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THE REGULATION AND PACKAGING OF SYNAPTIC VESICLES RELATED TO RECRUITMENT WITHIN CRAYFISH AND FRUIT FLY NEUROMUSCULAR JUNCTIONS: VARIATIONS IN LOW- AND HIGH-OUTPUT TERMINALS

Wenhui Wu
University of Kentucky, wuwenhui2@gmail.com

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Wenhui Wu, Student

Dr. Robin L. Cooper, Major Professor

Dr. David Westneat, Director of Graduate Studies

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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

Wenhui Wu

Lexington, Kentucky

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

Lexington, Kentucky

2013

ABSTRACT OF DISSERTATION

THE REGULATION AND PACKAGING OF SYNAPTIC VESICLES RELATED TO RECRUITMENT WITHIN CRAYFISH AND FRUIT FLY NEUROMUSCULAR JUNCTIONS: VARIATIONS IN LOW- AND HIGH-OUTPUT TERMINALS

Glutamate is the main excitatory neurotransmitter in the CNS and at the neuromuscular junctions (NMJs) of invertebrate. The characteristic similarities to CNS glutamatergic synapses in vertebrate and the anatomical simplicity of invertebrate NMJs favor the investigation of glutamatergic synaptic functions in this system. This dissertation mainly aimed to physiologically separate two functional vesicle groups, the reserve pool (RP) and readily releasable pool (RRP) within presynaptic nerve terminals of *Procambarus Clarkii* and *Drosophila melanogaster.* This was addressed in part by blocking the vesicular glutamate transporter (VGlut) with bafilomycin A1**.** Various frequencies of motor nerve stimulation, exposure time, and concentration of bafilomycin A1 were examined. The low-output tonic opener NMJs in crayfish exposed to 4μM bafilomycin A1 and 20Hz continuous stimulation decreased the EPSP amplitude to 50% in ~30min with controls lasting 3h. After activity and bafilomycin A1-induced synaptic depression, the EPSPs were rapidly revitalized by serotonin (5-HT, 1μM) in the crayfish preparations. The 5-HT action can be blocked almost completely with a PLC inhibitor, but partially with a cAMP activator. The higher output synapses of the larval Drosophila NMJ when stimulated at 1Hz or 5Hz and exposed to 4μM of bafilomycin A1 showed a depression rate of 50% within ~10min with controls lasting ~40min. After low frequency depression and/or exposure to bafilomycin A1 a burst of higher frequency (10Hz) can recruit vesicles from the RP to the RRP. Physiological differences in low- (tonic like) and high-output (phasic like) synapses match many of the expected anatomical features of these terminals, part of this dissertation highlights physiological differences and differential modulation and/or extent of the vesicles in a RP for maintaining synaptic output during evoked depression of the RRP in crayfish abdomen extensor preparation. With the use of bafilomycin A1, the tonic terminal is fatigue resistant due to a large RRP, whereas the phasic depresses rapidly upon continuous stimulation. Upon depression of the tonic terminal, 5-HT has a

large RP to act on to recruit vesicles to the RRP; whereas, the phasic terminal, 5-HT can recruit RP vesicles to the RRP prior to synaptic depression but not after depression.

KEYWORDS: neuromuscular junctions, reserve pool, readily releasable pool, bafilomycin A1, 5-HT

Wenhui Wu

Student Signature

July 10, 2013

Date

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By

Wenhui Wu

Robin L Cooper

Director of Dissertation

David Westneat

Director of Graduate Studies

July 10, 2013

Date

FOR MY LOVELY DAUGHTER, MANDY WU GUO
MY DEAR HUSBAND, HOUFU GUO
AND MY VENERABLE PARENTS,
QIULIANG WU AND QIAOYUN CHENG
ALL OF YOU MAKE MY LIFE WONDERFUL

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CHAPTER ONE

Background and Significance

General Introduction

The nervous system is considered as one of the most complex and important physiological systems in an animal. It controls and regulates the activities of all the other systems, in order to coordinate the whole body functioning as a single entity. One particular example is the indispensable role of the nervous system in the movement of skeleton musculature. This process can be simply illustrated in a neuronal circuit **(Figure1.1),** which generally contains four components: sensory neuron, central nervous system (CNS), motor neuron and skeletal muscle. By sensing either the internal or external stimuli, sensory neuron sends signals into CNS. Here the signals are intergraded. The CNS output is through motor neurons to control the activity of skeletal muscles.

The specific synapses where motor neurons communicate to muscle fibers are at neuromuscular junctions (NMJs) **(Figure 1.1).** The presynaptic terminal, the ending of the motor neuron, directly controls the postsynaptic muscle activity by regulating the amount of vesicular neurotransmitter released, in response to action potentials (AP) within the motor axon. Due to the fact that NMJs can be easily accessed in crayfish and their characteristic of non-spiking excitatory postsynaptic potentials (EPSPs) (Wiersma and Van Harreveld, 1938; Katz and Kuffler, 1946) fundamental synaptic processes can be examined. These preparations can provide significant insights to better understand more complicated synapses in mammalian CNS, because the NMJ at the crayfish is similar in regards to the graded electrical signal generated in postsynaptic dendrites in mammalian CNS, although the crayfish synapses are more accessible for direct monitoring of quantal events.

At the presynaptic terminal of a NMJ, an AP depolarization activates voltage gated Ca^{2+} channels near synaptic active zones. This triggers primed vesicles to fuse with the presynaptic membrane and release neurotransmitter. In the case of the crayfish and *Drosophila* NMJs, the transmitter is glutamate which produces depolarization of the muscle fiber through ligand-gated ionotropic receptors.

Vesicle pools

In the early 1960s presynaptic vesicles were placed into different groups based on their spatial distribution and physiological properties (Birks and MacIntosh 1961; Elmquist and Quastel 1965). Various nomenclatures have been used; therefore, no absolute definitions have been established with anatomical

distinctions (Li and Schwarz 1999; Kuromi and Kidokoro 2000; Palfrey and Artalejo 1998; Rizzoli et al. 2003; Rizzoli and Betz 2005; Rosenmund and Stevens 1996; Fdez and Hilfiker 2006). Vesicles are distributed inside a nerve terminal without a clear anatomical line to separate RRP from RP. Nevertheless, the physiological and anatomical separation of a RP and RRP is widely utilized for working models to explain and study synaptic transmission (Ruiz et al. 2011). RRP is defined as a group of vesicles close to the synaptic active zones which will fuse and release neurotransmitter responding to Ca^{2+} influx, while the RP is a group of vesicles slightly further away from the synaptic face. Kidokoro et al. (2004) defined an immediately releasing pool (IRP) at *Drosophila* NMJs as distinctly different from a RRP based on vesicles docked for initial fusion and as compared to ones that can undergo rapid exocytosis and endocytosis. Generally, the IRP is grouped within the RRP (Aravanis et al. 2003; Sudhof 2004) and for our purposes, we grouped these two pools together as we examined a longer term exocytosis and endocytosis processes. The vesicles in the RP are recruited under particular circumstances such as high frequency stimulation or in the presence of neuromodulators like 5-HT for crayfish NMJs (Wang and Zucker 1998; Quigley et al. 1999; Logsdon et al. 2006). With transmission electron microscopy (TEM), Johnstone et al. (2008, 2011) showed the structure of a crayfish nerve terminal with single vesicle resolution and an approach to estimate a range, with incorporating stereological errors, in measuring the location of vesicles in regards to the presynaptic membrane **(Figure 1.2)**. Rarely are errors in location or distance dealt with from measurements utilizing TEM; however, such considerations are valuable (Atwood and Cooper 1996a; Feuerverger et al. 2000; Kim et al. 2000). Since a number of disease states afflicting humans are related to presynaptic function, it is of interest to understand the fundamental properties that are most likely common to all animals as well as those that are different (Waites and Garner 2011).

Several experiments have used various ways to physiologically separate vesicle pools. Bykhovskaia et al. (2001) and Rosenmund and Stevens (1996) used high osmolarity shock to deplete RRP. Such osmotic pressure changes on the nerve terminal were shown in the 1960s to cause transmitter release (Hubbard et al. 1968). Millar et al. (2002) employed a rapid vesicle depletion technique (200Hz train stimulation) with a Cs^{+} substitution, to deplete docked vesicles in crayfish tonic and phasic extensor NMJs. Schneggenburger et al. (1999) also demonstrated, in synapses at the Calyx of Held, that only a small fraction of the RRP is used by a single presynaptic action potential under physiological conditions but with a large percentage of vesicles that can be induced to fuse to the membrane if presynaptic calcium rises quickly. Akbergenova and Bykhovskaia (2009) identified RRP in *Drosophila* NMJ from TEM micrographs. In their study, the recycled vesicles, loaded with FM1-43, after intense stimulation were primarily localized within a group close to the synapses. Also, using a mutation in *Drosophila* to block recycling of vesicles revealed two distinct pools of vesicles (Kuromi and Kidokoro 1998). In previous research in our laboratory, Logsdon et al. (2006) depleted the vesicles in RRP in crayfish opener NMJ by moderately stimulating a motor nerve axon in the presence of TBOA (DL-threo-β-

Benzyloxyaspartic acid), an inhibitor of a glutamate transporter (GluT) located on the plasma membrane. After synaptic depression occurred, it was demonstrated that the depressed state could be reversed by adding 5-HT. The interpretation is that 5-HT had the ability to promote the recruitment of vesicles from RP into RRP (Logsdon et al. 2006). Dudel (1965b) first showed that synaptic transmission is enhanced at the crayfish NMJ by 5-HT and since then there have been a number of studies addressing the potential mechanisms of action of 5-HT (Dixon and Atwood 1985, 1989; Enyeart 1981; Glusman and Kravitz 1982). The compiled findings indicated that two vesicle pools can be distinguished physiologically with one pool as the RRP and the other RP that can be recruited by 5-HT into the RRP.

Vesicle recycling

Generally, there are three different proposed processes for vesicle fusion with presynaptic membrane. The first process is exocytosis, in which one single vesicle fuses completely into plasma membrane. This results in the release of all the contained neurotransmitter (i.e. producing a quanta of neurotransmitter) which is followed by the formation of a new empty vesicle through endocytosis. In this case, the turnover and insertion of vesicular proteins into newly formed vesicles is critically determining the available vesicles for future neuronal activity. A second proposed model is called kiss-and-run. Here, instead of complete membrane fusion the vesicle only forms a transient pore, such that it releases some fractions of neurotransmitter. The neck of the fusion pore pinches off and the vesicles are repackaged with neurotransmitter. The third model is multiple-fusion pore which was first demonstrated in frog hair cells. Unlike single vesicular fusion, a multiple-fusion pore is characterized as several vesicles fuse together and release multi-quanta in response to a single AP in the terminal. Since a multiple-fusion pore model has not been described in crayfish preparation as quantal measures do not indicate this phenomenon to be present, I will only consider complete exocytosis and kiss-and-run in this proposal. Recently it is thought that multiple endocytotic vesicles can occur with rapid stimulation (Cheung et al., 2010), so I might have to be cautious in my observations with high stimulation frequencies.

No matter how the vesicles fuse, after release of neurotransmitter, both newly formed and recycled vesicles have to be packed with neurotransmitter, in order to be utilized in the subsequent responses. So, vesicular recycling is fundamentally controlling the synaptic efficacy.

It is postulated that new vesicles form the RP from an endosome and are packaged with a neurotransmitter. It is implied that the RP can be spared and may not intermix with the RRP upon high stimulation frequency (Kuromi and Kidokoro 1998). Based on this view, Logsdon et al. (2006) proposed for the crayfish opener excitatory motor neuron that with repetitively high frequency stimulation, vesicles can be quickly recycled within RRP and be depleted prior to

recruiting from the RP. Thus, synaptic depression occurs due to the depletion of filled vesicles in RRP, while most vesicles in RP remain fully packaged with neurotransmitter. When evoked EPSPs, that showed depression, are rescued with 5-HT, not only will the evoked EPSPs grow in amplitude but also the spontaneous quantal events become intermixed in size. Normal sized quantal events induced by 5-HT and reduced ones from the depressed state appear together as compared to just the smaller sized quanta responses during depression. A working model to explain this phenomenon was put forth, such that fully packaged vesicles from the RP are recruited, by actions of 5-HT, to the RRP which contains partially filled vesicles that are rapidly recycling during the high stimulation frequency.

Glutamate packaging in recycling vesicles

There are two main sources of glutamate that can be utilized to fill recycling vesicles in the nerve terminals. One is the glutamate directly taken back up from synaptic cleft through the plasma membrane transporter (GluT). This is the glutamate that was released during synaptic transmission. A second source of glutamate is the glutamate already in the cytoplasm within the nerve terminals that might not have been immediately taken up across the membrane by synaptic activity.

In the vertebrate central nervous system one common means of glutamate recycling is the synaptically released glutamate being taken up by glia cells and converted to glutamine followed by the glia cell releasing glutamine. This glutamine is then transported back into the nerve terminal. The glia recycling process involves excitatory amino acid transporters (EAATs) on the glia membrane. Once the glutamate is in the glia cells it is converted into glutamine by glutamine synthetase (GS). Then, glutamine is transported out of glial cells and the presynaptic terminal takes up glutamine through a glutamine transporter in the plasma membrane. In the nerve terminal, glutamine is converted back to glutamate. Five EAATs (1-5) have been identified so far, each of them has its preferential expression pattern (Rothstein et al., 1994; Danbolt, 2001). EAAT2 and EAAT3 are the major ones expressed in glia and neurons respectively in many brain regions, such as cerebralcortex and hippocampus (Lee et. al 2010). EAAT3 are located in presynaptic regions of both glutamatergic and GABAergic (Rothstein et al., 1994; Danbolt, 2001), which also plays an essential role in taking up extracellular glutamate. At the crayfish and *Drosophila* NMJs, as compared to the vertebrate CNS, glutamate does not cycle through a glutamine path via a glia cell. Therefore, instead of being transported through glial cells and glutamine transporters, glutamate in synaptic cleft is taken back up into the terminal only through Glutamate transporter (GluT) located in the presynaptic membrane.

Previously published research, from our research group (Logsdon et al., 2006), has shown an accelerated synaptic depression in the presence of the glutamate

transporter inhibitor TBOA. This past research was conducted in crayfish opener preparations with comparing the depression time in normal crayfish saline to terminals treated with TBOA. TBOA treated preparations were on average depressed 2 hours sooner. The results indicate a significant role of glutamate uptake from synaptic cleft in the process of refilling recycled vesicles. Because two sources of glutamate are available for vesicle packaging, it is reasonable that cytoplasmic glutamate may also be important. As a result, TBOA treatment does not seem like the most favorable drug to trigger a substantial synaptic depression in a fast manner. In my first project, I tested the potential role of bafilomycin A1, V-APTase inhibitor, in triggering synaptic depression in both crayfish and *Drosophila* NMJs (Chapter 4 and 5). I expected a better blockage in the repackaging of recycling vesicles by preventing the usage of internal glutamate within the nerve terminal prior to synaptic release and reuptake.

V-ATPase and Bafilomycin A1

The molecular mechanisms describing the process of neurotransmitter packaging in vesicles have been investigated over the years. Here I highlight the current working model. The vacuolar ATPase (V-ATPase hydrolyzes ATP and pumps cytoplasmic protons into a vesicle (Nelson, 1991, 1992). This elevates the proton electrochemical gradient inside of vesicle, which then energizes an exchange of a proton with cytoplasmic glutamate via a vacuolar glutamate anti-porter (VGLUT) (**Figure 1.3**). This process can be facilitated by chloride binding to the allosteric site in VGLUT as suggested by Juge (Juge et al, 2010). However, acetoacetate, a metabolite of fat, competes with Cl^- for the binding site, hinders glutamate transport. This would decrease glutamate content in each single vesicle. Eventually, postsynaptic responses are reduced due to the lower amount of packaged neurotransmitter. This paper proposed that the competitive binding of fat metabolites in VGLUT is a possible mechanism to explain why high fat diet can be used to release seizures in epilepsy patients. From this point of view, my first project, using bafilomycin A1 to trigger synaptic depression, can also serve as another way to decrease the synaptic strength by preventing repackage of recycling vesicles.

V-ATPase is a trans-membrane protein containing extracellular catalytic domain and intracellular proton transduction domain (**Figure 1.4**). It not only plays an extremely important role in packaging neurotransmitter in synaptic vesicles, but it is also responsible for the acidification of intracellular compartments such as endosome and lysosome. These processes are crucial for intracellular membrane traffic, protein degradation, clathrin-coated endocytosis and apoptosis (Zhang et al, 1994; Bowman et al, 2004).

Bafilomycin A1 belongs to macrolide antibiotics family in which “bafilomycins” and “concanamycins” were identified as specific V-ATPase inhibitors in 1988 (Bowman et al, 1988). This has provided scientists useful pharmacological tools to investigate the function of compartmental acidification. It is a compound used

in studies involving animal and plant tissues. It was suggested having the potential to treat various human diseases such as respiratory infection, pancreatic cancer, and forms of malaria. Therefore, my study of bafilomycin A1 in synaptic transmission in crayfish may aid to better understand the possible effects of this drug on the mammalian nervous system.

Pharmacological studies using V-ATPases from *Neurospora crassa*, chromaffin granules and corn vacuoles determined an affinity for bafilomycin A1 at nanomolar concentration (Bowman et al, 1988). However, concentrations ranging from nanomolar to micromolar have been used in different in vivo experiments. Due to its hydrophobic property, I would expect the drug to accumulate in plasma membrane easily which may affect its accessibility to the internal organelles. Tapper and Sundler (1995) suggested a higher concentration, rather than the one used to inhibit ATPases in the plasma membrane, is needed to inhibit lysosomal acidification in macrophages. In addition, Dijkstra et al. (1994) also reported that 10μM is able to achieve half maximal inhibition of Malpighian tubules in an ant species (*Formica)* (Drose and Altendorf, 1997). Cavelier and Attwell (2007) applied 4 μM bafilomycin A1 in a slice of a mouse brain with at least 2.5 hours incubation for conducting for their experiments.

To my knowledge no attempts, besides my preliminary trials, have applied bafilomycin A1 to crayfish preparations. It is universally toxic to all cellular V-ATPase proteins at nanomolar concentration. However, E type ATPase such as Ca^{2+} ATPase in sarcoplasmic reticulum and the Na-K pump in plasma membrane shows toxicity to bafilomycin A1 in the micromolar range. Also prolonged incubation causes cell death (Hall, 1994; Manabe et al, 1993). The past observations would suggest specific attention is needed in the use of bafilomycin A1. From this point of view, a dose-response curve related to physiological actions will provide me with a general idea about which concentration is relatively suitable to carry out the maximal V-ATPase inhibition and minimal toxicity to the crayfish and *Drosophila* preparations.

Phasic (high-output) and tonic (low-output) terminals

Basically, the role of motor neurons is to control animal behavior via communication with muscles. The “fast” and “slow” muscle contractions were first observed in crab and crayfish closer muscle (Lucas, 1907, 1917). Since then, similar contractile differentiation was described in many other muscle types such as crayfish abdomen flexors (Kennedy and Takeda, 1965a, b), crayfish limb extensors (Van Harreveld and Wiersma, 1936) as well as *Drosophila* abdominal longitudinal muscles (Kurdyak et al, 1994). “Fast” muscle contractions initiate quick response such as tail flip response observed in crayfish while “slow” contractions maintain the slow movements as with the maintenance of certain postures (Bradacs et al, 1997). Corresponding to the “fast” and “slow” muscle contractions, the “phasic” (high-output) and “tonic” (low-output) are broadly utilized to describe the motor neurons in crayfish system while in *Drosophila*, Is

and Ib are named based on the size of the terminal varicosities. The details of the terminal structures are described in chapter 2, 4 and 5.
Even though postsynaptic myofibrillar protein isoform expression is importantly contributing to the contractile differences, the different rate and timing of muscle contractions is also related to presynaptic differences in synaptic structure and synaptic strength. Synaptic structure directly affects synaptic strength which is determined by the efficacy of neurotransmitter release from the presynaptic nerve terminal, which in turn is affected by the number of releasable vesicles and the rate of vesicle recycling. The detailed known similarities and differences of these two motor neuron terminals in crayfish leg extensor are described in chapter 4. There has not been much emphasis in addressing vesicle cycling between RRP and RP in these types of nerve terminals. This can be done by depleting RRP with continuous stimulation while blocking the repackaging of glutamate into the vesicles, and then recruiting RP vesicles with 5-HT as I performed in crayfish abdomen extensor preparations. Based on my observations, I hypothesize that tonic terminals can tolerant continuous stimulation longer, and show more profound effect induced by 5-HT for recruiting a RP to the RRP.

5-Hydroxytryptamine

5-Hydroxytryptamine (5-HT) is a common biogenic amine found in both vertebrates and invertebrates. Besides being a neurotransmitter and neurohormone, it serves as an important neuromodulator in most animal species. 5-HT binds to its receptors, mostly transmembrane GPCRs, which then can activate or inhibit different second messenger cascades. 7 families, 14 subtypes of 5-HT receptors have been identified in vertebrate and four subtypes have been cloned and characterized in *Drosophila melanogaster* (Blenau and Baumann, 2001; Saudou and Hen, 1994). Physiological and pharmacological studies suggested that there might be similar large number of 5-HT receptors present in crustacean even though only two have been cloned and characterized (Clark et al., 2004; Spitzer et al., 2004, 2005).

At the NMJs of crayfish, *Procambarus clarkii,* vertebrate 5-HT_2-like receptors were physiologically identified (Tabor and Cooper, 2002). 5-HT is released into hemolymph from the nerve endings in the 2th thoracic roots and the pericardial organs (Beltz and Kravitz, 1983). It has been known to enhance synaptic transmission since 1960's (Dudel, 1965b), but the mechanism is not fully understood. cAMP was first suggested to be involved in 5-HT action by Battlelle and Kravitz(1978). Later, Dixon and Atwood (1989) demonstrated the role of IP3 in inducing 5-HT effects. It has also been shown that 5-HT can enhance the probability of releasing and increase the releasing sites (Southard et al., 2000; Strawn et al., 2000).

Since vertebrate 5-HT_2 receptor family activates phospholipase C (PLC) (Saudou and Hen, 1994) and similar receptors are present in crayfish NMJs, I examined

whether a PLC inhibitor can attenuate 5-HT effect on enhancing synaptic transmission. In the study described in chapter 4, a non-selective PLC inhibitor U73122 was used to address this topic. An inactive analog U73343 served as a negative control. U73122 is widely used in biochemical and physiological studies with the range from micro-molar to mili-molar, but it has not previously been used in crayfish preparations.

In summary

To test these hypotheses, the crayfish opener muscle preparation (intermediate type of terminals), *Drosophila* abdominal longitudinal muscle M6, M7 preparation (high-output terminals) and the abdomen extensor muscle preparation (high-output and low-output terminals) were utilized. The details of using these preparations as working models are illustrated within chapters 2, 3, 4 and 5.

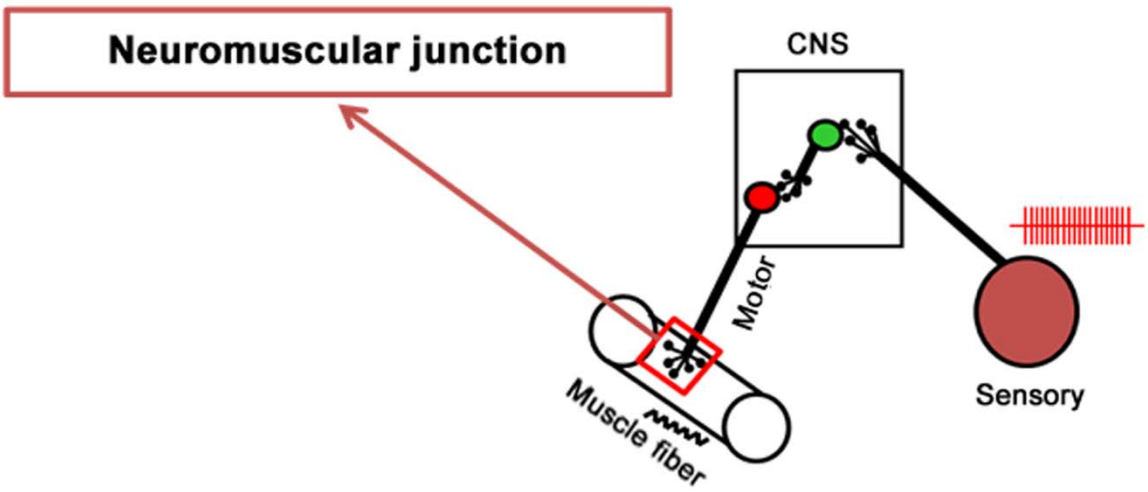

Figure 1.1: General schematic structure of neural circuit. A general neural circuit contains four components: sensory neuron, central nervous system (CNS), motor neuron and skeletal muscle.

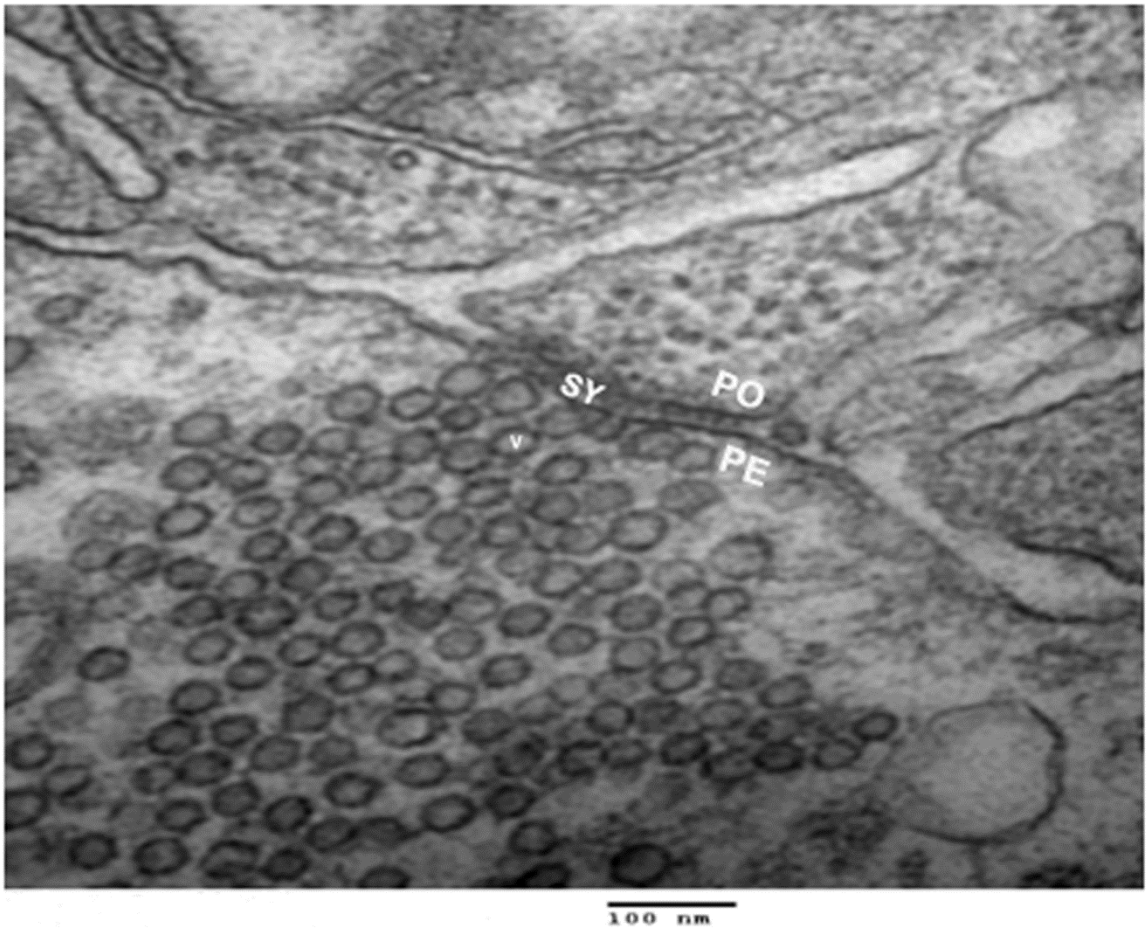

Figure 1.2: TEM view of synaptic terminal in crayfish extensor NMJ. V: vesicles; PE: presynaptic; PO: postsynaptic; SY: synapse. Synaptic vesicles are randomly distributed in the presynaptic terminal, with few dense body vesicles. (Provided by Dr. Andrew Johnstone while conducting his doctorate in the Cooper laboratory).

