2008

THE INFLUENCE OF Ca2+ REGULATION IN SYNAPTIC FACILITATION OF MOTOR NERVE TERMINALS IN CRAYFISH AND *DROSOPHILA* AS WELL AS IN THE PHYSIOLOGICAL REGULATION OF LARVAL *DROSOPHILA* HEART

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ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Arts and Sciences at the
University of Kentucky

By
Mohati Desai-Shah
Lexington, Kentucky

Director: Dr. Robin Lewis Cooper, Associate Professor of Biology

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Intracellular Ca^{2+} ions are highly regulated in animal cells for them to function normally. Since the tight regulation of $[Ca^{2+}]_i$ is so ubiquitous among cells, it is not surprising that altered function in $[Ca^{2+}]_i$ regulation is associated with a myriad of disease states in humans. This is particularly evident in pacing myocytes and nerve terminals related to synaptic transmission. A common thread through this dissertation is on the role of three regulators proteins that are common to many cell types. These are the plasmalemmal Na^+/Ca^{2+} exchanger (NCX), the Ca^{2+}-ATPase (PMCA) and the SERCA on the endoplasmic reticulum. In chapter 1 a historical overview is provided on how the understanding in the importance of Ca^{2+} came about. In Chapter 2, I address indirectly the function of residual $[Ca^{2+}]_i$ on the efficacy of synaptic transmission by quantal analysis but also develop novel means of assessing quantal analysis to assign a *n* and *p* value to particular synapses. Chapters 3 and 4 address the role of the three Ca^{2+} regulator proteins in short bursts of synaptic transmission related to short-term facilitation or depression depending on the type of neuromuscular junction (NMJ). Two key model NMJs I used were the crayfish (Chapter 3) and the larval *Drosophila* (Chapter 4). For comparative purposes in investigating the role of the three proteins in $[Ca^{2+}]_i$ regulation, I used the *Drosophila* larval heart preparation (Chapter 5). Throughout these studies, I used various pharmacological and ionic approaches to compromise the function of these Ca^{2+} channels. The results were unexpected in some cases due to non-specific effects of the pharmacological agent or ionic manipulations. In addition, a mutational line of *Drosophila* was used to asses SERCA function, but the results at the NMJ were not as expected. However, results with the mutation on the function of the heart were promising. The significance of these studies stresses that multiple approaches to compromise channels is warranted and the findings should be beneficial for future investigators to advance in mechanistic studies.

KEY WORDS: Synapse, Calcium, Drosophila, Heart, Neuromuscular

Mohati Desai-Shah
November 6, 2008

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November 6, 2008

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Chapter One

General Background on Synaptic Communication

All animals use the nervous system to sense their external and internal environment. Neurons communicate these signals with each other and with non-neuronal targets such as muscles. The communication is important as this allows the animal to respond to its environment and regulate bodily functions. The chemical communication between neurons and targets occurs at synapses. The chemical signals are referred to as transmitter and they are stored within vesicles contained within the presynaptic nerve terminal. For motor neurons, the increase in intracellular calcium $[Ca^{2+}]_i$ causes synaptic vesicles to fuse with the presynaptic membrane to release their contents (del Castillo & Stark, 1952; Dodge & Rahamimoff, 1967). The post-synaptic receptors determine how the chemical signal is relayed to the cell, such as being excitatory or inhibitory. If the postsynaptic cell depolarizes then we refer to the signal being excitatory whereas a response that can result in a hyperpolarization we generally refer to it as inhibitory. This is not always the case as second messenger cascades can result in excitatory or inhibitory phenomena without necessarily causing the membrane potential to change.

The neurotransmitter molecules that are stored in vesicles in the presynaptic nerve terminal are released by the fusion of the vesicles to the presynaptic membrane. This fusion of the vesicles occurs via a synaptic vesicle fusion cycle which is a complex process involving many proteins and other molecules. One of the major players in the synaptic vesicle fusion cycle is Ca^{2+} (Sudhof, 2004). The $[Ca^{2+}]_i$ is very low compared to that outside (for example, in the squid axon the $[Ca^{2+}]_i$ is about 0.1 μM, while the $[Ca^{2+}]_o$ is 10 mM, a 10^4 fold difference). This large difference in $[Ca^{2+}]$ provides a gradient for Ca^{2+} to enter into the cell (Hodgkin, 1964). This Ca^{2+} then causes the mobilization of both the reserve and the readily releasable pools of vesicles (Rosenmund & Stevens, 1996; Kuromi & Kidokoro, 1998). Moreover it is also responsible for the fusion of the vesicle to the presynaptic membrane which can then cause the neurotransmitter to be released into the synaptic cleft.

The involvement of Ca^{2+} in these processes, though very important is indirect. It is an accepted hypothesis that the reserve pool of vesicles is held in place in the cytoskeletal network and may be mobilized by a second messenger cascade involving IP3, such as in response to serotonin (5-HT) in crayfish. The mobilized vesicles are then released in a calcium dependent manner (Dixon & Atwood, 1989;

Dudel, 1965). There are many proteins that act as calcium sensors, which sense the change in $[Ca^{2+}]_i$ and initiate an intracellular change.

The trafficking of synaptic vesicles in the nerve terminal is closely regulated and it occurs via a synaptic vesicle fusion cycle which is briefly described here. The synaptic vesicles are bound and held in place in the cytoskeleton from which they need to be mobilized. This occurs due to the entry of Ca^{2+} into the nerve terminal. The Ca^{2+} indirectly causes the phosphorylation of proteins called synapsins which act to bind the vesicles to the cytoskeleton. On being phosphorylated the synapsins release the vesicles and allow them to be transported to the active zone to which they dock via various SNARE proteins like syntaxin and synaptobrevin. Thereafter, the tethering of proteins on the vesicle and synaptic face undergoes a series of reactions which prepares the vesicle for fusion with the presynaptic membrane. These vesicles are termed as “primed”. Once docked and primed, an integral membrane protein on the synaptic vesicle, synaptotagmin, is able to sense the $[Ca^{2+}]_i$ in the presynaptic terminal. Synaptotagmin has the ability to bind four Ca^{2+} ions and in turn undergo a conformational change that leads to complete fusion with among the membranes to allow transmitter release. In addition, it was shown in *Drosophila* that mutations in the calcium binding domains of synaptotagmin had an effect in vesicle numbers and vesicle size, suggesting it may play a role in vesicle structure as well (Loewen *et al*. 2006).

Thus, $[Ca^{2+}]_i$ plays an important role in vesicle fusion and neurotransmitter release mainly indirectly by causing the activation of other proteins involved in the synaptic vesicle cycle. Activation of these proteins occurs when $[Ca^{2+}]_i$ rises and they remain activated until the $[Ca^{2+}]_i$ is brought back down to basal levels. Thus, with bursts of electrical activity Ca^{2+} flows into the terminal, causing activation of Ca^{2+} sensing proteins which are involved in the synaptic vesicle cycle and thereby allows more docking/fusion of the vesicles. During maintained stimulation the $[Ca^{2+}]_i$ increases. Thus, more vesicles would be recruited to maintain transmission thoughout the maintained electrical activity.

After understanding how important calcium is in neurotransmitter release, it is not difficult to realize how a change in $[Ca^{2+}]_i$ can affect transmitter release. Synaptic plasticity is the alteration in synaptic transmission resulting from previous impulse activity and leading to a change in the strength of neuronal synapses. There are many events that can be categorized into this broad topic of synaptic plasticity, facilitation being one of them. Facilitation is an enhancement of neurotransmission by repetitive stimulation due to an increased probability of vesicle fusion and release in response to an action potential. It leads to an increase in postsynaptic response after

a train of pulses is administered. Facilitation can be divided into short-term (STF) and long-term facilitation (LTF). They differ in the duration of stimulation applied and the time the effect persists. The response is, in most cases studied, an excitatory response but there are inhibitory responses that can show a facilitatory nature as well. LTF occurs when prolonged trains of stimuli leads to an increase in a postsynaptic response which persists for minutes to hours even after the inducing activity has ceased. STF is the increase in postsynaptic response by a subsequent impulse following an impulse or a brief train of stimuli. In order to present how facilitation is understood today, I first provide a short historical perspective on how facilitation was shown by the force of muscle contraction and later by electrical recordings of muscle.

Some of the first instances of bioelectricity were showed by Italian anatomist and physician, Luigi Galvani, who performed a series of experiments starting around 1780, involving electric discharges and frogs. He found that a charge applied to the spinal cord of a frog could generate muscular spasms throughout its body. Charges could make frog legs contract even if the legs were no longer attached to a frog. While cutting a frog leg, Galvani's steel scalpel touched a brass hook that was holding the leg in place. The leg twitched. Further experiments confirmed this effect, and Galvani was convinced that he was seeing the effects of what he called animal electricity, the life force within the muscles of the frog. Later Alessandro Volta did similar experiments and applied weaker current stimuli and observed that the muscle reacts the same way. He found the minimum current/force that was required to activate the muscle. These were the first instances indicating that there is a threshold potential which if reached leads to the initiation of an action potential (this information was translated from French from a correspondence letter between Volta and Cavallo describing Galvani's discoveries; Galvani & Volta, 1793).

The first description of facilitation is in experiments done by Charles Richet. He describes stimulating a crayfish muscle and recording the time taken for the excitation to be observed (Archives de physiologie normale et pathologique, 1879). He points out that there is a lag between the time the stimulation is given and the time at which the excitation is observed. He had observed that when two stimuli are given the lag between the time-point of stimulation and the time the excitation is observed differs between the two stimuli. And these times depend on the period between the two stimuli. If the two stimuli are closer spaced then the time lag between the second stimulus and the excitation of the muscle by that stimulus is shorter than in the first case. In the literature, Richet does not describe the time between the two stimuli but he does mention that the time lag between the first

stimulus and the first excitation is about 0.008 sec and that between the successive stimuli and their corresponding excitations is about 0.004 sec, with 0.003 sec being the shortest duration. But, he did observe that if the two successive stimuli are farther apart then the opposite happens and the time lag actually increases.

In his book, *Physiologie des muscles et des nerfs*, 1881 (Physiology of the muscles and the nerves), Charles Richet describes an experiment wherein he demonstrates the influence of successive excitations of the muscle which resulted in what he describes as 'jolts' or twitches. He states that Helmholtz had previously observed that two excitations of equal magnitude, one closely following the other result in the merging of the second twitch with the first one. If the time between the two excitations is even less, then it will result in a single twitch which will be stronger than that provoked by a single excitation.

In his experiment describing the influence of seven successive excitations all of equal magnitude Richet observes that there is an increase in the excitability of the muscle. The first couple of excitations do not evoke a twitch; the third shows a very weak twitch. The next 4 excitations show prominent twitches, which increased successively in strength. The muscle facilitates in the strength of muscle contraction.

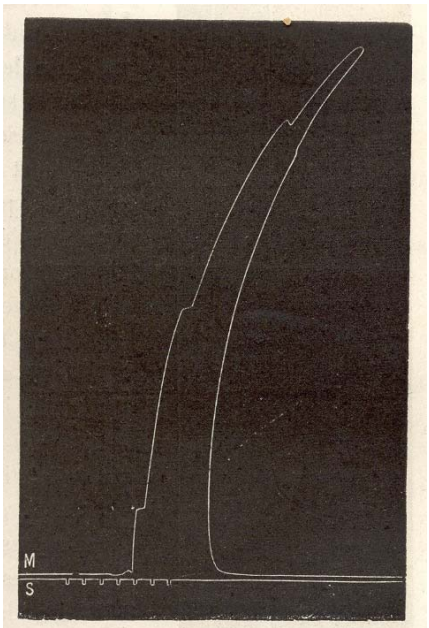

Fig. 32. — EXCITABILITÉ CROISSANTE.

Muscle d'écrevisse. — S. Indications des sept excitations électriques d'intensité égale. — Rythme uniforme.

Figure 1.1: First recorded STF: Figure showing the first instance of STF recorded from crayfish muscle by Charles Richet. Shown here is the result of 7 excitations

(stimuli), the first 3 of which do not show any significant twitches while the last 4 show facilitation in tension

In these experiments, myographic recordings showing tension development in the muscle were carried out and they measured the effect of the excitation on the muscle. These results, although they are not intracellular recordings, indicate the first instances of recorded STF, as far as I am aware.

After the first intracellular recording electrode was made by Ling and Gerard in 1949, soon afterwards the first intracellular recordings of STF were carried out by Fatt and Katz in 1953 in crab muscle fibers. Crustacean muscles are known to produce graded contractions (Katz & Kuffler 1946; Katz, 1949; Wiersma, 1949). This means that they produce powerful contractions without propagated action potentials. The figure from their 1953 manuscript (Figure 2) shows the graded potentials produced in crab muscle fibers in response to several current pulses.

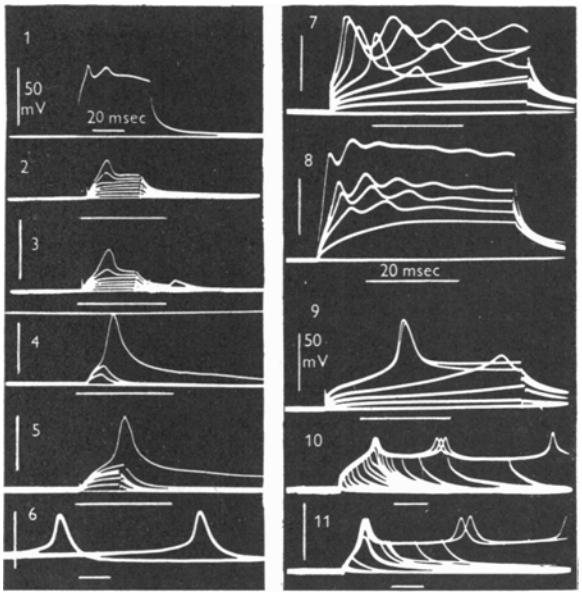

Figure 1.2: First intracellular recordings of STF: Original figure from Fatt & Katz, 1953 showing potentials in crab muscle fibers in response to several current pulses. Records 2-5 and 7-9 show pulses of constant duration and varying intensity. Records 10-11 show pulses of varying duration and constant intensity. (Record 6 shows injury discharge in the muscle fiber).

Fatt and Katz showed only a few years earlier, in 1951, that acetylcholine was the transmitter that depolarized the frog motor endplate and voltages would summate

(Fatt & Katz, 1951). Both the frog and crustacean NMJs appeared to be the models of choice to investigate the frontiers of facilitation for a number of years. This is most likely due to the robust nature of these experimental preparations. A typical intracellular response of STF in the opener muscle of the crayfish walking leg is shown in Figure 1.3.

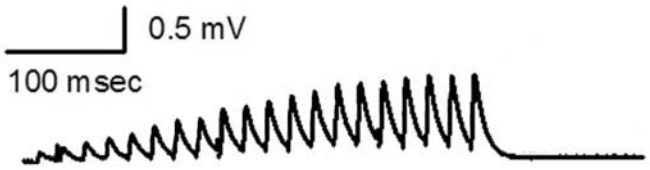

Figure 1.3: STF recorded from crayfish NMJ: The NMJ of the opener muscle in crayfish shows prominent STF in response to 20 stimulation pulses given at 40 Hz. Note the plateau during the end of the stimulus pulse

The basis of chemical synaptic transmission and properties that regulate STF, are well established, from insects to mammals, and are known to be dependent on the $[Ca^{2+}]_o$. Residual $[Ca^{2+}]_i$ due to previously evoked activity can accumulate, resulting in a greater $[Ca^{2+}]_i$ within the terminal and an increase in the amount of transmitter released, which is the basis for STF (Katz & Miledi, 1968). This regulation of $[Ca^{2+}]_i$ in the presynaptic terminal is necessary to maintain normal vesicle fusion and transmitter release. There are many mechanisms by which this residual $[Ca^{2+}]_i$ is removed. The residual $[Ca^{2+}]_i$ is altered by Ca^{2+} binding proteins and their dissociation rates (Augustine, 2001; Kretsinger & Nockolds, 1973; Lundh, 1998; Sheng *et al*. 1998). Ca^{2+} pumps and the sodium/calcium exchanger (NCX) can rapidly regulate $[Ca^{2+}]_i$, thus they have an effect on the rise and decay of $[Ca^{2+}]_i$. This residual $[Ca^{2+}]_i$ can also be correlated with vesicular release (Figure 1.4). The plasmalemma Ca^{2+} ATPase (PMCA) and the Ca^{2+} channels in the endoplasmic reticulum are two other important regulatory processes that control Ca^{2+} at the nerve terminal. Also, there are two Na^+/ Ca^{2+} exchangers that could play a role in clearing the $[Ca^{2+}]_i$. One is the NCX exchanger and the other is the mitochondrial Na^+/ Ca^{2+} exchanger (Zhong *et al*. 2001). While in *Drosophila,* the PMCA appears to be responsible for clearing Ca^{2+} which accumulates in motor nerve terminals with a larger impulse activity (Lnenicka *et al*. 2006). In the crayfish, the plasmalemmal NCX and the PMCA are observed to have important effects in controlling residual $[Ca^{2+}]_i$, whereas the mitochondrial Na^+/Ca^{2+} exchanger does not seem to have a significant role in STF (Zhong *et al*. 2001).

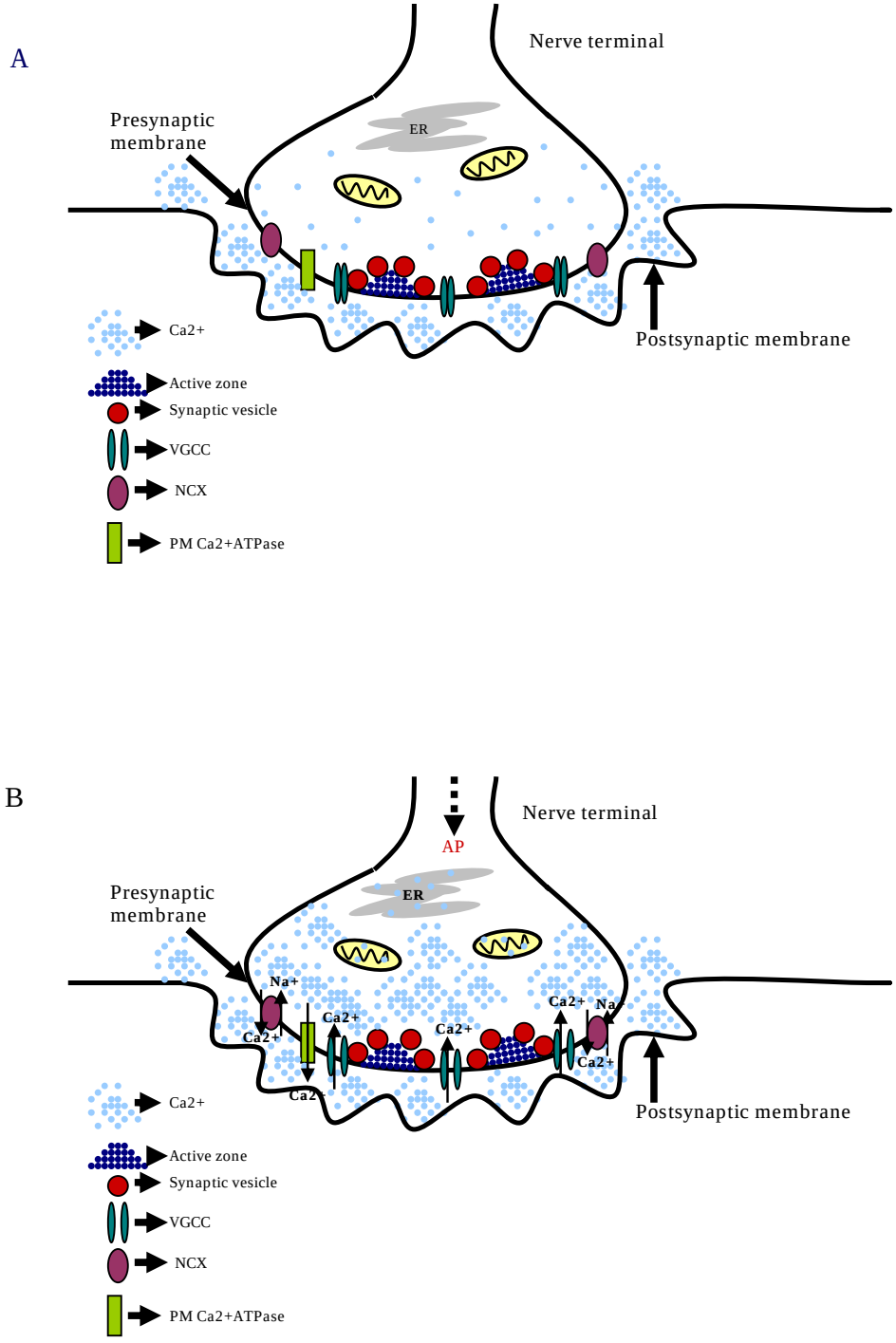

Figure 1.4: Cartoon of a NMJ at rest and after arrival of an action potential: (A) A cartoon depicting the various ion channels present at the presynaptic terminal. At rest the synaptic vesicles are docked and ready to release their contents. (B) The same terminal after the arrival of repetitive action potentials. There is an influx of Ca^{2+} from

the voltage gated Ca^{2+} channels. An increase of Ca^{2+} occurs within the terminal induced by a rapid series of stimuli. This causes an increase in the probability of vesicles to fuse to the presynaptic membrane and release neurotransmitter.

With repetitive stimulation, some motor nerve terminals (i.e. the crayfish tonic opener motor neuron) do not rapidly depress over a period of hours with short trains of stimulation at 40Hz but can continually demonstrate STF. When a short train of pulses at a given frequency (20 pulses at 40 Hz) is applied, the excitatory postsynaptic potentials (EPSPs) may reach a plateau in their amplitude during the pulse train (Figure 1.3). Even though the electrochemical driving gradient for the EPSPs is still sufficient to result in larger potentials they are restricted because of the amount of transmitter being released, thus a limitation is present which is not due to the lack in the driving potential for E_{Ca} or $[Ca^{2+}]$ (Logsdon *et al*. 2006; Sparks & Cooper, 2004). The $[Ca^{2+}]_i$ increases during the stimulation train, but it is presumed that an equilibrium is reached with the continual influx, buffering and exchange mechanisms to result in a steady state of transmitter release. With a transition to a higher stimulation frequency it is likely that a new equilibrium is established at a higher $[Ca^{2+}]_i$. The higher stimulation rate also results in a faster rise in $[Ca^{2+}]_i$, thus resulting in an increase in the mean quantal content and a faster rise of the EPSPs to the plateau level. I am interested in finding out what role the NCX plays and how important it is in establishing this equilibrium.

The NCX has been primarily studied in the cardiac muscle (cardiomyocytes) where it is the primary mechanism for trans-sarcolemmal Ca^{2+} efflux leading to contractile events. It is also found in several other tissues in varying abundance, for example, it is found in relative abundance in kidney, smooth muscle and brain while it is apparently low in liver, which also suggests the relative importance of Na^+/Ca^{2+} exchange in these different tissues (Philipson & Nicoll, 2000).

The NCX is a member of a large superfamily of membrane proteins, the members of which are defined by the presence of sequence motifs known as α repeats. This superfamily has been divided into four families (Philipson & Nicoll, 2000) which are as follows:

1) The NCX family: This family includes the most studied exchanger- the cardiac sarcolemmal exchanger, NCX1 whose stoichiometry is $3Na^+_{(in)}/1Ca^{2+}_{(out)}$. Other exchangers in this group include the mammalian NCX2 and NCX3 exchangers and their homologues from squid, *Drosophila* and *Arabidopsis*.

2) The NCKX family: These exchange 4 Na^+ for 1 Ca^{2+} and 1 K^+. The prototype is the NCKX1 which is abundantly found in the outer segments of rod photoreceptor cells.
3) The bacterial family: These bacterial proteins with α repeats are probably Ca^{2+}/H^+ exchangers.
4) The CHX exchangers: This family includes the Ca^{2+}/H^+ exchanger of yeast vacuoles.

For some of my studies, I have used the opener neuromuscular junction (NMJ) of a crustacean (i.e., crayfish) to address the role of the NCX in the development and maintenance of STF. The advantage of crustacean motor neurons for studying neurophysiology is the large size and identifiability of the individual neurons. The NMJs provide accessible synapses which show both short-term and long-term plasticity and have features common with many central synapses in vertebrates. The individual peripheral synapses, the large conducting axons, and the neuronal cell body can be studied with refined analytical techniques. Moreover, the crustacean motor neurons offer central type synaptic physiology, found in vertebrates, not available in more specialized synapses such as the squid ganglionic giant axon. The crayfish opener muscle is easy to dissect and the excitatory nerve is comparatively large (~ 20 μm in diameter), thus the preparation is easy for carrying out intracellular and focal synaptic recordings. I have demonstrated that there is a steady state in the amount of vesicles that are released during STF. The results support the idea that release is not saturated due to the lack in the ability of the terminal to release more transmitter or that there is a limitation in synaptic area for vesicle docking. Also the results support the notion that the NCX is crucial in shaping the extent of STF. Chapter 3 deals with studies that examine the plateau phase of STF and its regulation by the NCX at various stimulation frequencies.

In the study presented in Chapter 3, I proposed to decrease the efficiency of the NCX, which exchanges Ca^{2+} outside for Na^+ inside the nerve terminal, in order to understand its role in synaptic responses. I predict that reducing the function of the NCX would lead to an accumulation of Ca^{2+} inside the terminal. This will be approached by decreasing the amount of Na^+ in the bathing solution by $1/3^{rd}$ and $2/3^{rd}$ from normal levels. This study will help in understanding the role of the NCX in regulating short term facilitation.

It has been postulated that the increase in synaptic activity (and thereby STF) may partly be due to the recruitment and activation of previously inactive (silent) synapses (Atwood & Wojtowicz, 1999). There are various statistical methods and

parameters currently used to quantify synaptic efficacy. They are: ***m*** which is the mean quantal content that is commonly used as an index of the average number of events (or vesicles that fuse) per stimulus; ***n*** which represents the number of sites that release a vesicle; and ***p*** which is the probability of an event occurring at a release site (del Castillo & Katz, 1954). Various methods have been developed to measure *m* and *p*, which are not directly measurable (McLachlan, 1975; Korn & Faber, 1998). But when synaptic efficacy is very low these methods do not work well and in such cases the distribution is better fit using a Poisson distribution or a binomial distribution assuming *n*=1.

In addition, with using direct quantal counting methods two potential events both of which indicate a single evoked event would be counted identically, even if the two potentials appear distinctly different in the shapes of their membrane potential or current. Since differences in potential sizes or shapes of quantal events may indicate different synaptic sites firing, this may be useful information in determining the overall number of synaptic sites utilized. The sizes and shapes of the synaptic currents themselves also provide information about *n* and thus *p*. I address the possibility of synaptic recruitment in Chapter 2 by examining the characteristics in the quantal shapes as an index of a synaptic signature. This type of investigation can also be done after compromising the NCX and it will give us an idea of the total number of sites that are firing when the exchanger is compromised vs. when it is working at normal rate.

Direct structure-function studies (Cooper *et al*. 1995, 1996a, b) of discrete regions of motor nerve terminals have revealed that there can be many synapses (30 to 40), each with multiple active zones, and that the physiological measures of synaptic currents, with subsequent determination of *n* & *p*, may estimate *n*=1 despite quite large variations in the sizes and shapes of single evoked synaptic currents. The physiological and structural data would indicate that multiple sites are being utilized for vesicle fusion. Similar studies can be carried out in this case, which will tell us whether new sites are being recruited or the firing rates of the same sites increase. Moreover, it is well established that variation in quantal events occurs and that there are subcategories of quantal events that show a signature. It appears that synapses might give a particular signature and that if new sites are recruited it will show up as a new subcluster in the varied characteristics that can be measured for a population. Thus, for example, Viele *et al*. (2003) used clusters of the quantal EPSPs such as peak amplitude and area under the curve to produce precise estimates of *n* and *p* where counting methods were not refined enough. As the standardized approach of obtaining *n* and *p* from methods of directly counting quantal events (del Castillo &

Katz, 1954) and determining their distribution of occurrence underestimates the functional number of sites in this project. I propose to use these modern statistical methods that incorporate differences in current sizes and shapes to estimate *n* and *p*.

I also carried out comparative physiological studies on the NCX in crayfish and files at the NMJs (Chapter 4). The NCX in *Drosophila* has been characterized and both the NCX and the NCKX type of exchangers are found to be present in the fly. But, the family of the NCX exchangers present in the crayfish is not known. In order to compare the physiological functions of the exchangers in the two systems intracellular recordings were carried out and the effect of NCX on short-term facilitation in both were observed.

Moreover as mentioned above, along with the NCX, the PMCA and the SERCA also have a role in Ca^{2+} sequestration. Therefore, I hypothesize that reducing the working efficiency of these ion channels and observing STF, would help us answer the question as to which of these channels plays a more important role in $[Ca^{2+}]_i$ regulation. Intracellular recordings were carried out as described before for NCX, but in this case the efficiencies of the PMCA and the SERCA were compromised. These studies, like before, were done for both the crayfish and *Drosophila* NMJs. For compromising SERCA function, a drug that blocks the SERCA (i.e., thapsigargin) was used for the crayfish preparation. While for the *Drosophila* preparation, a special fly line, Kum^{170} that is mutant for the SERCA was used as well as a pharmacological apparoch. For diminishing PMCA function the pH of the bathing saline was increased to 8.8 from the normal pH (7.2 for *Drosophila*, 7.4 for crayfish). At this elevated pH the PMCA ceases to function (Lnenicka *et al*. 2006). Thus, by compromising/eliminating the function of these Ca^{2+} channels one after the other and observing synaptic transmission I can understand the role each of them has on $[Ca^{2+}]_i$ clearance and regulation in the nerve terminal.

Just like in the skeletal muscle, in cardiomyocytes Ca^{2+} plays an important role as a global signaling component controlling muscle action. Lewis Victor Heilbrunn in the 1930s and 1940s was the first to show that Ca^{2+} acts as an intracellular trigger for muscle contraction. It was also established to be an activator of the muscle contractile proteins (Hasselbach & Makinose, 1961). Moreover the sarcoplasmic reticulum regulates intracellular release and reuptake of Ca^{2+} in muscle (Ebashi, 1972). All this handling of calcium has to be carried out in a very tightly regulated manner. $[Ca^{2+}]_i$ is regulated by the different ion pumps, ion channels and ion exchangers that maintain a 10,000-fold lower (~ 100nM) $[Ca^{2+}]_i$ inside the cardiomyocyte in comparison to the $[Ca^{2+}]_o$. The contribution of the different ion channels, exchangers and pumps in regulation of heart rate has not been closely

studied in *Drosophila*. In this research study, I try to elucidate the differential roles of these different Ca^{2+} channels in regulation of *Drosophila* larval heart rate.

The *Drosophila* larval analog of the vertebrate heart is a tubular structure that runs along the entire length of the animal, it is known as the larval dorsal vessel. It is a simple tubular pump which by its peristaltic movements pumps hemolymph throughout the larval body (Curtis *et al*. 1999). It is divided into two regions: the more anterior one being the aorta and the posterior one being the true heart. The true heart contains at least one possible pacemaker (Rizki, 1978) which would be responsible in setting the rate and rhythm of the heart. Moreover in the larval stage the heart is myogenic. Here I have made an attempt to map the various regions of the heart for possible pacemaker activity by intracellularly recording action potentials from individual cardiomyocytes. The pacemaker cells would have distinctive action potentials just like in mammalian cardiac cells (Opthof, 2007) which would set them apart from the rest of the cardiomyocytes.

It was known for a long time that the heart requires Ca^{2+} for contraction. Ca^{2+} from the endoplasmic reticulum enters the cytoplasm resulting in contractions of the heart. This Ca^{2+} has to be then sequestered back into the ER in order continue the rapid and periodic contractions of the heart. This is carried out by the SERCA. In *Drosophila* cardiac muscle, it has been shown that the SERCA is enriched at the Z-line (Sanyal *et al*. 2005). It has also been shown that the SERCA is required for generating and maintaining cardiac contractions and maintaining the rhythmicity of the heart. Recently a particular *Drosophila* line, Kum^{170TS}, has become available in which the function of the SERCA has been perturbed. It is a temperature sensitive mutation in which the animals exhibit temporary paralysis after being subjected to a heat shock (5 min at 40°C). I used this fly line to determine the role of the SERCA in the maintenance and regulation of *Drosophila* larval heart rate. Moreover, I also studied the effects of the PMCA and NCX on the *Drosophila* larval heart rate.

The specific aims of this dissertation research are:

(1) To advance a previous analysis of the quantal responses in synaptic transmission, to one during STF in order to better estimate discrete *n*'s and to assign a *p* to each given site.
(2) To determine the role of the NCX, PMCA and SERCA on short-term facilitation and maintenance of transmission in motor nerve terminals in crayfish and their influence on short-term facilitation.

(3) To determine if similar physiologic profiles are present in *Drosophila* NMJs as for the crayfish NMJs in regards to the actions of NCX, PMCA and SERCA on synaptic transmission.

(4) To determine the roles of the NCX, PMCA and the SERCA in the regulation of HR in *Drosophila* larvae.

Addressing these aims is very important in understanding processes in Ca^{2+} regulation at the neuromuscular junction and in the heart. Several Ca^{2+} handling disorders have been described in mammals. In order to understand these disorders better, one can use invertebrate models like crayfish and flies, which can serve as good electrophysiological and genetic (fly) models, to study Ca^{2+} regulation on a fundamental level.

Chapter Two

Assessment of Synaptic Function during Short-term Facilitation in Motor Nerve Terminals in the Crayfish

INTRODUCTION

For nervous systems to function properly at various levels of excitation and inhibition, the efficacy of synapses is finely regulated and able to adjust in response to changing circumstances and requirements. However, unregulated variation in synaptic input when needing to relay a specific amount of information would provide an inappropriate signal for target cells to integrate. Due to the relative inaccessibility of postsynaptic dendrites and complexity of the central nervous system in general, most of our understanding in synaptic properties at a quantal level has been gained by studying accessible and relatively simple preparations, such as neuromuscular junctions (NMJ) (Bennett & Pettigrew, 1975; Nudell & Grinnell, 1983; Wilkinson & Lunin, 1994; Wilkinson *et al.* 1992). Historically, the frog neuromuscular junction was used but in physiological conditions the electrical events, due to nerve stimulation, produce action potentials that are conducted along the muscle. Further studies with lowered extracellular calcium or Ach-receptor blockers allowed more detailed studies to be made on the quantal nature of synaptic transmission (Del Castillo & Katz, 1954). Neuromuscular preparations in many invertebrates also serve as good models to investigate mechanisms underlying synaptic transmission since the postsynaptic evoked events are graded and do not spike, much like the dendrites of neurons in the vertebrate CNS. The advantage of NMJs is that the sites of release are able to be directly monitored without worrying about distortion due to electrical cable properties when recording in a cell body within a CNS preparation. NMJs within crayfish were investigated particularly for these purposes (Fatt & Katz, 1953a, b; Dudel, 1965, 1981; Dudel *et al.* 1983; Cooper *et al*. 1995a,b, 1996a,b,c; Atwood & Cooper, 1996b; Cooper & Ruffner, 1998; He *et al.* 1999; Southard *et al.* 2000).

Since synaptic strength is defined as the effectiveness of neurotransmission between pre- and post-synaptic cells, both pre- and post-synaptic factors can influence synaptic strength. Short-term plasticity of synapses, such as short-term facilitation (STF) which is a form of memory at a synaptic level, allows the gain of the system to be readily regulated without changing the hard wiring. In order to characterize transmission during STF, indexing the probability (p) and number of release sites (n) is valuable (McLachlan, 1978; Stricker, *et al.* 1994; Cooper *et al.*

1996b); however, to measure *n* and *p* over time when the basal amount of release is changing is a difficult task by conventional methods (Del Castillo & Katz 1954; Korn & Faber 1991; Viele *et al.* 2003).

Classically the analysis of quantal release and estimation of *p* comes from the average probability of release over time, but since each quantal occurrence is counted as an event regardless of its size or shape of the postsynaptic response this masks truly distinct synaptic sites. The traditional counting method would deem any two quantal events as identical in probability and site for release, even if the two currents (or potentials) appear distinctly different in their characteristics. Since differences in size or shape of the quanta are likely indicators that different synaptic sites are active, this can be useful information in determining if there is a change in the overall number of active sites during experimental manipulation (Viele *et al*. 2003). Given that many synapses have been serially reconstructed for the crayfish opener excitatory NMJ, the nature of synaptic size and its synaptic complexity are becoming well known (Atwood & Cooper, 1995, 1996a; Govind *et al.* 1995; Cooper *et al.* 1996b,c). Direct structure-function studies of discrete regions of motor nerve terminals have revealed that there can be many synapses, each with multiple active zones. Physiological measures of quantal synaptic currents and field potentials along with estimation of *n* and *p* for low frequency stimulation (1Hz) reveals n=1 despite quite large variations in the size and shape of single evoked synaptic currents. The physiological and structural data would indicate that multiple sites are being utilized for vesicle fusion. In addition, the standardized approach of obtaining *n* and *p* from methods of directly counting quantal events (Del Castillo & Katz, 1954) and determining their distribution of occurrence underestimates the functional number of sites. For these reasons, in an earlier study, we devised a means that incorporates differences in characteristics in the single quantal responses to estimate *n* and *p* for quantal subsets (Lancaster *et al.* 2007; Viele *et al.* 2003, 2006). We now extend this analysis to the quantal responses during STF to estimate discrete *n*'s and to assign a *p* to each given site. This type of analysis is a significant improvement over traditional analysis in determining the quantal parameters of release with multiple sites of release.

Note that this chapter of the dissertation has been published recently (Desai-Shah et al., 2008). The paper is reproduced here. This study was a team effort and was a subset of my dissertation project as a project in quantal analysis to assess synaptic function. The co-authors of the project participated in the following manner. Sparks and Hayden were undergraduate researchers that helped in data mining.

Srinivasan is currently an undergraduate and he helped not only in data mining but also in proof reading the manuscript in which he contributed significantly. Drs. Nadolski and Viele wrote code and processed the data for statistical comparisons. My role was to design the experimental procedures, experimentally collect data and compile the project into a manageable and publishable study with a thematic topic of examining quantal signatures.

METHODS

General

All experiments were performed using the first and second walking legs of freshly obtained crayfish, *Procambarus clarkii*, measuring 6-8 cm in body length (Atchafalaya Biological Supply Co., Raceland, LA). The animals were housed individually in an aquatic facility and fed dried fish food. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (Sparks & Cooper, 2004). Crayfish were induced to autotomize the first or second walking leg by forcefully pinching at the merus segment.

Physiology

To elicit an evoked response, the excitatory axon was selectively stimulated by placing a branch of the leg nerve (from the merus segment) into a suction electrode connected to a Grass stimulator (Dudel & Kuffler, 1961). STF was induced by giving a train of pulses at 10 second intervals at 20 or 40 Hz to the excitatory nerve. Intracellular EPSP and field EPSP (fEPSP) recordings were performed by standard procedures (Crider & Cooper, 2000; Cooper *et al.* 1995b; Dudel *et al*. 1983; Sparks & Cooper 2004). The fEPSPs were recorded by placing a focal recording electrode directly over a visualized varicosity on the nerve terminal. A vital fluorescent dye, 4-[4-(diethylamino) styryl]-N-methylpyridinium iodide (4-Di-2-Asp; Molecular Probes, Eugene, OR), was used to visualize the varicosities (Marigassi *et al.* 1987; Cooper et al. 1995a). All chemicals were obtained from Sigma chemical company (St. Louis, MO). Electrical signals were recorded on-line to a PowerLab/4s interface (ADInstruments, Australia).

Direct counts of the number of evoked quantal events in evoked release were used as an index of synaptic function. If only a single event occurred after the spike, it was counted as one; when double events occurred, they had counted as two, and

so on. In this study, 1000 trials of 10 pulses each were collected and recorded. An acquisition rate of 20kHz was used. The quantal events were individually examined to see whether they were single, double or more events. Mean quantal content (*m*) was determined by directly counting the events (Cooper *et al.* 1995b). For determining characteristics in the shape of quantal events only discrete single events were used. In some cases an event might ride on top of another event or start with very short synaptic delay and thus would lead to errors in separating out the signal of the action potential spike, produced by the nerve terminal in the recording, from the quantal event. Since multiple events could be counted as "events" they were used to determine mean quantal content but could not be used for assessment in the shape of the quantal event due to uncertainty in the true shape of the single event. Only distinct single events were processed for determining clusters of similar quantal properties.

Computational analysis

The data points for the single evoked quanta were copied from the full traces and placed into individual columns within an Excel spread sheet. The data points for each quanta were bracketed by the beginning of the quantal rise until the event returned to baseline. The files were transferred to text files. The routines herein are implemented in a statistical language called "S" using the freeware software "R" (R Development Core Team, 2005; see footnote). "R" is maintained by a consortium of statisticians and others, and may be downloaded at http://www.r-project.org/. We computed the time to the peak amplitude and peak amplitude for each single quanta within 500 traces for one experiment at 40Hz. To determine the number of active sites and the firing rate for each site (*n* and *p*) over the course of the ten pulses, we employed normal mixture models similarly to Viele *et al*. (2003). A normal mixture model attempts to cluster observations into separate normal (Gaussian) distributions. Mixture models are commonly used in model-based clustering (Raftery & Dean 2006). As with Viele *et al.* (2003), we selected the number of normal distributions required based on Bayesian Information Criteria (BIC, Kass & Raftery 1995). This is a Bayesian procedure which consistently estimates the correct number of components, meaning that as the sample size increases BIC will choose the true underlying number of components. Instead of a p-value measuring "statistical significance", BIC can be calibrated by "posterior probabilities" which measures the relative likelihood the data reflect each possible number of components. For more information, see Kass & Raftery (1995) or Gelman *et al*. (2004). In our context

differing components indicate differing active sites (in theory they could also mean the same site is having a different pattern of firing).

We performed the mixture analysis on the square root of the product of time to peak and peak amplitude. While at first this may seem an odd choice, it is justified in the literature. Previously, Viele *el al*. (2003) clustered the square root of the area under the curve (AUC) measures, with the square root justified by the intuitive notion that area is measured in squared units, and the statistical justification that the square root transformation produced better fits. Note here the AUC values were difficult to compute because the small amount of time between pulses made the baseline computation difficult. While this issue also affects peak amplitude, in the AUC any baseline error is multiplied over the length of the trace. Viele *et al.* (2006) demonstrated that AUC and the product of peak amplitude and rise time were highly correlated (in that paper ρ=0.96). Thus, our response of interest is sqrt (RP), where R is the time to peak amplitude and P is the peak amplitude. Note that mixture models are invariant to affine transformations of the data, thus the units are irrelevant to the number of components estimated.

For the data from each of the 10 pulses, we fit normal mixture models of 1, 2, 3, and 4 components using the EM algorithm (Dempster *et al.* 1977). We then computed the BIC value for each of the four fits (BIC is a combination of the likelihood with a penalty term based on the number of parameters). This BIC values were then converted to posterior probabilities (Kass & Raftery, 1995) and the model with the highest posterior probability was chosen. Thus, for each of the ten pulses we chose one of 1, 2, 3, or 4 components.

For the best fitting model, we also recorded the fitted mean and variance of the normal distribution components, and the estimated probability of firing. These best fits were then compared across the ten pulses to look for recruitment or changes in the active sites during STF.

RESULTS

The EPSP responses obtained by intracellular recording show a marked facilitation at 40Hz stimulation within the 10 pulse stimulus train (2.1A). The average of 20 trials shown here replicates the general trend of single trains (Figure 2.1B). There are differences in the degree of facilitation depending on the region of the opener muscle that is being monitored (Cooper *et al.* 1995a, Mykles *et al.* 2002). In this study, the terminals from central muscle fibers were used. It has been established that the majority of the facilitation during the STF is due to presynaptic

components, primarily by enhanced calcium build up (Katz & Miledi, 1968; Rahamimoff, 1968; Zucker & Lara-Estrella, 1983; Winslow *et al.* 1994). This build up of residual $[Ca^{2+}]_i$ enhances vesicular fusion and as expected mean quantal content (Sparks & Cooper, 2004). To index the degree of enhancement, we calculated mean quantal content (*m*) by counting the discrete number of evoked quantal responses for each stimulus pulse within the 10 pulse train for each trial. Figure 2.2 shows a representative trail of the individual fEPSP responses of a preparation stimulated at 40Hz with 10 pulse trains depicting the discrete quantal events recorded with a focal macropatch electrode over an indentified region of the nerve terminal (Figure 2.2A1). In some pulses, single evoked quantal events are observed while in others the failure of evoking a response or even two quantal events is observable. The probability of multiquantal responses is relatively low during the initial part of the stimulus train but increases toward the tenth pulse. However, the range in the area and peak amplitudes of the single quantal events from the earlier pulses covers the same range as what are deemed as single events for the tenth pulse, so we are confident in what is deemed a single or a doublet is accurate. In addition, the probability of vesicular fusion is still not extremely high for the tenth pulse so probabilistically one should not expect too many multiple events. The inset in Figure 2.2A2 is a multiquantal event with higher resolution. A 10 pulse series of stimuli are demarcated by the arrows in Figure 2.2B1. The magnified region of the trace shown in Figure 2B1 is depicted in Figure 2.2B2 for clarity to demonstrate that multiple evoked events and failures in evoking a response can be resolved. The number of events associated for each pulse within the 10 pulse data set and *m* for each pulse are shown in Table 2.1. With the discrete counts one can model various release probabilities (i.e., Binomial or Poisson). However, considering simultaneous estimation of *n* and *p* by discrete counts alone is notoriously unreliable and we had relatively low firing rates for many pulses we utilize functionals of the traces (e.g. peak amplitude, rise time, etc.) to estimate *n* and *p* (see Olkin *et al.* 1981, Casella & Berger 2001, or Viele *et al*. 2003. Olkin *et al*. provides an example on how changing a single data point by 1 can change estimates of *n* by 100).

