage of vesicles may employ a "kiss-and-run" mechanism of neurotransmitter release (Gandhi & Stevens, 2003; Verstreken et al., 2002). In this manner, vesicles transiently dock and bind the plasma membrane, opening a small pore to release neurotransmitter. These vesicles are then rapidly removed from the plasma membrane rather than being incorporated into it, avoiding the need for endocytosis.

1.2 Synaptic Transmission and pH

The following formula $pH = -\log(\gamma_H[H^+])$ defines pH, a measure of the acidity or alkalinity of a solution. The activity coefficient of H^+ is γ_H and is normally approximately 0.83 (Putnam, 2001). Due to the pH-sensitive nature of many proteins, all cells must regulate pH to function properly. Regulation of pHi is achieved through multiple mechanisms that the cell can employ in response to changes in its environment. This

includes both cytoplasmic pH as well as regulation of pH in intracellular organelles such as vesicles and mitochondria which may be different than pHi. One of the most basic mechanisms by which pH is regulated is through buffering, in which weak acids or bases in solution can bind protons and limit pH changes. Other mechanisms include exchangers or pumps that move protons across membranes in response to changes in pH or when maintaining a gradient, such as the necessary proton gradient required for ATP production in the mitochondria. Organelles such as lysosomes maintain acidic environments that are optimal for enzymatic degradation of its targets. The cell can also regulate pH through H^+ metabolism or flux of acids or bases across its membrane through transporters. Specifically, active transport mechanisms involved in the regulation of pH consists of cation-H^+ exchangers, bicarbonate transporters, proton pumps, or weak base cotransporters or exchangers (Putnam, 2001; Ruffin et al., 2014).

One of the most well-known exchangers is the sodium-hydrogen exchanger (NHE) that pumps out one proton in exchange for influx of one sodium ion across the cell membrane in response to acidification (Mahnensmith & Aronson, 1985). The NHE can be activated by acidification via binding of protons to an allosteric site on the exchanger that increases its activity (Uria-Ayellanal & Robertson, 2014). *Drosophila* have been shown to have three NHE family genes that are expressed throughout the body and play a role in the regulation of pHi (Ahearn et al., 2001; Giannakou & Dow, 2001).

The acidity or alkalinity of a cell or its environment is extremely influential on multiple cellular processes, such as metabolism, cell shape and motility, ion channel conductance, growth, and more (Aronson et al., 1982; Busa & Nuccitelli, 1984; Campos de Carvalho et al., 1984; Edmonds et al., 1995; Putnam 2001; Trivedi & Danforth, 1966).

The tightly regulated processes of synaptic transmission are notably affected by alterations in intracellular or extracellular pH, which may have major implications for modulating cell communication (Caldwell et al., 2013; Chen et al., 1998; Drapeau & Nachshen 1998; Ohki & Arnold, 1990; Rocha et al., 2008; Trudeau et al., 1999). However, the mechanisms of action by which these alterations occur remains unclear. It is possible that both vesicular packaging and vesicular fusion and release into the synaptic cleft are impacted. Additionally, the interaction of calcium in conjunction with changes in intracellular pH is not fully understood. In the snail, injection of calcium into neurons leads to rapid acidification, and it is suggested that this is due to Ca^{2+}/H^{+} exchange across the plasma membrane as well as the mitochondria (Meech & Thomas, 1976).

In *Drosophila*, inhibition of plasma-membrane calcium ATPase (PMCA) that exchanges intracellular calcium for extracellular protons slows the rate of intracellular acidification at the presynaptic terminal following electrical stimulation of a motor nerve (Caldwell et al., 2013). The current focus is the impact of pH alterations on evoked as well as spontaneous vesicle fusion events and its potential mechanisms of action. In studies investigating the impact of pHi alterations on spontaneous vesicular fusion events at the NMJ, it has been shown that a more acidic environment can increase fusion and subsequently increase synaptic transmission (Caldwell et al., 2013).

Two common methods utilized in studying the effects of decreased pHi on synaptic transmission include the use of propionic acid or ammonium chloride. Due to its ability to easily pass through cell membranes, propionic acid can rapidly acidify the intracellular environment of a neuron. Ammonium chloride, on the other hand, may be used to create an alkaline environment in the cell after an incubation period, allowing for compensatory

mechanisms in the cell to become active. Upon rapid removal of ammonium chloride, the compensatory mechanisms are still active for a short period, causing transient acidification intracellularly. In the current research lab conducting this thesis project, it has been shown previously in the crayfish model (Cooper et al., 2015) that this pre-pulse technique has increased both spontaneous and evoked nerve transmission. In contrast, acidification following propionic acid administration was shown to increase spontaneous vesicular fusion but reduce evoked responses. This indicates that the method of cellular acidification may impact the results obtained in such studies.

Use of genetically encoded pH indicators has shown the nerve terminal of the *Drosophila* NMJ undergoes acidification during stimulation *in vivo* (Rossano et al., 2013), identifying it as a useful model for the study of pH effects in synaptic transmission through endogenous pH changes rather than applied acids or bases. These pH shifts during high activity are predicted to have some effect on the processes underlying synaptic transmission, but the extent of its role during normal nerve firing is yet to be elucidated (Chesler, 2003; Makani & Chesler, 2010; Ruffin et al., 2014).

While propionic acid and ammonium chloride can be utilized to modulate pHi, the effects of these compounds on extracellular pH (pHo) and the effects on postsynaptic receptors at the motor endplate must also be considered. It is still not yet fully understood how pH affects the activity of glutamatergic receptors. Currently it is not feasible to alter pHi of the motor neuron independently from the muscle fibers on which it synapses. It is possible that acidification using propionic acid or removal of ammonium chloride could also alter glutamate receptor activity and thus have an effect on recordings of activity at the motor endplate independent of the effects of pHi changes at the presynaptic terminal.

In humans, it has been shown that the GluN1-GluN2A NMDA receptor is negatively regulated by protons such that acidification inhibits receptor opening by altering the receptor conformation (Zhang et al., 2018). It is not known how the quisqualate subtype receptors at the *Drosophila* NMJ (Lee et al., 2009) are affected by pHo or pHi.

In addition to the potential direct effects of pH on protein-protein interactions with vesicle fusion and recycling, packaging, ion channels, exchangers, pumps, and post- and pre-synaptic receptors, there is a direct effect on the resting membrane potential (Chesler, 2003). The mechanism of the change in resting membrane potential remains to fully resolved, but regardless of the mechanism, the change in membrane potential will have an impact on the activity of the Na-K pump, voltage-gated ion channels, driving gradients of ions, and potentially on vesicle fusion and receptivity of autoreceptors and postsynaptic receptor function. It is known that reducing pH of the bathing saline from 7.1 to 5 will depolarize the skeletal muscle membrane potentials of larval *Drosophila* and crayfish (Badre et al., 2005; Bierbower & Cooper, 2010). It should also be noted, however, that altering the pH in the bathing saline does not necessarily alter the pHi as protons do not passively pass through the membrane.

Consequences of heightened neural activity such as that seen during seizures may include transient alterations in pH due to production of CO_2 from highly metabolically active cells (Chesler, 2003). It is of interest to study the potential effects of altered pH on synaptic transmission that may apply in such cases. During the postictal phase, the period of time after a seizure occurs where cognition remains impacted, it is possible that seizure side effects may partially be due to lasting effects caused by changes in synaptic efficacy due to changes in pH. The impact of pH may also be notable in cases where breathing

difficulties and reduced ventilation are common symptoms, such as chronic obstructive pulmonary disease (COPD). In such cases CO_2 concentration may increase in the blood due to lack of proper ventilation and gas exchange across lung alveoli, thereby reducing pH and affecting cellular functioning. This may also be relevant in the use of CO_2 as an anesthetic, commonly used in many research models such as mice or *Drosophila*. Thus, the impact of extracellular and intracellular pH on synaptic transmission remains an important area of study.

1.3 *Drosophila* as a Model

The *Drosophila* model has widespread use for research in biology (Schneider, 2000; Shin et al., 2018; Yamaguchi & Yoshida, 2018). The fruit fly is relatively inexpensive and fly lines may be easily sustained for generations with low maintenance required. Large-scale production of flies is also possible as *Drosophila* have short lifespans and high turnover rates, such that multiple generations may be studied over a short amount of time (Hales et al., 2015). In addition, there is much known about the physiology and genetics of the *Drosophila* model that may be translated into knowledge useful for human research. Many fundamental biological concepts emerged from research that began with the fruit fly (Bellen et al., 2010). Furthermore, the *Drosophila* model is highly amenable to processes of genetic modification, making it an ideal model to use in genetic research in studies that wish to alter the expression of specific genes or utilize insertion of transgenes (Venken et al., 2016). The *Drosophila* NMJ is a common and useful model for the investigation of synaptic function (Ugur et al., 2016). The NMJ is glutamatergic and utilizes ionotropic glutamate receptors at the postsynaptic density on the body wall muscle fiber being targeted. Additionally, the synapses are large and recording from them is

relatively easy. Due to the small size of the muscle fibers used in electrophysiological recordings, it can be easier to address and evaluate quantal events. The *in situ* larval *Drosophila* NMJ can be maintained in physiological saline for days, making the preparations amenable for long-term studies (Ball et al., 2003). Furthermore, as much of the structure and function of the *Drosophila* larval NMJ is conserved among species, research findings utilizing this model can often be translationally applied. The layout and structure of the *Drosophila* nervous system is also extremely well-classified due to its relative simplicity. Neurons and their associated muscle fiber connections are identifiable and can be specifically targeted from preparation to preparation; this level of classification is lacking in many more complex models. Another beneficial aspect of the *Drosophila* neuromuscular junction as a model is its non-spiking nature. Excitatory junction potentials (EJPs) at the motor endplate are graded, with EJP amplitude correlating with levels of vesicular fusion and neurotransmitter release at the presynaptic membrane. This is advantageous for studies involving quantal analysis (Frank et al., 2013).

Previous work by Caldwell et al. (2013) has explored the effects of acidification on synaptic transmission by use of ammonium chloride and propionic acid in *Drosophila*. Caldwell et al. also examined the interaction of pHi alteration and calcium flux during synaptic transmission. This study acts as the foundation of the current thesis research. Their results indicated an increase in spontaneous vesicular fusion events upon application of propionic acid. Using the removal of ammonium chloride to allow for the study of miniature excitatory junction potentials (mEJPs) during transient compensatory acidification, results were mixed; some preparations showed an increase in mEJP frequency while others showed no change. The current research sought to expand upon

these findings by examining effects of pHi modulation on both spontaneous and evoked synaptic transmission. To study evoked activity at the NMJ, synaptic depression studies were utilized. Synaptic depression is characterized by analysis of EJP amplitude reduction over time during high stimulation. This amplitude reduction can result from impaired vesicular packaging and fusion such that less neurotransmitter is released across the synaptic cleft to act on the motor endplate. The impact of pHi on vesicular packaging and fusion can thus be partially addressed by such studies.

1.4 Summary of Project Aims

In Chapters 1 and 2 of this thesis I address the hypothesis that modulation of pHi affects both spontaneous and evoked synaptic transmission at the NMJ in *Drosophila melanogaster* via alterations in vesicular packaging and fusion. Furthermore, it is hypothesized that the effects of pHi changes are dependent upon the method of modulation used. To study these hypotheses, I utilized electrophysiological recordings from the *Drosophila* body wall muscle to measure quantal events and modulated pH with application of either propionic acid or ammonium chloride. I set out to determine the following:

1. the effects of propionic acid on spontaneous synaptic transmission
2. the effects of ammonium chloride withdrawal on spontaneous synaptic transmission
3. the effects of propionic acid on synaptic depression

The design of this thesis is as such: to provide a general background of synaptic transmission and its relationship with pHo and pHi in the course of Chapter 1. Chapter 2 is formatted as a publication of the research conducted in exploring the interplay between pH

modulation and synaptic transmission. Chapter 3, the final chapter, is an overview of the thesis as a whole, the significance of this research, a discussion of further questions to pursue, and the learning objectives met during the course of this project. It will also include a description of two additional research projects I was involved in during this program: one conducted on examining the effects of the drug tricaine mesylate (MS-222) on synaptic function in the *Drosophila*, crayfish, and crab model systems; and another in which a STEM-based module was developed as an approach for modeling and engineering circulatory system models for use in the classroom to encourage and enhance learning.

CHAPTER 2. THE EFFECT OF ACIDIFICATION AT THE NMJ

2.1 Introduction

Factors which alter synaptic transmission are diverse, from external factors (i.e. neuromodulators, temperature) to intracellularly initiated actions such as metabolism. Increased electrical activity of a neuron is known to result in intracellular acidification, which is a decrease in intracellular pH (pHi). The decrease in pHi can then influence the local extracellular pH (pHo). When electrical activity of a neuron is induced, resulting in a change in pH, it is assumed that the acidification is partly a result of the rise of CO_2 from cellular metabolism and the biochemical reaction of CO_2+H_2O producing HCO_3^- and H^+. The rapid diffusion of CO_2 across bilipid membranes can influence not only the intracellular environment but also affect the extracellular space. Furthermore, proton (H^+) flux across membranes can occur by various channels, exchangers, and pumps. Even with endogenous buffering of pHi and pHo to maintain cellular homeostasis, the cell and its environment undergo transient pH changes in relation to cellular activity (Boron 2004; Casey et al., 2010; Willoughby & Schwiening, 2002). The consequences of pH alteration and its effect on synaptic communication have been investigated for several years in various synaptic models (*Drosophila* NMJ- Badre et al., 2005; Caldwell et al., 2013; Crayfish NMJ- Bierbower and Cooper, 2013).

In addressing the effects of pHo and pHi alterations on synaptic transmission, several indices are used. These include alterations in membrane potential, effects on evoked transmission, the rate of occurrence of spontaneous quantal events, as well as ion and proton changes with electrical activity. Each of these indices can provide information on the mechanism of action for the effect of pH. Alterations in spontaneous quantal event

frequency upon acidification or alkalization, for example, may indicate an impact on vesicular fusion and neurotransmitter release. Effects on membrane potential could suggest pH sensitivity of sodium and potassium channels. In rat hepatocytes, it was found that altering pHi independent of pHo can alter the cell membrane potential, with its mechanism likely via alterations in the conductance of potassium across the plasma membrane (Fitz et al., 1989). Evoked transmission effects could be explained by both pre- and postsynaptic mechanisms. Changes in membrane potential can affect thresholds for voltage-gated channel opening and closing, and pHi changes may affect vesicular packaging of neurotransmitters or the ability of vesicles to dock at the presynaptic membrane and release neurotransmitter. Postsynaptic effects may include alterations in the sensitivity of postsynaptic receptors such that the effects of neurotransmitter binding are enhanced or suppressed. It may also be possible that pH changes affect ionic flux through voltage-gated channels or leak channels. Since large-scale, long-term pH changes are not generally seen during high neuronal activity, it is unlikely that pH drops enough to significantly impact the function of enzymes in the synaptic cleft that break down neurotransmitter. The transient alterations in pH, however, may impact cellular processes. For presynaptic effects, it has been shown that endocytosis can be affected by pH changes as well as vesicular packaging and calcium channel opening (Goh et al., 2011; Tombaugh & Somjen, 1997; Ybe et al., 1998). However, the full effects of transient pH changes on synaptic transmission remain to be completely revealed.