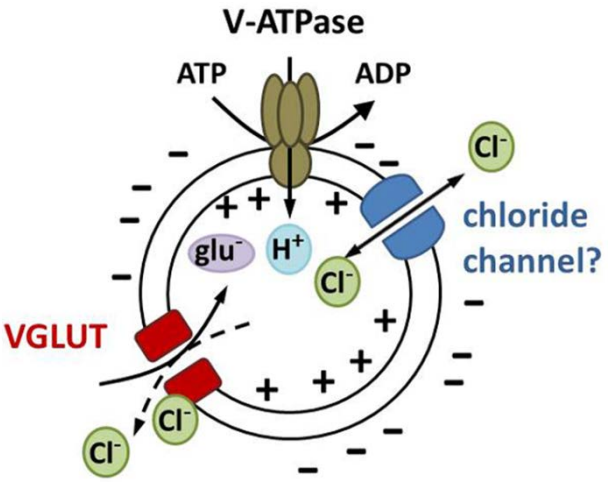

Figure 1.3: A schematic structure of single synaptic vesicle. V-ATPase pumps H+ into vesicle, the increased electro-chemical gradient exchange with a plasma glutamate through a VGLUT antiporter. This process is facilitated by chloride binding to the allestoric site in VGLUT. (Figure modified from Juge, N. et al, 2010)

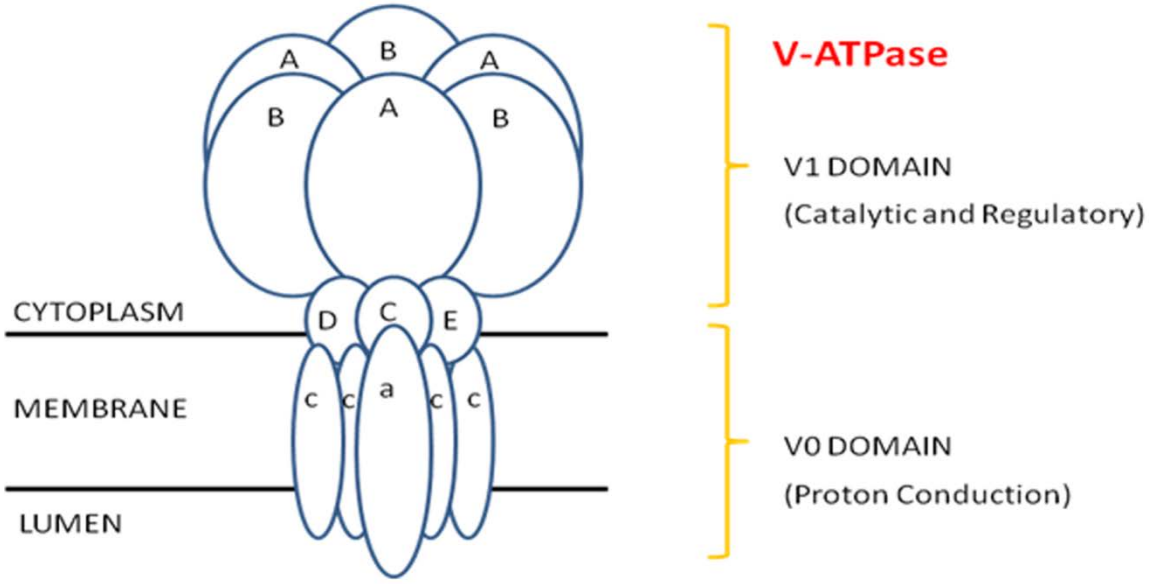

Figure 1.4: The schematic structure of vacuolar ATPase (V-ATPase). It contains two domains: V1 is the catalytic and regulatory domain and V0 is proton conduction domain. (Figure obtained from Nelson,1991,TiPS).

CHAPTER TWO

Physiological Recordings of High and Low Output NMJs on the Crayfish Leg Extensor Muscle

Introduction

This chapter has been published in Wu and Cooper (2010) and is reproduced here for completeness of the dissertation.

Motor neurons communicate with a muscle fiber at synapses which are collectively referred to as a neuromuscular junction (NMJ). NMJs can be accessed easily in most crayfish muscle preparations. Many of the crayfish NMJs demonstrate non-spiking excitatory postsynaptic potentials (EPSP) similar to the graded electrical signals generated in postsynaptic dendrites within the mammalian CNS or subthreshold responses noted in vertebrate NMJs (Wiersma and Van Harreveld, 1938; Katz and Kuffler, 1946). The crayfish NMJs can serve as fundamental synaptic models to provide general insights into synaptic transmission and synaptic differentiation.

Generally, motor units regulate aspects of animal behavior via the type of synaptic communication at NMJs and properties of the muscles. Since the first observation of "fast" and "slow" muscle contractions in crab and crayfish closer muscle (Lucas, 1907, 1917), similar muscular contractile differentiation has been described in other crayfish muscle types such as abdominal flexors (Kennedy and Takeda,1965a, b) and limb extensors (Van Harreveld and Wiersma, 1936). "Fast" contractions initiate quick responses. For example, the crayfish tail flip is a fast behavior. "Slow" contractions maintain slow movements and help maintain posture (Bradacs et al., 1997). Corresponding to "fast" and "slow" muscle contractions, "phasic/high output" and "tonic/low output" are broadly used to describe the motor neurons. The difference in rate and timing of muscle contraction is in part related to presynaptic differences in synaptic structure and synaptic strength (King et al., 1996). Myofibrillar protein isoform expression is also important in contractile differences, but in preparations like that of the leg extensor muscle, in which a given fiber is innervated by both types of motor neurons, the focus is on synaptic differences of the terminals, since the terminals share the same target cell (Mykles et al., 2002). An earlier study examined the two excitatory motor axons of the leg extensor and described the phasic and tonic phenotypes (Bradacs et al., 1997). In this report, we demonstrate how to perform the dissection and obtain recordings so that others can further investigate the properties of the synaptic differentiation of these nerve terminals.

Viewed with transmission electron microscopy, various series of sections obtained from the tonic and phasic terminals on crayfish leg extensor muscle revealed that tonic terminals contain more RRP vesicles than phasic terminals; the mitochondria are more prevalent in tonic neurons, and the synapses on phasic terminals are more complex than those on the low-output synapses, since they contain multiple active zones with varied spacing (Miller et al, 2002; Johnstone et al., 2008; King et al., 1996; Bradacs et al., 1997). The low-output tonic terminals are also more susceptible to enhancing synaptic transmission with the neuromodulator serotonin (5-HT) than are the phasic terminals (Cooper et al., 2003).

The fact that the tonic and phasic NMJ are present on the same muscle fiber makes it easier to assess presynaptic differences on a given muscle fiber and to address questions of muscle fatigue, synaptic depression and synaptic cross talk. Various questions remain to be addressed in this preparation, such as whether there are differences in the postsynaptic receptor density and glutamate receptor subtypes in postsynaptic targets for the tonic and phasic terminals, but a better understanding of the fundamental differences in the anatomy and physiology of these two motor units will aid in building a more profound knowledge base. The hope is that the fundamental principles learned in this synaptic preparation will be applicable to other synapses in various preparations and will enhance future investigations in this synaptic model of the crayfish.

Method

1. All the experiments are conducted on the first or second walking legs of midsize crayfish (Procambarus clarkii). The animals are individually housed in plastic containers with oxygenized water. The temperature of the animal room is in the range of 13°C-16°C. The animals are fed with dry fish food and the water changed on a weekly basis.

2. The distal aspect of the walking leg in a crayfish is anatomically divided into six segments (**Figure 2.1**). The leg extensor is located in the meropodite, and the nerve bundle that will be isolated is close to the meropodite ischiopodite joint. The tonic or phasic axon can be selectively stimulated as needed for physiological purposes after they are exposed.

3. Cooper and Cooper (2009) described some aspects of the initial dissection, including methods for exposing the excitor of the opener motor neuron within the meropodite region, but their description does not provide the care required for protecting the extensor muscle from damage, as it was not needed to address the opener muscle preparation. In order to protect the extensor, the first or second walking leg is removed from the crayfish, measuring 6-10 cm in body length (Atchafalaya Biological Supply Co., Raceland, LA), by inducing the animal to automize the limb with forceful pinching distal to the fracture plane in the ischiopodite segment. The leg is placed on the dissection plate with the lateral (outer side) facing the viewer. The leg is turned around until the viewer can be

sure the outside (lateral side) is facing up on the dissection plate, usually with the arched side up (**Figure 2.2**). Placing the leg on a piece of tissue paper makes it easier to turn the preparation while making these cuts.

4. With a scalpel blade breaker and holder, a sharp razor blade is used to etch the cuticle until just cutting through in the pattern shown in **Figure 2.3** for the meropodite segment. Care is taken not to cut too far distal on the dorsal to ventral cut by the meropodite-carpopodite joint. The preparation is placed in saline. The dissection dish should have a Sylgard (Dow Corning) coating on the bottom (1cm thick).The Sylgard is used so that insect pins can be stuck into it for holding the preparation still. At this point, a pin is stuck in the middle of the carpopodite segment and in the dorsal aspect of the ischiopodite segment (**Figure 2.4**). Dissected preparations are bathed in standard crayfish saline, modified from Van Harreveld s solution (1936), which is made with 205 NaCl; 5.3KCl; 13.5 CaCl2; 2H2O; 2.45 MgCl2; 6H2O; 5 HEPES and adjusted to pH 7.4 (in mM).

5. The cuticle is gently lifted in the distal region and the muscle fibers are cut away from the cuticle by making strokes towards the base of the leg. The cuticle can be lifted off.

6. The apodeme (tendon) is cut at the meropodite-carpopodite joint. A pin is placed on the inner surface of the flexor tendon to show where the cut is to be made (**Figure 2.5**). The tendon is then pinched where it was cut with tweezers and the flexor muscle pulled off by lifting it in a caudal direction (**Figure 2.6**), exposing the main leg nerve and the extensor muscle.

7. The main leg nerve is cut at the meropodite-carpopodite joint and carefully pulled back over the extensor muscle. The medial surface of the muscle is used throughout this study. The separation of the nerve to the extensor muscle from the main leg nerve can be enhanced by gently pulling the distal stump of the main leg nerve to the side of the preparation. When peeling the main leg nerve back over the extensor muscle, small branches of axon may need to be cut. These are branches from the inhibitory motor neuron to the extensor muscle. The larger bundle branching off from the main leg nerve near the proximal end of the meropodite is the small nerve bundle of interest. This nerve bundle can be seen with methylene blue staining (**Figure 2.8**) or with 4-Di-2-ASP fluorescent stain (**Figure 2.9**).

Physiological Profiles

1. To observe the excitatory postsynaptic potentials (EPSPs) of the tonic or phasic neurons, one of the isolated axons in the nerve bundle is stimulated by a suction electrode (**Figure 2.11**) connected to a Grass stimulator while intracellular potentials in the muscle are monitored (Johnstone et al., 2008). Stimulation at 70 Hz is applied to the tonic axon in order to promote a facilitated response for the low output NMJs, or a single pulse (1 Hz) is applied to the

phasic axon in order to obtain large EPSPs of the high output NMJs as shown in **Figure 2.12**. The EPSPs are recorded to a computer via a PowerLab/4s interface.

2. Investigation in the nature of synaptic facilitation and synaptic depression can be approached by various experimental paradigms with the low and high output NMJs. Facilitation of the low output NMJs depends on the frequency, as shown for the 20, 40 and 60 Hz pulses of approximately 20 stimuli (**Figure 2.13**).

3. The rate of synaptic depression to frequency of stimulation is also related to the high output NMJs. **Figure 2.14** shows a continuous 5 Hz of stimulation depressing the NMJ over 30 min. With higher stimulation frequency, the preparation will depress more rapidly (Bradacs et al., 1997).

Quantal Responses

1. A procedure similar to that described for the opener muscle of the crayfish (Cooper and Cooper, 2009) is used in this preparation. The quantal EPSPs directly over identifiable regions of the nerve terminal are recorded by placing the lumen of a macro-patch recording electrode on synaptic varicosities visualized with the vital dye 4-Di-2-Asp (5 μM, 5-min treatment, Cooper et al., 1995b; Magrassi et al., 1987). The spontaneous as well as evoked quantal responses can be recorded along the nerve terminals. The evoked and spontaneous synaptic potentials are recorded with the macro-patch electrode (Dudel, 1981; Wojtowicz et al.,1991; Mallart, 1993). Kimax glass (outer diameter: 1.5 mm) was pulled and fire-polished to produce patch tips with inside diameters ranging from 10 to 20 μm (**Figure 2.15**). The lumen of the electrode is filled with the bathing medium. The amplifier is the same as that used for the intracellular recordings mentioned above. Electrode and seal resistance can be determined by passing test current pulses through the electrode. In our experiments, seal resistances ranged from 0.3 to 1.0 MOhm and the electrode resistance ranged from 0.5 to 1.0 MOhm. Seal resistance can be monitored throughout the recording.

2. Direct counting of quantal events is possible with low stimulation frequencies. For each evoked response, the number of quantal events can be readily determined for the low output terminals (**Figure 2.16**). These direct counts can help estimate the mean quantal content (Del Castillo and Katz, 1954; Cooper et al., 1995b). Since the evoked high output NMJs produce multi-quantal evoked events, the mean amplitude or area of the deflections, along with the average peak amplitude or area of the spontaneous events, can be used to approximate the mean quantal content (Cooper et al., 1995b).

Discussion

We have demonstrated in this report how to dissect record and quantify synaptic responses in a unique crayfish neuromuscular preparation in which both high- and low-output terminals innervate the same muscle fiber. The neuromuscular preparations in the crayfish offer many advantages over vertebrate neuromuscular junctions, since only a few excitatory motor neurons are needed to innervate a muscle, and since the neurons are identifiable from preparation to preparation (Atwood, 1976). In addition, the excitatory neurotransmitter is glutamate, and the excitatory postsynaptic potentials (EPSP) are graded; thus, the biophysical properties of graded events are analogous to the dendrites of neurons within the CNS of vertebrates. The quantal currents, however, can be monitored directly at the postsynaptic sites (Cooper et al., 1995b).

Repetitive 5 Hz stimulation of the phasic nerve gives rise to large EPSPs that become greatly depressed after several minutes. This type of depression is common in arthropod phasic neuromuscular junctions (Atwood and Cooper, 1996b). The presence of the tonic terminals alongside the phasic terminals allows one to assess whether or not the postsynaptic target is greatly modified during and after depression of the phasic motor neuron. In addition, the low output terminals provide a nice preparation to investigate mechanisms that underlie synaptic facilitation (Desai-Shah et al., 2008; Desai-Shah and Cooper, 2009)

The high output terminals provide a playground to investigate modulation in the rate of synaptic depression and the recovery process. Accessible and viable preparations should help in deciphering the mechanisms behind synaptic depression. In this crayfish leg extensor preparation, exogenous application of serotonin is another tool to investigate recovery of synaptic depression and potentially a means of furthering investigation into the mobilization of synaptic vesicle pools. This preparation provides several experimental advantages, since individual muscle fibers are innervated by both phasic and tonic motor neurons.

Since serotonin increases the number of vesicles that are released with evoked stimulation, and since it promotes recovery during depression, it is apparent that there is some modulations of the vesicle pools for enhancing the probability of fusion with serotonin present (Johnstone et al., 2008; Logsdon et al., 2006; Sparks et al., 2004). Models that would explain the dynamics of the vesicle pools within the presynaptic nerve terminal during low frequency stimulation, as opposed to a depressed state, also need to be considered among the various experimental protocols that are possible with this preparation.

It has been noted in other systems that about 30% of the vesicle pool undergoes a rapid recycling, whereas the rest of the recycled vesicles go through a traditional slow recycling path through the endoplasmic reticulum (Harata et al., 2001; Tsien et al., 2001). Such dual paths may also be present in this system. If ATP is lacking in the depressed state of the presynaptic terminal, then docking and undocking might not be able to occur; thus, many more unloaded vesicles

would remain at the synaptic surface. Since more vesicles are rapidly released when the terminals are exposed to 5-HT, it is feasible that more are contained within the readily releasable pool (RRP). However, in the depressed state, with reduced ATP, the docking and undocking may be blocked even in the presence of 5 HT, which again leaves unloaded vesicles at the synaptic surface. Since the preliminary data suggests that more vesicles are released over time with prolonged 5 HT exposure and stimulation, the distribution of the vesicle pool from the fast and slow recycling paths may be skewed to have competent vesicles for re-release (from Johnstone et al., 2008; see reviews- Desai-Shah et al., 2008; Desai-Shah and Cooper, 2009)

With the techniques of focal macropatch recordings of postsynaptic currents and measures of single quanta from defined regions of the motor nerve terminal, one can ask questions to determine whether synaptic depression is occurring as a result of fewer vesicles being released or because of alterations of the function of postsynaptic receptors. It has been shown that the vesicles are more sensitive to fusion at the phasic NMJ of this preparation for an equal calcium exposure (Miller et al. 2005), which likely accounts for the higher mean quantal content of the phasic terminals (Msghina et al., 1998, 1999). In addition, differences in the calcium binding protein frequenin (Jeromin et al., 1999) and ultrastructure (King et al., 1996) contribute to the differential synaptic efficacy (Cooper et al., 2003).

Some prior studies have explored the muscle phenotype of the extensor muscle fibers (Bradacs et al., 1997; Cooper et al., 2003). Comparing the regulation of muscle differentiation for purely tonic and phasic fiber types in the crayfish to mixed fiber types, like for the leg extensor that is dully innervated, could provide clues to muscle phenotype expression and regulation (LaFramboise et al., 2000; Sohn et al., 2000; Griffis et al., 2001; Mykles et al., 2002).

Many fundamental questions remain to be addressed in neurobiology, and this preparation may aid in tackling some of them. A few topics of interest in the field today that might be approached with the leg extensor include: 1) Determining the cellular mechanisms that underlie synaptic depression within high-output terminals (Is the depression due to a reduction of Ca2+ entry, lack of a competent readily releasable vesicle (RRV) pool, and/or altered postsynaptic receptivity?) 2) Determining the mechanistic role of 5-HT when applied after the induction of synaptic depression to promote a faster recovery, and 3) Determining whether the shapes of the quantal currents that arise from stimulating phasic terminals are being altered during the induction of depression to address pre- and post-synaptic components of synaptic depression.

This NMJ is important to ongoing research and future investigators because it enables us to obtain pertinent information that addresses the underling mechanisms of synaptic performance as measured directly at the release sites. Current research in this area is providing information about the modulation of synaptic depression by 5-HT and the dynamics of the vesicle pools. Such topics pertain to the fundamental basics of synaptic transmission relevant to all neural systems.

Materials

1. Crayfish (Procambarus clarkii). Atchafalaya Biological Supply Co., Raceland, LA., USA.

2. Standard crayfish saline: Modified from Van Harreveld's solution (1936). (in mM) 205 NaCl; 5.3 KCl; 13.5 CaCl22H2O; 2.45 MgCl26H2O; 5 HEPES and adjusted to pH 7.4

3. Dissection tools: Fine #5 tweezers, fine scissors, knife blade holder, #26002-20 insect pins (all obtained from Fine Science Tools (USA), Inc., 373-G Vintage Park Drive, Foster City, CA 94404-1139)

4. Sylgard coated dissection dish, a recording dish either constructed with suction electrode or use a suction electrode and anther manipulator to hold a suction electrode.

5. Dissecting microscope with zoom function for intracellular recordings. For focal recording on visualized terminals a compound microscope with upright objectives (4 x and 20X) is used. One needs a Hg light source.

6. Standard intracellular amplifier and A/D board for on line recording to a computer. Electrical signals are recorded on line to a PowerLab/4s interface (ADInstruments, Australia). We use standard software from ADInstruments named Chart or Scope.

7. Chemicals: We use a vital fluorescent dye, 4 [4 (diethylamino) styryl] N methylpyridinium iodide (4 Di 2 Asp; Molecular Probes, Eugene, OR), to visualize the varicosities (Marigassi et al., 1987; Cooper et al., 1995b). All saline chemicals were obtained from Sigma chemical company (St. Louis, MO).

8. For intracellular recordings we use glass capillary tubing (catalogue # 30-31-0 from FHC, Brunswick, ME, 04011, USA) and for focal macropatch electrodes we use Kimax-51, Kimble Products Art. No. 34502, ID 0.8-1.1mm, length 100mm. The intracellular electrode should have a resistance of 20 to 30 mOhm. The macropatch electrode is constructed by breaking off the tip of the glass after a fine tip was made from an electrode puller. The broken off tip needs to be a clean perpendicular break about 20μM in diameter. The tip is then heat polished to about 10μM inner diameter. The shaft of the electrode is then run over a heating element to cause it to bend about 45 degrees with a gradual bend. This produces a flat or perpendicular electrode lumen over the nerve terminal as the angle with the micro-manipulator will produce about another 45 degrees to the preparation.

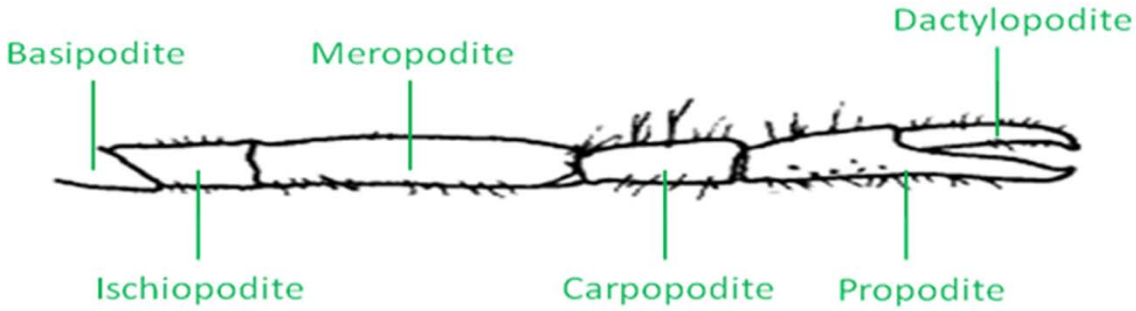

Figure 2.1: Schematic of a crayfish walking leg and the six distal segments.

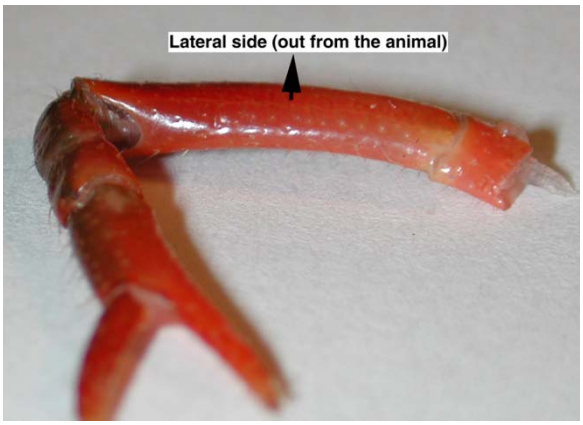

Figure 2.2: The lateral side of the meropodite. The lateral side of the meropodite, usually the side that is arched, is facing up on the dissection plate.

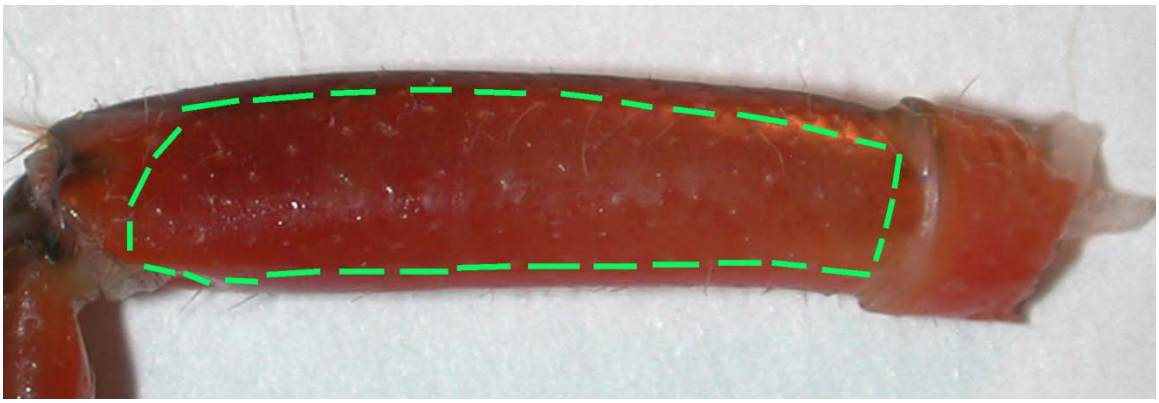

Figure 2.3: The suggested etching window of meropodite segment. The meropodite segment with lines as a suggested pattern for etching out the window of cuticle.

Figure 2.4: The meropodite segment with cut window being lifted off. Note the location of the dissection pins.

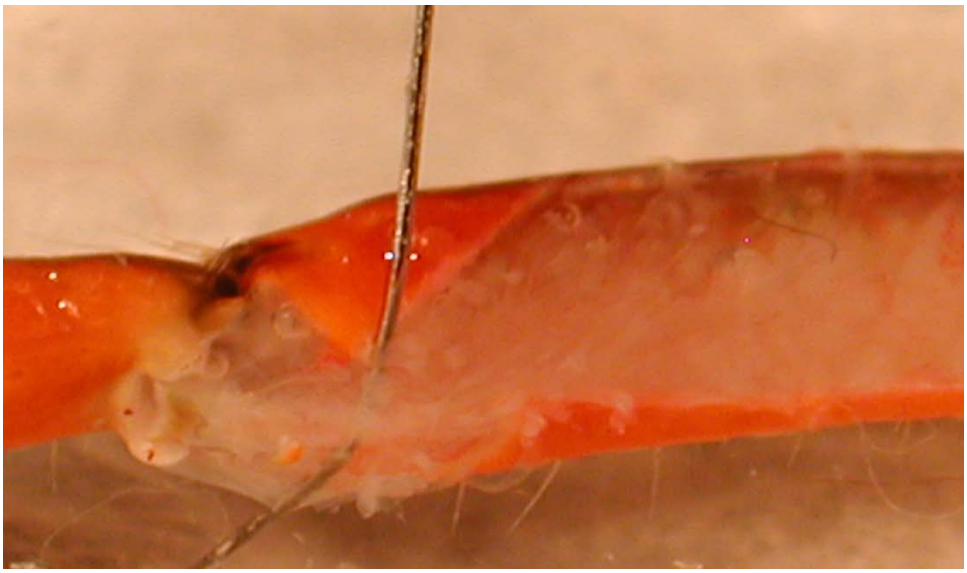

Figure 2.5: Separate the flexor with a pin. The apodeme of the flexor muscle is highlighted by displacing it from the flexor with a pin.

Figure 2.6: The apodeme of the flexor muscle is cut and removed with care as not to damage the main leg nerve.

Figure 2.7: The main leg nerve is cut and pulled back in a proximal direction.

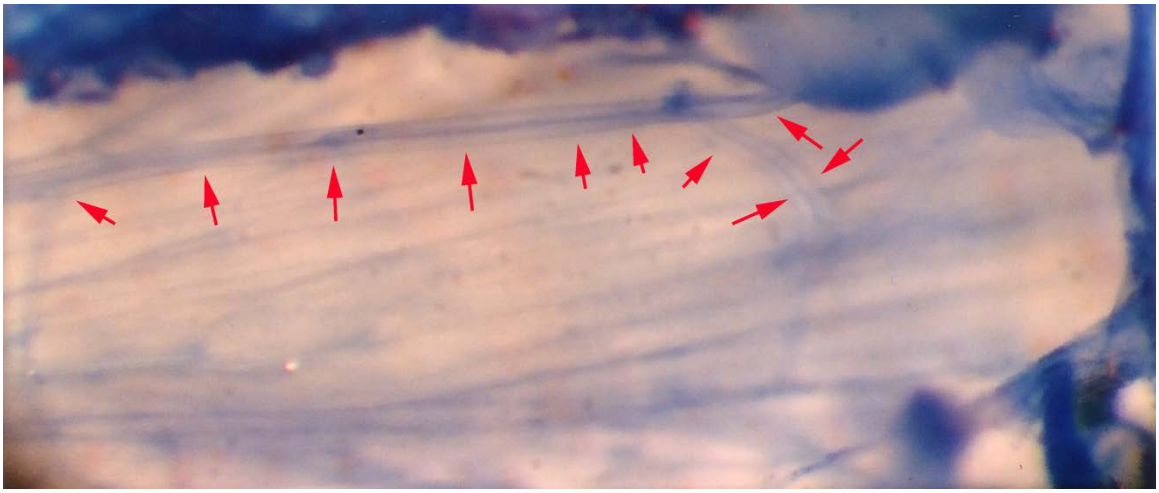

Figure 2.8: The extensor muscle stained with methylene blue. Note the axon branching and the two readily visible axons within the nerve. Red arrows demark the nerve track.

