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Different Mechanisms of Ca^{2+} Regulation That Influence Synaptic Transmission: Comparison Between Crayfish and Drosophila Neuromuscular Junctions

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ABSTRACT A brief historical background on synaptic transmission in relation to Ca^{2+} dynamics and short-term facilitation is described. This study focuses on the mechanisms responsible for the regulation of intracellular calcium concentration ($[Ca^{2+}]_i$) in high output terminals of larval *Drosophila* compared to a low-output terminal of the crayfish neuromuscular junction (NMJ). Three processes; plasmalemmal Na^+/Ca^{2+} exchanger [NCX], Ca^{2+}-ATPase (PMCA), and sarcoplasmic/endoplasmic Ca^{2+}-ATPase (SERCA) are important in regulating the $[Ca^{2+}]_i$ are examined. When the NCX is compromised by reduced $[Na^+]_o$, no consistent effect occurred; but a NCX blocker KB-R7943 decreased the excitatory postsynaptic potential (EPSP) amplitudes. Compromising the PMCA with pH 8.8 resulted in an increase in EPSP amplitude but treatment with a PMCA specific inhibitor carboxyeosin produced opposite results. Thapsigargin exposure to block the SERCA generally decreases EPSP amplitude. Compromising the activity of the above Ca^{2+} regulating proteins had no substantial effects on short-term depression. The Kum^{170TS} strain (with dysfunctional SERCA), showed a decrease in EPSP amplitudes including the first EPSP within the train. Synaptic transmission is altered by reducing the function of the above three $[Ca^{2+}]_i$ regulators; but they are not consistent among different species as expected. Results in crayfish NMJ were more consistent with expected results as compared to the *Drosophila* NMJ. It is predicated that different mechanisms are used for regulating the $[Ca^{2+}]_i$ in high and low output synaptic terminals. **Synapse 63:1100–1121, 2009.** © 2009 Wiley-Liss, Inc.

INTRODUCTION

Brief historical background of chemical synaptic communication

All animals use the nervous system to sense their external and internal environment and to regulate target tissues such as the skeletal muscles. Chemical communication between closely spaced neurons and/or targets occurs at synapses. The chemical signals are stored in vesicles present within the presynaptic nerve terminal. An increase in the intracellular calcium concentration; ($[Ca^{2+}]_i$) causes synaptic vesicles to fuse with the presynaptic membrane to release their contents (del Castillo and Stark, 1952; Dodge and Rahamimoff, 1967). Postsynaptic receptors determine how a chemical signal (excitatory or inhibitory) is relayed to the target cell.

The fusion cycle of synaptic vesicles is a complex process involving many proteins, and Ca^{2+} ion is one of the major players (Sudhof, 2004). The $[Ca^{2+}]_i$ is lower than the extracellular Ca^{2+}(($[Ca^{2+}]_o$), e.g., in the squid axon $[Ca^{2+}]_i$ is about 0.1 μM, and $[Ca^{2+}]_o$ is 10 mM (10^4-fold difference). This large difference in concentration creates a Ca^{2+} gradient and allows Ca^{2+} to enter the cell (Hodgkin, 1964), where it mobilizes both the reserve and the readily releasable pools

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of synaptic vesicles (Kuromi and Kidokoro, 1998; Rosenmund and Stevens, 1996). Moreover, Ca^{2+} is also responsible for the fusion of vesicles to presynaptic membrane and subsequent release of neurotransmitters into the synaptic cleft.

The involvement of Ca^{2+} in neurotransmission, although very important, is indirect. Many proteins act as Ca^{2+} sensors, which detect a change in $[Ca^{2+}]_i$ and initiate intracellular reactions. A synaptic vesicle is referred as a "primed" vesicle when it is destined for fusion with the presynaptic membrane by anchoring. Subsequently, an integral membrane protein synaptotagmin (on the synaptic vesicle) senses the $[Ca^{2+}]_i$ in the presynaptic terminal. Synaptotagmin can bind four Ca^{2+} ions and undergoes a conformational change leading to complete membrane fusion and neurotransmitter release from the vesicle. In addition, mutations in calcium binding domains of *Drosophila* synaptotagmin had been shown to have an effect on vesicle numbers and size, suggesting its role in vesicle structure (Loewen et al., 2006).

Thus, $[Ca^{2+}]_i$ plays an important role in vesicle fusion and neurotransmitter release indirectly by activating other proteins involved in the synaptic vesicle cycle. These proteins are activated by an increase in $[Ca^{2+}]_i$ and remain activated until the $[Ca^{2+}]_i$ returns to basal levels. Thus, with bursts of electrical activity Ca^{2+} flows into the terminal allowing fusion of more vesicles. Depending on the type of presynaptic nerve terminal, during maintained repetitive stimulation, $[Ca^{2+}]_i$ can continue to increase by each successive action potential (AP) because frequency of APs does not allow it to return to basal levels. This allows more vesicles to be recruited during maintained high frequency stimulation. This general scenario is the foundation for short-term facilitation (STF). This is one form of the synaptic plasticity because an alteration in the synaptic transmission resulting from previous impulse activity occurs that can lead to a change in the strength of neuronal synapses. Facilitation can be divided into STF and long-term facilitation (LTF). LTF occurs when prolonged trains of stimuli lead to an increase in postsynaptic responses which persists for minutes to hours even after the inducing activity has ceased, whereas the time scale of STF is in milliseconds to seconds.

Some of the first instances of bioelectricity were shown by the Italian anatomist and physician Luigi Galvani in 1780 by measuring electrical discharges in frogs. He showed that a electrical charge applied to the spinal nerves of a frog generates muscular spasms in the whole body. Alessandro Volta in similar experiments applied weaker current stimuli and observed muscle spasms and determined minimum current/force required to activate a muscle. These were the first observations showing that if a threshold potential is reached, it initiates an action potential (translated from French in a correspondence letter between Volta and Cavallo; Galvani and Volta, 1793).

Charles Richet first described facilitation is his experiments using a crayfish muscle. This is interesting as the first tension measures for facilitation were reported in crayfish, whereas the later experiments monitoring facilitation in electrical responses with intracellular recordings were first reported in crab skeletal muscle. Richet (1879) described stimulation of a crayfish muscle and recorded the excitation and found a lag between the time of stimulation and excitation. He also reported that the time of muscle contraction differs and when two stimuli are given with a lag. These times depended on the period between the two stimuli. The time lag between the first stimulus and the first excitation was about 0.008 s and that between the successive stimuli and their corresponding excitations was about 0.004 s, with 0.003 s being the shortest duration. Richet further observed that if the two successive stimuli are farther apart then the time lag actually increases.

Richet (1882; Physiologie des muscles et des nerfs or Physiology of the muscles and the nerves, 1881), in an experiment also demonstrated the influence of successive muscle excitations resulting in "jolts" or twitches. Helmholtz previously observed that two successive excitations of equal magnitude resulted in the merging of the second twitch with the first or in a stronger single twitch, if the two excitations were very close. Richet, using seven successive excitations of equal magnitude, observed an increase in muscle excitability; first two excitations did not evoke a twitch but the third showed a very weak twitch. The next four excitations showed prominent twitches with successively increased strength. Thus muscles facilitate the strength of muscle contraction. These myographic recordings demonstrate the development presence muscle tension (Fig. 1). These results, although not intracellular recordings, indicate the first instances of recorded STF.

The first intracellular recording electrode was made by Ling and Gerard (1949). The first intracellular recordings of STF were carried out by Fatt and Katz (1953) in crab muscle fibers. Crustacean muscles are known to produce graded contractions (Katz and Kuffler, 1946; Katz, 1949; Wiersma, 1949). Thus, electrical depolarizations can produce powerful contractions without propagated action potentials. Figure 2 (Fatt and Katz, 1953) shows graded potentials produced in response to several current pulses in crab muscle fibers.

Fatt and Katz (1951) had shown that acetylcholine was the transmitter that depolarized the frog motor endplate and that voltages would summate. Both the frog and crustacean NMJs were the models of choice for investigating the frontiers of facilitation for many

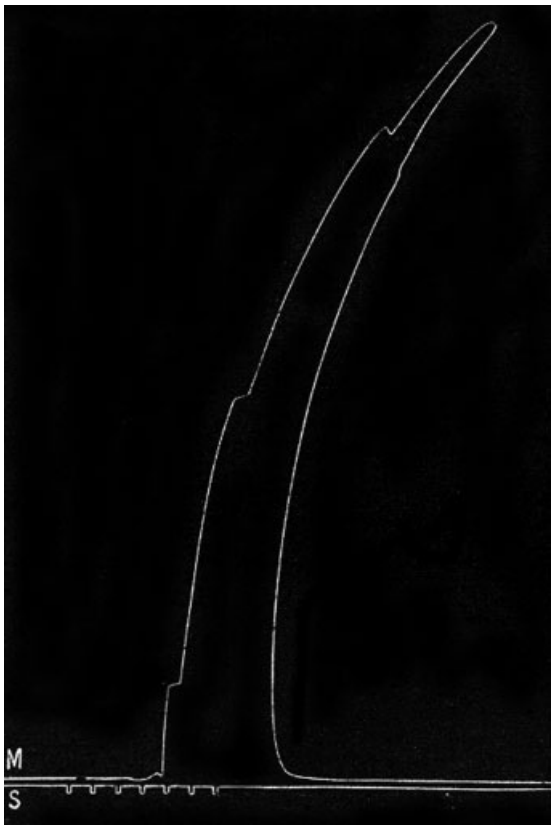

Fig. 1. First instance of STF recorded in crayfish muscle by Charles Richet. Results of seven excitations (stimuli), the first three do not show any significant twitches while the last four show facilitation in tension. Reproduced from Physiologie des muscles et des nerfs, 1882, Charles Richet.

years. This was 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 3.

The basis of chemical synaptic transmission and properties that regulate STF, are well established from insects to mammals, and are dependent on $[Ca^{2+}]_o$. Residual $[Ca^{2+}]_i$ due to previously evoked activity can accumulate, resulting in a higher $[Ca^{2+}]_i$ within the terminal and an increase in the amount of transmitter released, which is the basis for STF (Katz and Miledi, 1968). The residual $[Ca^{2+}]_i$ is altered by Ca^{2+} binding proteins and their dissociation rates (Augustine, 2001; Kretsinger and Nockolds, 1973; Lundh, 1998; Sheng et al., 1998). Ca^{2+} pumps and the sodium/calcium exchanger (NCX) can rapidly regulate $[Ca^{2+}]_i$ and thus effect the rise and decay of $[Ca^{2+}]_i$ (see reviews, Berridge, 1997, 2005; Berridge et al., 2000; Budde et al., 2002; Friel and Chiel, 2008; Thayer et al., 2002).

The plasmalemma Ca^{2+} ATPase (PMCA) and the Ca^{2+} pump in the endoplasmic reticulum (SERCA) are two other important regulatory processes. Besides the NCX exchanger, mitochondrial Na^+/Ca^{2+} exchanger (Zhong et al., 2001) may also have an important role. In *Drosophila*, PMCA is responsible for clearing Ca^{2+} which accumulates in the motor nerve terminals with larger impulse activity (Lnenicka et al., 2006). In crayfish, plasmalemmal NCX and PMCA have significant effects on 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).