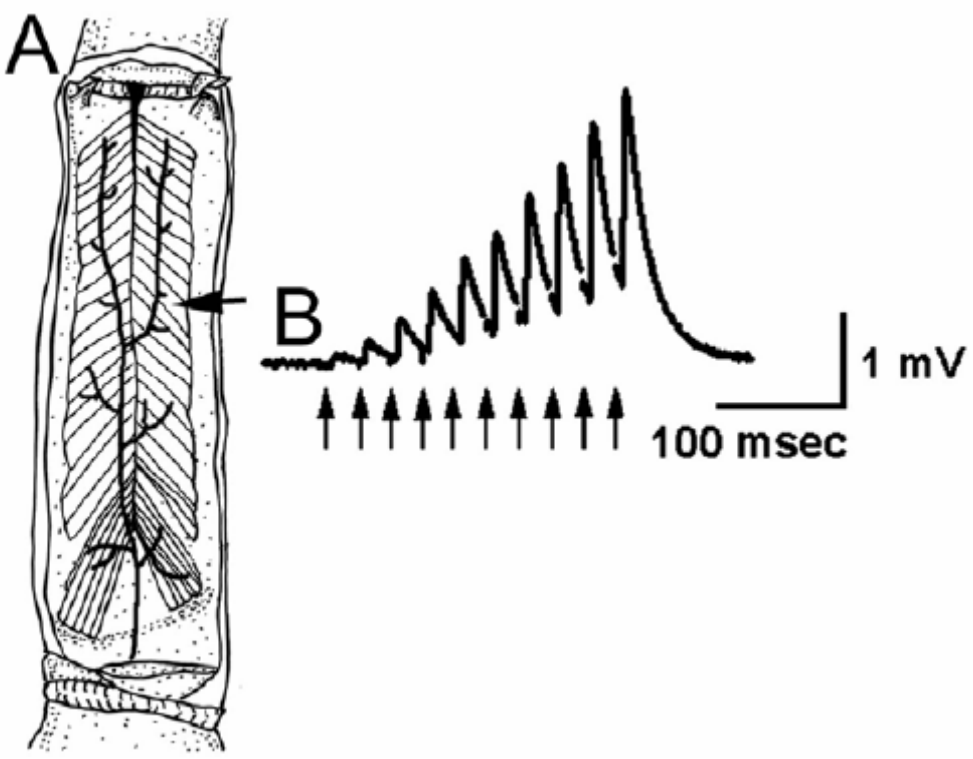

Figure 2.1: Crayfish NMJ preparation: (A) A schematic of the opener muscle in the crayfish walking leg showing the EPSP in response to a train of ten stimulation pulses given at 40 Hz. (B) The EPSP responses recorded intracellular from the central muscle fibers and the response shows a marked facilitation that occurs throughout the stimulation train. Arrows indicate each stimulation in the 10 pulse train.

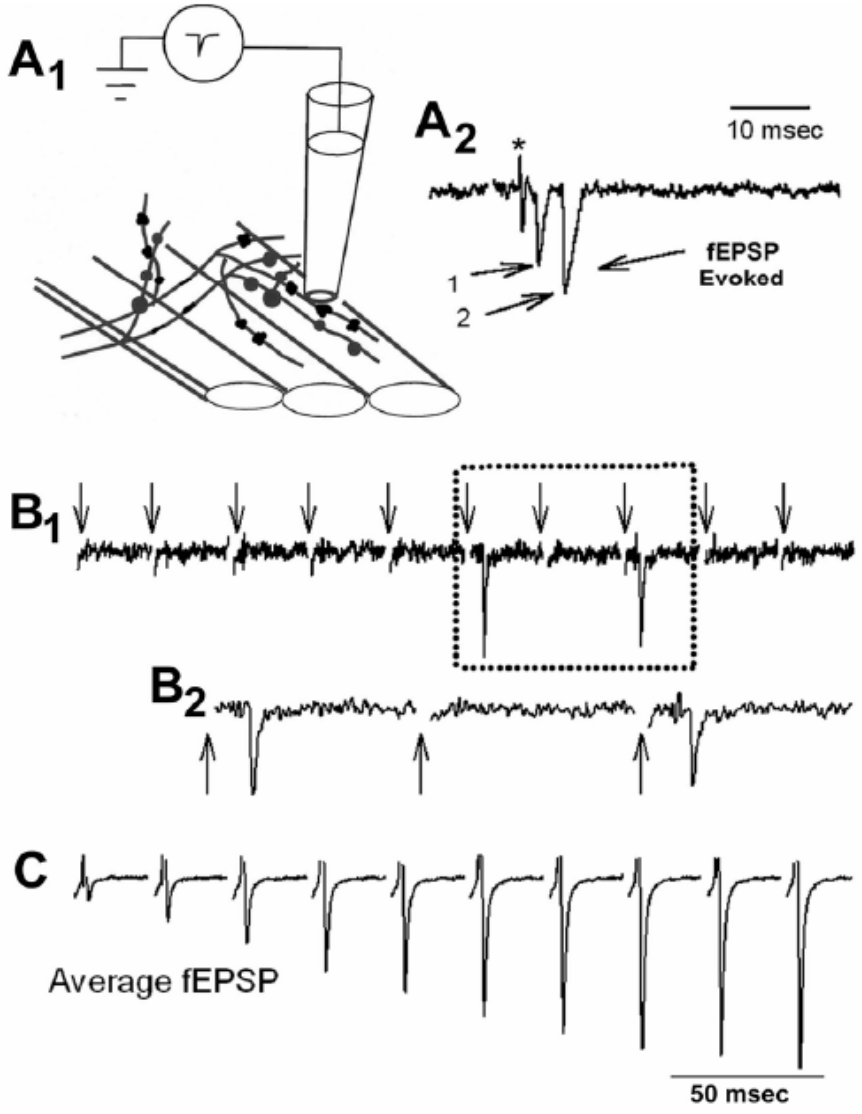

Figure 2.2: Focal recordings from crayfish NMJ: (A1) Recordings of individual evoked quantal events are obtained by use of a focal macropatch electrode placed over visualized varicosity on a muscle fiber. (A2) The quantal responses (focal EPSP or fEPSP) from evoked release are monitored. The * shows the extracellular spike (action potential) preceding the quantal response. Two quanta are recorded here with a short latency between responses. (B1) Individual fEPSP responses depict the discrete quantal events. The 10 stimulation pulses are shown by arrows. The events 6 and 8 show single evoked quantal events. Failure to evoke an event is noted for the other pulses. (B2) The inset shows the enlarged time scale within the dotted box in trace B1. (C) The average field potential (fEPSPs) from 500 stimulation trails recorded over a single varicosity mimics the whole muscle EPSPs in facilitation of

responses. The stimulus artifacts and the extracellular spikes were removed (blanks in the traces B and C) for ease is observing the evoked quantal events. The time scale bar shown in C is the same for the trace shown in B1.

Table 2.1: Quantal counts for each pulse: The discrete quantal counts for each pulse determined by direct observation. Mean quantal content (*m*) provides an index of presynaptic efficacy. All distributions were best fit by a *Poisson* distribution so *n* and *p* are un-realistic to determine.

Quanta	0	1	2	3	4	5	6	*m*
1st pulse	980	17	2	1	0	0	0	0.024
2nd pulse	946	51	3	0	0	0	0	0.057
3rd pulse	905	89	3	2	1	0	0	0.105
4th pulse	833	162	2	2	1	0	0	0.176
5th pulse	767	212	19	2	0	0	0	0.256
6th pulse	679	298	19	3	1	0	0	0.349
7th pulse	608	359	29	3	0	0	1	0.432
8th pulse	568	390	38	3	0	0	1	0.481
9th pulse	508	441	47	4	0	0	0	0.547
10th pulse	455	469	66	5	2	1	2	0.641

The rise in *m* over the pulse train is almost linear as shown with a linear least squares fit for one preparation, examined at 40hz (Figure 2.3). In another preparation, *m* was calculated for each pulse at 20 and 40Hz stimulation trains (Figure 2.4) and again a liner increase in *m* is observed for each stimulation frequency. This linearity may well change for different stimulation paradigms; however, with higher stimulation frequency the stimulus artifacts become mingled with the evoked responses which presents difficulty in obtaining measurements. Since the characteristics of each quantal event are used for further analysis, a good signal to noise ratio is required. The measures of the quantal events are illustrated on a single response in 2.5. The peak amplitude and rise time to peak were determined for each event that was deemed as a single event by direct observation. Once these measures were obtained, we proceeded with the various comparisons to examine novel quantal shapes that appeared to be recruited throughout STF.

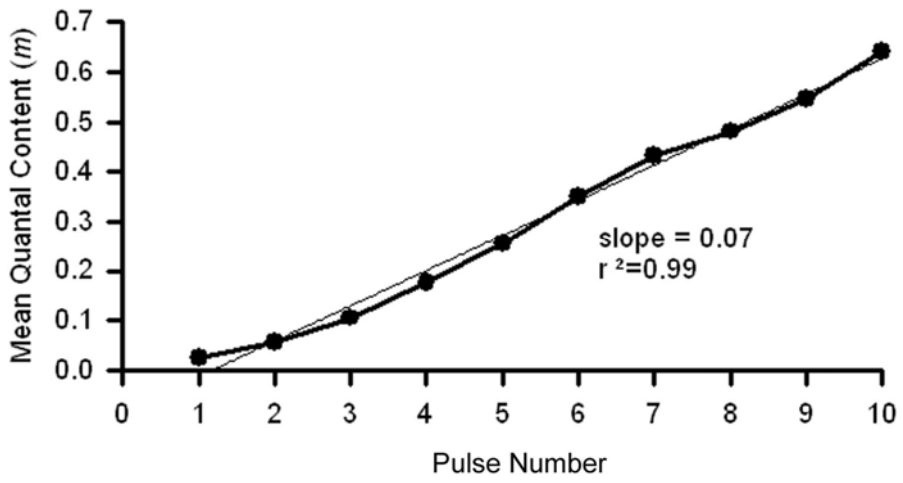

Figure 2.3: Mean Quantal Content during STF during 40 Hz stimulation: Synaptic efficacy determined by *m* from direct counts throughout STF. The curve was fitted by a linear least squares fit and the R2 value is reported. This preparation was stimulated only at 40 Hz.

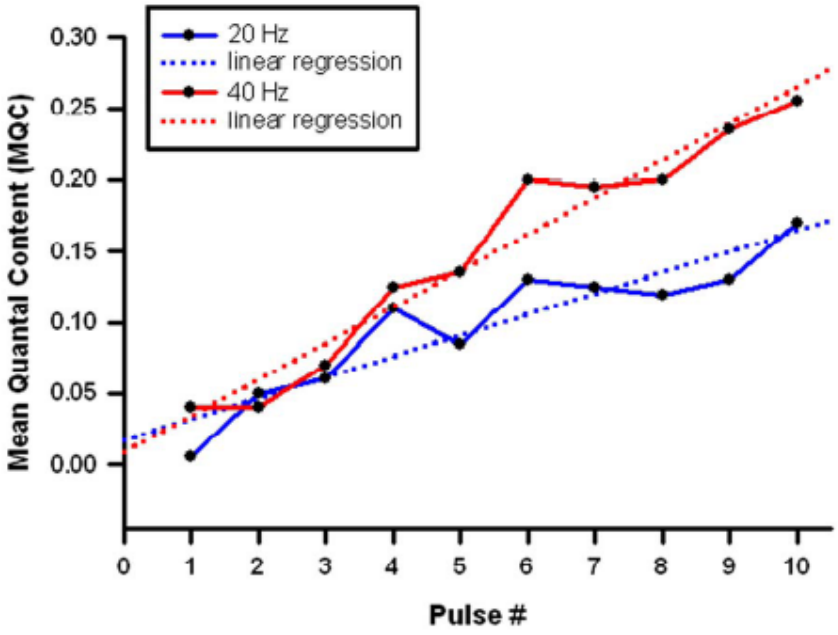

Figure 2.4: Mean Quantal Content for 20 Hz and 40 Hz stimulation: Synaptic efficacy determined by m from direct counts throughout STF. The curve was fitted by a linear squares fit. This preparation was stimulated at 20 and 40 Hz. The linear least squares fit and the R2 values are: 20 Hz is 0.85 and 40 Hz is 0.95.

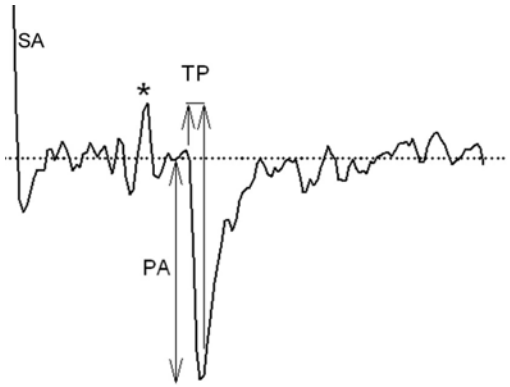

Figure 2.5: Characteristics of Quantal Events: The characteristic measures obtained from each quantal event are illustrated on this single response. The peak amplitude (PA) and the time to peak (TP) are shown. The stimulus artifact (SA) is followed by the extracellular spike (*) of the nerve terminal.

Table 2.2 shows the BIC values (in the left half of the table) for each pulse of the 1, 2, 3, or 4 potential fusion sites for the preparation depicted in Figure 2.3 (40Hz alone). These BIC values have also been converted to Bayesian posterior probabilities (in the right half of the table). Note BIC values are measured on the log scale, and thus their absolute difference, not relative difference, is important. Furthermore, BIC values are only comparable within each pulse, not across pulses as different pulses represent different datasets. For more information see Kass & Raftery (1995) or Viele *et al*. (2003). Values in bold indicate the best fit (the fit with the highest BIC and thus the highest posterior probability).

Table 2.2: BIC values and posterior probabilities: Bayesian information criteria (BIC) values and estimated posterior probability for each of 1, 2, 3, and 4 component models fitted separately to the data from each pulse. Bold entries indicate the best fit for that pulse

Pulse	BIC1	BIC2	BIC3	BIC4	Pr(1)	Pr(2)	Pr(3)	Pr(4)
1	12.46	**14.66**	8.62	10.77	0.097	**0.883**	0.000	0.018
2	28.30	**30.54**	28.53	23.47	0.086	**0.805**	0.108	0.000
3	85.69	**92.45**	85.40	82.42	0.000	**0.998**	0.000	0.000
4	94.49	104.30	**104.96**	100.81	0.000	0.338	**0.652**	0.010
5	72.09	82.52	**84.55**	78.69	0.000	0.116	**0.882**	0.000
6	47.44	75.30	**76.77**	69.64	0.000	0.187	**0.813**	0.000
7	100.05	99.31	**103.08**	94.85	0.045	0.022	**0.933**	0.000
8	61.83	78.32	**80.60**	76.86	0.000	0.091	**0.887**	0.021
9	54.07	**66.43**	58.16	56.34	0.000	**1.000**	0.000	0.000
10	**59.15**	58.36	53.47	52.06	**0.686**	0.311	0.000	0.000

Table 2.3 summarizes the best fits for each of the ten pulses (preparation stimulated at 40Hz alone). For each fitted component we report the mean μ (the fitted average value of sqrt (RP)), the standard deviation σ, and the firing rate p (here p refers to the probability a stimulus results in a firing at that site for the specific pulse, thus the probabilities do not add to 1, instead they add to the overall firing rate for the pulse). Note the probability of firing is low for the first pulse and increases dramatically for higher pulses.

Table 2.3: Estimated means, standard deviations and firing rates: Estimated means (μ), standard deviations (σ), and firing rates (relative to the total number of stimulations) for each normal density component (1, 2, or 3 indicating first, second, or third component) from the best fitting model for each pulse. The 1, 2, or 3 then relates to each potential release site. The results have been aligned in columns to indicate apparent similarity between the estimated components from pulse to pulse

	μ_1	σ_1	p_1	μ_2	σ_2	p_2	μ_3	σ_3	p_3
Pulse 1	0.444	0.088	0.044	0.782	0.010	0.004	-	-	-
Pulse 2	0.465	0.089	0.099	0.728	0.181	0.015	-	-	-
Pulse 3	0.454	0.071	0.189	0.615	0.146	0.081	-	-	-
Pulse 4	0.486	0.080	0.296	0.710	0.069	0.094	1.030	0.044	0.012
Pulse 5	0.496	0.089	0.293	0.718	0.134	0.231	1.145	0.301	0.012
Pulse 6	0.524	0.091	0.262	0.753	0.144	0.334	1.652	0.169	0.008
Pulse 7	0.580	0. 101	0.350	0.817	0.120	0.264	1.465	0.022	0.004
Pulse 8	0.569	0.103	0.408	0.815	0.155	0.313	1.460	0.164	0.011
Pulse 9	-	-	-	0.704	0.190	0.672	1.993	0.010	0.002
Pulse 10	-	-	-	0.744	0.204	0.760	-	-	-

Figure 2.6 shows the fits for these models. Each “pane” in Figure 2.6 shows a histogram of the observed firings for one of the ten pulses. The individual estimated normal distributions are shown as either red, green, or blue curves. Finally, the black curve is the sum of the colored curves and provides the overall mixture fit to the histogram. As can be seen in the figure, all the mixture fits provide reasonable approximations to the observed histograms, including the multiple observed peaks for the data in some pulses.

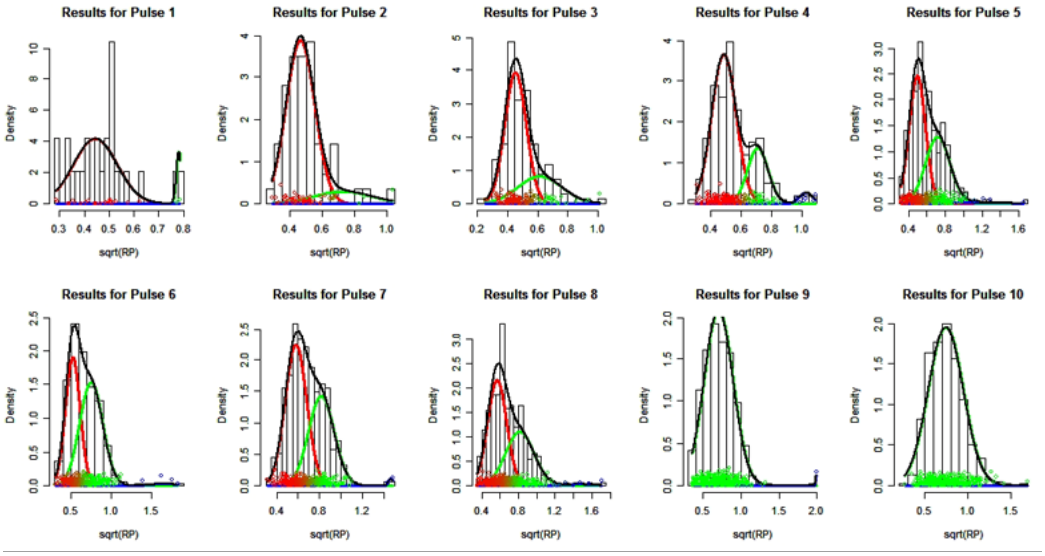

Figure 2.6: Mixture modeling plots for 40 Hz: Plots of the overall mixture fit and underlying fitted components for each of the ten pulses for 40 Hz (40hz only data).. Each histogram shows the sqrt(RP) data (square root of rise time multiplied by peak amplitude) for the corresponding pulse. The black line indicates the overall mixture fits, while the colored lines represent the underlying mixture components (the colored lines sum to the black line). The colored points indicate the estimated component membership of each data point. The y-axis “density” simply indicates the relative likelihoods of sqrt(RP) within each pulse.

The consistencies in Table 2.3 from pulse to pulse provide evidence concerning which sites are active at any given time. There is a site, active for pulses 1-8, with mean in the 0.45-0.60 range for sqrt (RP). The standard deviation for the results from this site is also consistent, ranging from 0.071-0.105. Note that the probability this site fires increases from a low 0.044 in pulse 1 to 0.408 in pulse 8. A second site appears to be recruited with a mean in the 0.728-0.817 range for sqrt (RP) for pulses 1-8. Finally, a third site is present for pulses 4-9 with a mean greater than 1. However, note this third site contains small probability and is typically represented by a small number of firings in each case (for example, probabilities of 0.01 correspond to 5 out of 500 traces).

It is quite unclear why the first site becomes inactive in pulses 9 and 10. It is also difficult to justify why the increasing probabilities of site 1 firing for pulses 1-8 suddenly drops to 0. Also, note that site 2 suddenly has a big jump in probability of firing going from pulse 8 to pulse 9, and a slight jump in standard deviation, possibly indicating the fitted results for sites 1 and 2 have combined (if two sites produce very

similar patterns of firing, the mixture model will not be able to discriminate them). Finally, site 1 has an increasing trend in the mean. It is possible that the mean for site 1 increases to the point that in pulses 9 and 10, it is not possible to distinguish separate firing patterns for sites 1 and 2.

Similarly, it is unclear in pulse 10 why the 3rd site is any less active than it was in pulses 4-9. With such a low firing rate, it is possible that we simply did not observe a sufficient number or size of firing for the algorithm to detect.

For the preparation which was stimulated at 20 and 40Hz (Figure 2.4), a similar analysis was performed for estimating the BIC values for each pulse of the potential fusion sites. These BIC values have been converted to Bayesian posterior probabilities for the 20Hz and 40Hz data (Table 2.4 and 2.6). The best fit parameters for each of the ten pulses (for the preparation stimulated at 20Hz and then at 40Hz) are shown in Table 2.5 and 2.7. For each fitted component, we followed the same procedure as presented for the other preparation only stimulated at 40Hz with a mean μ, the standard deviation σ and the firing rate p (here p refers to the probability a stimulus results in a firing at that site for the specific pulse). As with the other preparation, the probability of firing is low for the first pulse and increases dramatically slightly for higher pulses at both 20 and 40Hz stimulations. The graphs in Figures 2.7 (20Hz) and 2.8 (40Hz) show the fits for the best fit models as in Figure 2.6. In this particular preparation at 20Hz, there were not enough quantal events for the first 3 pulses in the train to obtain any best fit model; even when the stimulation was turned up to 40Hz the initial pulses did not prove enough single events to fit well into any particular distribution. The model does predict that n was best described by 3 sites for the middle pulses within the train for 20Hz stimulation but then dropped back to 2 sites for later pulses; however, the p values are higher for the 2 sites than the earlier pulses within he train. A similar phenomenon was observed for the 40Hz with n changing slightly but p increasing slightly for each n with the increasing number of pulses within the stimulation train.

Table 2.4: BIC values for 20 Hz data: 20 Hz data set for estimating BIC. Bayesian Information Criteria values and estimated posterior probability for each of 1, 2, 3, and 4 component models fitted separately to the data from each pulse. For pulses 1-3, an insufficient number of firings were recorded. Bold entries indicate the best fit for that pulse

Pulse	BIC1	BIC2	BIC3	BIC4	Pr(1)	Pr(2)	Pr(3)	Pr(4)
1	NA							
2	NA							
3	NA							
4	**-107.1**	-104.8	-104.3	-103.0	**0.917**	0.082	0.001	0.000
5	-110.4	**-106.2**	-108.5	-111.4	0.014	**0.888**	0.093	0.005
6	-148.3	-149.5	**-146.8**	-147.8	0.136	0.040	**0.602**	0.223
7	-148.8	-146.6	**-145.9**	-149.3	0.033	0.323	**0.623**	0.021
8	-144.5	**-140.4**	-144.7	-148.0	0.016	**0.971**	0.013	0.001
9	-155.6	**-150.9**	-155.0	-160.1	0.009	**0.974**	0.017	0.000
10	-194.6	**-192.7**	-193.7	-198.2	0.098	**0.657**	0.243	0.003

Table 2.5: Estimated *n* and *p* for 20 Hz pulse train: Estimates of n and p for the pulses within the 20 Hz train. Estimated means (μ), standard deviations (σ), and firing rates (relative to the total number of stimulations) for each normal density component (1, 2, or 3 indicating first, second, or third component) from the best fitting model for each pulse. The results have been aligned in columns to indicate apparent similarity between the estimated components from pulse to pulse

	μ_1	σ_1	p_1	μ_2	σ_2	p_2	μ_3	σ_3	p_3
Pulse 1	-	-	-	-	-	-	-	-	-
Pulse 2	-	-	-	-	-	-	-	-	-
Pulse 3	-	-	-	-	-	-	-	-	-
Pulse 4	86.45	10.64	0.095	-	-	-	-	-	-
Pulse 5	76.74	18.61	0.066	193.94	31.34	0.017	-	-	-
Pulse 6	72.45	16.59	0.082	141.65	45.56	0.019	119.91	1.05	0.020
Pulse 7	85.92	8.52	0.042	143.28	23.36	0.039	62.66	3.24	0.039
Pulse 8	68.40	12.17	0.079	136.89	29.29	0.038	-	-	-
Pulse 9	74.10	14.03	0.103	146.92	18.58	0.026	-	-	-
Pulse 10	86.61	17.95	0.137	161.23	18.31	0.025	-	-	-

DISCUSSION

In this study, we have shown that there is an effect on synaptic efficacy during STF by enhancement of presynaptic fusion of vesicles, and the underlying nature for

the increase appears to be due to both an increase in probability of release from low threshold release sites as well as a recruitment of new release sites. Understanding the subtleties of synaptic transmission related to the function and recruitment of synaptic sites in close proximity is problematic due to the spatial resolutions and 3D nature of nerve terminals.

One would predict differences in the mechanisms of synaptic plasticity during STF depending on the synaptic architecture. Many synapses in the vertebrate CNS and NMJs appear to have a grid of presynaptic dense bodies, which are presumed to be active zones (AZ), spread out on the synaptic surface (Atwood, 2006). Thus, with such a structure, it is feasible to assume that each site has an equal probability of release during a given frequency of stimulation and either a tuning-up or -down the probability of the entire grid occurs during STF. Perhaps, there are exceptions for the AZs along the edge of the synapse due to the lack of overlapping calcium clouds from neighboring AZs. However, synapses at the crayfish NMJ show a varied structural complexity with various sizes of synapses, numbers of AZs, as well as spacing among AZs. Such complexity is observed in insects and crustaceans (Atwood & Cooper, 1995; Cooper *et al.* 1995a, 1996a) and has been vital to understanding synaptic plasticity to account for mutational effects in synaptically relevant issues in the *Drosophila* NMJ model (Stewart *et al.* 1996).

Table 2.6. BIC values for 40 Hz data: 40Hz data set for estimating BIC. Bayesian Information Criteria values and estimated posterior probability for each of 1, 2, 3, and 4 component models fitted separately to the data from each pulse. For pulses 1-4, an insufficient number of firings were recorded. Bold entries indicate the best fit for that pulse

Pulse	BIC1	BIC2	BIC3	BIC4	Pr(1)	Pr(2)	Pr(3)	Pr(4)
1	NA							
2	NA							
3	NA							
4	NA							
5	-120.1	-116.4	**-113.1**	-113.2	0.001	0.018	**0.516**	0.465
6	**-189.6**	-190.4	-195.1	-200.2	**0.687**	0.310	0.003	0.000
7	-169.4	**-168.4**	-172.5	-175.6	0.274	**0.714**	0.012	0.001
8	-162.0	**-160.1**	-160.9	-165.7	0.100	**0.606**	0.292	0.002
9	-203.2	**-198.9**	-199.2	-202.6	0.008	**0.549**	0.430	0.014
10	-213.6	**-209.0**	-211.9	-215.8	0.010	**0.936**	0.053	0.001

Table 2.7: Estimated *n* and *p* for 40 Hz pulse train: Estimates of *n* and *p* for the pulses within the 40Hz train. Estimated means (μ), standard deviations (σ), and firing rates (relative to the total number of stimulations) for each normal density component (1, 2, or 3 indicating first, second, or third component) from the best fitting model for each pulse. The results have been aligned in columns to indicate apparent similarity between the estimated components from pulse to pulse

	μ_1	σ_1	p_1	μ_2	σ_2	p_2	μ_3	σ_3	p_3
Pulse 1	-	-	-	-	-	-	-	-	-
Pulse 2	-	-	-	-	-	-	-	-	-
Pulse 3	-	-	-	-	-	-	-	-	-
Pulse 4	-	-	-	-	-	-	-	-	-
Pulse 5	63.47	9.16	0.095	147.25	4.48	0.010	98.65	1.69	0.020
Pulse 6	77.39	32.29	0.190	-	-	-	-	-	-
Pulse 7	71.46	15.04	0.175	139.80	8.53	0.010	-	-	-
Pulse 8	63.21	13.57	0.159	123.66	17.04	0.016	-	-	-
Pulse 9	66.17	11.90	0.204	122.41	16.31	0.021	-	-	-
Pulse 10	67.48	13.10	0.218	130.74	16.16	0.017	-	-	-

Since the synapses occur on all sides of the terminal and are not planar, optical imaging by use of vesicle associated uptake dyes, such as FM1-43, would not allow optical assessment of multiple synapses simultaneously in these terminals. However, a physiological measure that characterizes discrete synapses at a quantal level is one possibility to assess multiple sites during STF as approached in this study. The variety in quantal responses has allowed us to group the quantal parameters for assessment in probability of occurrence and if new groupings appeared during STF. The analysis indicates that the overall rate of observing a firing increases for each successive pulse. In the preparation stimulated at 40Hz, only 3 active sites appear to have been used during the entire stimulus train. Sites 1 and 2 are active throughout pulses 1-8 (and site 1 is quite possibly active but not detectable in pulses 9-10), while site 3 is recruited at pulse 4. The results of the analysis also show that site 3 produces large values of sqrt (RP), but fires at a low rate. The other preparation, which was also stimulated for 10 pulses but at 20 and 40Hz, revealed that increasing the stimulation frequency increased the *p* more so than the *n* which accounted for the increase in *m* throughout the pulse train. The *p* increase for site 1 as compare to site 2. Given that a varicosity at this crayfish NMJ contains on the order of 20 to 40 synapses, it is surprising that *n* was not estimated to be higher during these pulse trains. Since there is such a linear increase in *m* within the pulse

trains for both preparations (40Hz alone and for the one stimulated at 20 and then 40Hz), we would have expected the same in the estimated *n* and *p* values with this analysis. This suggests that the quantal counting method and the method presented herein are not directly comparable. Also, these results could suggest that quantal signatures are not likely to be present for discrete synapses by this means of analyses. We also expected that the results for the 40Hz stimulation among the two preparations would have produced more similar results in estimates for *n* and *p* among the pulses since both recordings were made from primary varicosities along the nerve terminal string of varicosities (Cooper *et al.* 1996b)

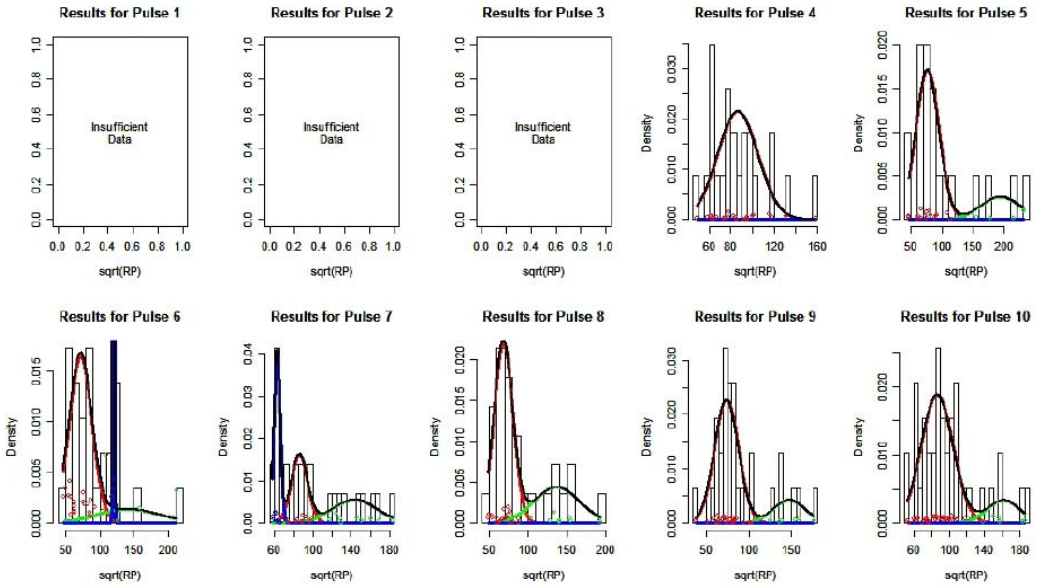

Figure 2.7: Overall mixture fit plots for 20 Hz: Plots of the overall mixture fit and underlying fitted components for each of the ten pulses during 20 Hz pulse train. Each histogramshows the sqrt(RP) data (square root of rise time multiplied by peak amplitude) for the corresponding pulse. The black line indicates the overall mixture fits, while the colored lines represent the underlying mixture components (the colored lines sum to the black line). The colored points indicate the estimated component membership of each data point. The y-axis “density” simply indicates the relative likelihoods of sqrt(RP) within each pulse.

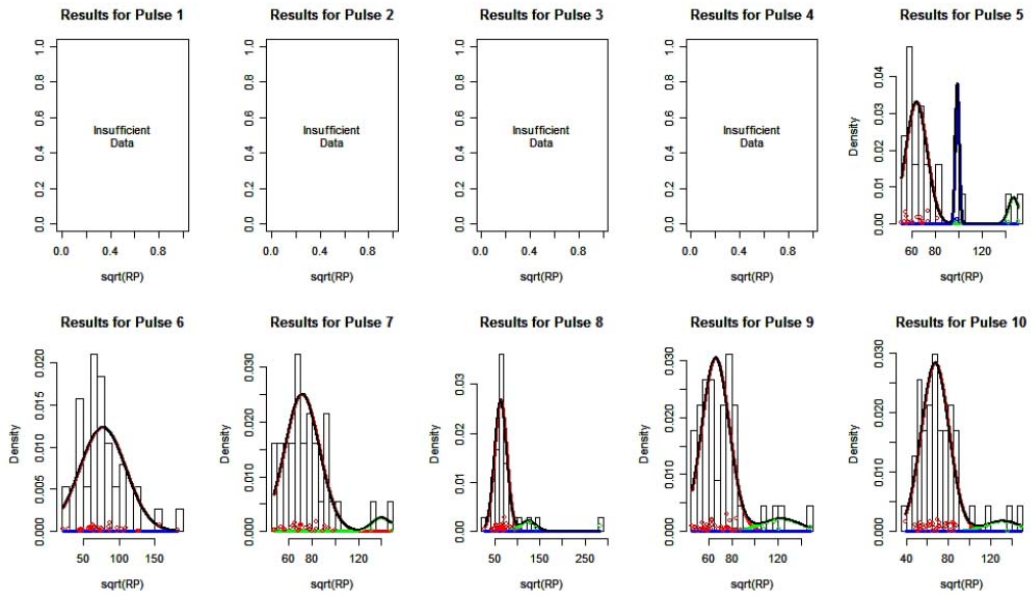

Figure 2.8: Overall mixture fit plots for 40 Hz: Plots of the overall mixture fit and underlying fitted components for each of the ten pulses during 40Hz pulse train. Each histogram shows the sqrt(RP) data (square root of rise time multiplied by peak amplitude) for the corresponding pulse. The black line indicates the overall mixture fits, while the colored lines represent the underlying mixture components (the colored lines sum to the black line). The colored points indicate the estimated component membership of each data point. The y-axis "density" simply indicates the relative likelihoods of sqrt(RP) within each pulse.

A potential error in these measures is a non-uniformity in size of vesicles that could produce varied size and shape of quantal responses for a given synapse; but since the synaptic vesicle diameters in the opener motor nerve terminal show little variation in size (Kim *et al.* 2000), we are fairly convinced that size does not account for the quantal variation observed. In addition, it is not likely that the rapidly recycling pool of vesicles did not have enough time to refill with glutamate in between pulse trains as this preparation can withstand being stimulated at 5Hz continuously for up to an hour before showing reduced quantal responses with use of a blocker for the presynaptic glutamate transporter (Logsdon *et al.* 2006). In fact, the range in quantal responses for any given pulse appears to be maintained throughout the experimental paradigm used in this current study. Recently it was noted that *Drosophila* larvae, which are highly active crawlers showed larger vesicles to appear after about 90 minutes of active crawling and that these vesicles were formed due to activity but became depleted over time (Steinert *et al.* 2006). If such were the case for the

stimulation paradigm used in our study then we would expect to show increased variationover time in the quantal responses for the 1st evoked pluses, which is not the case. Since the variation seen for each pulse is fairly consistent over time, but more variation occurs among different pulses (i.e., the 4^{th} to the 8^{th}) within a given train, indicates stability in recruiting similar threshold synapses. Recently, studies have shown that with high frequency stimulation, vesicles can be endocytosed by a bulk process thus allowing a large number of vesicular fusion events to occur at the synapses but without distorting the synaptic area (Clayton *et al.* 2008). In high-output NMJs of the crayfish on the extensor muscle, we did not observe bulk endosytosis with prolonged stimulation in a recent study (Johnstone *et al*. 2008), so we would not expect to find bulk endocytosis during these relatively short pulse trains for the low output NMJs used in this current study.

Some variation in the probability of release over the pulse train could be induced due to glutamate-ergic autoreceptor presynaptic feedback (Dudel & Schramm, 2003). However, this should not influence the characteristic quantal shape but the likelihood of occurrence (i.e. *p*). Such feedback regulation on the presynaptic terminal could, in part, cause the plateau in the EPSP amplitudes from the underlying leveling off in the mean quantal content within long pulse trains, such as 20 or 40 pulses, delivered at 40Hz.

One source of error in our study is the latency between two simultaneous events. The timing might be short enough to produce a large response that is multi-quantal. Such errors would likely occur in the later pulses of the stimulus train when the probability is enhanced. A means to possible address this issue would be to reduce the $[Ca^{2+}]_o$ or to decrease the bath temperature in order to shift the synapses to a lower probability. In our study, we essential changed the internal calcium by changing the stimulus frequency under the same external concentration. Such an approach was recently used to demonstrate in rat hippocampal neurons that the endocytic capacity is linked to the level of $[Ca^{2+}]_i$ (Balaji *et al.* 2008). Thus, with an increase of *p* for fusion within the STF train, there also needs to be an increase in the *p* of endocytosis. Similar studies of interest would be to document at the crayfish NMJ because of the large synaptic size and the known varied synaptic complexity differences for high- and low-output terminals at the capital NMJs (Atwood & Cooper, 1996b).

In his study, we tested if a lateral shift occurs in *n* and *p* obtained at the beginning of the pulse train to the later stimulus pulses within the train as $[Ca^{2+}]_i$ is raised by starting off with 20Hz and then moving to 40Hz. Within the stimulus train of 10 pulses, there was an incremental increase in both *n* and *p* as the stimulus train

increased for the same given stimuli within the train. In one recording the *p* increased substantially while *n* only varied by a value of 1 or 2. Currently, studies are under way to cause an earlier shift of *n* and *p* within the pulse train by raising $[Ca^{2+}]_i$ through blocking the sodium-calcium exchanger (NCX) (Desai-Shah *et al.* 2008)

The phenomenon commonly observed in the amplitude of EPSPs reaching a plateau during STF has not been fully addressed, but the balance in the influx through voltage gated channels, extrusion of calcium by pumps and the NCX are candidates because of a rapid time frame (100s of msec) (Juhaszova *et al.* 2000). In the STF induced in this study, the plateau is submaximal since a higher stimulation frequency results in yet higher amplitudes of EPSPs which reaches a new plateau. Thus, additional AZs would likely be recruited and the probability of the ones active at lower stimulation frequency would increase. When might all the AZs be recruited for a maximal *n* to be achieved? When this does happen, only the variation in the probability of each *n* will be increased for enhancing the gain of the system. However, if saturation in recruiting all the sites occurs at these NMJs is not known. With the analysis technique we present it might be possible to address such issues.

Presenting another means for examining the quantal nature of synaptic transmission opens novel avenues for further investigating the mechanisms of synaptic function. Studies are currently underway in the lab with similar methodical approaches at the *Drosophila* NMJs in mutant strains related to synaptic function and glutamate receptor sensitivity.

FOOTNOTE

R: A language and environment for statistical computing. R Development Core Team R Foundation for Statistical Computing, Vienna, Austria 2005;ISBN 3-900051-07-0; http://www.R-project.org

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Chapter Three

The Influence of the NCX, PMCA and SERCA on Short-term Facilitation and Maintenance of Transmission in Motor Nerve Terminals in Crayfish

INTRODUCTION

Chemical synaptic transmission is well established, from insects to mammals, to be dependent on the extracellular calcium levels (Katz & Miledi, 1968; Neher & Sakaba, 2008; Xing *et al.* 2005). A power relationship of 3 to 5 exists for the influence of calcium on transmitter release for a number of preparations (Dodge & Rahamimhoff, 1967; Stewart *et al.* 1994). The key is that transmission is enhanced with an increase in $[Ca^{2+}]_i$ in the presynaptic terminal. In addition, residual Ca^{2+} due to previous evoked activity can accumulate, resulting in a greater $[Ca^{2+}]_i$ which is the basis for short-term facilitation (STF) (Atwood & Wojtowicz, 1986; Cooper *et al.*1996b; Sherman & Atwood, 1971). The enhanced postsynaptic response during STF comes about by an increase number of evoked vesicular fusion (Del Castillo & Katz , 1954). Exactly how calcium, $[Ca^{2+}]_i$, promotes a vesicle to form a fusion pore with the presynaptic membrane is not fully understood, but there is support that a calcium binding protein, like synaptotagmin, can have an influence on the docking and release process (Neher & Sakaba, 2008). Calcium binding proteins also alter residual $[Ca^{2+}]_i$ that effects vesicle fusion (Cooper *et al.* 1996b; Winslow *et al.* 1994). The dissociation rate of Ca^{2+} ions from various calcium binding molecules may not be equal and thus, the effects from various Ca^{2+}-binding proteins maybe quite complicated. Pumps and the sodium/calcium exchanger (NCX) can rapidly regulate $[Ca^{2+}]_i$, thus having an effect on the rise and decay of $[Ca^{2+}]_i$ and effects in the dynamics of residual $[Ca^{2+}]_i$ on vesicular release (Atwood & Karunithi, 2002; Catterall & Few 2008).

The release of Ca^{2+} from internal stores such as smooth endoplasmic reticulum (ER), mitochondria, Golgi apparatus, synaptic vesicles, and calcium bound to molecules such as calmodulin (Brailoiu *et al.* 2002) can account for background $[Ca^{2+}]_i$ that might well play a role with sustained stimulation frequencies. Release of Ca^{2+} from ER can be regulated by inositol 1,4,5-trisphosphate (IP3) (Berridge, 1997, 2005; Berridge *et al.* 2000) and possible other mediators as mentioned by Dropic *et al.* (2005): activation of ryanodine receptors, cyclic adenosine diphosphate-ribose (cADPR), Ca^{2+}, NAADP, ryanodine, caffeine, or Ca^{2+} itself (the so-called Ca^{2+}-induced Ca^{2+}-release or CICR) (Bouchard *et al.* 2003; Brailoiu *et al.* 2002; Lee, 2000,

2001; Mattson *et al.* 2000; Petersen & Cancela, 1999). Since activation of ryanodine receptors within presynaptic nerve terminals is known to alter evoked transmitter release (Galante & Marty, 2003) as well as the size of the quantal unit at neuromuscular junctions (Liu *et al.* 2005), suggests that there is an uptake into the ER by the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA). SERCA could have a major role in controlling the residual $[Ca^{2+}]_i$ during STF. Dropic *et al*. (2005) demonstrated that adenophostin-A (a stable analog of IP3), caffeine and ryanodine enhanced transmitter release at the crayfish neuromuscular junction. Electron microscope spectroscopic imaging in frog motor nerve terminals revealed the presence of Ca^{2+}-loaded smooth ER, mitochondria, SER and synaptic vesicles (Pezzati *et al.* 2001). This opens the possibility for the potential presence of SERCA in other nerve terminals.

The NCX, PMCA and SERCA are all well established to have rapid effects on synaptic transmission and the associated facilitation and/or depression, for particular synaptic preparations, when their activity is inhibited (Jensen *et al.* 2007; Reuter & Porzig, 1995; Scheuss *et al.* 2006; Empson *et al.* 2007). $[Ca^{2+}]_i$ during stimulation is dependent on the electro-chemical driving gradients on the voltage-dependent Ca^{2+} channels ($Ca^{2+}{}_V$) but also on the distribution and density of these channels as well as others (i.e., K_{Ca} channel) that regulate depolarization of the membrane (Fakler & Adelman, 2008). Evidence suggests that within synapses the Ca^{2+} channels are localized within an area around the "active zone". The active zone (AZ) is somewhat a misnomer since they are not active till stimulated and not all AZs have equal thresholds for activation for vesicle fusion. They are recruited for transmission when stimulation is sufficient. Depending on the number of AZs within a synapse and the number of synapses within a length of nerve terminal, the amount of Ca^{2+} entry within the terminal during depolarization may be restricted locally or more diffuse. Structural differences of synapses such as the size of the AZ (i.e. the length of the dense bar) and spacing between AZs on the synapse can influence the resulting $[Ca^{2+}]_i$, thus regulating vesicular release (Cooper *et al.* 1995a,1996a, b). The synaptic complexity at the crayfish NMJ has been fairly well described by direct structure-function studies by use of serial electron micrographic reconstruction on physiologically recorded terminals (Atwood and Cooper, 1995, 1996a, b; Cooper *et al.* 1995a, b, 1996a). Furthering knowledge in understanding the Ca^{2+} regulation, during STF, allows one to build on this past knowledge in synaptic structure and efficacy to model the terminal in relation to vesicle kinetics and spatial organization of proteins and organelles that could have an impact on STF.