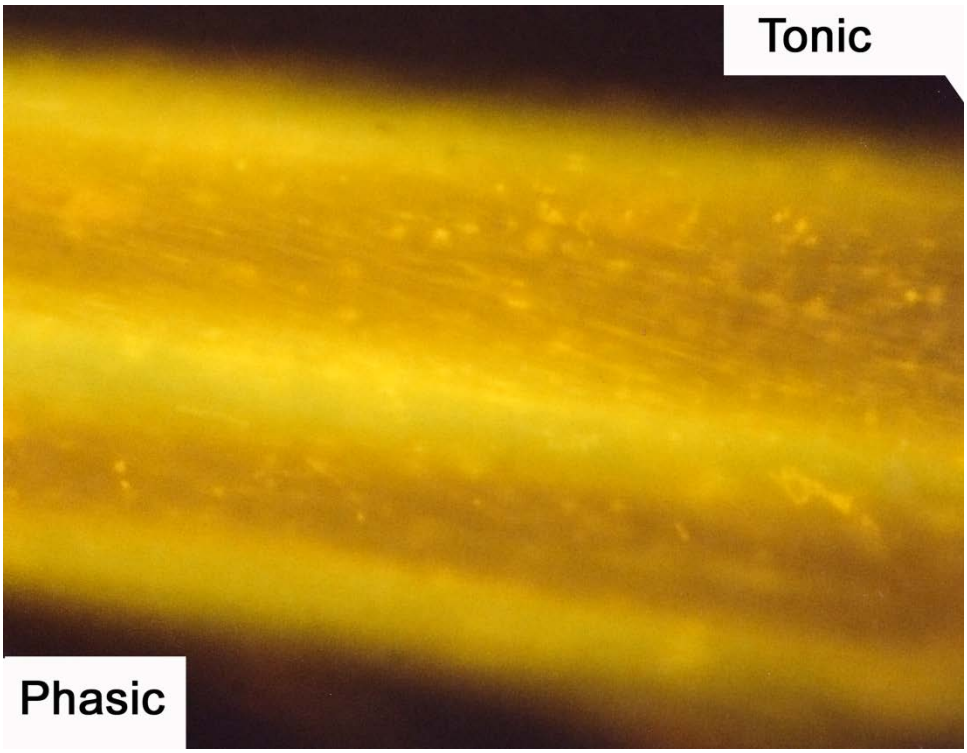

Figure 2.9: The axons of the motor nerve stained with 4-Di-2-ASP. The tonic axon is more brightly visible due to the increased mitochondrial content.

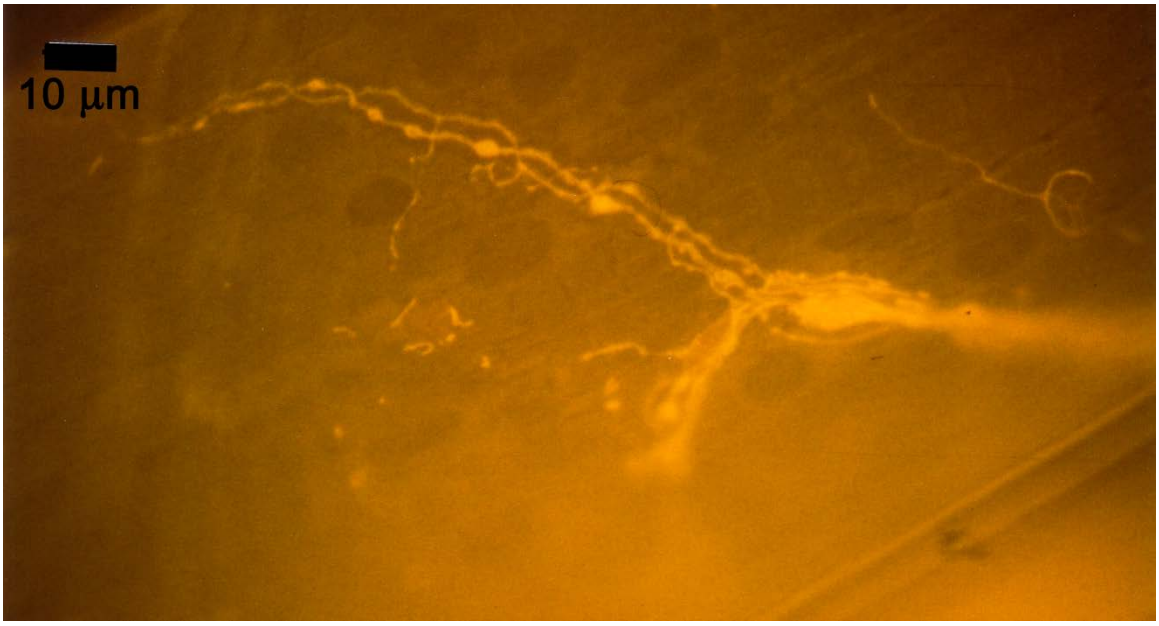

Figure 2.10A: Individual terminals of phasic and tonic neurons stained with 4-Di-2-ASP. Note the varicosities of the tonic terminals and the thin nature of the phasic terminals.

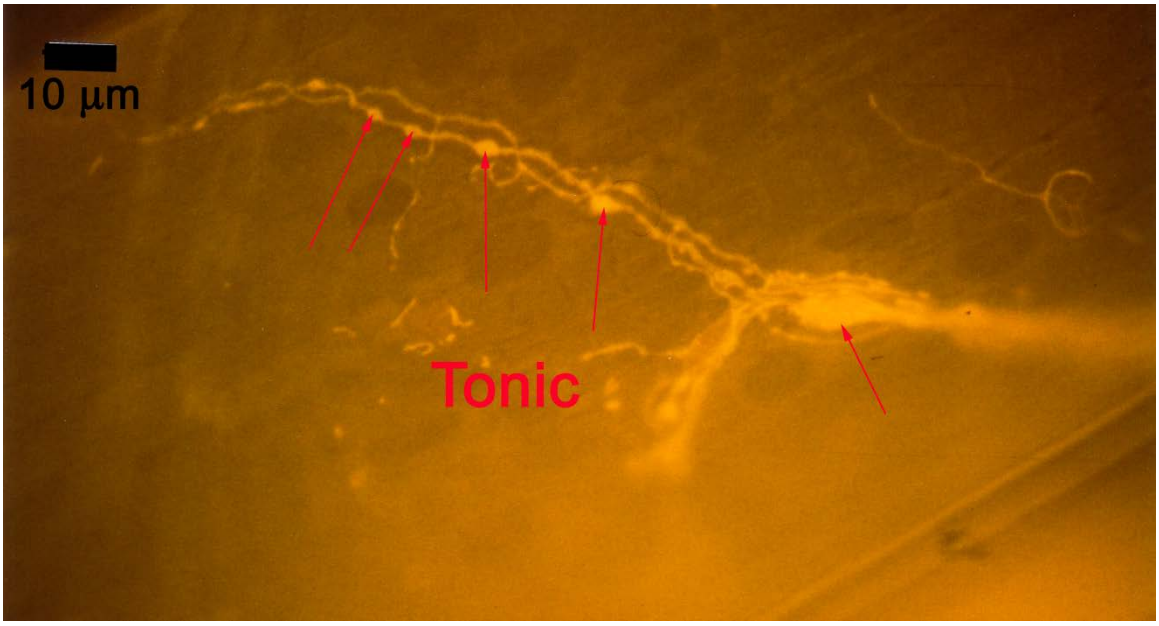

Figure 2.10B -Tonic noted

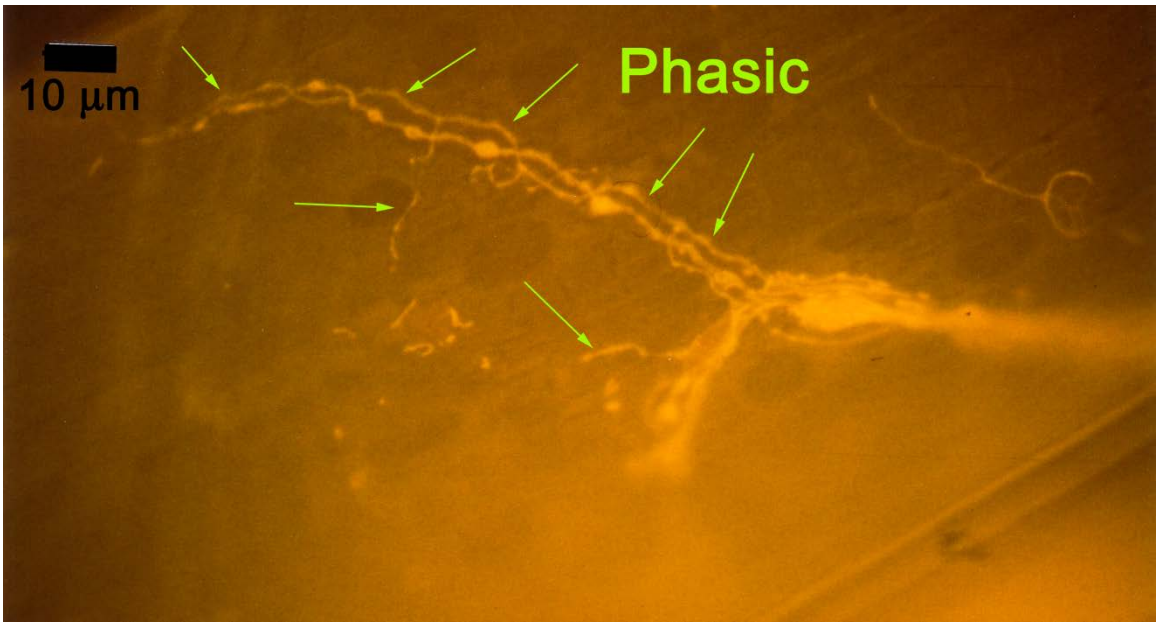

Figure 2.10C -Phasic noted

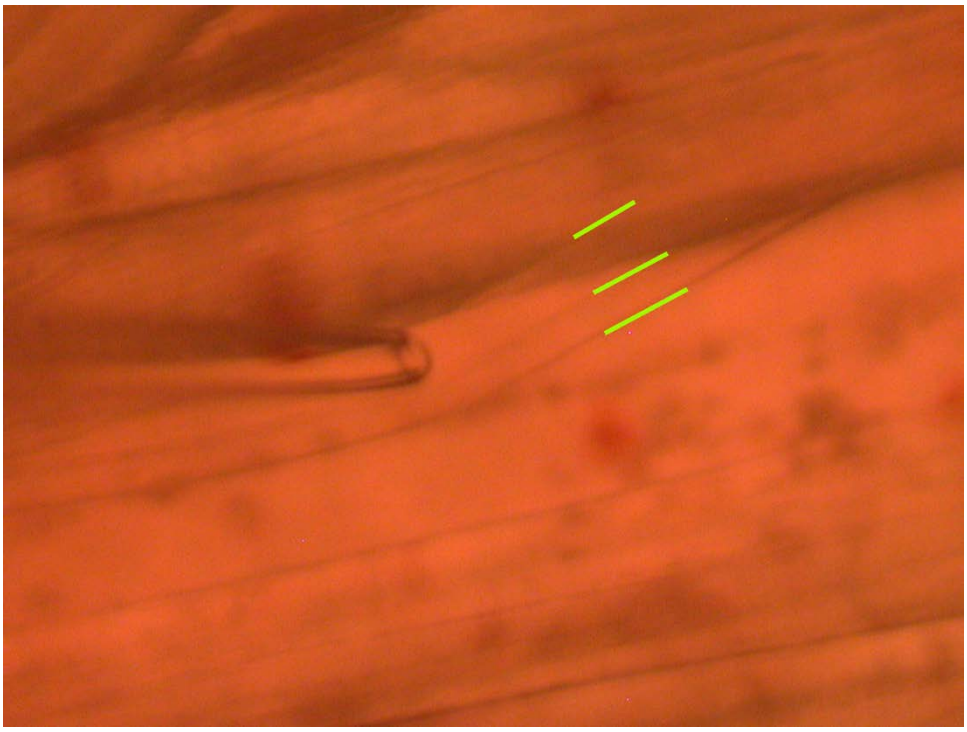

Figure 2.11: Stimulating electrode placed over a single axon within the nerve bundle. Note the green line outlining the 2 main axons.

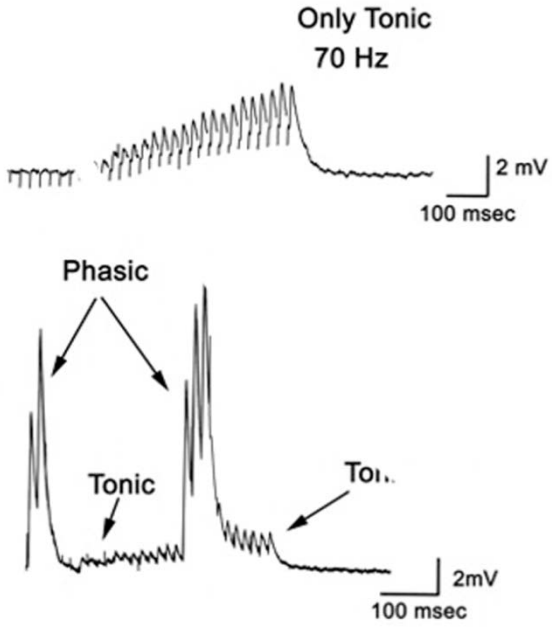

Figure 2.12: Postsynaptic potentials (EPSPs) of the tonic or phasic neurons as obtained by intracellular recordings.

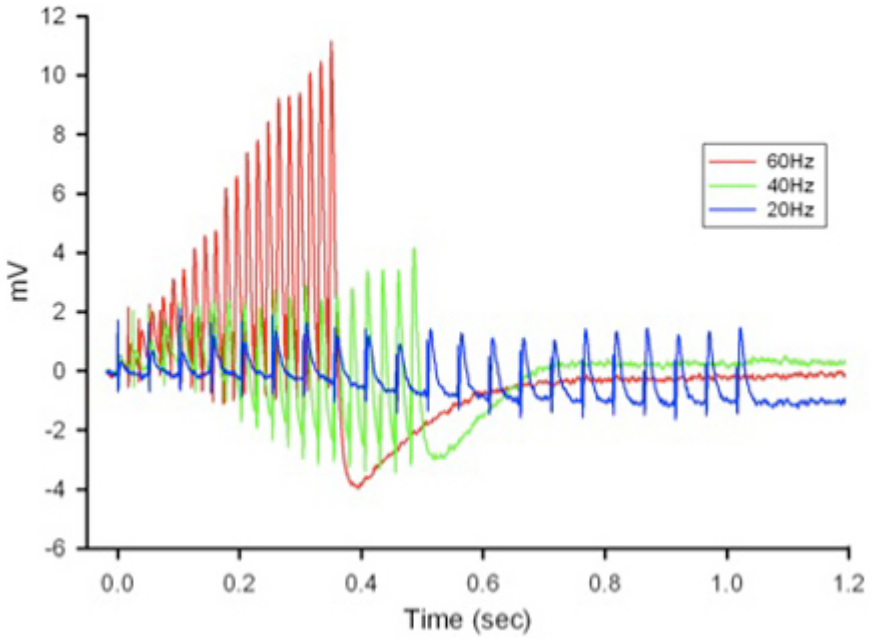

Figure 2.13: EPSPs in response to a train of stimulation pulses given at three different frequencies 20, 40 and 60 Hz in normal crayfish saline.

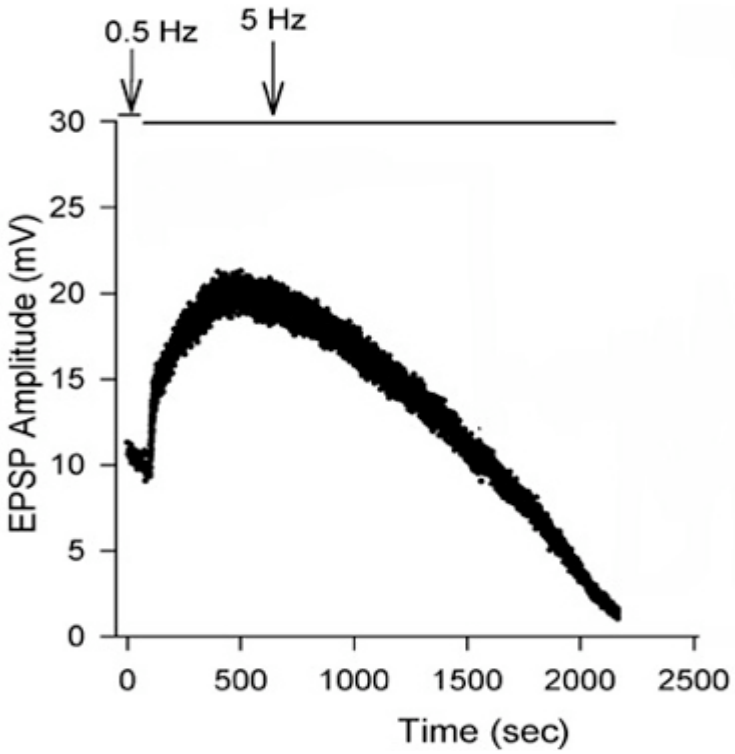

Figure 2.14: EPSP amplitude of the phasic response during 5 Hz stimulation to induce depression.

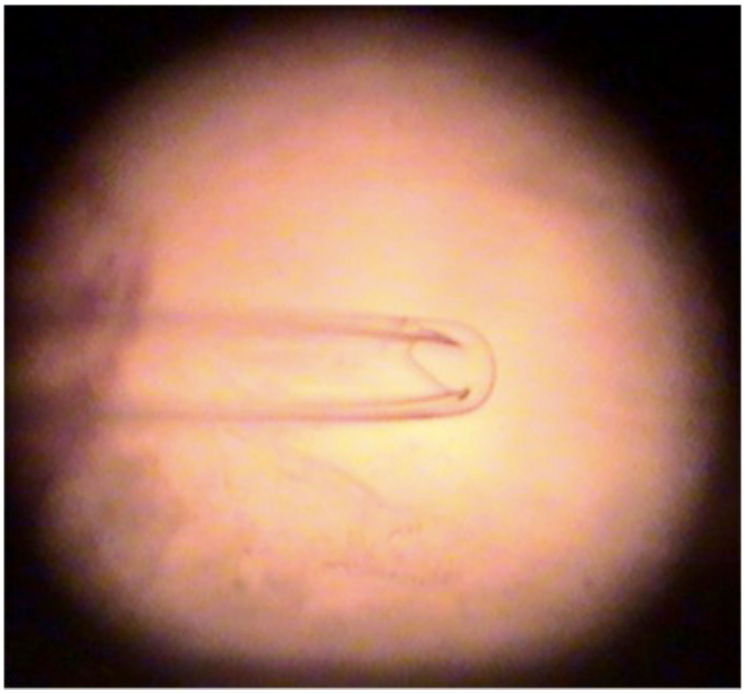

Figure 2.15: The lumen of a macro-patch recording electrode.

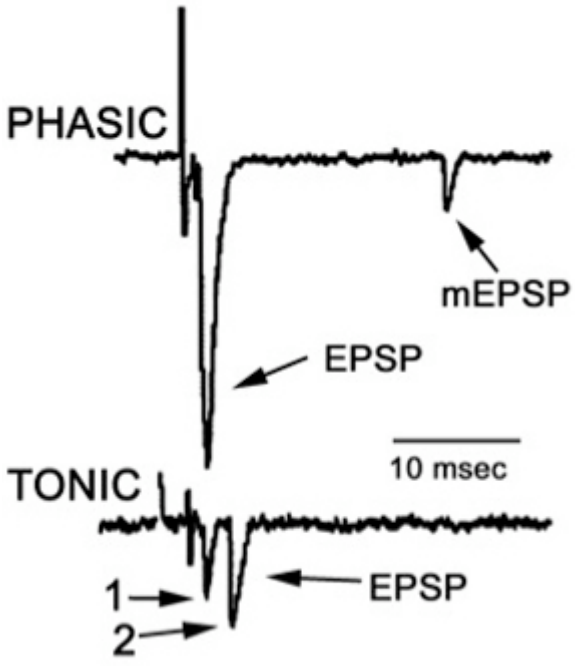

Figure 2.16 Focal traces recorded from a phasic and a tonic NMJ.

$$\text{Mean quantal content} = \frac{\sum (\#\text{ of failures})(0) + (\#\text{ of singles})(1) + (\#\text{ of doubles})(2)\ldots}{\text{Total number of stimulus trials}}$$

$$\text{Mean quantal content} = \frac{\text{Mean amplitude (or area) of evoked quantal events}}{\text{Mean amplitude (or area) of spontaneous quantal events}}$$

Figure 2.17 Equations for calculating mean quantal content.

CHAPTER THREE

Serotonin and Synaptic Transmission at Invertebrate Neuromuscular Junctions

General background of 5-hydroxytryptamine and receptors

This chapter has been published in Wu and Cooper (2012a) and is reproduced here for completeness of the dissertation.

5-Hydroxytryptamine (5-HT, serotonin) is a common biogenic amine found in both vertebrates and invertebrates as well as in plants (Azmitia, 2001, 2007). The precursor to 5-HT, tryptophan, is likely important in the early evolution of life and perhaps the early presence of tryptophan is a reason for 5-HT to be potentially the first neurotransmitter noted with the development of a nervous system (Azmitia, 2007). 5-HT acts as both a neurotransmitter and neurohormone and as a potent modulator of neurons and various tissues in many animal species (Bunin and Wightman, 1999). Generally 5-HT actions are elicited by transmembrane G protein coupled receptors (GPCRs), which then activate or inhibit different intracellular second messenger cascades. 5-HT receptors from some organisms have been classified based on sequence or pharmacology (Monastirioti, 1999; Tierney, 2001); for example in the vertebrates, 7 families (5-HT1-7), 14 subtypes have been identified, whereas in *Drosophila* four 5-HT receptors named 5-HT1Adro 5-HT1Bdro 5-HT2dro 5-HT7dro (Blenau and Baumann, 2001; Colas et al, 1999; Saudou et al, 1992; Saudou and Hen, 1994; Tierney, 2001; Witz et al, 1990) have been classified. 5-HT receptors appear to be present on invertebrate presynaptic nerve terminals and on muscle membranes; receptors of a cricket (*Gryllus domestica*) mandibular muscle have a similar pharmacological profile as a 5-HT2- like receptor subtype (Baines and Downer, 1991). Profiling the 5-HT receptor subtypes directly on skeletal muscle within invertebrates is an area of research that is lacking. The 5-HT4 and 5-HT7 receptors are shown to have alternate splice variants which increase the number of receptor subtypes and may alter the selectivity to pharmacological agents (Hoyer et al, 2002). In addition, 5-HT2 receptors can have different RNA-edited isoforms (Burns et al, 1997; Niswender et al, 1998).

With the use of the genetically modifiable model *D. melanogaster*, a number of studies have examined over-expression and under-expression of receptors subtypes on the effects of development, behavior and physiology as well as the general actions of 5-HT in *D. melanogaster* (Colas et al, 1995, 1999b; Dasari and Cooper, 2004, 2006; Dasari et al, 2009]. Based on physiological and pharmacological studies in crustaceans there may be a larger number of 5-HT receptors present than in *D. melanogaster* (Dropic et al, 2005; Sosa et al, 2004; Sparks et al, 2003, 2004; Tabor and Cooper, 2002; Tierney and Mangiamele, 2001; Tierney et al, 2004). Two receptors types have been cloned and characterized in crustaceans (Clark et al, 2004; Saudou and Hen, 1994; Spitzer

et al, 2008) and in a pond snail (Mapara et al, 2008). A 5-HT receptor 5-HT (apAC1) has been cloned, sequenced and characterized in *Aplysia* sensory neurons (Lee et al, 2009). 5-HT receptors are being cloned in a variety of invertebrates and surely more will be forth coming with the rapid development in genomic sequencing abilities. There are a plethora of reports on the effects of 5-HT for sensory and central neurons as well as on behaviors in invertebrates which are worthy of multiple reviews. However, for this brief review we focus on the physiological effects of 5-HT at the skeletal neuromuscular junctions in some of the key model invertebrates. The invertebrate neuromuscular junctions (NMJs) are very diverse across species and within species in structure and function (Atwood and Cooper, 1995, 1996b; Collins and DiAntonio, 2007; Gerschenfeld, 1973; Govind and Walrond, 1989; Westfall, 1996; Whim et al, 1993). The recent majority of reports on structure and function of NMJs are of *D. melanogaster* due to the genetic approaches and manipulations being utilized (Lee et al, 2009; Li and Cooper, 2001; Li et al, 2001, 2002).

Why focus on NMJs?

The synaptic communication between neurons and target cells depends on the specialized anatomy and physiology of the synapses (Sherrington, 1906). The regulation and modulation of neurotransmitter release is the basis of chemical synaptic transmission. For nervous systems to function properly, the efficacy of synapses are finely regulated and adjustable to respond to changing circumstance and requirement. Too high or too low synaptic input both result in inappropriate communication of target cells. Both pre- and postsynaptic factors can influence the synaptic strength. The amount of neurotransmitter released and the sensitivity of the postsynaptic membrane both are important for measuring synaptic strength. Each step in the process of synaptic transmission can be the target of many factors that lead to alteration of synaptic strength. For example, the phosphorylation state of SNARE proteins that are involved in vesicle docking, or the density of active zones where transmitter is released, can influence the number of quantal units released per impulse (presynaptic mechanism). Postsynaptically, the number of active receptors, the postsynaptic input resistance, the area and the ultrastructure of subsynaptic reticulum, all can alter the effectiveness of quantum release and thus influence synaptic strength.

The ease in accessibility to the synaptic sites at NMJs allows one to record intracellular or very close to synapses by extracellular recordings (focal macropatch over a varicosity) in order to minimize cable properties in signal decrement (Cooper et al, 1995a, 1996a,b). Such signal loss occurs with recordings in a neuron cell body to measure synaptic function in the dendritic trees. The localized recording over a NMJ allows one the ability to measure properties of single and multiple vesicular quanta for very precise quantal analysis (occurrences, size and shape) to index synaptic function (Cooper et al, 1995a; Lancaster et al, 2007; Viele et al, 2003, 2006). In addition, invertebrate NMJs are relatively stable for hours in a minimal saline at room temperature as

compared to mammalian NMJs. Since most muscles in invertebrates are innervated by relatively few motor neurons, for the most part, they are identifiable anatomically and physiologically from preparation to preparation (Atwood and Cooper, 1996b; Worden, 1998). Since the fine structure and detailed quantal analysis is feasible for many invertebrate NMJs, the acute and chronic actions of modulators on structure and function can be examined for their mechanistic actions (Cooper and Ruffer, 1998; Logsdon et al, 2006; Ruffner et al, 1999).

Insects

Given such a diverse group of animals within the class Insecta, it would not be surprising to find a wide range of anatomic and physiologic profiles in the innervation of skeletal NMJs. For example, the innervation of the genital chamber of the female cricket, *Acheta domestica*, shows 5-HT-immunoreactive nerve terminals that contact the muscle fibers which likely releases 5-HT in a type of volume transmission over the muscle as there are no defined synapses (Elekes and Hustert, 1988). Such 5-HT containing nerve endings are also present in earthworm skeletal muscles (Nishihara, 1967). However, no serotonin is associated with the oviducts or the innervation to the oviducts in the locust (Lange, 2004). Earlier studies did not elucidate if the effect of 5-HT was directly on the presynaptic terminal or on the muscle but reported overall changes in force of muscle contraction. In a locust leg muscle, 5-HT produces an overall decrease in force development (Hill and Usherwood, 1961) but the mechanism of action still needs to be determined. It is suggested that in some of the earlier studies with insects, the high concentrations of 5-HT used may indeed block synaptic transmission by impeding the postsynaptic receptors (Hill and Usherwood, 1961; Gerschenfeld, 1973).