A schematic representation of a nerve terminal upon stimulation by a train of action potentials is depicted in Figure 4.

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 40 Hz but can continually demonstrate STF. When a short train of pulses at a given frequency (20 pulses at 40 Hz) was applied, the amplitude of excitatory postsynaptic potentials (EPSPs) reached a plateau during the pulse train (Fig. 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 and 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 increaseing the mean quantal content and a rapid rise in EPSPs to a plateau level. We were interested in finding out the role of NCX, PMCA, and SERCA in establishing this equilibrium in two commonly used, crayfish and larval *Drosophila,* skeletal model neuromuscular preparations.

The crayfish and *Drosophila* models for investigating the role of NCX, PMCA, and SERCA in synaptic transmission

Currently, we are investigating the regulation of STF by NCX, PMCA and SERCA in the crayfish NMJ during STF (Desai and Cooper, 2008). Using quantal analysis, we have already shown a role of STF on synaptic efficacy in crayfish NMJ (Desai-Shah et al., 2008). In this report herein, our goal was to compare and contrast the *Drosophila* NMJ using similar experimental paradigms and pharmacological approaches, but in addition, using a mutational strain to compromise SERCA to compare pharmacological approaches. We used a SERCA blocking drug thapsigargin to compromise SERCA function, in a crayfish

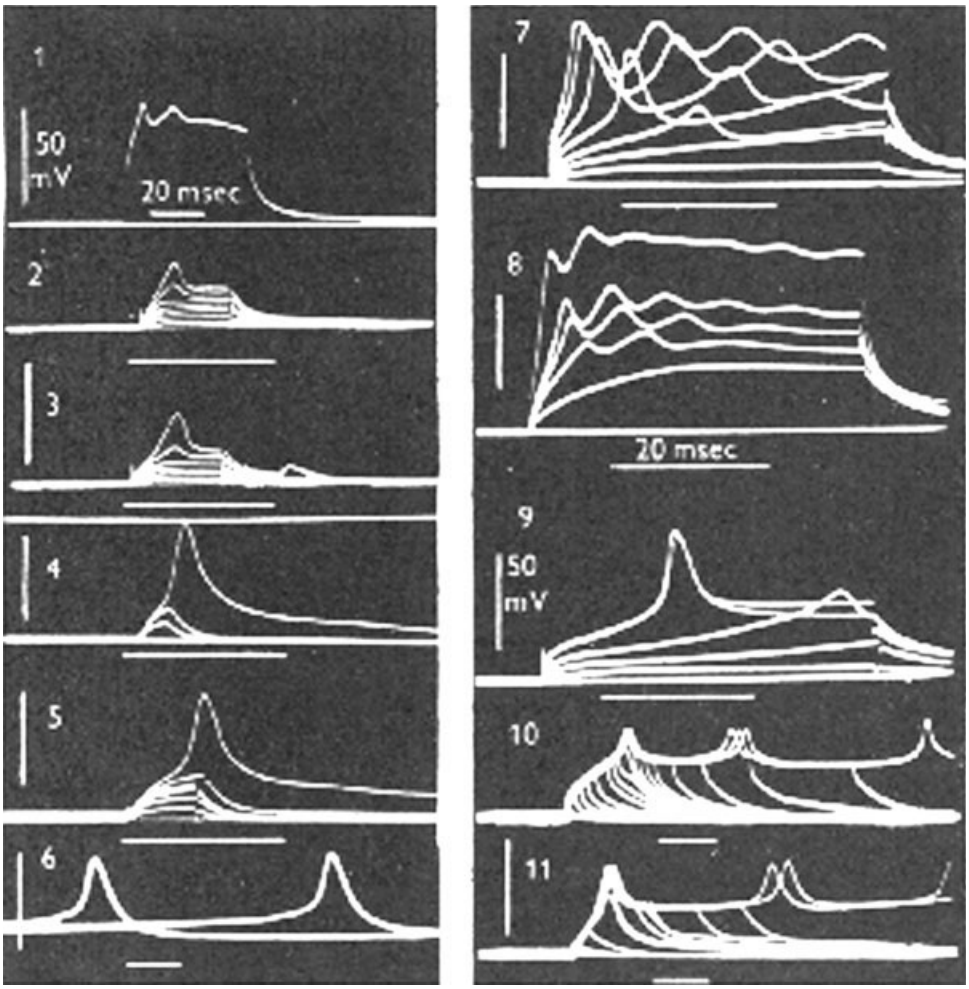

Fig. 2. Potentials in crab muscle fibers in response to several current pulses. Records 2–5 and 7–9 show pulses of constant duration and varying intensity. Records 10–11 show pulses of varying duration and constant intensity. (Record 6 shows injury discharge in the muscle fiber). (Reproduced with permission from Fatt and Katz, J Physiol, 1953, 120, 171–204, © Cambridge University Press).

preparation. While for the *Drosophila* preparation, a fly line, Kum^{170} (with a SERCA function mutation) was used in addition to thapsigargin. To stop PMCA function, pH of the bathing saline was increased to 8.8 (normal 7.2 for *Drosophila*, 7.4 for crayfish) (Lnenicka et al., 2006). Thus, by compromising/eliminating functions of the above Ca^{2+} regulators and observing their effect on synaptic transmission we can better understand their role on $[Ca^{2+}]_i$ clearance and regulation in the nerve terminal. We also describe the differences between types of synaptic efficacy in these NMJs which likely contribute to the differences in the roles of the NCX, PMCA, and SERCA in these types of motor nerve terminals.

The varied synaptic structures observed in *Drosophila* NMJs are comparable to other arthropods, particularly crustaceans, where the synaptic complexity accounts, in part, for the synaptic efficacy under a variety of stimulus conditions (Atwood and Cooper, 1996a,b; Stewart et al., 1996). Terminal morphology has been shown to regulate synaptic strength in various preparations (Atwood and Cooper, 1996a,b; Govind and Chiang, 1979; Kuno et al., 1971; Stewart et al., 1996; Walrond et al., 1993). Development of the motor nerve terminals in *Drosophila* is similar to crustaceans (Atwood et al., 1993; Atwood and Cooper, 1996b; Crossley, 1978; Li et al., 2002).

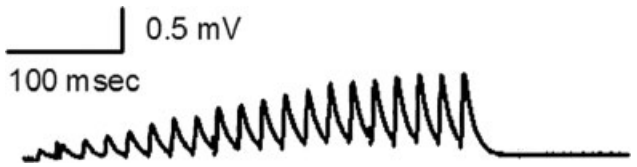

Fig. 3. The NMJ of the opener muscle in crayfish shows prominent STF in response to 20 stimulation pulses at 40 Hz. Note the plateau during the end of the stimulus pulse (data recorded by Desai-Shah and Cooper, this study).

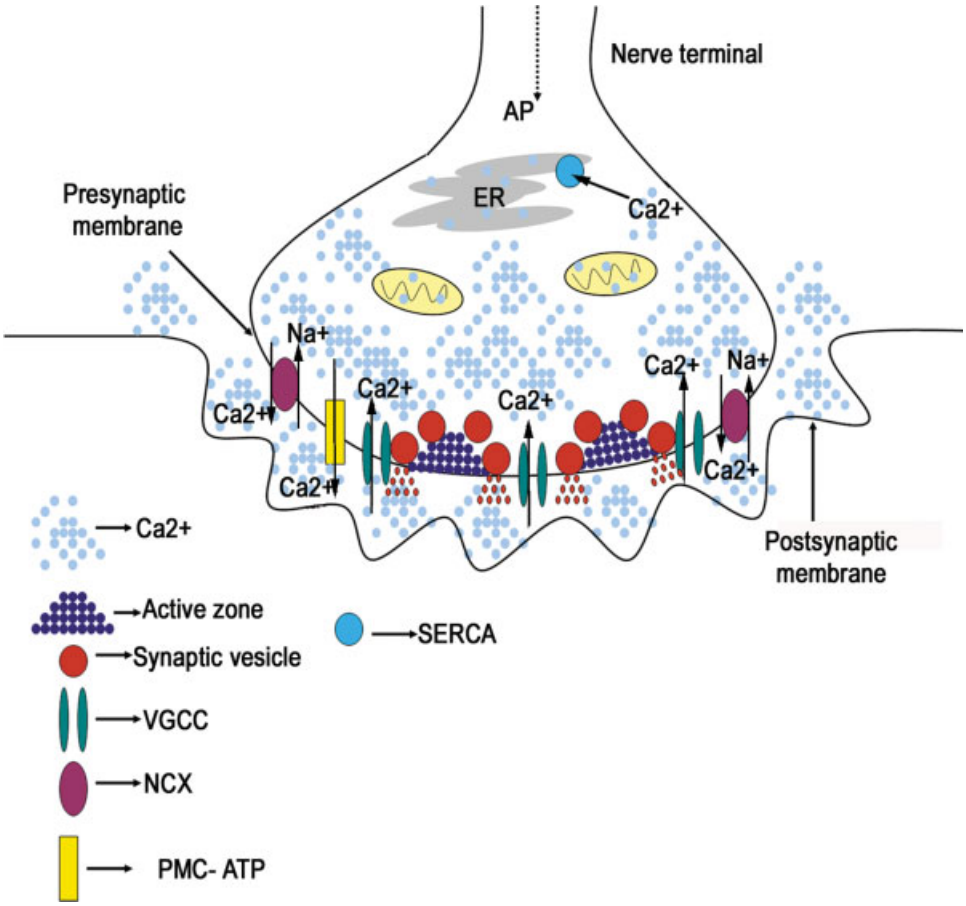

Fig. 4. A cartoon depicting various ion channels present in the presynaptic terminal after the arrival of repetitive action potentials. There is an influx of Ca^{2+} from the voltage gated Ca^{2+} channels. An increase in Ca^{2+} concentration occurs within the terminal induced by a series of rapid stimuli. This causes an increase in the probability of vesicles to fuse with presynaptic membrane and release neurotransmitter.

Genetics-based malformations of the synaptic function in humans, for particular defects, continue to be modeled on *Drosophila* synapses (Badre and Cooper, 2008; Fradkin et al., 2008; Lee et al., 2004; Lu et al., 2007; Ubhi et al., 2007) to learn more about the disease status and its effects on synaptic development (Xing et al., 2005) and function to enable us to offset or treat pathological conditions. Considering that *Drosophila melanogaster* is a model synaptic neuromuscular preparation, it is important to understand the regulation of $[Ca^{2+}]_i$ in this model (Atwood et al., 1993; Betz et al., 1993; Jan and Jan, 1976; Kurdyak et al., 1994; Li et al., 2002; Li and Cooper, 2001; Pawlu et al., 2004; Ruffner et al., 1999; Sigrist et al., 2002, 2003; Stewart et al., 1994, 1996).