There are multiple approaches used to measure pH intracellularly. For large cells, intracellular microelectrodes can be used. However, with the advent of pH indicators that are able to be genetically expressed to cell subtypes or to the surface of cells where

postsynaptic receptors are located, the understanding of pH changes within cells and even in the synaptic cleft between cells has been greatly enhanced. Using genetically encoded pH indicators (GEpHIs), it has been demonstrated in *Drosophila* that high stimulation of the motor nerve rapidly reduces intracellular pH from 7.30 to approximately 7.14 (Rossano et al., 2013). Chemical pH indicators have also been used but can be difficult to apply (Thomas et al., 1979).

The *Drosophila* neuromuscular junction (NMJ) is a commonly used model that is extremely useful for studies on synaptic transmission. The NMJ is readily accessible, with large synapses and identified muscle fibers to record from. Due to the small size of the muscle fibers, the measure of quantal responses is relatively easy. Additionally, genetic manipulation is commonly used in *Drosophila*, making it useful for studies involved in genetic research or for the use of such things as GEpHIs (Rossano et al., 2013). In addition to pH, ionic concentration can also be quantified using genetically encoded sensors or use of electrodes. The *Drosophila* NMJ allows for analysis of membrane potential and spontaneous as well as evoked synaptic transmission at the postsynaptic muscle, in addition to tracking of vesicle dynamics within the presynaptic terminal (Kuromi & Kidokoro, 1998; Ugur et al., 2016). Furthermore, due to the graded nature of excitatory junction potentials (EJPs) at the motor endplate, EJP amplitude can be correlated with amount of neurotransmitter released presynaptically (Cooper et al., 1995; Kurdyak et al., 1994).

There has been some research using the *Drosophila* model in the investigation of the effect of pH on synaptic transmission (Caldwell et al., 2013; Rossano et al., 2013; Sandstrom, 2011). Research by Sandstrom has found that modulation of pHo affects both spontaneous and evoked excitatory junctional currents (EJCs). Application of HCl was

used to acidify the NMJ extracellular space to 5.0 pH units and was found to significantly reduce the amplitude of EJCs. The finding that evoked rather than spontaneous EJCs were more significantly impacted by the pH change indicates that reduction in vesicular fusion and release of neurotransmitter is the main effect of acidification rather than alterations in the amount of neurotransmitter in each vesicle. Interestingly, alkalization of the nerve terminal to a pH of 8.5 was shown to initially increase EJC amplitude, followed by long-term synaptic depression with greater reduction upon washout. Caldwell et al. (2013) further investigated the effects of acidification at the *Drosophila* NMJ by specifically targeting its effects on vesicular dynamics. Acidification was achieved by two methods: application of propionic acid and withdrawal of ammonium chloride. Propionic acid easily crosses the plasma membrane and can acidify the interior of the cell. Ammonium chloride application and incubation is known to alkalize the interior of the presynaptic terminal, engaging compensatory mechanisms in the cell. Upon rapid withdrawal during washout, the compensatory mechanisms are still active, leading to transient acidification of the intracellular space. Using propionic acid, Caldwell et al. (2013) found that miniature endplate potential (mEPP) frequency was significantly increased, while washout led to a decrease in mEPP frequency. The membrane potential was also found to rapidly hyperpolarize upon propionic acid administration. Inhibition of the sodium-hydrogen exchanger (NHE), which is used to regulate pHi, caused an even greater increase in mEPP frequency for a longer duration, showing that blocking compensatory mechanisms increases the effects of intracellular acidification on synaptic transmission. Use of the ammonium chloride pre-pulse to acidify the presynaptic terminal showed more mixed

results. Only some preparations showed an increase in mEPP frequency, and frequencies were not affected by NHE inhibition.

In this study, the effect of altering pHi by propionic acid and ammonium chloride on spontaneous synaptic activity was investigated in various experimental paradigms. In addition, the effect of pH on the rate of evoked synaptic depression was investigated. These studies are significant in corroborating previous reported findings with similar techniques as well adding to the effect of synaptic depression with heightened activity in relation to pH.

2.2 Methods and Materials

2.2.1 Fly Stock Maintenance

Flies utilized were Canton-S (CS) strain *Drosophila melanogaster* obtained from Bloomington Fly Stock Center that have been maintained in the lab for many years. Third instar larvae were used for all electrophysiological studies. Flies and larvae were fed a standard cornmeal-agar-dextrose-yeast medium diet, housed at temperatures between 21 and 23° Celsius, and maintained on a 12:12 light:dark cycle.

2.2.2 Saline and Pharmacology

For all dissected preparations, a modified hemolymph-like saline 3 (HL3) [in mM: 1.0 $CaCl_2 \cdot 2H_2O$, 70 NaCl, 20 $MgCl_2$, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, 25 5N,N-bis(2-hydoxyethyl)-2-aminoethanesulfonic acid (BES)] at pH 7.1 was utilized as a bathing medium (deCastro et al., 2014; Stewart et al., 1994). For studies using propionic acid (PA), a stock solution of 13.26 M/L PA was utilized and diluted with HL3 saline (20 mM PA: pH 5.70; 40 mM PA: pH 4.75). All compounds were obtained from Sigma-

Aldrich (St. Louis MO, USA). The NH_4Cl (20 mM: pH 7.12; 40 mM: pH 7.15) and PA was made fresh prior to experimentation. Solutions were housed in closed vials during the course of the experiments.

2.2.3 Electrophysiological Recordings

Larvae were pinned through the cuticle at the anterior and posterior ends using dissecting pins. They were cut longitudinally on the dorsal side and the cuticle was peeled back and pinned on four corners. Internal organs were removed to expose the body wall muscles of the preparations. Segmental nerves were cut near the base of the brain and the nerves were maintained in the preparation to keep their connections to the muscle fibers. Immediately following dissection, postsynaptic recordings were taken with a sharp glass intracellular recording electrode filled with 3M KCl (40 mega ohm resistance).

Muscle 6 at abdominal segment 3 or 4 for was used for electrophysiological recordings. Recordings were performed at 20-21 °C with standard procedures (Lee et al., 2009). An AxoClamp-2 B amplifier (Molecular Devices, LLC. 1311 Orleans Drive, Sunnyvale CA, USA) was used to obtain membrane potentials. PowerLab/4s interface (ADI Instruments, Colorado Springs, CO, USA) was used to digitize the electrical signals. For studies using stimulation, a suction electrode was used to take up the proper segmental nerve and deliver stimulation (S88 Stimulator, Astro-Med, Inc., GRASS Co., USA). Evoked excitatory junction potentials (EJPs), spontaneous miniature excitatory junction potentials (mEJPs), and membrane potentials were observed and analyzed with LabChart 7.0 (ADInstruments, USA) (Figure 2.1).

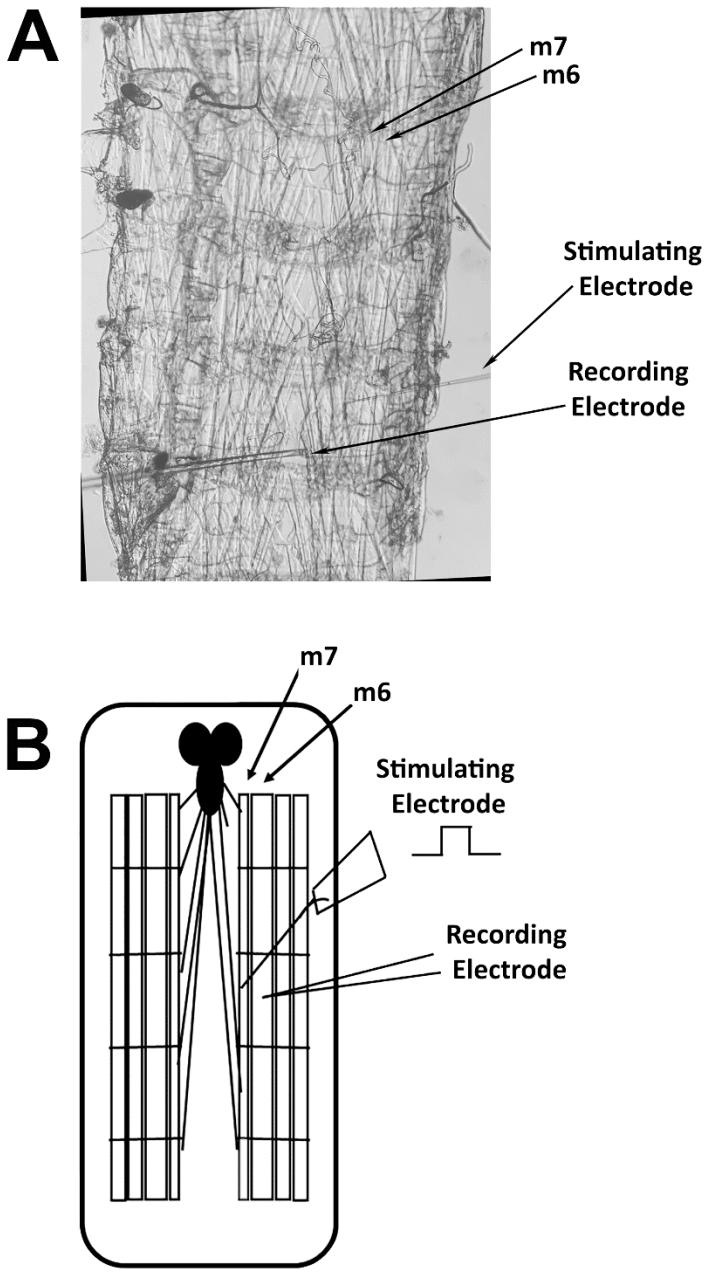

Figure 2.1 Semi-intact dissection setup

A) The semi-intact dissected larval *Drosophila* preparation. Recordings were taken from muscle m6. In studies using stimulation, a suction electrode (labeled stimulating electrode) delivers stimulation to the appropriate segmental nerve. B) A schematic representation of A).

2.2.4 Measures of Synaptic Transmission and Membrane Potentials

2.2.4.1 Propionic Acid

Spontaneous mEJPs were recorded for five minutes while bathing the dissected preparation in HL3 saline. The saline was then switched out with either 20 mM or 40 mM PA and allowed to bathe for another five minutes. The PA was then removed and the preparation was flushed with fresh saline twice. After recording for five minutes following washout, the saline was switched out with PA of the same concentration a second time and allowed to bathe the preparation for five minutes. The PA was removed and the preparation was flushed with saline two more times. The mEJPs were recorded for five more minutes following the final flushing (Figure 2.2A).

Studies were also performed utilizing cadmium ($CdCl_2$; 1 mM), a calcium channel blocker. Recordings were taken for five minutes while bathing the dissected preparation in HL3 saline. 1 mM $CdCl_2$ was added to the preparation and allowed to bathe for five minutes. The solution was then removed and the preparation flushed with 20 mM PA and 1 mM $CdCl_2$ combined and recorded for an additional five minutes (Figure 2.2B).

2.2.4.2 Ammonium Chloride

Spontaneous mEJPs were recorded for five minutes from the dissected preparation bathed in HL3 saline. The preparation was then incubated for 20 minutes in either 20 mM or 40mM NH_4Cl. Spontaneous events were recorded for an additional 10 minutes following rapid removal of NH_4Cl (Figure 2.2C).

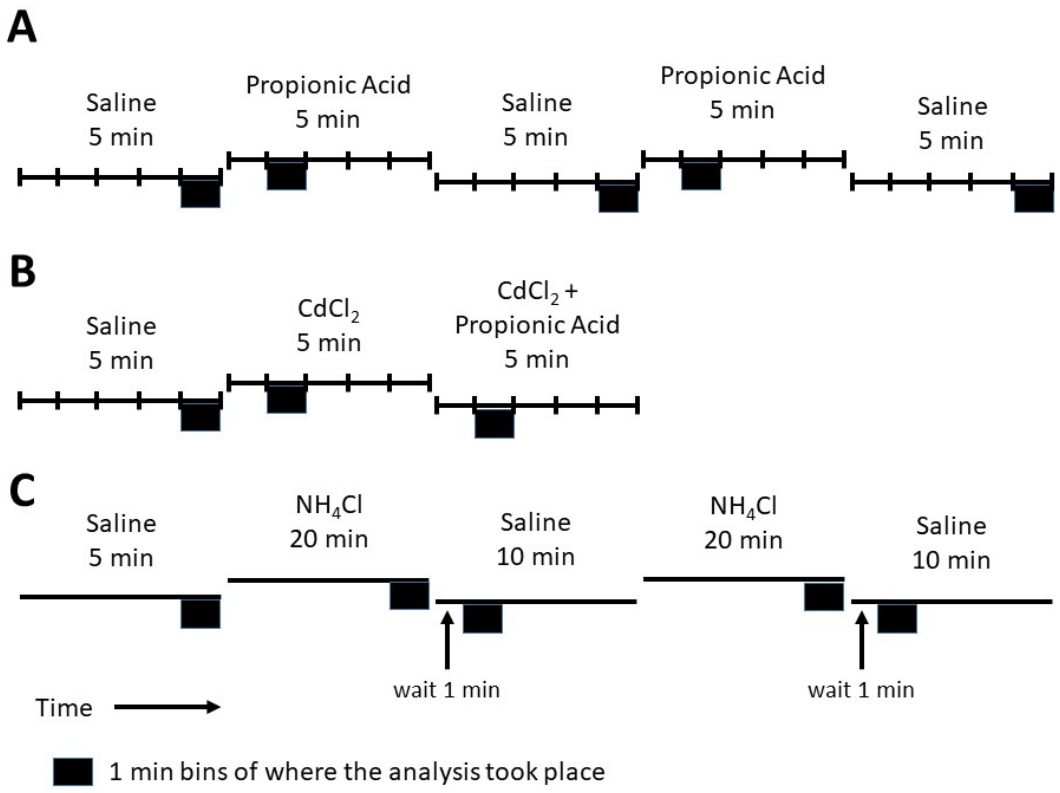

Figure 2.2 Timelines for experimental protocols and analysis windows of spontaneous mEJP studies

The timelines for each experiment in the mEJP studies are shown. The black boxes indicate the one-minute windows in which mEJP frequency was calculated. A) The protocol for the effect of propionic acid on spontaneous mEJPs. B) The protocol for the effect of $CdCl_2$ in combination with propionic acid on spontaneous mEJP frequency. C) The protocol for the effect of ammonium chloride on spontaneous mEJP frequency.