With repetitive stimulation in terminals which do not rapidly depress, such as for the crayfish tonic opener motor neuron, STF (Sherman & Atwood, 1971) as well as LTF (Wojtowicz & Atwood, 1985) can be induced. With short pulse trains, the excitatory postsynaptic potentials (EPSP) may reach a plateau in their amplitude (Crider & Cooper, 1999, 2000); however, with longer pulse trains or a higher frequency of stimulation a new plateau in the EPSP amplitude will be obtained. Even though the electrochemical driving gradient for the EPSP is still sufficient to result in larger potentials, they have reached a plateau in their amplitude. This is because the number of vesicles fusing is regulated but not limiting. The $[Ca^{2+}]_i$ increases during the stimulation train, but it is presumed that an equilibrium is reached with the continual influx, buffering and exchange mechanisms to result in a steady state of vesicle docking and fusion (Neher & Sakaba, 2008). This suggests vesicles rapidly refill and/or that they are replenished from a reserve pool as not to deplete filled vesicles for a readily releasable pool. With a transition to a higher stimulation frequency it is likely a new equilibrium is established at a higher $[Ca^{2+}]_i$. The higher stimulation rate also results in a faster rise in $[Ca^{2+}]_i$, thus resulting in a faster rise of the EPSPs to the plateau level. It was recently shown at the crayfish NMJ, of the opener muscle, that the change in the plateau of the EPSP amplitude was enhanced as the stimulation frequency was increased (Sparks & Cooper, 2004). This phenomenon may in part be due a lingering residual $[Ca^{2+}]_i$ or even to an increase in $[Ca^{2+}]_i$ induced enhancement by the NCX working in reverse due to the high influx through the evoked Na^+_V channels, thus resulting in a faster steady state process. Possible a limitation in the number of vesicles that can be released at the AZs within a short period accounts for a steady state. Such an anatomical limitation in the number of active zones may also be the rational for higher-output terminals associated with phasic motor nerves terminals which do not facilitate as much as lower-output tonic motor nerve terminals (Bradacs *et al*. 1997; Cooper *et al*. 2003). However a limitation in vesicular docking does not appear to be a factor for 20 pulse trains of 20, 40 and 60Hz stimulation rates since application of the neuromodulator 5-HT produces more vesicles to be released (i.e., an increased probability) during these evoked stimulus trains (Sparks & Cooper, 2004).

The purpose of this study was to determine if the release process during the plateau phase of STF is in part balanced by the NCX, PMCA and SERCA. In addition, I was interested to know to what extent these proteins function to induce STF. Since the crayfish is not amenable to genetic manipulation, as other model organisms such as the fruit fly (Chapter 4 of this dissertation), I used pharmacological and ionic manipulations to compromise the function of these three Ca^{2+} regulatory

proteins. I also compared different approaches to target the same protein to address if the approaches are complimentary to each other. One approach in dampening the function of the NCX is by reducing the driving gradient of Na^+ into the terminals by using a lower $[Na^+]_o$; however, I was concerned that this could have an impact on the shape of the action potential which would indirectly allow fewer Ca^{2+} ions to enter the nerve terminal by not evoking the $Ca^{2+}{}_V$ channels. To address this issue, I measured, with intracellular electrodes, the shape of the action potential in the pre-terminal axons before and during $[Na^+]_o$ manipulations.

A decrease in the amount of $[Na^+]_o$ that is required for the action potential or maintenance of the resting membrane potential of the cell needs to be considered in experiments in which $[Na^+]_o$ is lowered to compromise the NCX. Previous experiments by Hodgkin & Katz (1949) in the giant axon of the squid have shown that reducing the $[Na^+]_o$ produced a significant change in the amplitude of the action potentials while alterations in the resting membrane potential was comparatively small. These experiments were done in the squid, a seawater animal, where the $[Na^+]_o$ is more than twice that for the fresh water crayfish, so the results cannot be extrapolated directly to the crayfish without being first tested. I carried out experiments to ascertain if the resting membrane potential of the neuron is reduced as to cause a hindrance in $Ca^{2+}{}_V$ channel opening in the presence of an action potential. In addition, with reduced $[Na^+]_o$ the height of the AP would likely diminish relative to the reductions in $[Na^+]_o$. If the action potential is reduced below the threshold for opening the $Ca^{2+}{}_V$ channels then the probability of vesicular fusion will be affected, thus introducing an indirect variable to examining a compromised NCX on STF. This was approached in these studies by determining the size of the action potential and the change in resting membrane potential at various reduced $[Na^+]_o$ levels. It has been previously shown that the largest peak in calcium current (I_{Ca}) during presynaptic depolarization occurs near -10mV and the reversal potential for I_{Ca} is between +25 to +40 mV. Moreover, the first presynaptic depolarization that produced a Ca^{2+} current (I_{Ca}) was detected at about -35 to -30 mV (Wright *et al.* 1996; see Fig. 1). So as long at the resting potential stayed more negative than about -40mV the channels should not open during the resting state and if the amplitude remained above -10 mV the there would be enough potential difference to open the Ca^{2+} channels and reach a peak response during an action potential to result in transmitter release.

The crayfish opener NMJ serves as a good model for synaptic physiology since the postsynaptic responses are graded, shows pronounced STF, and the amplitude of the EPSPs are directly related to the presynaptic efficacy. The

significance of this type of study is that many of the phenomenon that our observed by experimental manipulations can be addressed mechanistically by directly measuring $[Ca^{2+}]_i$ within the terminal and quantal release can be directly correlated at synaptic sites which is not possible in most vertebrate synapses used to study STF and LTP. The hope is that the fundamental principles learned in this experimentally amenable preparation will apply, or at least help, in understanding the underlying nature of all chemical synapses.

METHODS

General

All experiments were performed using the first and second walking legs of freshly obtained crayfish, *Procambarus clarkii*, measuring 6-10 cm in body length (Atchafalaya Biological Supply Co., Raceland, LA). Animals were housed individually in an aquatic facility and fed dried fish food. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205 NaCl; 5.3 KCl; 13.5 CaCl2.2H2O; 2.45 MgCl2.6H2O; 5 HEPES adjusted to pH 7.4). Crayfish were induced to autotomize the first or second walking leg by forcefully pinching at the merus segment.

Chemicals

All chemicals were obtained from Sigma chemical company (St. Louis, MO) with the exception of thapsigargin and *KB-R7943* which were obtained from Tocris (Ellisville, MO) and 5,6-carboxyeosin from Marker Gene Technologies Inc. (Eugene, OR).

To reduce the driving force for the NCX a saline with a reduction of $[Na^+]_o$ by two-thirds was used. The Na^+ was substituted by choline chloride to balance osmolarity. In order to inhibit the PMCA, the pH of saline is raised to 8.8 from its normal value of 7.4. Thereafter, in order to study a compromised NCX and PMCA, the concentration of Na^+ in the saline is reduced by $2/3^{rds}$ and pH held at 8.8. Reduction in function of the SERCA was approached by exposure to thapsigargin (TG) (10 μM) for 10 minutes while providing stimulus pulse trains (Rumpal & Lnenicka, 2003). I also compared the approach of reduced $2/3^{rds}$ $[Na^+]_o$ to application of the NCX blocker (*KB-R7943*; 40 μM). *KB-R7943* is known to block the efflux but not the influx of Ca^{2+} through the NCX in rat myocytes (5 μmol/L within 15 seconds,

Satoh *et al.* 2000). To examine the difference of inhibiting the PMCA by pH8.8 and a pharmacological approach, I used carboxyeosin (CE). It was shown that 5 μM is sufficient to inhibit PMCA in rats and in guinea pigs (Mackienwicz & Lewartowski, 2006). Therefore, I used this concentration and incubated the preparation for 10 minutes.

Physiology

To elicit an evoked response, the excitatory axon was selectively stimulated by placing a branch of the leg nerve (from the merus segment) into a suction electrode connected to a Grass stimulator (Dudel & Kuffler, 1961). STF was obtained by giving a train of 10 or 20 pulses at 10 or 20 second intervals, respectively, to the excitatory nerve. The frequency of stimulation within the train varied (40, 60 and 80 Hz). Intracellular EPSP and field EPSP recordings were performed by standard procedures (Crider & Cooper, 2000; Cooper *et al.* 1995b; Dudel, 1983; Sparks and Cooper 2004).

In order to examine if the reduced $[Na^+]_o$ had an effect on the shape of the presynaptic action potential the preterminal of the excitatory axon, of the walking leg opener muscle, was impaled with sharp intracellular electrode filled with 3 M KCl. The neuromuscular junctions was exposed in situ and bathed in a physiological saline. The motor nerve, in the merus segment, was stimulated by placing it into a suction electrode which was connected to a stimulator. Stimulations were given at a frequency starting from 0.5 Hz and if the recording started in normal saline with the stimulation just slightly greater than threshold of activation. The stimulation intensity had to be increased as the $[Na^+]_o$ was reduced. The amplitudes of the action potentials produced were measured from digital records obtained at a 20 KHz acquisition rate. Initially the preparation was maintained in normal crayfish saline and stimulated. After every 200 stimulations (delivered at 0.5Hz) the external saline was replaced by one with a lower $[Na^+]_o$. Six salines with different Na^+ concentrations were used. When Na^+ was reduced, the osmolarity was maintained by replacing it with choline chloride. The six different salines, with the amounts of NaCl and choline chloride used for each, is shown in Table 3.1. An agar bridge (1.5% agar in normal crayfish saline) was used to keep the action potential amplitudes from fluctuating when the saline were changed.

Table 3.1: Crayfish saline composition: Composition of saline with reduced Na^+ and compensated with choline chloride

	100% Na+ (Normal Saline)	80% Na^+	66.66% Na^+	50% Na^+	33.33% Na^+	20% Na^+
NaCl	205 mM	164 mM	136.65 mM	102.5 mM	68.32 mM	41 mM
Choline Chloride	-	41 mM	68.3 mM	102.5 mM	136.53 mM	164 mM

Electrical signals were recorded on VHS tape (Vetter, 400), and on-line to a Power Mac 9500 via a MacLab/4s interface.

Analysis

To index facilitation, the ratio in the peak amplitude of the EPSP for one of the preceding pulses from the last one within the stimulus train was used. A unitary value of 1 was subtracted from the ratio to provide a facilitation Index (FI). The subtraction of one ensures that if no facilitation is present (i.e., the amplitudes of the responses are the same), FI will be zero. In addition, the amplitudes of the plateau EPSPs were compared within preparations at different stimulation frequencies by procedures previously described (Crider & Cooper, 2000).

Statistics employed were either the Student's *t*-test or a Wilcoxon paired rank sum test (a non-parametric test).

RESULTS

Opener muscle and STF

The opener muscle is divided in three general regions: distal, central and proximal. Even though the entire open muscle is innervated by a single motor neuron, the NMJs are structurally different and have regional specific differences in synaptic efficacy in these three general regions (Cooper *et al*. 1995a, b). The muscle fiber phenotype type has also been shown to be different in these regions (Mykles *et al.* 2002). For these reasons, I used the most distal fibers for these studies (Figure 3.1A). The STF induced by 20 or 30 pulses delivered at 20, 30, or 40 Hz to the opener motor neuron produces a train of EPSPs that initially facilitate and then a quasi-stable state in vesicular fusion and recycling occurs which produces a plateau

in the amplitude of the response. The opener muscle preparation and the responses for 20 or 30 pulses delivered at 20, 30, and 40 Hz stimulation trains are shown in Figure 3.1 B & C. The rate of the increase in the EPSP amplitude and the amplitude of the plateau increases as the stimulation rate increases.

Given the amplitudes of the EPSPs are fairly consistent after the 20th pulse in the 20 Hz stimulation paradigm one might predict that the rate of vesicle recycling might have reached a maximum; however, stimulating at a higher frequency 30 Hz or 40 Hz the EPSPs amplitudes increase and reach a new plateau level (Figures 3.1 B & C). Thus, the rate of recycling has a large capacity at the lower stimulation paradigms, but is tightly regulated to a continuous rate of fusion during the stimulation when a plateau in the amplitudes has been obtained.

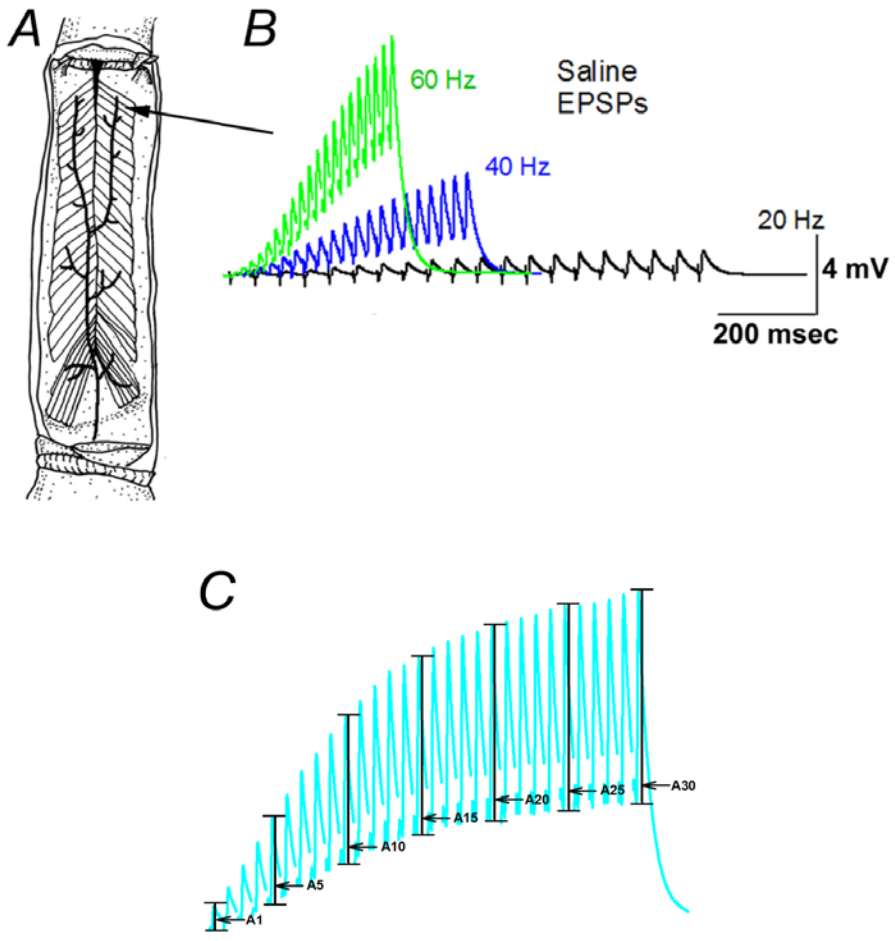

Figure 3.1: Crayfish opener muscle preparation and representative EPSPs: (A) The schematic of the opener muscle in the crayfish walking leg in which the distal muscle fibers were recorded with an intracellular electrode (black arrow). (B) EPSPs in

response to a train of 20 stimulation pulses given at three different frequencies 20, 40 and 60 Hz in normal crayfish saline. (C) The measurement in the amplitudes of the EPSPs was obtained by the difference in the potential prior to the event to the peak, as shown for the 1st, 5th, 10th, 15th, 20th, 25th and 30th events.

The changes in the plateau level in the EPSP amplitude with frequency of stimulation is even more pronounced when the bathing saline is switched to one containing 2/3rd $[Na^+]_o$. The depression in the amplitudes is substantial for all the amplitudes within the train. However, only subsets of the EPSPs are analyzed for quantitative purposes (i.e., 1st, 5th, 10th, 15th, 20th, 25th and 30th events). The change in the EPSP amplitudes for each preparation was decreased in 8 out of 8 preparations for all EPSP amplitudes measured ($P<0.05$, Wilcoxon rank-sum test). In comparing the responses among preparations a percent change from saline exposure to a reduced NCX state was determined. A percent is used since the absolute EPSP amplitudes varied among preparations. The means in the percent differences for the various events within the train were decreased (Figure 2; $P<0.05$, Student's paired *t*-test).

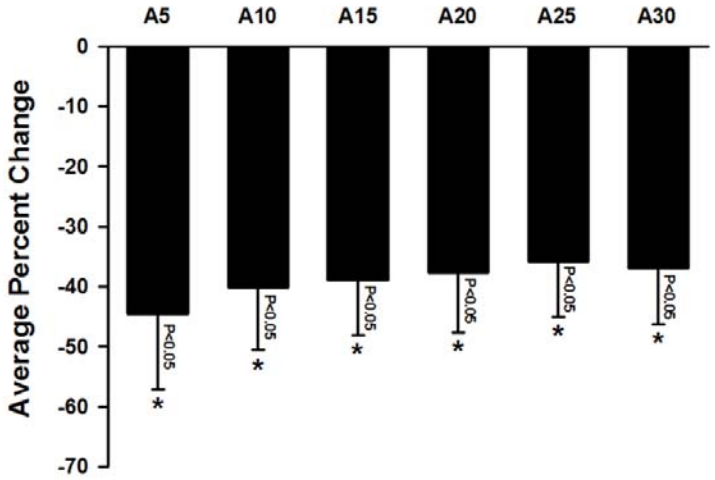

Figure 3.2: Effect of low $[Na^+]_o$ on EPSP amplitude: Composite figure showing percent change in amplitude before and in the presence of 2/3rd reduced $[Na^+]_o$. (Error bars indicate ±SEM; N=8 preparations).

The rate of reaching a plateau response is indicated by the facilitation index taken at various EPSPs within the train (Figure 3.3 A). Also the facilitation index suggests that the response reaches a plateau by about the 10th event in normal

$[Na^+]_o$ and with choline chloride containing saline. Thus, the effect of reducing the NCX results in an alteration early in the initiation of the train as well as during the sustained maintenance of the STF. Since there is substantial variation in the FI for A30/A5 the individual preparations are compared for their absolute change (Figure 3.3 B). Here 7 out of 8 preparations increased in FI with the reduced $[Na^+]_o$. To further compare subtleties in the FI, I compared the percent change in the various FI values (Figure 3.4). No significant differences are present.

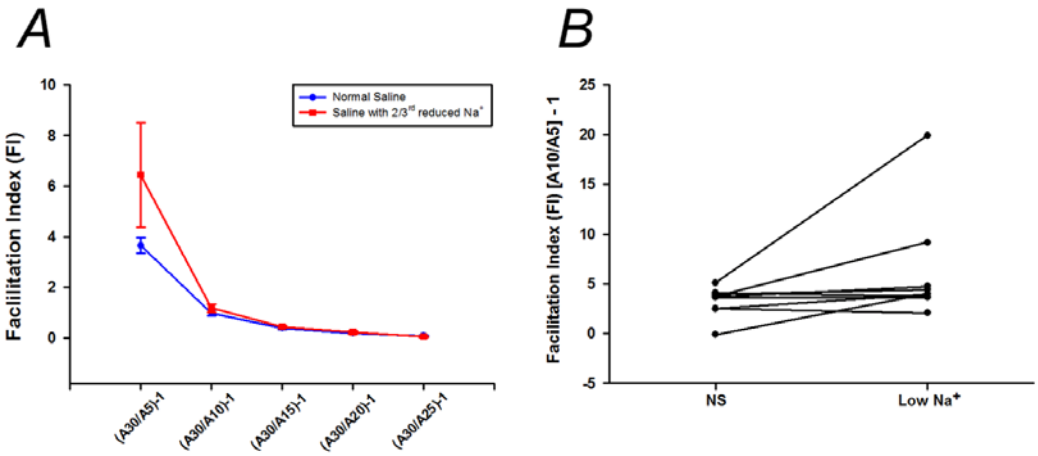

Figure 3.3: Effect of low $[Na^+]_o$ on Facilitation index (FI): FI in presence of normal and 2/3rd reduced (low) $[Na^+]_o$.

The FI is compared for a subset of the EPSPs with a 30 pulse train (A). There is only a significant difference in FI among the A30/A5 index (B). (Error bars indicate ±SEM; N=8).

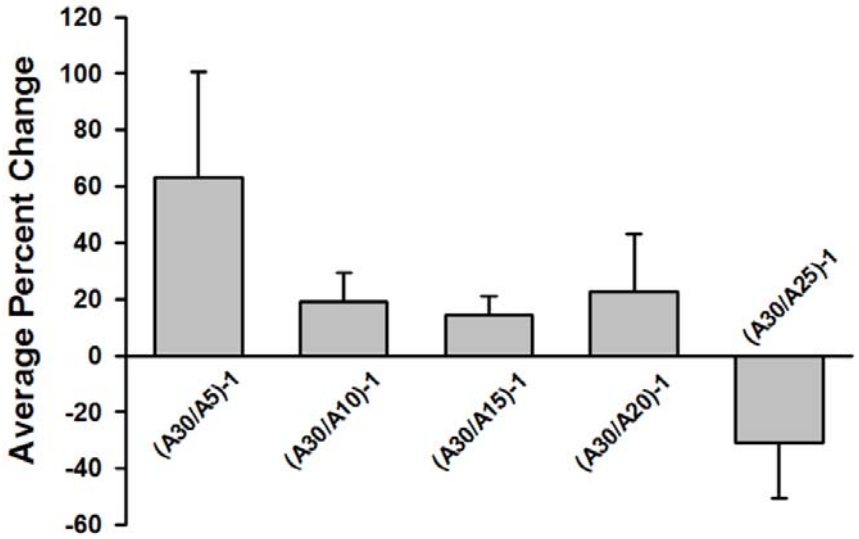

Figure 3.4: Percent change in facilitation index (FI) after decreasing $[Na^+]_o$: Composite figure showing percent change in the FI before and in the presence of 2/3rd reduced $[Na^+]_o$. FI = [(A30/Ax)-1] (where x is amplitude indicated). (Error bars indicate ±SEM; N=8).

Effects on the action potential when lowering $[Na^+]_o$

In order to study the influence of decreasing $[Na^+]_o$ on the size of the action potential in the presynaptic neuron, intracellular recordings were carried out on the crayfish tonic motor neuron which innervates the opener muscle in the first walking leg, while changing the $[Na^+]_o$. The axon (preterminal) was impaled as close to the terminal string of varicosities as possible (Figure 3.5 A). The action potential was typically in the range of 60 to 80 mV in amplitude with approximately -74 mV for a resting membrane potential (Figure 3.5 B). The $[Na^+]_o$ was decreased successively from 100% to 20% in respect to the concentration in normal crayfish saline (205 mM). The amplitude was seen to drop on an average by 35% when the $[Na^+]_o$ was reduced to 20%. The resting membrane potential (RP) was also measured at each of the different $[Na^+]_o$. At each $[Na^+]_o$ the RP only dropped (more negative) by a couple of mVs. Generally the RP became more negative as the $[Na^+]_o$ was decreased. Although the maximum drop in RP was not consistent for a particular $[Na^+]_o$ but the RP did drop in 5 out of 5 experiments when the $[Na^+]_o$ was reduced from the initial concentration ($P<0.05$, Wilcoxon rank-sum test). The effects of lowering $[Na^+]_o$ on the amplitude of the action potential is shown for a representative preparation (Figure 3.5 C). Note the linear drop in amplitude up to the 2/3^{rds} reduction. Further reduction in

$[Na^+]_o$ resulted in a drastic drop in the action potential amplitude. A composite of five preparations revealed the same trend (Figure 3.6); however, there is a significant reduction ($P<0.05$, One Way Repeated Measures ANOVA at $2/3^{rds}$ $[Na^+]_o$. This reduction is still above the level to activate the $Ca^{2+}{}_V$ channels.

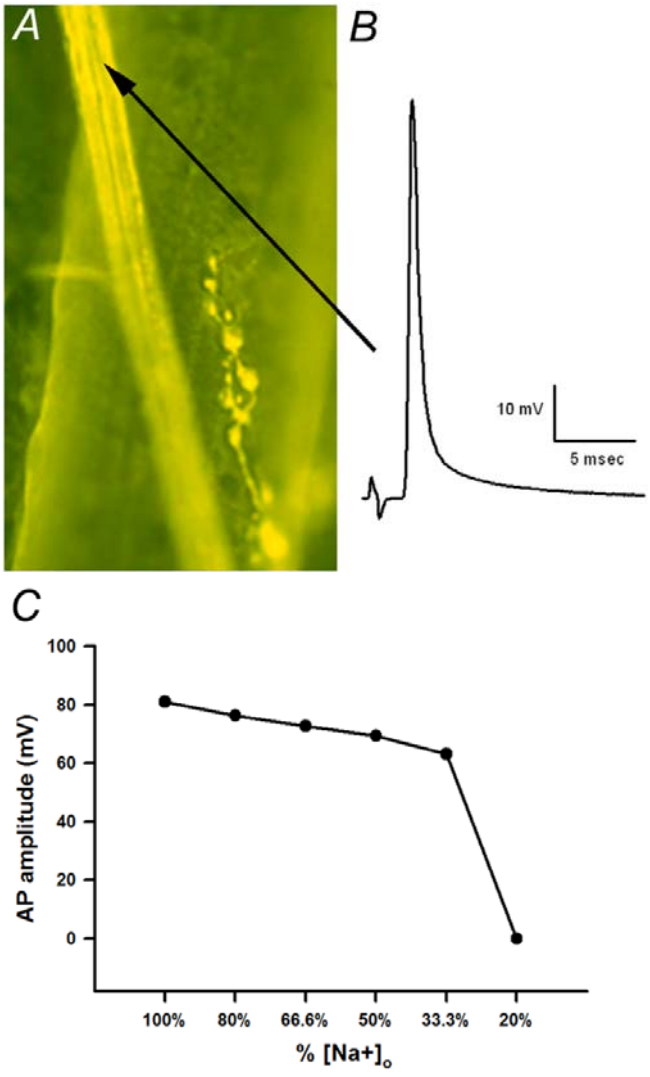

Figure 3.5: Recording amplitudes of APs in presence of low $[Na^+]_o$: (A) Light micrograph showing the axon innervating the crayfish walking leg. The preparation was stained using 4,2-DiASP for representative purposes, but the physiological preparations were not stained prior to recording. Note the string of varicosities on the muscle showing that indeed the axon is preterminal. The black arrow indicates the region where the axon is impaled with a recording intracellular electrode. (B) The action potential trace recorded from the excitatory nerve. (C) The change in the

amplitude (in mV) of the action potential as the $[Na^+]_o$ is changed for a representative preparation. The $[Na^+]_o$ is expressed in percent of $[Na^+]_o$ in normal crayfish saline which is 205mM.

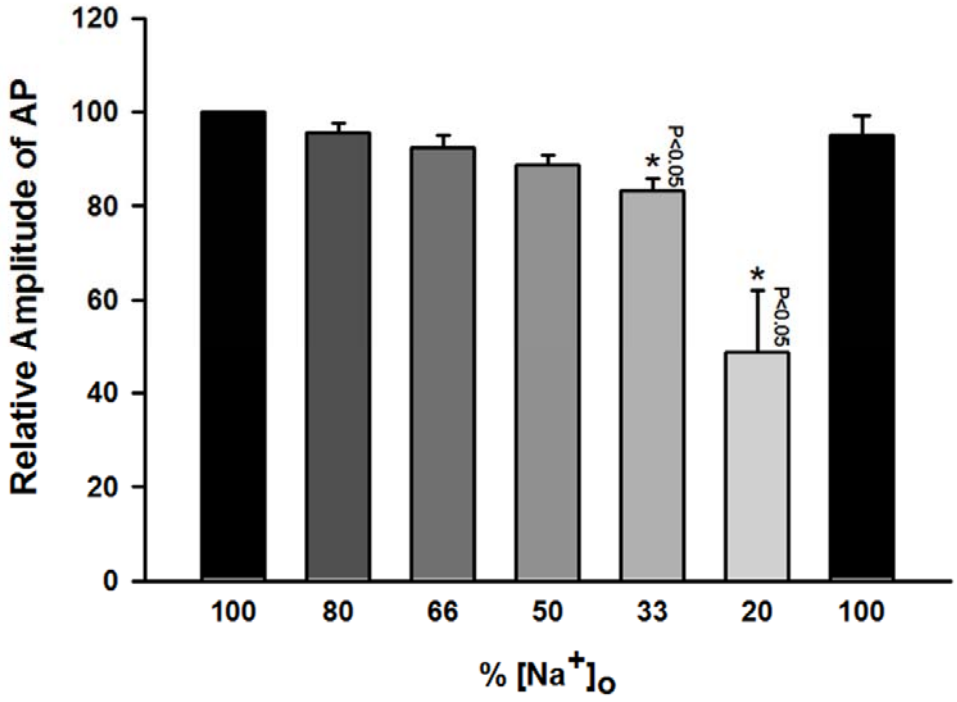

Figure 3.6: Effect of low $[Na^+]_o$ on AP amplitude: The average percent change in amplitude of action potentials recorded at different $[Na^+]_o$. The $[Na^+]_o$ is expressed in percent of $[Na^+]_o$ in normal crayfish saline which is 205mM. There is a linear reduction in action potential amplitude as the $[Na^+]_o$ is reduced up to a 2/3rd reduction. The change is not significant until the $[Na^+]_o$ is reduced to 33% of the original $[Na^+]_o$. When $[Na^+]_o$ is reduced by 2/3rds (to 33%) of the original concentration the action potential amplitude decreases by ~17%. When the $[Na^+]_o$ is reduced to 20% of the original concentration the amplitude of the action potential drops to about 50%. ($P<0.05$, Student's paired *t*-test, N=5)

Theoretical calculations

In order to determine if the reduced $[Na^+]_o$ follows a predicted decrease in the resting membrane potential a theoretical estimation was made. Previously ion permeabilities of Na^+ and K^+ were determined in the crayfish giant axon by Strickholm and Wallin (1967), and the results of the study indicated that when $[K^+]_o$=

5.4 mM the value of P_{Na}/P_K is 2.4 X 10^{-3}. Moreover, an updated value was determined by Strickholm (1981) states P_{Na}/P_K value as 1.44 X 10^{-3}. Using the values of P_{Na}/P_K one can calculate the change in resting membrane potential with changes in the $[Na^+]_o$. These theoretical values were then compared to the experimentally determine values (Table 3.2).

Table 3.2: Observed and theoretical values for the resting membrane potential in crayfish axon when $[Na^+]_o$ is varied.

% Na+ in Saline	100%	80%	66.6%	50%	33.3%	20%
Theoretical RP	-96.3	-96.57	-96.75	-96.98	-97.21	-97.39
Observed RP	-73.4	-75.6	-78.0	-79.8	-77.2	-77.6

It should be kept in mind though that these values for P_{Na}/P_K have been determined in the crayfish giant axon while the experimental results have been obtained from the axon of the opener muscle in the walking leg of the crayfish, so there might be some discrepancy. But the point of emphasis here is that the resting membrane potential does not vary by more than 5 mV in each of the cases. This demonstrates that a change in $[Na^+]_o$ does not greatly affect the membrane potential. This was also shown earlier by Strickholm and Wallin (1967), where they varied $[Na^+]_o$ at normal or depolarized membrane potentials and in both cases found no appreciable effect on the membrane potential occurred. The calculations I used for the Goldman-Hodgkin-Katz equation in order to calculate the permeability of ions is:

$$E= (RT/nF) \ln (P_{Na}[Na^+]_o + P_K [K^+]_o) / (P_{Na}[Na^+]_i + P_K [K^+]_i)$$

The values I used were : R= 8.3145 $VCmol^{-1}K^{-1}$; T= 273.15 + 21°C= 294.15 K; $[Na^+]_i$ =17.4 mM; $[K^+]_i$ = 256 mM; $[Na^+]_o$ =variable; $[K^+]_o$ =5.3 mM; P_{Na}/P_K=1.44X10^{-3}.

Action of KB-R7943 in compromising NCX

The reduction in $[Na^+]_o$ is one approach to dampen the ability of the NCX to extrude Ca^{2+}, however I also wanted to try a known pharmacological approach to block NCX function as in other animal models (Wu et al., 2008). Since I did compare, both the reduced $[Na^+]_o$ and this pharmacological approach on the *Drosophila* NMJ (Chapter 4 of this dissertation) and the *Drosophila heart* (Chapter 5 of this dissertation), I was interested to continue this comparison.

The action of *KB-R7943* is not consistent with the results for reduced $[Na^+]_o$ in depressing the EPSP amplitudes throughout the response train. *KB-R7943* effects were of the same increasing trend in 6 out of 6 preparations for EPSP amplitudes (Figure 7A; P<0.05; Wilcoxon rank-sum test). The EPSP amplitudes that were significantly different by a Student's paired *t*-test are indicated on the graph with an asterisk**.** The calculated FI did not show any significant effect on any FI measures (Figure 3.7 B).

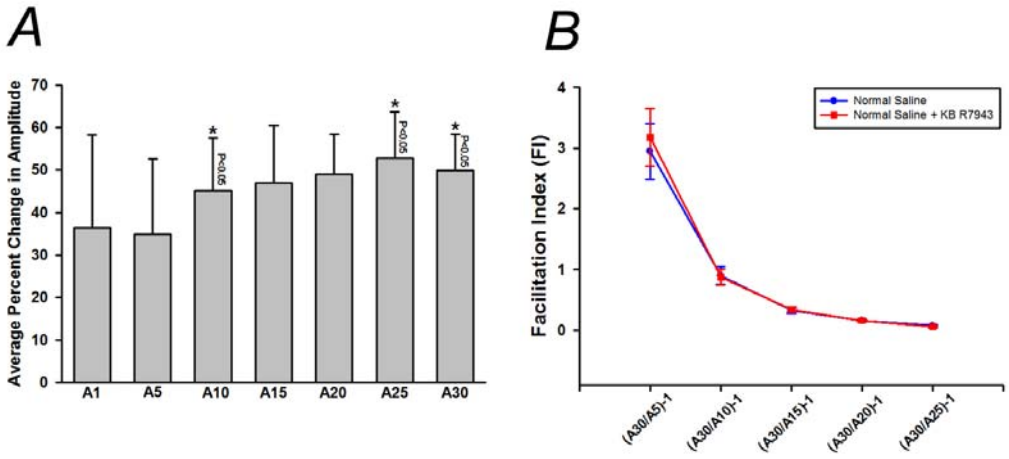

Figure 3.7: Effect of NCX blocker- *KB-R7943* on EPSP amplitudes and FI: (A) Graph shows the average percent change in amplitudes recorded in the presence of the NCX inhibitory drug *KB-R7943*. *KB-R7943* causes an increase in all EPSP amplitudes (P<0.05; Wilcoxon rank-sum test), but the 10th, 25th and 30th pulses showing a significant increase as grouped data (Student's paired *t*-test, n=6, P values as indicated on graph). (B) The facilitation index (FI) in presence of normal saline and saline with *KB-R7943* did not significantly change. Error bars indicate ±SEM.

The effect on transmission and STF with a compromised PMCA

In order to block the function of the PMCA the bathing media was switched to one from a normal pH 7.4 to one at pH 8.8 while continuing to provide trains of stimuli. The initial EPSP within the train rapidly increased. This trend was observed 6 out of 6 times for all EPSP measures within the response train (P<0.05; Wilcoxon rank-sum test). The percent change in the amplitudes concurred with the absolute change in the EPSP (Figure 3.8 A1; P<0.05 paired Student's *t*-test). No significant

effect was observed for the FI (Figure 3.8 A2). Using CE to pharmacological block the PMCA function did not produce the same result as raising the pH to 8.8. CE produced a decrease in EPSP amplitudes for some of the preparations. The trend was not significant. The percent change in the EPSP amplitudes also indicates the varied response in the action of CE (Figure 3.8 B1). Likewise there is no effect on FI (Figure 3.8 B2).

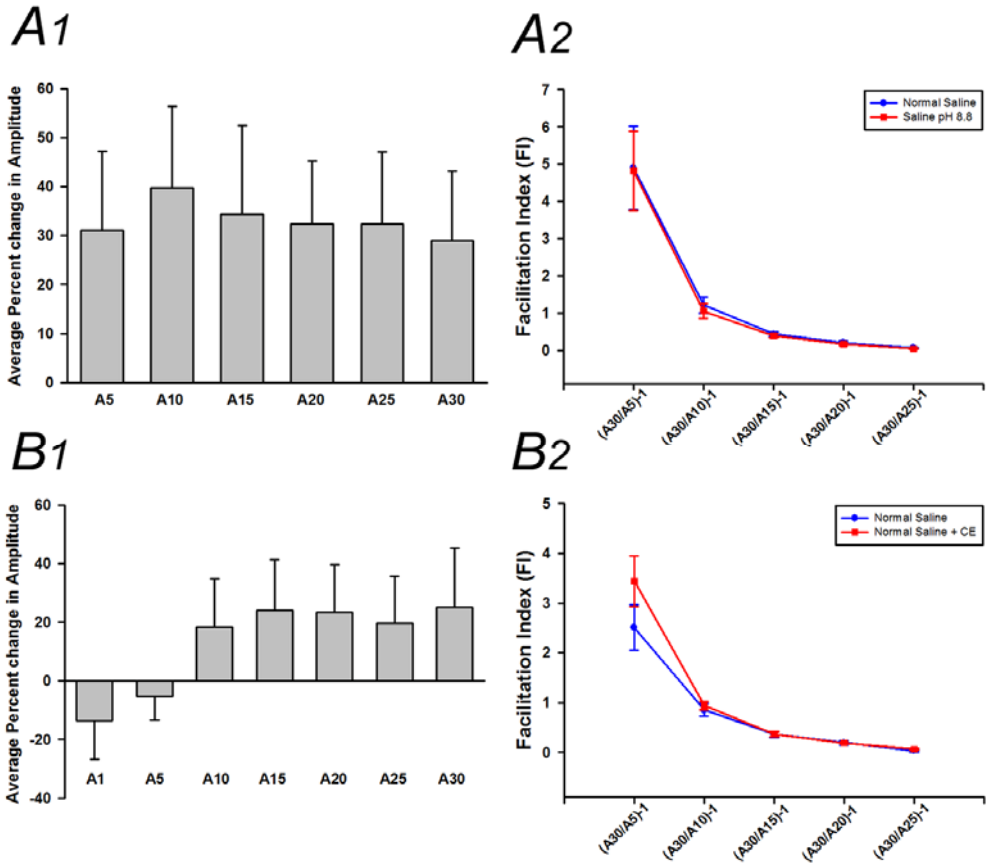

Figure 3.8: Effects of inhibiting PMCA (by two different methods) on EPSP amplitudes and FI: The average percent change in EPSP amplitudes in the presence of saline with pH 8.8 (to inhibit the PMCA) (A1) and the PMCA inhibitory drug carboxyeosin (CE) (B1). The results for the pH 8.8 and CE were not similar. The amplitudes increase for pH 8.8 exposure ($P<0.05$, Student's paired *t*-test, N=6) but not for the actions of CE. A2 and B2 show the facilitation index (FI) for these two treatments pH 8.8 and CE respectively. There is no significant change in the FI with these two treatments by either parametric Student's *t*-test or Wilcoxon rank-sum. Error bars indicate ±SEM.

The effect on transmission and STF with a compromised SERCA

The Ca^{2+}pump on the ER (SERCA) was targeted by a pharmacological approach with application of thapsigargin (TG) (10 μM). The preparations were first examined in normal saline and then incubated with TG for 15 min without nerve stimulation. The amplitude of the EPSPs increased in 6 out of 6 preparations ($P<0.05$; Wilcoxon rank-sum test) by TG for the various EPSP amplitudes measured. The percent change in the EPSP amplitudes is significant by paired Student's *t*-test for a subset of amplitudes within the train (Figure 3.9 A). No significant effect was observed for the FI (Figure 3.9 B).

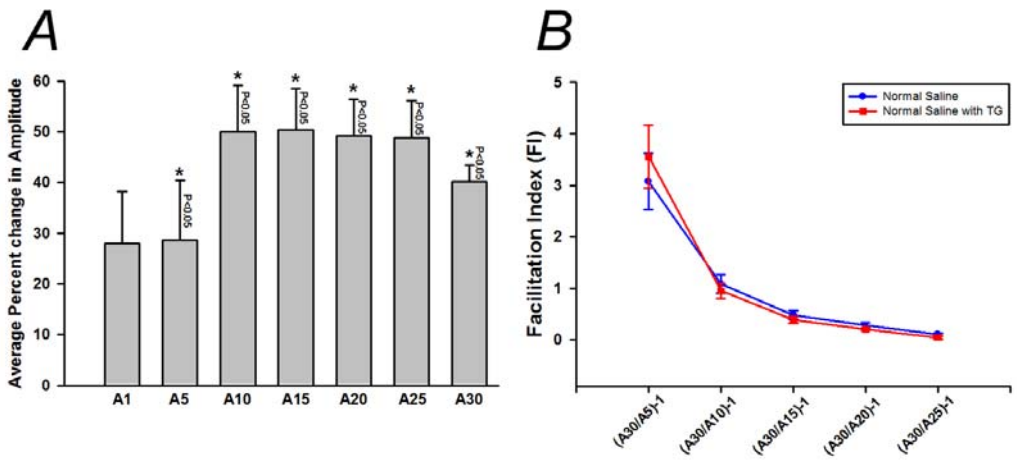

Figure 3.9: Effect of inhibiting SERCA (with thapsigargin) on EPSP amplitudes and FI: (A) The percent change in EPSP amplitudes in the presence of saline with thapsigargin (TG), a SERCA inhibitor. A significant increase in amplitude of EPSPs is observed throughout the EPSP train starting with the 5th EPSP (Student's paired *t*-test, n=6, $P<0.05$); however, by Wilcoxon rank-sum test for each EPSP amplitude, there was a significant increase throughout the train. (B) The facilitation index (FI) in presence of TG is not significantly different from saline control. Error bars indicate ±SEM.

The combined effects in reduced function of the NCX, PMCA and SERCA

With use of the pharmacological approaches and ionic approaches a composite effect on the channels could be compared for synergistic or antagonist effects. Initially the effect of TG, 2/3rds reduced $[Na^+]_o$ and pH 8.8 was examined.

The effects on the EPSP at the start of the train were mixed among the preparations with some increasing and others showing a decrease. The percent change in the EPSP amplitudes further along in the train increased. The effect was significant after the 10th EPSP within the train (Figure 10A; P<0.05, Student's paired *t*-test, N=6). The facilitation index (FI) did not significantly change (Figure 3.10 B).

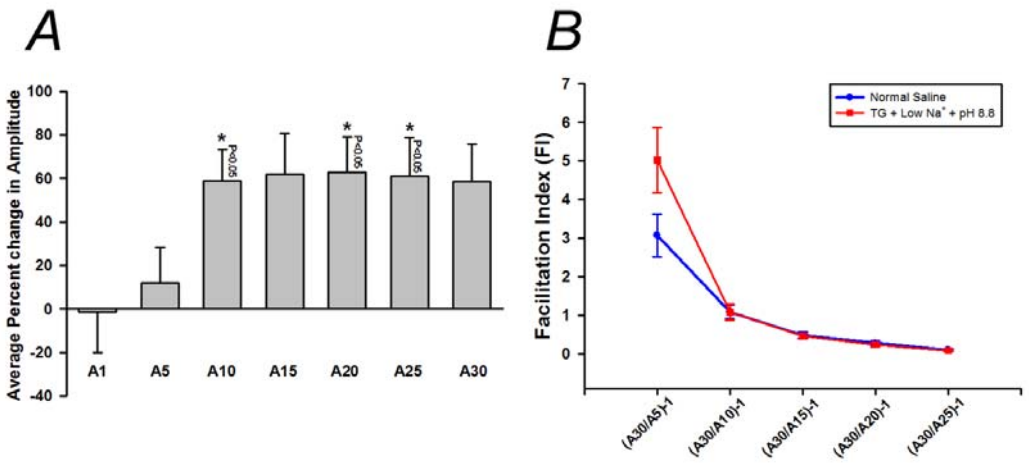

Figure 3.10: Effect of inhibiting all three, the NCX, PMCA and SERCA on EPSP amplitudes and FI: (A) The average percent change in EPSP amplitudes in the presence of saline with thapsigargin (TG), 2/3rd reduced $[Na^+]_o$ and pH 8.8 (to inhibit all three Ca^{2+} channels; SERCA, NCX and PMCA). Inhibiting all three channels causes and increase in the EPSP amplitudes, with pulses 10 (A10), 20 (A20) and 25 (A25) showing a significance (P<0.05, Student's paired *t*-test, N=6,). (B) The facilitation index (FI) when all the three channels are inhibited did not show a significant change. Error bars indicate ±SEM.

Comparing the effects of pharmacological agents and ionic treatments to the altered function in the NCX and PMCA are compared individually. The effect of inhibiting PMCA by pH 8.8 treatments and exposure to CE produced somewhat similar effects in increasing the EPSP amplitudes except for the initial EPSP amplitudes of the train (Figure 3.11 A). There is no significant difference in the effects of the two compounds using a non-paired Student's *t*-test. A paired Student's *t*-test is not valid since different preparations are being compared. To compare different approaches in targeting the NCX, one with 2/3 reduced $[Na^+]_o$ is compared to treatment with *KB-R7943*. The two conditions produced opposite results in the percent change of EPSP amplitudes all throughout the train (Figure 3.11 B).

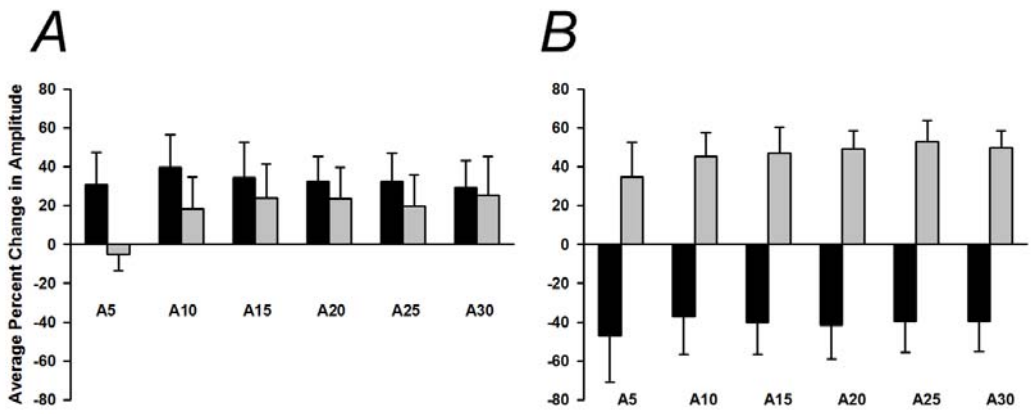

Figure 3.11: Comparing effects of inhibiting PMCA and NCX by two different methods: (A) Comparing the average percent change in amplitude of EPSPs on blocking the PMCA by two different treatments. Both treatments show an increase in EPSP amplitudes. pH 8.8- Black bars; CE- Gray bars. (B) Comparing the average percent change in amplitude of EPSPs on inhibiting the NCX by two different treatments. 2/3rd reduced $[Na^+]_o$- Black bars; *KB-R7943*- Gray bars.