The NMJ of larval *Drosophila* is an ideal preparation to investigate Ca^{2+} regulation within the presynaptic nerve terminal in regards to the efficacy of synaptic transmission because the EPSPs are graded (like most crayfish skeletal muscles) and refined quantal analysis is readily ascertainable. In addition, innervation of the 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 autoregulation in the observed Ca^{2+} responses. Surprisingly little attention has been focused on the mechanisms of Ca^{2+} regulation and short term facilitation/depression in the NMJs of the *Drosophila* larva model.

In the *Drosophila* larval NMJ, synaptic transmission is enhanced or depressed depending on the $[Ca^{2+}]_i$ load within the presynaptic terminal. In the frog and crayfish NMJs (Atwood and Wojtowicz, 1986; Cooper et al., 1996b; Matveev et al., 2006; Sherman and Atwood, 1971), residual Ca^{2+}, due to previous evoked activity, can accumulate and is the basis for STF at low $[Ca^{2+}]_o$. This can account for the maintenance of short-term depression (STD) at high efficacy crayfish (Bradacs et al., 1997; Cooper et al., 2003) and in *Drosophila* NMJs (Kurdyak et al., 1994; Stewart et al., 1994).

Like other nerve terminals, the general view at the *Drosophila* NMJ is that $[Ca^{2+}]_i$, promotes vesicle fusion via synaptotagmin (Neher and 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, if rapidly delivered, 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 is 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 and Few, 2008; Mochida et al., 2008; Tsujimoto et al., 2002). Therefore, if a substantial population of vesicles are docked, in the RRP, the first stimulus of a train can result in many vesicles fusing to the membrane, such that not enough vesicles are available for the 2nd and/or subsequent stimuli, this can thus result in presynaptic depression. Lowering the percentage of vesicles that release glutamate in these high output terminals, by reducing $[Ca^{2+}]_o$ can transform STD into STF at NMJs (Atwood and Karunanithi, 2002; Cooper et al., 2003).

In *Drosophila*, the PMCA is responsible for clearing Ca^{2+} that accumulates in the motor nerve terminals with large impulse activity (Lnenicka et al., 2006). In the crayfish, the plasmalemmal NCX and PMCA have important effects in regulating residual $[Ca^{2+}]_i$. There has yet to be a study which examines the role of all three Ca^{2+} channels with regards to STF or STD using *Drosophila* NMJs.

One study on the mechanism of calcium regulation at the NMJ of the *Drosophila* larva revealed that if the Ca^{2+}- binding protein fequenin is overexpressed there is an enhancement of facilitation (Rivosecchi et al., 1994). Additionally, a temperature sensitive dysfunctional Ca^{2+} channel in the 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 development. Adults also showed a lack in homeostatic compensation with reduced Ca^{2+} channel function (Badre and Cooper, 2008). Another unpredicted result was when the SERCA was inhibited, by a temperature sensitive mutation at *Drosophila* NMJs, the EPSPs amplitude decreased instead of increasing (Sanyal et al., 2005).

In this study, the individual role of the NCX, PMCA, and SERCA in synaptic transmission during bursts of motor nerve activity that induces STD and STF at the *Drosophila* NMJ was investigated. In the discussion section, we compared the primary findings of this study with the information we are now gathering for the crayfish NMJ. We felt that it is also important to compare different approaches to target the same proteins to learn whether they are complementary 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.

MATERIALS AND METHODS

General procedures for the crayfish preparation

Some of the experimental protocols for the crayfish NMJ are reported in Desai-Shah et al., (2008). 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 $CaCl_2{\cdot}2H_2O$; 2.45 $MgCl_2{\cdot}6H_2O$; 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 used for the crayfish preparation

All chemicals were obtained from Sigma chemical company (St. Louis, MO) with the exception of thapsigargin and KBR7943 which were obtained from Tocris (Ellisville, MO) and 5,6-carboxyeosin from Marker Gene Technologies (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. To inhibit the PMCA, the pH of saline is raised to 8.8 from its normal value of 7.4. Thereafter, to study a compromised NCX and PMCA, the concentration of Na^+ in the saline is reduced by two-thirds and pH held at 8.8. Reduction in function of the SERCA was approached by exposure to thapsigargin (TG) (10 μM) for 10 min while providing stimulus pulse trains (Rumpal and Lnenicka, 2003). We also compared the approach of reduced two-thirds $[Na^+]_o$ 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 s, Satoh et al., 2000). To examine the difference of inhibiting the PMCA by pH 8.8 and a pharmacological approach carboxyeosin (CE) was used. It was shown that 5 μM is sufficient to inhibit PMCA in rats and in guinea pigs (Mackie-

TABLE I. Composition of crayfish 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	NA	41 mM	68.3 mM	102.5 mM	136.53 mM	164 mM

wicz and Lewartowski, 2006). Therefore, we used this concentration and incubated the preparation for 10 min.

Physiology used for the crayfish preparation

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 and Kuffler, 1961). STF was obtained by giving a train of 10 or 20 pulses at 10 or 20 s 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 and Cooper, 2000; Cooper et al., 1995b; Dudel, 1983; Sparks and Cooper, 2004).

To examine if the reduced $[Na^+]_o$ had an effect on the shape of the presynaptic action potential, the preterminal of the excitatory axon in the walking leg opener muscle was impaled with sharp intracellular electrode filled with 3 M KCl. The neuromuscular junctions were 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 responses were obtained at a 20 KHz acquisition rate. Initially the preparation was maintained in normal crayfish saline and stimulated. After every 200 stimulations (delivered at 0.5 Hz) 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 are shown in Table I. An agar bridge (1.5% agar in normal crayfish saline) was used to keep the action potential amplitudes from fluctuating when the saline was changed. Electrical signals were recorded on-line to a Power Mac 9500 via a MacLab/4s interface.

Analysis for the crayfish preparation

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 and Cooper, 2000). Statistics employed were either the Student's *t*-test or a Wilcoxon paired rank sum test (a nonparametric test). In all cases, we used * for $P < 0.05$ while ** is for $P < 0.001$.

General procedures for the *Drosophila* preparation

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 third instar staged larvae, flies were pulsed to lay eggs for 2 h and then the eggs were incubated at 21°C. Staging of fly larvae and the dissection technique have been previously reported (Ball et al., 2003; Campos-Ortega and Hartenstein, 1985; Li et al., 2002).

Physiology used for the *Drosophila* preparation

Physiological saline HL3 (in mM): 1.0 $CaCl_2 \cdot 2H_2O$, 20 $MgCl_2$, 70 NaCl, 5 KCl, 10$NaHCO_3$, 5 trehalose, 115 sucrose, 5 BES (*N*,*N*-bis[2-hydroxy-ethyl]-2-aminoethanesulfonic acid) and adjusted to pH 7.2, was used for initial dissections (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 because the BES buffer required a pH adjusted from a lower value. Thus, to avoid adding any NaOH we started with basic saline and raised its pH to 8.8 with HCl.

The recording techniques for intracellular EPSP measures have been previously described (Dasari and Cooper, 2004; Sparks et al., 2004; Stewart et al., 1994). The compound amplitude of 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 −50 mV or more negative were used. The HL3 dissection medium was com-

pletely 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) except thapsigargin and KB-R7943 which were obtained from Tocris (Ellisville, MO) and 5,6-Carboxyeosin (CE) from Marker Gene Technologies (Eugene, OR).

Analysis for the *Drosophila* preparation

Facilitation/depression was measured by an index of 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), to ensure 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 a online 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 by 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 experiment.

RESULTS

Larval *Drosophila* NMJ preparation

The m6 muscle used in these studies within the larval skeletal muscle preparation is depicted in Figure 5A. This preparation is planar when stretched at the four corners. A segmental nerve branching from the CNS is transected and drawn into a suction electrode so it can be stimulated selectively for a particular segment. In this study, the 4th segmental nerve was stimulated at 20 Hz to produce 10 EPSP responses (Fig. 5B). In the standard HL3 saline, with 1 mM $[Ca^{2+}]$, initial EPSP is normally larger than the following EPSPs in the train. The rate of decrease in amplitudes is dependent on the stimulation frequency. In this case, we used amplitudes of the 1st, 5th, and 10th EPSPs within the train to index changes and to calculate facilitation or depression in amplitudes. Generally, in standard HL3 saline the facilitation measure (i.e., [(A10/A1) − 1] = FI) has a negative value, and thus it can be referred to as negative facilitation. We refer negative facilitation as short-term depression (STD) to be consistent with depression of transmission in 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 vesicles in the readily releasable pool (RRP) are used within the 1st pulse and there is not enough time to replenish this pool with a rapid 40 Hz stimulation train (Atwood and Cooper, 1995, 1996a,b). Thus, the efficacy of synaptic release during a stimulus train would depress to a greater extent over time as the $[Ca^{2+}]_i$ increases in this high output fly NMJ, resulting in a more negative value using 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 the EPSPs amplitudes and STD index. Just measuring one pulse in the train during STD may give misleading information, as the amplitudes can vary during the stimulus train (Crider and Cooper, 1999). So we chose the 10th, 5th, and the 1st EPSPs as the measures for STD for various experimental manipulations (Fig. 5B).

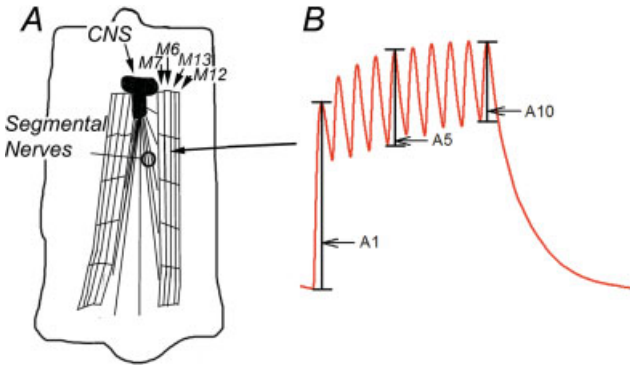

Fig. 5. (**A**) Schematic of the *Drosophila* larval neuromuscular junction preparation. A third instar larva is cut open on its dorsal side to expose the ventral abdominal muscles shown here as M6, M7, M12, and M13. A segmental nerve innervating these muscles is drawn into a suction electrode and stimulated. (**B**) Representative EPSP train of 10 pulses recorded from the M6 when a segmental nerve was stimulated at 20 Hz. The measurement of the amplitudes A1, A5, and A10 are indicated. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Actions of compromising NCX

One approach used in this study to reduce the function of NCX was to lower $[Na^+]_o$ by using a HL3 saline with less NaCl. However, in the later studies where both PMCA and NCX were compromised together pH of saline was raised to be 8.8 (to reduce PMCA function) without adding NaOH because it would alter $[Na^+]$. Therefore, we used Tris-Base and adjusted it to pH 8.8 with HCl. Because Tris-Base saline resulted in abnormal EPSP amplitudes, we compared it to preparations dissected and bathed in standard HL3 saline, and then switched to Tris-Base HL3 saline instead of one with BES buffer (see Methods). Results clearly show that Tris-Base saline (pH 7.2) reduced the amplitude in the 1st and 5th EPSPs