2.2.4.3 Quantal Analysis

Quantal events were considered as synaptic activity above two times the average background noise level and with a sharp rise time. Frequency of the mEJPs were measured by manually placing a cursor at the base and the peak of each event in set time windows (Figure 2.3).

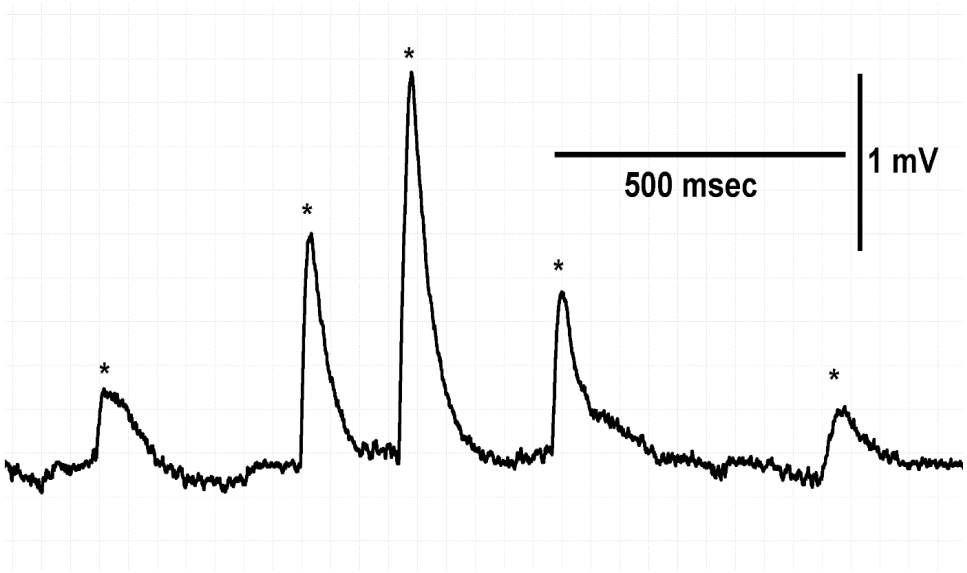

Figure 2.3 An example of spontaneous quantal events

A representative trace of spontaneous quantal events, seen as spikes, versus the baseline recording. Quantal events are marked by asterisks (*). The events are counted as synaptic activity above two times the average background noise level. The spikes have a characteristic fast rise followed by slow decay.

2.2.4.4 Membrane Potentials

Resting membrane potentials (RPs) were analyzed for the propionic acid and ammonium chloride studies on spontaneous mEJPs. Membrane potential values were taken from the end of each saline or treatment condition and compared across conditions.

2.2.4.5 Evoked EJPs

The evoked EJPs were induced by stimulating the segmental nerve with a fire polished glass suction electrode and Grass S-88 stimulator. Amplitudes were measured manually by placing a cursor at the base and peak of the EJP using the LabChart software. EJP amplitudes were corrected for non-linear summation using Martin's correction factor, for the EJP amplitudes larger than 15% of the resting potential by assuming a reversal potential of 0 mV and a membrane capacitance factor of 0.55 (Kim et al., 2009; McLachlan and Martin, 1981).

2.2.5 Depression Studies

Third instar larvae were utilized to determine the amount of time it takes for 50% depression of EJPs amplitudes upon continuous stimulation. To determine the minimum voltage necessary to produce maximal EJP response upon stimulation, the segmental nerve was stimulated at 0.5 Hz while continually adjusting the voltage supplied. Upon determining the minimum voltage, 20 mM PA was applied to the preparation and allowed to bathe for 1 minute while stimulated at 0.5 Hz. The stimulation rate was then increased to 5 Hz to produce continuous EJPs at a high rate. As the membrane potential rapidly depolarizes upon addition of PA, the initial EJP amplitude was determined as the amplitude of the first EJP following stabilization of the membrane potential. Continuous stimulation was applied until the average amplitude of the EJPs being produced were 50% of the initial

EJP amplitude. The time it took from the start of stimulation to 50% depression was recorded. A timeline of the protocol can be seen in Figure 2.4.

To determine the possible effects of the changing membrane potential on the initial depression study recordings, the experiment was repeated while voltage clamping the membrane potential at -60 mV. Voltage was maintained by injecting current during all periods in which it is clamped at -60 mV.

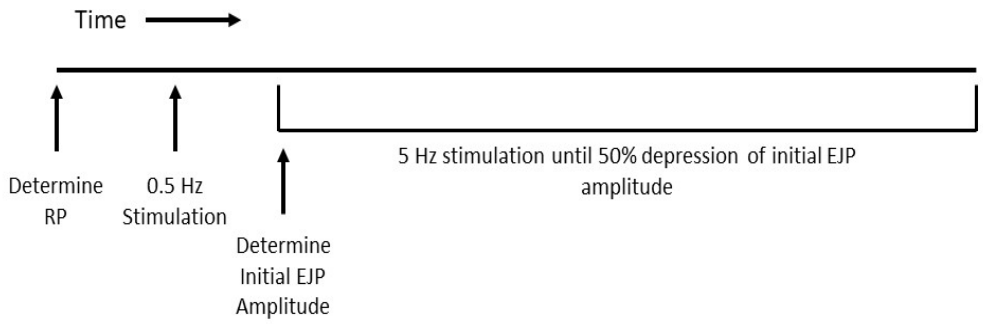

Figure 2.4 Protocol timeline for 50% synaptic depression

Using an intracellular recording electrode, the resting membrane potential of the muscle fiber is determined. Half Hz stimulation frequency is applied to the associated segmental nerve through a suction electrode to determine the minimum voltage necessary for maximal response of the muscle such that all nerves are recruited. Stimulation frequency is then increased to 5 Hz and the initial EJP amplitude is determined from the average of the first five full EJPs. Stimulation at 5 Hz is continued until the average amplitude of EJPs is 50% of the initial EJP amplitude.

2.2.6 Statistical Analysis

A paired t-test or repeated measures ANOVA was used to compare the difference in number of spontaneous quantal events before and after exchanging solution with saline containing PA or NH_4Cl. Difference in the resting membrane potentials were measured before and after added these same compounds and analyzed by a repeated measures ANOVA.

A repeated measures ANOVA was used to examine the before and after data as a repeated measure and if the data sets were normally distributed. A Bonferroni test was used as a post hoc test following the ANOVA to compare the relative changes induced by the compounds within an experimental paradigm to determine significant differences. This analysis was performed with Sigma Stat software. A p of ≤ 0.05 is considered as statistically significant with an asterisk (*).

2.3 Results

2.3.1 Propionic Acid

A representative trace for the effect of 20 mM propionic acid on spontaneous quantal events illustrates a notable pattern of depolarization upon application of propionic acid, along with an increase in mEJP frequency (Figure 2.5A). Subsequent washout with fresh saline was shown to repolarize the membrane potential and reduce mEJP frequency. A second application of propionic acid and washout with saline repeated the pattern of depolarization and the increase in the frequency of the mEJPs followed by repolarization and reduction in the EJP frequency. A similar pattern was observed when using 40 mM propionic acid (Figure 2.5B). It is important to note, however, that in many of the preparations it was difficult to keep the intracellular electrode recording in the muscle fiber.

Particularly during 40 mM propionic acid application, many preparations underwent muscle contraction causing oscillations in RP values. In such cases the intracellular electrode is more prone to popping out of the muscle fiber and can thus make recording difficult as well as causing damage to the fiber.

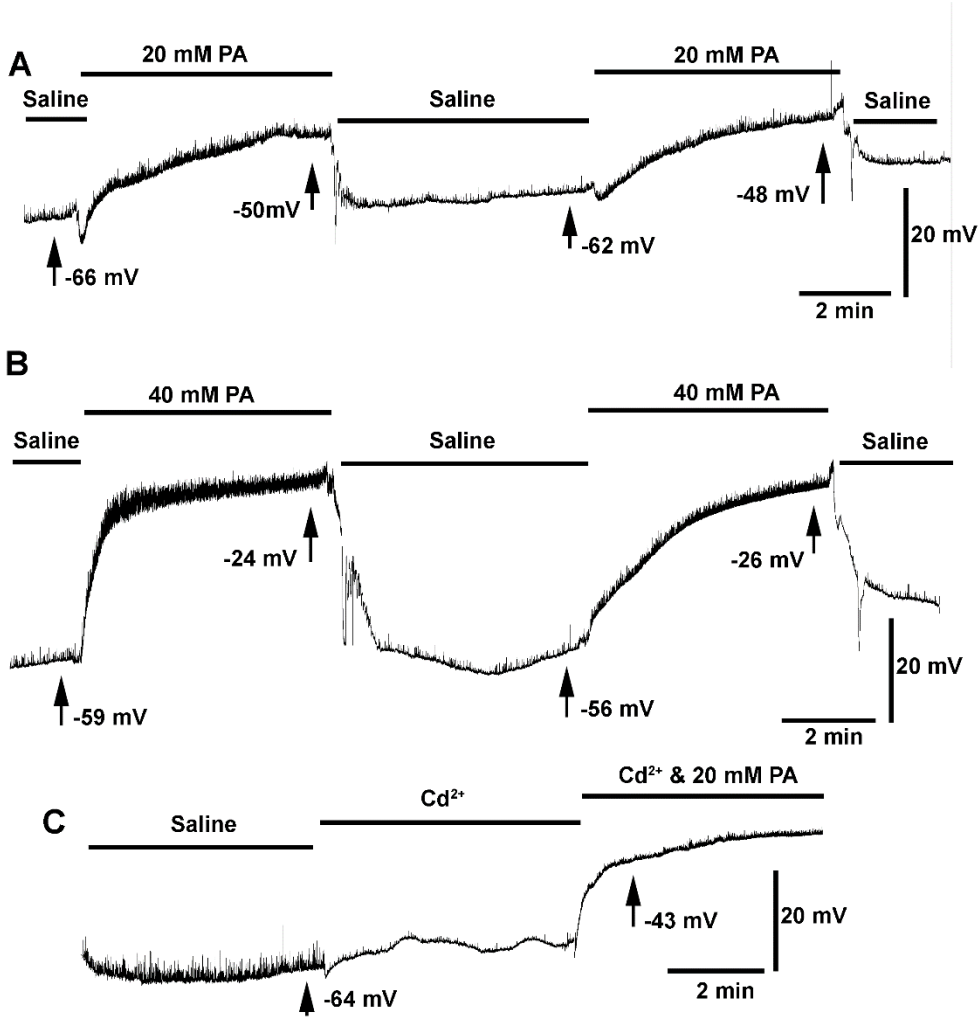

Figure 2.5 Representative traces for the effect of propionic acid on spontaneous quantal events

A) Representative traces of spontaneous quantal events during 5 minutes of saline, 5 minutes after application of 20 mM propionic acid, 5 minutes after washout with saline, 5 minutes after a second treatment with 20 mM propionic acid, and 5 minutes after a final washout with saline. B) Representative trace of spontaneous quantal events using the same protocol as for A) but with 40 mM propionic acid. C) Representative trace of spontaneous quantal events during 5 minutes of saline, 5 minutes after application of 1 mM $CdCl_2$, and 5 minutes after application of 1 mM $CdCl_2$ and 20 mM propionic acid.

The mean RP was significantly depolarized after the first application of 20 mM propionic acid (-43.67 ± 2.23 mV; one-way ANOVA with a Bonferroni post hoc analysis) compared to the initial saline condition (-58.33 ± 2.64 mV) ($p < 0.001$). RP became significantly more negative following washout (-51.67 ± 2.32 mV) ($p = 0.029$). RP was then significantly depolarized after a second application of 20 mM propionic acid (-37.67 ± 2.03 mV, $p < 0.001$). The second and final washout made the RP significantly more negative (-47.17 ± 2.32 mV, $p = 0.007$). Thus, application 20 mM propionic acid depolarized the RP but washout with saline was able to repolarize the RP. It is important to note that the final washout RP was still significantly depolarized compared to the initial saline value ($p = 0.001$). Figure 2.6A depicts the trends in percent change of the RP between each condition.