DISCUSSION

In this study I demonstrated that there is a steady state in the amplitude of the EPSPs during prolonged STF. This is likely due to a continuous recycling of vesicles which equates to a homeostatic balance of the $[Ca^{2+}]_i$ during the maintained STF. Perturbing one or a combination of the SERCA, PMCA and NCX channels resulted in an alteration in the amplitudes of the EPSPs, but not necessarily FI. Also the results support the notion that all three channels shape the extent of STF within relatively short high frequency bursts. The results support the idea that release is not saturated due to the lack in the ability of the terminal to release more transmitter or that there is a limitation in synaptic area for vesicle docking during the plateau phase of STF. The plateau of release during the stimulus train is likely a response to steady state of influx, buffering, and efflux of calcium ions. The efflux is likely due, in part, to the SERCA, PMCA and NCX channels. There are differences in the effects on the EPSP amplitudes depending on how the NCX is compromised. When $[Na^+]_o$ is reduced by 2/3rd to retard Ca^{2+} efflux, there is a general reduction in the EPSP amplitude, when I had expected an increase due to a build-up of $[Ca^{2+}]_i$. The application of the NCX inhibitor (*KB-R7943*) revealed the expected results in producing an increase in the EPSP amplitudes. The pharmacological inhibitor of the PMCA mimicked the same

trend as other conventional approach of increasing pH to 8.8, with both producing an increase in the EPSP amplitudes. Although pH 8.8 was more consistent in raising the EPSP amplitudes throughout the stimulus train. The ER appears to be a major contributor in $[Ca^{2+}]_i$ handling in these motor nerve terminals since inhibition of the SERCA with TG produced the most substantial increase in EPSP amplitudes throughout the pulse train during STF. On average all of the manipulations of $[Ca^{2+}]_i$ did not have a large impact of facilitation, instead the EPSP amplitudes as a whole tended to increase or decrease in unison throughout the pulse train. This produced small changes in the ratios used to calculate FI, resulting in negligible effects on the FI.

Since the presynaptic action potential is slightly reduced (17%) in amplitude by the lowering $[Na^+]_o$, this can have some effect in the duration of the depolarization remaining above the threshold for the $Ca^{2+}{}_V$ channels. This might reduce enough of the Ca^{2+} influx during the early phase of STF as well as reduce the residual $[Ca^{2+}]_I$ which could be responsible for maintaining a lowered EPSP throughout the rest of the train. The net result is a non significant change in facilitation except for reduced $[Na^+]_o$ for the first EPSP measure and even in this set of data the change is not that pronounced. The action potential was typically in the range of 60 to 80 mV in amplitude with approximately -74 mV for a resting membrane potential, which for these terminals is substantially above the threshold for the P-type Ca^{2+} channels present (Araque *et al*. 1994; Wright *et al.* 1996). The resting membrane potentials for the axons are in the range of -60 to -80 mV which would result in the action potentials being substantially over the -40 mV threshold of activation for these $Ca^{2+}{}_V$ channels (Wright *et al*. 1996). Also, the potential would exceed the voltages needed to cover most all of the I_{Ca} range for the channels as shown in I-V curve (Fig. 3.12 B; taken from Wright *et al*. 1996). However, when the $[Na^+]_o$ is reduced by 2/3rd the action potential is reduced to about 66 mV in amplitude which would then still be past the I_{Ca} maximum but would not cover the full range of inward I_{Ca} as shown for the I-V relationship. Thus, possibly the full influx of Ca^{2+} is compromised by the reduced action potential amplitude. However, the reduced amplitude may have impacted the width of the action potential and shortened the duration of I_{Ca}. In the superimposed action potential traces, obtained from the squid axon, with reduced $[Na^{2+}]_o$ and raised choline chloride (as used in this current study to control for osmolarity with reduced Na^+) the action potential broadens (Fig. 3.13 A; taken from Wright *et al.* 1996). Such mixed actions as a reduced amplitude but broader action potential could result in even a greater Ca^{2+} influx. I also measured the widths of the action potential, but in the crayfish axons, with reduced $[Na^{2+}]_o$ and raised choline chloride (Fig. 3.13

B). Note the width is not as prolonged as in the squid axon. Therefore, the total I_{Ca} is likely retarded in my experiments with 2/3rd reduced $[Na^{2+}]_o$. This is also suggestive by examining the I_{Ca} curves in Figure 3.12 A. The slightest reduction in duration of the action potential is on a steep slope in the influx of the I_{Ca}.

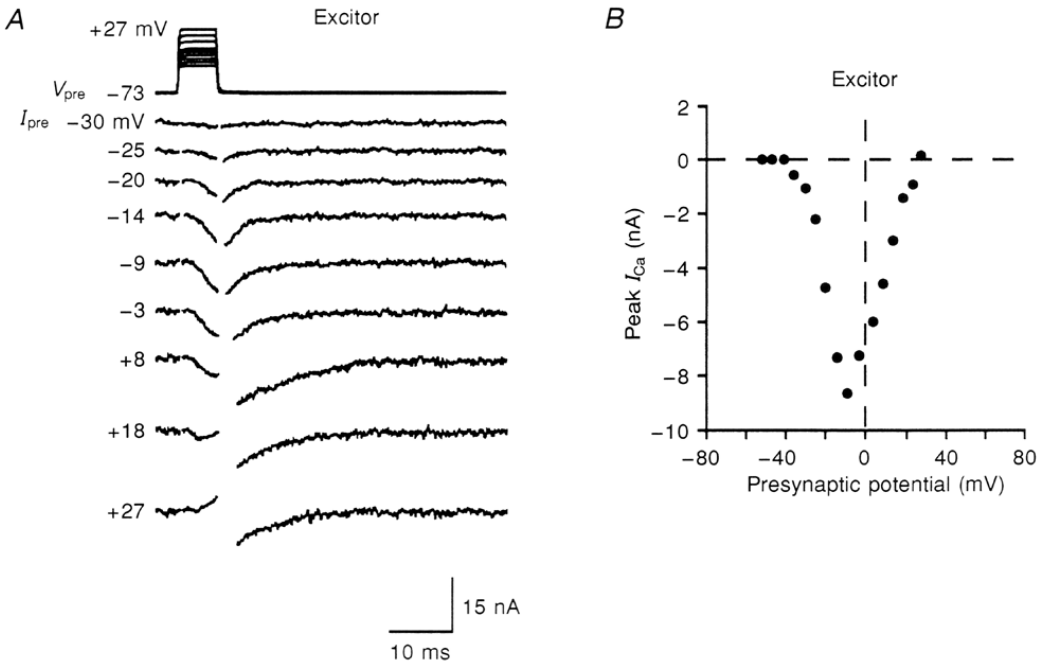

Figure 3.12: Ca^{2+} currents from the excitor axon of the crayfish opener: The figure is reproduced from (Figure 3). (A) The Ca^{2+} currents from the excitor axon of the crayfish opener muscle are shown for a series of depolarizations of 6 msec. (B) The I-V relationship for the experiment shows in (A) is plotted. This data was obtained in the same species of crayfish and the same type of NMJ as used in the current study.

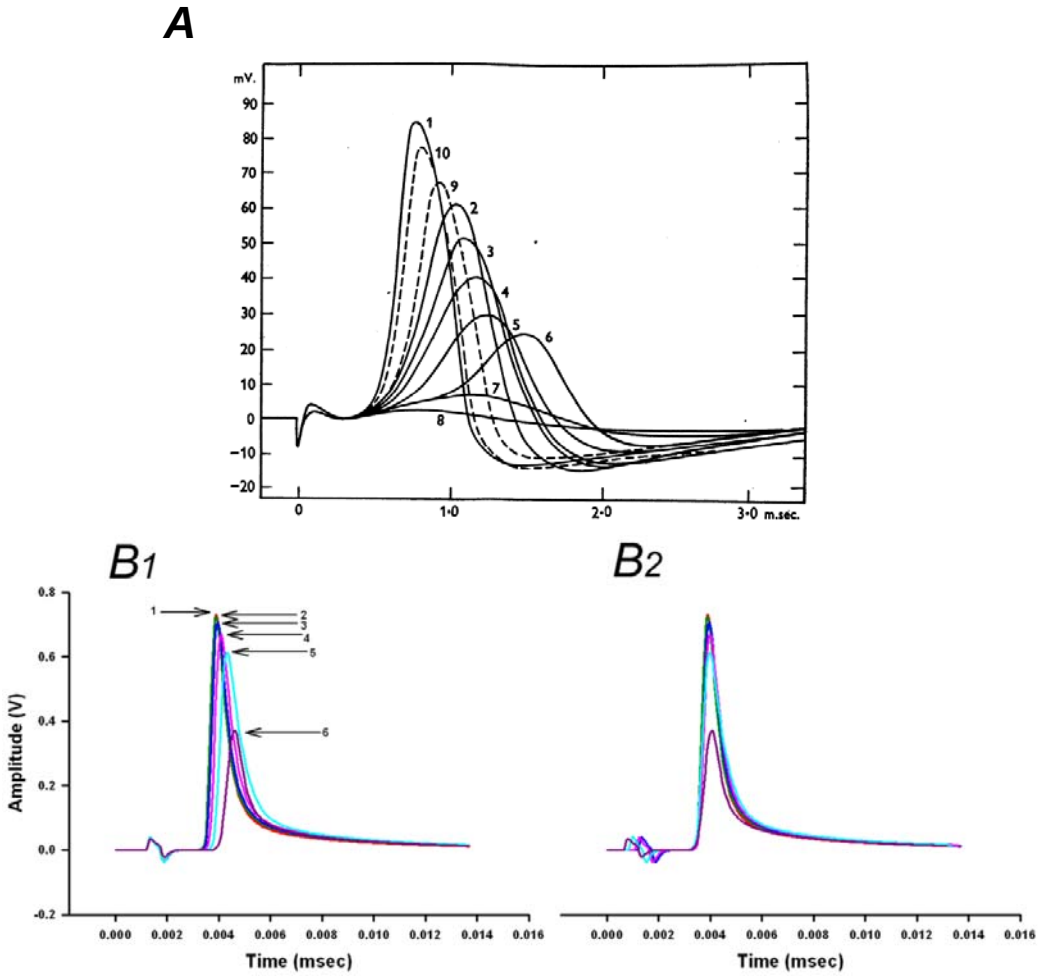

Figure 3.13: Action potentials from squid and crayfish axons in presence of low $[Na^+]_o$: (A) The figure is reproduced from Hodgkin and Katz, 1949 (Figure 3). The action potentials are obtained from the squid axon while the bathing solution is exchanged to a lower concentration of Na^+ (50% over time) (1 is sea water at t=0; 2 t=30s; 3 t=46s; 4 t=62s; 5 t=86s; 6 t=102s; 7 t=107s; 8 t=118s; 9 is 30s after reapplication of sea water followed by 10 at t=90s). As the $[Na^+]_o$ is reduced the osmolarity is maintained by raising the choline chloride concentration. (B1) The superimposed action potentials, obtained in the crayfish axon from this current study, revealed a reduction in the amplitude but not in broadening over a range in $[Na^+]_o$. The percentage values of Na^+ are: 1= 100%; 2= 80% ; 3= 66.6%; 4= 50%; 5=33.3% and 6= 20%). (B2) The action potentials are shifted so that the peak is synchronized to compare the widths.

It is interesting to note the similarities in the amplitudes of the action potential of the squid and crayfish axon; however, the response to lowered $[Na^+]_o$ is strikingly

different. The earlier report by Hodgkin and Katz (1949) did not state an effect on the RP with reduced $[Na^+]_o$. Even though each axon preparation had a slightly different range in resting membrane potentials as the $[[Na^+]_o$ was manipulated, a same general trend occurred for 5 out of 5 preparations ($P<0.05$ nonparametric Wilcoxon rank-sum test), in that the resting membrane potential became more negative with the reduction in $[Na^+]_o$ (Fig. 13). Although, some preparations showed a maximal drop in the potential at a 50% $[Na^+]_o$ reduction. Such a phenomena would incline one to suggest that there is likely a I_{Na} leak at the normal $[Na^+]_o$ used and that upon reduction of $[Na^+]_o$ the driving gradient is reduced with the membrane potential being driven to the E_K for the cell. However, why the potential increases in some preparations at even lower $[Na^+]_o$ is not readily explained.

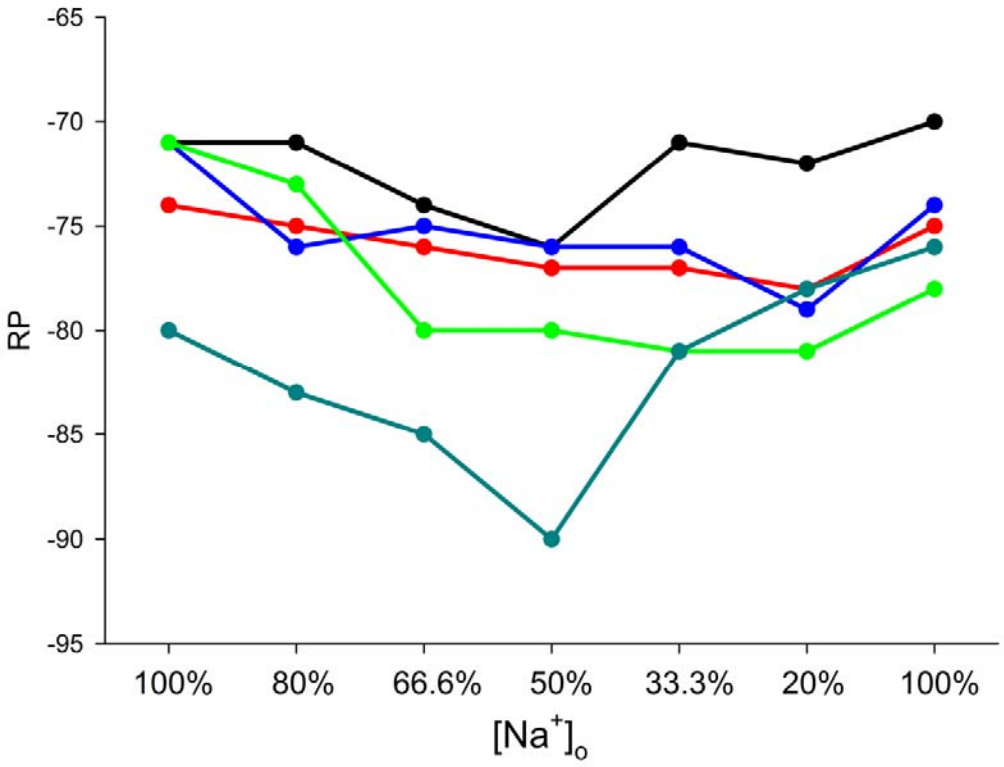

Figure 3.14: Resting membrane potentials in presence of varying $[Na^+]_o$: Resting membrane potentials in motor nerve axon of the crayfish opener as the $[Na^+]_o$ is lowered and raised.

The slight shifts in the I_{Ca}, from altering the ionic composition of the Na^+, likely produces indirect effects on STF, which complicates dissecting apart the direct effect of compromising the NCX. There may be other non-direct effects I have not

accounted for in reducing $[Na^+]_o$ such as the possibility in altered screening potential around other ion channels which could impact the shape of the presynaptic action potential or even the influx of I_{Na} through the postsynaptic glutamatergic ionotropic receptors. To address the second possibility, one could record the size of postsynaptic spontaneous quantal events or use as means to measure the currents induced by glutamate exposure to the muscle fiber for determining if the ionic flux of the postsynaptic receptors is altered.

Fortunately, I used various approaches to address the role of NCX and PMCA and did not rely on a single method. The opposing results of lowered $[Na^+]_o$ to application of *KB-R7943* was not expected. In growth cones of crayfish motor neurons *KB-R7943* was shown to have nonspecific effects by increasing the resting $[Ca^{2+}]_i$ in normal saline (Rumpal & Lnenicka, 2003). If this occurred in my study, I would have expected an increase in the background Ca^{2+} level to have an impact on the FI but facilitation was no different before and during exposure to *KB-R7943* (Figure 7B). As stated in the study by Rumpal & Lnenicka (2003), *KB-R7943* is known to have nonspecific effects in other systems, (blocking L-type Ca^{2+} channels, Na^+ channels, and N-methyl-D-aspartate (NMDA) channels) (Arakawa *et al.* 2000; Matsuda *et al.* 2001; Sobolevsky & Khodorov, 1999). Both the approaches in blocking the PMCA (raised pH or application of CE) produced similar results. These results allow confidence for future investigations in using CE, *KB-R7943* and even TG, since the results are as expected for blocking the SERCA. In this study, I used a mixture of ionic approaches to examine a combined role of the Ca^{2+} channels as I was not confident that a mixture of three pharmacological organic agents would not interact or confound indirect effects. In addition, invertebrates have shown not to conform to all pharmacological profiles as describe for vertebrates. For example, m-chlorophenylpiperazine (m-CPP) which is known as a non-selective agonist to vertebrate 5-HT1 and 5-HT2 family receptors but also antagonizes 5-HT2B receptors in some models and is known to block Na^+_V in crayfish axons (Sparks *et al*. 2003). Paradoxial results in application to ouabain, a well known compound for blocking the Na^+/K^+-ATP pump appeared not to have an effect on *Drosophila* Malpighian tubules. But, this was due to the unique nature of the cells to actively excrete this organic compound (Torrie *et al*. 2004). Even within the vertebrate brain there are different PMCA and NCX isoforms that account for the variable responses in Ca^{2+} extrusion (Jensen *et al.* 2007). Thus, there might be different forms of feedback regulation or modulation of these different isoforms. Since it is not yet known which isoform exists, or even if there are multiple forms, at the crayfish motor nerve terminals I can only speculate to potential differences in regulation of these channels. It is known that in

Drosophila, the NCX is inhibited by $[Ca^{2+}]_i$, where as other NXC forms are not (Hryshko *et al.* 1996). Also it was established that *KB-R7943* is a weaker inhibitor (4-5 times) of the NCX in *Drosophila* (CALX1.1) than for the NCX in canines (Isaac *et al.* 2002). Similar activity studies needs to be performed in the crayfish model to ideally know the efficiency of pharmacological agents on the NCX, PMCA and SERCA.

Rumpal & Lnenicka (2003) conducted a study on two type of crayfish motor neurons grown in culture and demonstrated that the NCX exchange has a significant action in extruding large Ca^{2+} loads from the growth cones in both tonic and phasic motor neurons. They did compromise the NCX function by lowering $[Na^+]_o$ so there could be other consequences, as mentioned above, in neuronal function. However, the study also documented when the NCX was compromised that the PMCA was able to extrude the $[Ca^{2+}]_i$ sufficiently. It was also noted in the crayfish neurons that mitochondria can play a role in sequestering Ca^{2+} for high $[Ca^{2+}]_i$ and that the mitochondria releases it slowly as the $[Ca^{2+}]_i$ decays. In our studies, we have not addressed the role of the mitochondria's ability in sequestering Ca^{2+} to a role in STF. It would be of interest to compromise SERCA, NCX, PMCA as well as the other $[Ca^{2+}]_i$ regulators (e.g., vesicle sequestering of Ca^{2+}; Ca^{2+} binding proteins); however, it is difficult to tackle all possibilities without inducing indirect actions on vesicle docking and other ionic channels. There could be other ionic exchangers in these neurons such as the K^+-dependent NCX (NCKX) that has a greater role in clearing Ca^{2+} in the Calyx of Held than NCX, SERCA and the PMCA (Kim *et al*. 2005). The NCKX is also known to have a significant role in rat neurons (Lee et al., 2002) and in some *Drosophila* cells (Winkfein *et al.* 2004). Thus, it is a challenging task to model all the factors and time domains in the multifaceted approaches occurring within the nerve terminal to regulate Ca^{2+}.

In order to attempt to mechanistically explain the results obtained in this study I designed relatively simple hypothetical models to explain the changes in the EPSP amplitudes as well as the occurrences in STF (Figure14). If the high capacity NCX is inhibited alone and if the PMCA and the SERCA are not able to compensate quick enough during STF, the EPSP amplitudes increase throughout the stimulus train and the new plateau is reached. Thus, a new homeostatic setting in regulation for SERCA and PMCA as well as other Ca^{2+} buffering sources occurs. In this scenario, the high affinity, but low capacity, PMCA would have a role in maintaining the new Ca^{2+} equilibrium just as would the SERCA. When all three channels are working normally, there could even be a Ca^{2+} induced inhibition of the NCX, thus leaning more on the other mechanisms to maintain the synaptic balance. However, the inhibition of the NCX or SERCA or PMCA all increased the amplitudes of the EPSPs early in the

stimulus train and throughout the 30 pulses. This would suggest that all 3 share a strong rapid component to bring $[Ca^{2+}]_i$ back into check. Both the NCX and SERCA had a larger impact on the initial facilitation when they were inhibited than the PMCA, so likely the PMCA does not have as great of role in rapidly buffering the rapid rise in $[Ca^{2+}]_i$ with the induction of STF. This also supports the results in the Rumpal & Lnenicka (2003) study on motor nerve growth cones in which Ca^{2+}-imaging revealed a larger $[Ca^{2+}]_i$ when the NCX was inhibited as compared to the PMCA. A more complex model would need to include various Ca^{2+} binding proteins with their on/off rates, potentially mitochondrial as well as vesicle buffering, other NCX/SERCA/PMCA isoforms, account for different density/amounts of the proteins, Na/K pump and even potentially the presence of KNCX, the types of channels (P-$Ca^{2+}{}_V$, $K^+{}_{Ca}$) present and modulation/regulation of the channels (e.g., phosphorylation, Ca^{2+} feedback inhibition). There is still a lot of work ahead to integrate the possibilities in the contributions of these other variables.

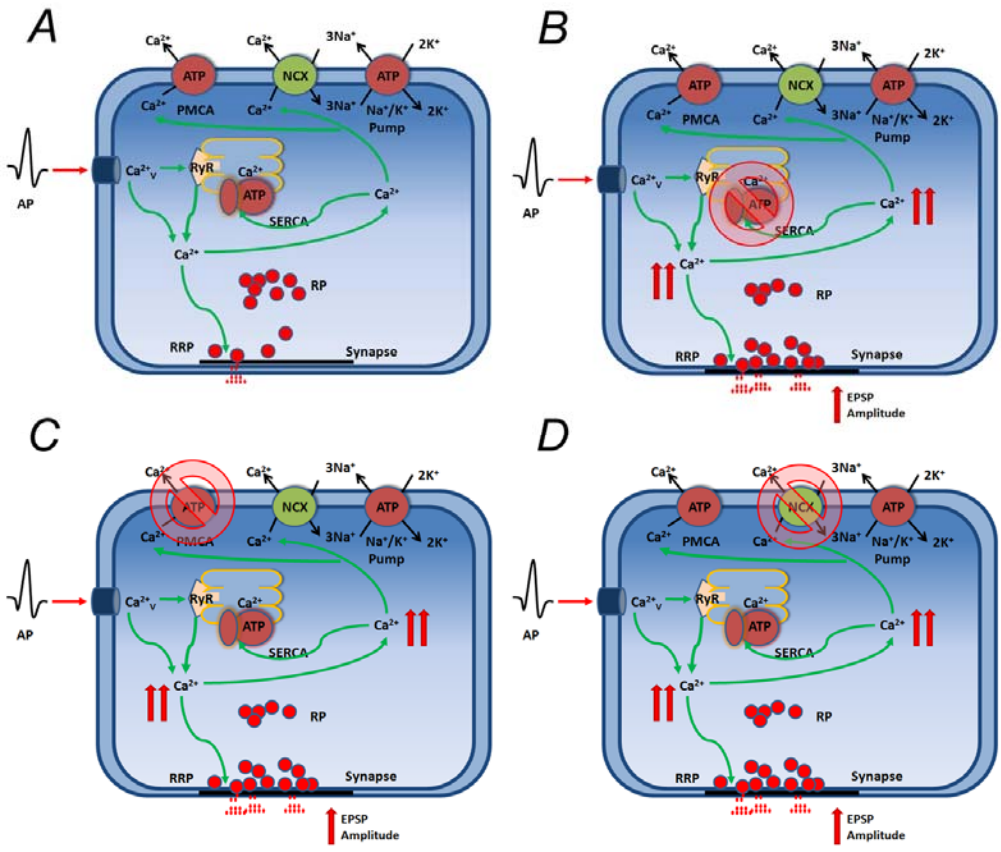

Figure 3.15: Model of the presynaptic nerve terminal at the crayfish NMJ. These low-output synapses have few vesicles docked within the ready releasable pool

(RRP) and many in the reserve pool (RP). Upon starting a short train of stimulations the first action potential will result in a few of vesicles to fuse and release transmitter. The subsequent nerve terminal depolarizations will have more vesicles that can be primed; therefore, synaptic facilitation is observed by monitoring in the postsynaptic muscle fiber. Reducing the function of one or the other Ca^{2+} buffering mechanisms results in changes in the probability of vesicle fusion.

The contributions of this study are furthering the understanding of three major $[Ca^{2+}]_i$ regulatory process on synaptic transmission and the impact for the onset and maintenance of STF as well as directly on vesicle dynamics (Awatramani *et al*. 2005; Hosoi *et al.* 2007; Lee et al., 2008). The results indicate that NCX, PMCA and SERCA all effect the fundamental mechanisms that allow STF to occur based on the residual $[Ca^{2+}]_i$ model. Since STF is one form of memory, the alteration in regulation can lead the way to dissecting underlying mechanisms for more prolonged forms of memory or synaptic plasticity, such as long term facilitation (LTF). In fact, the same preparation used in this study is the preparation in which LTF/LTP was first described (Sherman & Atwood, 1971). One can now build on my findings and others to address the potential role of these Ca^{2+} regulator pathways in LTF. In addition, presenting the effects of various pharmacological and ionic approaches demonstrates that various methods are possible to manipulate the channels. This allows other investigators to advance the range of techniques available to them. I also demonstrated that the action potential shape in the preterminals of a motor neuron is different is respect to lowered $[Na^+]_o$ as compared to the classic model squid axon commonly used for a biophysical reference. I feel the crayfish NMJ preparation can continue to provide a playground for electrophysiological investigations of synaptic transmission.

As for future directions of this research, I feel it would be important to conduct Ca^{2+}-imaging studies to examine the rate of rise and decay of $[Ca^{2+}]_i$ upon individually manipulating the NCX, PMCA and SERCA as well as in various combinations or all three channels. In addition, the role of these channels on the development of LTF or transition from STF to LTF as well as their action on low-frequency depression (Pahapill *et al.* 1987) is important to investigate. Regional differentiation in buffering $[Ca^{2+}]_i$ within a single neuron is of interest as one can address the mechanistic differences to account for local synaptic regulation (Atwood & Cooper, 1995, 1996b). The opener motor neuron varies in structure and function depending on the target muscle, suggesting local retrograde regulation from the muscle on the nerve terminals (Cooper *et al.* 1995a, 1996a; Mykles *et al.* 2002; LaFramboise *et al.* 2000). The information gained can also be used to address

differences in high and low-output terminals not just among the opener NMJs but among other neurons (e.g., phasic and tonic NMJs) as established junctions or ones undergoing rapid organizational changes during development. The regional differences along a given terminal need to be examined for differential $[Ca^{2+}]_i$ regulation as the terminals demonstrate a developmental time course from older regions to newer ones as the terminals grow with the animal. A homeostatic regulation of $[Ca^{2+}]_i$ with temperature has not been well addressed in regards to synaptic transmission and STF. Given the species of crayfish I used in this study is ecologically fit to survive in hot to cold environments suggests that they may have unique abilities to regulate synaptic transmission to acclimate to changes in temperature which could yield discoveries in conditioning mammalian synapses to survive prolonged cold adaptations. Dysfunction in NCX, SERCA, and PMCA is known to be associated with various disease states in mammals (Mata & Sepúlveda, 2005). The possibility of computationally addressing diseases and treatments, that impair synaptic transmission, may possibly be experimentally tested in electrophysiologically amenable and identifiable single neurons with a prolific background of historic information in synaptic anatomy and physiology is an exciting potential to reach.

Chapter Four

Comparative Physiological Studies on the NCX, PMCA, SERCA in Relation to Synaptic Transmission at *Drosophila* NMJs

INTRODUCTION

The neuromuscular junction (NMJ) of larval *Drosophila* is an ideal preparation to investigate questions related to Ca^{2+} regulation within the presynaptic nerve terminal in regards to efficacy of synaptic transmission since the excitatory postsynaptic potentials (EPSPs) are graded, and refined quantal analysis is readily ascertainable. In addition, the innervation of one or two excitatory motor neurons to a single, large target cell (i.e. a muscle fiber), without the complication of an inhibitor neuron, should allow clear interpretations of the targeted action and the processes of the auto-regulation in the observed Ca^{2+} responses. Surprisingly little concentration has focused on mechanisms of Ca^{2+} regulation and short term facilitation/depression at the model NMJs of the *Drosophila* larva.

Since synaptic transmission is enhanced or depressed, at the *Drosophila* larval NMJ, depending on load of $[Ca^{2+}]_i$ within the presynaptic terminal during repetitive stimulation, it is important to know how $[Ca^{2+}]_i$ is regulated so the underlying mechanism can be investigated. As for the frog and crayfish NMJs, residual Ca^{2+}, due to previous evoked activity, can accumulate and thus result in a greater overall $[Ca^{2+}]_i$. This is the basis for short-term facilitation (STF) at low efficacy crayfish NMJs (Atwood & Wojtowicz, 1986; Cooper *et al*. 1996; Matveev *et al*. 2006; Sherman & Atwood, 1971) and can account for the maintenance of short-term depression (STD) at high efficacy crayfish NMJs (Bradacs *et al.* 1997; Cooper *et al*. 2003) and *Drosophila* larval NMJs (Stewart *et al.* 1994; Kurdyak *et al*. 1994).

The general view is that $[Ca^{2+}]_i$, promotes vesicle fusion via synaptotagmin (Neher & Sakaba, 2008; see review Lisman *et al.* 2007) and if the readily releasable vesicle pools (RRP) are not extensively used within the first stimulus of a stimulus train, then the subsequent stimulations can build on the residual $[Ca^{2+}]_i$ which adds to the vesicles already primed. This promotes even more vesicles to fuse as the binding to synaptotagmin appears to be a cooperative (Brose *et al.* 1992; Kertz *et al.* 2007). It is also suggested that the $[Ca^{2+}]_i$ could lead to a greater influx of Ca^{2+} by a "facilitation sensor" (Catterall & Few, 2008; Mochida *et al.* 2008; Tsujimoto *et al*. 2002). If there is a substantial population of vesicles docked, in the RRP, the first stimulus of a train can result in so many vesicles fusing to the membrane that there

are not enough available for the 2nd and/or subsequent stimuli, and thus a presynaptic depression occurs. Lowering the percentage of vesicles that release in these high output terminals, by reducing $[Ca^{2+}]_o$ can transform STD into STF at NMJs (Atwood & Karunanithi, 2002; Cooper *et al*. 2003). It was demonstrated in *Drosophila* larval motor nerve terminals that the duration of fusion pore for a vesicle depends on the $[Ca^{2+}]_i$ (Pawlu *et al*. 2004). Thus, depending on the buffering and extrusion rates of Ca^{2+} during rapid stimulation the synaptic efficacy can be variable.

There are a numerous regulators for free Ca^{2+} within the nerve terminal (see recent review- Budde *et al*. 2002; Friel & Chiel, 2008; Thayer *et al*. 2002). Here I focus on three major Ca^{2+} channels that would likely impact $[Ca^{2+}]_i$ during STF or STD in order to learn more of their interactive nature and their contribution in synaptic transmission during stimulus trains. Pumps and the sodium/calcium exchanger (NCX) can rapidly regulate $[Ca^{2+}]_i$, thus having an effect on the rise and decay of $[Ca^{2+}]_i$ which directly impacts the dynamics in residual $[Ca^{2+}]_i$ (Atwood & Karunanithi, 2002; Catterall & Few, 2008). The sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) that pumps Ca^{2+} into the ER is important not only in muscle but is known to have a role in neurons (Berridge, 1997, 2005; Berridge *et al*. 2000). The third channel I examine is the plasmalemma Ca^{2+}-ATPase (PMCA). In *Drosophila,* the PMCA appears to be responsible for clearing Ca^{2+} which accumulates in motor nerve terminals with large impulse activity (Lnenicka *et al*. 2006). In the crayfish, the plasmalemmal NCX and the PMCA are observed to have important effects in controlling residual $[Ca^{2+}]_i$, whereas the mitochondrial Na^+/Ca^{2+} exchanger does not seem to have a significant role in STF (Zhong *et al*. 2001; however see chapter 3 in this dissertation). There has yet to be a study which examines the role of all three channels with regards to STF or STD using *Drosophila* NMJs.

The larval *Drosophila* NMJ serves as a genetic and physiological model for unraveling properties of synaptic develop and function. It has served well for investigating localization of synaptically relevant proteins in both pre- and post-synaptic sites (Atwood & Karunanithi, 2002; Featherstone *et al.* 2005; Guerrero *et al.* 2005; Rasse *et al*. 2005; Pawlu *et al*. 2004; Littleton & Ganetzky, 2000). The makeup of the postsynaptic glutamate receptor subtypes and pharmacology on the muscle have recently being identified and how they regulate synaptic responses (Bhatt & Cooper, 2005). Accountability for differences in quantal size among nerve terminals due to vesicle size (Karunanithi *et al*. 2002) and fusion pore kinetics (Pawlu *et al*. 2004) are setting the stage for such possible parallel studies in mammalian synapses (Lisman *et al*. 2007). The varied synaptic structures observed at *Drosophila* NMJs are comparable to other arthropods, particularly crustaceans, where it is known that

the synaptic complexity accounts in part for synaptic efficacy in a variety of stimulus conditions (Atwood & Cooper, 1996a, b; Stewart *et al*.1996). Several pieces of evidence show that terminal morphology can regulate synaptic strength in various preparations (Kuno *et al.* 1971; Govind & Chiang, 1979; Stewart *et al*. 1996; Atwood & Cooper, 1996a, b; Walrond *et al*. 1993). Development of the motor nerve terminals in *Drosophila* is also similar as to those in crustaceans (Atwood *et al.* 1993; Atwood & Cooper, 1996b; Crossley, 1978; Li *et al*. 2002).

Genetic based malformations of synaptic function in humans, that are accounted for by particular defects, continue to be modeled at *Drosophila* synapses (Badre & Cooper, 2008; Fradkin *et al*. 2008; Lee *et al*. 2004; Lu *et al*. 2007; Ubhi *et al*. 2007) in hopes of learning more about disease states and their effects on synaptic development (Xing *et al.* 2005) as well as function in order to be able to offset or treat pathological conditions afflicting humans. Considering that *Drosophila melanogaster* is a true model organism, with a known genome and has been used successful rapid induction of mutations in various studies of development and synaptic mechanisms it is important to understand the regulation of $[Ca^{2+}]_i$ within this model synaptic neuromuscular preparation (Pawlu *et al*. 2004; Li *et al.* 2002; Betz *et al.* 1993; Atwood *et al.* 1993; Kurdyak *et al.* 1994; Jan & Jan 1976; Stewart *et al.* 1994, 1996; Sigrist *et al*. 2002, 2003; Li & Cooper, 2001; Ruffner *et al*. 1999).

One study that has focused on mechanisms of calcium regulation at the NMJ of the *Drosophila* larva revealed that there are differences in the Ca^{2+}- binding protein fequenin between nerve terminals and if it is over expressed there is an enhancement of facilitation (Rivosecchi *et al.* 1994). Additionally, a temperature sensitive dysfunctional Ca^{2+} channel in motor nerve terminals results in reduced synaptic transmission (Kawasaki *et al.* 2004; Kuromi *et al*. 2004). However, even over developmental time from 1st to 3rd instar with reduced Ca^{2+} channel function, the nerve terminal does not compensate to reach homeostasis in synaptic efficacy (Rieckhof *et al*. 2003; Xing *et al.* 2005). One would have predicted a possible reduction in rapid extrusion of Ca^{2+} as a homeostatic mechanism to maintain synaptic strength in this mutational line (cacophony) during larval. Adults also showed a lack in homeostatic compensation with reduced Ca^{2+} channel function (Badre & Cooper, 2008). Also, oddly enough, when the SERCA is inhibited at *Drosophila* NMJs, by use of temperature sensitive mutation, the EPSPs decrease in amplitude instead of increase as one would likely predict (Sanyal *et al.* 2005).

In this study, I examined individually the role of the NCX, PMCA and SERCA in synaptic transmission during bursts of motor nerve activity that induces STD and STF. In addition, I was interested to know the combined role that two or more of

these Ca^{2+} channels had on transmission. Because there is a mutational phenotype of a dysfunctional SERCA, induced by heat shock, I used this *Drosophila* line as well as pharmacological and ionic manipulations to compromise the function of these three Ca^{2+} regulatory proteins. It was important to also compare different approaches to target the same protein to learn if the approaches are complimentary to each other. This study also provides information for future experiments to build on to learn more about the underlying mechanism of Ca^{2+} regulation within nerve terminals in general.

METHODS

Canton S (CS) flies, a wild type of *Drosophila*, were used for comparison to *Kum*^{170TS} (Sanyal *et al.* 2005). Both strains were maintained on a standard cornmeal-dextrose-agar-yeast medium. The *Kum*^{170TS} strain was provided by Dr. Sanyal (Emory University, School of Medicine). To obtain early 3rd instar staged larvae, flies were pulsed to lay eggs for 2 hours and then the eggs were incubated at 21°C. Staging of fly larvae and the dissection technique have been previously reported (Campos-Ortega & Hartenstein, 1985; Li *et al.* 2002; Ball *et al.* 2003).

The physiological saline HL3 was used for initial dissections (in mM): 1.0 CaCl2 2H2O, 20 MgCl2, 70 NaCl, 5 KCl, 10NaHCO3, 5 trehalose, 115 sucrose, 5 BES (N,N-bis[2-hydroxy-ethyl]-2-aminoethanesulfonic acid) and adjusted to a pH of 7.2 (Stewart et al., 1994). All experiments were performed at room temperature (20-25°C). However, in experiments in which the pH needed to be raised to 8.8 and the $[Na^+]_o$ reduced, a Tris-base buffer was used since the BES buffer required a pH adjust from a lower value. Thus, to avoid adding any NaOH I started with a basic saline and brought the pH to 8.8 with HCl.

The recording techniques have been previously described for intracellular EPSP measures (Stewart *et al.* 1994; Dasari & Cooper, 2004; Sparks *et al.* 2004). The compound amplitude of the excitatory postsynaptic potentials (EPSP) elicited by Is and Ib motor nerve terminals in segment 3 of muscle m6 was monitored (Kurdyak *et al.* 1994; Ruffner *et al.* 1999). The identified m6 muscle was used in each preparation. Only preparations with a resting membrane potential of -50mV or greater were used. The HL3 dissection medium was completely replaced by HL3 media containing the pharmacological compounds or different ionic composition. The media was rapidly exchanged within 30 s. All chemicals were obtained from Sigma chemical company (St. Louis, MO) with the exception of thapsigargin and KB-R7943 which

were obtained from Tocris (Ellisville, MO) and 5,6-Carboxyeosin from Marker Gene Technologies Inc. (Eugene, OR).

Facilitation/depression was measured by an index in which the ratio in the peak amplitude of the EPSP for one of the preceding pulses from another within the stimulus train. A unitary value of 1 was subtracted from the ratio to provide a Facilitation Index (FI). The subtraction of one ensures that if no facilitation is present (i.e., the amplitudes of the responses are the same), FI will be zero. If FI is negative then by definition this will produce STD. Electrical signals were recorded on line to a computer via a MacLab/4s interface.

Depending on the experimental design either a Student's *t*-test (paired or unpaired) or a two-way ANOVA followed with various posthoc analyses were used. Some data is presented as a percent change to compare an experimental manipulation or differences over time as explained within the Results section for each given experiment.

RESULTS

The preparation

The m6 muscle that was primarily used in these studies within the larval skeletal muscle preparation is depicted in Figure 4.1 A. The preparation is planar when stretched at the 4 corners. This allows the segmental muscles to be readily identified. The segmental nerves branching from the CNS are easily transected and drawn into a suction electrode to be stimulated selectively for a particular segment. In this study, the segmental nerve was stimulated at 20 Hz to produce 10 EPSP responses as shown in Figure 4.1 B. With the standard HL3 saline, with 1 mM [Ca^{2+}], the initial EPSP within the train is normally larger than the following EPSPs in the train. The rate in the decrease amplitudes is dependent on the stimulation frequency. In this case, I used the amplitude of the 1^{st}, 5^{th} and 10^{th} EPSPs within the train to index changes in the EPSPs as well as to calculate the facilitation or depression in the amplitudes. General in standard HL3 saline the facilitation measure, (i.e., [(A10/A1)-1] =FI) would be a negative value, so this can be referred to as negative facilitation. To be consistent for a depression of transmission I refer to negative facilitation as short-term depression (STD) throughout this study. The likely reason for the depression in this NMJ as compared to the low output NMJs of other arthropod preparations (i.e., crayfish opener NMJ) is that these synapses are of a

high output type and many of the vesicles in the readily releasable pool (RRP) are used within the 1st pulse and there is not enough time to replenish the pool with a rapid 40 Hz stimulation train (Atwood &Cooper, 1995, 1996a, b).

Thus, since the efficacy of synaptic release during a stimulus train, as for this high output fly NMJ, should depress to a greater extant over time as the $[Ca^{2+}]_i$ increases. So this should be measured by a more negative value using the STD index. Since compromising the NCX, PMCA or the SERCA are expected to have an effect on $[Ca^{2+}]_i$, the two measures to quantify alterations in synaptic transmission are amplitudes of the EPSPs and the STD index. Just measuring one form of STD may give misleading information, as the amplitudes can vary over the stimulus train (Crider & Cooper, 1999), so I choose to use the 10th, 5th and 1st EPSPs as measures for STD in the various experimental manipulations.

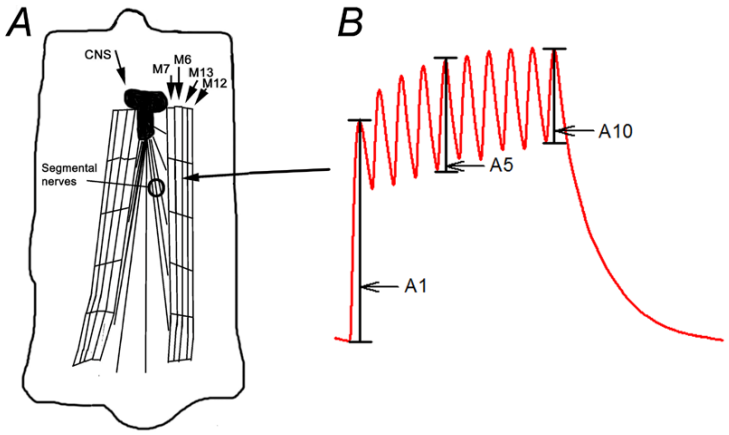

Figure 4.1: *Drosophila* larval NMJ preparation: (A) Schematic of the *Drosophila* larval neuromuscular junction preparation. A third instar larva is cut open on its dorsal side to expose the ventral abdominal muscles shown here as M6, M7, M12, M13. A segmental nerve innervating these muscles is drawn into a suction electrode and stimulated. (B) Representative EPSP train of 10 pulses recorded from M6 when stimulating of a segmental nerve at 20 Hz is shown. The measurement of the amplitudes A1, A5 and A10 is indicated.

Action of compromising NCX

One approach used in this study to reduce the function of the NCX was to lower $[Na^+]_o$ by using a HL3 saline with less Na^+. However, in later studies in which the PMCA and the NCX are compromised together, the pH in the saline is raised to

pH 8.8 (to reduce PMCA function) and I did not want to do so by adding NaOH. In order to get around this, I used a Tris-Base and adjusted pH down to 8.8 with HCl. I noticed that the saline made with Tris-Base resulted in odd EPSP amplitudes, so I set out to compare preparations dissected and bathed in standard HL3 saline and later switched to HL3 made with Tris-Base instead of the BES buffer (see Methods). The results are very clear that the Tris-Base saline at pH 7.2 reduced the amplitude in the 1st and 5th EPSPs in 5 out of 5 preparations (Figure 4.2 A; $P<0.05$; Wilcoxon rank-sum test). This is also observed in the percent changes in the amplitudes among the preparations (Figure 4.2 B; Student's Paired *t* test)The 10th EPSP did not show a significant change in the pooled data but did in the comparisons among individual preparations (Figure 4.2 A; 5 out of 5, $P<0.05$; Wilcoxon rank-sum test). The measures of FI did not show any significant changes; however, the mean values did decrease, but there was a large variation among the preparations (Figure 4.2 C).