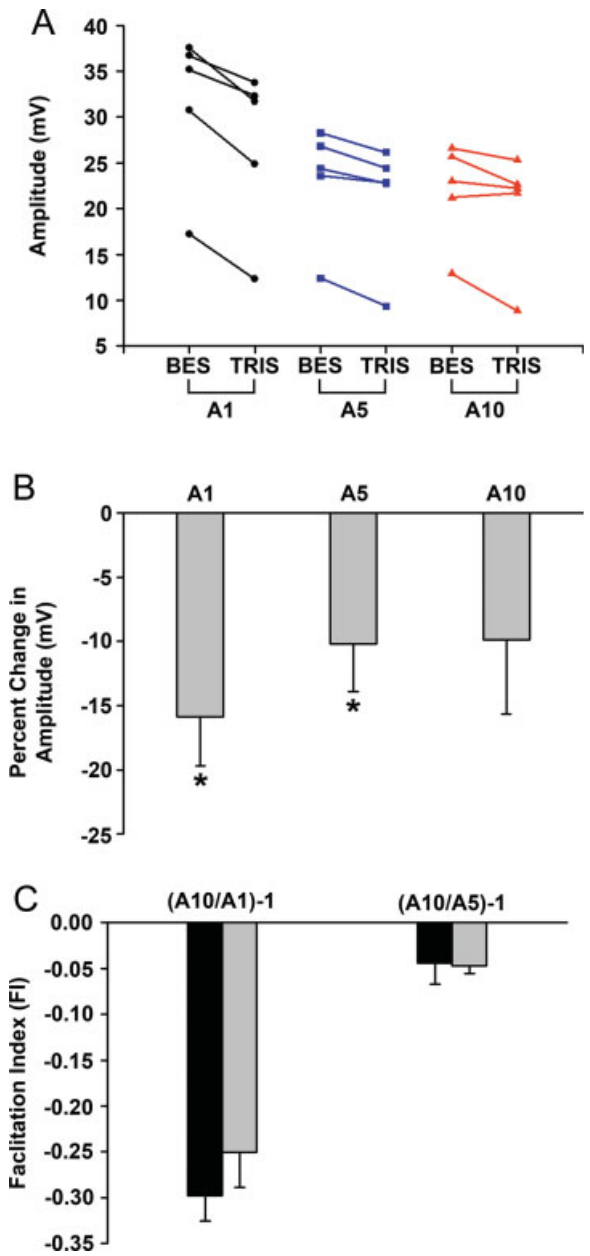

Fig. 6. (**A**) The effect on the EPSP amplitudes in changing the bathing saline in BES buffer to Tris-Base buffer (pH 7.2 for both). Amplitudes 1, 5, and 10 events within the 10 pulse train are shown. There is a significant reduction in amplitudes in TRIS as compared with BES. (**B**) The percent change in 1, 5, and 10 amplitudes. (**C**) Black bars indicate FI in normal saline with BES buffer and gray bars indicate FI in normal saline with TRIS buffer. The FI of EPSPs in BES and TRIS was not significantly different between the two salines. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in 5/5 preparations (Fig. 6A; $P < 0.05$; Wilcoxon rank-sum test). This was also observed in the percent changes in the amplitudes among the preparations (Fig. 6B; Student's Paired *t*-test). Pooled data of the 10th EPSP did not show a significant change but comparisons between individual preparations showed differences (Fig. 6A; five of five, $P < 0.05$; Wilcoxon rank-sum test). FI measurements did not show any significant change; but the mean values decreased, but there were large variations between preparations (Fig. 6C).

Despite the reduced amplitudes in the EPSP, with Tris-Base buffer, we used it in our studies to examine compromised PMCA at pH 8.8. To first examine the effects of reduced $[Na^+]_o$ to comprise NCX activity, EPSP amplitudes were measured before and in saline with 2/3rds reduced $[Na^+]_o$ and percent changes were determined. Percent is used since absolute EPSP amplitudes varied between preparations. The means of percent differences of the various EPSPs within the response train (1st, 5th, and 10th) and for individual preparations are shown. To highlight a preparation with a very large increase in the EPSP amplitude a box is placed around the values shown (Fig. 7A). Because some preparations showed increases while others decreases in the EPSPs, no significant trend was observed when examining a before and an after effect (Fig. 7B). Likewise there was no significant effect in percent changes in amplitudes (Fig. 7A1). However, after removing the large outlier from the data set, the percent change in the 5th EPSP amplitude did show a significant change (Fig. 7A2; Student's paired *t*-test). The facilitation index, at various EPSPs within the train, revealed no significant effect of lowering $[Na^+]_o$. Thus, the effect of reducing the $[Na^+]_o$ to compromise the NCX did not show a consistent or reliable effect.

Action of KB-R7943 in compromising NCX

A reduction in $[Na^+]_o$ is one approach to dampen the ability of the NCX to extrude Ca^{2+}, however we also wanted to try a known pharmacological approach to block NCX function as in other animal models (Wu et al., 2008). Since we did compare, both the reduced $[Na^+]_o$ and this pharmacological approach on the crayfish NMJ (Desai and Cooper, 2008) and for experiments on the *Drosophila* larval heart, we were 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 all six of six preparations for the 5th and 10th EPSP amplitudes, but not the 1st EPSP amplitude within the train (Fig. 8A; $P < 0.05$; Wilcoxon rank-sum test). The percent change in the EPSP amplitudes also revealed a significant effect of KB-R7943 in reducing the amplitudes for the 5th and 10th EPSP amplitudes (Fig. 8B; Student's paired *t*-test) calculated FI (Fig. 8C) did not show any significant effect on either of the two FI measures, [(A10/A1) − 1] or [(A5/A10) − 1].

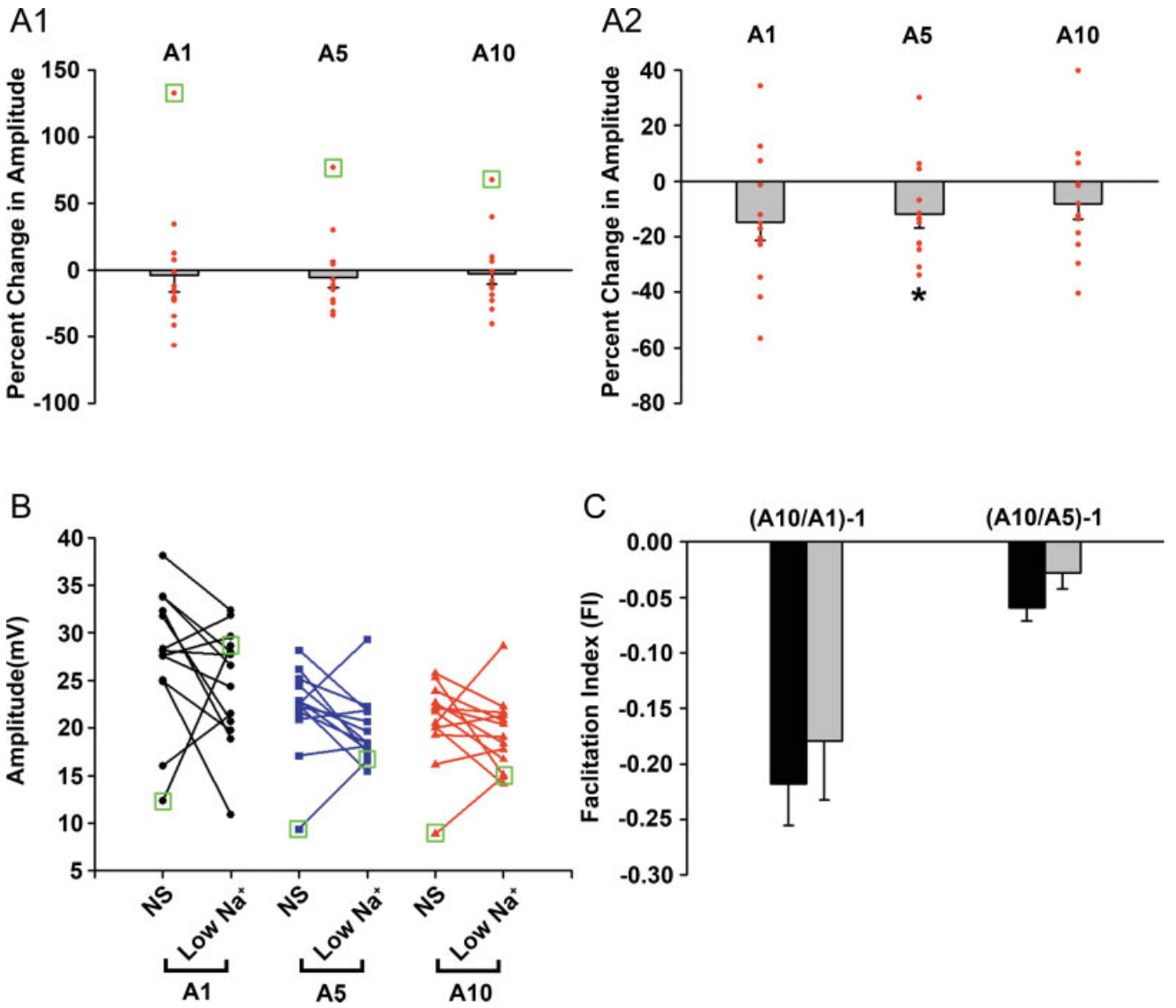

Fig. 7. Compromising the NCX with reduced $[Na^+]_o$. (**A1**) The percent change in the amplitudes of the 1st, 5th, and 10th EPSPs did not reveal a significant effect. The red dots show individual preparations and since one preparation (in green box) revealed a large increase the data was reassessed with the results of the one preparation removed (**A2**) (green box In A1). This resulted in significant effect in the percent change in the 5th EPSP amplitude. The before and after effect, for individual preparations is shown in (**B**) (one outlier shown with green open boxes). FI was not significantly affected by reduced $[Na^+]_o$ as shown in (**C**) where black bars indicate FI in normal saline and gray bars indicate FI in saline with two-third reduced Na^+. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The effect on transmission with a compromised PMCA

To block the function of PMCA, the bathing media was switched to from normal pH 7.2 to pH 8.8 while continuing to provide trains of stimuli. The initial EPSP within the train rapidly increased. This trend was observed five of five times for all three EPSP measures within the response train (i.e., A1, A5, and A10) (Fig. 9A; $P < 0.05$; Wilcoxon rank-sum test). The percent change in the amplitudes concurred with absolute changes in the EPSP (Fig. 9B; $P < 0.05$; Student's paired *t*-test). No significant effect was observed for the FI (Fig. 9C).