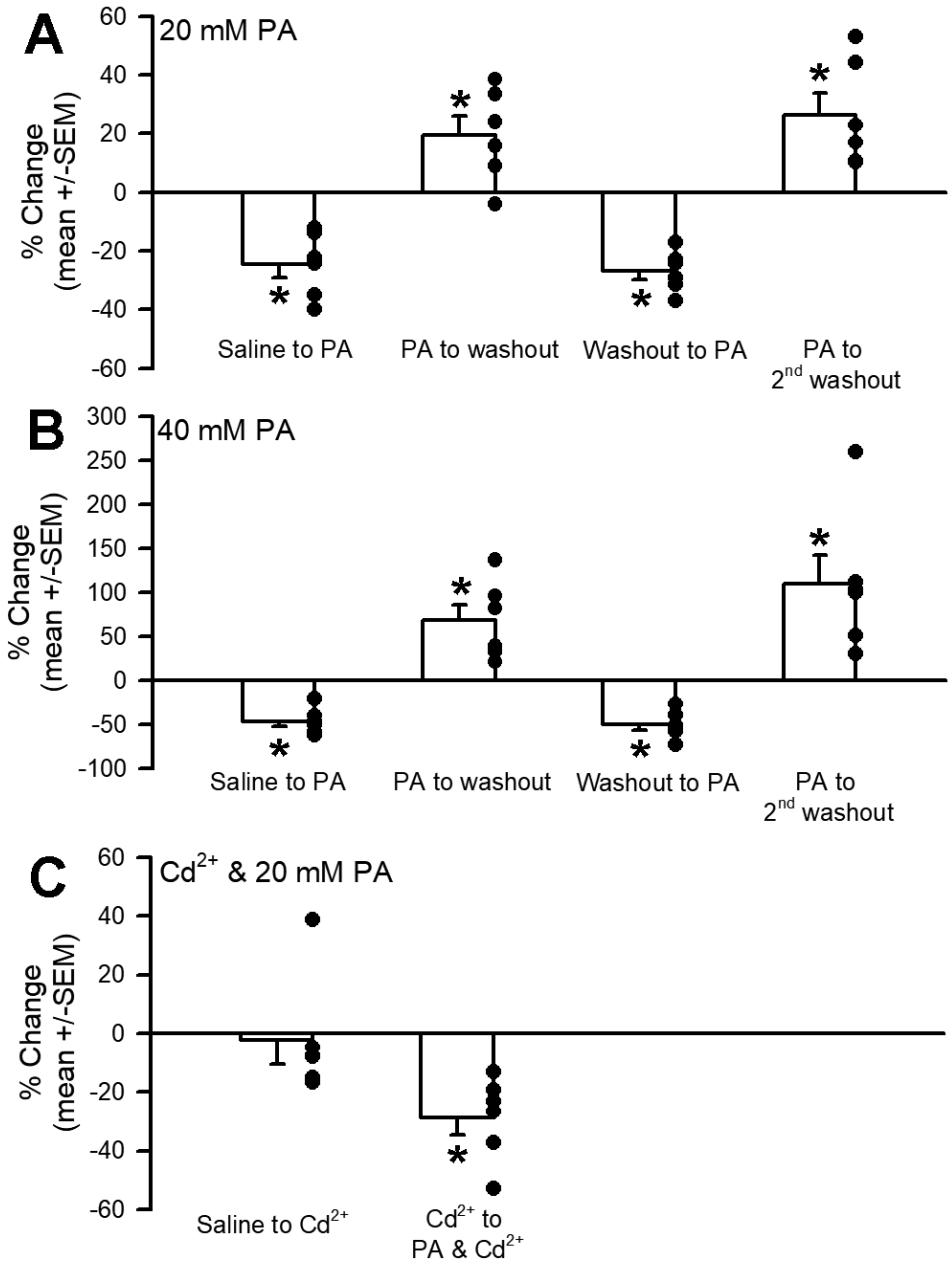

Figure 2.6 Percent change in membrane potential values with propionic acid

Percent change values in membrane potentials were calculated between each changing condition. Membrane potential values were taken from the end of each condition. A) The average ± SEM percent change in membrane potentials between the initial saline condition and after application of 20 mM propionic acid, between the first application of

20 mM propionic acid and the first washout, between the first washout and the second application of 20 mM propionic acid, and between the second application of 20 mM propionic acid and the final washout. B) The average ± SEM percent change in membrane potentials using the same protocol as A) but with 40 mM propionic acid. C) The average ± SEM percent change in membrane potentials between the initial saline condition and after application of 1 mM $CdCl_2$, and between the 1 mM $CdCl_2$ condition and after application of 1 mM $CdCl_2$ with 20 mM propionic acid. Significance of $p < 0.05$ is denoted by an *. Significance values are calculated based on the raw data.

The mean RP was also significantly depolarized after the first application of 40 mM propionic acid (-34.17 ± 3.60 mV) compared to the initial saline condition (-64.33 ± 4.04 mV, $p < 0.001$, one-way repeated measures ANOVA and the Bonferroni post hoc analysis). The RP then became significantly more negative following the first washout (-54.50 ± 2.25 mV, $p < 0.001$). Application of 40 mM propionic acid for a second time significantly depolarized the RP (-27.17 ± 3.61 mV, $p < 0.001$). The second and final washout made the RP significantly more negative (-51.67 ± 1.78 mV, $p < 0.001$). It is important to note that the final washout RP is still significantly depolarized compared to the initial saline value ($p = 0.017$). Therefore, similar to treatment with 20 mM propionic acid, 40 mM propionic acid application depolarized RP but washout was effective in repolarizing the RP, although not necessarily to initial RP values. Figure 2.6B depicts the trends in percent change of the RP between each condition. A comparison of the percent change in membrane potential between saline and propionic acid application for 20 mM versus 40 mM propionic acid indicates that 40 mM propionic acid depolarized RP to a significantly greater extent than 20 mM propionic acid (N=6, $p = 0.019$, Student's t-test).

The mean frequency of mEJPs was not significantly different between the initial saline condition (1.47 ± 0.26 Hz) and first treatment with 20 mM propionic acid (2.06 ± 0.71 Hz, N=6, $p > 0.05$, one-way repeated measures ANOVA and the Bonferroni post hoc analysis). There was a significant decrease in mEJP frequency between the first 20 mM propionic acid treatment and the first washout (0.72 ± 0.15 Hz, N=6, $p = 0.010$). There was then a significant increase in mEJP frequency after the second application of 20 mM propionic acid (1.60 ± 0.40 Hz, N=6, $p < 0.05$, paired T-test). There was no significant

difference in mEJP frequency after the final washout (0.65 ± 0.20 Hz, N=6, $p > 0.05$). Figure 2.7A depicts the trends for percent change in mEJP frequency across all conditions.

To determine if the increase in the frequency of mEJPs was due to propionic acid promoting an influx of extracellular calcium, a separate set of preparations were exposed to $CdCl_2$ (1 mM) to block voltage-gated calcium channels on the plasma membrane. Figure 2.5C depicts a representative trace for the effect of propionic acid on spontaneous quantal events in the presence of $CdCl_2$. The trace illustrates a pattern of mEJP frequency reduction following addition of $CdCl_2$, followed by depolarization upon addition of 20 mM propionic acid. There was no significant difference in the mean RP between the initial saline condition (-64.50 ± 3.53 mV) and after application of 1 mM $CdCl_2$ (-61.67 ± 1.33 mV, repeated measures one-way ANOVA and the Bonferroni post hoc analysis). Application of 1 mM $CdCl_2$ in combination with 20 mM propionic acid led to significant depolarization (-43.67 ± 3.23 mV) compared to both the initial saline condition and the 1 mM $CdCl_2$ condition. Figure 2.6C depicts the trends in percent change of the RP between each condition. The mean frequency of mEJPs was significantly decreased between the initial saline condition (2.98 ± 0.19 Hz) and treatment with $CdCl_2$ (1.31 ± 0.11 Hz, N=6, $p < 0.05$, paired T-test). For a comparison of mEJP frequency between the $CdCl_2$ condition and $CdCl_2$ combined with 20 mM propionic acid, there was not a significant difference (N=6, p=0.063, Wilcoxon Signed rank test was used as the Shapiro-Wilk test of normal distribution failed) (Figure 2.7B).

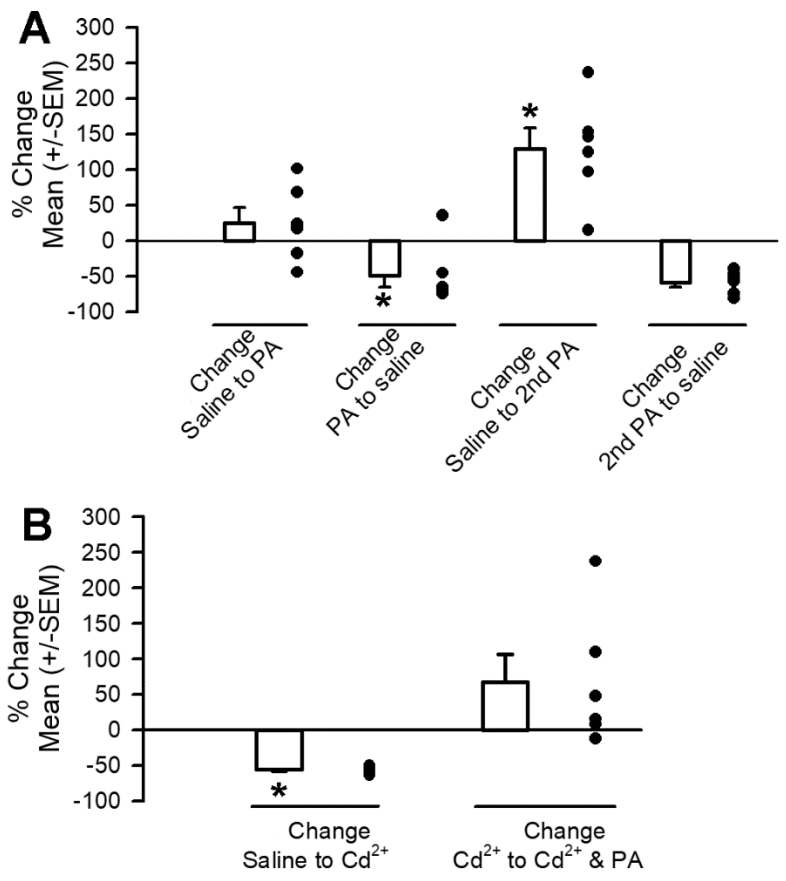

Figure 2.7 Percent change in spontaneous quantal event frequency with propionic acid

Percent change values in spontaneous quantal event frequency were calculated between each changing condition. A) The average ± SEM percent change in spontaneous quantal event frequency between the initial saline condition and after application of 20 mM propionic acid, between the first application of 20 mM propionic acid and the first washout, between the first washout and the second application of 20 mM propionic acid, and between the second application of 20 mM propionic acid and the final washout. B) The average ± SEM percent change in spontaneous quantal event frequency between the initial saline condition and after application of 1 mM $CdCl_2$, and between the 1 mM $CdCl_2$ condition and after application of 1 mM $CdCl_2$ with 20 mM propionic acid. Significance of $p < 0.05$ is denoted by an *. Significance values are calculated based on the raw data.

Time to 50% EJP amplitude depression was utilized as a means of examining the effect of propionic acid on evoked synaptic transmission. A representative trace for 50% EJP amplitude depression in saline as a control depicts a lengthy rundown time (Figure 2.8A). In contrast, a representative trace for 50% EJP amplitude depression following administration of 20 mM propionic acid in a non-voltage-clamped preparation illustrates a rapid rundown to 50% of the initial EJP amplitude (Figure 2.8B). This pattern is repeated in voltage-clamped preparations that are clamped to -60 mV (Figure 2.8C).

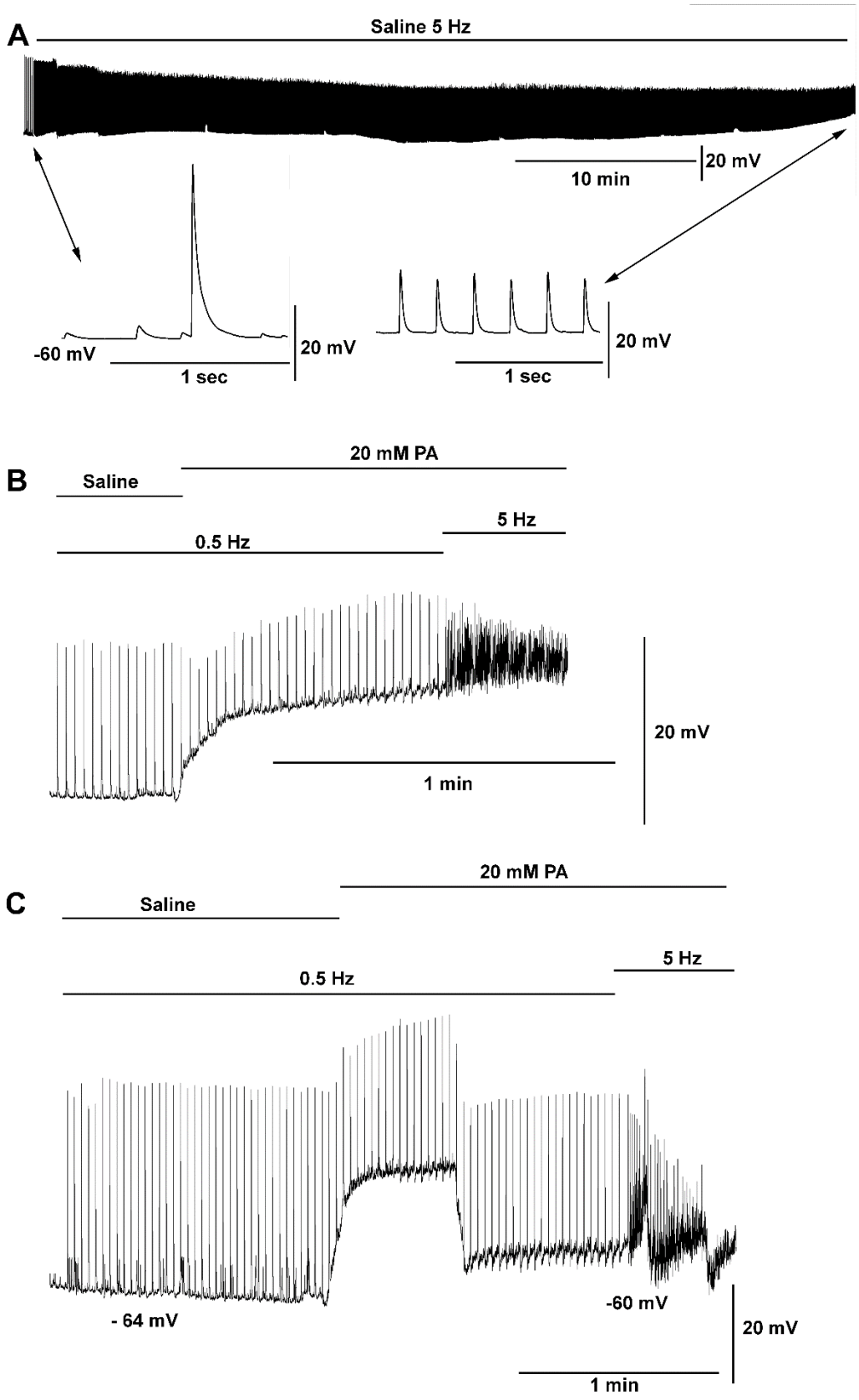
A
Saline 5 Hz
10 min
20 mV
-60 mV
20 mV
1 sec
20 mV
1 sec
B
20 mM PA
Saline
5 Hz
0.5 Hz
20 mV
1 min
C
20 mM PA
Saline
5 Hz
0.5 Hz
- 64 mV
-60 mV
20 mV
1 min

Figure 2.8 Representative traces for time to 50% EJP amplitude depression

For each preparation, the initial EJP amplitude was determined from the first full EJP upon the start of 5 Hz stimulation. Each preparation was observed until the average amplitudes of the evoked EJPs were 50% of the initial EJP amplitude from that preparation. Note the difference in scale bars for each trace. A) Representative trace of the time to 50% EJP amplitude depression in saline only. B) Representative trace of the time to 50% EJP amplitude depression in a non-voltage clamped preparation after addition of 20 mM propionic acid. C) Representative trace of the time to 50% EJP amplitude depression in a voltage-clamped preparation after addition of 20 mM propionic acid.