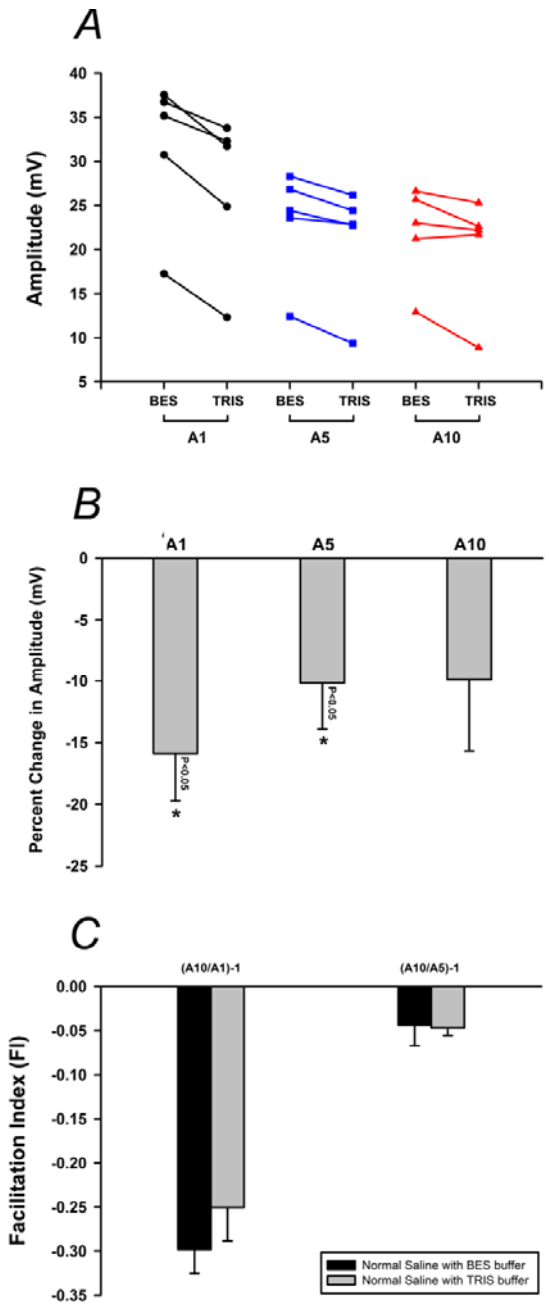

Figure 4.2: Differences in EPSP amplitudes and FI in TRIS and BES saline: (A) The effect on the EPSP amplitudes in changing the bathing saline from normal saline made with BES buffer to one made with a Tris-Base buffer. Both salines are at pH7.2. Amplitudes 1, 5 and 10 events within the 10 pulse train are shown. There is a significant reduction in amplitudes in TRIS as compared with BES. (B) The percent change in 1, 5 and 10 amplitudes. (C) The facilitation index of EPSPs in BES and TRIS was not significantly different between the two salines.

Despite the reduced amplitude in the EPSPs, with the Tris-Base buffer, I needed to continue using it as future studies with a compromised PMCA (pH8.8) were to be examined. In order, to first examine the effects of a reduced $[Na^+]_o$ for a comprising the NCX, the amplitudes of the EPSPs were measured before and in a saline with 2/3rds reduced $[Na^+]_o$. In comparing the responses among preparations, a percent change from saline exposure to a reduced NCX state was determined. A percent is used since the absolute EPSP amplitudes varied among preparations. The means in the percent differences for the various EPSPs within the response train (1^{st}, 5^{th}, 10^{th}) are shown as well as the values for individual preparations. The reason to show the individual preparations is to highlight the one preparation that had a very large increase in the EPSP amplitude (double circle shown in Figure 4.3 A1). Since some preparations had increases and others decreases in the EPSPs, there is no significant trend when examining a before and after effect (Figure 4.3 B). Likewise there is no significant effect when examining a percent change in the amplitudes (Figure 4.3 A1). However, after removing the large outlier from the data set, the percent change in the 5^{th} EPSP amplitude did show a significant change (Figure 3A2; Student's paired *t*-test). The facilitation index, taken at various EPSPs within the train, revealed no significant effect in lowering the $[Na^+]_o$. Thus, the effect of reducing the $[Na^+]_o$ to compromise the NCX did not show a consistent or reliable effect.

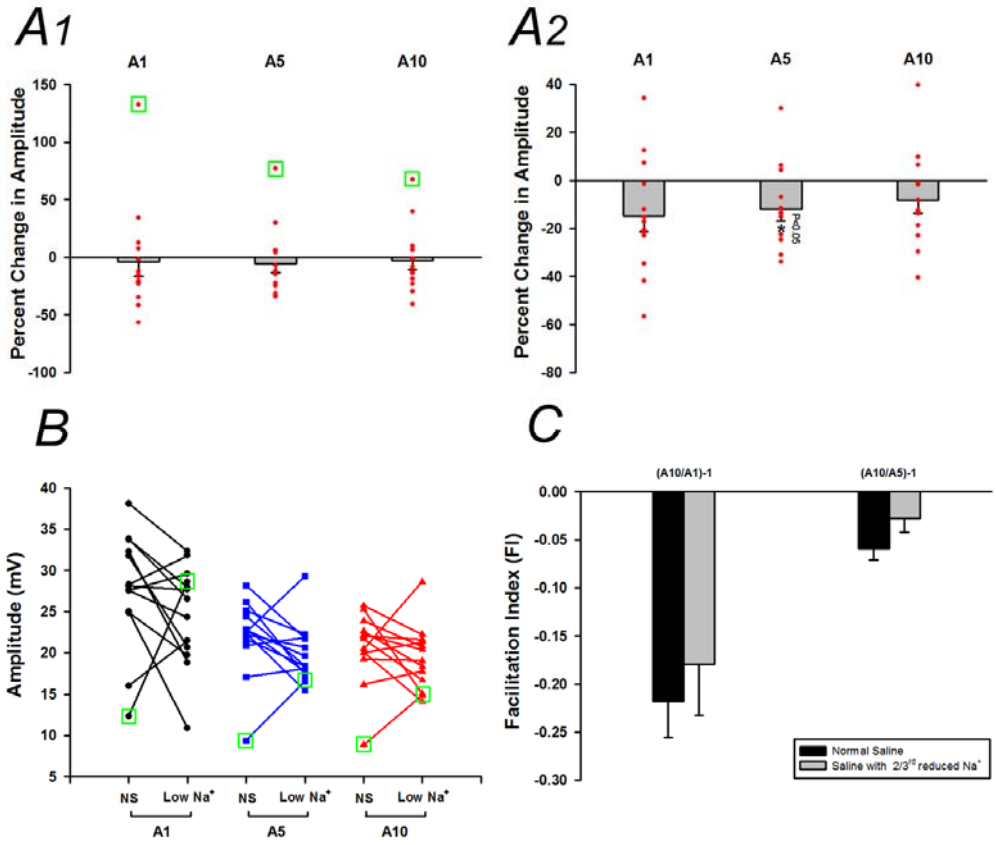

Figure 4.3: Effect of compromising NCX (with low $[Na^+]_o$) on EPSP amplitudes and FI: Compromising the NCX with reduced $[Na^+]_o$. (A1) The percent change in the amplitudes of the 1st, 5th and 10th EPSPs did not reveal a significant effect. The red dots show individual preparations and since one preparation (in green box) revealed a large increase the data was reassessed with the results of the one preparation removed (A2). This preparation is depicted with a green box in A1. In removing the data from one preparation there was only a significant effect in the percent change in the 5th EPSP amplitude. The before and after effect, for individual preparations, is shown in B. The one outlier is again shown with green open boxes. The FI was not significantly affected by reduced $[Na^+]_o$ (C)

Action of KB-R7943 in inhibiting NCX

The reduction in $[Na^+]_o$ is one approach to dampen the ability of the NCX to extrude Ca^{2+}, however I also wanted to try a known pharmacological approach to block NCX function as in other animal models (Wu *et al*. 2008). Since I did compare, both the reduced $[Na^+]_o$ and this pharmacological approach on the crayfish NMJ

(Chapter 3 of this dissertation) and the *Drosophila* heart (Chapter 5 of this dissertation), I was interested to continue this comparison at the *Drosophila* NMJ.

The action of *KB-R7943* was more consistent than the results with reduced $[Na^+]_o$ in depressing the EPSP amplitudes throughout the response train. Similar effects were present in 6 out of 6 preparations for the 5^{th} and 10^{th} EPSP amplitudes but not the 1^{st} EPSP amplitude with the train (Figure 4A; $P<0.05$; Wilcoxon rank-sum test). The percent change in the EPSP amplitudes also revelad a significant effect of *KB-R7943* in reducing the amplitudes for the 5^{th} and 10^{th} EPSP amplitudes (Figure 4.4 B; Student's paired *t*-test) calculated FI (Figure 4.4 C) did not show any significant effect on either of the two FI measures, [(A10/A1)-1] or [(A5/A10-1].

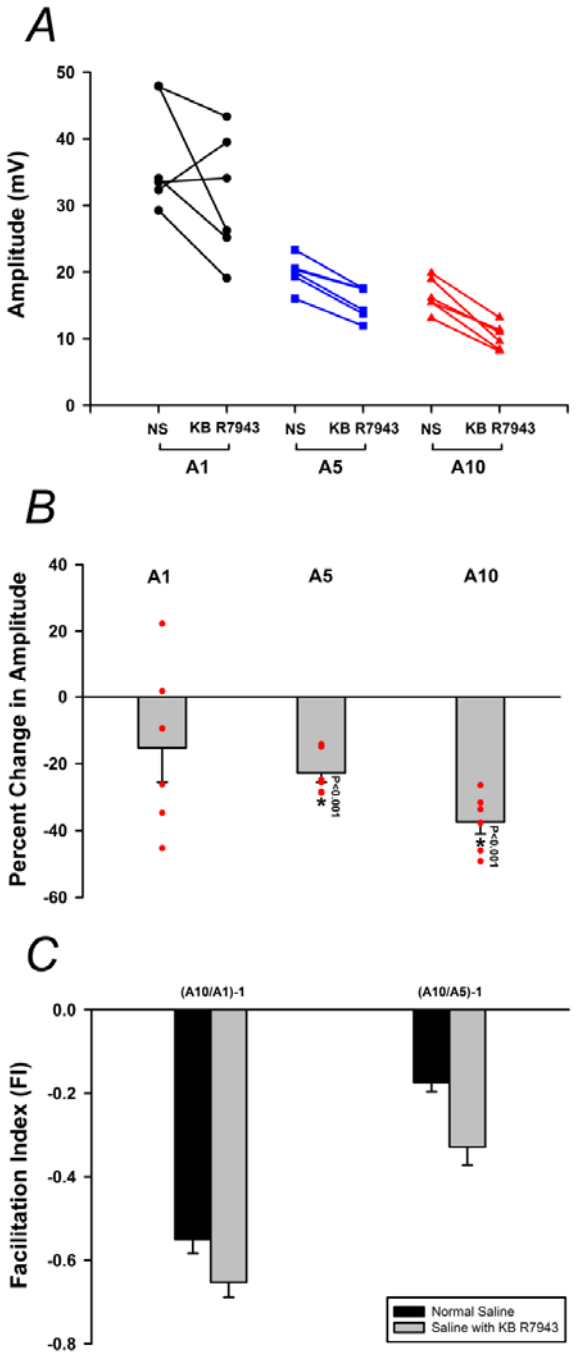

Figure 4.4: Effect of inhibiting NCX (with *KB-R7943*) on EPSP amplitudes and FI: The action of *KB-R7943* in inhibiting the NCX: (A) *KB-R7943* reduced the amplitude for the 5th and 10th EPSPs as examined for changing in individual preparations (A) and for the percent changes among preparations (B). The percent change for individual preparations is shown as red dots superimposed on the mean (± SEM) bar chart. No effect occurred for the facilitation index (C).

The effect on transmission with a compromised PMCA

In order to block the function of the PMCA the bathing media was switched to one from a normal pH 7.2 to one at pH 8.8 while continuing to provide trains of stimuli. The initial EPSP within the train rapidly increased. This trend was observed 5 out of 5 times for all three EPSP measures within the response train (i.e, A1, A5 and A10) (Figure 4.5 A; $P<0.05$; Wilcoxon rank-sum test). The percent change in the amplitudes concurred with the absolute change in the EPSP (Figure 4.5 B; $P<0.05$; Student's paired *t*-test). No significant effect was observed for the FI (Figure 4.5 C).

Using CE to pharmacological block the PMCA function did not produce the same result as raising the pH to 8.8. CE produced a decrease in EPSP amplitudes in 5 out of 6 times for the 1^{st}, 5^{th} and 10^{th} EPSP measures within the response train (Figure 4.6 A). The percent change in all three EPSP amplitudes is significantly different (Figure 4.6 B; $P<0.05$; Student's paired *t*-test) No significant effect was observed for the FI (Figure 4.6 C).

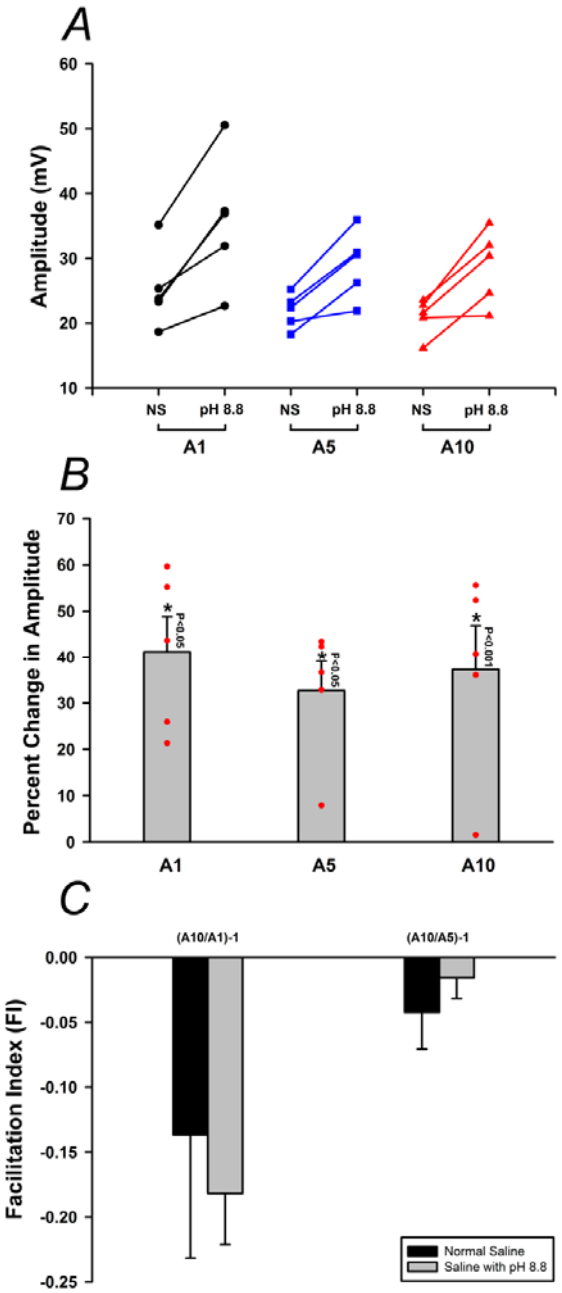

Figure 4.5: Effect of inhibiting PMCA (with pH 8.8) on EPSP amplitudes and FI: The action of raising pH from 7.2 to 8.8 in compromising the PMCA. The increase in pH produced a consistent trend in increasing the 1st, 5th and 10th EPSPs in individual preparations (A) and for the percent changes among preparations (B). The percent change for individual preparations is shown as red dots superimposed on the mean (± SEM) bar chart. No effect is occurred for the facilitation index (C).

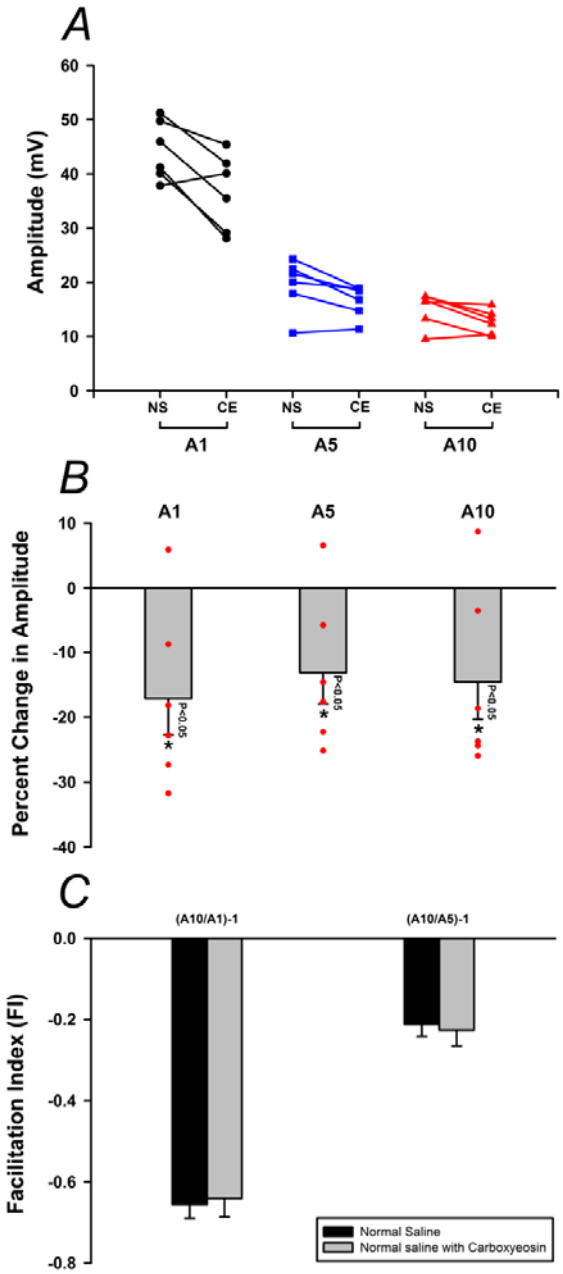

Figure 4.6: Effect of inhibiting PMCA (with carboxyeosin) on EPSP amplitudes and FI: The action of 5, 6-Carboxyeosin (CE) in compromising the PMCA. CE reduced the amplitude for the 1st, 5th and 10th EPSPs in 5 out of 6 individual preparations (A) and for the percent changes among preparations there is a significant affect (B). The percent change for individual preparations is shown as red dots superimposed on the mean (± SEM) bar chart. No effect is occurred for the facilitation index (C).

The effect on transmission with a compromised SERCA

The Ca^{2+} pump on the ER (SERCA) was targeted by a pharmacological approach with application of TG (10 μM) and by use the mutational *Kum* line (when heat shocked the SERCA protein is rapidly inactivated) (Sanyal *et al*. 2005). The preparations were first examined in normal saline and then incubated with TG for 15 min without nerve stimulation. The nerve roots were again stimulated to assess the effect of the EPSP amplitudes. The amplitude of the EPSPs is depressed in 5 out of 6 preparations by TG for the 1^{st} and 10^{th} EPSPs but in 6 out of 6 for the 5^{th} EPSP (Figure 4.7 A). The percent change in the EPSP amplitudes is significant for the 1^{st} and 5^{th} EPSP amplitude but not the 10^{th} (Figure 4.7 B). To insure the reduced amplitudes were due to maintaining an intracellular recording during the incubation period the neighboring muscle (m7) fiber was examined. These also showed reduced EPSP amplitudes with normal resting membrane potentials. So the effect was not due to a general rundown by retaining an intercellular recording electrode within the muscle fiber.

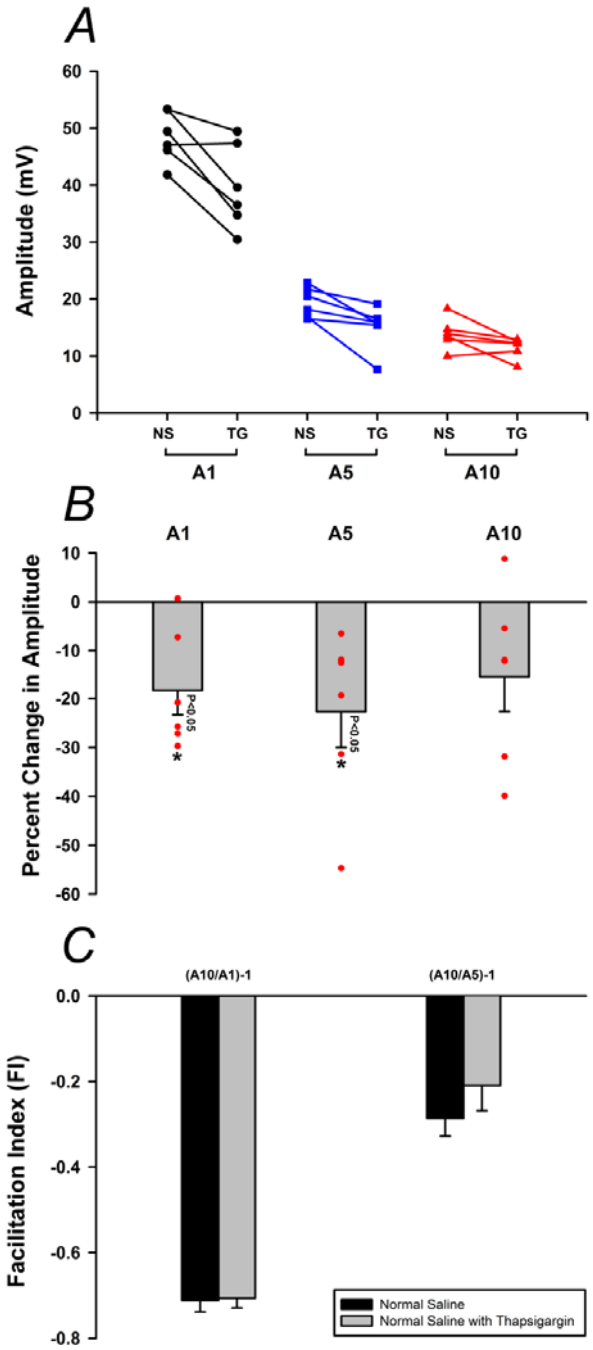

Figure 4.7: Effect of inhibiting SERCA (with thapsigargin) on EPSP amplitudes and FI: The action of thapsigargin (TG) in compromising the SERCA. TG reduced the amplitude for the 5th and 10th EPSPs in 5 out of 6 individual preparations and 6 out of 6 for the 1st EPSP (A). For the percent changes among preparations there is a significant affect for the 1st and 5th EPSP amplitudes (B). The percent change for individual preparations is shown as red dots superimposed on the mean (± SEM) bar chart. No effect occurred for the facilitation index (C).

The combined effects in reduced function of the NCX, PMCA and SERCA

With use of the *Kum*^{170TS} temperature sensitive strain I could compare the pharmacological approach to the SERCA mutation. However, the appropriate controls for the effect of heat shock were required to differentiate the action of heat shocking the preparation. For controls, I used the CS strain and recorded the EPSP at room temperature and then raised the temperature by exchanging the saline bath with saline heated to 40°C while measuring the EPSP amplitudes. The 1st EPSP amplitude increased in 4 out of 5 preparations upon heat shock (Figure 4.8 A). The 5th and 10th EPSPs showed a mixed response among the preparations. The FI measures did not significantly change (Figure 4.8 B). In a separate set of experiments the same procedure was used but this time the heat shock was combined with a pH of 8.8 and 2/3^{rds} reduced [Na^+] in the bath. In this transition, 5 out of 6 preparations showed a decrease in the 1st, 5th and 10th EPSP amplitudes (Figure 4.8 C). The FI measures did not significantly change for these conditions (Figure 4.8 D).

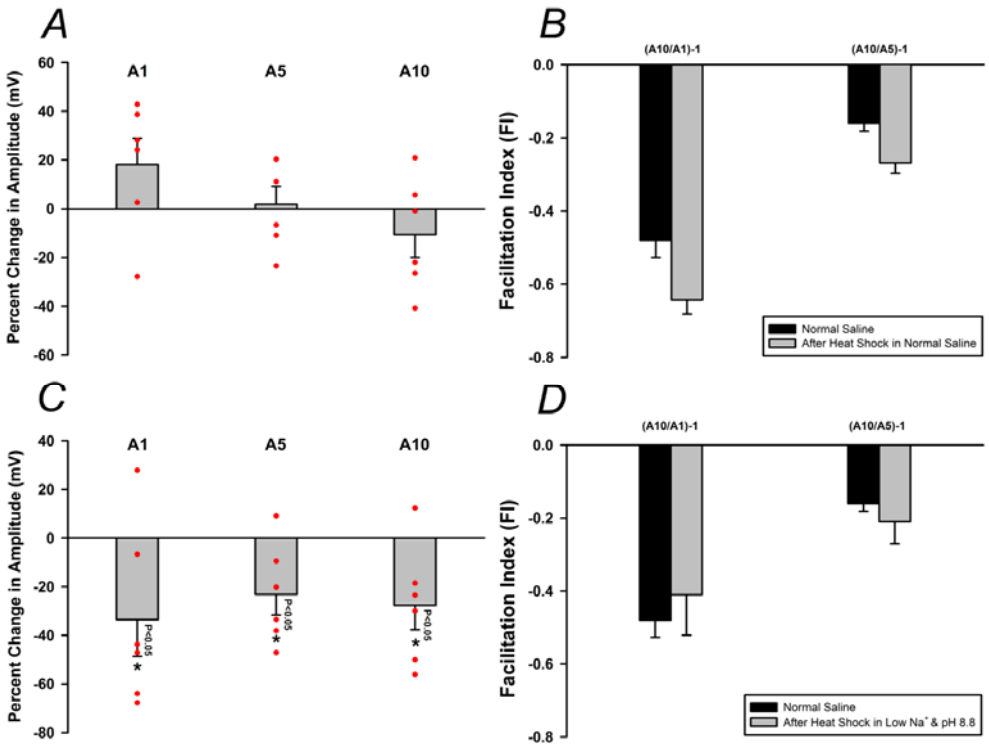

Figure 4.8: Action of heat shock and compromising PMCA and NCX in the genetic background control CS strain: (A) Examination of heat shock alone on the control CS strain of *Drosophila*. Heat shock did not produce a consistent trend in altering the

EPSP amplitudes. The individual preparations are shown as dots superimposed on the mean (± SEM) percent change bar chart. (B) The facilitation index did not show a significant effect either with heat shock. The results before and during heat shock are shown. The effect of heat shock, 2/3^{rds} reduced $[Na^+]_o$ and pH 8.8 on the percent change in the EPSP amplitudes resulted in a decrease in 5 out of 6 preparations (C) with no general trend in altering the FI from before and during the combined exposure (D).

The same procedures were repeated for the *Kum* strain as for the CS, but this time there was a trend to decrease the amplitudes of the EPSPs during the heat shock. In 5 out of 6 preparations, the 1st, 5th and 10th EPSP amplitudes showed a decrease in the percent change (Figure 4.9 A). There was no consistent trend from before to after heat shock for the FI index (Figure 4.9 B). In a separate set of experiments the same procedure was again used but this time the heat shock was combined with a pH of 8.8 and 2/3^{rds} reduced $[Na^+]$ in the bath for the *Kum* strain. In this transition 6 out of 6 preparations showed a decrease in the 1st EPSP percent and 5 out 6 for the 5th and 10th EPSP amplitudes decreased in the percent change (Figure 4.9 C). The FI measures did not significantly change for these conditions and there was substantial variation among the preparations (Figure 4.9 D).

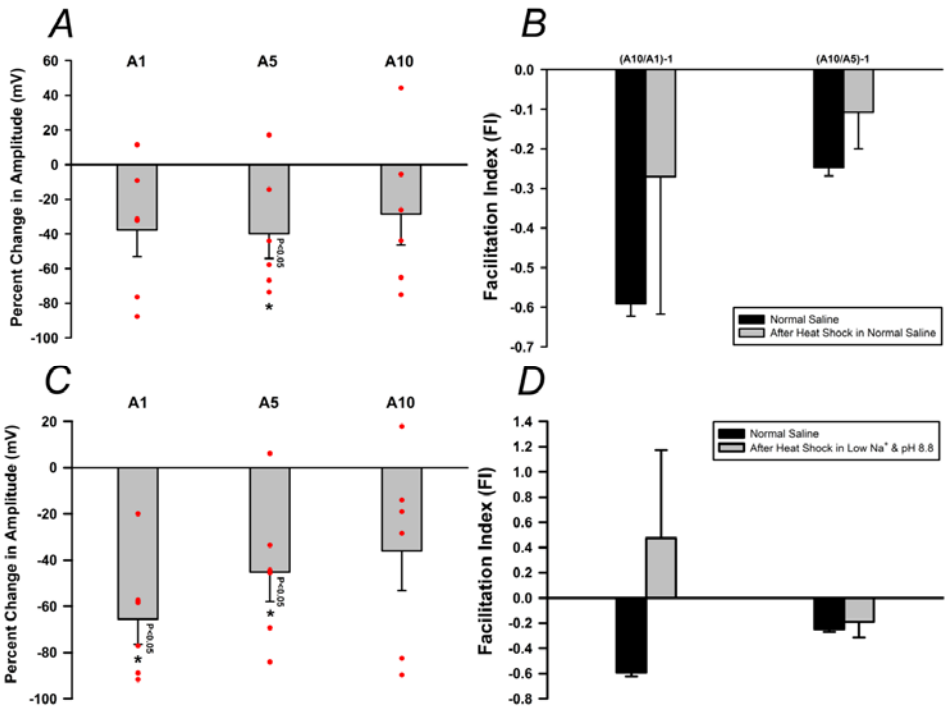

Figure 4.9: The action of heat shock and compromising PMCA and NCX in the *Kum* strain: (A) Examination of heat shock alone on the *Kum* strain. Heat shock did tend to

reduce the percent change for 5 out of 6 preparations, the 1st, 5th and 10th EPSP amplitudes The individual preparations are shown as dots superimposed on the mean (± SEM) percent change bar chart. (B) The facilitation index did not show a significant effect either with heat shock. The results before and during heat shock are shown. The effect of heat shock, 2/3rds reduced $[Na^+]_o$ and pH 8.8 on the percent change in the EPSP amplitudes resulted in a decrease in 6 out of 6 preparations for the 1st EPSP, but only 5 out 6 preparations for the 5th and 10th EPSPs (C) with no general trend in altering the FI from before and during the combined exposure (D).

In order to isolate the average effect in the difference in percent change for amplitudes, due to heat shock, among the controls from the changes measured in the *Kum* strain, the differences in the average effect were subtracted. The panel in Figure 4.8 A was subtracted from Figure 4.9 A and the result is shown in Figure 4.10 A. The errors were taken from the data set with the largest value and placed on the subtracted result. The net effect is about a 55% reduction in the percent change for the 1st EPSP, about 42% for the 5th amplitude and about an 18% for the 10th EPSP amplitude. The effects of inhibiting the PMCA and NCX along with heat shock for CS and *Kum* was also compared by subtracting out the heat shock effect measured in CS. The differences in Figure 4.8 C and Figure 4.9 C are shown in Figure 4.10 B. The average difference for the percent change in the 1st and 5th EPSP is a decrease of about 32% and 22% respectively and 8.5% for the 10th EPSP amplitude. So it appears, in general, that *Kum* mutation demonstrated a greater effect in reducing the EPSP amplitudes then just the effect of heat shock alone.

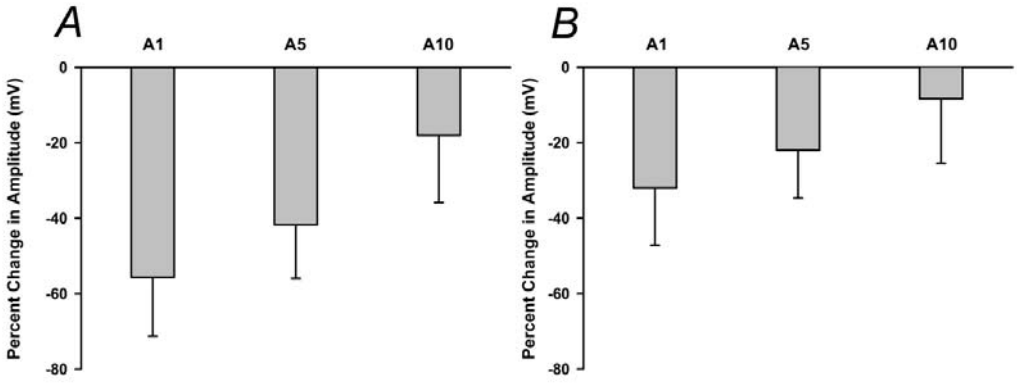

Figure 4.10: Net effect of heat shock on *Kum*: The effect of heat shock is subtracted from controls and the *Kum* strain to obtain an average effect of the SERCA mutation. (A) The effect of heat shock on the percent change in EPSP amplitudes for *Kum*. The

effect on CS controls was subtracted from the effect on *Kum* to obtain these values. (B) The effect of heat shock with 2/3rds reduced $[Na^+]_o$ and pH 8.8 on the percent change in the EPSP amplitudes is compared for the effect of the mutation only by the difference in controls and the *Kum* strain.

DISCUSSION

This study demonstrated that in these high output synapses, present on the larval *Drosophila* muscle, are sensitive to manipulations of the NCX, PMCA and SERCA in respect to synaptic physiology and plasticity. In physiological $[Na^+]_o$, a rapid stimulation train of the nerve produces STD. This depression is characterized by an initial large amplitude EPSP followed by subsequently smaller amplitude EPSPs. Since a key to STD is the amount of presynaptic Ca^{2+} entry during neural stimulation it is not surprising that regulation of $[Ca^{2+}]_i$ is important throughout the stimulus train for maintenance of STD. Specifically, a compromised NCX, by a lowered $[Na^+]_o$, resulted in no significant effect on all the EPSP amplitudes throughout the pulse train. It was expected that STD would be reduced if the initial amplitude within the EPSP train was to be reduced but this was not the case. However, blocking the NCX channel by KBR produced different results by generally decreasing the initial EPSP and subsequent EPSPs within the train. This did not result in significant changes in STD. The reduction in function of the PMCA by raising pH or exposure to CE produced opposite results. The SERCA function appears to be similar to the PMCA since compromising it with TG mimicked the altered responses of the PMCA when exposed to CE. However, using the mutant heat sensitive Kum^{170TS} line to block SERCA function I discovered that the background control line, when heat shocked, also resulted in altered EPSP amplitudes just as the *Kum* line. After controlling for the decrease in the background line, *Kum* still produced a reduction in the EPSP amplitudes. One would assume then that the inhibition of SERCA could be unmasked in this line, but with a substantially reduced EPSP this would have an impact on the development and maintenance of STD. The combined effects of lowered $[Na^+]_o$, pH 8.8 and TG did have an effect on the EPSP amplitudes to a greater extent than any individual treatment. The multiple blockages of these 3 channels on STD did not show significant differences. Thus, I suggest that there is a synergistic effect in compromising more than one Ca^{2+} buffering mechanism within these motor nerve terminals.

The varied responses in the initial EPSP and the rest of the EPSP amplitudes within the response train, when $[Na^+]_o$ is lowered, is not an effect I associate with a specific action on the reduction in the efficacy of the NCX. Most likely a direct effect on a reduction of the action potential amplitude in the motor neuron could be occurring just enough to be at a critical level to effect $Ca^{2+}{}_V$, where sometimes the channel open time and potential inactivation is not consistent. Just as observed in Chapter 3 for the crayfish motor neuron, the potentially reduced amplitude may not only have an effect on the $Ca^{2+}{}_V$ channel activation, but also on the duration of the depolarization which would result in less influx of Ca^{2+}. However, it is not possible, at present, to record within the preterminal motor axon in the *Drosophila* as one can perform in crayfish preparations due to diameter of the axon and the associated sheath. In fact, I am not aware of intracellular recordings from preterminal axons in intact vertebrate motor neurons. A reduction in the EPSP amplitude should have resulted in fewer vesicles being depleted in the 1st response so one should expect relatively enlarged second EPSP amplitude. This was not observed. The second approach to retard the function of the NCX, with *KB-R7943*, which did give the expected result of enhanced STD is likely due to $[Ca^{2+}]_i$ building up in the nerve terminal throughout the repetitive stimulation. It is hard to know if the *KB-R7943* should be relied on as a pharmacological drug of choice since the compound is known to have non-specific effects. In addition, there could be various NCX, PMCA and SERCA isoforms that may produce varied responses (Jensen *et al.* 2007) and they might share the same gene sequence but be produced by alternative splicing or even post translational modification. So, until the proteins are purified and analyzed for potential modifications I cannot be sure in my studies that I have been examining only a single form of these channels. The different forms of feedback regulation or modulation of channel isoforms is known. For example, in *Drosophila* the NCX is inhibited by $[Ca^{2+}]_i$ where as other NCX forms are not (Hryshko *et al*. 1996). This can even impact the application of pharmacological agents, as it was shown that *KB-R7943* is a weaker inhibitor (4-5 times) of the *Drosophila* NCX (CALX1.1) than an NCX isolated in canines (Isaac *et al*. 2002).

Since it is established that *KB-R7943* can have nonspecific effects in other systems by blocking L-type Ca^{2+} channels, Na^+ channels, and N-methyl-D-aspartate (NMDA) channels (Arakawa *et al*. 2000; Matsuda *et al.* 2001; Sobolevsky & Khodorov, 1999; Rumpal & Lnenicka, 2003) it would worth designing experiments to specifically address this issue either as was done for actions on Ca^{2+} induced inactivation of NCX by expressing the *Drosophila* NCX in a frog oocyte (Hryshko *et al.* 1996) or in a cell line where its function can be addressed without complications of

other exchangers and unknown channels. The two approaches in blocking the PMCA (raised pH or application of CE) produced opposite results. The results presented here will allow future studies to consider if using CE, *KB-R7943* and even TG are valid. As I approached with the crayfish NMJ, the mixture of ionic approaches to examine a combined role of the Ca^{2+} channels helped to compare with three pharmacological organic agents and the mutational *Kum* strain. However, the reduced EPSP amplitudes in background controls does complicate comparisons since this altered the amount of vesicles initially used in the RRP which impacts STD and interpretations of the SERCA contribution. I do think direct application of the drugs on the exposed NMJ is preferred over injecting into the larva to examine behavioral changes as an index of NMJ function as one is hard pressed to know if the actions are in the CNS or at the NMJ. Many hormonal studies directed at understanding their role on the heart in pupa as well as in larvae were preformed with whole animal injections. I address these issues in the next chapter (Chapter 5) that deals with the larval heart. However as an example, paradoxial results in application to ouabain, a well known compound for blocking the Na^+/K^+-ATP pump, appeared not to have an effect on *Drosophila* malpighian tubules. But this was due to the unique nature of the cells to actively excrete this organic compound (Torrie *et al.* 2004). Studies need to be performed in with isolated *Drosophila* neurons to better identify the pharmacological profiles of the NCX, PMCA and SERCA.

In order to attempt to mechanistically explain the results obtained in this study I designed relatively simple hypothetical models to explain the changes in the EPSP amplitudes as well as the occurrences in STD (Figure13). If the high capacity NCX is inhibited alone and if the PMCA and the SERCA are not able to compensate quickly enough during STF, the EPSP amplitudes increases throughout the early part of the stimulus train and the new plateau is reached. Thus, a new homeostatic setting, in regulation for SERCA and PMCA as well as other Ca^{2+} buffering sources, occurs. In this scenario, the high affinity, but low capacity, PMCA would have a role in maintaining the new Ca^{2+} equilibrium just as would the SERCA. When all three channels are working normally, there could even be a Ca^{2+} induced inhibition of the NCX, thus leaning more on the other mechanisms to maintain the synaptic balance. However, the inhibition of the NCX by *KB-R7943* and PMCA by CE depressed synaptic transmission just as inhibition of the SERCA by TG or with the *Kum* mutation exposed to heat shock. One possibility is that very high $[Ca^{2+}]_i$ occurred when these channels are blocked and a Ca^{2+} induced inhibition of the $Ca^{2+}{}_V$ occurs. This does not fit with the inhibition of the PMCA by pH 8.8 which resulted in an increase in vesicle release. Perhaps at the fly NMJ pH 8.8 is resulting in other effects

besides what I intended. We need to know more about the types of Ca^{2+} channels at these nerve terminals (P-Ca^{2+}_V, K^+_{Ca}) and if they are modulated by phosphorylation or Ca^{2+} feedback inhibition. There is still a lot of work ahead to integrate the possibilities in the contributions of these other variables.

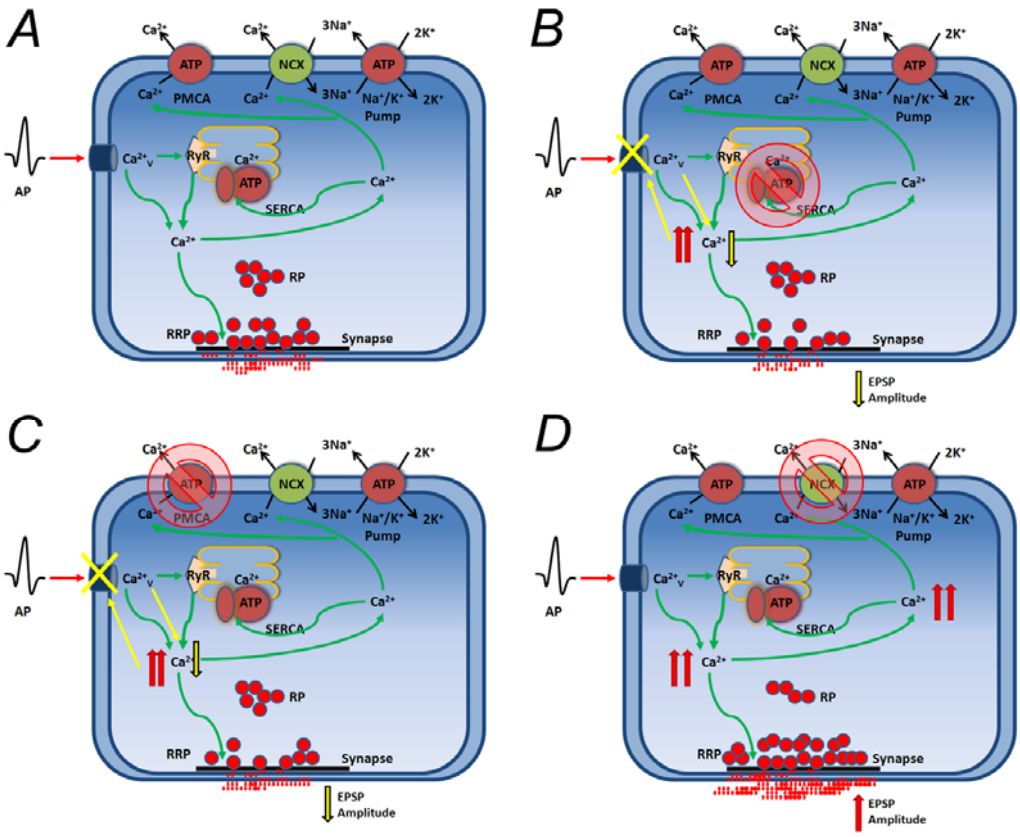

Figure 4.11: Model of the presynaptic nerve terminal at the *Drosophila* NMJ. These high-output synapses have many vesicles docked within the ready releasable pool (RRP) and few in the reserve pool (RP). Upon starting a short train of stimulations the first action potential will result in the majority of vesicles to fuse and release transmitter. The subsequent nerve terminal depolarizations will have fewer vesicles primed; therefore, synaptic depression is observed by monitoring in the postsynaptic muscle fiber. Reducing the function of one or the other Ca^{2+} buffering mechanisms results in changes in the probability of vesicle fusion.

Ca^{2+} clearance is controlled by many specialized mechanisms in neurons and the three major processes are the ones I tackled in this study. The NCX, PMCA and SERCA are all known to be modulated and differentially regulated (Thayer *et al.* 2002). In my study, compromising SERCA by use of the heat shock has raised some

intriguing questions of why the EPSPs are reduced so variable in the control CS line as well as in the *Kum* line. In the first report by Sanyal *et al.* (2005), the EPSPs in the larvae and adult fly muscle were noted to decrease substantially in the *Kum* line upon heat shock. Since initiating action potentials in the muscle were damped in the heat shocked *Kum*, the authors suggested that the *Kum* is reducing the function of $Ca^{2+}{}_V$ channels. They did not report variable changes in EPSP amplitudes as I have in control flies with heat shock. In fact, they did not report any effect of temperature on wild type (control) adults or larvae. Neal *et al*. (2006) demonstrated less variability in synaptic transmission following heat shock which was linked to stabilization of the channels due to the expression of heat shock proteins.

Function of NXC would not appear to have a primary role to produce a change in the first EPSP within the train since Ca^{2+} has not even been induced to build up. Also the effect a potentially raised $[Ca^{2+}]_i$ was not able to be compensated by the PMCA or the SERCA. This does raise the question of the specificity of *KB-R7943*, but the use of *KB-R7943* did at least produce a consistent effect on synaptic transmission. I feel further investigation is needed to know how specific *KB-R7943* is to the *Drosophila* NCX in the motor nerve terminals. It would also be of interest to know if there might be a Na^+/Ca^{2+}-K^+ exchanger (NCKX) present in these neurons since its expression is known to be present in the ventral nerve cord in stage 16 embryos (Winkfein *et al.* 2004). There could even be different isoforms of the NCX in these neurons which would produce varied responses depending on their density and sensitivity to pharmacological agents (Ruknudin *et al.* 1997; Isaac *et al.* 2002). The *Drosophila* NCX form termed CALX1.1 is recognized for it stability to be negatively regulated by Ca^{2+} in comparisons to other NCX proteins (Isaac *et al.* 2002; Hryshko *et al.* 1996). The ER is now established to have a prominent role in nerve terminals as well as dendrites in buffering Ca^{2+}. Release of Ca^{2+} via the ryanodine receptors, which could come from internal Ca^{2+} via Ca^{2+} induced Ca^{2+} release (CICR), can have a prominent effect on synaptic transmission or STF and STD (Lauri *et al.* 2003; Galante & Marty, 2003). Likewise, the Ca^{2+} release via IP3 receptors on ER has been shown to alter neuronal synaptic activity (see review- Bardo *et al.* 2006). The role of the ER is transmission and neuronal plasticity has not be investigated to the same extent at the *Drosophila* NMJ as in vertebrate CNS preparations but it is known that the normal function of the ER is essential for larval development (Sullivan *et al.* 2000).