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

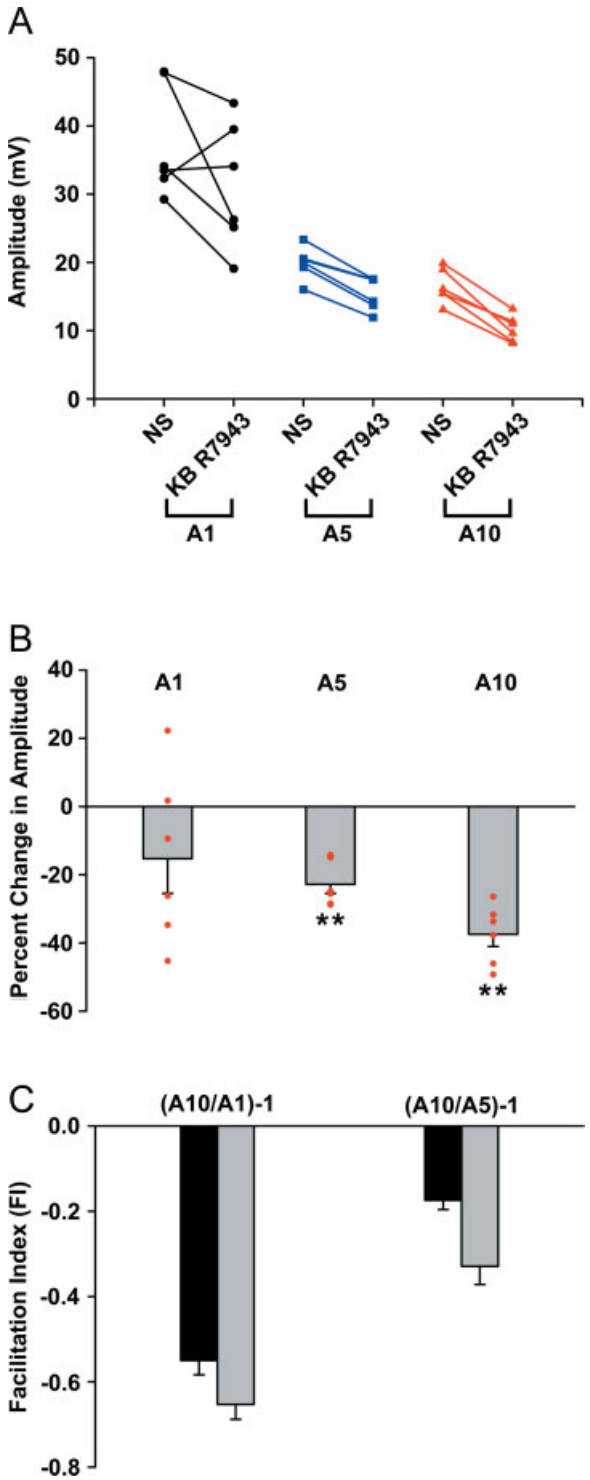

Fig. 8. The effect of KB-R7943 on compromising the NCX. (**A**) KB-R7943 reduced the amplitude for the 5th and 10th EPSPs; individual preparations (A) and for percent changes among preparations (**B**). The percent change for individual preparations is shown as red dots superimposed on the mean (±SEM) bar chart. No effect occurred for the facilitation index (**C**) where black bars indicate FI in normal saline and gray bars indicate FI in saline with KB R7943. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

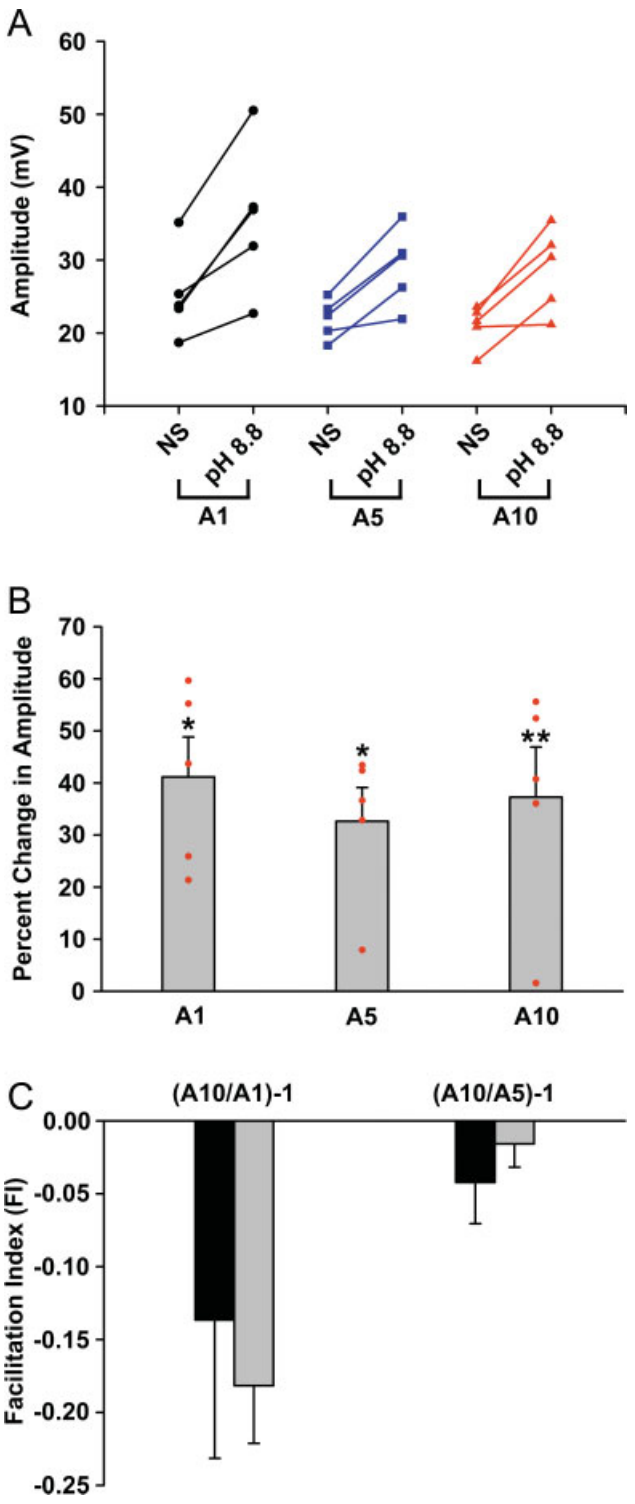

Fig. 9. The effect of raising pH from 7.2 to 8.8 in compromising PMCA. An increase in pH produced a consistent trend in increasing the 1st, 5th, and 10th EPSPs in individual preparations (**A**) and for the percent changes among preparations (**B**). The percent change for individual preparations is shown as red dots superimposed on the mean (±SEM) bar chart. No effect occurred for the facilitation index (**C**) where black bars indicate FI in normal saline and gray bars indicate FI in normal saline with carboxyeosin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

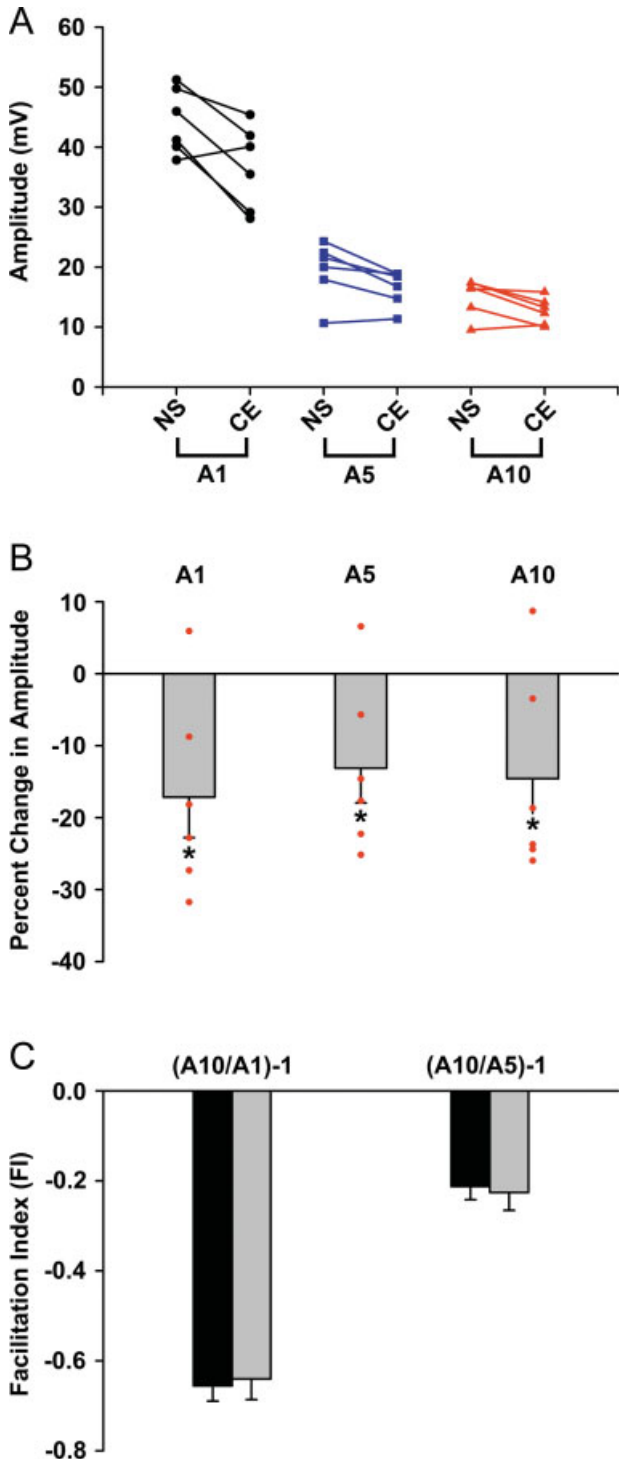

Fig. 10. The effect of 5,6-carboxyeosin (CE) in compromising the PMCA. CE reduced the amplitude for the 1st, 5th, and 10th EPSPs in five of six individual preparations (**A**) and for the percent changes among preparations there is a significant affect (**B**). The percent change for individual preparations is shown as red dots superimposed on the mean (±SEM) bar chart. No effect occurred for the facilitation index (**C**) where black bars indicate FI in normal saline and gray bars indicate FI in normal saline with carboxyeosin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The effect of compromised SERCA on transmission

A Ca^{2+} pump on the ER (SERCA) was targeted by a pharmacological approach with application of 10 μM thapsigargin (TG) and using a mutational Kum line (the SERCA protein is rapidly inactivated when heat shocked) (Sanyal et al., 2005). 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 five of six preparations by TG for the 1st and 10th EPSPs but in six of six for the 5th EPSP (Fig. 11A). The percent change in the EPSP amplitudes was significant for the 1st and 5th EPSP amplitude but not the 10th (Fig. 11B). To insure that the reduced amplitudes were due to maintaining intracellular recording during the incubation period, neighboring muscle (m7) fiber was also examined. This (m7) also showed reduced EPSP amplitudes with normal resting membrane potentials. So the effect was not due to a general rundown by retaining and intercellular recording electrode within the muscle fiber.