Despite the intense investigations in synaptic structure and plasticity in *D. melanogaster* related to genetic and mutational manipulations, there are few reports on the modulation of synaptic efficacy by peptides or modulators at the skeletal NMJ (Badre et al, 2005; Chen and Ganetzky, 2012; Cooper and Neckameyer, 1999; Dunn and Mercier, 2005; Middleton et al, 2006; Nagaya et al, 2002; Ruffner et al, 1999). As for the influence of 5-HT at the NMJ, the scantiness of studies is likely due to the mild effects observed by using 5-HT itself as well as pharmacological agonists/antagonists of 5-HT receptors. However, application of 5-HT to the intact larval CNS does enhance the drive of motor neurons (MN) (Dasari and Cooper, 2004). The most commonly studied *Drosophila* neuromuscular junctions are those in the most prominent ventral longitudinal abdominal muscle fiber muscles 6 and 7 (Crossley, 1978), which have the simplest innervation pattern among the *Drosophila* body wall muscles. Both electrophysiological and morphological studies imply that each of these two muscles is innervated by only 2 axons (Jan and Jan, 1976; Sink and Whitington, 1991). Application of 5-HT to these NMJs appears to slightly depress synaptic strength (Dasari et al, 2007; Sparks and Cooper, 2004). We are not aware of any attempt to investigate actions of 5-HT on adult skeletal NMJs. However, with the

recent advent of designer receptors exclusively activated by designer drugs (DREAD) in motor neurons allows one to examine mechanisms of activating second messenger cascades as if receptors for modulators existed on presynaptic nerve terminals or on the muscles themselves (Majeed et al, 2012; Nichols et al, 2012).

Crustaceans

The NMJs in crustaceans offer many advantages for addressing mechanism of action in modulation of synaptic efficacy at NMJs, but crustaceans do fall short in being able to genetically modify the properties for investigations. Potentially approaches with RNAi might be practical to address more species-specific manipulations in synaptic function in a variety of crustaceans (Estrada et al., 2007; Estrada et al., 2007; Kato et al, 2011; Pekhletski, 1996). The same physiological and anatomical advantages of the *Drosophila* NMJs apply for the crustaceans, but in addition, the wide range in known diversity in synapses within crustaceans makes them attractable for comparative studies in commonalities of mechanisms in low-and high-output synapses or ones that facilitate or depress rapidly (Atwood and Cooper, 1995, 1996b; Wu and Cooper, 2010). The parallels to vertebrate central synaptic physiology of phenomenon described at crustacean NMJs are likely one reason of continual interest to a wide variety of researchers investigating synaptic transmission. In addition, the historical contribution of crustaceans in synaptic physiology is unsurpassed (Atwood, 1967, 1976; Cooper and Cooper, 2009; Wiese, 2002). The ability to combine direct structure and function in defined labeled synapses offers the ability to unravel synaptic structural complexity with function (Atwood and Cooper, 1995, 1996b; Cooper et al., 1995a, 1996a, b; Johnstone et al., 2011).

It was demonstrated as early as 1954 that 5-HT enhances synaptic transmission at the crustacean NMJs (Fischer and Florey, 1983; Florey and Florey, 1954) and that the effect was likely a presynaptic enhancement of mean quantal content came afterwards (Dudel, 1965). The 5-HT that modulates most crustacean skeletal NMJs does so through the exposure of hemolymph. 5-HT is released from nerve endings in thoracic roots and from the pericardial organs into the hemolymph (Beltz and Kravitz, 1983). Thus, 5-HT is accessible to all the exposed NMJs. The excitatory as well as inhibitory NMJs are enhanced in transmission by 5-HT (Vyshedskiy et al, 1998; Wang and Zucker, 1998). The quantal effects are explained by increased probability of vesicular fusion during evoked transmission likely caused by an increase in the number of vesicular vesicles being docked and possibility their sensitivity of fusing due to enhanced Ca^{2+} sensitivity or presence of free Ca2+ within the terminals (Dudel, 1988). However, several studies have shown that a presynaptic rise in free Ca^{2+} is not substantial enough to account as a primary mechanism of 5-HT's action (Dixon and Atwood, 1985; Delaney et al, 1991; Glusman and Kravitz, 1982; Vyshedskiy et al, 1998). Since there is a steep rise in sensitivity to Ca^{2+} for enhancing synaptic efficacy at crustacean NMJs (Dudel, 1981) a low release from internal

stores may account well enough for part of the effect (Dropic et al, 2005). This notion of an internal release of Ca^{2+} is also supported by experiments conducted by Glusman and Kravitz (1982) in which they showed that a calcium-free bath, along with EGTA and high $MgCl_2$, 5-HT could still cause spontaneous release of transmitter for lobster NMJs. The enhanced spontaneous and evoked fusion events relates to an increase in 'n' (number of sites) and 'p' (probability of release) to explain the enhanced 'm' (mean quantal content; m=np) after exposure to 5-HT (Southard et al, 2000; Sparks and Cooper, 2004; Strawn et al, 2000). An interesting observation, but not yet explained mechanistically, is that 5-HT produced an effect with low or zero extracellular calcium at a crayfish NMJ but 5-HT's effect depended on extracellular sodium concentration (Dixon and Atwood, 1985).

Low- and high-output NMJs in crayfish and crab show differential responses to 5-HT (Cooper et al, 2003; Djokaj et al, 2001; Johnstone et al, 2008). This could be due to the larger reserve pool of vesicles in tonic (low-output) terminals than the phasic (high-output) terminals and the fact that higher-output synapses in crustaceans have more complex synapses containing more active zones in close apposition on synapses than lower output synapses (Cooper et al, 1996b; Bradacs, 1997; Millar et al, 2005; King et al, 1996).

NMJs investigated in lobster and crab revealed similar findings to those of the crayfish. 5-HT also enhances both excitatory and inhibitor NMJs that have been examined in *Homarus americanus* (lobster) (Hamilton et al, 2007; Harris-Warrick and Kravitz, 1984). 5-HT also promotes the force of nerve-evoked contractions of the gastric mill muscle of the crab, *Cancer borealis* (Jorge-Rivera et al, 1998).

The differential responses and cellular mechanism of 5-HT's action at crustacean NMJs is likely accounted for by the density and receptor subtypes on the presynaptic terminals. Vertebrate 5-HT_2-like receptors were physiologically identified for *Procambarus clarkii* at NMJs (Sosa et al, 2004; Sparks et al, 2003, 2004; Sparks and Cooper, 2004; Tabor and Cooper, 2002). Since this subtype of receptor has been sequenced in a crab and crayfish (Spritzer et al, 2008) these may be the subtypes present at the NMJs; however the blockers for the vertebrate 5-HT_2-like receptors could not block the entire 5-HT enhancement of synaptic enhancement (Tabor and Cooper, 2002). Also 5-HT_2 agonists did not mimic the responses fully at the crayfish NMJ (Tabor and Cooper, 2002), so potential affinity in binding 5-HT and pharmacological agents differ in crustaceans to vertebrate subtype receptor analogs. The pharmacology of monoamines in the cardiac ganglion of lobsters also does not mimic vertebrate classifications (Berlind, 2001). Care needs to be taken in assuming the pharmacology of mammalian 5-HT receptors applies to invertebrates (Sparks et al, 2003).

Given there is at least some pharmacological and sequence similarity to vertebrate 5-HT_2 receptor subtype present in crayfish and that injection of an IP3 analog (adenophostin-A) in the presynaptic motor nerve terminals enhances release (Dropic et al, 2005), a potential mechanism is that 5-HT receptors on the

presynaptic membrane mediate activation of G coupled receptors which leads to activation of phospholipase C (PLC) which in turn produces 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) (Berridge and Irvine, 1989). The production of IP3 can directly result in Ca^{2+} release from internal stores (i.e., ER) through IP3 receptors on the ER (Hisatsune et al, 2005).

Since vertebrate 5-HT2 receptor family activates phospholipase C (PLC) (Saudou and Hen, 1994) a similar receptor activated cascade is possible at the crayfish NMJs, Such mechanisms are established in other systems (Berridge and Irvine, 1989; Mattson et al, 2000; Petersen and Cancela, 1999) and given that caffeine and ryanodine actions are in concurrence with IP3 receptors potentially on the ER in crayfish presynaptic motor nerve terminals (Dropic et al, 2005) we have to consider this mechanism as a likely possibility. The rise is Ca2+, even a slight rise, could activate calmodulin and in turn activate CaM-Kinase (CaM-K), which can lead to phosphorylation of proteins such as synapsin. The possibility is that vesicles would then be able to leave the tethers to the cytoskeleton and dock to the presynaptic membrane, which is also a phosphorylation step (Chi et al, 2003; He et al., 1999; Tolar and Pallanck, 1998). The increased docked vesicles could be subjected to the calcium influx and release from internal stores (Yang et al., 1999). This would account for the increase in the occurrence of spontaneous quantal events and enhanced evoked responses with 5-HT exposure. In the invertebrate *Aplysia*, it was shown that exposure of neurons to 5-HT results in phosphorylation of synapsins (Fiumara et al, 2004). cAMP was also suggested to be involved in 5-HT action (Battelle and Kravitz, 1978; Dixon and Atwood, 1989; Goy and Kravitz, 1989; Lee et al, 2009). cAMP has been shown to activate Protein Kinase A (PKA) which then can lead to phosphorylation of transcriptional factors such as CREB. Such action can regulate synthesis of proteins used in synaptic transmission (Geppert et al, 1997; Bolshakov et al, 1997; Yao et al, 2006). It has also been suggested that the cAMP and calmodulin pathways may work together and promote transcription (Dash et al, 1991). When phosphatases are inhibited at the crayfish NMJ the effect of 5-HT is enhanced, thus demonstrating the significance of phosphorylation (Swain et al, 1991) which is known to occur with exposure to 5-HT at crustacean NMJs (Goy et al, 1984).

In a recent study addressing the potential mechanisms of 5-HT, as well as stimulation of the motor nerve terminal, in recruiting vesicles from a reserve pool (RP) to a readily releasable pool (RRP) within the presynaptic nerve terminals of crayfish NMJs, we developed a model to account for the observations and previous reports. In a current study, we inhibited the packaging of glutamate by blocking the vesicular glutamate transporter (VGlut) with the drug bafilomycin A1 (Bowman et al, 2004; Cavelier and Attwell, 2007; Juge et al, 2010). In this way, the rapidly recycling vesicles within the RRP will be empty with repetitive stimulation. However, if the RP is spared from being recruited by low frequency stimulation and if they are already packaged with transmitter, prior to exposure to bafilomycin A1, then 5-HT should be able to recruit these RP vesicles to the RRP and synaptic transmission restored temporarily. This is exactly what was observed indicating that the RP and RRP can be physiologically differentiated

into distinct functional groups and that 5-HT recruits the RP into action (Wu and Cooper, 2012b). To deplete or use up the packaged RRP vesicles, continuous stimulation was provided since the opener NMJ preparation is low-output and fatigue resistant. A high frequency of 40 Hz was used for comparative purposes to 20 Hz continuous stimulation. As expected, preparations stimulated at 40 Hz depressed faster than the ones stimulated at 20 Hz and there was a reduced effect for the 40 Hz stimulated preparations to exposure of 5-HT. This suggests that a higher stimulation frequency is able to recruit some of the RP to the RRP. This is illustrated in a model (**Figure 3.1**)**.** To address if PLC is an intermediate step within the cascade of events activated by 5-HT mediated responses, we used a PLC inhibitor (U73122) and an inactive analog (U73343) to serve as a negative control (Bleasdale et al, 1990). We found that the treatment of U73122 caused a significant decrease of 5-HT effect on synaptic transmission. This result confirmed the involvement of PLC signaling cascade in inducing the enhancement of synaptic transmission by 5-HT at a different physiological condition. There are observations in other preparations that indicate the presence of two distinct vesicle pools: RRP and RP. In the cat superior sympathetic ganglion, Prado et al. (1992) separated the two pools by electrically stimulating the nerve to deplete the RRP of acetylcholine, and then recruit RP vesicles by tityustoxin. Using FM 1-43 dye, the two pools have been identified in a temperature-sensitive mutant *Drosophila* line, *shibire*, and later in WT (Betz et al, 1992; Kuromi and Kidokoro, 2000). However in our study with the crayfish NMJ, a novel approach with bafilomycin A1 was used together with continuous stimulation to deplete the RRP, and then 5-HT was applied to recruit RP vesicles and the recruitment involves a PLC signaling cascade. A mechanistic illustration is detailed in **Figure. 3.1**.

There does not appear to be a substantial direct effect on crustacean skeletal muscle to account for an increase in EPSP or IPSP amplitude due to an increase in input resistance of the fibers (Battelle and Kravitz, 1978; Florey and Florey, 1954; Grundfest and Reuben, 1961; Kravitz et al, 1980). A small increase in input resistance, by exposure to 5-HT, accounts for a slight increase in the EPSP amplitude for superficial flexor muscle fibers of crayfish (Strawn et al, 2000). More substantial alteration in input resistance can occur in crustacean neurons due to 5-HT exposure (Cooper et al, 2003) so there could be some effect on the presynaptic motor nerve terminals.

In comparison to the smooth muscle in the intestine of vertebrates, the muscles of the crayfish hindgut are striated with gap junctions and generate intrinsic pacemaker activity (Brenner, 1999; To et al, 2004). Application of 5-HT (Musolf, 2007) and octopamine (Orchard and Lange, 1985) to GI tract increases the frequency and strength of contractions. 5-HT and dopamine are highly concentrated in CNS and GI tract and they are directly responsible for the peristalsis and muscle contraction (Cooper et al, 2011; Musolf , 2007)

Annelids

The leech has served as a model organism in neurobiology for many years (Nicholls et al, 1981) but few studies have directly focused attention at NMJs in the leech and even fewer on the effects of 5-HT in synaptic transmission at NMJs. However, studies have examined the effect of 5-HT on the drive of motor neurons and innervation patterns (Brodfuehrer et al, 1995; Stuart, 1970; Ort et al, 1974; Kuffler, 1978; Cline, 1983; O'Gara et al, 1999; Sawasa and Coggeshall, 1976). 5-HT exposure has a relaxing effect on skeletal muscle in the leech (Schain, 1961) and enhances muscle force and work production during locomotion and feeding (Gerry and Ellerby, 2011). This is physiological relevant since Retzius neurons do directly innervate skeletal muscle in the leech and these cells do release 5-HT (Kuffler, 1978; Mason and Kristan, 1982; Manson et al, 1979; Yaksta-Sauerland and Coggeshall, 1973). In the earthworm and polychaete (*Sabellastarte magnifica*) muscle contraction is reduced by 5-HT (Hidaka et al, 1969; Del et al, 1969) which lead to the idea that 5-HT might be acting as inhibitory transmitter in these preparations (Díaz-Miranda et al, 1992).

Gastropods

A few studies with gastropods have been approached for the direct effect of 5-HT at the NMJ. 5-HT produces facilitation for an evoked response in buccal muscle within *Aplysia* (Fox and Lloyd, 2002). The presynaptic actions of 5-HT is to enhance transmitter release (Lotshaw and Lloyd, 1990). Like for some of the actions in annelids, 5-HT can also produce muscle relaxation and reduce force in *Aplysia* (Evan et al, 1999). Such effects on muscle contraction and force maybe dependent on 5-HT concentration and the species studied, since in *Aplysia brasiliana* 5-HT increases a Ca2+ influx that promotes muscle contraction used for swimming (Laurienti and Blankenship, 1997).

Other invertebrates

In a sea urchin (*Parechinus*), 5-HT apparently had no effect at the NMJ (Boltt and Ewer, 1963). However in a sea cucumber (*Apostichopus japonicas*), 5-HT inhibited evoked contractions induced by acetylcholine and there appears to be 5-HT innervation directly to muscles of the body wall (Inoue et al, 2002).

Summary

Although headway has been made in describing the various actions of 5-HT at NMJs in invertebrates, the cellular mechanisms of these actions are still lacking. Additional pharmacological and molecular profiling in a variety of invertebrate preparations will increase our knowledge of both the uniqueness and similarities among the invertebrates. As history has taught us in physiology, and in particular neurobiology, what is learned in invertebrate preparations paves the way to new views and mechanistic cellular understanding of complex processes within the vertebrates.

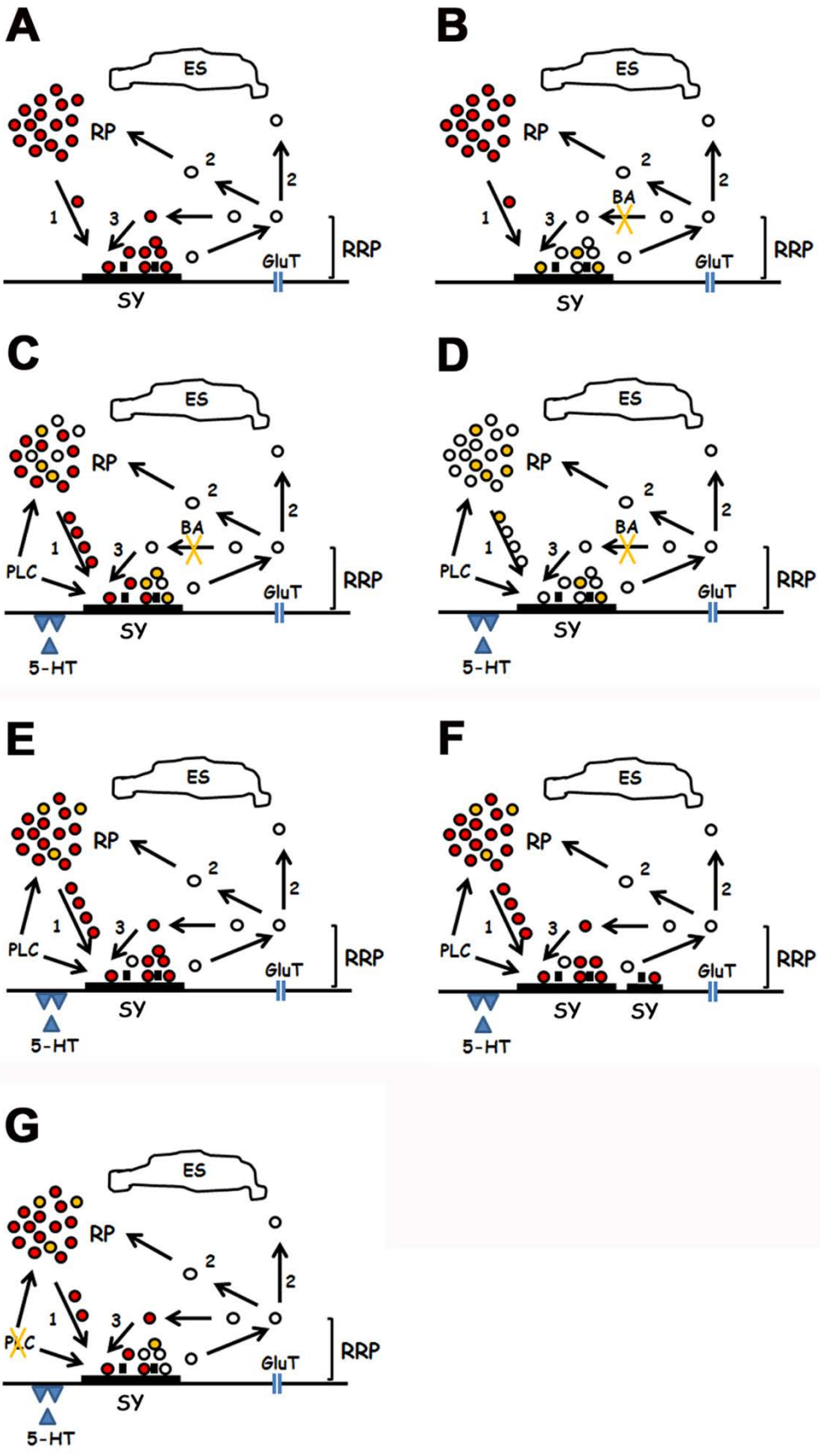
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Figure 3.1: Schematic illustration of 5-HT in recruiting vesicles from a reserve pool. (A) Two vesicle recycling pathways have been proposed. In a resting synapse, vesicles in RP slowly join in to the RRP (1), and then recycle back to RP either through or bypass endosome (2). This is called slow recycling loop. However, in an active synapse, in addition to the slow recycling loop, vesicles in RRP recycle quickly within the RRP (3) which is named quick recycling loop. Recycling vesicles are refilled with glutamate to be able to participate in the coming synaptic activities. (B) In an active synapse treated with Bafilomycin A1, vesicles in RRP can be used up in time with stimulation because recycling vesicles can no longer be refilled. Synaptic depression occurs sooner than the one without Bafilomycin A1 treatment. (C) If 5-HT is added after depression, 5-HT possibly activates PLC signaling cascade and recruits RP vesicles to revitalize the synaptic transmission in a fast manner. (D) In time, synaptic depression occurs again because most RRP and RP vesicles are empty. Yellow colored vesicles represent partially full of glutamate. (E) Even when RRP vesicles are not depleted by Bafilomycin A1, 5-HT can also recruit RP vesicles into RRP via one possible mechanism (PLC). (F) It is also possible that 5-HT can activate silence synapse most likely in low-output terminals. (G) The PLC activation of 5-HT effect is confirmed with PLC non-selective inhibitor. RP, reserved pool; RRP, readily releasable pool; SY, synapse; GluT, glutamate transporter; BA, Bafilomycin A1.

CHAPTER FOUR

The Regulation and Packaging of Synaptic Vesicles as Related to Recruitment within Glutamatergic Synapses

Introduction

This chapter has been published in Wu and Cooper (2012b) and is reproduced here for completeness of the dissertation.

In the early 1960s presynaptic vesicles were placed into different groups based on their spatial distribution and physiological properties (Birks and MacIntosh, 1961; Elmquist and Quastel, 1965). Various nomenclatures have been used; therefore, no absolute definitions have been established with anatomical distinctions (Rosenmund and Stevens, 1996; Palfrey and Artalejo, 1998; Li and Schwarz, 1999; Kuromi and Kidokoro, 2000; Rizzoli et al., 2003; Rizzoli and Betz, 2005; Fdez and Hilfiker, 2006). Vesicles are distributed inside a nerve terminal without a clear anatomical line to separate readily releasable pool (RRP) from reserve pool (RP). Nevertheless, the physiological and anatomical separation of a RP and RRP is widely utilized for working models to explain and study synaptic transmission (Ruiz et al., 2011). RRP is defined as a group of vesicles close to the synaptically active zones which will fuse and release neurotransmitter responding to Ca^{2+} influx, while the RP is a group of vesicles slightly farther away from the synaptic face. Kidokoro et al. (2004) defined an immediately releasing pool (IRP) at *Drosophila* neuromuscular junctions (NMJs) as distinctly different from a RRP based on vesicles docked for initial fusion and as compared to ones that can undergo rapid exocytosis and endocytosis. Generally, the IRP is grouped within the RRP (Aravanis et al., 2003) and (Sudhof, 2004]) and for our purposes, we grouped these two pools together as we examined longer term exocytosis and endocytosis processes. The vesicles in the RP pool are recruited under particular circumstances such as high frequency stimulation or in the presence of neuromodulators like 5-HT for crayfish NMJs ((Wang and Zucker, 1998; Quigley et al., 1999; Logsdon et al., 2006). With transmission electron microscopy (TEM), Johnstone et al., (2008, 2011) showed the structure of a crayfish nerve terminal with single vesicle resolution and an approach to estimate a range, with incorporating stereological errors, in measuring the location of vesicles in regard to the presynaptic membrane. Rarely are errors in location or distance dealt with from measurements utilizing TEM; however, such considerations are valuable (Atwood and Cooper, 1996a; Feuerverger et al., 2000; Kim et al., 2000). Since a number of disease states afflicting humans are related to presynaptic function, it is of interest to understand the fundamental properties that are most likely common to all animals as well as those that are different (Waites and Garner, 2011).

Several experiments have used various ways to physiologically separate vesicle pools. Rosenmund and Stevens (1996) and Bykhovskaia et al. (2001) used high osmolarity shock to deplete RRP. Such osmotic pressure changes on the nerve terminal were shown in the 1960s to cause transmitter release (Hubbard et al., 1968). Millar et al. (2002) employed a rapid vesicle depletion technique (200-Hz train stimulation) with a Cs^+ substitution, to deplete docked vesicles in crayfish tonic and phasic extensor NMJs. Schneggenburger et al. (1999) also demonstrated, in synapses at the Calyx of Held, that only a small fraction of the RRP is used by a single presynaptic action potential under physiological conditions but with a large percentage of vesicles that can be induced to fuse to the membrane if presynaptic calcium rises quickly. Akbergenova and Bykhovskaia (2009) identified RRP in *Drosophila* NMJ from TEM micrographs. In their study, the recycled vesicles, loaded with FM1-43, after intense stimulation were primarily localized within a group close to the synapses. Also, using a mutation in *Drosophila* to block recycling of vesicles revealed two distinct pools of vesicles (Kuromi and Kidokoro, 1998). In previous research in our laboratory, Logsdon et al. (2006) depleted the vesicles in RRP in crayfish opener NMJ by moderately stimulating a motor nerve axon in the presence of TBOA (dl-threo-β-benzyloxyaspartic acid), an inhibitor of a glutamate transporter (GluT) located on the plasma membrane. After synaptic depression occurred, it was demonstrated that the depressed state could be reversed by adding 5-HT. The interpretation is that 5-HT had the ability to promote the recruitment of vesicles from RP into RRP (Logsdon et al., 2006). Dudel (1965) first showed that synaptic transmission is enhanced at the crayfish NMJ by 5-HT and since then there have been a number of studies addressing the potential mechanisms of 5-HT's action (Enyeart, 1981; Glusman and Kravitz, 1982; Dixon and Atwood, 1985, 1989). The compiled findings indicated that two vesicle pools can be distinguished physiologically with one pool as the RRP and the other RP that can be recruited by 5-HT into the RRP.

It is postulated that new vesicles form the RP from an endosome and are packaged with neurotransmitter. It is implied that the RP can be spared and may not intermix with the RRP upon low-stimulation frequency (Kuromi and Kidokoro, 1998). Based on this view, Logsdon et al. (2006) proposed for the crayfish opener excitatory motor neuron that with repetitive trains of stimulation, vesicles can be quickly recycled within RRP and be depleted prior to recruiting from the RP. Thus, synaptic depression occurs due to the depletion of filled vesicles in RRP, while most vesicles in RP remain fully packaged with neurotransmitter. When evoked excitatory postsynaptic potentials (EPSPs), that showed depression, are rescued with 5-HT, not only will the evoked EPSPs grow in amplitude but also the spontaneous quantal events become intermixed in size. Normal-sized quantal events induced by 5-HT and reduced ones from the depressed state appear together as compared to just the smaller sized quanta responses during depression. A working model to explain this phenomenon was put forth, such that fully packaged vesicles from the RP are recruited, by actions

of 5-HT, to the RRP which contains partially filled vesicles that are rapidly recycling during the high-stimulation frequency.

There are two main sources of glutamate that can be utilized to fill recycling vesicles in the nerve terminals. Glutamate can be directly taken back up from the synaptic cleft through the plasma membrane transporter (GluT). This is the glutamate that was released during synaptic transmission. At the crayfish and *Drosophila* NMJs, as compared to the vertebrate CNS, glutamate does not cycle through a glutamine path via a glia cell. A second source of glutamate is the glutamate already in the cytoplasm, within the nerve terminals, that may not have been immediately taken up across the membrane during synaptic activity. The NMJ on the opener muscle in crayfish is used here to compare the previous findings related to glutamate uptake and to build on studies that addressed actions of 5-HT in enhancing synaptic transmission (Dudel, 1965; Dixon and Atwood, 1989, Crider and Cooper, 2000; Sparks et al., 2004). The NMJs on this tonic muscle are relatively low in synaptic efficacy as compared to higher output phasic motor nerve terminals in the crayfish (Cooper et al., 1995a; Cooper et al., 2003) or even those of the larval *Drosophila* NMJs (Cooper et al., 1995b) presented herein. Previous results indicate a significant role for glutamate uptake from synaptic cleft occurs in the process of refilling recycled vesicles. TBOA-treated NMJs preparations of the crayfish opener muscle showed a depression 2 h sooner, measured by EPSP amplitudes, as compared to controls. TBOA treatment did not block cytoplasmic glutamate from packaging in the vesicles nor could one address the issue if the vesicles were recycling within the RRP and RP. The purpose of this current study is to address this point by the use of bafilomycin A1.

As shown in past studies, 5-HT effect on enhancing synaptic transmission is partially induced by inositol 1,4,5-trisphosphate (IP3)-signaling pathway (Dixon and Atwood, 1989). In the study herein, we confirmed that blocking PLC, which in turn could lead to a decrease in IP3 formation, substantially blocked the 5-HT-induced response. Given that the 5-HT receptors at this nerve terminal appear to share pharmacological similarities to the mammalian 5-HT_2 subfamily (Tabor and Cooper, 2002), the mechanism of PLC activating IP3 formation for the downstream effect in promoting synaptic transmission is practical (Dropic et al., 2005). The novel aspect in this study compared to previous studies is the RP is being recruited following depression of the RRP and the mechanism of recruitment from a depressed synaptic state is being addressed.