The mean time to 50% EJP amplitude depression was significantly decreased in the non-voltage clamped condition (N=6, $p<0.001$, Kruskal-Wallis one way of variance on ranks, all pairwise comparisons Dunn's method) compared to the saline condition (2962 ± 400 seconds, ± SEM). There was also a significant decrease in time to 50% EJP amplitude depression between the saline and voltage-clamped condition (56 ± 45 seconds, ± SEM, N=5, $p<0.007$, Kruskal-Wallis one way of variance on ranks, all pairwise comparisons Dunn's method). There was no significant difference in the time to 50% depression between the non-voltage clamped and voltage-clamped 20 mM propionic acid preparations (N=6, $p=0.792$, Mann-Whitney rank sum test was used as the Shapiro-Wilk test of normal distribution failed) (Figure 2.9).

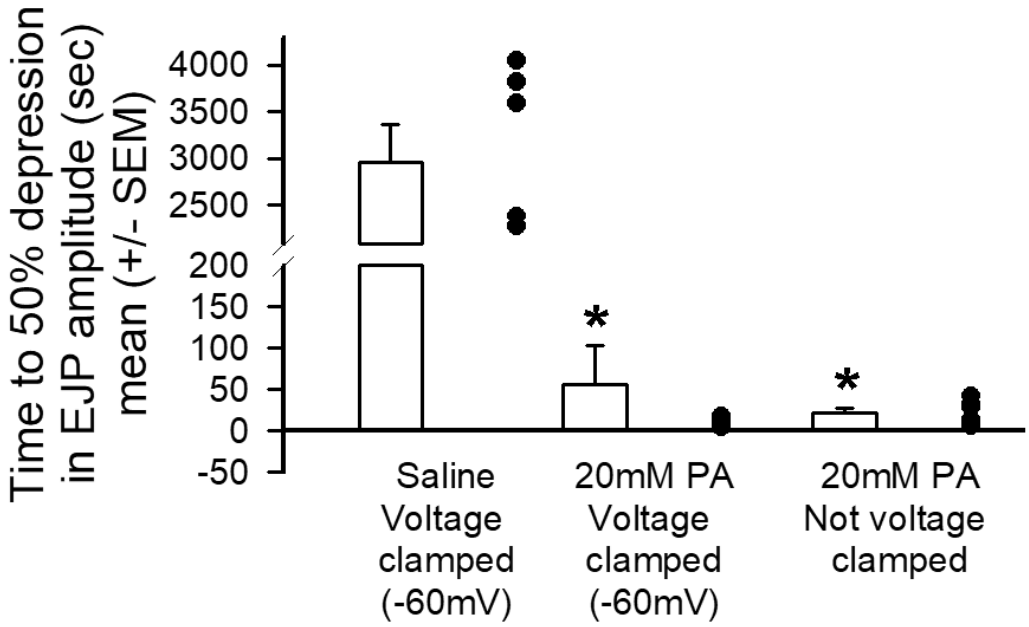

Figure 2.9 Time to 50% EJP depression using 5 Hz stimulation

The mean ± SEM time (in seconds) for 50% EJP amplitude depression during 5 Hz stimulation for saline controls, after addition of 20 mM propionic acid for non-voltage-clamped preparations, and after addition of 20 mM propionic acid for voltage-clamped preparations. * indicates a significant difference ($p < 0.05$) from the saline condition.

2.3.2 Ammonium Chloride

Application of ammonium chloride leads to alkalization of the cell, whereas rapid withdrawal induces transient acidification. A representative trace for the effect of 20 mM ammonium chloride on spontaneous quantal events shows depolarization and mEJP frequency reduction after addition of ammonium chloride, whereas its removal causes repolarization and further reduction in the frequency of mEJPs (Figure 2.10A). A similar pattern is seen for the 40 mM ammonium chloride condition (Figure 2.10B).

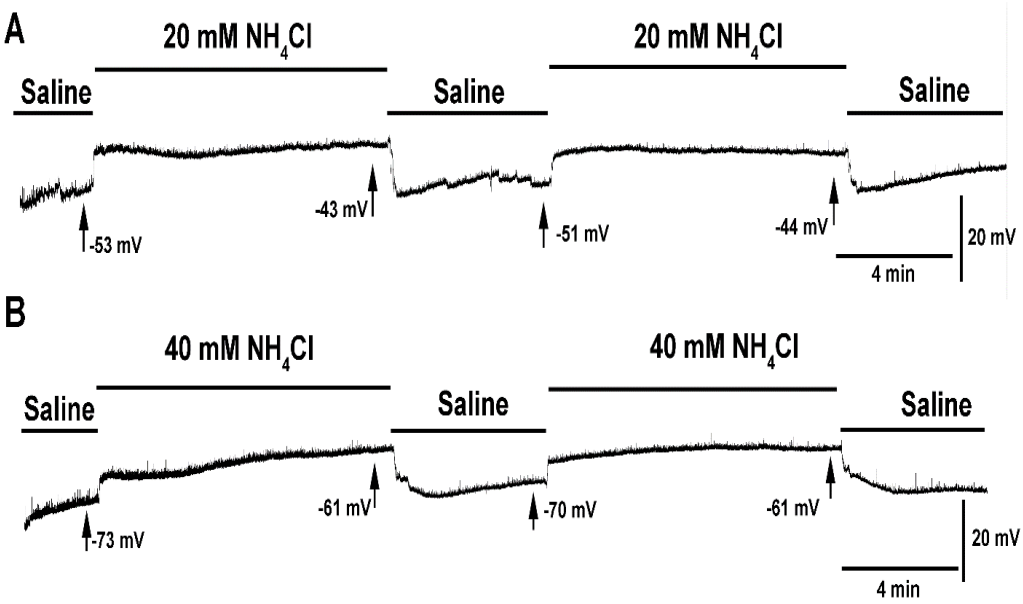

Figure 2.10 Representative traces for the effect of ammonium chloride on spontaneous quantal events

A) Representative traces of spontaneous quantal events during 5 minutes of saline, 20 minutes after application of 20 mM ammonium chloride, 10 minutes after washout with saline, 20 minutes after a second treatment with 20 mM ammonium chloride, and 10 minutes after a final washout with saline. B) Representative trace of spontaneous quantal events using the same protocol as for A) but with 40 mM ammonium chloride.

RP was significantly increased after the first application of 20 mM ammonium chloride (-51.50 ± 3.42 mV) compared to the initial saline condition (-61.00 ± 2.76 mV, $p = 0.028$, a one-way ANOVA and the Bonferroni post hoc analysis). Analysis by ANOVA was unable to compare the difference in mean RP between the first application of ammonium chloride and the first washout due to non-normality. Analysis by paired t-test indicated no significant difference between the two conditions ($p = 0.452$). RP was significantly increased after a second application of 20 mM ammonium chloride (-44.17 ± 2.32 mV, $p = 0.012$). The second and final washout significantly hyperpolarized the RP (-55.50 ± 2.09 mV, $p = 0.006$). Thus, application 20 mM ammonium chloride depolarized the RP but washout with saline was able to repolarize the RP. Figure 2.11A depicts the trends in percent change of the RP between each condition.

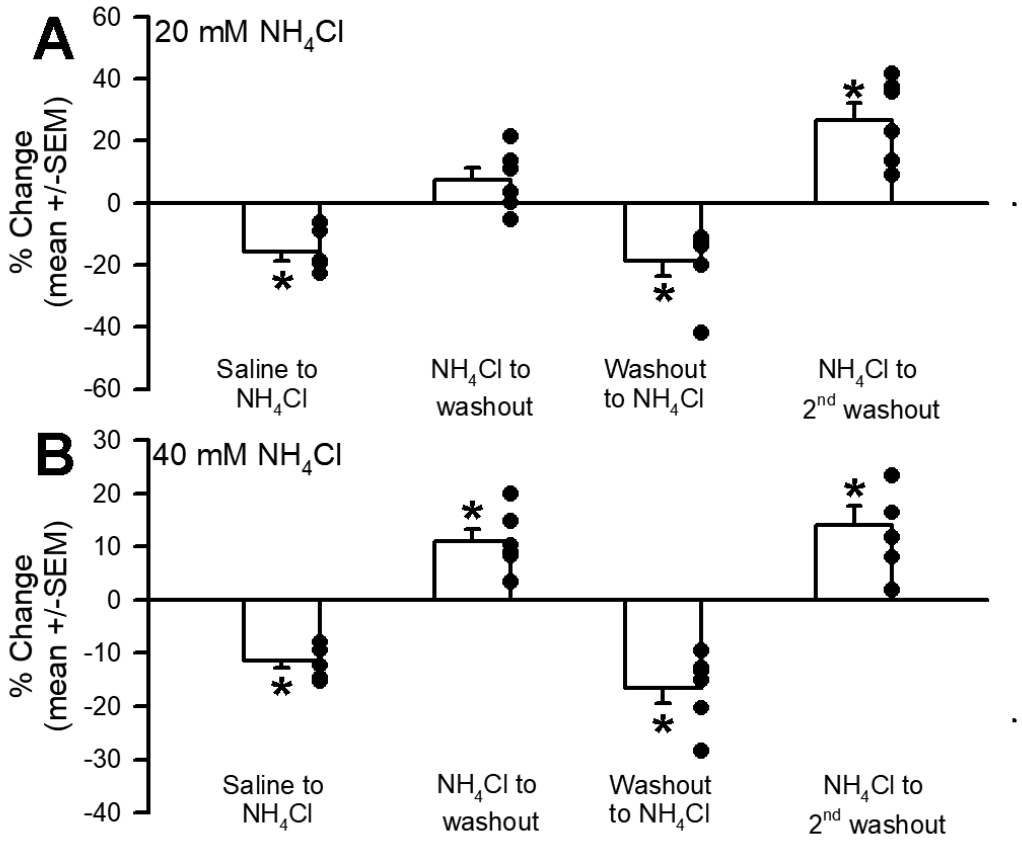

Figure 2.11 Percent change in membrane potential values with ammonium chloride

Percent change values in membrane potentials were calculated between each changing condition. Membrane potential values were taken from the end of each condition. A) The average ± SEM percent change in membrane potentials between the initial saline condition and after application of 20 mM ammonium chloride, between the first application of 20 mM ammonium chloride and the first washout, between the first washout and the second application of 20 mM ammonium chloride, and between the second application of 20 mM ammonium chloride and the final washout. B) The average ± SEM percent change in membrane potentials using the same protocol as A) but with 40 mM ammonium chloride. Significance of $p < 0.05$ is denoted by an *. Significance values are calculated based on the raw data.

The RP was significantly depolarized after the first application of 40 mM ammonium chloride (-57.00 ± 2.88 mV) compared to the initial saline condition (-64.50 ± 3.41 mV, $p = 0.011$, one-way repeated measures ANOVA and the Bonferroni post hoc analysis). The change in RP between the first application of 40 mM ammonium chloride and the first washout (-63.17 ± 3.10 mV) was very near significance ($p = 0.051$). Application of 40 mM ammonium chloride for a second time significantly depolarized the RP (-52.67 ± 3.08 mV, $p < 0.001$). The second and final washout made the RP significantly more negative (-59.83 ± 3.06 mV, $p = 0.016$). Thus, there was a similar pattern of depolarization after application of ammonium chloride and a repolarization upon washout for both 20 mM and 40 mM concentrations of ammonium chloride. A comparison of the percent change in membrane potential between saline and ammonium chloride application for 20 mM versus 40 mM ammonium chloride indicate that there was no significant difference in the extent of RP depolarization between doses ($p = 0.18$). Figure 2.11B depicts the trends in percent change of the RP between each condition.

In analyzing the effects of 20 mM ammonium chloride on spontaneous quantal events, the mean frequency of mEJPs was found to be significantly decreased between the initial saline condition (2.61 ± 0.29 Hz, ± SEM) and after application of 20 mM ammonium chloride (1.43 ± 0.26 Hz, N=6, $p < 0.05$, a one-way ANOVA and the Bonferroni post hoc analysis). There was no significant change in mEJP frequency following the first removal of 20 mM ammonium chloride (0.85 ± 0.14 Hz, N=6, $p > 0.05$). There was also no significant difference in mEJP frequency upon the second application of 20 mM ammonium chloride (0.62 ± 0.29 Hz, N=6, $p > 0.05$, paired T-test) or after the final washout (0.49 ± 0.12 Hz, N=6, $p > 0.05$, paired T-test).

The effects of 40 mM ammonium chloride application and withdrawal showed slightly different trends to that seen in the preparations exposed to the lower concentration. The mean frequency of mEJPs was not significantly different between the initial saline condition (3.69 ± 0.52 Hz) and the first application of 40 mM NH_4Cl (3.97 ± 0.43 Hz, N=6, $p > 0.05$, paired T-test). However, there was a significant decrease in mEJP frequency following ammonium chloride removal (1.79 ± 0.22 Hz, N=6, $p < 0.05$, a one-way ANOVA and the Bonferroni post hoc analysis). There was no significant change in mEJP frequency after a second application of ammonium chloride (2.89 ± 0.30 Hz, N=6, $p > 0.05$). Removal of ammonium chloride for the second time resulted in a significant reduction in mEJP frequency (1.19 ± 0.26 Hz, N=6, $p < 0.05$). Figure 2.12 depicts the trends for percent change in mEJP frequency across conditions.