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

With use of the Kum^{170TS} temperature sensitive strain we compared our 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, we used a 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 four of five preparations upon heat shock (Fig. 12A). The 5th and 10th EPSPs showed a mixed response between the preparations. The FI measures did not change significantly (Fig. 12B). 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 two-thirds reduced [Na^+] in the bath. In this transition, five of six preparations showed a decrease in the 1st, 5th, and 10th EPSP amplitudes (Fig. 12C). The FI measures did not significantly change under these conditions (Fig. 12D).

Same procedures were repeated for the Kum strain as for the CS, and the trend was a decrease in the amplitudes of EPSPs during heat shock. In five of six preparations, the 1st, 5th, and 10th EPSP amplitudes showed a decrease in percent change (Fig. 13A). There was no consistent trend from before to after heat shock for the FI index (Fig. 13B). In a separate set of experiments using the same procedure except

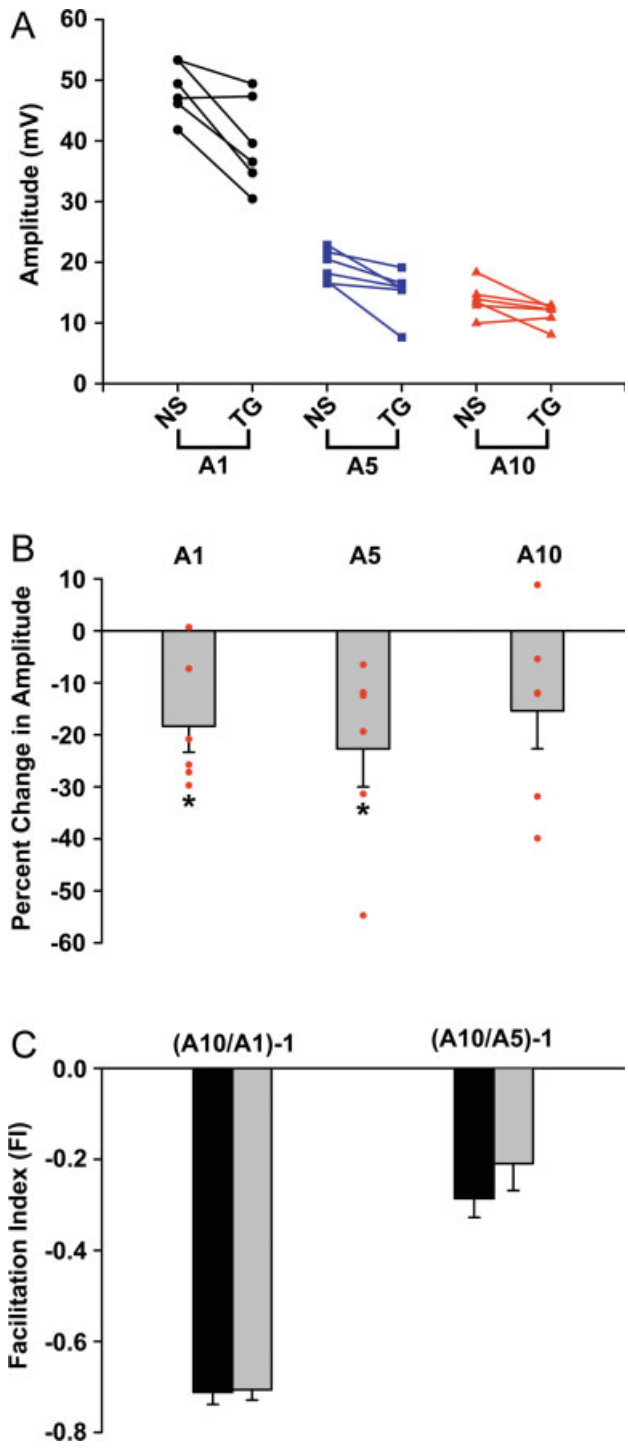

Fig. 11. The action of thapsigargin (TG) in compromising the SERCA. TG reduced the amplitude for the 5th and 10th EPSPs in five of six individual preparations and six of six for the 1st EPSP (**A**). For the percent changes among preparations there is a significant affect for the 1st and 5th EPSP amplitudes (**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**) where black bars indicate FI in normal saline and gray bars indicate FI in normal saline with thapsigargin. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

that heat shock was combined with a pH of 8.8 and two-thirds reduced $[Na^+]$ in the bath for the Kum strain.

In this transition, six of six preparations showed a decrease in the 1st EPSP percent change and five of six of the 5th and 10th EPSP amplitudes decreased in the percent change (Fig. 13C). The FI measures did not significantly change for these conditions. Also substantial variations between preparations were observed (Fig. 13D).

To isolate the average effect in the differences in percent change in amplitudes, due to heat shock, the controls from the changes measured in the Kum strain, the differences in the average effect were subtracted. The panel in Figure 12A was subtracted from Figure 13A and the result is shown in Figure 14A. Errors were from the data set with the largest value and placed on the subtracted result. The net effect was 55% reduction in the percent change for the 1st, 42% for the 5th, and 18% for the 10th EPSP amplitude. The effects of inhibiting the PMCA and NCX along with heat shock for CS and KUM were also compared by subtracting the heat shock effect measured in CS. The differences in Figures 12C and 13C are shown in Figure 14B. The average differences in the percent change in the 1st and 5th and 10th EPSP were a decrease of about 32%, 22%, and 8.5%, respectively. Thus in general, Kum mutation demonstrated a greater effect in reducing the EPSP amplitudes than the effect of heat shock alone.

DISCUSSION

In this report, we briefly reviewed early historical studies about STF and presented novel results in *Drosophila* larval NMJ model for comparisons to other synaptic preparations where the NCX, PMCA, and SERCA function was inhibited. These high output synapses are sensitive to manipulations of the NCX, PMCA, and SERCA with respect to synaptic physiology and plasticity. In physiological $[Na^+]_o$, a rapid nerve stimulation train produces STD. This depression is characterized by an initial large amplitude EPSP followed by subsequent smaller amplitude EPSPs. Because a key to STD is the amount of presynaptic Ca^{2+} entry during the terminal depolarization, it is not surprising that regulation of $[Ca^{2+}]_i$ is important throughout the stimulus train for the maintenance of STD. Our findings revealed that a compromised NCX, by lower $[Na^+]_o$, had no significant effect on the EPSP amplitudes throughout the pulse train. It was expected that STD would be reduced if the initial amplitude within the EPSP train was reduced but this was not the case. However, blocking NCX by KB-R7943 produced contrary results by generally decreasing the initial and subsequent EPSPs within the train. However, this did not result

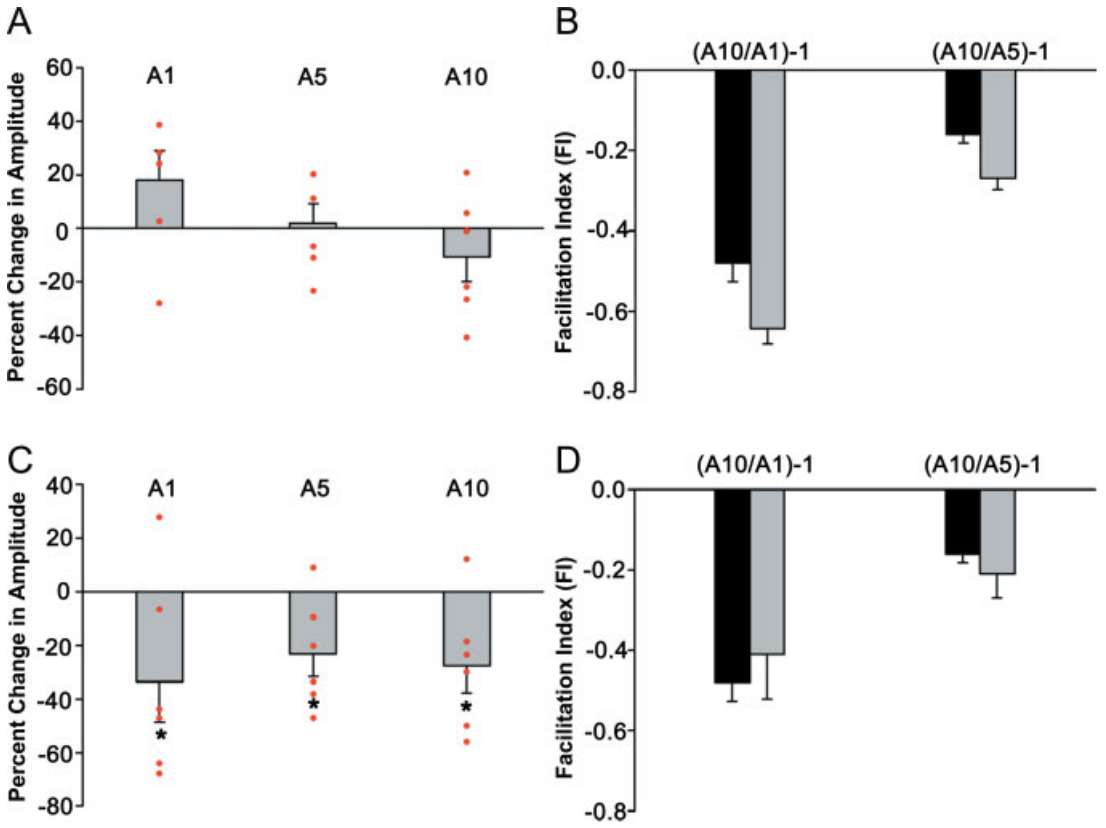

Fig. 12. The effect of heat shock and compromising PMCA and NCX in the genetic background control CS strain. (**A**) Examination of heat shock alone on the control CS strain of *Drosophila*. Heat shock did not produce a consistent trend in altering the EPSP amplitudes. The individual preparations are shown as dots superimposed on the mean (±SEM) percent change bar chart. (**B**) Black bars indicate FI in normal saline and gray bars indicate FI after heat shock in normal saline. 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, two-thirds reduced $[Na^+]_o$ and pH 8.8 on the percent change in the EPSP amplitudes resulted in a decrease in five of six preparations (**C**) with no general trend in altering the FI from before and during the combined exposure (**D**) where black bars indicate FI in normal saline and gray bars indicate FI after heat shock in low Na^+ and pH 8.8. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in significant changes in STD. A reduction in function of PMCA by raising the pH to 8.8 or exposure to CE produced opposite results. SERCA function appears to be similar to PMCA since compromising it with TG mimicked the altered responses of the PMCA when exposed to CE. However, using a heat sensitive Kum^{170TS} mutant line to block SERCA function, we discovered that both the background control and the Kum line, when heat shocked, resulted in altered EPSP amplitudes. After controlling for the decrease in the background line, Kum still produced a greater reduction in EPSP amplitudes than controls. One would assume then that the inhibition of SERCA could be unmasked in this line, but with a substantially reduced EPSP it was expected to impact 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 blocking of these three protiens on STD did not show significant differences. Thus, we 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 and subsequent EPSP amplitudes within the response train, when $[Na^+]_o$ is lowered, is not an effect we associate with a specific action on the reduction in the efficacy of the NCX. Most likely a direct effect of a reduction in the action potential amplitude in the motor neuron could be occurring just enough to be at a critical level to affect $Ca^{2+}{}_V$ channels, where sometimes the channel open time and potential inactivation is not consistent. Just as observed for the crayfish motor neurons, potentially reduced amplitude may not only effect the $Ca^{2+}{}_V$ channels activation, but may also effect the duration of depolarization which would result in less influx of Ca^{2+} overall. However, at present, it is not possible to record within the preterminal motor nerve axon in the *Drosophila* unlike in crayfish preparations due to axon diameter and its associated sheath. A reduction in the EPSP amplitude should have