These high output synapses of the fly NMJ would require fast buffering as not to have prolonged vesicle fusion events following an action potential. Also, STD would be prolonged, since in this preparation the depression is due to fewer vesicles

being docked and available for the subsequent pulses. So with reduced extrusion or uptake by the ER one should expect a more pronounced depression, it is probably not possible to overcome the structural limitations in the number of vesicles that are docked. Since, I did not measure any consistent change in the STD index, for treatments with *KB-R7943*, CE, pH 8.8 or in the *Kum* with heat shock, the vesicles are likely docked but may have increased in recruitment from the RP to the RRP in compensation to offset STD.

Modulation of the onset of STD and the recovery process can help in deciphering the mechanisms of synaptic depression and plasticity. It would be beneficial to learn more on the role of free $[Ca^{2+}]_i$ on this process which would lead also to the regulation in the buffering of Ca^{2+}. The high output synapses with complex synaptic structure maybe more prone to STD than lower output synapses but only if the vesicle docking is also enhanced at the high output sites. Otherwise with fewer vesicles docking sites bound initially then more would have the potential to dock and STF would be promoted as residual $[Ca^{2+}]_i$ increased. This type of differential regulation in STF and STD has been demonstrated at the opener and extensor NMJ of the crayfish where serial reconstruction of physiological recorded terminals were performed (Cooper *et al.* 1995b, c; Johnstone *et al*. 2008). Imaging of vesicle dynamics in living terminals would help to delineate potential mechanisms in differences of vesicle RP pools being recruited to RRP based on activity and synaptic complexity; however, one has to insure physiological correlation as it is known that vesicles can recycle empty at the *Drosophila* NMJ (Kidokoro *et al*., 2004). STD is very likely not a rundown of ATP stores but partially based on synaptic structure and vesicle dynamics. Logsdon *et al*. (2005) showed that glutamate depleted RRP vesicles were rapidly mixed with RP vesicles by 5-HT action at the crayfish NMJ.

Lnenicka *et al*. (2006) showed with imaging of Ca^{2+} signals in *Drosophila* nerve terminals that the primary source for the terminal in buffering the $[Ca^{2+}]_i$ after a series of stimuli is the PMCA. It would be of interest if the *Kum* strain would show even a greater expression of the PMCA when the animals are raised at a temperature to comprise the SERCA function without killing the larvae. The calcium binding protein, frequenin, would also be of interest to see if its expression could be differentially regulated depending on Ca^{2+} load of the terminals over time, since it is known to be present in the *Drosophila* nerve terminals (Rivosecchi *et al.* 1994).

The NMJ of *Drosophila* larvae have become somewhat of a hot bed to investigate vesicle recycling because of the availability of mutational studies. The role of Ca^{2+} is significant for understanding the kinetics of vesicle mobility, fusion and modulation of the storage pools within the nerve terminal (Msghina *et al.* 1999;

Harata *et al.* 2001; Pyle *et al.* 2000; Matthews, 2004; Parsons *et al.* 1999). So, it is not surprising that modifications in NCX, PMCA and SERCA would have a significant role. It was shown in *Drosophila* NMJs on m6 and m7 that CICR mobilized even the dense core vesicles through actions on CaMKII (Shakiryanova *et al.* 2007). Since these high output NMJ do show STD, there is likely a significant role of the RP to be mobilized to the RRP with repetitive train stimulation, as shown for hippocampal neurons (Goda & Stevens, 1998), but how much Ca^{2+} is directly involved is still not fully resolved. Ca^{2+} does have a role in even the endocytic process (Balaji *et al.* 2008; Palfrey & Artalejo, 1998). Some studies have shown that the vesicle can act as a sink for Ca^{2+} and thus the size of the RRP could alter the Ca^{2+} sparks with an action potential at the synaptic face. However, at the *Drosophila* NMJ the vesicles have been shown to have little significance on the Ca^{2+} buffering (Macleod *et al*. 2004) but it does not mean that they do not influence the Ca^{2+} clouds as they restrict the volume and essentially increase the concentration of ions in the remaining restrictive locations (Cooper *et al*. 1995a).

It would be of interest to learn if chronic manipulation of NCX, PMCA or *Kum* would result in developmental abnormalities of nerve terminal growth and or modifications at a synaptic level. The NMJ of larval *Drosophila* is an ideal preparation to investigate developmental questions related with synaptic function as the terminals grow along with the muscle in order to maintain overall synaptic efficacy, particularly at these NMJs since the EPSPs are graded. The rate of larval growth, thus NMJ growth, can be regulated by temperature and mutations allowing additional insight into regulatory mechanisms. Possible over compensation by one or multiple mechanisms would occur with chronically higher background $[Ca^{2+}]_i$ levels to maintain a normal $[Ca^{2+}]_i$ so as to maintain synaptic homeostasis (Burrone & Murthy, 2003). When $Ca^{2+}{}_V$ channels were chronically compromised the nerve terminal did not compensate to maintain synaptic efficacy (Xing *et al*. 2005). Perhaps the same is true for a higher load of $[Ca^{2+}]_i$. The impact of excess $[Ca^{2+}]_o$ on the induction of long term depression (LTD), which is known to be Ca^{2+} dependent in the cerebellum of rodents (Ito, 1989; Gall *et al.* 2005) and *Drosophila* NMJs (Guo & Zhong, 2006), would be of interest to investigate mechanisms of synaptic plasticity. It is known that external Ca^{2+} is important in membrane stabilization (Stefani & Steinbach, 1969) and has clinical relevance for humans (Bezprozvanny & Mattson, 2008), but I am not aware of any studies addressing the membrane stabilization from a internal viewpoint of raised $[Ca^{2+}]_i$. I would assume that the cell is so well tuned to maintain $[Ca^{2+}]_i$ at a low level, that higher than normal levels would induce many effects and many would be indirectly related to Ca^{2+} but through activation of various cascades, such as CaM

Kinase II (Fink *et al.* 2003) which could alter nerve terminal morphology. Direct actions would undoubtedly have a role such as Ca^{2+} induced inactivation of the $Ca^{2+}{}_V$ channels, activation of K_{Ca} channels (Fakler & Adelman, 2008) and actions on SNARE proteins that work as a Ca^{2+} sensor for vesicle fusion (see review by Catterall & Few, 2008). Mitochondrial function may also be impaired since these organelles can buffer Ca^{2+} and effect vesicle dynamics (Verstreken *et al.* 2005). Any chronic manipulation in $[Ca^{2+}]_i$ levels by use of the *Kum* line would not only alter the nerve terminal but likely the soma as well which would then likely alter gene regulation (West *et al.* 2001). The actions could be broad in this sense from mechanisms that promote LTP through CRE and CREB, as in the hippocampus of rodents (Lee *et al*. 2006), to inducing cell death through regulated apoptosis (Fernández-Gómez *et al*. 2008). A high Ca^{2+} load in neurons of the CNS, in mammalian models, is one major causes of neuronal tissue damage after reperfusion of occluded blood vessels (Kristian & Siesjo, 1998). So, one would need to be cautious in interpreting chronic manipulations in Ca^{2+} regulatory processes such as with SERCA, NXC or PMCA.

The developmental and acute compensatory actions at the NMJ would be of significant interest if defined gene knockouts or protein suppression by mRNAi can be described by functional consequences. One has to understand the normal function to learn if it is perturbed or to study pathological states. There are a number of neurological disorders associated with impaired function to regulate $[Ca^{2+}]_i$ (Mata & Sepúlveda, 2005).

Future studies that could build in what was reported in this study to help in further understanding of the mechanisms would be to use a Ca^{2+} indicator in larval presynaptic terminals of NMJs. This would allow one to examine for differential regulation in function of the NCX, PMCA and SERCA along a single nerve terminal and among neurons of different synaptic efficacy. To compare the differences along a given terminal in a 3rd instar larvae to various developmental stages could shed some light on to why there is variation in synaptic structure and function over development and how it is maintained. Long term manipulation studies will likely have to be approached with genetic drivers. The consequences on LTD by compromising the NCX, PMCA and/or SERCA in this preparation may also help to determine the mechanistic factors behind LTD. There are many new avenues to proceed from the observations and speculations proposed in this study.

Chapter Five

Roles of the SERCA, PMCA and NCX in Regulation of Heart Rate in *Drosophila* Larvae

INTRODUCTION

Insects like vertebrates use the heart to promote circulation of the hemolymph or blood; however in insects the hemolymph is not necessarily used for gas exchange since tracheal delivery can be directly to target tissue. The mechanisms in function and development of the heart are strikingly similar for insects and vertebrates. For these reasons and because it is relatively easier to work with invertebrate preparations they have become increasing used as model systems for studying cardiac function at a cellular level. *Drosophila melanogaster* is one of the most widely studied invertebrate cardiac systems, at a genetic level for altered function. The *Drosophila* cardiac muscle is similar enough that particular functions are relevant to model cardiac function and disease states in other animals including humans (Bier and Bodmer, 2004). Besides being a strong model for genetic studies on deleterious genes or malformations for development in morphology, *Drosophila* also serve as a good model for physiological function at a cellular level. A number of ion channel mutations discovered in mammals also have homology with ones found in *Drosophila* and the disease states can be mimicked in this tractable genetic organism (Ocorr *et al*. 2007a, b; Dowse *et al*. 1995; Johnson *et al*. 1998; Ganetzky, 2000; Bier and Bodmer, 2004). Little is known about the Ca^{2+} regulation in the *Drosophila* heart as only a few studies have made use of the larval heart in this regard. Here, I have made an attempt to elucidate the role of the different Ca^{2+} ion channels namely the NCX, the PMCA and the SERCA on the larval heart rate (HR).

The majority of studies on *Drosophila* heart have been targeted toward development in the embryo (Azpiazu and Frasch, 1993; Bodmer, 1993, Bodmer *et al*. 1990; Zaffran *et al.* 2006) and physiological function in the pre-pupal, pupal or adult stages (Ashton *et al.* 2001; He and Adler, 2001; Molina and Cripps, 2001; Ponzielli *et al.* 2002; Sláma and Farkaš, 2005; Wessells and Bodmer, 2004). The pupal stage is very dynamic during metamorphosis since hormones and biogenic amines are fluctuating and the heart is undergoing structural transformation. Thus, this stage is hard to control for variables when investigating physiology of the myocytes (Dulcis *et al.* 2005; Johnson *et al.* 2002; Miller, 1997; Papaefthimiou and Theophilidis, 2001). Likewise, since the adult heart is neurogenic, this complicates addressing the

function of the intrinsic cardiac pacemaker and ionic regulation in the intact heart (Dulcis and Levine, 2003, 2005). The heart is known to be myogenic in the larval stage and can readily be removed or left *in situ* while being bathed by a defined physiological saline to limit compounding variables such as hormones or neural modulation (Nichols *et al.* 1999; Dowse *et al.* 1995; Johnson *et al.* 1997; Dasari and Cooper, 2006; Feng *et al.* 2004). The myogenic nature of the larval heart is comparable to the mammalian heart in that there are pacemakers that drive the rest of the heart to pump fluid in a direction to be effective in bathing organs, despite the fact that *Drosophila* has an "open" circulatory system. The chronotropic and inotropic nature of the *Drosophila* larval heart could serve as a rapid means to test fundamental principles and pathological effects for mammalian heart function as well as a helping to develop a comparative model for cellular physiology such as ionic regulation of pacemaker cells.

The *Drosophila* heart, known as the dorsal vessel, is a continuous tube extending from the last abdominal segment to the dorso-anterior region of the cerebral hemisphere. The heart is divided into anterior aorta and posterior heart (Rizki, 1978; Dasari & Cooper, 2006). There are a variety of cell types in the heart and they are differentially regulated (Gajewski *et al.* 2000; Molina & Cripps, 2001; Zaffran *et al.* 2006). The HR varies throughout larval stages depending on whole animal activity (feeding and crawling) and the HR tends to slow down during pupation (Dasari & Cooper, 2004*b*; Sláma & Farkaš, 2005). The larval heart is very susceptible to biogenic amines and peptides which could vary in the hemolymph depending on food source or intrinsic state of the animal (Dasari & Cooper, 2006; Johnson *et al.* 1997; Johnson, 2000; Nichols *et al.* 1999; Zornik *et al.* 1999).

The cellular mechanism of action of the neurotransmitters and cardiac modulators in larva have not been described to date as there has not been a sufficient understanding of the ionic currents and channel types present in the larval heart that contribute and regulate pacemaker activity. In addition, this highly tractable genetic system and potential model for human diseases has not been made use of to study the significance of calcium regulation in myocytes in regards to the combined importance of the Sodium Calcium Exchanger (NCX), the Plasma Membrane Ca^{2+}-ATPase (PMCA) and the Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA). A number of ailments in dysfunction of mammalian atrial pacemaker cells and myocytes in general are known to be related to an altered function in one or more of these calcium regulatory processes (Morgan, 1991; Chien, 1999; Hove-Madsen *et al*., 2004).

The general notion is that the NCX has a low affinity but high capacity for Ca^{2+} turnover, whereas the PMCA has a high affinity but a low capacity. In this regard, the NCX comes into play with large $[Ca^{2+}]_i$ and the PMCA fines tunes $[Ca^{2+}]_i$ (Herchuelz, 2007). As noted in a recent review (Terracciano *et al*. 2007), inhibiting NCX can improve function in mammalian heart failure while other studies demonstrated over expression of NCX decreases the progression of systolic and diastolic contractile dysfunction. This implies that the approach needed to manipulate the NCX function and expression levels by gene therapy for human heart failure requires further investigation. Even the role of the PMCA and density of expression are not completely understood for mammalian cardiomyocytes. The current view is that the PMCA does not have a significant role in the excitation-contraction of cardiomyocytes (Bers *et al.*1996), but likely has an important role in development of the heart (Hammes *et al.* 1998; Cartwright *et al*. 2005). On the other hand, the SERCA in cardiomyocytes is primarily explained by its role within the ionic currents that make up the pacemaker potential and thus pacing of the heart. However, the density of the SERCA is known to regulate the rate of muscle relaxation-contraction by the extant of removing $[Ca^{2+}]_i$ in heart muscle as well as in slowly and rapidly contracting skeletal muscle (Rome *et al.* 1996)

The scheme of ionic currents within a cardiac cycle for a mammalian pacemaker cell (i.e., SA node) is generally described with the background $[Ca^{2+}]_i$ continually increasing and decreasing. Starting in diastolic depolarization with a slow release of Ca^{2+} by ryanodine receptors (RyRs), from the SR, leads to a rise in $[Ca^{2+}]_i$. The SERCA pumps Ca^{2+} back into the SR and the NCX removes $[Ca^{2+}]_i$ in exchange for Na^+ ions across the plasma membrane of the cell. The influx of Na^+ ions can lead to a depolarization of the plasma membrane. Thus, opening low voltage-gated T-type Ca^{2+} channels (V_{Ca}) (Hüser *et al.* 2000) and potentially voltage-gated Na^+ channels. The influx of Ca^{2+} acts on the RyRs to cause the ER to dump Ca^{2+} which results in a calcium induced inhibition of the RyRs. Until the $[Ca^{2+}]_i$ is reduced by the SERCA and NCX, the RyRs stay inhibited but will start leaking Ca^{2+}, as $[Ca^{2+}]_i$ returns to a low level to then repeat the cycle (Subramani & Subbanna, 2006). In the mammalian heart, the pacing sinus node cells do not contain a K^+ current (I_{K1}) which is thought to be one reason the pacing cells do not show a resting membrane potential (Opthof, 2007). However, we assume based on gross anatomy of the heart (Gu & Singh, 1995) the pacing cells in the larval *Drosophila* heart are also contracting myocytes which can generate action potentials and thus, likely have pronounced voltage-gated Na^+ currents. The influx of Na^+ as well as Ca^{2+} must be tightly regulated to prime the cell for the next cycle. In short, as in mammalian hearts (Bers,

2002*b*; Philipson & Nicoll, 2000), the NCX and SERCA in *Drosophila* likely play major roles in the cardiac electrical activity of the pacemaker and are in coordination with each other (Kapur & Banach, 2007; Vinogradova *et al.* 2005; Sanders *et al.* 2006).

The action of the SERCA on HR in larval *Drosophila* has been examined with the use of a heat shock sensitive mutation which results in the function of the protein being compromised (Sanyal *et al*. 2006). This mutation is postulated to result in a reduced amount of calcium being taken up by the sarco-endoplasmic reticulum within the heart to account for the reduced HR (Sanyal *et al.* 2006). However, it is not known what compensatory mechanisms come into action by the NCX and PMCA when the SERCA is compromised resulting in a rise of $[Ca^{2+}]_i$.

Thus, the approach used in this study is to compromise the NCX and the PMCA, while the SERCA is inactive, to observe the interplay of the three main players of intracellular calcium regulation on pacemaker activity and contractility. I also used this fly line developed by Sanyal *et al*. (2005) to determine the role of the SERCA in the maintenance and regulation of *Drosophila* larval heart rate. This line is named *Kum*^{170TS} (now onwards referred to as *Kum*) after an ancient Indian mythological hero known as *Kum*bhakarna who slept for months at a time (Sanyal *et al.* 2005). The larval heart preparation provides an additional model besides the skeletal NMJ to investigate the role of the SERCA, NCX and PMCX on intracellular calcium regulation. By knowing more about the ionic currents and shaping of the action potentials in myocytes in various species, one can hope to get a handle on the known ionic dysfunctions associated to specific genes responsible for various diseases (Harrell *et al.* 2007; Mangoni *et al.* 2006; Wessells & Bodmer, 2004).

METHODS

Staging of the flies

Canton S (CS) flies, a wild type of *Drosophila*, were used for comparison to *Kum* (Sanyal *et al.* 2005). Both strains were maintained on a standard cornmeal-dextrose-agar-yeast medium. The *Kum*^{170TS} strain was provided by Dr. Sanyal (Emory University, School of Medicine). To obtain staged larvae, flies were pulsed to lay eggs for 2 hours and then the eggs were incubated at either 18°C, 21°C or 28°C.

Monitoring heart rate in the intact larva

The movement of the trachea is commonly used to monitor *Drosophila* larval HR because of the clear contrast of the structures (Dasari & Cooper, 2006; White *et al*. 1992). Early 3rd instar larvae were glued ventrally on a glass slip using super-glue in such a way that mouth hooks are free to move. Care was taken not to glue the spiracles so that only ventral aspect within the mid-length was adhered to the glass. Food was placed over their head as to keep the larva busy eating (Dasari & Cooper, 2004*b*). The larvae were glued down and HR measured for 1 min. For heat shock experiments, the larva and glass cover slip were placed in a glass Petri dish that floated on top of water in a temperature controlled water bath.

Monitoring heart rate in the dissected larva

The general dissection technique and HL3 saline content has been previously reported (Dasari & Cooper, 2006, 2007). The HL3 saline was derived from direct measures with ion sensitive electrodes of larval hemolymph. This saline maintains normal function of larval neuromuscular junctions and the CNS (Stewart *et al.* 1994). In brief, the HL3 saline was prepared from component reagents (Sigma) and contained: 1 mM $CaCl_2.2H_2O$, 20 mM $MgCl_2$, 70 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, and 5 mM BES (N,N-bis [2-Hydoxy-ethyl] -2- aminoethane-sulfonic acid). The HL3 was freshly controlled for pH and temperature prior to experimentation, as the pH will drift during storage. In experiments in which the $[Na^+]_o$ was reduced, the HL3 saline was made with TRIZMA base and the pH was adjusted with HCl (1 M) as not to alter the $[Na^+]_o$ by adjusting pH with NaOH.

The modifications to the HL3 saline for particular experiments are described in the Results for those particular experiments. For heat shock experiments, the dissected larvae were pinned out on the dissection dish covered with the HL3 saline and placed on a glass Petri dish that floated on top of water in a temperature controlled water bath. Within the time frame used in these experiments no changes in pH of the HL3 saline were noted during the heat shocks.

Varying external calcium concentration

Varying the $[Ca^{2+}]_o$ to higher levels in order to load the myocytes with Ca^{2+} during electrical depolarization and contraction allowed us to examine how the NCX,

PMCA and SERCA would process the increase $[Ca^{2+}]_o$ load placed on them. The basal HR was monitored in both CS and *Kum* lines while the bathing media was exchanged to increasing $[Ca^{2+}]_o$ (1, 2, 4, and 8 mM). Each concentration was allowed to bathe the preparation for 1 min prior to recording the HR, and exchanging the media for the next higher concentration.

Chemicals

All chemicals were obtained from Sigma chemical company (St. Louis, MO) with the exception of thapsigargin and *KB-R7943* which were obtained from Tocris (Ellisville, MO) and 5,6-Carboxyeosin from Marker Gene Technologies Inc. (Eugene, OR).

Intracellular recordings

Intracellular recordings were performed in cardiomyocytes with a 30-60 MΩ resistance, 3 M KCl-filled microelectrode. The amplitudes of the depolarizing potentials and the resting membrane potential were monitored with a 1 X LU head stage and an Axoclamp 2A amplifier. Various regions of the heart and the aorta were surveyed for electrical events. Electrical signals were recorded on-line to a PowerLab/4s interface (ADInstruments, Australia) at an acquisition rate of 20 kHz. Some preparations were dye filled by pressure injection of Texas Red-dextran 3,000 KD (Molecular Probes; Eugene, OR). Blebbistatin (10 μM; Sigma) was used in some preparations to stop muscle contraction while recording electrical events.

Statistical analysis

Depending on the experimental design either a Student's *t*-test (paired or unpaired) or a two-way ANOVA followed with various *posthoc* analysis were used. Some data is presented as a percent change to compare an experimental manipulation or differences over time as explained within the Results section for each given experiment.

RESULTS

Heart rate recording

The anatomical arrangement of the heart tube in larvae is very conducive for monitoring heart rate within the intact animal since it can readily be observed on the dorsal midline (Figure 5.1 *A*). The exposed preparation is also anatomically advantageous since one can readily expose the heart tube by dissecting from the ventral side of the animal. Exposing the heart is needed in order to be able to examine the effects of pharmacological agents and directly measure electrophysiological properties of myocytes. The internal organs are easily removed in a filleted larva that is pinned so the dorsal side is facing downward (Figure 5.1 *B*). The division between the heart and the aorta can be easily seen by the narrowing of the heart (Figure 1*C*). The lymph glands provide good demarcation of the heart tube for aiding in monitoring heart rate. The heart and aorta are outlined in Figure 1D for clarification.

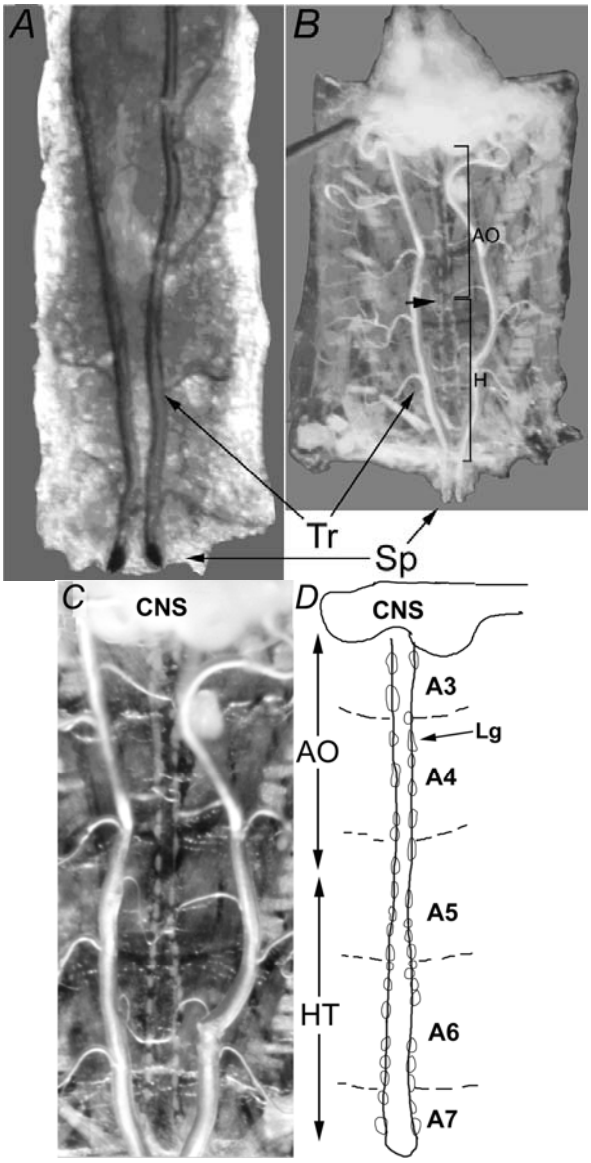

Figure 5.1: *Drosophila* larval heart preparation: (*A*) Dorsal view of an intact 3rd instar *Drosophila* larva. The movement of trachea due to pulling of the attachments from heart is used to observe the heart rate. (*B*) Ventral dissection of a 3rd instar to view the heart directly. Pinning of the animal on its back after dissection is used to directly apply the compounds on the heart with or without the CNS intact. (*C*) Enlarged view of the in situ heart tube and (*D*) a schematic view of the heart and aorta as shown in (*C*) for the larval segments. (Tr, Trachea; Sp, Spiracles; HT, Heart; AO, Aorta; Lg, lymph gland).

The first sets of experiments were to examine if the HR within the intact animal is substantially different in the *Kum* strain as compared to controls (CS strain)

at room temperature and following heat shock (41°C) for 5 min. I also addressed if there is difference in recovery after heat shock by measuring HR again 30 min after the animals are returned to room temperature. In these experiments, individual larvae were monitored so that statistical analysis could be performed considering individual larvae. There is a wider degree of variation of the *Kum* group as compared to the controls after heat shock (Figure 5.2 *A* & *B*) (values are coefficient of variation; heat shock=HS; for CS= 0.19; CS after HS= 0.26; CS 30 min after HS = 0.14; *Kum*= 0.13; *Kum* after HS= 0.53; *Kum* 30 min after HS= 0.4). In comparing the percent difference from the initial value and heat shock for both groups there is a greater decrease for the *Kum* larvae than for controls (Figure 5.2 *C*; $P<0.02$; Student's un-paired *t*-test). There is also a significant decrease in HR in both fly lines after heat shock. (Figure 5.2 *C*, $P<0.001$ for both CS and *Kum*, Student's paired *t*-test)

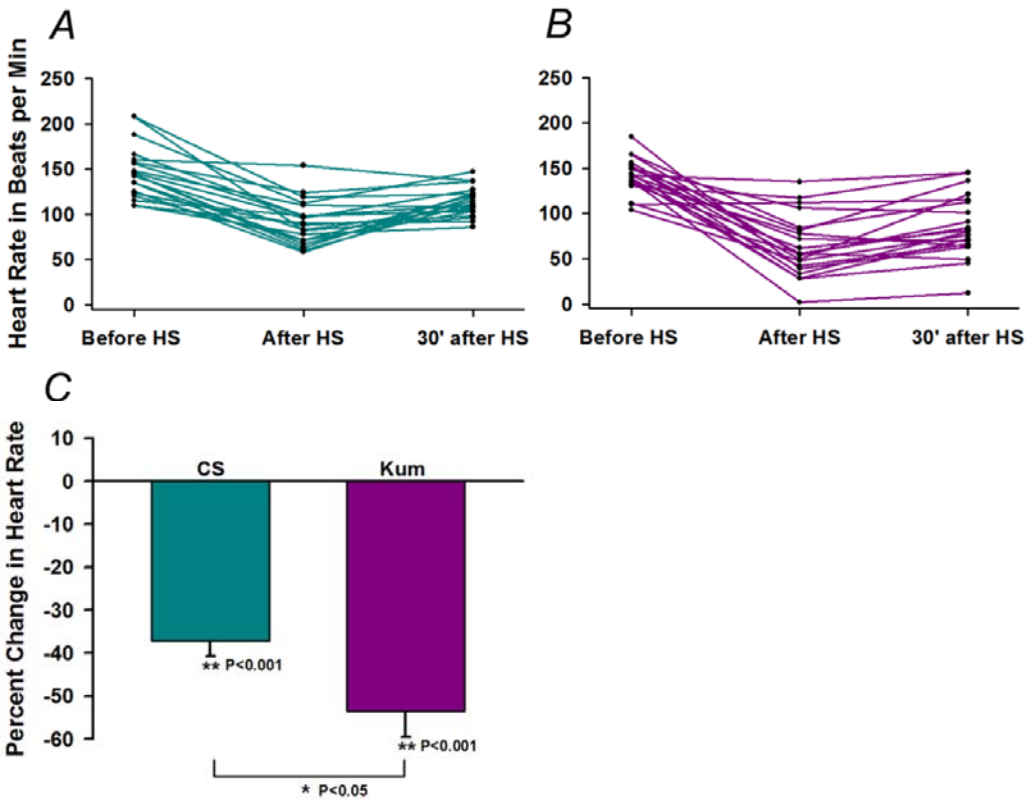

Figure 5.2: Effect of heat shock on HR for intact CS and *Kum* strains grown at 21°C: HR in intact preparations of flies grown at 21°C (n=20 each) before heat shock, immediately after heat shock and 30 min following heat shock (recovery). Blue bars (*A*) indicate CS flies while the purple bars (*B*) indicate *Kum* flies. (*C*) The percent change in HR after heat shock. There is a significant decrease in HR of both CS and

Kum flies after heat shock (*P*<0.001 for both CS and *Kum*, Student's paired *t*-test, n=20 for each set). Moreover the *Kum* has a significantly greater drop in HR than the CS after heat shock (*P*<0.05, Student's unpaired *t*-test)

The whole larvae assay of the *Kum* and controls allowed me to examine the effect of an altered SERCA function on HR, but to address the role of the PMCA and the NCX the heart has to be exposed so that the bathing media can be changed. Since dissection and exposure to the specially designed *Drosophila* physiological saline (e.g. HL3 saline) could have consequences on the initial HR, the rate was measured before and after heat shock as performed for intact larva with the exception that the larvae were dissected and bathed in HL3 saline.

Exposing the *in situ* heart to saline resulted in an increase in the variation of initial HR in both control and *Kum* larvae. In addition, the initial rate tended to be lower than for the intact larvae (Figure 3 *A*, *B*). During heat shock there was little change in the mean HR for the *Kum* strain which is different than for intact animals (Figure 2*B* and Figure 3*B*). There is also a smaller degree in the reduction of HR upon heat shock for the control in these exposed heart preparations as compared intact control larvae (Figure 2*C* and Figure 3*C*). As a result of these changes upon dissection no significant difference between the controls and *Kum* strain with heat shock is noted.

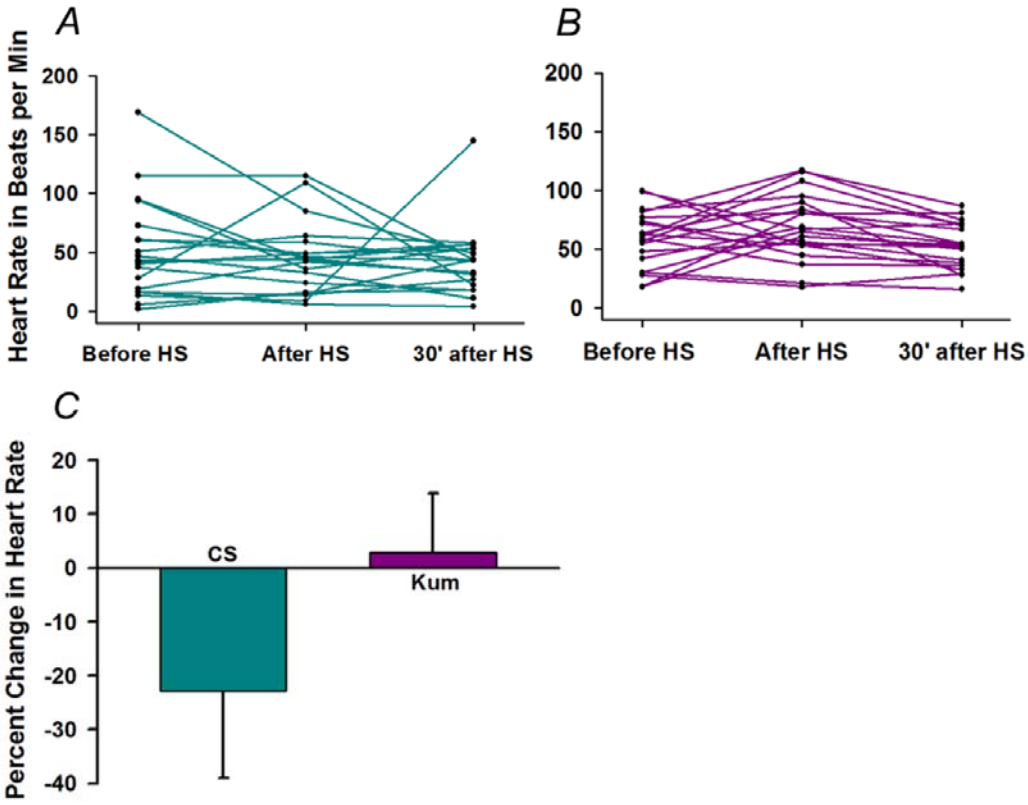

Figure 5.3: Effect of heat shock on HR for dissected CS and *Kum* strains grown at 21°C: HR in dissected preparations of flies grown at 21°C (n=20 each) before heat shock, immediately after heat shock and 30 min following heat shock (recovery). Blue bars (*A*) indicate CS flies while the purple bars (*B*) indicate *Kum* flies. (*C*) The percent change in HR rate after heat shock (not significant; paired and unpaired Student's *t*-tests; n=20 for each CS and *Kum* sets)

Since the *Kum* strain is a heat shock line that reduces the SERCA function, we were concerned that room temperature (21°C) might be a high enough temperature to produce an effect on altered function of the SERCA protein while the larvae were developing. To address this issue, we raised flies for 2 generations at 18°C and the larvae produced were used to examine the effects of heat shock in intact as well as dissected (saline exposed) larva. The percent change to heat shock (41°C) for intact larvae is significantly different between controls and the *Kum* strain (Figure 4.4). But no significant difference is present after HS between larvae grown at 21°C and those grown at 18°C (P=0.55, Student's un-paired *t*-test). However, the dissected preparations, grown at 18°C, also show no significant difference between

the controls and the *Kum* strain. Moreover, the dissected controls and *Kum* larvae do not show any difference before and after HS.

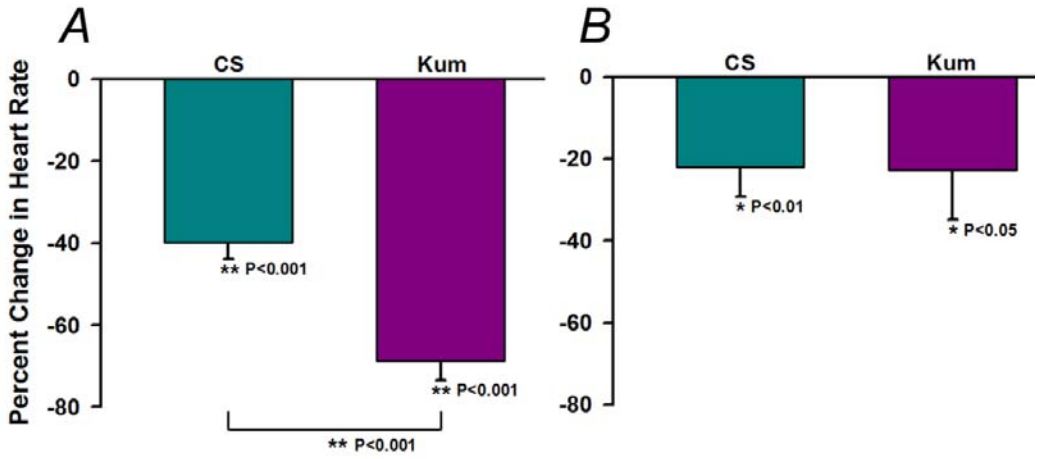

Figure 5.4: Effect of heat shock on HR for CS and *Kum* strains grown at 18°C: Larvae cultured at 18°C to address effects on HR for the temperature sensitive *Kum* and effects of dissection. (*A*,*B*) The percent change in HR after heat shock for flies grown at 18°C: (*A*) intact preparations, (*B*) dissected preparations. A significant decrease in HR is present for CS and *Kum* flies after heat shock in both intact and dissected preparations (Student's paired *t*-test, n=20). A significant decrease is present in the *Kum* as compared to CS after heat shock in the intact preparations (*A*) (*P*<0.001, Student's unpaired *t*-test), where as there is no significant change in *Kum* compared to CS in the dissected preparations.

I also tested the effect of chronic exposure at the permissive temperature to activate the heat shock effect in the SERCA mutation by raising adult flies at 28°C and examining the larvae of their off spring. As in previous experimental paradigms, both intact and dissected preparations were examined for the effect heat shock at 40°C. As shown above, for the flies raised at 21°C and 18°C there is a significant reduction in HR upon exposure to heat shock in both control larvae as well as the *Kum* strain (Figure 5.5 *A*). There is also a significantly greater reduction in HR for the *Kum* as compared to controls (*P*< 0.05, unpaired Student's *t*-test). The dissected preparations again showed a wide variation in HR upon heat shock with no significant difference between the control and *Kum* larvae (Figure 5.5 *B*).

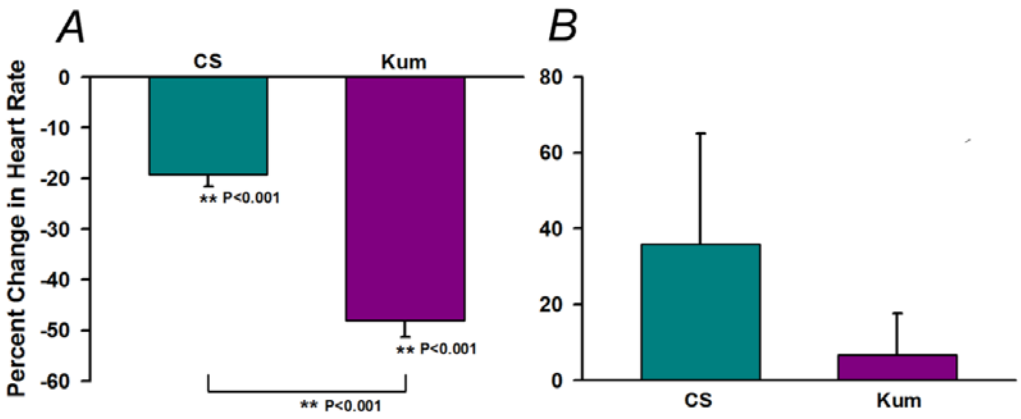

Figure 5.5: Effect of heat shock on HR for CS and *Kum* strains grown at 28°C: (*A*) Intact preparations and (*B*) dissected preparations are compared. There is significant decrease in HR in both CS and *Kum* flies after heat shock in intact preparations ($P<0.001$ for both CS and *Kum*, Student's paired *t*-test, n=20 for both). Moreover there is a significant decrease in the *Kum* as compared to CS after heat shock in the intact preparations (*A*) ($P<0.001$, Student's unpaired *t*-test). The dissected preparations do not show any significant change (*B*).

To address the role of the PMCA on HR, as well as the combination of compromising the PMCA and the SERCA, a series of experiments were conducted. Only dissected preparations are feasible to block the PMCA as the bathing media either requires pharmacological agents introduced or by adjusting the pH to 8.8. For this set of experiments, the larvae were raised at 21°C and dissected open. There is a significant reduction in HR for both controls and *Kum* ($P<0.001$; Student's paired *t*-test) when the bathing saline is changed from pH 7.2 to pH 8.8 (Figure 6; first two bars). Moreover, there is no significant difference between controls and *Kum* at pH 8.8 ($P<0.05$; Student's paired *t*-test). The same preparations were heat shocked at 40°C for 5 min to combine the effects of blocking both the PMCA and the SERCA. There is a significant increase in HR for CS after heat shock as compared to the rate while exposed to pH 8.8. However, the rate was lower than the HR at pH 7.2. So, for the CS flies there is a greater decrease in HR on inhibiting the PMCA but the HS actually helped retard this decrease. In the *Kum* flies, the HR came back to almost a normal rate (No significant change; Student's paired *t*-test) after the combined treatments. Furthermore, there is no significant difference between controls and *Kum* after the combined treatments to reduce the SERCA and PMCA function.

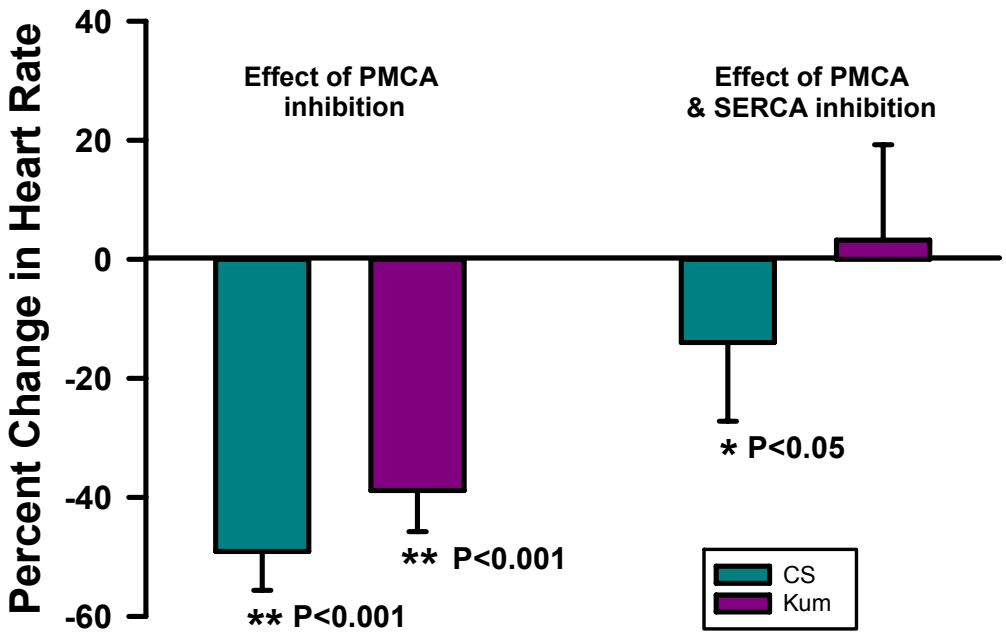

Figure 5.6: Effect of inhibiting the PMCA and the combined effect of inhibiting the PMCA and SERCA on HR: The first pair of bars, on the left, depicts the change in HR for CS (blue) and *Kum* (purple) after blocking the PMCA at pH 8.8. A significant decrease in HR occurs for both CS and *Kum* larvae after inhibiting the PMCA (*P*< 0.001 for both CS and *Kum*, Student's paired *t*-test, n=20). Only in the CS is the effect of reducing both the SERCA and PMCA significant (right set of bars, Student's paired *t*-test,).

Since I addressed the effects of PMCA and the SERCA on HR, I next examined the role of the NCX on HR. To start off, I examined the effect of a compromised NCX by itself before combining a compromised PMCA and the SERCA. The NCX is best compromised by reducing the driving gradient of Na^+ into the cell by lowering the extracellular Na^+ (personal communication Dr. Harold Reuter, Bern Switzerland with my mentor). I examined a reduced $[Na^+]_o$ by $1/3^{rd}$ and $2/3^{rds}$ on HR. The NCX is compromised in its normal function of exchanging Na^+ into the cell for Ca^{2+} out when the driving gradients are optimal. However, when $[Na^+]_o$ is reduced then the gradient is reduced and as a result $[Ca^{2+}]_i$ could increase over time. The effect on HR can be addressed acutely or over longer periods. One issue with reduced $[Na^+]_o$ is that if the level is reduced too drastically, the excitability of the

myocytes might not be pronounced enough to allow the pacemaker potential to still function. The Ca^{2+} enters the myocytes though voltage-gated calcium channels (Ca^{2+}_V). So the cells need to be depolarized enough to activate these channels. Much to my surprise even with 2/3^{rds} reduced $[Na^+]_o$, the heart beats vigorously. There is no significant increase in HR with 1/3rd reduced $[Na^+]_o$; however a 2/3^{rds} reduction in $[Na^+]_o$ induced a significant increase in HR (Figure 5.7, P<0.05, Student's paired *t*-test, n=10). (In a following section, I address the effect of a reduced $[Na^+]_o$ on the resting membrane potential).

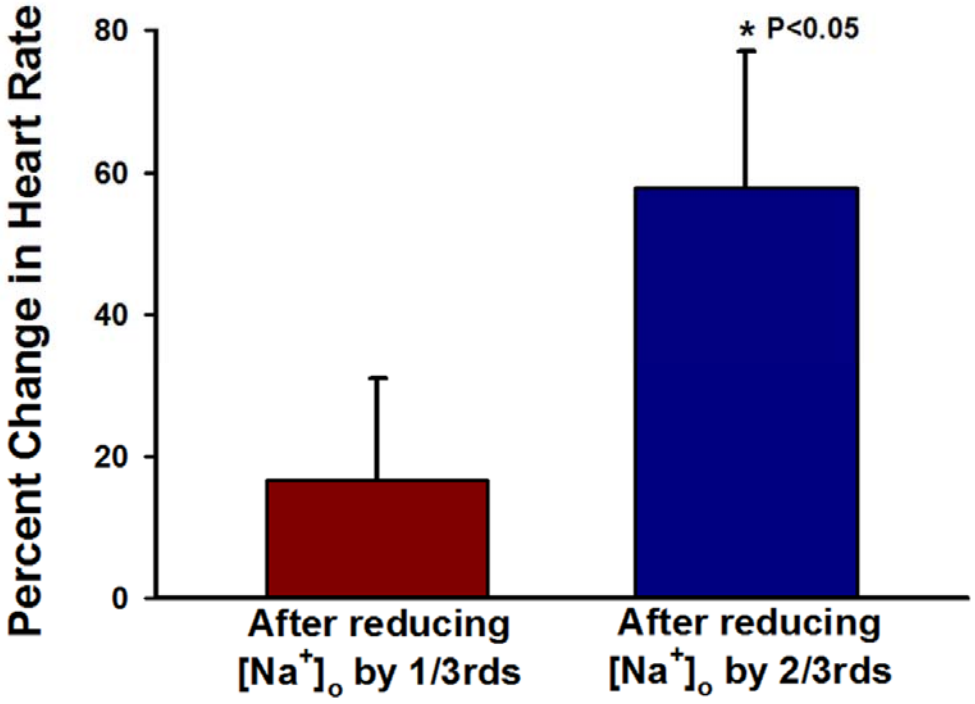

Figure 5.7: Effect of compromising NCX (low $[Na^+]_o$) on HR: Change in heart rate after reducing the $[Na^+]_o$ by 1/3rd and 2/3rds. There is a significant increase in heart rate when $[Na^+]_o$ is reduced by 2/3rd (P<0.05, Student's paired *t*-test, n=10).