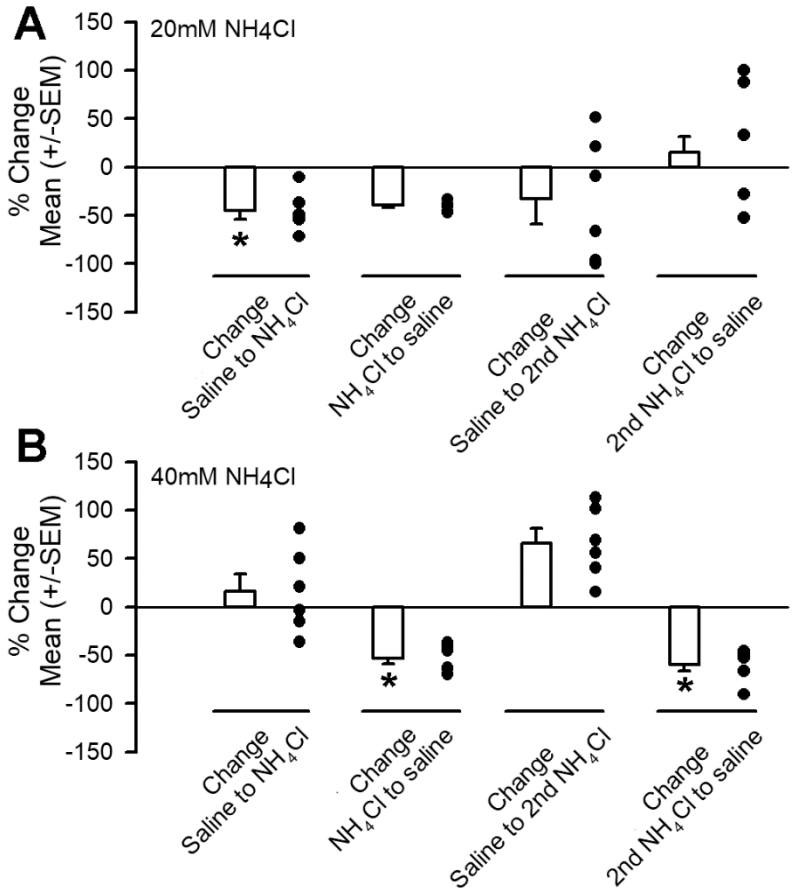

Figure 2.12 Percent change in spontaneous quantal event frequency with ammonium chloride

Percent change values in spontaneous quantal event frequency were calculated between each changing condition. A) The average ± SEM percent change in spontaneous quantal event frequency between the initial saline condition and after application of 20 mM ammonium chloride, between the first application of 20 mM ammonium chloride and the first washout, between the first washout and the second application of 20 mM ammonium chloride, and between the second application of 20 mM ammonium chloride and the final washout. B) The average ± SEM percent change in spontaneous quantal event frequency using the same protocol as A) but with 40 mM ammonium chloride. Significance of $p < 0.05$ is denoted by an *. Significance values are calculated based on the raw data.

2.4 Discussion

There are several important findings from this study to be addressed. Propionic acid was shown to depolarize the membrane potential of the cell, with repolarization occurring following flushing with fresh saline. The extent of depolarization was found to be greater with a higher concentration of propionic acid, suggesting a dose-dependent effect. Analysis of spontaneous quantal events before and after propionic acid administration indicates that, at a 20 mM concentration, propionic acid can cause an increase in mEJP frequency which is decreased following washout. Inhibition of calcium channels with $CdCl_2$ prevents the increase in mEJP frequency. At 40 mM propionic acid, the resultant frequency of mEJPs was so high as to prevent accurate analysis; while the data could not be quantified, there was a notable increase in mEJP frequency in all preparations observed. This effect was reversed upon removal of propionic acid.

Time to 50% EJP amplitude depression was the method utilized to examine the effect of pH on evoked synaptic transmission. The 50% EJP depression protocol provides insight into presynaptic effects of pH through alterations in vesicular packaging and fusion. Due to the graded nature of EJPs at the *Drosophila* NMJ, EJP amplitude is dependent upon the amount of neurotransmitter released from the presynaptic cell to act at the glutamatergic receptors at the postsynaptic membrane. Effects on EJP amplitude can thus indicate alterations in the amount of neurotransmitter reaching the motor endplate, which can be due to changes in vesicular dynamics. 5 Hz stimulation was applied to the preparations to simulate high firing activity, which over time leads to depletion of vesicles available in the readily releasable pool (RRP) and recruitment of vesicles from the recycling and reserve pools. It is also important to note the effect of resting membrane potential (RP) changes in the postsynaptic cell for this experimental paradigm. As RP changes, the driving gradient

of ions is altered. This altered driving gradient itself can affect the amplitude of EJPs independent of the effects involving vesicular dynamics at the presynaptic membrane. While all cells in electrophysiological recordings will slowly exhibit RP changes over time, with depolarization occurring as the membrane is compromised for long recordings, the effect of propionic acid is known to further depolarize the RP. To account for this, voltage-clamping can be used during 50% EJP depression studies. Voltage-clamping of the membrane potential is achieved by injecting current into the membrane to keep the membrane potential at a specific voltage, thus removing the changing driving gradients as a factor for EJP amplitude alterations. Six saline control preparations were compared with six preparations in which 20 mM propionic acid was applied and each condition was voltage-clamped to -60 mV. Six 20 mM propionic acid preparations were also performed to indicate the effects in an intact system without voltage-clamping the muscle. Compared to the saline conditions, 20 mM propionic acid application led to extremely rapid rundown of EJP amplitude. This was shown for both the voltage-clamped and non-voltage-clamped preparations. This indicates that pHi likely has some effect on vesicular dynamics, such that vesicles are unable to be repackaged or recycled and high stimulation rapidly depletes the vesicle pools available. However, the effects of propionic acid on postsynaptic receptors cannot be ruled out as a potential explanation for these results.

As a second mechanism of intracellular acidification, ammonium chloride was studied. Incubation of a cell in ammonium chloride leads to alkalization of the cell, which engages multiple compensatory response mechanisms to limit the change in pHi. Upon rapid removal of ammonium chloride with washout, the interior of the cell is transiently acidified before the compensatory responses return the pHi to equilibrium (Boron & De

Weer, 1976). This is suggested to occur in part as ammonium enters the cell slowly and dissociates into a proton and ammonia (NH_3). Ammonia then exits the cell, combining with an extracellular proton to replenish ammonium outside the cell and maintain a constant gradient of flow. The loss of protons inside the cell is suggested to account for the intracellular acidification (Boron & De Weer, 1976). Interestingly, application of ammonium chloride at both 20 mM and 40 mM concentrations was shown to depolarize the cell, whereas its rapid removal causing transient acidification lead to repolarization towards the initial RP. Thus, both alkalization by ammonium chloride and acidification with propionic acid were associated with membrane depolarization, but acidification after ammonium chloride washout was associated with repolarization. This may be explained by the transient nature of the acidification; it is possible that restoration to pH equilibrium occurred so rapidly as to not have an effect on the RP.

The effects of ammonium chloride on spontaneous quantal events is more complex. At the lower concentration, mEJP frequency is only decreased after application of ammonium chloride. However, a second application and washout had no significant effects on mEJP frequency. At the 40 mM concentration, the first application of ammonium chloride had no effect, but washout led to a reduction in mEJP frequency. Interestingly, however, in comparison to the lower concentration, a second application of ammonium chloride showed a non-significant trend towards an increase in mEJP frequency. A second washout then significantly reduces mEJP frequency, indicating that 40 mM ammonium chloride removal and transient acidification acts to reduce mEJP frequency while having no effect on frequency during the incubation period. This indicates that the direction of the effect of pHi through this method of acidification also differs based on the concentration

utilized. A possible explanation for this could be that, in the case of 40 mM ammonium chloride application, a second dose may have an effect as the intracellular environment had not returned to a normal status in regards to effects on channels and/or protein structure. The second dose may then act as somewhat of an additive effect, although the RP was able to be recovered before the second application of ammonium chloride. The difference in the direction of mEJP frequency alteration between concentrations is interesting and requires further investigation. It is possible that there is a bell curve effect on proteins and vesicle dynamics by ammonium chloride; it has been shown previously that both 5 mM and 50 mM ammonium chloride application in rat hippocampal cultures inhibits vesicle release (Lazarenko et al., 2017). Ammonium chloride was also shown to elevate intracellular calcium levels, potentially due to blocking of mitochondrial calcium uptake; these effects were long-lasting and independent of the alterations in pH and membrane potential (Lazarenko et al., 2017), which suggests potential issues when using this method to study the effects of pHi acidification on synaptic transmission.

A difference from previous studies (Caldwell et al., 2013; Lindgren et al., 1997; Sandstrom, 2011) is the use and analysis of a second bout of acidification within each preparation. Such a method can further elucidate potential compounding effects of acidification and the reversibility of the effects with multiple applications. It also provides information and insight into how preparations respond differently on the first compared to the second administration as well as how these patterns may differ based on the concentration of the compound used. While acidification via propionic acid leads to a consistent pattern of RP depolarization and hyperpolarization along with mEJP frequency increase and reduction following washout at both 20 mM and 40 mM concentrations,

ammonium chloride seems to differ in its responses based on concentration as well as whether it is the first or second application. This may indicate potential issues that arise in the use of ammonium chloride for such an assay.

The effects of both propionic acid and ammonium chloride on the RP of the cell may indicate pH sensitivity of sodium and potassium leak channels or potentially the sodium-potassium ATPase pump. The RP is generated based on the varying concentrations of charged ions inside and outside of the cell membrane as well as the relative permeability of the membrane to those ions. The equilibrium potential of any specific ion can be calculated using the Nernst equation, which gives a measure of the voltage required at which there will be no net flow for that ion given differential ionic concentrations on either side of the membrane. However, in a cell, multiple types of ions are found inside and outside of the cell and contribute to the cell's resting potential. The resting membrane potential of any given cell can be calculated by using the Goldman-Hodgkin-Katz equation if ionic concentrations on either side of the cell and the cell's permeability to those ions are known.

Differential ionic concentrations on either side of the membrane are created by ion pumps and transporters. The resting membrane potential of the average cell is largely dependent upon the action of the sodium-potassium ATP pump, which exchanges sodium inside of the cell with potassium outside of the cell to keep the interior potassium concentration high and the sodium concentration low. Additionally, ion leak channels are necessary for the development of the resting membrane potential by allowing ions to flow down their concentration gradient, creating a circuit wherein channels are considered resistors. In this manner, potassium slowly leaks out of cells down its concentration

gradient through cation leak channels and is continuously pumped back in through the sodium-potassium ATP pump, while sodium leaks inside the cell and is pumped back out. Since the membrane is much more permeable to potassium than sodium due to the higher permeability of the monovalent cationic leak channels to potassium, the concentration of potassium inside and outside the cell is the dominant factor for determining a cell's resting membrane potential.

Both intracellular and extracellular pH has been shown to alter the activity of the sodium-potassium ATPase pump (Breitwieser et al., 1987; Eaton et al., 1984; Salonikidis et al., 2000; Zade-Oppen et al., 1979). In the squid axon, it was found that the pump functions maximally at a pH between 7.2 and 7.4, whereas both acidification and alkalization led to a reduction in pump activity (Breitwieser et al., 1987). This was also seen in rabbit urinary bladder cells (Eaton et al., 1984) and sheep erythrocytes (Zade-Oppen et al., 1979). Furthermore, intracellular acidification has been shown to increase potassium selectivity on the cytoplasmic side of the pump, potentially increasing competition for sodium binding (Cornelius et al., 2018). Possibly, intracellular acidification through propionic acid administration or removal of ammonium chloride inhibits the function of the pump enough to cause depolarization as the ionic gradients of sodium and potassium are slowly lost. Additionally, pH may affect sodium and potassium leak channels themselves that are crucial for maintenance of the RP. In rat hepatocytes, it was found that intracellular acidification led to depolarization of the RP, a result attributed to the accompanied reduction in potassium flux observed (Fitz et al., 1989). Another effect to consider is the status of the NHE that is thought to be involved in the modulation of pHi. At acidic intracellular pH, the NHE is implicated in restoring pHi as it pumps out protons

in exchange for extracellular sodium (Cha et al., 2009; Koliakos et al., 2008). The influx of sodium may affect the RP by altering the ionic gradient across the membrane. In *Drosophila*, it has been shown that mEJP frequency increase upon addition of propionic acid is enhanced by use of the NHE blocker EIPA, demonstrating that slowing the restoration of pHi to equilibrium prolongs the effects of acidification (Caldwell et al., 2013). Other methods of pH regulation in the cell may also be at play; buffers and other transporters such as those for bicarbonate can also help restore pH after perturbation from equilibrium. The complete mechanisms that the cell employs to sense and restore pH alterations are highly complex and not yet fully identified.

The pHo and pHi are both altered during normal physiological function of synaptic transmission, and even greater changes in pH occur during pathological conditions. In the case of increased metabolic activity, both pre- and post-synaptic tissues will undergo pHi changes which will undoubtably effect pHo as CO_2 can readily pass through bilipid membranes. The effect of pHo is partially dependent on how it affects intracellular changes, but pHo could also have an effect on membranes of the pre- and post-synaptic cells independently of pHi. However, the activity of presynaptic neurons adds an additional dimension as the synaptic vesicles are packaged based on a proton pump and the exchange of transmitter into the synaptic vesicles. When these vesicles fuse with the presynaptic membrane, transient reductions in pHo occur along with neurotransmitter diffusion and binding to postsynaptic receptors. How this rapid change in pHo affects postsynaptic receptors is still an area of investigation. In addition, as pHo decreases from vesicle fusion, this likely has an effect on the membrane potential of both pre- and post-synaptic membranes locally in the neighborhood of the synaptic cleft.

When pHo is decreased by application of HCl-adjusted saline, the pHi of cells is not likely to be affected as much as pHo as protons do not passively flux across the membrane. With depolarization of the post-synaptic membrane by a decrease in pHo, it is possible there could be a decrease in the Mg^{2+} block of NMDA receptors within a central nervous system. There has been some research into the effects of pHo on GABAergic neurotransmission in rats, finding that acidification leads to a reduction in spontaneous GABAergic quantal frequency and amplitudes as well as a reduction in chloride currents whereas alkalization has the opposite effect (Chen et al., 2014; Huang & Dillon, 1999). Additionally, pHo has been shown to affect cholinergic receptors by altering channel currents, desensitization time, and channel gating (Li & McNamee, 1992; Palma et al., 1991). How pHo and membrane depolarization affect other receptor subtypes has not be fully investigated. In addition, depending on the type of neurotransmitter and how that neurotransmitter is recycled or metabolized, pH may have a greater effect on certain synaptic junctions compared to others. For example, at cholinergic transmission sites acetylcholinesterase (AChE) acts to degrade acetylcholine in the synaptic cleft. However, acetylcholine itself inhibits AChE activity through a process called substrate inhibition (Reed et al., 2010). In this manner, neurotransmission in which high levels of acetylcholine release are required can occur without acetylcholine immediately be degraded before reaching the postsynaptic cell. While at higher pH (7-8) wild-type AChE undergoes substrate inhibition, at low pH (5-5.75) AChE actually exhibits substrate activation (Masson et al., 2002). Thus, the pH sensitivity of different enzymes and receptors at specific synapses can vary greatly and have a plethora of effects. In the modulation of glutamate in vertebrates, for example, glutamate uptake from astrocytes through excitatory

amino acid transporters (EAATs) is part of the recycling process where glutamate is converted into glutamine before being released and taken up by the presynaptic neuron. Glutamatergic uptake has also been identified in *Drosophila* astrocytes with EAAT inhibition resulting in prolonged post-synaptic currents induced by the actions of glutamatergic interneurons (MacNamee et al., 2016). Knockdown of the *Drosophila* EAAT, EAAT1, results in deficits in locomotion in larvae, suggesting an important role in the recycling of glutamate at the NMJ (Stacey et al., 2010). Interestingly, in Müller cells of the salamander retina, decreased pHo has shown to inhibit the uptake of glutamate, suggesting that EAAT is inhibited by acidic conditions; however, a decreased pHi was not shown to greatly affect glutamate uptake (Billups & Attwell, 1996). If glutamatergic uptake is affected in *Drosophila* in the same manner, acidification may work to increase glutamatergic signaling at the neuromuscular junction. However, the time to decrease EJP amplitude upon acidification is so rapid that it is not likely a key contributor as previous studies in crayfish using TBOA, an EAAT blocker, have shown that TBOA takes a longer time to have an effect on EJP amplitude (Logsdon et al., 2006).