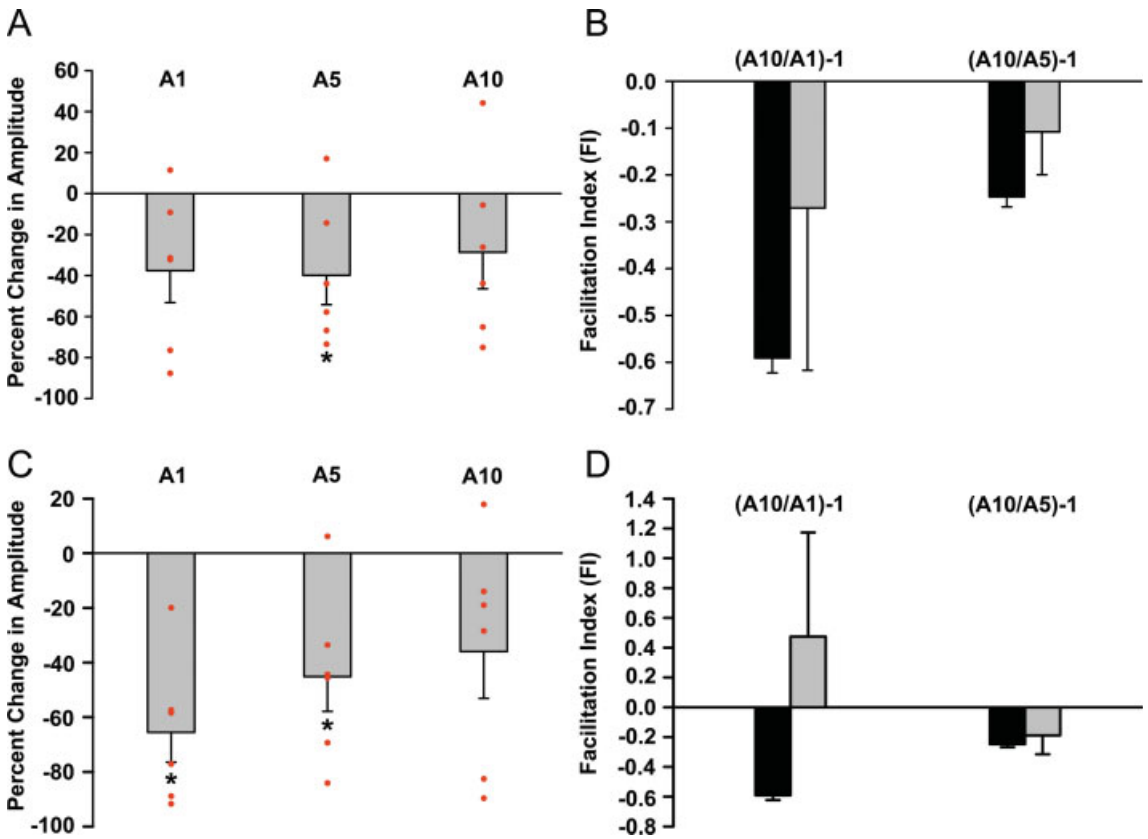

Fig. 13. The effect of heat shock and compromising PMCA and NCX on the Kum strain. (**A**) Examination of heat shock alone on the Kum strain. Heat shock tended to reduce the percent change in five of six preparations, the 1st, 5th, and 10th EPSP amplitudes. The individual preparations are shown as dots superimposed on the mean (±SEM) percent change bar chart. (**B**) Black bars indicate FI in normal saline and gray bars indicate FI after heat shock in normal saline. Facilitation index did not show any significant effect with heat shock. The results before and during heat shock are shown. The effect of heat shock, two-thirds reduced $[Na^+]_o$ and pH 8.8 on the percent change in the EPSP amplitudes resulted in a decrease in six of six preparations for the 1st EPSP, but only five of six preparations for the 5th and 10th EPSPs (**C**) with no general trend in altering the FI from before and during the combined exposure (**D**) where black bars indicate FI in normal saline and gray bars indicate FI after heat shock in low Na^+ and pH 8.8. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

resulted in fewer vesicles being depleted in the 1st response, so one should expect a relatively, enlarged second EPSP amplitude (i.e., facilitation). This was not observed. The second approach to retard NCX function with KB-R7943 did give the expected result of enhanced STD and is likely due to $[Ca^{2+}]_i$ building up in the terminal during repetitive stimulation. However, it is hard to know if KB-R7943 is suitable as a pharmacological drug of choice since it is known to have nonspecific effects.

In addition, there could be various NCX, PMCA, and SERCA isoforms that may produce varied responses and have differening pharmacological properties (Jensen et al., 2007). Although their gene sequences maybe the same, there could be alternative splicing or even post translational modification. Therefore, until these proteins are purified and analyzed for potential modifications, we cannot be sure in these studies that only a single form of these proteins is present in the nerve terminals. Different forms of feedback regulation or modulation of channel isoforms is known. For example, in *Drosophila,* NCX is inhibited by $[Ca^{2+}]_i$ where as other NCX forms are not (Hryshko et al., 1996). This can impact the application of pharmacological agents, as it was shown that KB-R7943 is a weaker inhibitor (four to five times) of the *Drosophila* NCX (CALX1.1) than an NCX isolated in canines (Isaac et al., 2002). Because 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; Rumpal and Lnenicka, 2003; Sobolevsky and Khodorov, 1999) it would be worth to design experiments to specifically address this issue as was done for actions on Ca^{2+} induced inactivation of NCX by expressing the *Drosophila* NCX in a oocyte (Hryshko et al., 1996) or in a cell line where its function can be addressed without complications of other exchangers and unknown channels.

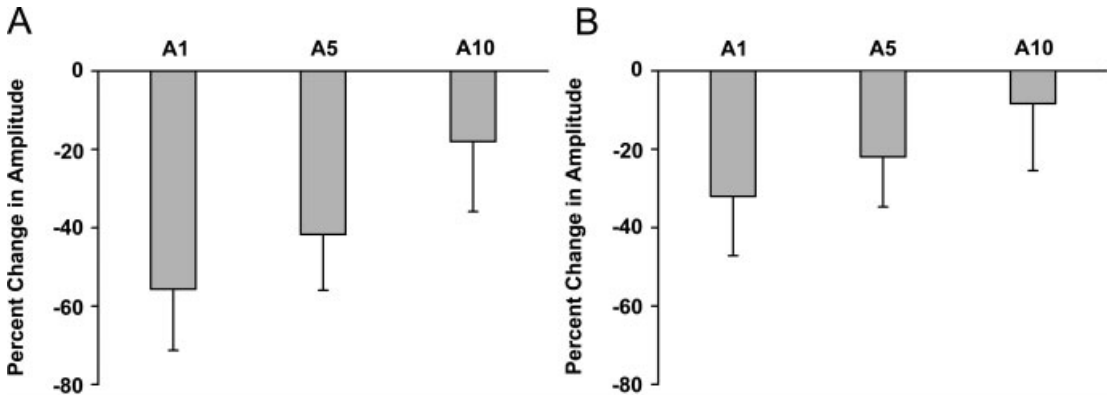

Fig. 14. The effect of heat shock is subtracted from controls and the Kum strain to obtain average effect of the SERCA mutation. (**A**) Effect of heat shock on percent change in EPSP amplitudes for KUM. Effect on CS controls was subtracted from the effect on Kum to obtain these values. (**B**) The effect of heat shock with two-thirds 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.

The two approaches (raised pH or application of CE) for blocking PMCA produced opposite results. We feel it is important to present these findings to allow future studies to consider whether using CE, KB-R7943 or TG in *Drosophila* is appropriate, as well as encouraging others to investigate the mechanisms behind our observations. Direct application of drugs on the exposed NMJ is preferrable than 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. Paradoxical results in application of quabain on *Drosophila* Malpighian tubules occurred due to the unique nature of the cells to actively excrete this organic compound (Torrie et al., 2004). Further studies are needed in isolated *Drosophila* neurons to better identify the pharmacological profiles of the NCX, PMCA and SERCA (Yuan and Lee, 2007).

To attempt to mechanistically explain the results obtained in this study a relatively simple hypothetical model explains the changes in the EPSP amplitudes as well as the occurrences in STD (Fig. 15). 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 a new plateau is reached. Thus, a new homeostatic setting occurs, to regulate SERCA, PMCA, and other Ca^{2+} buffering sources. In this scenario, the high affinity, but low capacity, PMCA and SERCA would have a role in maintaining the new Ca^{2+} equilibrium. When all three transport mechanisms are working normally, there could be a Ca^{2+} induced inhibition of the NCX, thus leaning more on the other mechanisms to maintain the synaptic balance. However, an inhibition of the NCX by KB-R7943 and of PMCA by CE depressed synaptic transmission just as the inhibition of SERCA by TG or with a Kum mutation exposed to heat shock. One possibility is that very high $[Ca^{2+}]_i$ resulted when these protiens are nonfunctional and due to a Ca^{2+} induced inhibition of the Ca^{2+} channel. This proposal is not consistent with an inhibition of PMCA by pH 8.8 which resulted in an increase in vesicle release. Perhaps in the fly NMJ pH 8.8 has other effects beside inhibiting PMCA. We need to know more about the types of Ca^{2+} channels in these nerve terminals ($P\text{-}Ca^{2+}{}_V$, $K^+{}_{Ca}$) and whether 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.

Clearance of Ca^{2+} is controlled by many specialized mechanisms in the neurons and three of the major processes are discussed in this study. NCX, PMCA, and SERCA are modulated and differentially regulated (Thayer et al., 2002). In our study, compromising SERCA by heat shock has raised some intriguing questions of why the EPSPs are reduced and 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. Because initiating action potentials in the muscle were dampened in the heat shocked Kum, they suggested that the function of $Ca^{2+}{}_V$ channels was reduced. They did not report variable changes in EPSP amplitudes as we have shown in the wild type (control) flies by heat shock. In fact, they did not report any effect of temperature on control adults or larvae. Neal et al. (2006) demonstrated less variability in synaptic transmission following heat shock and linked it to the stabilization of channels due to the expression of heat shock proteins.