The larval *Drosophila* NMJs also utilize glutamate as a neurotransmitter (Lee et al., 2009); however, synaptic transmission is not enhanced by exposure to 5-HT (Dasari and Cooper, 2004). We used the larval *Drosophila* NMJ as a comparison for the effects of bafilomycin A1 on the crayfish preparation. Kidokoro et al. (2004) used 20 μM at the *Drosophila* NMJ which is high compared to studies with vertebrate brain slices. Such high concentration appears to be toxic for the crayfish NMJs. Thus, we used lower concentrations throughout our studies. Since we examined actions of bafilomycin A1 with crayfish, a model species for

comparative studies, various concentrations and stimulation paradigms were utilized.

Experimental procedures

General

The experiments on crayfish were carried out in the first or second walking legs of midsize animals (*Procambarus clarkii*), measuring 6–10 cm in body length. They were individually housed in plastic containers with aerated water and fed dry fish food. The temperature of the animal room was controlled at 20–21 °C. The water was changed on a weekly basis. Crayfish were induced to autotomize the first or second walking leg by forceful pinching at the ischiopodite segment. The details on the dissection of the opener muscle preparations are in video format (Cooper and Cooper, 2009). Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205 NaCl; 5.3 KCl; 13.5 $CaCl_2$ $2H_2O$; 2.45 $MgCl_2$ $6H_2O$; 5 Hepes adjusted to pH 7.4). The larval *Drosophila* preparations were performed as previously described (Li et al., 2001) for early 3rd instars. The standard HL3 saline was used for physiological measures (Stewart et al., 1994; Ball et al., 2003). All experiments conformed to a named local as well as International Guidelines on the ethical use of animals and that all efforts were made to minimise the number of animals used and their suffering.

Pharmacology

All chemicals were obtained from Sigma Chemical (St. Louis, MO). Bafilomycin A1 (B1793) solution was made by dissolving 10 μg powder in 20 μl DMSO (99.9%), then adding crayfish or *Drosophila* saline to obtain the desired concentrations. The solution was stored at −20 °C no longer than 3 months. Various concentrations ranging from 4 nM to 16 μM were used. The crayfish preparations without incubation were compared to the ones with 2.5-h incubation. In the group without incubation, electrical stimulation was applied immediately after switching from saline to bafilomycin A1 containing saline. The groups with 2.5-h incubation in bafilomycin A1 were electrically stimulated after the 2.5 h. During the 2.5-h incubation time, the bafilomycin A1 solution was mixed in the recording dish every 30 min. 5-HT (1 μM) was made in crayfish saline from frozen stock of 1 mM. The bafilomycin A1 saline was used to make up the 5-HT containing saline. 5-HT was only examined in the crayfish preparations as 5-HT does not enhance synaptic activity at the *Drosophila* NMJs. U73122 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione and U73343 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione were obtained from Tocris Bioscience (Minneapolis, MN). Five millimolar U73122 and U73343 stock solutions were made by dissolving the

chemicals in DMSO (99.9% pure). The desired concentrations were obtained by adding crayfish saline. The solutions were freshly made for all the trials. The preparations were incubated for 1 h while the train stimulation was applied. A 500 nM 5-HT solution made from 1 mM stock was added to the preparations after 1-h incubation with either U73122 or U73343.

Physiology

The excitatory axon innervating the opener muscle in the crayfish was selectively stimulated in the merus segment by placing a branch of the leg nerve into a suction electrode connected to a Grass stimulator (Dudel and Kuffler, 1961a). Continuous stimulation at 20 or 40 Hz was applied to the excitatory nerve to compare the effects of bafilomycin A1. A train-stimulation of 20 EPSPs at 40 Hz every 10 s was applied to examine the effect by the PLC inhibitor and the inactive PLC inhibitor analog. Intracellular EPSP recordings were performed by standard procedures (Cooper et al., 1995a; Crider and Cooper, 2000). To be consistent, the distal muscle fibers were always used in this study, because anatomical, physiological properties of opener muscle differ in proximal, medial, and distal regions (Cooper et al., 1995a; Mykles et al., 2002). The synaptic responses at the larval *Drosophila* NMJs were recorded by standard procedures (Lee et al., 2009). All the experiments were performed at room temperature (20–21 °C). Continuous stimulation at 1 and 5 Hz was applied to the excitatory nerve to examine the bafilomycin A1 effect. Miniature end-plate potentials (mEPPs) were recorded for 5 min before applying 5-Hz stimulation either in saline or 8 μM bafilomycin A1 and for 10 min after 50% depression occurred. To separate RRP from RP, a similar stimulation paradigm as presented by Kuromi and Kidokoro (1998) and Kuromi and Kidokoro (2000) was used. Preparations were stimulated at 1 or 5 Hz for 30 min followed by 10-Hz stimulation for 10 s, then returned to 1 Hz for at least 20 s. Both EPSPs and mEPPs were measured by intracellular recordings. Electrical signals were recorded online to a computer via a PowerLab/4s interface.

Analysis

On examining the effect of bafilomycin A1, the amplitude of the last EPSP every 1 or 10 s in various experimental paradigms was measured (software Chart Version 5; ADInstruments) for the crayfish responses. When the largest facilitated EPSP responses were present, 60 events were averaged and normalized to 100%. Because of the variation in the time to peak facilitation among preparations, the time it took from the beginning of the stimulation to the time to reach 50% of the maximum amplitude was used as an index for depression time. The mean time of 50% decline was compared among different experimental conditions. In the crayfish preparations, after the EPSP amplitudes were less than 50% and nearly undetectable from background noise, the saline was exchanged to 1 μM 5-HT. The mean time of 50% decline at 20-Hz

continuous stimulation with 2.5-h incubation in 8 μM, 4 μM, 400 nM, 4 nM, bafilomycin A1 solutions as well as a control saline were used to provide a dose–response relationship. For the experiments of PLC inhibitor the 20th EPSP in a pulse train for totally 20 consecutive trains before and during exposure to 5-HT were compared. A percentage change was measured. In *Drosophila* preparations, the analysis of the responses for depression experiments was similar with the exception that only 1 and 5 Hz stimulation paradigms were used. The percentage change was measured by comparing the average amplitude of 100 mEPPs before applying stimulation and after 50% depression or comparing the average amplitude of 20 EPSPs before and after 10-Hz stimulation. The coefficient of variation (C_V) was determined by the ratio of the standard deviation to the mean. Statistical significance was assessed by paired, unpaired *t*-test or Mann–Whitney rank sum test. One, two and three asterisks indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. All the data are presented as mean ± SEM.

Results

Depression at crayfish NMJs for 20- and 40-Hz stimulation

The depression time to 50% depression was taken as an index for measuring the effect of bafilomycin A1 on synaptic transmission as compared to controls. A 20-Hz continuous stimulation was used in the opener muscle preparation as performed in previous experimentation using this preparation (Logsdon et al., 2006). A 40-Hz continuous stimulation was also used for comparative purposes to determine if depression time would be decreased as compared to 20-Hz continuous stimulation in the absence and presence of bafilomycin A1.

The viability of crayfish neuromuscular preparations in minimal saline is well known. Even with nerve stimulation, excised preparations are robust in nature. When exposed to crayfish saline with 20-Hz continuous stimulation, the average depression time to 50% of the peak EPSP amplitude is 192 min ($n = 5$, ±33 min SEM, **Figure 4.1A1 and C**). After depression was induced, exposure to 5-HT revitalized synaptic transmission in all cases with the 20-Hz stimulation paradigm (**Table 4.1**). This rejuvenation demonstrates the nerve terminals are not dead or badly damaged by the prolonged time of stimulation.

Preparations continuously stimulated at 40 Hz in crayfish saline had an average 50% depression time of 42 min ($n = 7$, ±8 min SEM, **Figure 4.1A2 and C**). After depression, only three preparations showed recovery of synaptic transmission with the presence of 5-HT (**Table 4.1**). This is probably because RP vesicles were already recruited to the RRP at a high frequency of stimulation. These results indicated that 40-Hz stimulation depresses synaptic transmission quicker than 20 Hz ($p = 0.003$, Mann–Whitney rank sum test), which is consistent with the expectation because vesicular recycling is facilitated at a higher stimulation frequency.

Effect of stimulation rate and exposure time to bafilomycin A1

In examining the effects of bafilomycin A1 to produce a 50% depression in EPSP amplitude, two different exposure paradigms were used. Six preparations at 20-Hz continuous stimulation in 4 μM bafilomycin A1, without a preincubation, took 62 min on average to depress to 50% (*n* = 6, ±20 min SEM, **Figure 4.1B1 and C**). Four out of six preparations were rejuvenated with 5-HT after depression (**Table 1**).

In contrast to 20-Hz continuous stimulation, preparations were continuously stimulated at 40 Hz, in the presence of 4 μM bafilomycin A1 without pre-incubation. In this set of experiments it took an average of 30 min (*n* = 6, ±8 min SEM, **Figure 4.1B2** and **C**) to depress the EPSP amplitudes to 50% but in only one out of six preparations did the EPSPs reappear with the 5-HT exposure (**Table 1**). Therefore, 20-Hz continuous stimulation was used for all the following experiments in crayfish preparations since they still had a response to 5-HT after depression.

Since there are no previous reports on the action of bafilomycin A1 at the crayfish NMJ, we examined various concentrations and exposure times. The use of 4 μM bafilomycin A1 with 2.5-h incubation was used for brain slices of rodents (Cavelier and Attwell, 2007). We examined if an incubation time of 2.5 h was necessary or if acute application was sufficient to have an effect on the recycling RRP. In this series of studies, 4 μM, 20-Hz continuous stimulation with 2.5-h prior incubation was compared to 4 μM, 20-Hz continuous stimulation without prior incubation but allowing the preparation to remain in saline for 2.5 h as for the sham control preparations.

With 20-Hz continuous stimulation and 2.5-h incubation in 4 μM bafilomycin A1, it took on average 31 min to depress the EPSP amplitude to 50% (*n* = 6, ±4 min SEM, **Figure 4.1C**); an enhancement of the EPSP amplitude induced by 5-HT after depression was observed in five out of six preparations (**Table 1**). By contrast, a 20-Hz continuous stimulation without incubation in 4 μM bafilomycin A1 took on average 62 min to depress to 50% (*n* = 6, ±20 min SEM, **Figure 4.1C**). As expected, there was a large degree of variation among these preparations. After depression, only four out of six preparations showed EPSPs regaining their amplitude with exposure to 5-HT (**Table 1**). These results showed that the synaptic transmission in the group without 2.5 h of incubation took longer to depress (almost double the time) than the group with 2.5 h of incubation. In addition, the results of this group without incubation were highly variable in the time to depression as compared to the group with incubation. The results of 2.5 h of pre-incubation appeared to be more reliable than without incubation for the effect of bafilomycin A1. This paradigm was used as standard procedure for obtaining a dose–response relationship to synaptic depression time.

Comparing the effect of bafilomycin A1 treatment to time of synaptic depression indicated that depression induced at 20 Hz is quicker (p = 0.004, Mann–Whitney rank sum test) with exposure to bafilomycin A1 (**Figure 4.1C** and **Table 1**). The higher stimulation rate of 40 Hz also induced depression more rapidly as compared to 20 Hz when not being exposed to bafilomycin A1. Also, with 40 Hz and exposure to bafilomycin A1, the depression rate was rapid (**Figure 4.1C**). One interpretation is that bafilomycin A1 can efficiently block vesicles refilling under repetitively high frequency stimulation. Also, we discovered that 2.5 h incubation resulted in less variability in the depression rate most likely because of an even distribution of the drug inside of the nerve terminal prior to rapid stimulation.

Dose–response relationship

Since these are novel experiments of bafilomycin A1 exposure in the crayfish model preparation, constructing a dose–response relationship helped in determining a practical dosage range for a specific cellular function. For example, it is conceivable that bafilomycin A1 might block ATP production by acting on mitochondrial ATPase H^+ pump, and, therefore, has a spurious action on synaptic transmission. However, an enhanced action of 5-HT on synaptic transmission after the induction of synaptic depression suggests that inhibition of ATP production was not a potential side effect for a given concentration. Given that bafilomycin A1 was used at *Drosophila* NMJs to address a similar topic (Kidokoro et al., 2004), we expected that this compound would also be suitable for other arthropod NMJs (i.e., crayfish).

Four micromolar bafilomycin A1

Four micromolar bafilomycin A1 was used with the standard stimulation paradigm described above in section ‘Effect of stimulation rate and exposure time to bafilomycin A1’. A total of six preparations were examined. On average, it took about 31 min to depress to 50% from the peak amplitude (n = 6, ±4 min SEM, **Figure 4.2B1**); after substantial depression was induced, 5-HT still enhanced the amplitude in EPSPs in five out of six preparations.

Eight micromolar bafilomycin A1 is toxic to crayfish NMJs

To test whether higher concentrations can work more effectively, the concentration of bafilomycin A1 was doubled from 4 to 8 μM. Two preparations failed to show synaptic responses during the incubation. In order to test the time to damaging the health of the NMJ and whether the lack of responsiveness was caused by the failure of neurotransmission or other side effects of this drug, three preparations were stimulated for 1 s every 10 min while being exposed to 8 μM

bafilomycin A1 (**Figure 4.2A1** and **A2**). The EPSPs in all three preparations failed within 1.5 h, however, the EPSPs recorded right before failures were normal. We interpret these results as a toxic effect rather than a failure of presynaptic neurotransmission to block the packaging of vesicles.

Four nanomolar and 400 nM bafilomycin A1

Two lower concentrations of bafilomycin A1 (4 nM and 400 nM) were also examined for the effect on vesicular repackaging inhibition. With 4 nM bafilomycin A1, the average depression time to 50% is 111 min (*n* = 5, ±19 min SEM, **Figure 4.2B1**). All the preparations showed synaptic recovery with 5-HT after depression. The average 50% depression time is 30 min (*n* = 5, ±9 min SEM, **Figure 4.2B1**) for 400 nM. All the preparations showed synaptic recovery with 5-HT after depression.

Overall results of dose-response effect

In normal crayfish saline, the average depression time to 50% peak amplitude is 192 min (*n* = 5, ±33 min SEM, **Figure 4.2B1**). After depression, 5-HT always produced an enhanced response. The 2.5 h of pre-incubation without stimulation for the following concentrations 8 μM, 4 μM, 400 nM, 4 nM of bafilomycin A1 and sham control followed by continuous 20-Hz stimulation was used to graph a dose–response curve of the time required to depress the EPSPs to 50%. As mentioned above, 8 μM of bafilomycin A1 exposure is lethal so data with 8 μM bafilomycin A1 are not included in the dose–response curve. The incubations at 4 μM took about 30 min while control preparations lasted on average 3 h. The 400 nM bafilomycin A1 had an effect similar to 4 μM. The depression time from 4 nM bafilomycin A1 occurred between the times noted for control and 400 nM treatments. Within the dose range examined, the suggested inhibition of vesicular refilling by bafilomycin A1 was correlated with the concentration of drug exposure (**Figure 4.2B2**). It appears that the RP vesicles were protected as the addition of 5-HT always resulted in an enhanced response after synaptic depression was induced.

PLC inhibitor attenuates 5-HT effect on synaptic transmission

The PLC non-selective inhibitor U73122 was used to test the possibility of 5-HT mediating an enhanced recruitment of RP vesicles via a PLC intermediate step. The inactive analog of U73122, U73343, served as a negative control. Doses ranging from nanomolar to millimolar have been employed in various studies of mammalian models. No previous reports are present for these compounds being examined at the crustacean NMJ. Ten micromolar was reported to be sufficient in reducing IP3 production in human platelet cells (Bleasdale et al., 1990). We used

5 and 10 μM, but no significant reduction of 5-HT effect was observed (data not shown). Therefore, 50 μM was used for both U73122 and U73343.

The preparations were recorded in crayfish saline for 1 h before being switched to a 500 nM 5-HT solution. The average amplitude of 20th EPSP in a pulse train for 20 consecutive trains was compared before and after adding 5-HT. The average percentage change increased by 266% (*n* = 5, ±42% SEM, **Figure 4.3A1** and **A2**). Instead of crayfish saline only, the preparations were stimulated and recorded while being bathed in 50 μM U73122 solution for 1 h before being exposed to 5-HT in crayfish saline. The average amplitude in the 20th EPSPs for 20 consecutive trains was compared before and after adding 5-HT. The average percentage change was only 14% (*n* = 6, ±6% SEM, **Figure 4.3B1** and **B2**). As a negative control, the preparations were recorded in U73343 (50 μM) solution for 1 h before the 5-HT exposure. The average amplitude of 20th EPSPs for the pulse train was compared before and after adding 5-HT. The average percentage change was 191% (*n* = 5, ±23% SEM, **Figure 4.3C1** and **C2**).

Actions of bafilomycin A1 at the Drosophila NMJ

Low frequencies (1 and 5 Hz) continuous stimulations were used due to the high output property of the *Drosophila* NMJs. Pre-incubation with 4 μM bafilomycin A1 did not result in any significant difference from the ones without pre-incubation (data not shown). There was no significant difference in depression rate for 5 Hz (*n* = 5, 32 min, ±6 min SEM) and 1 Hz (*n* = 5, 37 min, ±3 min SEM) when exposed to normal fly saline only (Figure 4.4).

As a comparison to the crayfish preparations, 4 μM bafilomycin A1 was first examined at both the 1-Hz and 5-Hz stimulation paradigm. In the presence of 4 μM bafilomycin A1 the 5 Hz resulted in rapid depression (*n* = 8, 5 min, ±1 min SEM); however, due to the variability, there is no significant difference in the 1-Hz stimulation rate (*n* = 5, 3 min, ±1 min SEM) (**Figure 4.4**). However, the difference in rate of depression is profound with exposure to bafilomycin A1 ($p < 0.001$, Student's *t*-test). This rapid rate of synaptic depression substantiates the paralyzed effect within 2 min of whole larval injection of bafilomycin A1 (~1 μM) (Denker et al., 2011a). To test whether higher concentrations work more effectively, the concentration of bafilomycin A1 was doubled from 4 to 8 μM. Surprisingly, the *Drosophila* preparations did not have a rapid cessation at 8 μM as for the crayfish preparation. All the preparations examined maintained their resting membrane potentials. The mean time of depression appeared to be reduced, however not significantly, at 5 Hz (*n* = 8, 9 min, ±0.6 min SEM) compared to 1 Hz (*n* = 5, 11 min, ±2 min SEM). Exposure to 8 μM did not depress terminals any faster than did the 4 μM exposure (Figure 4.4). To further confirm the possibility of saturating the rate of depression at 4 μM concentration, a higher dose of 16 μM was examined at 1-Hz stimulation (11 min, ±0.5 min

SEM). No significant difference in time to depression was present between 4 and 8 μM exposure.

The spontaneous vesicular pool at the Drosophila NMJ

The mEPPs are produced by single (quanta) vesicular fusion events (Fatt and Katz, 1952). Therefore, the size of mEPP can be used as an indirect measure in the amount of neurotransmitter content released during vesicle fusion as well as receptivity to the transmitter. One would expect the glutamate content of recycled vesicles to decrease if the vesicle is not completely empty after evoked depression in the presence of bafilomycin A1, because the refilling process is impeded. However, intracellular recordings of mEPPs in Drosophila muscle did not show significant difference between saline and bafilomycin A1 treatments. The percentage change in the amplitude as well as the coefficient of variation (C_V) after depression was similar in saline changed to saline (control group) or saline changed to bafilomycin A1 (experimental group). There was a similar degree in rundown in the amplitude of the spontaneous single quantal events for both conditions (**Figure 4.5**). This is shown as a negative percent change and a positive C_V.

Separation of RP and RRP physiologically at the Drosophila NMJ

Following a similar stimulation paradigm as presented by Kuromi and Kidokoro (1998) and Kuromi and Kidokoro (2000), which was designed to test if RP vesicles can be recruited into the RRP, a high frequency stimulation (10 Hz) was given for 10 s and then returned to a lower stimulation frequency (1 or 5 Hz) while monitoring the EPSP amplitudes. Unlike crayfish NMJs, 5-HT has no effect on enhancing synaptic transmission at these *Drosophila* NMJs (Dasari and Cooper, 2004) so the high frequency stimulation paradigm was used to recruit from the RP. The two stimulation paradigms used are depicted in **Figure 4.6A**. The nerve was stimulated at low frequency (1 or 5 Hz) for 30 min in the presence or absence of 4 μM bafilomycin A1, followed by high frequency stimulation at 10 Hz for 10 s then back to 1 or 5 Hz for at least 20 s. The average amplitude in 20 EPSPs before and after the 10-Hz stimulation was compared.

Consistent with the general findings of Kuromi and Kidokoro (2000), preparations not exposed to bafilomycin A1 showed no difference in the EPSP amplitudes before and after tetanic stimulation (10 Hz) for both paradigms (1 Hz – 10 Hz – 1 Hz or 5 Hz – 10 Hz – 5 Hz, **Figure 4.6B** and **D**). With bafilomycin A1, tetanic stimulation can enhance synaptic transmission from the evoked depressed state (**Figure 4.6C** and **D**). This suggests that bafilomycin A1 treatment depletes glutamate in RRP vesicles during the 30 min low frequency stimulation and the 10 Hz tetanic stimulation can recruit RP vesicles that are full of glutamate to recover the synaptic activity.

Discussion

The ATPase specific inhibitor bafilomycin A1 has been used in various studies involving packaging of vesicles in mice hippocampal neurons (Cavelier and Attwell, 2007). However, the effect of bafilomycin A1 on vesicular packaging and synaptic transmission in the invertebrate model organism *P. clarkii* has not been previously examined in relation to recruiting a RP with neuromodulation. Bafilomycin A1 exposure at 20 μM on *Drosophila* NMJs was reported earlier and was shown to have substantial effects in reducing evoked synaptic transmission (Kidokoro et al., 2004). In depressed NMJs of the *Drosophila*, in the presence of bafilomycin A1, a burst of 30-Hz stimulation was able to rejuvenate vesicular release for a short period. Thus, a RP was indicated to be independent of the RRP. Likewise, in nerve terminals of the crayfish opener NMJ after the induction of evoked depression in the presence of bafilomycin A1, evoked responses reappeared with modulation by 5-HT. Thus, a physiological separation in the RRP and RP is present for both preparations. Our results also extend the findings that the quantal events by minis appear to be a different pool of vesicles compared to the evoked vesicle pool in the larval *Drosophila* NMJ preparation.

As anticipated, the crayfish preparations stimulated at 40 Hz depressed faster than the ones stimulated at 20 Hz. However, recovery of synaptic activity by 5-HT following depression was more variable in preparations stimulated at 40 Hz compared to the 20 Hz paradigm. Two out of several possibilities could explain the larger variation at the higher frequency stimulation paradigm. First, it is possible that at 40 Hz the RP vesicles were recruited into RRP to support the functional demand; thus, fewer vesicles remained in RP which could be recruited by 5-HT. Secondly, in the presence of bafilomycin A, the accelerated rate of vesicular turnover at 40 Hz reduced the RRP of vesicles that were able to be repackaged with transmitter.

The opener crayfish preparation is lower in synaptic efficacy than the larval M6 *Drosophila* NMJ (Cooper et al., 1995b); however, they both appear to have a means of protecting a reserve pool of vesicles that can be pulled into action when a demand is present if the terminals are not highly depressed due to high frequency stimulation. The RP not only is distinguishable from the RRP, but also another discrete pool that maintains spontaneous events is likely present. At least for the *Drosophila* NMJs, when the RRP is functionally depleted of filled vesicles for evoked transmission, minis still occur. The size of the crayfish muscles used in this study did not allow for spontaneous events to be clearly distinguishable for quantitative purposes, with intracellular recordings, to compare with the small *Drosophila* muscles. Possibly with focal macropatch electrode recordings over nerve terminals would be an approach to address this issue at the crayfish NMJs or to use smaller preparations. The larval *Drosophila* muscles used are relatively small which aids in a higher whole membrane resistance allowing the spontaneous event to be readily observed. The larval *Drosophila* NMJ does show rundown in evoked release with the HL3 saline (Stewart et al., 1994). Also, there is some attenuation in the amplitudes of the mEPPs as well if one starts recording within a few minutes of a rapid dissection

(total time from start of dissection to recording ~5 min). Kidokoro et al. (2004) noted that in transected segmental nerves, the same NMJ preparation as used in current study, vesicle recycling is reduced as compared to intact segmental nerves. In our study, we were required to drive the axons without intrinsic motor nerve activity induced from an intact CNS. Since the controls and the experimentally treated preparations were handled in a similar manner, direct effect of the experimental manipulation can be compared. It is likely that HL3 is not an optimally suited saline. Other salines, such as HL6, are being examined for NMJ physiology (Macleod et al., 2002). Given that HL6 does contain amino acids, we did not wish to add such cofounding variables to these experimental conditions used in our study with transmitter repackaging. We suggest, this remaining pool of vesicles that were still fusing after the RRP depletion is arriving from the RP vesicles to the synaptic face. In a model for the *Drosophila* NMJ, we suggest a dynamic RP in which vesicles can be recruited depending on the stimulation frequency and even after the RRP is depressed, some RP vesicles could be pulled into action for asynchronous and evoked coordinated fusion.

The presence of mEPPs after depression of evoked transmission does bring up an interesting topic to the source of the mEPPs in relation to evoked vesicular events. In vertebrate CNS slice preparations, it appears the spontaneous occurring events arise from an independent pool of vesicles from the evoked pool (Sara et al., 2005, Fredj and Burrone, 2009; Chung et al., 2010); however, other studies, suggest that the spontaneous events are from the same pool as the evoked (Wilhelm et al., 2010).

The recovery of the depressed crayfish preparations with exposure to 5-HT suggests that mitochondria ATP production is not impaired by bafilomycin A1 because vesicle docking and recycling is an ATP-dependent process (Tolar and Pallanck, 1998). Incubating preparations for 2.5 h might allow bafilomycin A1 to distribute more evenly inside the terminals, which theoretically would result in more consistent results, compared to acute exposure to bafilomycin A1. This postulation is supported as the variation in depression rates among preparations was greater when preparations were not incubated. Also, with incubation, further fusion events are not compromised, as exposure to 5-HT still enhances release. Likewise, the *Drosophila* NMJs likely have sufficient mitochondrial function after exposure to bafilomycin A1 and low frequency depression since high frequency stimulating bursts still allow vesicular fusion events to occur.

It would be of interest to know if similar cellular mechanisms in recruitment of the vesicles from the RP to the RRP occur for these two preparations. Potentially different cellular mechanism might lead to the same end result, such as phosphorylation of synapsins to increase the pool of free vesicles for docking or possibly actions on calcium influx and release from internal stores (Yang et al., 1999; Dropic et al., 2005). This internal release of Ca^{2+} could activate calmodulin in turn activating CaM-kinase (CaM-K), which can phosphorylate proteins to either activate or deactivate them. In this case, CaM-K could phosphorylate the integral vesicle membrane protein synapsin. In *Aplysia* neurons exposure to 5-

HT has been shown to cause phosphorylation of synapsins (Fiumara et al., 2004). This could then result in vesicles being freed from the cytoskeleton to promote docking to the presynaptic membrane. Phosphorylation of mediator proteins involved directly with the docking machinery, such as t-SNARE, v-SNARES, NSFs or MUNC proteins, could also be occurring along with mobilizing vesicles from the RP (for crayfish studies see – Dixon and Atwood, 1989; Tolar and Pallanck, 1998; He et al., 1999; Southard et al., 2000). Possibly, an equivalent form of Bruchpilot-like proteins is distributed in the crayfish and *Drosophila* motor nerve terminals that might form divisions among vesicles in their movements (Kittel et al., 2006) and Ca^{2+} buffering which not only alters fusion but the endocytosis processes as well (Yamashita, 2012). There may indeed be various overlapping cellular processes utilized by 5-HT to pull vesicles from the RP to the RRP as well as independent processes related to electrical activity of the terminal. It was demonstrated that $[Ca^{2+}]_o$ is needed for synaptic release at the frog (Katz and Miledi, 1968) and crustacean NMJs (Fatt and Katz, 1953) and that higher $[Ca^{2+}]_o$ increases synaptic communication. The rise in $[Ca]_i$ from the prolonged Calcium conductance after a series of action potentials is also implied as being one mechanism which rises residual $[Ca]_i$ to account for short-term facilitation at the crayfish NMJs (Fuchs and Getting, 1976; Parnas et al., 1982; Wojtowicz and Atwood, 1984; Atwood and Wojtowicz, 1986; Dudel, 1989a, b; Cooper et al., 1996b). However, at very high concentrations of $[Ca^{2+}]_o$ synaptic transmission can be depressed quickly. The $[Ca^{2+}]_i$ can activate calmodulin in turn activating CaM-K as well as potentially activating a Ca^{2+}-induced Ca^{2+}-release (CICR). Release of Ca^{2+} from ER can be regulated by inositol 1,4,5-trisphosphate (IP3) (Berridge, 2005). Activation of ryanodine receptors within presynaptic nerve terminals is known to alter evoked transmitter release (Liu et al., 2005). One might suggest the rate of uptake into the ER by the sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and release by CICR will alter vesicle recruitment from the RRP. For review of vesicle pools and various experimental approaches to address their function at the *Drosophila* NMJ see Kidokoro et al. (2004). The role of $[Ca^{2+}]_i$ sequestering and release by the ER and extrusion by pumps and exchangers in crayfish and *Drosophila* NMJs was recently addressed (Desai-Shah and Cooper, 2009, 2010).