Depending on the postsynaptic target, if a graded response is used to transfer the electrical signal along the postsynaptic membrane or if an action potential is to be initiated at the synaptic site then voltage-gated channels would be present. The effect of lowered pHo from vesicle fusion and cellular metabolism, resulting in membrane depolarization, could affect the voltage-gated channels. Such transient drops in pHo could cause some voltage-gated channels to start to open. However, if the effect is not robust enough, it could lead to inactivation of channels such as for voltage-gated sodium channels. With such gradual depolarization and inactivation, this could thus raise the threshold for initiation of

an action potential. For the presynaptic membrane the same phenomena may apply, but in addition the presynaptic membrane includes voltage-gated Ca^{2+} channels which have a large impact on vesicular fusion. The membrane depolarization may aid in altering the status of the voltage-gated channels in the presynaptic membrane. With high neural activity and a decrease in pHo, this may also have impact on repetitive activity of the nerve terminal (e.g. voltage-gated sodium channel inactivation, opening or offsetting the status of voltage-gated Ca^{2+} channels). Of course, many other proteins may be affected by the drop in pH such as the sodium-calcium exchanger (NCX), calcium pumps, or the Na^{+}/K^{+} ATPase pump; it has not yet been fully investigated on how pHo as well as pHi fully affects these proteins and the complete interactions involving all of their individual effects. The NCX has been shown to be inhibited during both extracellular acidification and alkalization (Egger & Niggli, 2000) and inhibited during intracellular acidification (Doering et al., 1996). The large number of proteins involved in Ca^{2+} sensing, vesicle docking, and vesicle fusion have yet to be investigated for the interaction of various pHi conditions that are associated with physiological or pathological neuronal activity. The lack of research in addressing the effect of pH on the SNARE-SNAP protein interaction is an area ripe for further studies.

Given that the frequency of spontaneous vesicular fusion increases upon acidification of the intracellular compartments of the presynaptic neuron using propionic acid, this suggests a possible increase in calcium entry and possibly enhanced SNARE-SNAP or even Ca^{2+} sensitivity of synaptotagmin. In the preparations involving the use of $CdCl_2$, a calcium channel blocker, the effect of 20 mM propionic acid on mEJP frequency was inhibited. Thus, the effect of propionic acid is dependent upon calcium entry at the

presynaptic membrane and likely does not increase calcium release from intracellular stores. As calcium is required for synaptotagmin to change conformation and allow vesicular docking and fusion to the presynaptic membrane, this suggests that propionic acid is not necessarily increasing vesicular fusion through pH effects on the proteins involved in such processes or indicates that its effects are dependent on the presence of calcium as well. However, an alternative explanation for the findings in this $CdCl_2$ experiment is that the results may in part be due to the greatly reduced amplitudes of mEJPs after addition of $CdCl_2$. It is possible that mEJPs were reduced to such an extent that they were not above the background noise level, potentially resulting in missing mEJPs.

Evoked synaptic transmission is depressed very rapidly under intracellular acidic conditions with propionic acid. This may be due to the depletion of readily releasable vesicles as a result of the high rate of spontaneous fusion events. However, there could also be an impact on vesicular recycling kinetics and even packaging of vesicles. Considering that glutamate is suggested to be transported into the vesicle in exchange for protons using VGLUT, a proton gradient must be present. Given that exposure to propionic acid reduces pHi, this may also reduce the driving gradient of the antiporter and thus reduce packaging of glutamate in recycling vesicles. However, it is also important to note that propionic acid could penetrate the membranes of vesicles as well as other organelles, such as the mitochondria, and such effects have not yet been addressed in isolation due to difficulty in experimentation to be able to address such individual factors.

Propionic acid and ammonium chloride both can acidify the interior of the cell through different mechanisms. The *Drosophila* cytosolic pH has been reported in the range of 7.2-7.3 (Rossano et al., 2013; Caldwell et al., 2013). The extent of intracellular

acidification via propionic acid or removal of ammonium chloride depends on the concentration used (Boron & De Weer, 1976; Lindgren et al., 1997), but even during high stimulation independent of applied compounds the pHi can drop by 0.1-0.2 pH units (Caldwell et al., 2013; Rossano et al., 2013). The time courses for both propionic acid and ammonium chloride also differ. Propionic acid is shown to rapidly increase mEJP frequency upon application in the current study as well as in previous research (Caldwell et al., 2013; Lindgren et al., 1997) and can maintain NMJ acidification for up to two hours (Lindgren et al., 1997). In crab muscle, 100 mM propionic acid is shown to drop pHi by 0.6 pH units (Sharp & Thomas, 1981). In the squid giant axon, ammonium chloride application rapidly increases pHi, but long-term incubation will eventually lead to a plateau of pHi and a slow re-acidification (Boron & De Weer, 1976). Washout then results in rebound acidification of pHi, with a drop of 0.07 pH units associated with use of 10 mM ammonium chloride in squid (Boron & DeWeer, 1976) while the same concentration in guinea pig hippocampal neurons resulted in acidification by anywhere from 0.12 to 0.54 pH units (Bonnet & Wiemann, 1999). It is important to note, however, that many studies lack definitive pHi measurements using these compounds; it may be beneficial for future studies to measure and state the pHi values observed with use of these compounds in the specific model organism being studied.

While both acidification methods offer an approach to acidifying the cell's interior, there are some associated issues with each. Propionate can easily pass through lipid membranes, and its effects on not only cytoplasmic pH but also other intracellular compartments such as the mitochondria or vesicles must be considered. It is unknown the complete extent which propionate is able to acidify the interior compartments of the cell or

what effects this might have. Furthermore, acidification using propionic acid is not able to avoid extracellular acidification as well. The full impact of alkalization prior to acidification using ammonium chloride is also unknown; possibly this can have an effect on how the cell responds to acidification. Additionally, in the use of the ammonium chloride pre-pulse technique, issues have arisen with non-responding cells in both guinea pig hippocampal slices (Bonnet & Wiemann, 1999) as well as *Drosophila* body wall muscle cells (Caldwell et al., 2013). There is also not yet a way to apply propionic acid or ammonium chloride to the motor nerve in isolation while studying synaptic transmission at the NMJ, so their potential effects on the muscle itself must also be considered. The culturing of motor neurons from the dissociated CNS of *Drosophila* has been successful (Darya et al., 2009; Rohrbough et al., 2003; Wiemerslage et al., 2013; Wu et al., 1983) which may at least allow for the study of synaptic transmission indirectly through used of pH dye indicators and dyes that reveal vesicle dynamics, such as FM1-43 (Lindgren et al., 1997).

Both ammonia and propionate can increase in the blood of humans and other mammals under pathological conditions and rare diseases (Jafari et al., 2013). Thus, the use of ammonium chloride and propionic acid to experimentally manipulate pHi offers a useful approach to address the effects of pHi on cellular function in addition to the use of CO_2. However, the effects of CO_2 may have additional actions besides altering pHi and pHo and can be preparation-dependent. It was reported that the presence of CO_2 blocks glutamatergic synapses at the larval *Drosophila* NMJ and crayfish NMJs independent of the decrease in pH (Badre et al., 2005; Beibower & Cooper, 2010). While Caldwell et al. (2013) found that application of 24 mM HCO_3^-/5% CO_2 had no effect on mEJP frequency,

this disparity may be in part explained by the lower concentration used compared to the previous studies. The direct effect of CO_2 on glutamatergic synapses in mammalian CNS preparations remains to be investigated.

It is important to note that synapses in mammals would not normally be exposed to ammonia, except possibly in some pathological conditions (Cooper & Kuhara, 2014; da Fonseca-Wollheim, 1995). The effect of ammonia in regulating blood pH has been a topic of interest for many years (Nash & Benedict, 1921). It has been shown not to be a major mechanism to regulate blood pH but is important in acid-base homeostasis in the renal system (Atkinson & Bourke, 1987; Weiner & Verlander, 2017). However, ammonium can increase in the blood and cross the blood brain barrier and can therefore have a direct effect on central synapses as well as throughout the body, such as at NMJs and other tissues.

This study served to address and expand upon findings from previous research in the study of pHi effects during acidification of the presynaptic nerve terminal. While many of the results corroborate previous findings, there are some differences to be addressed. In the study by Caldwell et al. (2013) that examined the effects of both propionic acid and the ammonium chloride pre-pulse technique on synaptic transmission, they found that propionic acid administration led to hyperpolarization of the RP, contrary to what was found in the current study. This can be partially explained by the use of the saline buffer BES and potentially the resting membrane potential of the cells they examined. The saline buffer BES in the current study was used at a concentration of 25 mM whereas Caldwell et al. (2013) used 15 mM; BES may have potentially buffered pH changes to a greater extent during application of propionic acid or ammonium chloride. Furthermore, the RP of cells in the Caldwell study seemed to be more depolarized than those obtained in the current

study, which could potentially affect the direction of polarization upon intracellular acidification. Preliminary data does support the findings that alkalization of the extracellular space using NaOH hyperpolarizes the muscle whereas acidic pHo using HCl causes depolarization of the RP; these findings corroborate those of Caldwell et al. (2013), however more data and analysis is required and planned for future studies. In both the study by Caldwell and the current findings, propionic acid was shown to significantly increase the frequency of spontaneous quantal events. Inhibition of the NHE by Caldwell et al. (2013) indicated a significantly greater increase in mEJP frequency. It is likely that the NHE helps to restore the interior of the cell to equilibrium pH through exchange of protons with extracellular sodium, and its inhibition slows this process.

The effects of ammonium chloride administration are more complex. Both 20 mM and 40 mM concentrations exhibited a pattern of depolarization upon application with hyperpolarization following washout, in concurrence Caldwell et al. (2013). Interestingly, however, the opposite trend occurs in rat hepatocytes (Fitz et al., 1989). There also seem to be dose-dependent effects in the direction of mEJP frequency alteration as well as differences in response on the first versus the second application at each dose. Caldwell et al. (2013) reported a mEPP frequency increase in 4 out of 9 preparations after removal of 20 mM ammonium chloride, whereas in the current study 20 mM ammonium chloride application, but not removal, resulted in a reduction in mEPP frequency. A possible explanation for these contradictory results may be that the higher BES buffer in the saline dampened the acidification homeostasis while incubating in NH_4Cl and thus the pHi did not rise or even decrease as much as would be expected. Future studies would need to be conducted with measures of pHi in heavily buffered saline conditions. Furthermore, mEJP

amplitudes were greatly reduced after the addition of ammonium chloride. In some traces small deflections in the membrane potential were noted to be shaped similarly to a quantal event, but were not greater than twice the background noise in amplitude to be considered a true quantal event. Due to this sharp reduction in mEJP amplitudes noticed, it is possible that the true effects of ammonium chloride on mEJP frequency were masked. Other potential explanations for this difference in results could be exposure times for ammonium chloride as well as times to experimental dissection. Caldwell et al. (2013) incubated in ammonium chloride for 10 minutes compared to the 20 minutes used in the current study, and there is no report on the timings of experimental dissection and the time between dissection and recording. Due to rundown and degradation of preparations, it is possible that differences in such timings can affect results. Another point to consider is that they recorded from both muscle 4 and muscle 6 in their analysis of mEJP frequencies, with seemingly no distinction or separation in the results; the current study only analyzed mEJP frequencies from muscle 6. It is possible that differences in the muscles used caused disparities in the results obtained.

Previous studies in *Drosophila* (Caldwell et al., 2013) did not address the effect of acidification on evoked synaptic transmission. This is relevant in the case of modeling the effects of acidification during high neural activity, such as in pathological conditions involving seizures or slightly enhanced neural activity. The finding that acidification greatly decreases the time to synaptic depression indicates alteration of vesicular dynamics. This is in line with the findings that propionic acid administration increases mEJP frequency as well; if acidification increases the rate of vesicular fusion and impedes vesicular recycling and repackaging, mEJPs would be expected to increase in frequency at

low or no stimulation as fusion is increased whereas high stimulation would result in greater numbers of fusion events and rapid depletion of the vesicle pools. This is supported by the findings at the lizard NMJ showing that acidification with propionic acid inhibits vesicular recycling by blocking endocytosis (Lindgren et al., 1997).

While this study addresses the acute effects of altered pH on synaptic transmission, there are likely long-term effects as well. A slight depolarization of pre- and post-synaptic cells could alter the effect of communication in synaptic homeostasis. The rationale assumed is that the occurrence of spontaneous synaptic transmission is a means of communicating with the target cells to form and maintain synaptic junctions (Alger, 2002; Davis & Murphey, 1994; Fitzsimonds & Poo, 1998; Regehr et al., 2009). With postsynaptic depolarization due to alterations in pH, the cell may assume a high frequency of spontaneous quantal events and/or evoked transmission which could alter retrograde communication to the presynaptic terminals. In addition, the local depolarization at synaptic junctions by quantal events would be joined by whole cell depolarization, which may alter the local nuclei in muscle cells and expression of particular proteins (Menon et al., 2013; Newlands et al., 1998; Rudolf et al., 2019). Likewise, increased quantal events and release of neurotransmitters from the enhanced frequency of spontaneous events could also alter the normal action of auto-receptors on presynaptic terminals, strengthening synaptic connections (Bailey et al., 2004; Choi et al., 2014; Kaneda & Kita, 2005; Kauwe & Isacoff, 2013), as well as volume transmission to other targets in the vicinity (Kinney et al., 2013; Taber & Hurley, 2014).