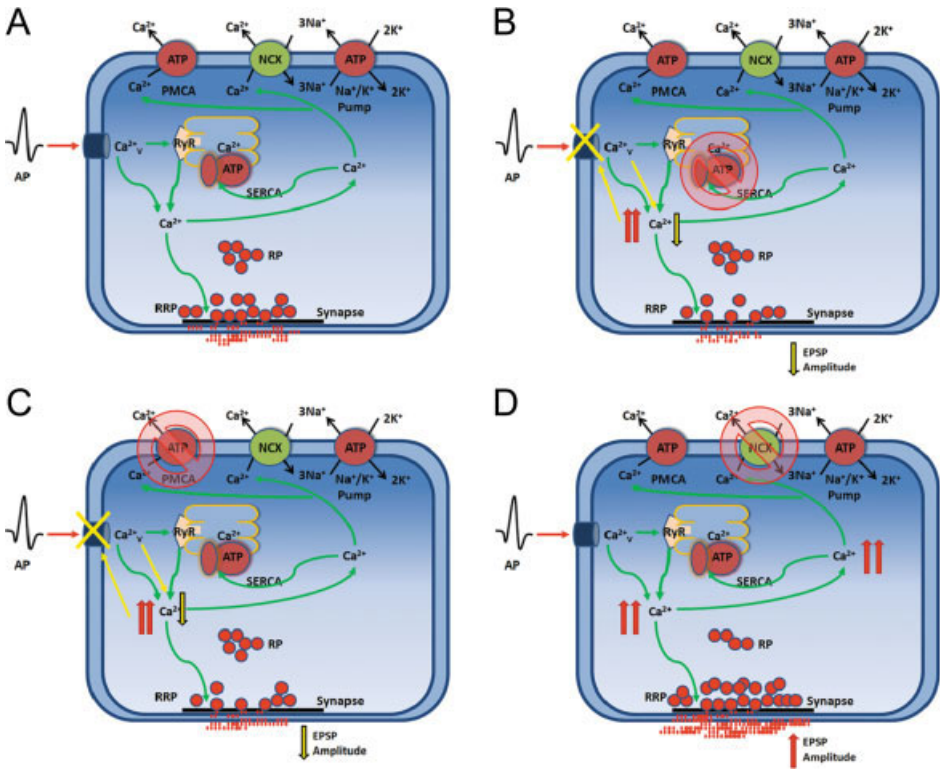

Fig. 15. Model of a presynaptic nerve terminal at the *Drosophila* NMJ. These high-output synapses have many vesicles docked within the ready releasable pool (RRP) and few in the reserve pool (RP). During a short train of stimulations the first action potential will result in the fusion of a majority of the vesicles and release transmitter. Thus subsequent nerve terminal depolarizations will have fewer vesicles primed; therefore, synaptic depression is observed 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.

Functions of NCX may not have a primary role in producing a change in the first EPSP within the train since Ca^{2+} build up was not yet induced. Also the effect in a potentially raised $[Ca^{2+}]_i$ was not able to be compensated by the PMCA or the SERCA. This does raise the question about the specificity of KB-R7943, but its use at least produced a consistent effect on synaptic transmission. *Drosophila* NCX form termed CALX1.1 is recognized for its stability to be negatively regulated by Ca^{2+} in comparison to other NCX proteins (Hryshko et al., 1996; Isaac et al., 2002). ER is now established to have a prominent role in buffering Ca^{2+} both in the nerve terminals and dendrites. Release of Ca^{2+} via ryanodine receptors, which could come from internal Ca^{2+} via Ca^{2+} induced Ca^{2+} release, can have a prominent effect on synaptic transmission or STF and STD (Galante and Marty, 2003; Lauri et al., 2003). Likewise, Ca^{2+} release by IP3 receptors in the ER has been shown to alter neuronal synaptic activity (see review Bardo et al., 2006). The role of the ER in transmission and neuronal plasticity has not been investigated to the same extent in the *Drosophila* NMJ as in vertebrate CNS preparations, but its normal function is essential for larval development (Sullivan et al., 2000).

These high output synapses of the fly NMJ require fast buffering to avoid prolonged vesicle fusion events following an action potential. Also, STD would be prolonged, because in this preparation the depression is due to fewer vesicles being docked and available for subsequent pulses. So with the reduced extrusion or uptake of Ca^{2+} by the ER, one should expect a more pronounced depression if it is at all possible to overcome the structural limitations in the number of vesicles to dock and be primed. Since there is no consistent changes in the STD index for treatments with KB-R7943, CE, pH 8.8 or in the Kum with heat shock, vesicles are likely to dock but may have increased recruitment from the RP to the RRP to compensate the offset in STD. The high output synapses with complex synaptic structure maybe more prone to STD than lower output synapses but only if vesicle docking is also enhanced at the high output sites. Otherwise with initially fewer vesicles docking sites bound, more vesicles 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 reconstructions of the physiologically recorded terminals were performed (Cooper et al., 1995a,b,c, 1996a; Johnstone

et al., 2008). Imaging of vesicle dynamics in living terminals could help to delineate potential mechanisms in differences in vesicle RP pools being recruited to RRP based on activity and synaptic complexity; however, one has to insure physiological correlation since vesicles can recycle empty at the *Drosophila* NMJ (Kidokoro et al., 2004). STD is very likely not a rundown of ATP stores, but maybe partially based on the synaptic structure and vesicle dynamics. Logsdon et al. (2006) showed that glutamate depleted RRP vesicles were rapidly mixed with RP vesicles by 5-HT action at a depressed crayfish NMJ.

Lnenicka et al. (2006) showed with imaging of Ca^{2+} signals, within the *Drosophila* nerve terminals, that the primary source for $[Ca^{2+}]_i$ buffering in the terminal 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 higher temperature to comprise SERCA function without killing the larvae. A calcium binding protein frequenin is present in the *Drosophila* nerve terminals (Rivosecchi et al., 1994) could also be of interest to see if its expression is differentially regulated depending on Ca^{2+} load of the terminals over time.

The NMJ of *Drosophila* larvae has 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 (Harata et al., 2001; Matthews, 2004; Msghina et al., 1999; Parsons et al., 1999; Pyle et al., 2000). So, it is not surprising that modification in NCX, PMCA, and SERCA to have a significant role. It was shown in *Drosophila* NMJs on m6 and m7 that CICR mobilized the dense core vesicles through actions on CaMKII (Shakiryanova et al., 2007). Because these high output NMJ show STD, there is likely to be a significant role of the RP to be mobilized to the RRP with repetitive train stimulation, as shown in hippocampal neurons (Goda and Stevens, 1998), but how much Ca^{2+} is directly involved is still not fully resolved. Ca^{2+} plays a role in the endocytic process (Balaji et al., 2008; Palfrey and 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, vesicles have been shown to have little significance in 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., 1996a,b).

It would be of interest to learn if chronic manipulation of NCX, PMCA, or SERCA would result in developmental abnormalities in nerve terminal growth and or modifications at the synaptic level. NMJ of larval *Drosophila* is an ideal preparation to investigate developmental questions related with synaptic function as the terminals grow along with the muscle 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 and Murthy, 2003). When $Ca^{2+}{}_V$ channels were chronically compromised, nerve terminal did not compensate to maintain synaptic efficacy (Xing et al., 2005). Perhaps the same is true for a higher load of $[Ca^{2+}]_i$. 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 could then alter gene regulation (West et al., 2001). The actions could be broad in this sense from mechanisms that promote LTP or LTF 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). So, one would need to be cautious in interpreting chronic manipulations in Ca^{2+} regulatory processes such as with SERCA, NCX, or PMCA.

Physiological studies are commonly compared across species to look for general phenomena and mechanisms of function; however, one should be cautious in comparisons of pharmacological and various other experimental manipulations. Even within species comparisons need careful consideration as it is becoming well known that many receptor subtypes are possible because of alternative splicing which may show varied pharmacological profiles (Kishore and Stamm, 2006; Krobert and Levy, 2002; Walther et al., 2003).

As a result of the various procedures to target the NCX, PMCA, and the SERCA, in *Drosophila*, we learned that some of the results obtained were not as predicted. So, we are in a conundrum in choosing which procedures might be working as predicted and which ones might have caused nonspecific effects related to the original target of interest. One approach is to compare with other arthorpod species and determine if similar findings are obtatined. From our recent work with crayfish NMJ, we predict that compromising the NCX function in the crayfish and *Drosophila* nerve terminals with lowered $[Na^+]_o$, is risky since the $Ca^{2+}{}_V$ channels might be teetering in reducing the amount of time and total Ca^{2+} current that enters the presynaptic nerve terminal with 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.

This was not as drastic in the crayfish NMJ, which is likely due to the fact that the NMJs in the *Drosophila* preparation are of a high-output type as compared to the low-output terminals in the crayfish preparation (Atwood and Cooper, 1995). As for the future studies to address this synaptic explanation, it would be beneficial to examine the effect of lowered $[Na^+]_o$ on high-output synapses in a crayfish NMJ. Extensor muscles in the walking leg of the crayfish would be one such 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 of 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). These are ligand-gated ion channels (Dudel et al., 1992; Heckmann and Dudel, 1997) where Na^+ is the primary ion to produce depolarization of the muscle. Also, some Ca^{2+} and a small amount of K^+ flux occurs across the ionotropic receptor (Dudel et al., 1992). The receptor has a reversal potential of 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.

When PMCA was compromised by pH 8.8 and CE application opposite results were obtained in *Drosophila* NMJ preparation, but both procedures gave the same results at the crayfish NMJ in enhancing the EPSP amplitudes. 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.

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 decreased EPSP amplitudes in the *Drosophila* NMJ, but in the crayfish NMJ the EPSPs increased in amplitude as expected if $[Ca^{2+}]_i$ increased. This is a multicomplex issue at the *Drosophila* NMJ because we now have reason to think that the Kum mutation is not specific for the SERCA function and that heat shock produces substantial alteration in the synaptic transmission with many unknown potential mechanisms. As addressed in the first report by Sanyal et al. (2005), the 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 we have in control flies with heat shock. In fact, they did not report any effect of temperature on the wild type (control) adults or larvae. The reason why TG decreased EPSP amplitudes in *Drosophila* NMJ and not in crayfish NMJ, is not known. Since it is not feasible to obtain recovery from exposure to TG in the time frame needed, there could be nondescript 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 in the bundles of fibers for the opener muscle. Also the terminal varicosities are more exposed in the *Drosophila* NMJs. Perhaps due to its lipid soluble nature, TG had a greater effect on the *Drosophila* NMJs and organelles than the crayfish and resulted in problems not detected in our experiments. The resting membrane potential of the muscle fibers in both crayfish and *Drosophila* was not altered by TG exposure. The actions at the crayfish NMJ were as expected and we 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.

The comparative approach using two NMJ models is helpful to emphasize species differences and to be cautious of extraplolating observations to mammalian

TABLE II. Summary of results for the crayfish walking leg opener muscle and Drosophila NMJs

	Crayfish NMJ		*Drosophila* NMJ	
Treatment	EPSP amplitude	Facilitation	EPSP amplitude	Depression
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 three channels	↑	NS	↓	NS

NS, non significant effect.

preparations. There are many new avenues to proceed from the observations and speculations proposed in this study.

A composite of results for the crayfish walking leg opener muscle and *Drosophila* NMJs are presented in Table II.

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