To address the combined effect of inhibiting the NCX, PMCA, and SERCA all the three above mentioned effects were combined. In dissected preparations, the NCX was compromised with 2/3rd reduced $[Na^+]_o$ saline. Then saline with 2/3rd reduced Na^+ and pH 8.8 was added to inhibit both the NCX and the PMCA. Lastly, the preparation with inhibited NCX and PMCA was heat shocked for 5 min at 41°C to also inhibit the SERCA (Figure 5.8).

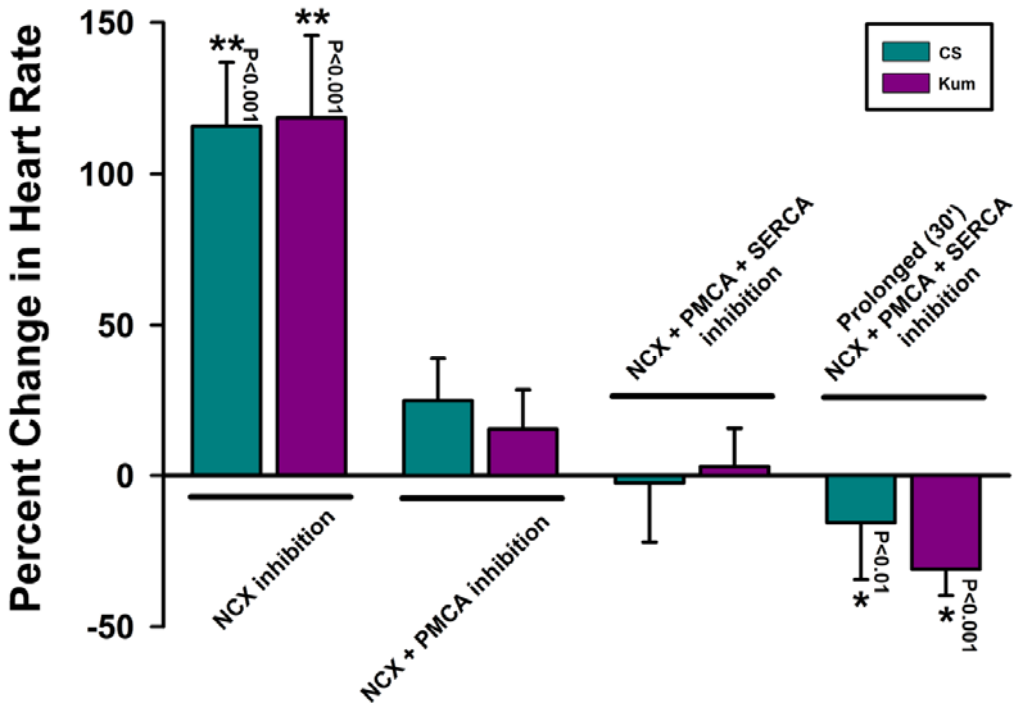

Figure 5.8: Combined effect of inhibiting all three channels: The first pair of bars indicates the percent change in HR of CS and *Kum* on reducing the NCX function and as before there is a significant increase in HR for both. There is no difference between CS and *Kum*. The second set of bars indicates the change in HR when both the NCX and the PMCA are inhibited. Surprisingly CS and *Kum* are not significantly different. The third set of bars indicates the percent change in HR when the NCX, the PMCA and the SERCA are inhibited. There is no significant change in HR between CS and *Kum* and no significant effect as compared to baseline. The last set of bars shows the percent change in HR 30 min after heat shock in saline with low $[Na^+]_o$ and pH 8.8. There is no significant change in HR between CS and *Kum* but there is significant difference as compared to baseline (Student's paired *t*-test for comparing HR before and after treatment and Student's unpaired *t*-test for comparing between CS and *Kum*, *P* values as indicated in graph, n=20 for each set).

Varying external calcium concentration

Prior to examining if altering $[Ca^{2+}]_o$ had an effect on HR, I investigated if HR varied over time when exposed to 1 mM $[Ca^{2+}]_o$ for up to 40 min. Twelve larvae were monitored every 10 min and the distribution in the absolute rates was compared. There were no significant effects among the various time points over the 40 min period (Figure 5.9).

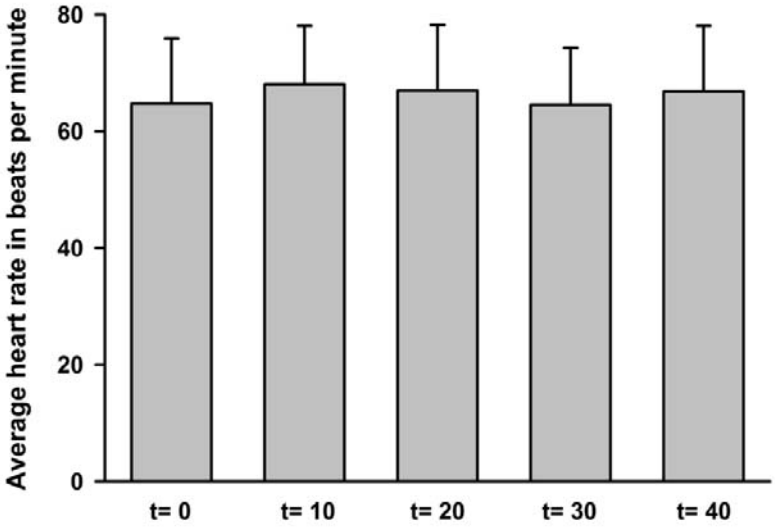

Figure 5.9: HR measured over time: HR in exposed hearts to saline containing 1 mM $[Ca^{2+}]_o$ over a 40 min window. Every 10 min the HR was counted for 2 min and averaged for beats per minute.

Varying the $[Ca^{2+}]_o$ to higher levels in order to load the myocytes with Ca^{2+} during electrical depolarization and contraction allowed me to examine how the NCX, PMCA and SERCA would process the increased $[Ca^{2+}]_o$ load placed on them. The basal HR was monitored in both CS (Figure 5.10 *A*) and *Kum* (Figure 5.10 *B*) lines while the bathing media was exchanged to decreasing as well as increasing $[Ca^{2+}]_o$ (1, 0.5, 2, 4, and 8 mM; with 1 mM being the normal HL3 concentration). Each concentration was allowed to bath the preparation for 2 min prior to exchanging the media to the next concentration. There is a substantial dose-dependent effect of increasing HR with rising $[Ca^{2+}]_o$ (ANOVA $P<0.05$; n=20) except when exposed to 8 mM (Figure 5.10- 12.23 ±13.15 % change for Ca^{2+}). Upon initial exposure to 8 mM the HR increased but subsequently decreased. In some cases, when exposed to 4 or 8 mM the heart would rapidly beat and flutter before slowing down. The HR was

monitored for 2 min and an average beat per minute is reported. The same effect on HR was observed for the various $[Ca^{2+}]_o$ in both CS (Figure 5.10 A_2) and *Kum* (Figure 5.10 B_2).

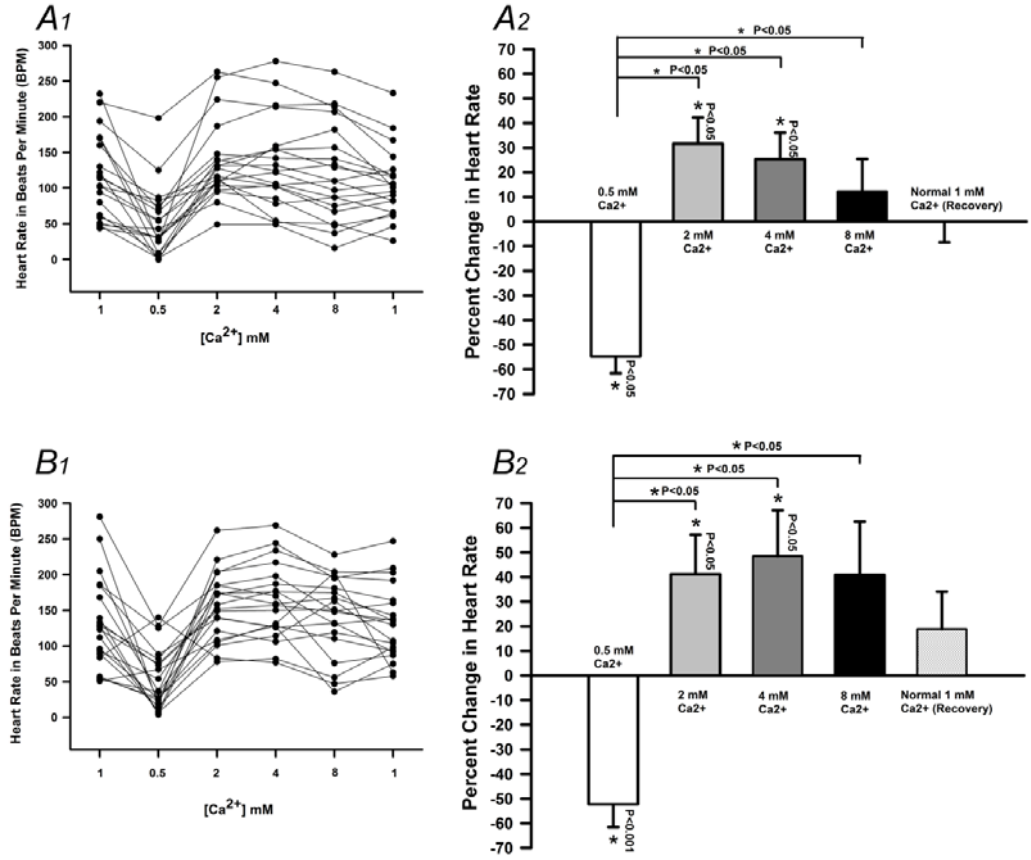

Figure 5.10: The effects of varying $[Ca^{2+}]_o$ on HR in CS (*A*) and *Kum* (*B*): The individual larvae were monitored for the effects of $[Ca^{2+}]_o$ on HR. The left panels show the HR in individuals for each change in $[Ca^{2+}]_o$. The right panels depict the percent change in HR in various $[Ca^{2+}]_o$ as compared to HR when exposed to the initial 1 mM $[Ca^{2+}]_o$. The asterisks/P values on top of the individual bars indicate a significant difference between that particular $[Ca^{2+}]_o$ and normal 1 mM $[Ca^{2+}]_o$. Both the CS (A_2) and *Kum* (B_2) show a significant difference when the $[Ca^{2+}]_o$ is reduced to 0.5 mM as well as when increased to 2 mM and 4 mM (Student's paired *t*-test, *P* values as indicated in graph, n= 20, for each CS and *Kum*). The asterisks/*P* values on lines between bars indicate a significant difference between the different percent changes. As indicated there is a significant difference between the percent change in HR at 0.5 mM $[Ca^{2+}]_o$ and that at 2 mM, 4 mM and 8 mM $[Ca^{2+}]_o$ (One way RM ANOVA with

Tukey's post-hoc analysis, *P* values as indicated in graph). The far right hand bar shows the recovery back to 1 mM $[Ca^{2+}]_o$.

Pharmacological approach to examine NCX, PMCA and SERCA

In order to examine if the *Kum* mutation, with a defective SERCA, was mimicked by a pharmacological approach of blocking the SERCA, I used thapsigargin (TG) at 1 *μ*M and 10 *μ*M with several minutes of incubation. TG is known to block SERCA function at 100 nM to 1 *μ*M range (Rumpal and Lnenicka, 2003). Within the first 2 min there is no significant difference; however, every time measured afterwards for up to 17 min there was a significant reduction for both concentrations of TG (Figure 5.11; *P*<0.05). Further studies were conducted with 10 *μ*M TG for up to 42 min (Figure 5.12). The effect of TG continued to produce a reduction in HR; however, the decrease was not as pronounced over the longer incubation times.

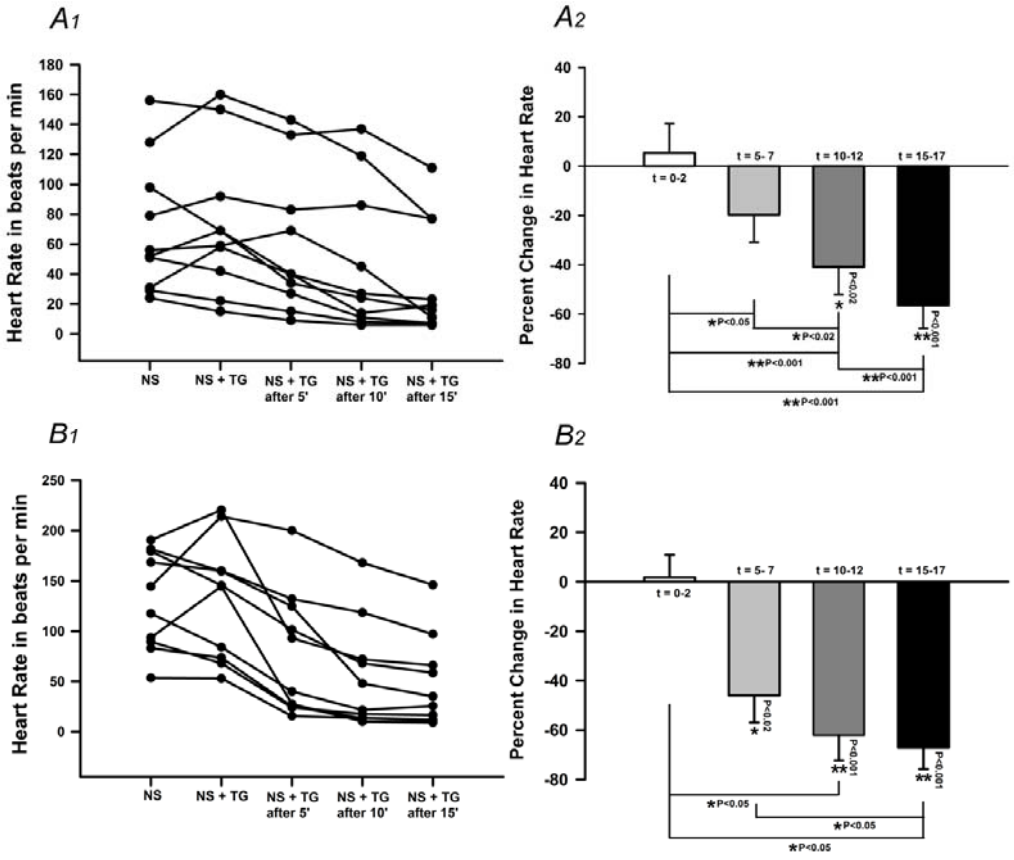

Figure 5.11: Effect of blocking SERCA (with thapsigargin) on HR: The effects of the SERCA blocker thapsigargin (TG) 1 *μ*M (top panel) and 10 *μ*M (bottom panel) on HR

for individual CS larvae over time for up to 17 min. Right panels indicate the percent change in HR before and during exposure to TG over a time periods. The change is compared to the HR prior to exposure of TG. The various time periods in which measures were taken were calculated as an average BPM. The asterisks/*P* values on top of the individual bars indicate a significant difference between HR at that particular time point in presence of TG to normal saline. There is a significant decrease in HR as compared to normal saline after 10 min of adding 1 *μ*M TG and the decrease becomes more prominent with time. With 10 *μ*M TG a significant decrease occurs after 5 min and the HR decreases even further with time (Student's paired *t*-test, n=10). The asterisks/*P* values on lines between bars indicate a significant difference between the different percent changes (*P* values as indicated in graphs are determined by one way repeated measures ANOVA with *Holm-Sidak posthoc* analysis in *B* and *Tukey's posthoc* analysis for *D*; n =10 for both 1 *μ*M and 10 *μ*M TG).

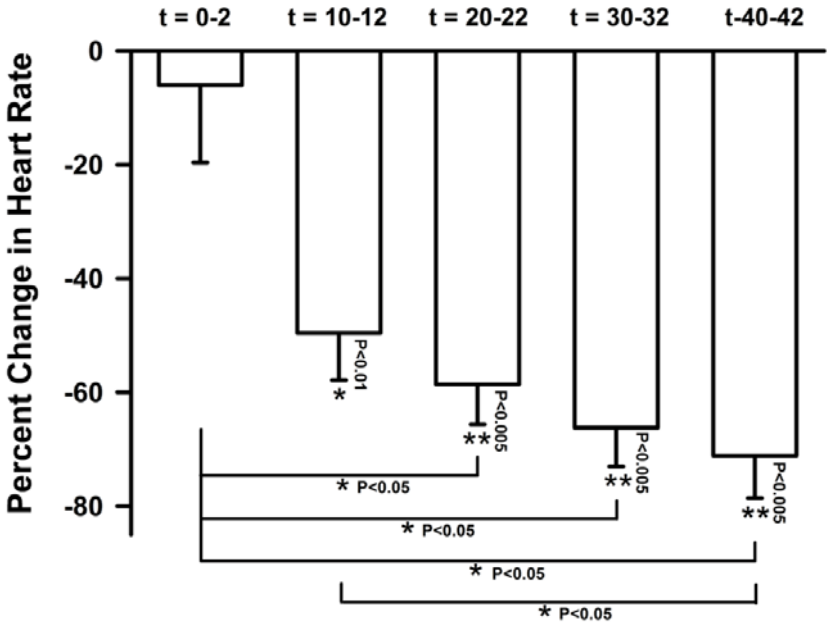

Figure 5.12: Effect of prolonged (42 min) 10 *μ*M TG exposure on HR: The asterisks/*P* values on below the individual bars indicate a significant difference between HR at that particular time point in presence of TG to normal saline. There is a significant decrease in HR after 10 min of adding 10 *μ*M TG and the decrease becomes more prominent with time. (Student's paired *t* test, *P* values as indicated in graph, n=10). The asterisks/ *P* values on lines between bars indicate a significant difference between the different percent changes. (*P* values as indicated in graphs

are determined by one way repeated measures ANOVA with *Tukey's posthoc* analysis; n =10)

I also compared the approach of 2/3rd reduced $[Na^+]_o$ to application of the NCX blocker (*KB-R7943*) at two different concentrations (Figure 5.13; 20 *μ*M-top panel and 40 *μ*M-bottom panel). *KB-R7943* is known to block the efflux but not the influx of Ca^{2+} through the NCX in rat myocytes (5 *μ*mol/L within 15 sec, Satoh *et al*. 2000). In frog oocytes, the expressed *Drosophila* NCX form was inhibited by *KB-R7943* for both inward and outward exchange currents (Omelchenko *et al*. 2003). The difference in the effects between 20 and 40 *μ*m is not significant. However, the low concentration initially caused a slight average decrease in HR after 5 min, where as the higher concentration did not show a significant effect until 10 min later (Figure 5.13, right panels). Since the decrease in the HR continued to occur after 10 min further studies were conducted for up to 40 min of incubation with 40 *μ*M of *KB-R7943* (Figure 5.14).

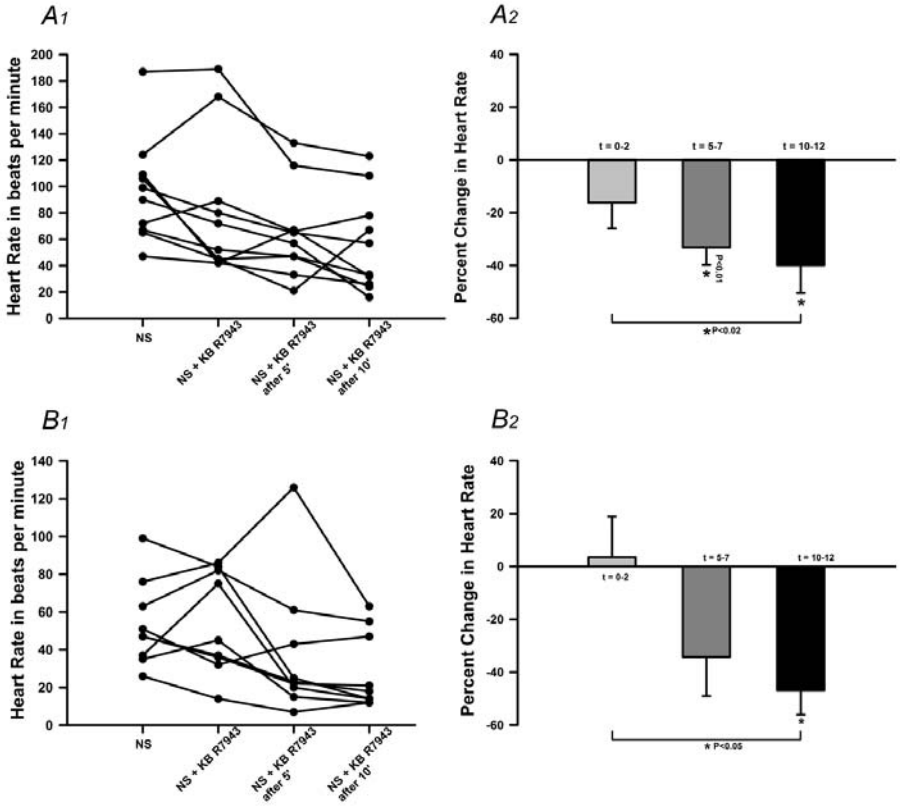

Figure 5.13: Effect of blocking NCX (with *KB-R7943*) on HR: The effects of the NCX blocker *KB-R7943* (20 *μ*M and 40 *μ*M) on HR in for individual CS larvae over time. Top panel is for 20 *μ*M and bottom panel for 40 *μ*M. Right side indicates the percent

change in HR before and during exposure to *KB-R7943* over a time period of 12 min. The change is compared to the HR prior to exposure to *KB-R7943*. The various time periods in which measures were taken were calculated as an average BPM. The asterisks/*P* values on top of the individual bars indicate a significant difference between HR at that particular time point in presence of *KB-R7943*. Student's paired *t*-test, n=10 for 20 *μ*M and n=9 for 40 *μ*M. The asterisks/ *P* values on lines between bars indicate a significant difference between the different percent changes. P values as indicated in graphs are determined by one way repeated measures ANOVA with *Holm-Sidak posthoc* analysis for both 20 *μ*M and 40 *μ*M, n=10 for 20 *μ*M and n=9 for 40 *μ*M.)

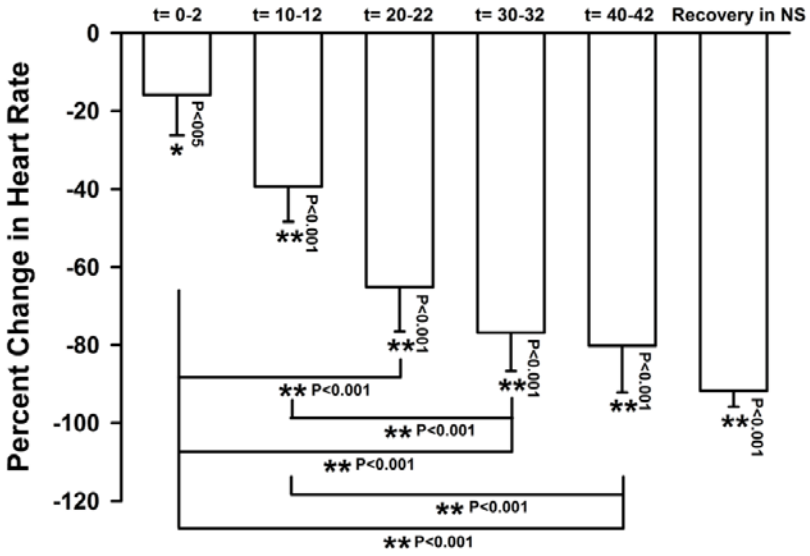

Figure 5.14: Effect of prolonged (42 min) 40 *μ*M *KB-R7943* exposure on HR: The asterisks/*P* values below the individual bars indicate a significant difference between HR at that particular time point in presence of *KB-R7943* and that in normal saline. There is a significant decrease in HR right after adding 40 *μ*M and the decrease becomes more prominent with time. (Student's paired *t* test, *P* values are indicated in graph, n=10). The asterisks/ *P* values on lines between bars indicate a significant difference between the different percent changes as determined by one way repeated measures ANOVA with *Holm Sidak's posthoc* analysis; n =10

To examine the difference of inhibiting the PMCA by pH 8.8 and a pharmacological approach I used CE. It was shown that 5 *μ*M is sufficient to inhibit PMCA in rats and in guinea pigs (Mackienwicz & Lewartowski, 2006). Therefore, I

used this concentration and incubated the preparation for 40 min. I observed a significant decrease in HR after 10 min (Figure 15). The decrease continued to be more pronounced the longer the preparation is incubated. Exchanging the bathing medium back to saline without CE did not produce a reversal of the effect within a 10 min window.

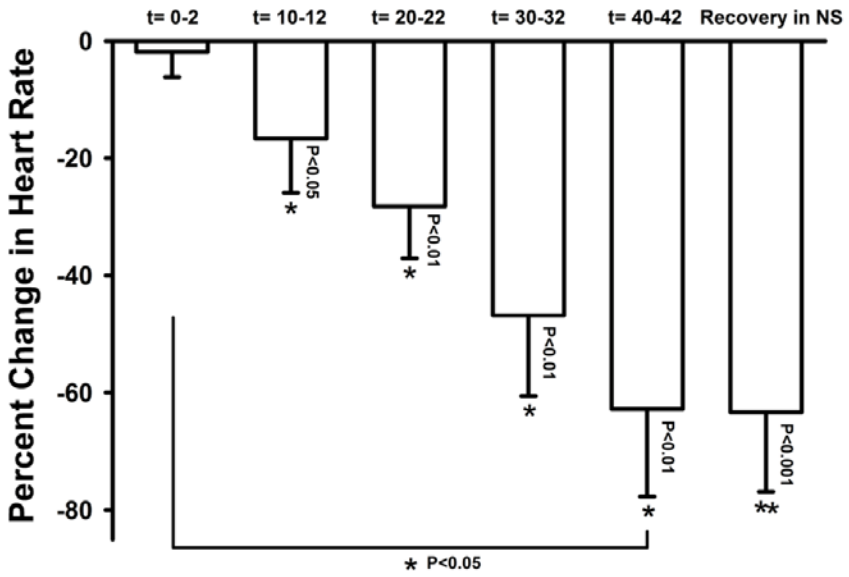

Figure 5.15: Effect of prolonged (42 min) 5 *μ*M CE exposure on HR: The asterisks/*P* values below the individual bars indicate a significant difference in HR before and after a particular time point in the presence of CE. There is a significant decrease in HR as compared to normal after 10 min of adding 5 *μ*M CE and the decrease becomes more prominent with time (Student's paired *t*-test, *P* values as indicated in graph, n=10). The asterisks/*P* values on lines between bars indicate a significant difference between the different percent changes as determined by one way repeated measures ANOVA with *Tukey's posthoc* analysis; n =10.

Comparing the effects of pharmacological agents, ionic treatments and a mutational approach to the altered function of calcium regulation on HR is shown in Figure 5.16. The effect of inhibiting PMCA by pH 8.8 treatments and exposure to CE produced the same effect in reducing HR for CS larva. Both treatments are significant in reducing HR. The change in HR in normal $[Na^+]_o$ and with 2/3 reduced $[Na^+]_o$ is compared to the change induced by normal $[Na^+]_o$ and treatment with *KB-R7943* (40 *μ*M). Both conditions produced an increase in HR. Lastly, comparing the change in HR for *Kum* with heat shock and CS treated with TG only, for 1 mM $[Ca^{2+}]_o$

in the bathing media, there was no difference in the treatment groups between each other and the treatment itself.

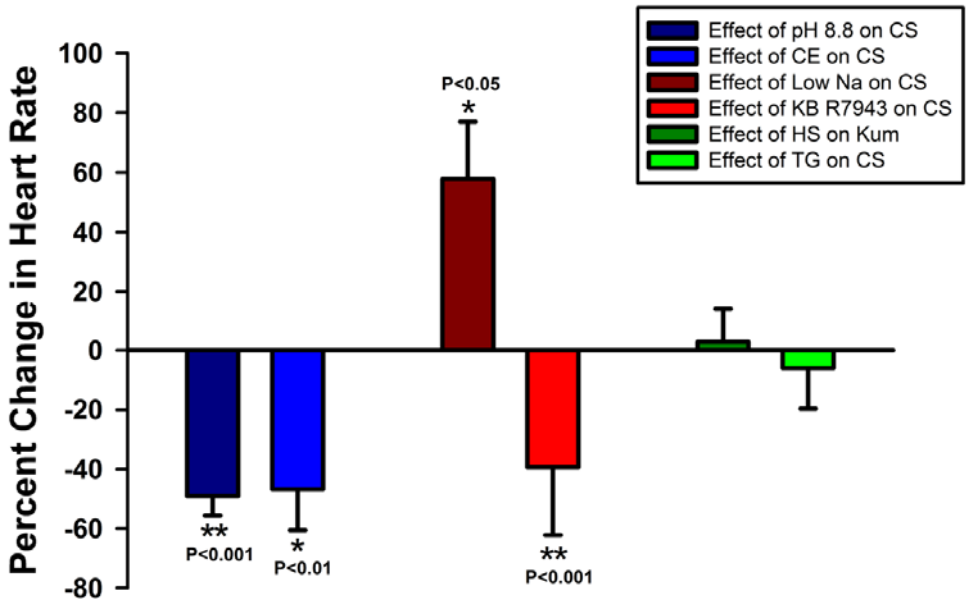

Figure 5.16: Comparing pharmacological inhibition of channels with other methods of inhibition: The blue bars show the two different methods used for inhibiting the PMCA. The dark blue bar shows the percent change in HR from normal after inhibiting the PMCA at pH 8.8 and the lighter blue bar shows the percent change in HR from normal after inhibition of PMCA using 5 μM CE. There is no significant difference between the changes in HR by these two methods. (Student's unpaired *t*-test, n=20 for inhibition with pH 8.8 and n=10 for inhibition with CE, *P* values>0.05 therefore not significant). The red bars show the two different methods used for inhibiting the NCX. The dark red bar shows the percent change in HR from normal after compromising the NCX at low $[Na^+]_o$ and the lighter red bar shows the percent change in HR from normal after inhibition of NCX using 40 μM *KB-R7943*. There is no significant difference between the changes in HR by these two methods. (Student's unpaired *t*-test, n=10 for both, *P* values>0.05 not significant). The green bars show the two different methods used for inhibiting the SERCA. The dark green bar shows the percent change in HR from normal after inhibiting the PMCA in *Kum* flies by HS and the light green bar shows the percent change in HR from normal after inhibiting the SERCA using 10 μM TG. There is no difference between the change in HR by these two methods (Student's unpaired *t*-test, n=20 for SERCA inhibition in *Kum* using HS and n=10 for inhibition with TG, *P* values>0.05 not significant). The

asterisks/*P* values by the individual bars indicate a significant difference between HR with that particular effect to HR in normal saline.

Mapping of the heart for electrical responses

My intentions were to also examine the influence of the PMCA, NCX and SERCA on the shape of the action potential in pacemaker myocytes. In addressing this task, I first mapped the heart myocytes for regions that produced pacemaker potentials in the heart tube. As far as I am aware surveying the heart has not been tackled before in a contracting *Drosophila* larval heart. Lalevée *et al.* (2006) did examine the action potentials produced in the caudal heart using Schneider's insect culturing medium. This medium is known to damage skeletal muscle and produce abnormal shape excitatory postsynaptic potentials. In my study, I used the defined physiological saline HL3 which is known to match the larval hemolymph in ionic composition (Stewart *et al*. 1994). In order to obtain an understanding in the variation of electrical potentials generated and conducted along the heart several larval hearts were mapped for electrical responses during rhythmic responses. As shown in Figure 5.17, for 3 larval hearts there is substantial variation in the amplitude and shapes of the action potentials. From this survey of potentials, it is apparent that the large spiking action potential occurs in the most caudal region of the heart tube and the smaller potentials arise from both the caudal and distal regions of the heart. I also noted only smaller potentials occurred in the aorta (data not shown).

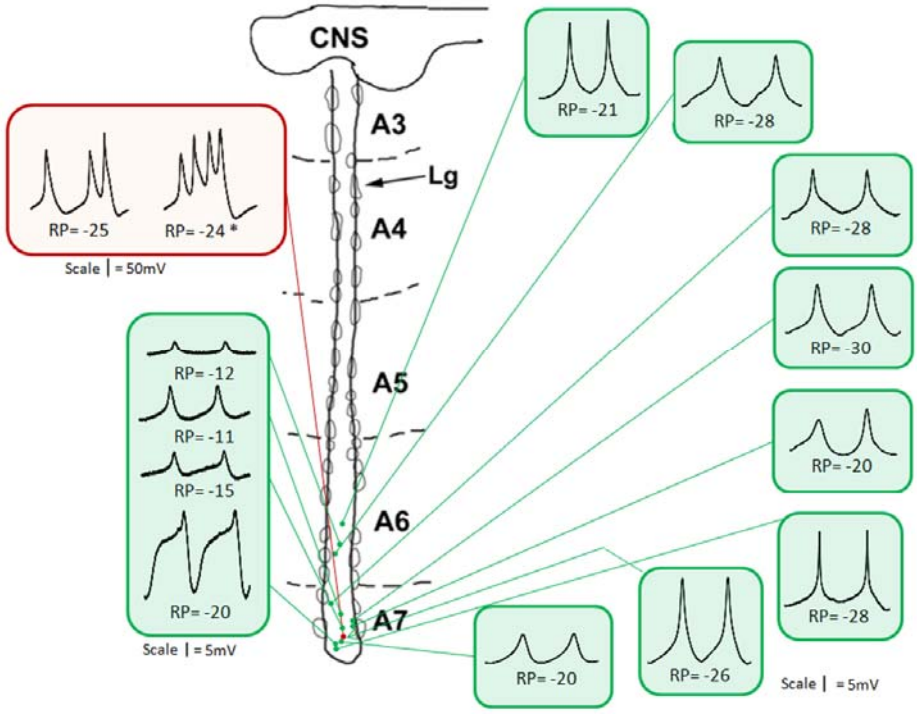

Figure 5.17: Intracellular recordings from *Drosophila* larval heart: Traces show intracellular recordings (action potentials) from *Drosophila* larval heart with the membrane potentials noted below them. The lines point to the region of the heart from where the recordings were obtained. Each individual box indicates recordings from a different larva. All the recordings are in normal saline except the one marked with asterisk which is in presence of blebbistatin containing normal saline to uncouple excitation and contraction. It is evident that different regions in the caudal heart show action potentials of different shapes as well as amplitudes and have different membrane potentials. (Amplitude scales indicated for each preparation in figure).

The resting membrane potentials were consistently around -20 mV for most recordings. The resting membrane potential from various regions of the heart was taken while the cells were not contracting. One problem with recording during the contractions is that the cells become damaged and they lose their resting membrane potential rather quickly. The composite map (Figure 5.17) shows the range of resting membrane potentials that were deemed to be reliable in none contracting myocytes or from initial recordings prior to the cell starting to contract.

In about 50% of the preparations immediately after impaling the myocyte with the microelectrode the cell will stop pacing. In order to help increase the cells to

maintain pacing I examined the effect of switching the bathing media from 1 to 8 mM $[Ca^{2+}]_o$ (Figure 5.18). The action potentials did not show a consistent trend in the change in the amplitude with the switch to the 8 mM. The shape of the action potentials was compared by normalizing the peak amplitudes. No consistent trend in altering the width or rise time is observed by switching to a higher $[Ca^{2+}]_o$ bathing medium (Figure 5.19). The slow gradual depolarization (#1 on the traces), the rapid rise time (2 to 3), the amplitude (3 before amplitude normalization), the decay time (3 to 4), and after polarization time (5) did vary among the preparations. However, in some preparations the general normalized shape did not vary from 1 to 8 mM $[Ca^{2+}]_o$ despite differences in their amplitude (see preparations 3 and 4 in Figures 5.18 and 5.19).

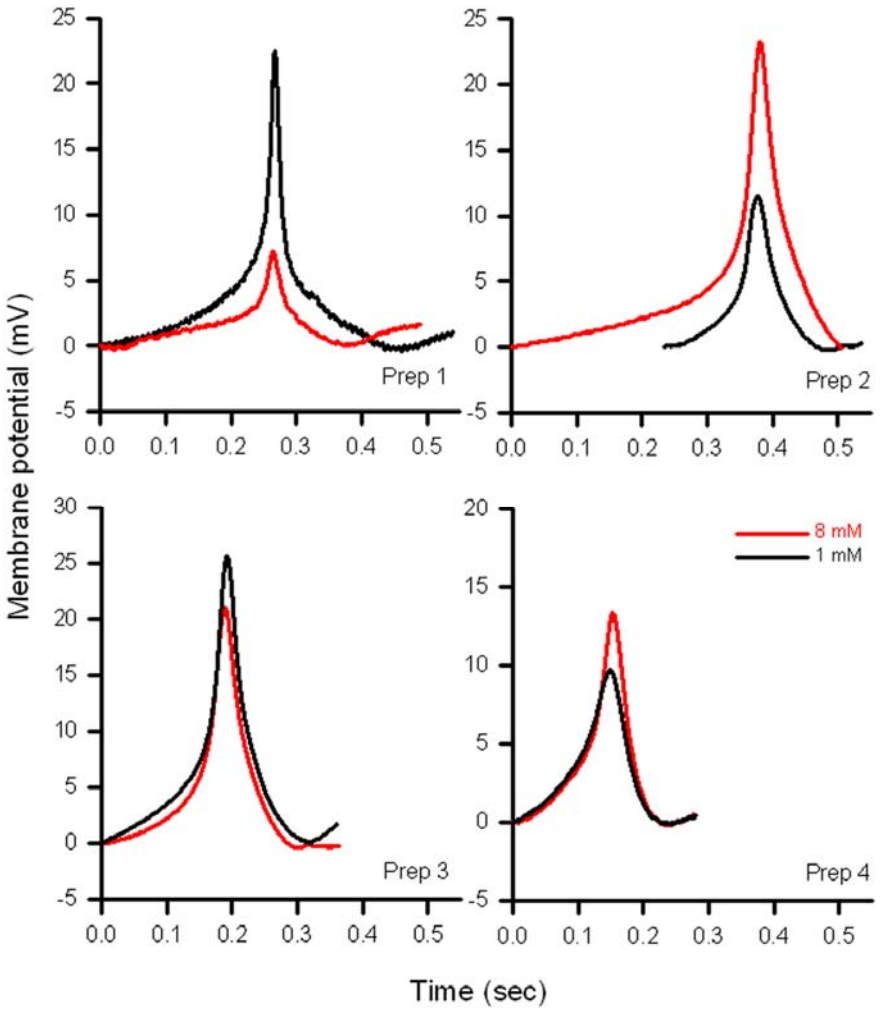

Figure 5.18: Heart intracellular recordings in normal and high $[Ca^{2+}]_o$: Four representative recordings of action potentials recorded in four different larval hearts in the caudal region of the heart. The initial recordings were made in 1 mM $[Ca^{2+}]_o$

(black line) for a few minutes prior to switching to 8 mM $[Ca^{2+}]_o$ (red line). There is not a consistent trend in the action potential amplitude with an increase in $[Ca^{2+}]_o$. Also the initial action potential amplitude varies within and among preparations. The resting membrane potential also varied among preparations. Each is adjusted to start at 0 mV for comparisons in the magnitude of the action potential. For these recordings the resting membrane potentials were: preparation 1 -27 mV; preparation 2 -30 mV; preparation 3 -26 mV; preparation 4 -28 mV.

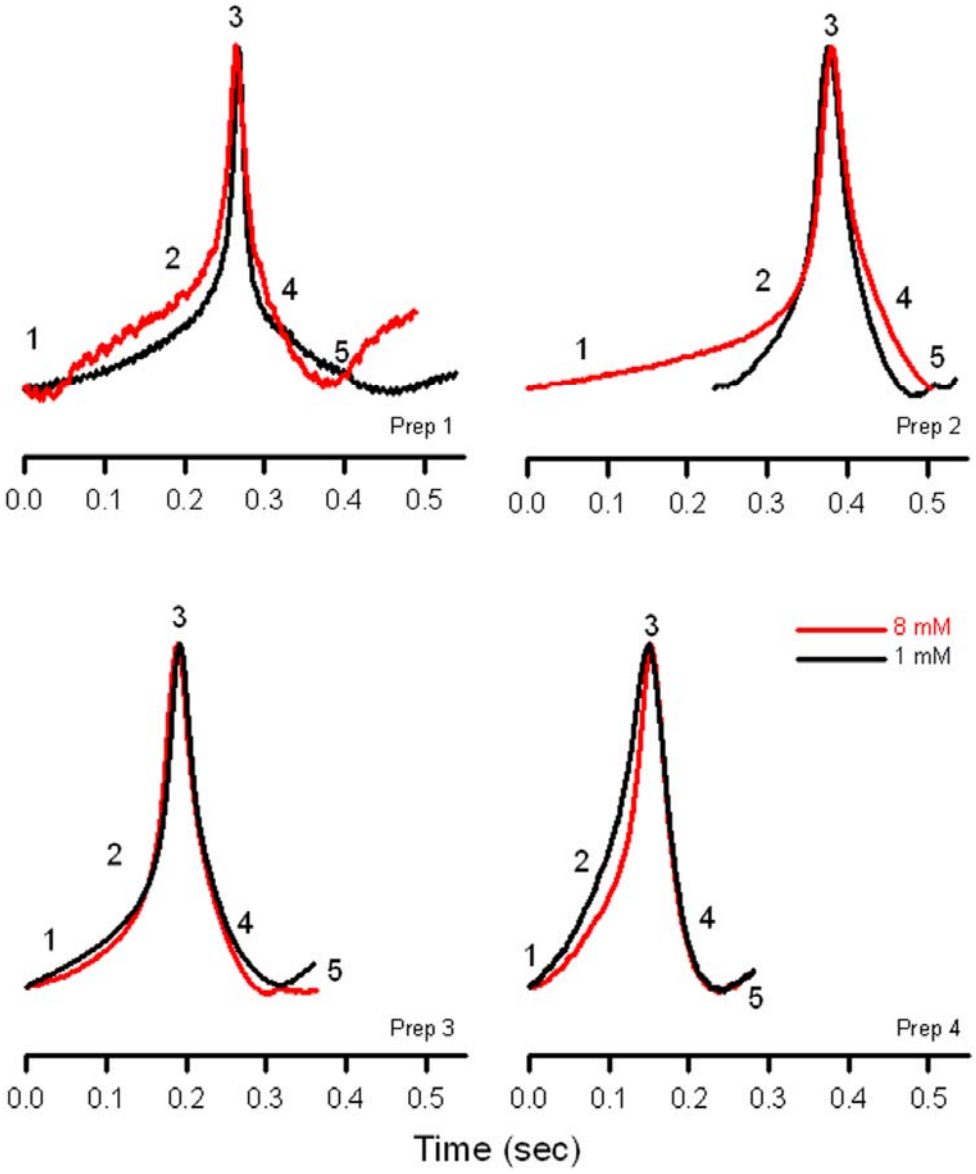

Figure 5.19: Normalized traces shown in Figure 5.18: The same four representative recordings of action potentials shown in Figure 5.18; however, the amplitudes are normalized for comparisons in the characteristic shapes of the action potential with

varying $[Ca^{2+}]_o$. The initial recording is made in 1 mM $[Ca^{2+}]_o$ (black line) for a few minutes prior to switching to 8 mM $[Ca^{2+}]_o$ (red line). There is not a consistent trend in the change in shape of the action potential amplitude with an increase in $[Ca^{2+}]_o$. In preparation 2, the increase in $[Ca^{2+}]_o$ resulted in a prolonged rise time from rest (1) to the threshold (2) but in preparation 1 the rise time is slower. From threshold (2) to the peak amplitude (3) the rise times are very similar as well as the decay time from the peak (3) to the repolarization (4). There is no consistent trend for the effect of a exposure to a higher $[Ca^{2+}]_o$. The duration of the action potential in all recordings is similar (100 msec). The time from repolarization (4) to initiation of the next action potential (5) depended on the heart rate.

Changes in the interpulse interval in mammalian pacemaker potentials are indicative of alterations in the ionic currents that impact chronotropy. One postulation is that activation of the voltage-dependent T-type Ca^{2+} current in neonatal ventricular myocytes is what stimulates the sacroplasmin to release Ca^{2+} during the diastolic phase. However, the voltage-dependent T-type Ca^{2+} current appears to be reduced in adult myocytes so the higher threshold L-type Ca^{2+} channels have a larger role for pacing (Bers, 2002*a*; Lipsius *et al*. 2001). I have not distinguished which types of voltage-gated calcium channels could be present in the *Drosophila* larval heart. So, if $[Ca^{2+}]_o$ is raised then Ca^{2+} current (I_{Ca}) would be enhanced and the diastolic phase would shorten. I examined if this occurred in the myocytes but did not obtain a consistent trend due to changing $[Ca^{2+}]_o$ from 1 to 8 mM (Figure 5.20). Preparation 2 shown in Figure 19 produced a nice slow depolarization with a clear threshold for initiating an action potential even in 8 mM $[Ca^{2+}]_o$. Preparation 1 showed an increase in heart rate and preparation 2 a decrease in rate with higher $[Ca^{2+}]_o$. Whereas preparations 3 and 4 showed no significant change with altered $[Ca^{2+}]_o$. It should be noted that the membrane potential shapes may also contain artifactual deformation in these recordings as the heart is pulsating which causes movements of the tip of the intracellular electrode.