2.5 Future Directions

I identified and expanded upon effects of acidification through alterations of vesicular dynamics on both spontaneous and evoked synaptic transmission. However, additional avenues of experimentation for this study using the larval *Drosophila* model would be to examine the sensitivity of glutamate receptors to a decrease in pHo by using saline adjusted with HCl compared to the effects of propionic acid and the transient effects of ammonium chloride. This could be approached with tight patch clamp techniques with excised membranes (DiAntonio et al., 1999; Heckmann & Dudel, 1997) or voltage clamping the muscle fiber and examining the shapes of quantal events in which many ionotropic glutamate receptors are activated. However, this is subject to assuming pH would not affect the fusion pore or packaging of the vesicles and that the amount of glutamate released with vesicle fusion remained the same. As the current study suggests acidification affects vesicle dynamics, the use of iontophoretic application of glutamate over NMJs, with voltage clamped fibers, would be a potential approach to address this topic. To account for the rapid depression of evoked EJPs under acidic conditions, it would be of interest to know if this result is due to the rapid decrease in the readily releasable pool of vesicles being able to recycle and refill or if the vesicles are able to recycle but repackaging is compromised. This could be addressed in part with using vesicle-associated dyes such as FM1-43 and examining the loading and unloading rates in the presence of acidic conditions. It is known that depolarization and increased quantal events leads to rapid de-staining (Lindgren et al., 1997), but one could still compare if there are differences in this process under acidified conditions. Raising extracellular potassium ($[K^+]o$) will depolarize terminals and result in increased vesicle fusion events (Liley, 1956). Examining vesicle repackaging by the size of quantal events, and examining recycling of vesicles with

FM1-43, under conditions with raised $[K^+]o$ with and without acidification may be one approach to address this point. For the analysis of action potentials, the *Drosophila* model does not allow axons to be penetrated with an intracellular electrode due to the small axon diameter. Thus, to directly measure the threshold and shape of the action potentials in such conditions, extrapolation from other models such as the squid or crustacean models would potentially allow insight into the *Drosophila* model for the effects within axons.

While this study has contributed to development of a working model for the effects of pH on synaptic transmission, there are many further avenues of experimentation to address. These include more in-depth analysis of pH effects for presynaptic function, such as through effects on vesicle loading, re-packaging, recycling, docking and fusion, and sensitivity to Ca^{2+} binding to synaptotagmin. There are also potential effects on voltage sensitivity of ion channels with evoked events, Ca^{2+} buffering, the NCX, calcium pumps, ATP production related to mitochondrial function, and the potential interaction of autoreceptors. Other questions involve whether the shape or threshold of excitability in inducing an action potential or the shape of the action potential itself altered due to effects on subsets of ion channels.

One must also consider the effects of pH on postsynaptic function. To be explored further are pH effects on receptor binding affinity and conformation, receptor kinetics, receptor desensitization, removal of desensitization, retrograde feedback to the presynaptic terminal, and long-term effects in synaptic structure and make up. Effects of pH in the synaptic cleft may also play a role. For example, in some cases the removal of transmitter from the cleft may also be pH-dependent. This is seen for AChE, and pH in the cleft could also potentially affect CNS glial uptake of glutamate (Billups & Attwell, 1996).

Another avenue of research would involve computational modeling of a complete synaptic junction. In this manner, one could add components together as they are addressed individually. The model can then be referred to in order to corroborate findings with experimental investigation, in addition to further developing the model as new aspects are experimentally explored and addressed; thus, a combination of forward and reverse engineering can be used to refine the model. Models of the synapse have been built in the investigation of neurotransmission (Wang et al., 2016) but further development is necessary (Brea & Gerstner, 2016). Presented below is a simplified diagram of a presynaptic bouton and some of the many transporters, exchangers, channels, and ions that may be affected by intracellular pH changes. All work in concert and further investigation into the effects of all these combining factors is required for a complete understanding of how pHi affects synaptic transmission (Figure 2.13).

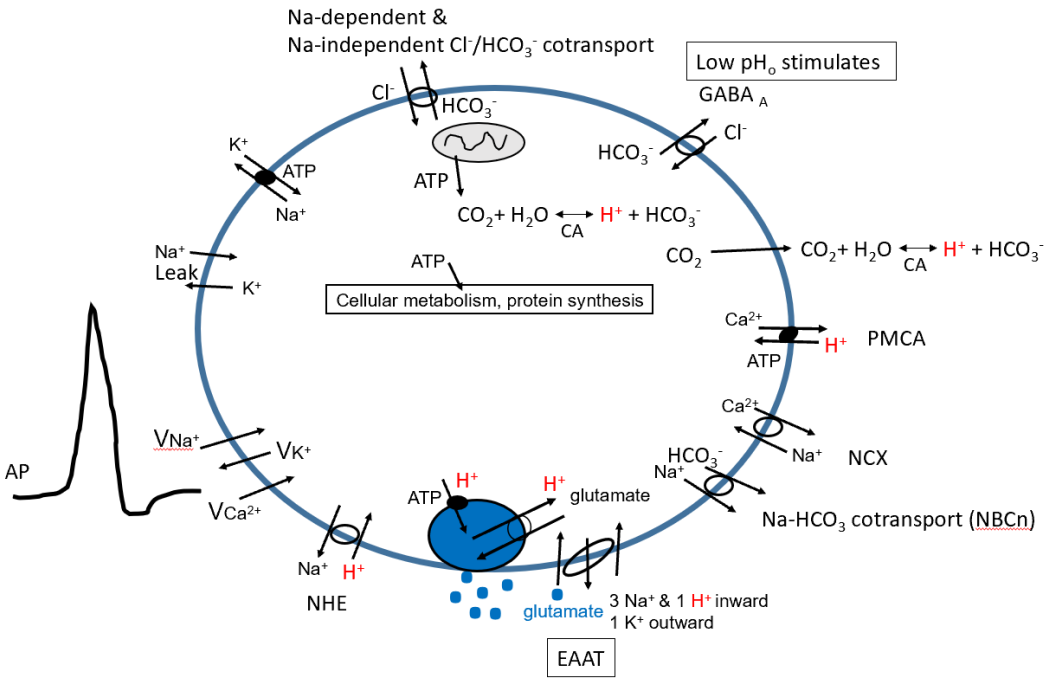

Figure 2.13 A model of a presynaptic bouton

A simplified model of a presynaptic bouton of a motor neuron with some of the many transporters, exchangers, channels, and ions that may be implicated in the effects of pH on synaptic transmission. Included is a vesicle (shown as a filled blue circle) to depict the fusion and release of glutamate into the synaptic cleft.

CHAPTER 3. CONCLUSIONS

3.1 Overview of Thesis

In summary, this thesis addresses many of the previous findings on how pH affects synaptic transmission and sought to expand upon those reports. By specifically targeting the effects of intracellular acidification, this research was able to gain insight into how transient pH fluctuations that occur naturally during the course of synaptic transmission may feedback on the process itself or how abnormal pH alterations in pathological conditions can affect neuronal communication. It was found that acute cytoplasmic acidification in the presynaptic terminal at the *Drosophila* NMJ affects both spontaneous and evoked synaptic transmission, with effects depending on the method of acidification utilized. It is likely that a reduction in pH in the presynaptic terminal reduces or inhibits vesicular recycling or repackaging while also increasing the rate of vesicular fusion, indicated by the increased frequency of spontaneous mEJPs and decreased time to 50% evoked EJP amplitude depression upon application of propionic acid. However, the effects of ammonium chloride on mEJP frequency are mixed and must be further investigated to parse out a full understanding of its effects. These two commonly used methods of intracellular acidification can produce differing results, so care must be taken when choosing one of these methods for research. These findings are significant in further developing a model of the effects of intracellular acidification while also being useful in corroborating or identifying conflicting results from previously published reports. Replication is extremely important in research and serves to validate findings and ensure the integrity of scientific knowledge. In carrying forward with this project, the study of how extracellular acidification and alkalization affects synaptic transmission is underway

for comparison to the results presented in this thesis using propionic acid and ammonium chloride. Future studies that use pH indicators in the nerve terminal while altering pH with sodium hydroxide and hydrochloric acid as compared to propionic acid and ammonium chloride would be informative.

Through this program I have greatly developed my skills in literature review, conducting research, analyzing the data I obtained, and effectively communicating my findings through writing. In the process of literature review, I have learned how to pick out the most important and relevant results without getting lost in the details. In research, I have gained valuable understanding and skills in both interpreting and conducting electrophysiological studies as well as technical dissection and microscope techniques. In data analysis I have furthered my understanding of running statistics and identifying the relevant information in the data I gathered. Finally, in the writing of this thesis I have been able to combine all of these skills in such a manner as to present a whole, unified project that conveys these important findings in the context of previous research.

3.2 Additional Projects

Two additional projects were conducted and finalized over the course of this Master's program. The first research project was developed for an upper-level neurophysiology laboratory course in the Biology department. In this project, along with a student participating in an undergraduate summer research program (KBRIN), we studied the effects of the compound tricaine mesylate (also known as MS-222) on several physiological measures in three invertebrate models: the blue crab (*Callinectes sapidus*), the red swamp crayfish (*Procambarus clarkii*), and the fruit fly (*Drosophila melanogaster*) (Stanley et al., 2020a). MS-222 is a known sodium channel blocker and is commonly used

as an anesthetic for fish. This study sought to examine and characterize its effects in invertebrates for potential use as an anesthetic in such models. In the crab PD chordotonal organ, a sensory organ that utilizes stretch receptors, it was found that incubation with MS-222 reduced neural firing activity during static and dynamic displacement of the crab leg. Washout led to a gradual return of synaptic activity. In the next model studied, the crayfish, both sensory and motor activity were examined. The muscle receptor organ (MRO) is a proprioceptive organ that contains two types of sensory neurons, one type that is slowly adapting and another that is rapidly adapting. Similar to that seen in the crab PD organ, incubation in MS-222 led to greatly reduced neural activity that was able to return upon washout of the compound. To study synaptic transmission, the neuromuscular junction of the crayfish was examined in the crayfish walking leg opener muscle. Action potentials were evoked in the excitatory axon via stimulation and EJPs were recorded in the associated muscle fiber. As the evoked EJPs in this preparation rapidly facilitate under high frequency stimulation, the amplitude of the 25th EJP was analyzed for the effects of MS-222. It was found that the EJP amplitude is significantly reduced during MS-222 incubation but is able to return upon washout. Action potentials were also recorded from the motor neuron itself to see how the shape of the action potential changed upon incubation with MS-222. The amplitude was found to be reduced with a return after washout of the compound. Synaptic transmission at the NMJ was also analyzed in *Drosophila* using body wall muscle 6. Similar to that seen in the crayfish walking leg opener muscle, EJPs were depressed when exposed to MS-222 and returned after washout. Additionally, of note is the finding that the larval heart ceased to beat upon exposure to MS-222 which was also reversed after washout. In the laboratory course, students were able to perform these

experiments on their own preparations in order to learn how such research is conducted and how data is analyzed. In the publication, data reproducibility was also evaluated by comparing student data analysis on a representative data set with analysis by individuals with prior training and experience. It was evident that analysis by different individuals can result in large variability in results obtained. This raises concerns about reproducibility and the different methods used to analyze data, causing the same data set to generate completely different results based on the individual performing the analysis. Overall, this study suggests that MS-222 can potentially be used as an anesthetic based on its reversible action on both sensory and motor nerve activity in different invertebrate models. Further studies that perform behavioral measures on the whole animal with injection or bathing in MS-222 will allow for greater understanding and characterization of its effects on a larger scale. The full publication "The effects of tricaine mesylate on arthropods: Crayfish, crab and *Drosophila*" can be found in volume 20 of *Invertebrate Neuroscience* (Stanley et al., 2020a).

The second project I was involved in was the development of a STEM-based module for use in the classroom that involves creating circulatory system models to enhance student learning and link the findings to real health issues. I aided in developing the publication of this project and presented it at the Association for Biology Laboratory Education (ABLE) conference at the University of Ottawa in the summer of 2019. The module involves exercises for students to engage in by relating the physical models they build of the circulatory system to a real-life example of cardiovascular function and health issues. The lessons involve studying fluid dynamics to relate to atherosclerosis and impeded blood flow due to plaques. Another model examines pressure differences and how

they are affected by clots or constrictions, relating to the ankle-brachial index (ABI) procedure that is used as a measure of arterial disease. Elastic recoil is modeled to examine the effects of blood vessel stiffening. Lastly, the effect of fluid viscosity on flow through tubing relates to the consequence of blood thickening. Thus, this module serves to enhance understanding of the circulatory system by relating it to real-life issues in a hands-on approach to learning. The full publication "STEM & Health: Stressors on the Circulatory System" can be found in volume 41 of *Advances in Biology Laboratory Education* (Stanley et al., 2020b).

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PROFESSIONAL PUBLICATIONS

1. Stanback, M., Stanback, A.E., Akhtar, S., Basham, R., Chithrala, B., Collis, B., Heberle, B.A., Higgins, E., Lane, A., Marella, S., Ponder, M., Raichur, P., Silverstein, A., **Stanley, C.**, Vela, K. and Cooper, R.L. (2019). The effect of lipopolysaccharides on primary sensory neurons in crustacean models. *IMPULSE* 2019.
2. **Stanley, C.E.**, Mauss, A., Borst, A. and Cooper, R.L. (2019). The effects of chloride flux on Drosophila heart rate. *Methods and Protocols 2*(3): 73.
3. Adams, R., **Stanley, C.E.**, Piana, E. and Cooper, R.L. (2019) Physiological and behavioral indicators to measure crustacean welfare. *Animals 9*(11), 914.
4. **Stanley, C.E.**, Adams, R., Nadolski, J., Amrit, E., Barrett, M., Bohnett, C., Campbell, K., Deweese, K., Dhar, S., Gillis, B., Hill, C., Inks, M., Kozak, K., Larson, A., Murtaza, I., Nichols, D., Roberts, R., Tyger, H., Waterbury, C. and Cooper, R.L. (2020). The effects of tricaine mesylate on arthropods: Crayfish, crab and *Drosophila. Invertebrate Neuroscience 20*, Article number: 10
5. **Stanley, C.**, Krall, R.M., Zeidler-Watters, K., Johnson, D., Blackwell, R.R., and Cooper, R.L. (2020). STEM & health: stressors on the circulatory system. Article 82 In: McMahon K., editor. Advances in biology laboratory education. Volume 41. Publication of the 41st Conference of the Association for Biology Laboratory Education (ABLE).