In order to illustrate the general findings presented in this study we present a simple model (**Figure 4.7**) that highlights the recruitment of the RP to the RRP by 5-HT and/or Ca^{2+} for the crayfish NMJ and by Ca^{2+} for the *Drosophila* NMJs. The *Drosophila* NMJs are not sensitive to 5-HT; however, there are likely other neuromodulators and second messenger cascades that may enhance transmission. A recent review of 5-HT actions on NMJs of invertebrates indicates various species that do have a response to applied 5-HT (Wu and Cooper, 2012a). It would be of interest to know if similar receptor subtypes and secondary messengers are utilized among these species. The presence of intracellular Ca^{2+} in this model occurs through voltage-gated Ca-channels. The presence of minis (i.e., spontaneous quantal events not associated with depolarization of the terminal) may be occurring due to vesicles fusing at the same synaptic sites

recruited during electrical depolarization of the terminal or other regions of the synapse (Chung et al., 2010).

Given that inhibition of PLC reduced the effect of 5-HT in recruiting presumable RP vesicles, a likely scenario is that the 5-HT receptors are coupled, through G protein-coupled receptors to PLC. The PLC can activate a number of further cascades such as DAG and IP3. The IP3 might then activate the Ca^{2+} response from the ER and this Ca^{2+} could even then activates CaM-K. A similar situation has been demonstrated for neurons in *Aplysia*, such that exposure of 5-HT resulted in an increase of IP3 levels that correlated with an increase in synaptic activity (Jin et al., 2007). Future studies with compounds such as a kinase inhibitor (staurosporin), a phosphatase inhibitor (okadaic acid) or cytoskeletal disrupting agents would be interesting to determine the impact in recruiting the RP to the RRP after synaptic depression is induced.

In short, this study aided in understanding a dose range of applying bafilomycin A1 on crayfish and *Drosophila* preparations as well as the effects of putatively blocking glutamate repackaging in recycling vesicle pools. The ability to physiologically differentiate the RRP and RP with varied experimental techniques provides additional insight into the underlying mechanisms of how these pools are utilized within nerve terminals. Future investigation into variations of how the pools function in various types of nerve terminals will shed additional light on the molecular regulation in synaptic vesicle dynamics. Currently we are comparing high output phasic type of nerve terminals in crayfish preparations and high output *Drosophila* NMJs in populations of vesicles within the RP and RRP in their recycling regulation. Recently, it was demonstrated that only a small percentage of vesicles undergo exocytosis even at high output motor nerve terminals (Denker et al., 2011a, b). It would be of interest to further investigate why there are fewer reserve vesicles in high output synapses and what the function of a large RRP is when a large percentage of them are apparently not used before synaptic depression occurs

TABLE 4.1: The 50% depression time and the number of preparations with increased amplitude after 5-HT

Stimulation	No. Prep	Time Depress to 50% (min)	w/5-HT
0 µm; 20Hz continuous	5	192.46	5 / 5
4µm; 2.5h; 20Hz continuous	6	30.51	5 / 6
4µm; 0h; 20Hz continuous	6	62.08	4 / 6
0 µm; 40Hz continuous	7	42.87	3 / 7
4µm; 0h; 40Hz continuous	6	30.07	2 / 6

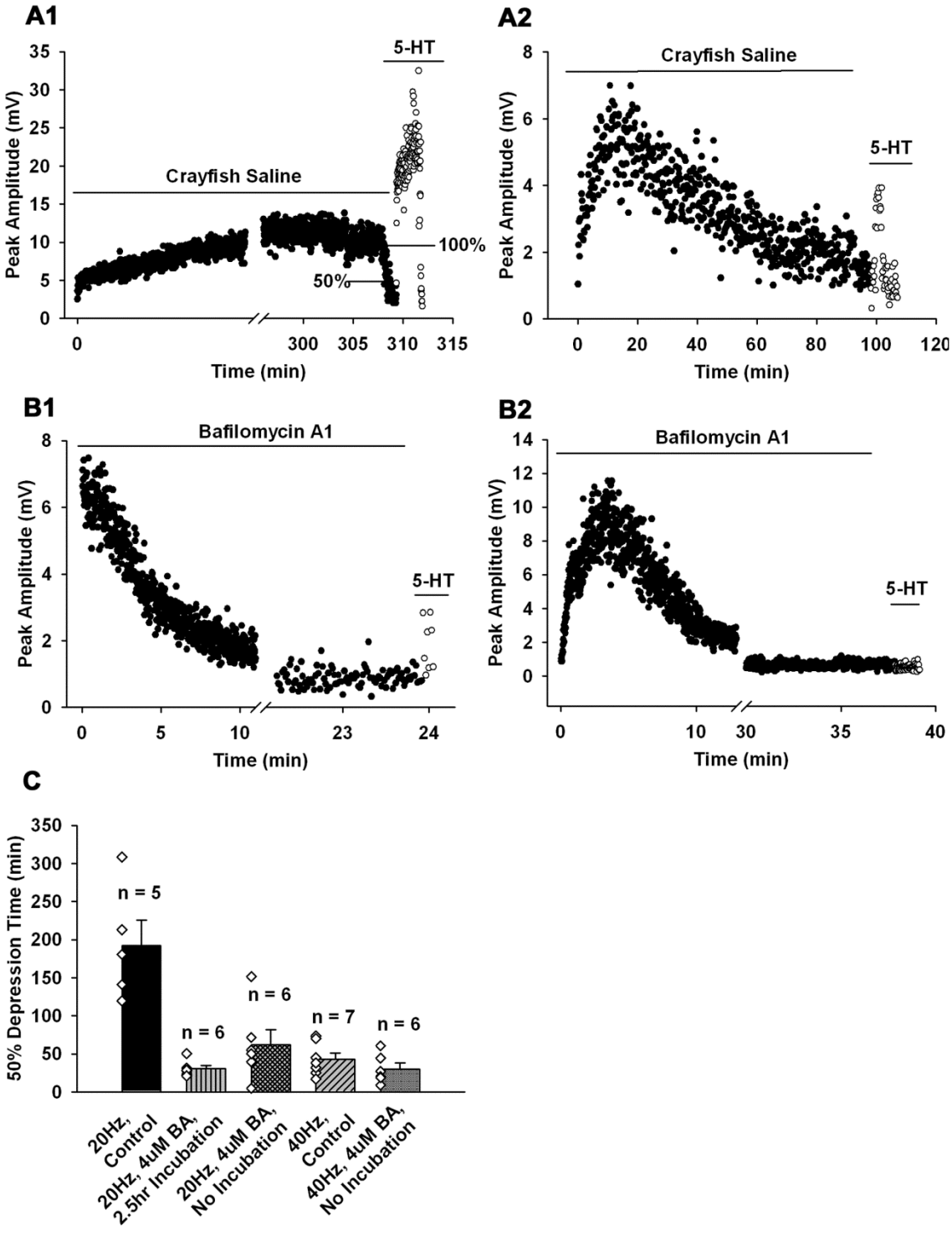
A1
5-HT
Crayfish Saline
100%
50%
Peak Amplitude (mV)
Time (min)
A2
Crayfish Saline
5-HT
Peak Amplitude (mV)
Time (min)
B1
Bafilomycin A1
5-HT
Peak Amplitude (mV)
Time (min)
B2
Bafilomycin A1
5-HT
Peak Amplitude (mV)
Time (min)
C
50% Depression Time (min)
n = 5
n = 6
n = 6
n = 7
n = 6
20Hz, Control
20Hz, 4uM BA, 2.5hr Incubation
20Hz, 4uM BA, No Incubation
40Hz, Control
40Hz, 4uM BA, No Incubation

Figure 4.1: Effect of stimulation rate and exposure time to bafilomycin A1 at 20-Hz and 40-Hz continuous stimulation. (A) Representative scatter plot of the EPSP peak amplitudes (mV) at 20-Hz (A1) and 40-Hz (A2) continuous stimulation in crayfish saline. Filled circles are EPSPs in crayfish saline. After substantial depression in the EPSP amplitude a 5-HT containing saline replaced the saline bath. EPSPs reappeared as indicated in open circles. (B) Representative scatter plot of the EPSP peak amplitudes (mV) at 20-Hz (B1) and 40-Hz (B2) continuous stimulation in 4 μM bafilomycin A1 without pre-incubation. After substantial depression in the EPSP amplitude a 5-HT containing saline replaced the saline bath. EPSPs reappeared as indicated in open circles. (C) Composite results comparing 50% depression times. Bar graph representing the various experimental conditions (*n* represents the sample size, BA is bafilomycin A1).

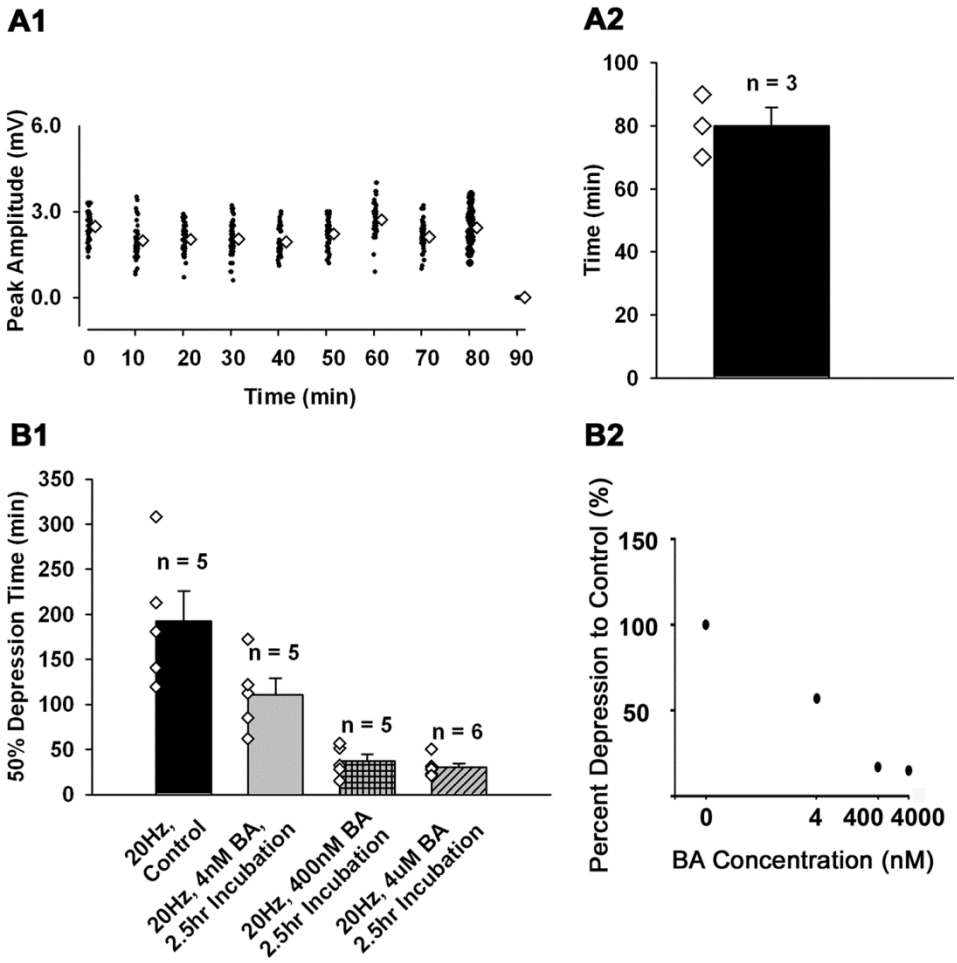

Figure 4.2: Dose–response relationship. (A) Representative scatter plot (A1) of the EPSP peak amplitudes (mV) during the incubation with 8 μM bafilomycin A1 in which the preparations were stimulated for 1 min measured every 10 min. Filled circles are individual EPSPs and the open diamonds are the average amplitudes. Bar graph (A2) is the time when preparations stopped producing EPSPs. The average time for all three preparations is 80 min (±6 min SEM). The open diamonds represent the three individual preparations. (B) Bar graph (B1) of the 50% depression time in relation to the concentration of bafilomycin A1 exposure. All the experiments were performed with 20-Hz continuous stimulation preceded by 2.5-h pre-incubation in 0 nM, 4 nM, 400 nM, and 4 μM bafilomycin A1 solution (*n* represents sample size, BA is bafilomycin A1). Dose–response relationship (B2) measures the rate to 50% depression with 0 nM, 4 nM, 400 nM, and 4 μM bafilomycin A1 exposure. Eight micromolar bafilomycin A1 is toxic and is not depicted in the graph.

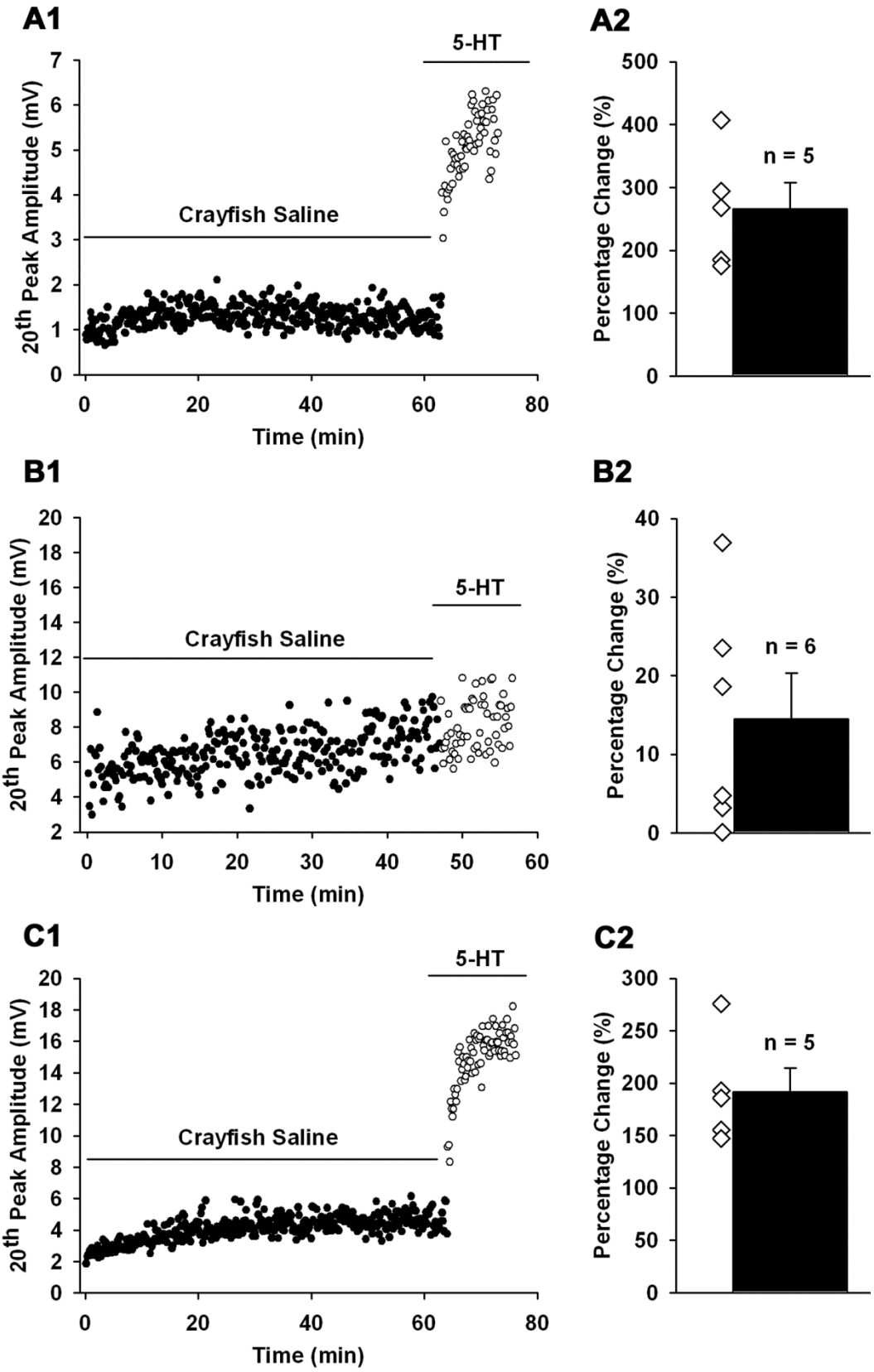
A1
5-HT
Crayfish Saline
20th Peak Amplitude (mV)
Time (min)
A2
Percentage Change (%)
n = 5
B1
5-HT
Crayfish Saline
20th Peak Amplitude (mV)
Time (min)
B2
Percentage Change (%)
n = 6
C1
5-HT
Crayfish Saline
20th Peak Amplitude (mV)
Time (min)
C2
Percentage Change (%)
n = 5

Figure 4.3: The effect of U73122 and U73343 on 5-HT induced synaptic transmission. A representative scatter plot for one preparation depicts the amplitude of 20th EPSP in each pulse train through an hour of control (A1), U73122 (B1) and U73343 (C1) treatment followed by 5-HT treatment. 5-HT enhances the amplitude significantly in control and U73343 treated preparations but not U73122 treated group (open circles). In control, U73122 and U73343 treated group, the average percentage change is 266% (*n* = 5, ±42% SEM) (A2), 14% (*n* = 6, ±5.8% SEM) (B2) and 191% (*n* = 5, ±23% SEM) (C2) respectively. The open diamonds is the percentage change for each individual preparation.

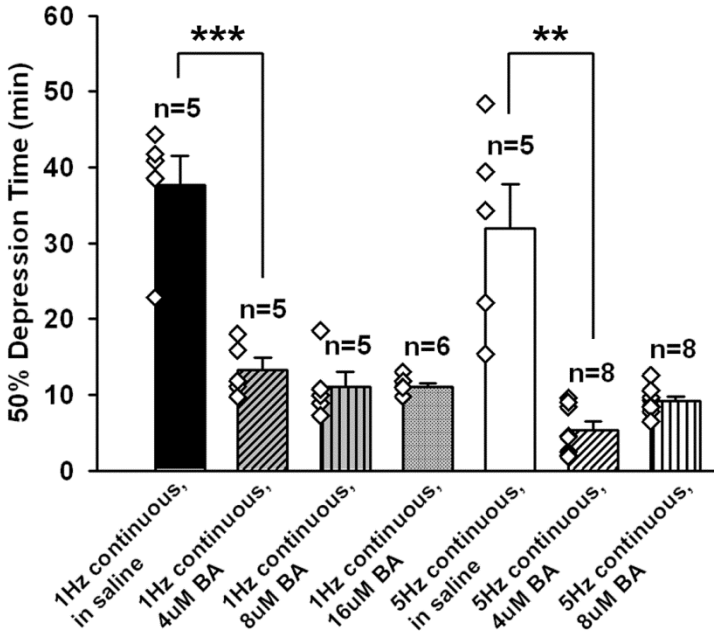

Figure 4.4: Actions of bafilomycin A1 at the *Drosophila* NMJ. Bar graph of the 50% depression time in minutes (min) when the preparations were treated with 4 μM, 8 μM bafilomycin A1 for both the 1-Hz and 5-Hz stimulation paradigms (16 μM only at 1 Hz) (*n* represents sample size, BA is bafilomycin A1). The diamonds are the 50% depression time of each individual preparation. Two and three asterisks indicate $p < 0.01$ and $p < 0.001$ respectively.

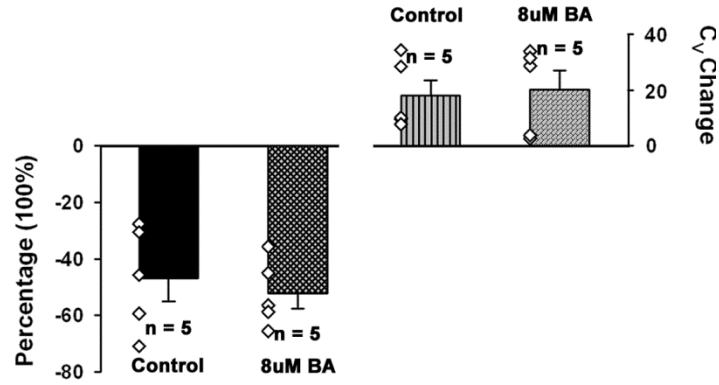

Figure 4.5: The spontaneous vesicular pool at the *Drosophila* NMJ. The percentage change of the average amplitude of 100 mEPPs before and after 50% depression and the coefficient of variation (C_V) are shown. Preparations in saline and in 8 μM bafilomycin A1 are compared. The diamonds represent each individual preparation.

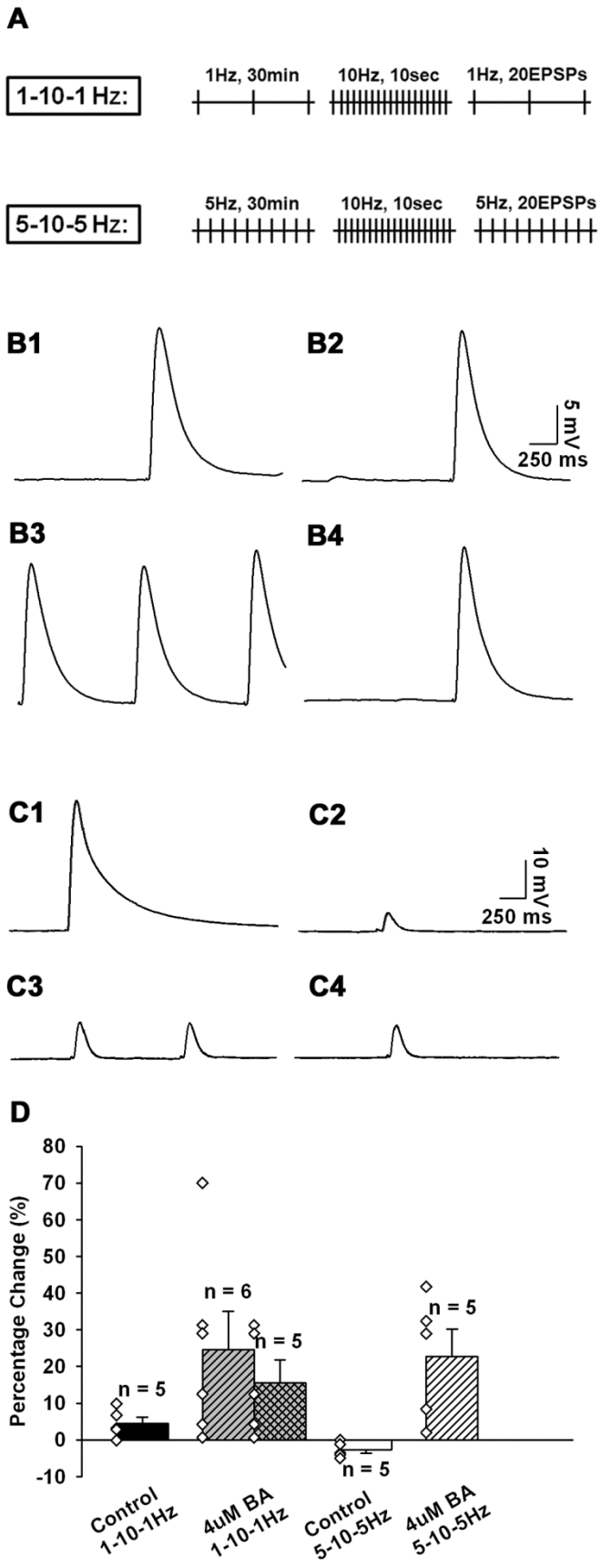
A
1-10-1 Hz:
1Hz, 30min
10Hz, 10sec
1Hz, 20EPSPs
5-10-5 Hz:
5Hz, 30min
10Hz, 10sec
5Hz, 20EPSPs
B1
B2
5 mV
250 ms
B3
B4
C1
C2
10 mV
250 ms
C3
C4
D
Percentage Change (%)
80
70
60
50
40
30
20
10
0
-10
n = 5
n = 6
n = 5
n = 5
n = 5
Control 1-10-1Hz
4uM BA 1-10-1Hz
Control 5-10-5Hz
4uM BA 5-10-5Hz

Figure 4.6: Separation of RP and RRP physiologically at the *Drosophila* NMJ. (A) The stimulation paradigm used to separate RRP and RP physiologically. 1-Hz and 5-Hz baseline stimulation are compared. The nerve was stimulated at low frequency (1 and 5 Hz) for 30 min in the presence or absence of 4 μM bafilomycin A1, followed by high frequency stimulation at 10 Hz for 10 s then retuned back to 1 or 5 Hz for at least 20 s. (B, C) Representative traces of 1 Hz–10 Hz–1 Hz in control (B) and 1 Hz–10 Hz–1 Hz in preparation treated with 4 μM bafilomycin A1 (C). The trace at 5 Hz–10 Hz–5 Hz group shows the similar pattern. B1, C1 show one EPSP at the beginning of 1-Hz stimulation. After 30 min, the amplitude in control does not change (B2) while bafilomycin A1 treated preparation depresses (C2). With 10 s of 10-Hz tetanic stimulation an enhancement in the EPSP amplitude did not occur in control group (B3) but did so for the bafilomycin A1 treated preparation (C3). The enhancement continues even when stimulation was returned to 1 Hz (C4), whereas the control preparation did not show as much change (B4). (D)The percentage change (average amplitude of 20 EPSPs after 10 Hz compared to the average of 20 EPSPs before 10 Hz) with 1 Hz–10 Hz–1 Hz and 5 Hz–10 Hz–5 Hz stimulation paradigms with and without exposure to 4 μM bafilomycin A1. In both stimulation paradigms, 4 μM bafilomycin A1 causes larger percentage change in EPSP amplitude.