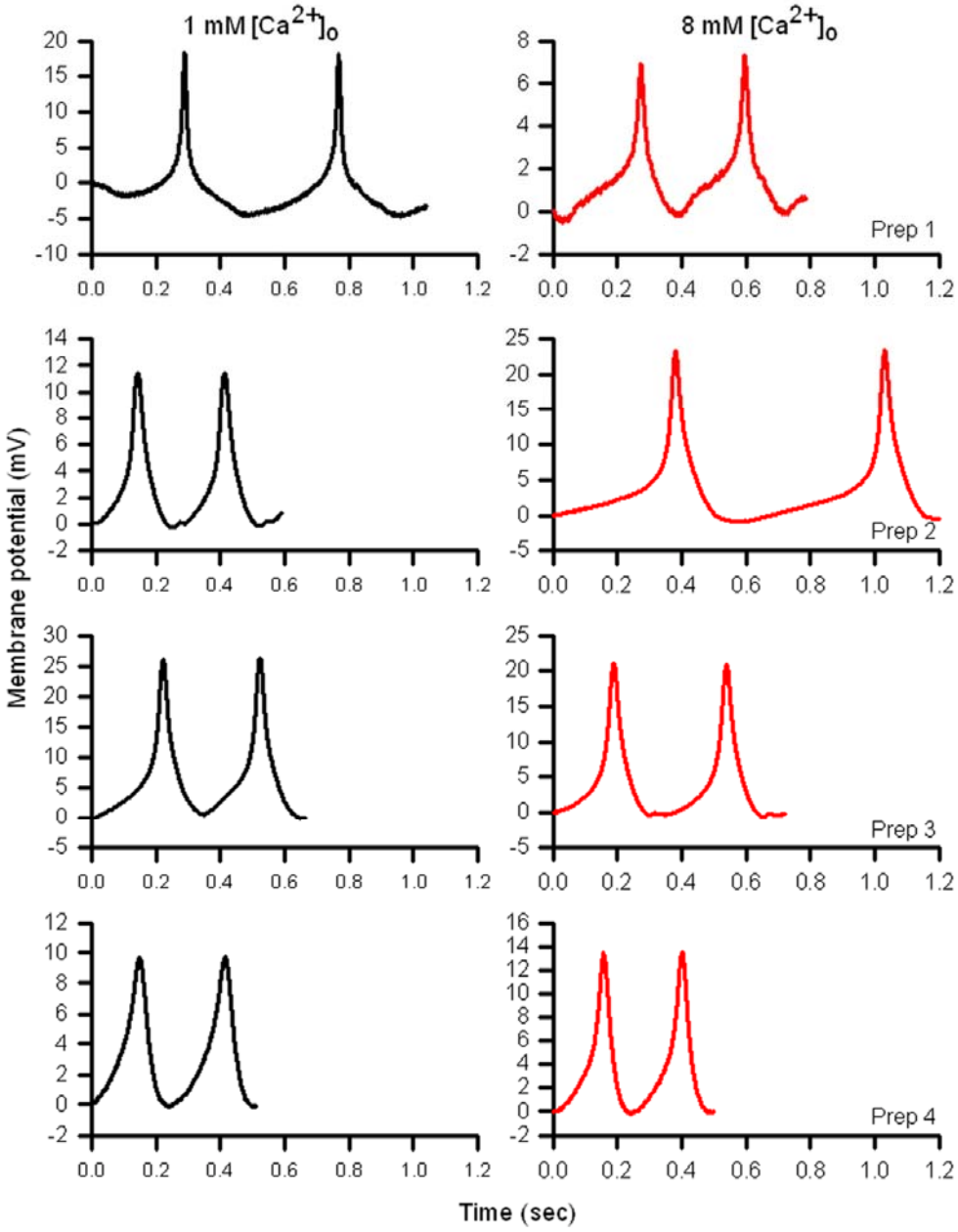

Figure 5.20: Same traces with similar time scales: The same four representative recordings of action potentials shown in Figures 5.18 and 5.19; however, plotted at the same time scale with different scales for membrane potential as to enhance comparisons in intrapulse potentials depending on heart rate. The heart rate with varying $[Ca^{2+}]_o$ is noted not to produce consistent results while maintaining a intracellular recording. The initial recording is made in 1 mM $[Ca^{2+}]_o$ (black line) for a few minutes prior to switching to 8 mM $[Ca^{2+}]_o$ (red line). There is not a consistent trend in the shape of the interpulse potential depending on heart rate nor in altered $[Ca^{2+}]_o$.

To determine if the NXC, PMCA and the SERCA had a role in shaping the action potential, the muscle contractions needed to be blocked while recording from pacing myocytes. Since it appears that waves of contractions start in the caudal end of the heart for intact larvae (non-dissected) these caudal cells were targeted to record from; however I recorded this time after obtaining a response in blebbistatin incubated preparations. Blebbistatin uncouples the myosin and actin in the contractile unit. The resting membrane potential before and after application of blebbistatin did not change.

In the next series of experiments, the dissected preparations were first exposed to normal HL3 saline containing blebbistatin (note: the buffer was TRIS-base to avoid adding NaOH to obtain a pH of 8.8 when needed). Only CS preparations were used in these experiments. As shown in Figure 16 (top left panel) the potentials are similar in threshold and amplitude before and after blebbistatin. In order to see of the cell electrical pacing would mimic the earlier effects observed by counting the contractions, I repeated the ionic manipulations while measuring the electrical events with intracellular recordings (Figure 20). After the heart stopped beating an electrically pacing cell was obtained in the most caudal region of the heart in HL3 saline. Then the bathing solution was exchanged to one with 2/3rd reduced $[Na^+]_o$. Some of the preparations showed an increase while others showed a decrease in amplitude of the action potential. In addition, all preparations shortened the interpulse interval (diastolic time) (P<0.05, Wilcoxon rank-sum). After switching the media to pH 8.8 and 2/3rd reduced $[Na^+]_o$ (to block PMCA and reduce the NCX function) the interpulse interval (diastolic time) increased as compared to just reduced $[Na^+]_o$ (P<0.05, Wilcoxon rank-sum), but there is no consistent trend in an alteration in the amplitude or shape of the action potential (Figure 20).

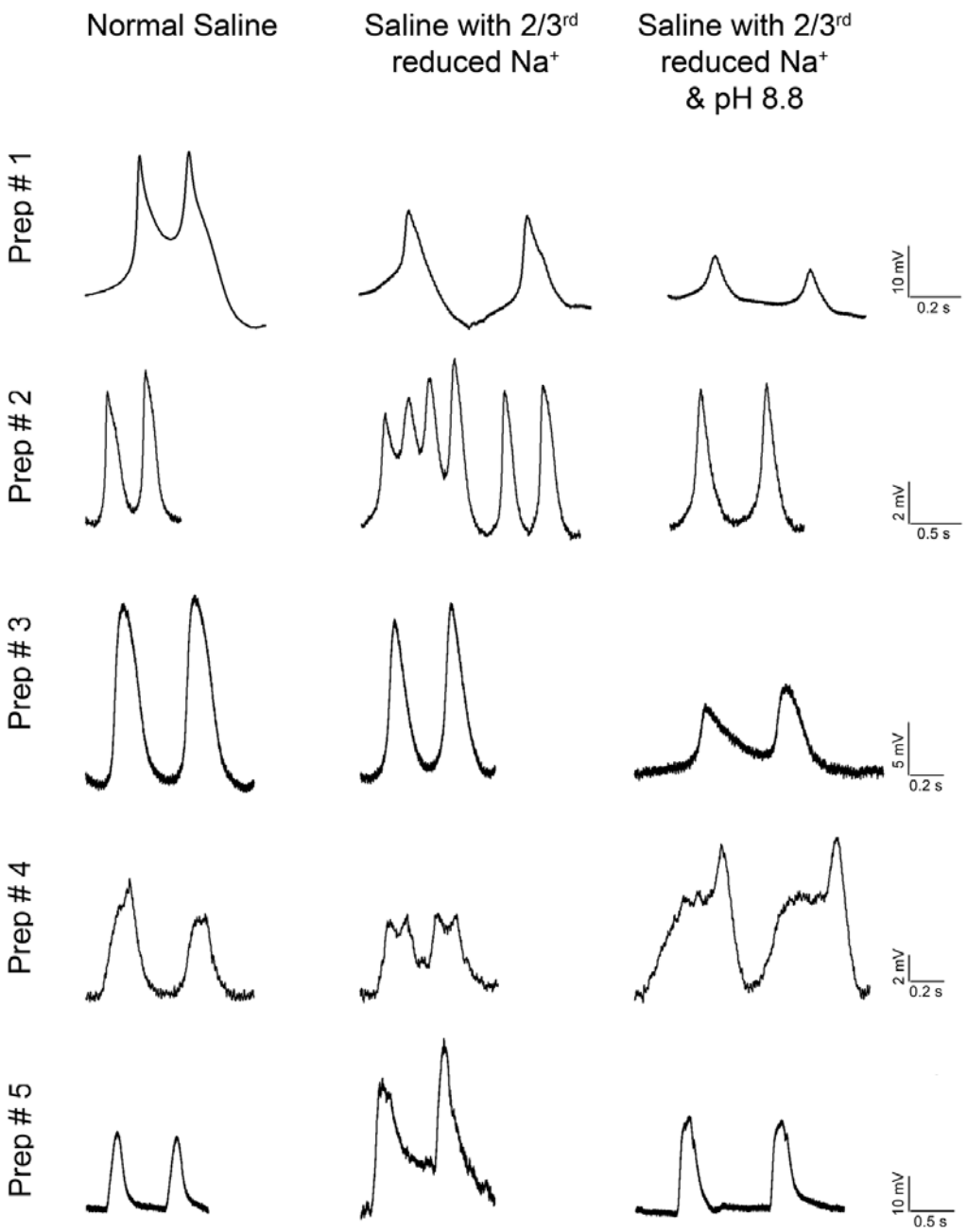

Figure 5.21: Heart intracellular recordings in normal and high $[Na^+]_o$: Intracellular recordings in 5 different CS *Drosophila* larval hearts. The recordings were initially carried out in normal HL3 saline (left panels) then switched to a saline containing 2/3rd reduced Na^+ (to compromise the NCX; middle panels). Thereafter the saline was exchanged with one at pH 8.8 and 2/3rd reduced Na^+ (to compromise the NCX as well as inhibit the PMCA; right panels). The time scale and amplitudes are consistent for each row (each preparation; scale bars shown on right for each row).

DISCUSSION

In this study, I have shown that compromising the SERCA in intact *Drosophila* larvae by use of a heat sensitive mutant strain (*Kum*^{170} or *Kum*) that HR slows down more than for control larvae at 21°C. In order to expose the heart directly to pharmacological agents, without needing to worry about endogenously released compounds within the intact larva, dissected preparations were used. However, the HL3 bathing media does not appear to be optimal for larval heart, as it is for maintenance of larval skeletal NMJ, since there is substantial variation among dissected preparations as compared to intact larva. The approach of heat shocking dissected preparations also did not mimic the result for intact larva. In examining, the potential long term effects of a dysfunctional SERCA on HR, larvae raised at 18, 21 and 28°C were examining as intact and dissected preparations. The intact preparations of larvae raised at the three temperatures all showed a greater reduction for the *Kum* line as compared to controls. Due to the large variations observed in dissected preparations no conclusive findings were apparent for the *Kum* as compared to controls upon heat shocking to compromise the SERCA. In compromising the PMCA, by exposure to pH 8.8, there is a significant reduction in HR for both the control and *Kum* lines without any difference among controls and *Kum* strains before or after heat shock. Thus, inhibiting the PMCA results in a decrease in HR, where as the SERA, in dissected preparations, does not appear to have a substantial effect. In reducing the function of the NCX to an efflux of $[Ca^{2+}]_i$ for an influx of $[Na^+]_o$, both controls and *Kum* strains had an increased HR with reduced $[Na^+]_o$ by 1/3 and 2/3; however, upon heat shocking controls and *Kum* lines no additional effects were apparent. From these results, one would imply that both the NCX and PMCA are important in regulating HR, where as the SERCA does not have as pronounced role. However, in the intact preparations the loss of the SERCA function does have an impact on HR, which might be accounted for by other modulatory roles of contents in the hemolymph for intact animals. In examining a range of $[Ca^{2+}]_o$ on HR there is an increase in HR as extracellular Ca^{2+} rises up to 8 mM. Pharmacological approaches to alter the PMCA, SERCA and NCX paralleled the results obtained by ionic and mutational approaches. This suggests that the effects or lack of effects, by one or the other approach is real in the dissected preparations. Mapping electrical events of the larval heart revealed that myocytes in the caudal region have both large and small amplitude action potentials. The myocytes proved to be difficult to hold intracellular recordings for prolong times due to the movements; however, stopping contractions by the use of blebbstatin revealed

that the myocytes continued to pace in the absence of contraction and various ionic manipulations are possible.

The surprising results in this study are that reduction in the PMCA (by pH 8.8) and the SERCA (by the *Kum* mutation) in dissected preparations did not result in an increase in HR and stronger contractions but a reduction in HR. These calcium channels are meant to lower $[Ca^{2+}]_i$, when they are not fully operational, one would assume $[Ca^{2+}]_i$ to increase even in the acute time periods used in this study. Possibly the dynamics of $[Ca^{2+}]_i$ has some indirect effects on rate via an influence on the NCX or coordination of the various underlying ionic currents to cause the rate to slow down. As expected, a compromised NCX (by low $[Na^+]_o$) caused an increase in HR. However, if the PMCA and/or the SERCA is subsequently inhibited this increase is drastically reduced. The reduction is not as great when PMCA or SERCA are inhibited individually. Thus, the rise in HR when NCX is reduced attenuates the decrease caused by inhibiting the SERCA and PMCA. This raises the question how selective raising pH to 8.8 targets the PMCA and not other cellular functions. Also, heat shock itself reduced HR even in dissected controls, which implies a possibility of other effects in the *Kum* line than just actions on the temperature sensitive nature of the SERCA function. Even in intact larva, heat shocking resulted in a decrease in HR for CS and *Kum* lines. So, it remains to be understood why heat shock reduces HR in CS and does not increase HR in the *Kum* line. However, Sanyal *et al.* (2005) also reported a decrease in HR in intact *Kum* larva with heat shock. This SERCA mutant had unexplained actions in adult *Drosophila* muscle with spike broadening. Sanyal *et al.* (2005) suggested that the SERCA may alter the function of the voltage-gated calcium channels. In addition, I was surprised by the effect of heat shock as HR general increases with temperature (Ashton *et al.* 2001; Johnson *et al.* 1997); however, the acute heat shocks produced a decrease in HR. This does raise many intriguing questions with using heat shock mutants in general for manipulating other physiological functions commonly used in the *Drosophila* community. If HR is reduced then locomotive assays could be compromised or possibly perfusion to the head and limbs in adults might be reduced.

The pharmacological approach I used was meant to target the same Ca^{2+} channels or the ionic manipulations or the *Kum* mutant line. One reason to test TG, to block SERCA, is that heat shocking the *Kum* larvae might not be fully knocking out function of the SERCA. In addition, pH 8.8 might only partially compromise PMCA as compared to action of CE. The amount of reducing $[Na^+]_o$ has a dose-dependent effect on NCX, so I used *KB-R7943* as an independent measure. Using these pharmacological compounds in the *Drosophila* larva also provides information how

well they work in *Drosophila* as a species so potential applications can be used on other tissues in *Drosophila* (i.e., NMJ, CNS).

The varied methodological approaches used in this study of addressing the role of the SERCA, PMCA, and NCX was beneficial as one can be more confident of the results being specific to the targeted site in question since the results are similar among the methods used. The increase in HR by 2/3rd reduced $[Na^+]_o$ had a slighter greater effect than *KB-R7943* at (40 μM); however, the average increase in HR with 1/3rd reduced $[Na^+]_o$ matched the effects of *KB-R7943* well. We did find that all the procedures among dissected larvae, except varying $[Ca^{2+}]_o$, produced a large variation in the absolute rate but a trend to increase or decrease was for the most part consistent. This is one of the main reasons to show a percent change in the effects of compromising a Ca^{2+} channel.

The variation in HR is substantial but each preparation was followed individually for the effects of NCX, PMCA as well as for the SERCA. What consequences a variable rate might have on the effect in altering channel function is not known. For example, if the rate was fast initially and then if the NCX is inhibited this could cause Ca^{2+} to influx, via the NCX as compared to a condition with a low HR initially. The Ca^{2+} leak from ER would also promote NCX to work driving Ca^{2+} out and Na^+ into the cell. So, in theory inhibiting the NCX, PMCA or SERCA in high or a low HR can have differential effects. One reason for us to show the HR in individual larva with each manipulation was to allow this to be assessed. However, we did not observe such substantial differences among our manipulations based on the initial HR.

It is apparent that the physiological saline (HL3; Stewart *et al.* 1994) is not optimal for the heart to be maintained at the same rate as in the intact larvae. The heart is myogenic and does not require neural innervation in the larva, so one can rule out direct neural influence. This is a bit surprising since the saline performs extremely well for skeletal NMJs and even for culturing the skeletal NMJ within dissected larvae for a few days (Ball *et al.* 2003). Considering there are numerous peptides and biogenic amines in the hemolymph, they may influence the inotropic and chronotropic nature of the heart within the intact preparation; however, these also introduce uncontrolled variability in accessing the Ca^{2+} channel regulation on HR within an intact larva. Thus, a greater effort is needed to develop a saline that would be well defined for the larval heart to be maintained so various pharmacological treatments can be assayed. Salines were screened for HR based on different ionic compostions and one that was similar to HL3 did produce good rates (Gu & Singh, 1995). Despite the HL3 saline producing a reduced HR, as compared to intact larva,

we felt we could still investigate the effects of perturbing calcium regulation on HR as a before and during exposure to pharmacological compounds and/or reduced $[Na^+]_o$ and/or altered pH. In addition, I was able to investigate the role of the SERCA along with the actions of a compromised NCX and PMCA which was not previous attempted. Sanyal *et al.* (2006) did examine *Kum*^{170} in larval hearts and found similar results that heat shock reduced HR in *Kum*^{170}. The CS control larva had about 60 BPM in HL3 saline; however, in this previous report did not mention the substantial variation among preparations as I observed using fresh HL3 saline. As was noted in an earlier study (Dasari & Cooper, 2006), the transected heart tube into the true heart and aorta became very sensitive to any alterations in changing of the bathing media which made it difficult to assay differential effects of the aorta and the true heart to exposure of modulators like 5-HT. Because of this past observation, in this current study I left the heart tube intact while exchanging the saline. Reasons that heart and aorta might have different pacing action potential shapes and rhythm could be due to some cells not acting as a dominate pacemaker but as latent pacemaker cells as postulated to occur in mammalian hearts (Lipsius *et al.* 2001). These latent pacemaker cells may even be a reason for atrial arrhythmias in mammals (Lipsius *et al.* 2001). Since the saline lacks hormones, biogenic amines as well as other compounds one reason for the drop in HR for the dissected preparations might be due to the lack of driving (i.e., modulating) the dominate pacemaker cells so latent ones are unmasked. One factor that could be responsible for a reduced HR in the dissected preparations as compared to the intact preparations is that back pressure on the heart is reduced in the dissected larvae. I am not aware if there are stretch activated channels in the *Drosophila* heart.

I have not addressed several other factors that could have a significant role on HR. One should consider if other exchangers are present in the cardiomyocytes, such as the NCKX (Kiedrowski, 2007) known to be in the fly genome. Likewise, I do not know if different isoforms of the PMCA are present which could result in some being inhibited at pH 8.8 and others not (Prasad *et al.* 2007). In fact, activity could have a role in phosphorylation of key proteins, such as the NCX, which then impact HR (Ruknudin & Schulze, 2002). In rodents, the unphosphorylated PLM form will inhibit the Na^+-K^+-ATPase (Cheung *et al.* 2007). In this earlier report, it was shown that PLM impacts cardiac contractility by modulating the function of the NCX and Na^+-K^+-ATPase. The pH shift I used to 8.8, in order to inhibit the PMCA, could have indirect effects on other ion channels. It is known that switching pH from 7.4 to pH 9.5 produces a negative voltage shift in the I-V relationship for I_{Na+} currents without altering the amplitude in isolated rat ventricular cells (Yatani *et al.* 1984). Identifying

the subtypes of ion channels present in the myocytes would be helpful. Since TTX does not even block the Na^+ channels in the *Drosophila* heart, as HR is not slowed by TTX. This could indicate that the voltage gated Na^+ channels might not have a significant role in pacing Gu & Singh (1995) or that they might be of a TTX insensitive subtype. It is known that the L-type Ca^{2+} channel blocker in mammals also reduced HR in *Drosophila* and K^+ channel blockers that function on larval muscles also reduced HR in larva (Gu & Singh, 1995; but also see Johnson *et al.* 1998). So some of the channels can be identified by classical pharmacological approaches but the more one can learn how they are modulated would be of interest. In fact, one does not even know yet if Ca^{2+} activated K^+ channels are present in these myocytes; however, they are not known to be present in cardiac myocytes of mammals (Bers, 2002a). Since the L-type Ca^{2+} channel blocker has an effect of *Drosophila* heart, this would support the notion that the larval heart functions as the mammalin adult heart in that L-type Ca^{2+} channel is key in pacing (Bers, 2002a) as compared to the T-type Ca^{2+} channel. I do not know what starts the beating after a quiescent period in the *Drosophila* heart. Possibly modulating the degree of the ER's background leak of Ca^{2+} through RyR is the key or even IP3 mediated receptors on ER. Since the larval heart is sensitive to 5-HT modulation (Dasari & Cooper, 2006) IP3 receptors may have a role in background $[Ca^{2+}]_i$. Quantitative genetic analysis effects on HR were examined for multiple deficiencies. Some mutations were shown to increase HR while others decreased HR (Ashton *et al.* 2001).

The large action potentials recorded in the caudal myocytes are likely responsible for the pacemaker activity of the larval heart as this is the location where waves of contractions begin. Based on cross sections and SEM topography (Johnstone & Cooper, 2006) there are few cells that make up the heart tube in this region. Such large action potentials would suggest a large Ca^{2+} entry if voltage-gated Ca^{2+} channels are present on the plasma membrane as in mammalian cardiomyocytes that can pace (ie., purkinje cells) (Noble *et al.* 2007). This would also suggest that these cells have a sufficient means to return $[Ca^{2+}]_i$ to resting stores in order to maintain a rhythm. The initialization of the Na^+ spike could be explained as for ventricular cells of mammals. As mentioned in the Introduction, the slow leak of Ca^{2+} by ryanodine receptors (RyR), can be a trigger the NCX to function and then produce a feedback inhibition of the SR release. The depolarization lead by the NCX may lead to the opening voltage-gated L-type Ca^{2+} channels and possibly voltage-gated Na^+ channels to account for the large action potential. The large influx of Ca^{2+} could directly inhibit the leak of the RR and provide time for the SERCA as well as the NCX, and possible the PMCA, to return $[Ca^{2+}]_i$ to low levels. Thus,

removing the RyR inhibition and resetting the cell for another cycle. At this time I do not know if there are voltage-gated K^+ channels present to help in repolarization of the action potentials as there are in contractile cardiomyocytes in mammalian hearts (Opthof, 2007). Na^+-K^+ pump removes $[Ca^{2+}]_i$ indirectly by maintaining the $[Na^+]_i$ gradient. It is likely that slowing down the Na^+-K^+ pump will results in a higher $[Ca^{2+}]_i$ level in the muscle, which will eventually lead to stronger contractions, but this has not been examined yet in larval *Drosophila*. Since inhibiting the PMCA at *Drosophila* NMJ in larvae by pH 8.8 produces a longer lasting $[Ca^{2+}]_i$ from single action potentials as well as trains (Lnenicka *et al.* 2006) it would be of interest to directly measure $[Ca^{2+}]_i$ with indicators in myocytes. Given that the resting membrane potential is around -20 mV would indicate that the myocytes are permeable to Na^+ and that the Na^+-K^+ ATP pump is not working at a level to keep the cell membrane at a more negative level as in neurons. It would be of interest to determine where the E_K is for these myocytes in order to learn why these cells have a small resting membrane potential. In fact, the membrane potential can influence Na^+-Ca^{2+} exchange and if the $[K^+]_o$ is not optimal in the HL3 this could explain a depolarized state for the myocytes (Baczkó *et al*. 2003). It is known that cardiac fibroblasts in the human heart are not electrically excitable but are mechanosensitive. The resting membrane potential of these cells is around -16 mV. It is stated by Kamkin *et al*. (1999) that "… rhythmic contractions of the myocardium associated with stretch of the surrounding tissue produce reversible changes in the membrane potential of cardiac fibroblasts. These mechanically induced potentials (MIPs) follow the rhythm of myocardial contractions." It is unlikely that the potentials I recorded are from supportive fibroblasts as when the heart is not contracting, during blebbistatin treatment, the potentials are still able to be recorded.

Based on the results obtained in this study, I propose a model for the role of NCX, PMCA and SERCA within the heart cycle (Figure 21). The base mechanism is hypothetically explained by an overcompensation of PMCA or SERCA when one or the other is compromised. I did not included the potential Na^+ and K^+ ionic currents in the model to account for the cardiac cycle nor feedback inhibition on the RyR or the voltage-gated Ca^{2+} channel by raised $[Ca^{2+}]_i$. Many possible scenarios exists from the observed measures but until direct measures of $[Ca^{2+}]_i$ can be made with Ca^{2+}-sensitive indicators or isolated ionic currents they would be very speculative. Possible computational models (Korhonen *et al.* 2008; Rapila *et al.* 2008) might be of help here in postulating feasible models based on selective blocking of one or a combination of these Ca^{2+} channels as approached in this study.

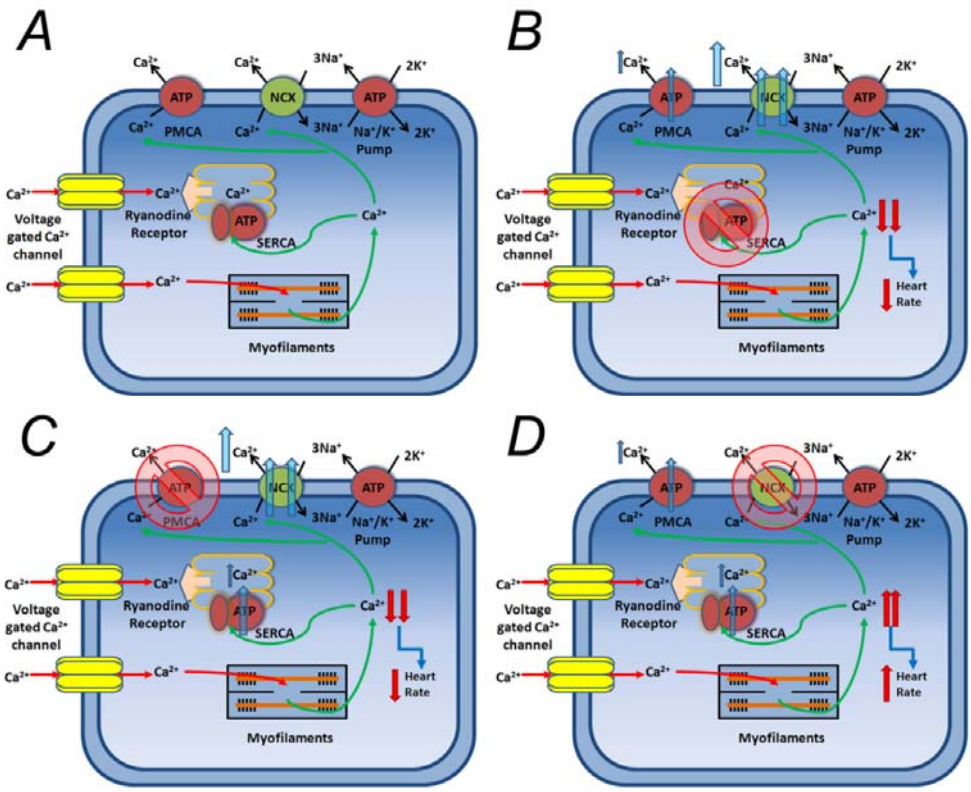

Figure 5.22: Schematic model for the effects of blocking the NCX, PMCA and SERCA on HR (*A*). When either of the three Ca^{2+}-ion channels are blocked the other two channels have an increased load to reduce the $[Ca^{2+}]_i$ in order to bring the HR back to normal or below a normal rate. (*B*) When the SERCA is blocked in *Kum* mutants, by means of heat shock, the increase in $[Ca^{2+}]_i$ causes the NCX and the PMCA to work even more efficiently and reduce the $[Ca^{2+}]_i$ below normal levels causing a decrease in HR. (*C*) In case of the compromising the PMCA, the reduction of HR is large, possibly indicating that the NCX and SERCA compensate more than necessary as the PMCA plays an important role in regulation of HR. This might suggest that there could be a rapid signaling mechanism among SERCA and the PMCA when one is inhibited so that the other can work more efficiently for $[Ca^{2+}]_i$ removal. (*D*) When the NCX is partly blocked, the other channels are not signaled to remove the $[Ca^{2+}]_i$ as quickly. But when the NCX is completely blocked with the drug *KB-R7943*, the HR decreases suggesting that now the PMCA and SERCA increase their efficiency and reduce $[Ca^{2+}]_i$.

Several follow up studies to these results would help in delineating the potential mechanisms in the interaction of the various Ca^{2+} buffering mechanisms. One idea is to use cultured myocytes for whole cell patch recordings and better

isolate ionic currents involved in pacing. Also determining specifically the dominant and latent pacemaker cells as postulated to occur in mammalian hearts would be of interest (Lipsius *et al.* 2001). Moreover responses in function to chronically altering one or all of the channels NCX, PMCA, SERCA and various treatments ranging from gene therapy to pharmacological agents would be insightful. Isolating the location of these proteins, possibly by antibody-gold staining with transmission electron microscopy, would help to describe regional cell types and function. Knowing the mechanisms of how modulators, such as 5-HT, alter HR and shapes of the action potential will help in knowing why the intact larvae demonstrate more stable HRs than the saline bathed preparations. The pupal heart is sensitive to a number of modulators, 5-HT, octopamine, norepinephrine, dopamine and acetylcholine that accelerate HR (Johnson *et al.* 1997; Dasari & Cooper, 2004*a*). The actions of these modulators depend on the developmental stage. While some of these compounds are likely inactive in larval stage, they could be active in pupa and adult (Zornik *et al*. 1999). It would be interesting to deterinmine if the larval heart would no longer be sensitive to 5-HT when CaM Kinase II is inactivated to learn how the HR is regulated by this modulator. Increased levels of cGMP increases HR so it is likely that cGMP would be a potentially activated cascade for a modulator role (Johnson *et al.* 2002). It is interesting that not all the pacemaker cells in mammalian heart are sensitive to vagal stimulation (i.e., Ach) since this could induce cardiac arrest. Thus, having some cells not responsive allows them to be driven by increase in electrical activity but not turned off when pacing cells are in a "standstill" by inhibitory modulation (Opthof, 2007). It would be of interest to examine if there is uniformity in responsiveness to modulators for individual myocytes along the entire dorsal tube in larval *Drosophila*. It would also be of interest to investigate developmental regulation to chronotropic and ionotropic modulations of HR. Such differences my help to explain why HR slows from 1st instar to adult (Sláma & Farkaš, 2005). Hopefully, as specific ionic recordings are forth coming in myocytes, the cellular responses of inhibiting NCX, SERCA and PMCA can be determined.

ACKNOWLEDGMENTS

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Chapter Six

Overview

Since the discovery that Ca^{2+} was important in the function of frog hearts by Sidney Ringer (1883; see Miller, 2004) it was likely on the mind of the earlier physiologists that started examining contraction of skeletal muscle, by nerve stimulation, as to what fluids maintained their preparations the best; however, I found it difficult to find descriptive text specifically on this matter by Galvani (~1780s) and Volta (~1793). Around Ringer's time, Charles Richet (1879) in stimulating a crayfish muscle must have had good insight as to what solutions produced good responses in muscle contraction. These solutions, besides just using sea water, were probably known to the researchers investigating the electrical properties of the squid axons (1950's). When synaptic physiology was seriously investigated as to mechanisms, the role of $[Ca^{2+}]_o$ became the center of attention (Fatt and Katz,1953; Katz and Miledi, 1968) and continued throughout time (Dodge and Rahamimoff, 1967) to this day (Catterall and Few, 2008). With the advent of directed mutational studies and genetic screening, molecular alterations in the Ca^{2+} channels is now able to be examined as to the functional significance on synaptic transmission (Kawasaki et al., 2000; Xing et al., 2005) and heart function (Badre et al., 2008; Sanyal et al., 2006).

Since it is well established now that intracellular Ca^{2+} ions are tightly regulated in many animals for allowing a heart to pace to nerve terminals controlling the degree of synaptic transmission, a focus has been on how is $[Ca^{2+}]_i$ monitored and maintained. The plasmalemmal Na^+/Ca^{2+} exchanger (NCX), the Ca^{2+}-ATPase (PMCA) as well as the SERCA on the endoplasmic reticulum are three important Ca^{2+} regulatory process in controlling $[Ca^{2+}]_i$ and thereby are of primary interest to learn their role in the process. The conventional approach in examining the mechanism or importance of ion channel function is to block the channel and examine the effects. With use of gene or mRNA regulation it has also be possible to examine the effects of over expression of proteins. Most all recent physiological studies build on past findings and methodological procedures. However, one should be cautious as to cross species comparisons of pharmacological and various other experimental manipulations when using previously published results. Even within species comparisons needs careful consideration as it is becoming well known that many receptor subtypes are possible because of alternative splicing and the receptors show varied pharmacological profiles. As in vertebrates there are differential expressed tryptophan hydroxylase genes centrally and peripherally which

produce different enzyme isoforms for the biochemical pathway in synthesis of dopamine in *Drosophila* (Walther *et al.*, 2003). Even when the gene sequence is the same, there may be alternative splicing differentially resulting in one form more sensitive to drug treatments. Enzyme splicing variants which show various drug affinities are due to various 5-HT receptor subtypes and such a phenomena is now commonly observed in multiple species (Kishore and Stamm, 2006; Krobert and Levy, 2002). This is now a hot bed of interest in human medicine as people are being genomically profiled for specific pharmacological treatments (López-Pedrera et al., 2008; Shlien et al., 2008).

One asset to my studies is that I used multiple approaches to compromise the channels under investigation instead of just using one standard procedure. I feel this will be very beneficial to future investigations using not only the crayfish and *Drosophila* models, but all models. I hope the results presented will bring awareness to the potential problems of choosing one method that has been published and basing so many other experiments and effort on that one procedure. As a result of my various procedures to target the NCX, PMCA and the SERCA, some of the results obtained were not as predicted. So now I am in a conundrum with choosing which procedures might be working as predicted and which ones might have caused non-specific effects related to the original target of interest. As for the compromising the NCX function in the crayfish and *Drosophila* nerve terminals with lowered $[Na^+]_o$ I feel the procedure is risky since the $Ca^{2+}{}_V$ channels might be teetering in reducing the amount of time and total Ica that enters the presynaptic nerve terminal with the repetitive stimulation. This is a potential problem as even the initial EPSP amplitude, in the 10 pulse train for the *Drosophila* NMJ, is reduced in amplitude (Chapter 4). This was not as drastic in the crayfish NMJ, which this is likely due to the fact that the NMJs in the *Drosophila* preparation are a high-output type as compared to the low-output terminals in the crayfish preparation used (Atwood and Cooper, 1995). As for a future study to address this synaptic explanation, it would be beneficial to examine the effect of lowered $[Na^+]_o$ on high-output synapses for a crayfish NMJ. The extensor muscle in the walking leg of the crayfish would be such a preparation as each muscle fiber is innervated by a very high-output nerve terminal as well as a low-output one (Bradacs et al., 1997; Cooper et al., 2003). If the lowered $[Na^+]_o$ is truly reducing the EPSPs by an action on the $Ca^{2+}{}_V$ channels this would be seen by a smaller phasic-like EPSP on the extensor muscle with single axon stimulations instead of trains. Potentially imaging for free Ca^{2+} responses within the terminals would also help to address the issues surrounding this low $[Na^+]_o$ procedure. In addition, it could be possible that there is a postsynaptic contribution to this observed phenomenon. The

pharmacological profile of the glutamate receptors at the *Drosophila* NMJ are of a quisqualate type (Bhatt and Cooper, 2005). They are also ligand-gated ion channels (Dudel et al. 1992; Heckmann and Dudel, 1997) which Na^+ is the primary ion to produce depolarization of the muscle. Also, some Ca^{2+} and a small amount of K^+ flux do occur across the ionotropic receptor (Dudel et al. 1992). The receptor has a reversal potential around -10 mV. So, if $[Na^+]_o$ is so low as to decrease the driving gradient of the postsynaptic depolarization, then the EPSPs would be reduced. This can be addressed by examining the amplitude of spontaneous quantal events, measured with an intracellular electrode, while gradually reducing the $[Na^+]_o$. The spontaneous events could be monitored in either or both NMJ preparations (i.e., crayfish or *Drosophila*).

As for obtaining the opposite results in compromising the PMCA by pH 8.8 and CE application within the *Drosophila* NMJ preparation I am at a loss (Chapter 4). Both procedures gave the same results at the crayfish NMJ, in enhancing the EPSP amplitudes as would be expected if $[Ca^{2+}]_i$ increased. So why would the *Drosophila* preparation be so different with pH 8.8 enhancing and CE reducing EPSP amplitude? One idea that comes to mind is that CE might be partially blocking the postsynaptic glutamate receptors in the *Drosophila* preparation but not at the crayfish NMJ. Both postsynaptic receptors are of a quisqualate type (Bhatt and Cooper, 2005; Shinozaki and Ishida, 1981; Shinozaki and Shibuya, 1974) but they might be different subtypes with varying affinity to CE. To address this possibility, the amplitude of spontaneous quantal events, measured with an intracellular electrode, while exposing the NMJ to various dosages of CE would be one approach to determine potential postsynaptic actions on glutamate receptivity. The spontaneous events could be monitored in both the crayfish and *Drosophila* NMJs.

In examining the role of the SERCA in synaptic transmission, there are confounding results with actions on altering the EPSP amplitudes between the *Drosophila* and crayfish NMJs. Thapsigargin exposure to block the SERCA generally decreases EPSP amplitudes at the *Drosophila* NMJ, but at the crayfish NMJ the EPSPs increase in amplitude as one would expect if $[Ca^{2+}]_i$ increased. This is a multi-complex issue at the *Drosophila* NMJ since I now have reason to think that the *Kum* mutation is not specific to the SERCA function and that heat shock produces substantial alteration in synaptic transmission with many unknown potential mechanisms. As addressed in Chapter 4, the first report by Sanyal et al., (2005) revealed that EPSPs in the larvae and adult fly muscle decreased substantially in the *Kum* line upon heat shock. The authors suggested that the *Kum* reduced the function of $Ca^{2+}{}_V$ channels without any documentation and went on to postulate that SERCA

and Ca^{2+}_{V} channels are closely tied, potentially in a physical manner. They did not report variable changes in EPSP amplitudes as I have in control flies with heat shock. In fact, they did not report any effect of temperature on wild type (control) adults or larvae. As to why TG decreased EPSP amplitudes at the *Drosophila* NMJ and not the crayfish NMJ, I do not know. Since it is not feasible to obtain recovery from exposure to TG in the time frame needed, there could be non-descript actions at the *Drosophila* NMJ. The *Drosophila* NMJ is more fragile than the crayfish NMJ as there is not as much support around the single isolated muscle fibers in the larva as the bundles of fibers for the opener muscle. Also the terminal varicosities are more exposed on the *Drosophila* NMJs. Perhaps the nature of the lipid soluble nature of TG had a greater effect on the *Drosophila* NMJs and organelles than the crayfish and resulted in problems undetected in my experiments. The resting membrane potential of the muscle fibers in both the crayfish and *Drosophila* was not altered by TG exposure. The actions at the crayfish NMJ were as expected and I have no reason to think that the effects are not realistic in compromising the SERCA except for the fact the same results are not observed at the *Drosophila* NMJ.

I was fortunate to be in the laboratory as another PhD student (Dr. Sameera Dasari) was completing her studies on the *Drosophila* larval heart and its sensitivity to modulators. Since I had this *Kum* mutational strain and was addressing the actions on synaptic transmission I thought it would be interesting to examine the potential effects on the heart rate in the larvae. I quickly became interested in the pacing of the heart and its ionic regulation. Ca^{2+} has such a key role in cardiac contractility and the free Ca^{2+} is regulated mainly by the same three proteins that I was investigating at the NMJs, so it was a natural transition to look into the actions of compromising their function one at a time or in combination to learn how they impacted heart rate.

In the heart study, I learned again that not all published works are so forth coming in addressing the issues which can cause problems for future investigators trying to repeat studies or build on them. I quickly learned that the intracellular potentials reported by Lalevée et al. (2006) in the caudal heart were very variable and this was not even mentioned in the previous study. In addition, they used Schneider's insect culturing medium which results in damage of skeletal muscles as well as produces abnormal shape excitatory postsynaptic potentials in skeletal muscles. So to understand the variation of electrical potentials I had to survey electrical responses over the heart. I was pleasantly surprised that compromising the PMCA, NCX and SERCA did alter HR. Although, I have not been able to determine why reducing PMCA function reduced HR. A reduced function of NCX did produce the expected increased HR. The net results indicate that the NCX and PMCA are

important in regulating HR, where as the SERCA does not have as pronounced of a role. However, in intact preparations the loss of SERCA function, by mutation, did have an impact on HR. Pharmacological approaches to alter PMCA and SERCA paralleled the results obtained by ionic and mutational approaches.

As a composite of results, I have three general models that have been presented at the end of Chapters 3, 4 and 5 for potentially explaining the main findings. The net results are presented in Table 6.1.

Table 6.1: Summary of results. (NS- non significant effect)

Treatment	**Crayfish NMJ**		**Drosophila NMJ**		**Drosophila Heart**
	EPSP Amplitude	**Facilitation**	**EPSP Amplitude**	**Depression**	**↑ or ↓ in HR**
Low Na (compromising NCX)	↓	NS	↓	↓	↑
KB-R7943 (blocking NCX)	↑	NS	↓	↑	↓
pH 8.8 (inhibiting PMCA)	↑	NS	↑	↑	↓
Carboxyeosin (blocking PMCA)	↑	NS	↓	NS	↓
Heat Shock (inhibiting SERCA)	---	---	↓	↓	↓
Thapsigargin (blocking SERCA)	↑	NS	↓	↓	↓
Inhibiting all 3 channels	↑	NS	↓	NS	NS

In addition, in each chapter I have addressed the purpose of the experiments that were performed and the significance of the findings to the scientific community. Within each chapter, I also address potential future projects for each set of findings. Overall, I think long term studies in compromising the function of one or all three of the proteins would provide useful information in homeostatic control for the NMJs and development of the terminals as well as actions on the heart. Improved methods in quantal analysis (Chapter 2) can open new horizons in examining altered synaptic function to correlate with structural differences in synapses due to developmental factors or pharmacological treatments. I do think that there is a clinical significance to

fundamental research on basic mechanisms in model organisms that can be rapidly examined to test predictions.

The comparative nature taken in this dissertation at the two NMJs and heart was very helpful to the way in which I will approach future scientific endeavors.

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SCIENTIFIC PUBLICATIONS

Research Articles

1) **M. Desai-Shah**, K. Viele, G. Sparks, J. Nadolski, B. Hayden, V.K. Srinivasan and R.L. Cooper. Assessment of Synaptic Function During Short-Term Facilitation in Motor Nerve Terminals in the Crayfish. The Open Neurosci J 2008; 2: 24-35. *Link to article:* http://www.bentham.org/open/toneurj/openaccess2.htm
2) **M. Desai-Shah**, Papoy A. R., Ward M. and Cooper R. L. (2009) Roles of the SERCA, PMCA and NCX in calcium regulation in the *Drosophila* larval heart. *(In Review)*
3) **M. Desai-Shah** and Cooper R.L. (2009) Variable means in Ca^{2+} regulation which influences synaptic transmission: Comparisons between Crayfish and *Drosophila* NMJs. Review article (*In Review*)

Abstracts (2008)

(● indicates mentored undergraduate)

1. ●*Papoy, A.R., **Desai, M.S**. and Cooper, R.L. (2008) Roles of the SERCA, PMCA and NCX in calcium regulation in the Drosophila larval heart. Univ. of KY, Showcase of Scholars (3rd annual undergraduate research event).

2. ●*Shrinivasan, V., **Desai, M.S**., Viele, K. and Cooper, R.L. (2008) Determining the characteristics of quantal events during short-term facilitation. Univ. of KY, Showcase of Scholars (3rd annual undergraduate research event).

3. ●*Papoy, A.R., **Desai, M.S**. and Cooper, R.L. (2008) Roles of the SERCA, PMCA and NCX in calcium regulation in the Drosophila larval heart. National Conferences on Undergraduate Research, 2008 (NCUR 22). Salisbury University, Salisbury, Maryland

4. ●*Ward, M**., Desai, M.S.,** Papoy, A. R. and Cooper, R. L. (2008) Roles of the SERCA, PMCA and NCX in calcium regulation in the Drosophila larval heart. Oral presentation. Kentucky Academy of Sciences, Lexington KY

5. ●*Shrinivasan, V., **Desai, M.S**., Viele, K. and Cooper, R.L. (2008) Assessment of Synaptic Function During Short-Term Facilitation in Motor Nerve Terminals in the Crayfish. Kentucky Academy of Sciences, Lexington KY

6. ***M. S. Desai** and R. L. Cooper (2008) Roles of the Sodium Calcium Exchanger (NCX), the Plasma Membrane Ca^{2+}-ATPase (PMCA) and the Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA) in synaptic transmission at the crayfish and *Drosophila* neuromuscular junctions. Society for Neuroscience, Annual Meeting 2008, Washington D.C.