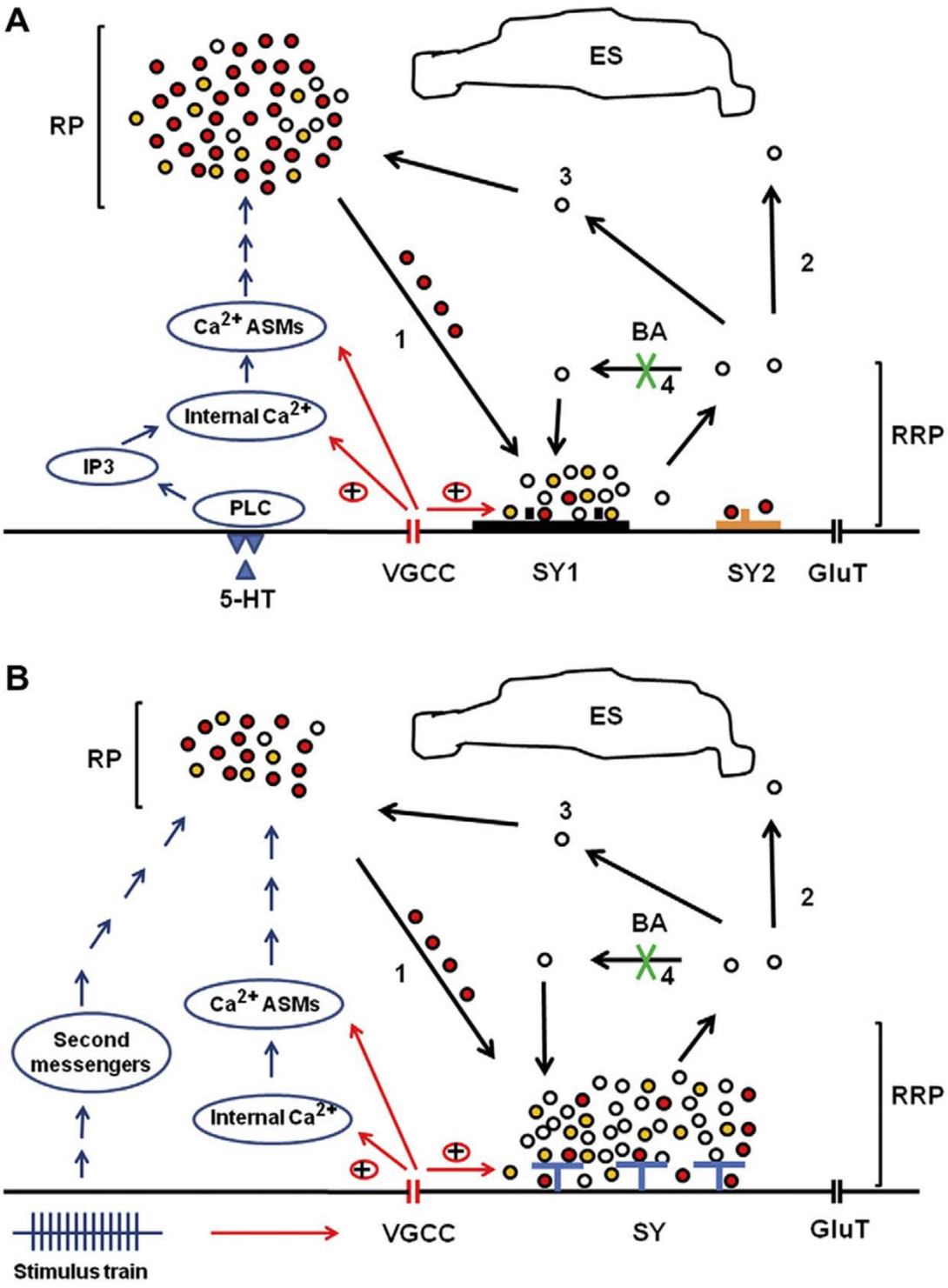
A
RP
ES
3
2
1
BA
4
Ca2+ ASMs
Internal Ca2+
IP3
PLC
5-HT
RRP
VGCC
SY1
SY2
GluT
B
RP
ES
3
2
1
BA
4
Second messengers
Ca2+ ASMs
Internal Ca2+
RRP
VGCC
SY
GluT
Stimulus train

Figure 4.7: Mechanistic diagram of vesicle recycling between RP and RRP and the effect of 5-HT or electrical stimulation on recruiting RP vesicles in crayfish and *Drosophila* NMJs. Synaptic vesicles are separated into RRP and RP. Two vesicle recycling pathways have been proposed. In a rest synapse, vesicles in RP can slowly join into the RRP (1), and then recycle back to RP either through or bypass endosome (2 or 3). This is called slow recycling loop. However, in an active synapse, in addition to the slow recycling loop, vesicles in RRP recycle quickly within the RRP (4) which is named quick recycling loop. Recycling vesicles are refilled with glutamate. However, this refilling process can be disrupted by bafilomycin A1 treatment (as shown by an empty vesicle in pathway 4). Synaptic depression occurs sooner with bafilomycin A1 treatment because vesicles in RRP can be depleted of transmitter in time with stimulation while the recycling vesicles can no longer be refilled. (A) In crayfish low output terminals, more RP vesicles comparing to RRP is proposed. After bafilomycin A1 induced synaptic depression, 5-HT is able to recruit RP vesicles to rejuvenate the synaptic activity. One of the possible mechanism is that 5-HT activates IP3 signaling pathway which stimulates Ca^{2+} release from the internal stores such as ER. The elevated $[Ca^{2+}]_i$ then activates other second messengers like CaM kinase. After several steps of cellular activity, RP vesicles are pulled into RRP. In addition, external Ca^{2+} coming through VGCC can increase $[Ca^{2+}]$ in the internal stores, activate Ca^{2+} activated second messengers, also increase the cooperativity of vesicle fusion via synaptotagmin. 5-HT may also activate low probability synapses like SY2. The low probability synapses are likely ones with less complex synaptic structure (Cooper et al., 1996). (B) In *Drosophila* high output terminals, more vesicles are present in RRP. After bafilomycin A1 induced synaptic depression, higher frequency stimulation can pull the RP vesicles into RRP. In this model $[Ca^{2+}]_i$ is elevated by repetitive high frequency stimulation which then increases $[Ca^{2+}]_i$ in the ER (internal stores) and directly activates Ca^{2+} activated second messengers as well as increases the cooperativity of vesicle fusion. Red filled vesicles are one full of transmitter. Orange are partly filled vesicles and blank ones are empty. RP, reserve pool; RRP, readily releasable pool; VGCC, voltage-gated calcium channel; SY, synapse; GluT, glutamate transporter; BA, bafilomycin A1; ES, endosome; Ca^{2+} ASMs, calcium activated second messengers.

CHAPTER FIVE

Physiological Separation of Vesicle Pools in Low- and High-Output Nerve Terminals

Introduction

This chapter has been published in Wu and Cooper (2013) and is reproduced here for completeness of the dissertation.

Chemical synaptic communication at neuromuscular junctions (NMJ) generally occurs by synaptic vesicles, packaged with a transmitter, fusing with the presynaptic plasma membrane to release the transmitter into a synaptic cleft for the postsynaptic receptors to receive and respond accordingly. How vesicles fuse with the presynaptic membrane is an active area of investigation on a comparative scale as it is assumed there are a variety of ways this process may occur from kiss-and-run to full exocytosis of vesicles (Aravanis et al., 2003; Fredj and Burrone, 2009; Rizzoli et al., 2003; Rizzoli and Betz, 2005; Rosenmund and Stevens,1996; Sudhof, 2004). The recycling and repacking of the vesicles are also of substantial interest particularly given that there appear to be different pools of vesicles for various functions within presynaptic nerve terminals and unique processes as well as similarities among various animal species (Atwood and Cooper, 1996a,b; Denker et al., 2011a,b; Rizzoli and Betz, 2005; Sudhof, 2004).

Recently it was suggested that synaptic vesicles may not just serve as a means of packaging transmitter but also providing essential proteins as a buffer source for use when needed (Denker et al., 2011a,b). In addition, a large influx of Ca^{2+} can depress synaptic transmission (Heuser et al., 1971; Katz and Miledi, 1969; Ohta and Kuba, 1980). Also, acidification within the nerve terminals depresses vesicles endocytosis (Lindgren et al., 1997). These processes may serve as potential negative feedback mechanisms. Such observations raise questions about the functional needs of reserve pool (RP) and readily releasable pool (RRP) of vesicles and their role. To determine the functional differences, in terms of vesicle recycling and recruitment in the RP and the RRP between phasic (high-output) and tonic (low-output) motor nerve terminals, the packaging of neurotransmitter was pharmacologically blocked in recycling vesicles and the action of the well-established modulator serotonin (5-HT) that enhances synaptic efficacy at crustacean NMJ was investigated in this study.

Crustaceans have played a major contribution for investigating structure and function relationships in synaptic transmission that have aided in understanding synapses in general for all animals (Atwood, 1976,1982a,b, Atwood and Cooper 1995,1996a,b; Cooper et al., 1995a,b, 1996a,b; Denker et al., 2011a; Jahromi and Atwood, 1974; Johnstone et al., 2008, 2011; Walrond et al., 1993). An advantage of many NMJs in the crayfish is that they are graded in transmission

as many crustacean muscles do not produce action potentials (Atwood, 1967, 1976). This allows one to follow a rise or decrease in synaptic efficacy over time as well as influences in modulation of the synaptic function with quantal analysis (Cooper et al., 1995b, 2003; Dudel and Kuffler,1961b; Djokaj et al., 2001).

Selective axonal stimulation first studied in crayfish leg extensor yielded two different types of muscle contraction: one a fast twitch-like, the other one with a slower response but depression resistant (Lucas 1907, 1917; Blaschko, et al. 1931; Wiersma, 1933; Van Harreveld and Wiersma, 1936). Later the same contractile pattern and physiology was also identified in crayfish abdomen extensor and flexor musculature (Parnas and Atwood, 1966; Kennedy and Takeda, 1965a,b). Physiological and histological studies suggested that this difference is not only due to the types of motor neurons (phasic/high-output and tonic/low-output), but also the structure of postsynaptic targets (fast and slow muscles) (Baierlein et al., 2011).

The morphological and physiological differences of tonic and phasic nerve terminals have been studied in the crayfish model (Atwood 1963, 2008) and particularly well in the leg extensor for comparisons (King et al, 1996; Bradacs et al, 1997, Msghina et al, 1998). In this leg preparation both types of nerve terminals innervate the same postsynaptic muscle fiber and give rise to stark differences in postsynaptic responses. An advantage of this preparation is that the target is the same fiber so comparisons in neuronal communication can be probed. In this preparation the small varicosities have a high mean quantal content and the synapses depress relatively quickly within the phasic terminals. However, the larger varicosities of the tonic nerve terminal have a low mean quantal content and show marked facilitation with resistance to depression (Wu and Cooper, 2010). Given that the nerve terminals in this preparation do innervate the same fiber there might be feedback from the fiber being stimulated by one neuron to the other non-stimulated neuron or an alteration in receptor sensitivity. So, for our current study we chose to use distinctly separate muscles in the crayfish abdomen that fit phasic and tonic profiles to avoid interaction of the muscle activity.

The abdominal extensor musculature has been well described in *Procambarus clarkii* and other closely related species (Pilgrim and Wiersma, 1963; Parnas and Atwood, 1966; Sohn et al, 2000). All three deep extensor muscles (medial - DEM, lateral 1 - DEL1, lateral 2 - DEL2) are composed of phasic type of muscle fibers (fast-contracting fibers) with short sarcomeres less than 5 µm (Parnas and Atwood, 1966). DEM is a twisted helix muscle. Most of the DEL1 and DEL2 muscle fibers are straight. Kennedy and Takeda (1965a,b) had identified the superficial extensors medial - SEM and lateral - SEL) and their innervation profiles. Both the superficial muscles contain tonic muscle fibers (slow-contracting fibers) with longer sarcomeres ranging 9 µm to 11 µm (Parnas and Atwood, 1966) (see **Figure 5.1** for details).

A recent study of the tonic NMJs on the crayfish opener muscle in the walking leg did demonstrate that blocking the vesicular glutamate transporter (VGlut) with

bafilomycin A1 depressed synaptic transmission faster than without its presence and that the rate of synaptic depression is stimulation dependent (Wu and Cooper, 2012b). Also, a working dose of 4μM bafilomycin A1 was demonstrated to work well without functional damage for crayfish NMJs. The opener NMJ has a substantial RP which can be recruited by 5-HT application after the induction of synaptic depression, suggesting a functional separation in vesicles of the RRP and RP. In this study, the high-output terminals on the DEL1 muscle fibers were compared to the low-output terminals in SEL muscle fibers within the same segment of abdominal musculature. Based on previous reports of the morphological and physiological characteristics of the tonic and phasic terminals, a slower depression of the tonic terminals with or without bafilomycin A1 treatment was predicted. Also, we expected that treatments with bafilomycin A1 would depress both the terminals faster than without the drug; however, we also bafilomycin A1 predicted phasic terminals would show a much greater rate of depression than tonic terminals in the presence of bafilomycin A1. Given the recent results of tonic terminals on the leg opener NMJs responding to 5-HT after the induction of depression, we expected to be able to recruit vesicles from RP in the tonic terminal to a greater extent than the phasic terminals in the abdominal preparations. Working models in the functional difference of the vesicle pools in low and high output terminals are presented.

Experimental procedure

General

All the experiments were carried out in the midsize crayfish (*Procambarus clarkii*) measuring 6-10 cm in body length. They were individually housed in plastic containers with oxygenized water. The temperature of the animal room was controlled at 20°C.

Dissection

The thorax was removed by a cross-section between thorax and abdomen. To expose the abdomen extensor muscles, the ventral side was removed after cutting along the lateral midline on both sides of the abdomen, to reveal the ventral surface of the extensor musculature. The dorsal part was completely spread out and pinned down in a Sylgard dish to reduce twitching during stimulation. All the connective tissues and residual flexor muscles were removed to better visualize DEL1, DEL2, DEM and SEL. Only DEL1 and SEL in segment A2, A3, A4 were used through the whole experiments (**Figure 5.1**). Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (in mM: 205 NaCl; 5.3 KCl; 13.5 $CaCl_2 2H_2O$; 2.45 $MgCl_2 6H_2O$; 5 HEPES adjusted to pH7.4)

Pharmacology

All chemicals were obtained from Sigma-Aldrich Chemical (St. Louis, MO). BA(B1793) solution were made by dissolving 10µg powder in 20µl DMSO (99.9%), then adding crayfish saline to the desired concentrations based on experimental conditions. The solution was stored at -20°C and used within 3 months. 4µM bafilomycin A1, 2.5hrs incubation was used based on the dose-response relation found in leg opener preparation of crayfish (Wu and Cooper, 2012b). The preparations were incubated in bafilomycin A1 solution for 2.5hrs, then they were stimulated using a suction electrode. During the 2.5hrs incubation time, the bafilomycin A1 solution was circulated in the dish every 30mins. 1µM 5-HT was made in crayfish saline from frozen stock of 1 mM 5-HT. It was added to the preparation to exchange with bafilomycin A1 solution after synaptic depression occurred.

Physiology

Only segments A2, A3 and A4 were used. The nerve bundles were stimulated in one segment above for phasic muscle (DEL1) recording or in the same segment for tonic muscle (SEL) recording by a suction electrode connected to a Grass stimulator. The effects of bafilomycin A1 on the rate of synaptic depression were compared between the phasic and tonic nerve terminals using continuous stimulation at 5Hz. In the 5-HT sensitivity assay prior to depression, the nerve was stimulated at 0.5Hz while DEL1 in the same segment was recorded immediately for 1min or less in saline then after the application of 5-HT. EPSPs were recorded following standard procedures (Baierlein et al., 2011; Cooper et al, 1995a; Crider and Cooper, 2000). Electrical signals were recorded online to a computer with Chart or Scope version 5 software (ADInstruments) via a PowerLab/4s (ADInstruments) interface, respectively.

Analysis

The amplitude of the last EPSP every 5mins or 1min in tonic preparations and ever 1 sec in phasic preparations was measured. Scatter plots of the EPSP amplitudes were graphed. The time to reach a 50% reduction in the peak EPSP amplitude was used as an index to calculate the half depression time. The averages of times to 50% decline were compared between phasic and tonic nerve terminals. For the 5-HT effect before synaptic depression, the percentage EPSP amplitude change before and after adding 5-HT was calculated

Results

Tonic nerve terminals

When the segmental nerve that innervates SEL muscle fibers was stimulated at 5 Hz in crayfish saline, the average time to 50% depression is 384.4 min (n=5, ±

13.7 min SEM, **Figure 5.2**). Five out of five preparations showed the restoration of synaptic transmission after 5-HT (1 µM) application ($p<0.05$ non-parametric rank sum test). Preparations responded in varying degrees to the 5-HT application; however, the amplitude of EPSPs was markedly increased in comparison with the depressed state (see **Figure 5.2A** example).

Tonic nerve terminals in 4µM bafilomycin A1

The average time to 50% depression is 58.9 min (± 11.8 min SEM, n=5, **Figure 5.2B**) using continuous stimulation at 5Hz in the presence of 4µM bafilomycin A1. As for the preparations bathed in crayfish saline that depressed, all the preparations exposed to 4µM bafilomycin A1 showed an enhanced effect to 5-HT as indicated by an increase in the EPSP amplitude ($p<0.05$ non-parametric rank sum test).

Phasic nerve terminals in crayfish saline

The segmental nerve was stimulated continuously at 5Hz while the very anterior part of DEL1 muscle in the next posterior segment was recorded. By recording in the next segment helps to insure recording the activity from a single phasic motor neuron and not a group of phasic neurons that innervate the same fibers within a segment (See Cooper et al., 1998). The average time to 50% depression time is 7.2 min (± 1.2 min SEM, n=5, **Figure 5.2C**). After depression, not one of the five preparations showed the rejuvenation of synaptic transmission with exposure to 5-HT, as illustrated in the representative scatter plot (**Figure 5.2C**).

Phasic nerve terminals in 4µM Bafilomycin A1

In the presence of 4µM bafilomycin A1, the phasic nerve terminals depressed much faster. The average time to 50% depression time is 3.8 min (± 0.6 min SEM, n=5, **Figure 5.2D**). Like in crayfish saline alone for these phasic terminals, exposure to 5-HT did not enhance the synaptic transmission in any of the 5 preparations.

Overall results

In both tonic and phasic preparations, 4µM bafilomycin A1 can depress the terminals markedly faster (**Figure 5.3**). After synaptic depression, exposure to 5-HT enhanced synaptic transmission in the tonic NMJs. However, the phasic NMJs which were either exposed or not to bafilomycin A1 did not show an increase in EPSP amplitude after 5-HT application.

The phasic NMJs do respond to 5-HT exposure prior to depression, but after depression occurs there is no enhancement (**Figure 5.4**). This is a similar finding

in 5-HT effects for other phasic NMJs for crayfish (Cooper et al., 2003; Johnstone et al., 2008). In five out of five preparations the phasic NMJs produced an enhanced response to 5-HT application prior to the induction of depression. These five preparations were only used for the 5-HT sensitivity assay prior to depression. These preparations had minimal stimulation before application of 5-HT with a 0.5Hz rate of stimulation for a minute or less in saline and then exposed to a saline containing 5-HT. An average percent change in the EPSP amplitude in response to application of 5-HT for these phasic NMJs is an increase of 82.36% (± 19.91 SEM, n=5).

Discussion

In this study we have demonstrated that bafilomycin A1 produces synaptic depression sooner then without exposure to the drug at the NMJs. Synaptic depression is rapid for the phasic terminals as compared to the tonic terminals; however, after depression of the tonic terminals application of 5-HT rejuvenates the EPSP responses as the amplitude increases. After the high-output phasic terminals fully depress there are apparently no RP vesicles to recruit to the RRP, because no further enhancement by 5-HT was observed. Prior to depression of the phasic terminals, 5-HT does enhance the synaptic responses. Thus, the evidence is suggestive that the high output terminals utilize what might be considered a RP during evoked stimulation. In this case the RP might be considered as a 5-HT sensitive pool as long as synaptic depression has not already occurred. The high-out terminals make use of all available vesicles for maintaining evoked release during the late stages of depression. However, the tonic terminals remain sensitive to enhancing transmission by 5-HT even after substantial depression. A model to explain this phenomenon is that the RP are present in tonic terminal and still available for modulation after depression; however, the RP is small in the phasic terminal and the RP is being used during evoked stimulation so there are few, if any, available for modulation after synaptic depression occurs.

Bafilomycin A1 belongs to a family of antibiotics. The “bafilomycins” and “concanamycins” were identified as specific vacuolar ATPase inhibitors (Bowman et al, 1988) and have proved to be useful pharmacological tools to investigate the function of compartmental acidification, since the hydrogen ion pump is blocked by bafilomycin A1, which prevents repackaging of the synaptic vesicle with transmitter after exocytosis-endocytosis cycle (Cavelier and Attwell, 2007; Kidokoro et al., 2004). The recovery of the tonic nerve terminals by 5-HT after they are treated with bafilomycin A1 and subsequent depression implies that mitochondria ATP production is not impaired by bafilomycin A1 within the time frame of these experiments because vesicle docking and recycling is an ATP dependent process (Tolar and Pallanck, 1998). Longer exposure times may lead to effects not investigated in this study. We have shown previously that 8 µM bafilomycin A1 appears to have some unwanted effects in a crayfish NMJ as the nerve would fail to conduct electrical signals; although, motor neurons function in

Drosophila larvae exposed to 16 µM (Wu and Cooper, 2012b). Generally, bafilomycin A1 appears to work as suggested for crayfish NMJs (Wu and Cooper 2012a,b) since the results are fitting in a dose dependent manner and for incubation times as well as parallel actions on synaptic responses observed for *Drosophila* NMJs (Denker et al., 2011a; Kuromi and Kidokoro, 2000) and mammalian brain slices (Cavelier and Attwell, 2007). So this compound can likely be utilized in other crustacean models for similar experimental manipulations.

The action of 5-HT at NMJs in a variety of invertebrates reveals a range of responses from excitation to inhibition depending on the species (Wu and Cooper, 2012a). However, at crayfish NMJs 5-HT has always been demonstrated to enhance synaptic transmission (Florey and Florey, 1954; Fischer and Florey, 1983; Dudel 1965), for the inhibitory (GABA) as well as excitatory (glutamate) motor neurons (Johnstone et al., 2008; Vyshedskiy et al., 1998; Wang and Zucker, 1998). The tonic like walking leg opener muscle in crayfish responds to 5-HT with an increase in the EPSP amplitudes of the excitatory nerve. This effect is mostly due to an increase in the mean quantal content related to more synaptic vesicles docking and fusing (Cooper et al., 2001; Djokaj et al., 2001; Logsdon et al., 2006; Sparks and Cooper, 2004). Likewise, in the walking leg of crayfish, the extensor muscle is innervated by phasic and tonic terminals and both motor nerve terminals increase in synaptic efficacy with 5-HT application (Cooper et al., 2003). However the tonic terminal shows a higher sensitivity to 5-HT as compared to the phasic even when the basal synaptic output is decreased by lowered extracellular Ca^{2+} (Cooper et al., 2003). Even though the phasic terminals do not reach their maximal output in vesicle fusion with lower evoked release, with a lowered bathing calcium, the EPSP responses do not appear to reach a maximal level in synaptic output with 100 nM 5-HT. In addition, the percent increase in synaptic output was similar for normal bathing saline (13.5 mM Ca^{2+}) as for the saline containing a reduced Ca^{2+} (6.75 mM), suggesting that a given activation of receptors or involvement of second messenger systems paralleled the two conditions.

Recently it was demonstrated that the 5-HT mediated responses at crayfish NMJs is in part mediated by PLC. Blocking PLC activation by pre-treatment of U73122 (50µM) solution drastically reduced the enhancement of the EPSP amplitude observed with 5-HT application (Wu and Cooper, 2012b). Investigation of PLC inhibition and 5-HT treatment for phasic terminals has not been approached yet; however, we expect a dampened response with such inhibition. The phasic terminals in the leg extensor do show an enhancement in the EPSP amplitude prior to synaptic depression by application of 5-HT (Johnstone et al., 2008), just as for the abdominal extensor muscle used in the herein study. Likewise, little enhancement occurs by 5-HT application after synaptic depression is induced in either of these phasic motor terminal preparations. A descriptive model helps to visualize what appears to be the differences in recruitment of vesicles from a dynamic RP, or for argument sake, vesicles that are sensitive to being recruited by modulation of downstream effects induced by 5-HT for tonic and phasic like motor nerve terminals. **Figure 5.5** and **5.6**

highlight a physiological model of RP and RRP vesicle utilization with evoked stimulation based on the observations to date in tonic low-output and phasic high-output NMJs of the crayfish model. Due to the large enhancement in the EPSP amplitude induced by 5-HT prior or after synaptic depression occurs, the prediction is that there is a larger RP of vesicles for the tonic terminals. This is also supported by the fact that bafilomycin A1 inhibits the recycling pool from repackaging with neurotransmitter and a RP can still be recruited by 5-HT. However, for the phasic terminals if synaptic depression is induced by evoked stimulation with or without bafilomycin A1 treatments there is little if any RP to be modulated by downstream actions of exposure to 5-HT. Considering, that high-output terminals in multiple types of crayfish preparations are slightly enhanced, as compared to tonic terminals, in synaptic efficacy by 5-HT prior to evoked synaptic depression may indicate that a small RP of vesicles exists in phasic terminals but are rapidly utilized during evoked activity of the terminal. Given that treatment with bafilomycin A1 produces rapid depression in high-output terminals is indicative that a large percentage of vesicles are utilized for each terminal stimulation.

As to why such differences in sensitivity to 5-HT occurs for evoking an enhancement of the EPSP amplitudes between the tonic and phasic terminals further investigation into 5-HT receptor density, subtypes, and second messenger recruitment as well as potential intracellular Ca2+ release from the endoplasmic reticulum needs to be addressed. Different 5-HT receptor subtypes use varying cellular cascades is possible (Hensler, 2002) and it is known that there are differences in the amount of the calcium binding protein frequenin (Jeromin et al., 1999) in tonic and phasic terminals of crayfish as well as differences in and the amount of Ca^{2+} influx with evoked stimulation (Msghina et al., 1999). The varying degree of Ca^{2+} entry with stimulation may have a role in the recruitment of RP into the RRP for the phasic terminals since the Ca^{2+} influx is greater (Msghina et al., 1999). For potential roles of second messenger systems induced by 5-HT or Ca^{2+} in crayfish motor nerve terminals see earlier reports (He et al., 1999; Tabor and Cooper, 2002; Wu and Cooper, 2012a,b). The effect of 5-HT increasing EPSP amplitude on non-depressed and even depressed tonic terminals may also be accounted for by an increased in docking of RRP. In this case, the probability of vesicular fusion could account in part for a larger EPSP (Southard et al., 2000; Strawn et al., 2000). This can potentially occur by various mechanisms as outlined in a review on 5-HT effects on invertebrate NMJs (Wu and Cooper, 2012a).

As for potential mechanisms in evoked stimulation induced recruitment of vesicles, in the absence of exogenous neuromodulation, see reports by Akbergenova and Bykhovskaia,(2009), Aravanis et al., (2003), Denker et al., (2012a,b), Desai-Shah and Cooper, (2009, 2010), Fiumara et al., (2004), Kuromi and Kidokoro (2000), Sudhof (2004) and Yamashita (2012). The usage of the RRP and RP of these tonic and phasic NMJs in the crayfish abdomen are fitting to their physiological profiles for the musculature involved in rapid tail flips or slow movements in postural control (Cooper et al., 1998; Mykles et al., 2002).

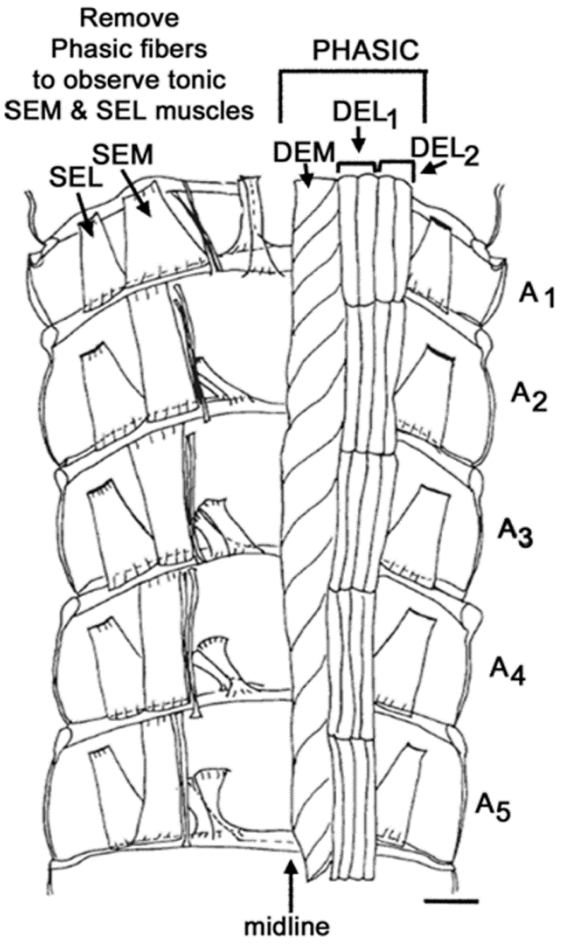

Figure 5.1: Schematic presentation of crayfish abdomen extensor musculature. Each side of each segment contains deep extensor medial muscle (DEM), deep extensor lateral muscle 1 (DEL1), deep extensor lateral muscle 2 (DEL2), superficial extensor lateral muscle (SEL), superficial extensor medial muscle (SEM). On the left side of the figure, dorsal SEL and SEM is viewed by removing DEM, DEL1, and DEL2. DEM, DEL1 and DEL2 are phasic muscles whereas SEM and SEL are tonic in nature. A1-A5 refers to abdomen segments. Scale bar = 2.35 mm. The figure is modified from Sohn et al, 2000.

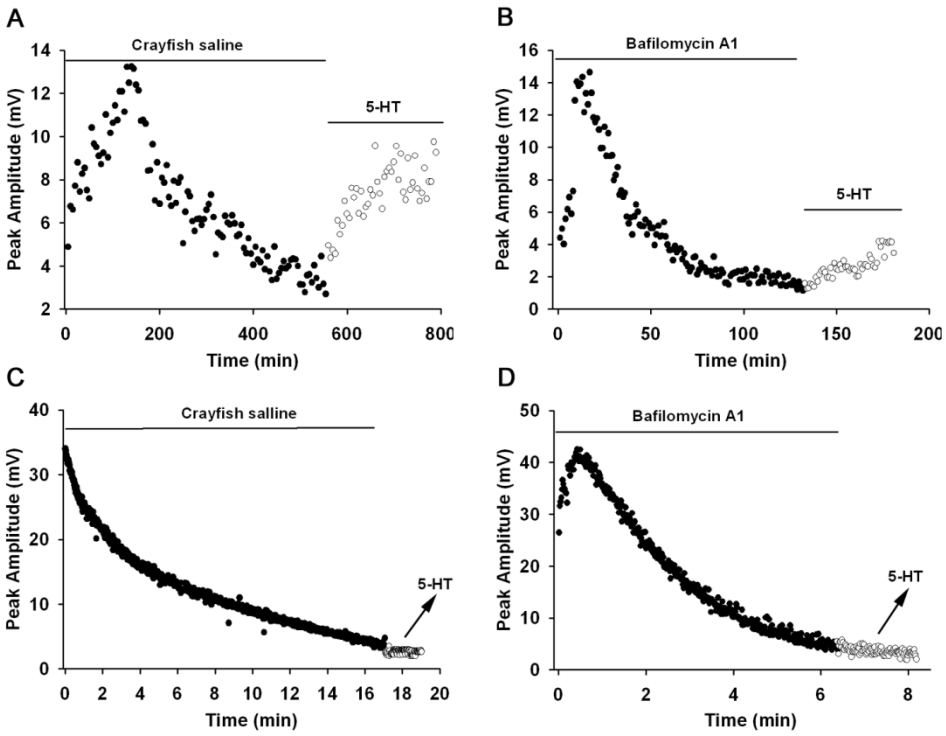

Figure 5.2: The synaptic transmission of abdominal tonic and phasic motor nerve terminals in crayfish saline and a 4 M bafilomycin A1 containing saline. (A) Representative scatter plot of EPSP amplitude (mV) over time serve as a saline control for tonic terminals. After substantial depression was observed, 5-HT containing saline was exchanged for the saline bath. The 5-HT exposure resulted in the amplitudes of the EPSPs increasing. (B) The synaptic activity of abdomen tonic nerve terminals in 4 M bafilomycin A1. The EPSPs depress sooner than controls and after substantial depression exposure to 5-HT also produced an increase in the EPSP amplitude. (C) The synaptic activity of
abdomen phasic nerve terminals in crayfish saline depress rapidly as indicated by the decline in EPSP amplitude. After substantial depression was observed exposure to 5-HT did not produce an increase in the EPSP amplitude. (D) The EPSP amplitudes produced by the abdomen phasic nerve terminals decreased faster than saline controls when incubated with 4 M bafilomycin A1. As with the saline control preparations, the EPSPs did not restore with subsequent exposure to 5-HT after depression occurred.

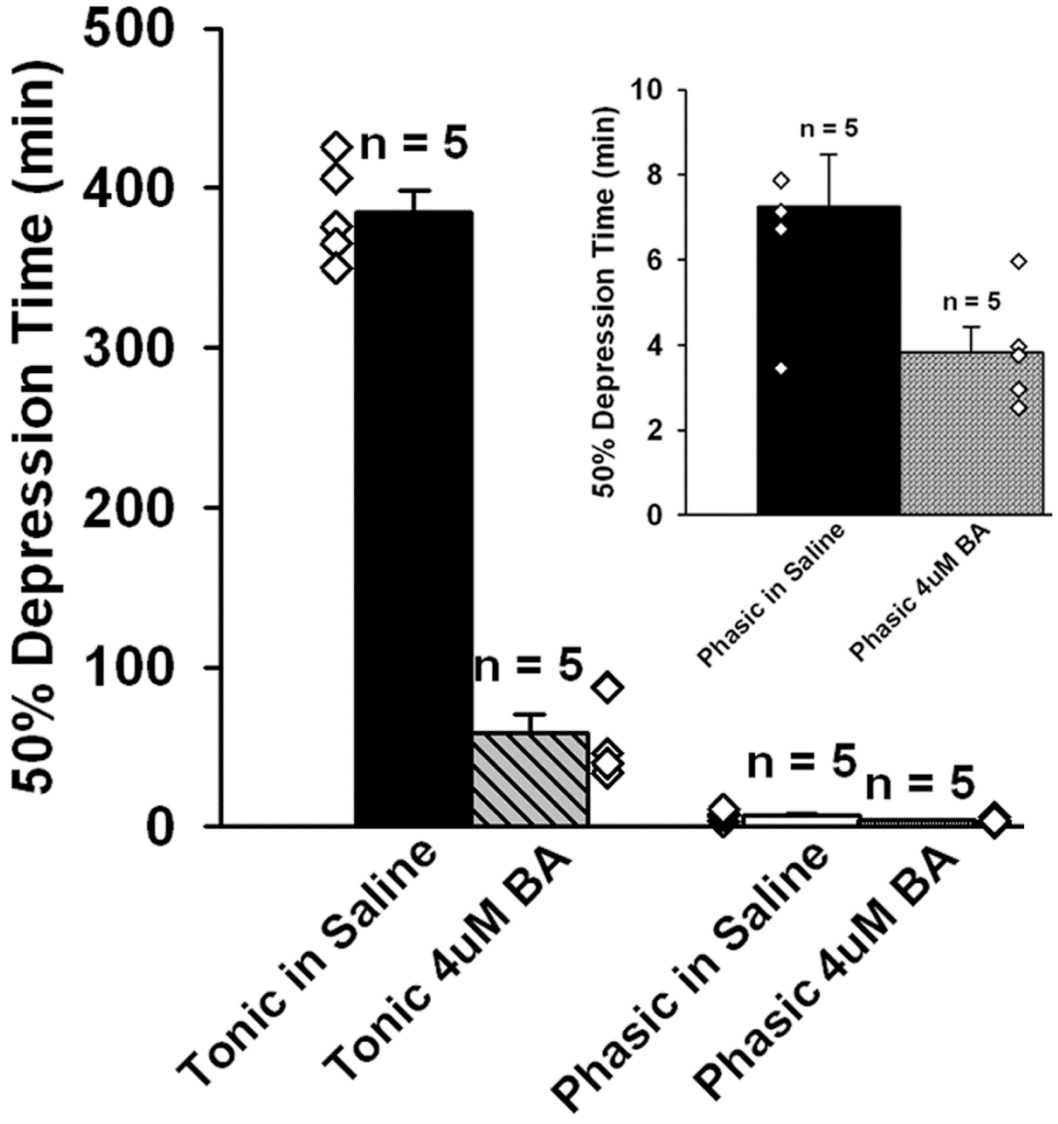

Figure 5.3: Bar graphs depicting the average time to 50% depression time in the EPSP amplitude for 5 preparations. The average depression time is 384.4 min (±13.7 min SEM, n = 5) for tonic NMJs exposed to saline only. When the tonic NMJs are incubated in bafilomycin A1 the depression time is 58.9 min (±11.8 min SEM, n = 5). The phasic terminals treated as a saline control depressed on average 7.2 min (±1.2 min SEM, n = 5). When the phasic terminals were treated with bafilomycin A1 they depressed on average 3.8 min (±0.6 min SEM, n = 5). The diamonds are the values for each individual preparation. The enlarged inset in the bar chart for the phasic terminals illustrates the small amount of time. The main graph is presented with the same time scale for the phasic and tonic NMJs for ease of comparison.

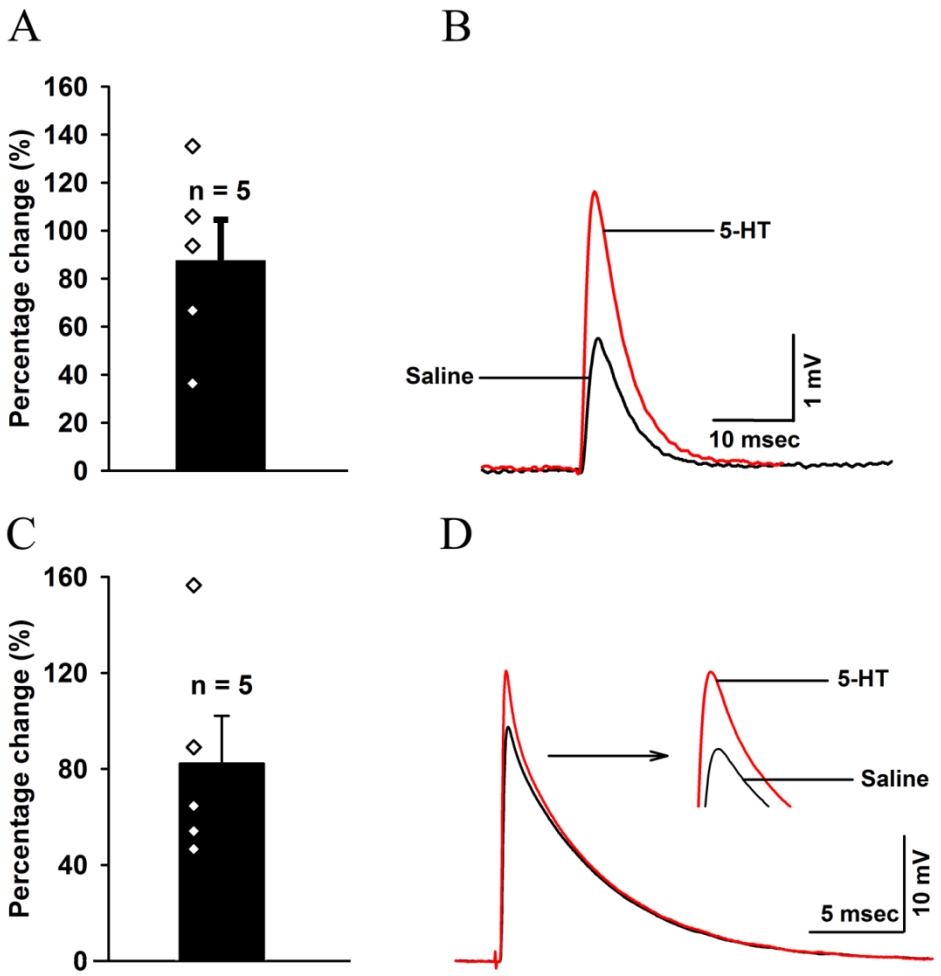

Figure 5.4: The tonic low-output and phasic high-output NMJs response to 5-HT prior to evoked depression. Five out of five preparations show an enhancement in the EPSP amplitude to application of 5-HT for both terminal types (A-tonic, C-phasic).The increase in peak amplitude of the EPSP, due to 5-HT exposure (red traces), is obvious prior to synaptic depression in the representative traces shown (B-tonic, D-phasic).

Tonic Low-output Terminal

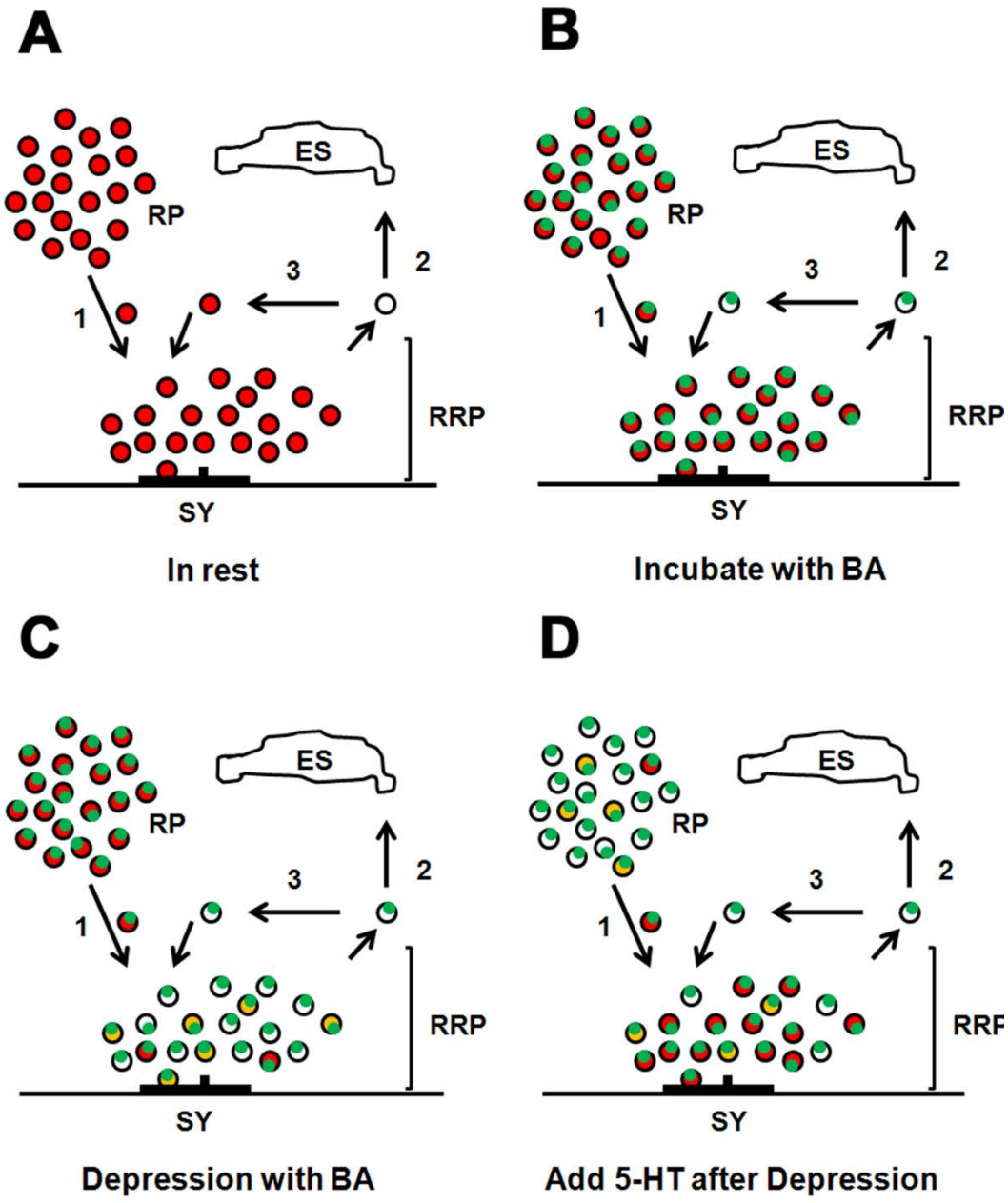

Figure 5.5: Model of vesicle recycling between RP and RRP and the effect of bafilomycin A1 and 5-HT on transmitter release with the associated vesicle dynamics in low-output NMJs. (A) Synaptic vesicles are separated into RRP and RP over a synapse and with electrical stimulation vesicles in RP can slowly join in to the RRP (1), and then recycle back to RP either through or bypass endosome (2 or 3). (B) In an active synapse, in addition to the slow recycling loop, vesicles in RRP recycle quickly within the RRP which is a quick recycling loop (3). Recycling vesicles are refilled with glutamate (red vesicles are filled with glutamate) but incubating with bafilomycin A1 (green dots represent bafilomycin A1) the recycling vesicles cannot refill (clear vesicles). (C) With repetitive stimulation the vesicles in the RRP are depleted of glutamate and with bafilomycin A1 the packaging is depressed at a considerably faster rate. (D) However, application of 5-HT recruits vesicles from the RP to the RRP. In the presences of bafilomycin A1 the RRP and vesicles recycling to the RP will become depleted of transmitter. The effect of 5-HT increasing EPSP amplitude may also be accounted for by an increase in docking of RRP. These low output synapses have a larger RP to recruit from than the high-output synapses. RP, reserve pool; RRP, readily releasable pool; SY, synapse; BA, bafilomycin A1; ES, endosome.

Phasic High-output Terminal

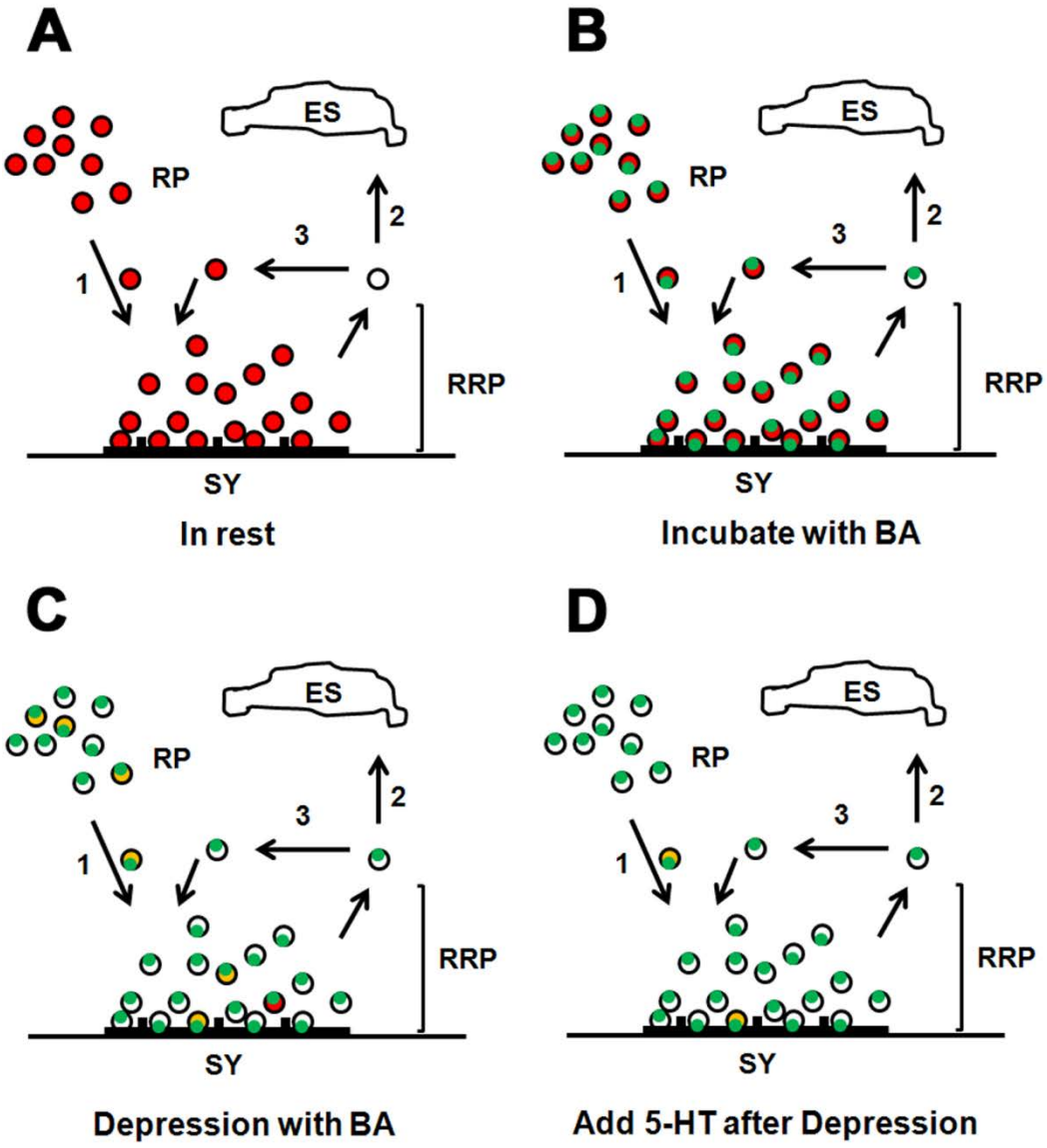

Figure 5.6: Model of vesicle recycling between RP and RRP and the effect of bafilomycin A1 and 5-HT on transmitter release with the associated vesicle dynamics in high-output NMJs. (A) Synaptic vesicles are separated into RRP and RP over a synapse and with electrical stimulation vesicles in RP can slowly join in to the RRP (1), and then recycle back to RP either through or bypass endosome (2 or 3). (B) In an active synapse, in addition to the slow recycling loop, vesicles in RRP recycle quickly within the RRP by a quick recycling loop (3). Recycling vesicles are refilled with glutamate (red vesicles are filled with glutamate) but incubating with bafilomycin A1 (green dots represent bafilomycin A1) the recycling vesicles cannot refill (clear vesicles). Since the high-output synapses have more vesicles in the RRP a larger postsynaptic effect is observed for the initial stimulations of the terminal. The effect of 5-HT in increasing the EPSP amplitude on non-depressed may also be accounted for by an increase in docking of RRP. (C) With repetitive stimulation the vesicles in the RRP are depleted of glutamate and with bafilomycin A1 the packaging is depressed at a considerably faster rate. Since more vesicles are recycling, the RRP pool is affected at a greater rate that for low-output synapses. (D) However, application of 5-HT recruits vesicles from the RP to the RRP (A or B plates). After synaptic depression occurs the vesicles in RP as well as the RRP are depleted of glutamate. Hence, 5-HT has little effect in recruiting any packaged vesicles from the RP. RP, reserve pool; RRP, readily releasable pool; SY, synapse; BA, bafilomycin A1; ES, endosome.

CHAPTER SIX

General Discussion and Future Direction

General Discussion and Conclusion

In this series of studies I have focused on addressing the fundamental principles in regulating chemical synaptic transmission, specifically in vesicular recycling and grouping. This was approached by physiologically depressing RRP with the aid of pharmacological blockage of vesicle repackage (bafilomycin A1), followed by recruiting RP vesicles which were spared during the depletion of RRP with either high frequency stimulation or the application of neuromodulator (5-HT). The results were in favor of the demonstration that RRP vesicles are responsible for the initial and basic synaptic activity, while RP vesicles back up the synaptic transmission when RRP vesicles can no longer fulfill the synaptic demand.

As an ATPase specific inhibitor, bafilomycin A1 has been used in studying various preparations such as mice hippocampal neurons (Cavelier and Attwell, 2007; Kuromi and Kidokoro, 1998). However the effect of bafilomycin A1 specifically on vesicular recycling and synaptic transmission in the invertebrate model organisms *Procambarus clarkii* and *Drosophila melanogaster* has not been previously examined. Various frequencies in stimulating the motor nerve and exposure time to bafilomycin A1 were used to determine ranges for physiological experimentation. In crayfish opener preparation bafilomycin A1 drastically reduces the time approaching synaptic depression. In bafilomycin A1 exposed terminals which are synaptically depressed, the presence of 5-HT can induce enhanced synaptic output which is assumed to be the consequence of recruiting RP vesicles. The action of 5-HT can be blocked by a PLC inhibitor. Thus, it is postulated that one possible mechanism of the 5-HT action is 5-HT recruits the RP to the RRP via a PLC mediated pathway likely through an IP3 mediated second messenger cascade. In *Drosophila* body wall NMJ preparations, bafilomycin A1 also significantly facilitates the rate of synaptic depression. Tetanic stimulation (10Hz) after induced depression is able to recruit RP vesicles to revitalize synaptic activity. However, no difference is found on the frequency or the amplitude of minis with the treatment of bafilomycin A1 comparing to control, which probably suggests that spontaneous activity is contributed by a different group of vesicles. Possibly by depressing evoked synaptic transmission and continually repetitively stimulating the nerve terminal possible even in higher extracellular [Ca^{2+}].

When comparing the high-output and low-output terminals in crayfish abdomen extensor preparation, both terminals show significant decrease in the time approaching 50% depression in bafilomycin A1 treated group. However, 5-HT only resumed the synaptic transmission in low-output terminals after synaptic depression. Thus, it is likely that bafilomycin A1 is sufficient in facilitating synaptic depression without affecting RP in low-output terminals. The 5Hz stimulation seems to be high enough to recruit vesicles from RP even before synaptic depression in high-output terminals.

Although these studies were conducted in two invertebrate preparations, they function as representative models for the bases of synaptic transmission in all animals as the fundamentals in vesicle docking, recycling and packaging as well as in synaptic efficacy have been shown repetitively to be similar in all synaptic preparations. Due to the complexity and vulnerability of vertebrate nervous system, conducting experimentation in invertebrate model systems may greatly contribute to better understanding of synaptic transmission in vertebrates.

In short, these studies aided to better understand a working range of concentrations for bafilomycin A1 on crayfish and *Drosophila* preparations as well as its effects on blocking glutamate repackage in recycling vesicles. Physiologically differentiating RRP and RP with various experimental techniques provides additional insight into the underlying mechanisms about how these pools are utilized within nerve terminals. The investigation in identifying the physiological uniqueness of different nerve terminals may help unravel their functional differences in behavior.

Chapter 3 of this thesis provided detailed review in the history of investigating 5-HT action in various invertebrate NMJs. Interestingly, the representative model system of crustacean and insects, crayfish and *Drosophila melanogaster,* exhibit different responses to the modulation of 5-HT at the NMJ. EPSP amplitudes increase about 400% by briefly (~5min) exposing non-depressed crayfish NMJs to 1 µM 5-HT (Dixon and Atwood, 1985) as compared to slightly depressing synaptic strength or having no effect at all, on *Drosophila* NMJs.

With the growing number of investigations in synaptic plasticity and distribution of 5-HT receptor subtypes in the *Drosophila* CNS, utilizing the genetic manipulation, one will likely learn more in time in terms of expression pattern, pharmacological properties and second messenger systems of 5-HT receptors as compared to crayfish NMJs. Despite the lack of response to 5-HT at the NMJ in *Drosophila*, probably due to the absence of a 5-HT receptor on postsynaptic muscle fibers or on the presynaptic nerve terminal, the ability to conduct physiological assays in the sensory-CNS-motor unit in *Drosophila* allows one to address physiological

actions on skeletal muscles which affect behavior of the animal (Dasari and Cooper, 2004; Dasari et al., 2009). However, understanding the mechanistic action in alterations by 5-HT of the vesicles pools and effect on synaptic efficacy, the crayfish NMJ offers advantages that are not yet possible with ease in the *Drosophila* CNS model.

In contrast, since the first observation of its strong presynaptic action in crayfish NMJs (Dudel, 1965), the mechanism of 5-HT in facilitating synaptic strength has been well studied in several invertebrate NMJs pharmacologically especially in 1980's. Both crayfish and lobster opener muscle preparations showed two phases of 5-HT action in the non-depressed terminals: fast-decay phase and slow decay long lasting phase (Dixon and Atwood, 1989; Goy and Kravitz, 1989). The interplay of phosphatidylinositol and cAMP signaling systems was suggested to count for such effect of 5-HT, in which the early intense phase was produced by IP3 induced internal Ca^{2+} release and the slow long lasting phase was contributed by the PKC and cAMP activity (Dixon and Atwood, 1989; Enyeart, 1981; Kravitz et al., 1981). In Chapter 4 of this thesis, it was shown, similar to the result in the non-depressed terminals, that non-selective PLC inhibitor U73122 almost completely blocks 5-HT's effect on enhancing synaptic transmission in electrically depressed crayfish opener NMJs. Thus, I embarked on a short preliminary study to address if cAMP could enhance vesicle fusion after synaptic depression. I present this data and experimental method here in Chapter 6 as the previous chapters are reproductions of published papers.

cAMP action in depressed nerve terminals

The experiment described was to test whether activation of cAMP can revitalize synaptic activity after the motor nerve terminal is undergoing synaptic depression. It is well established that application of forskolin, an activator of adenylate cyclase (Seamon and Daly, 1983), will raise intracellular cAMP levels (Dixon and Atwood, 1989; Yoshihara et al., 2000).

Intracellular recording was employed to measure the EPSP amplitudes in the opener muscle fiber stimulated at 20Hz continuously. The detailed physiology and preparation procedure is similar as the one described in Chapter 4. Forskolin was obtained from Sigma Chemical (St. Louis, MO). 100 µM forskolin was made by dissolving 1mg powder in 73µl DMSO (0.3%), then adding crayfish saline to obtain the final concentration. Forskolin solution was applied onto the preparations 1min after stimulation (control group) and after 50% depression was approached (depression group). The average amplitude of 50 EPSPs in series was used to calculate the percentage change from the initial EPSPs or a before

and during exposure to forskolin. In the control group, after adding forskolin the EPSPs were measured immediately after 1hr incubation (control with 1hr incubation). The percentage change in the EPSP amplitude was compared among the three groups (control with 1hr incubation, control without incubation and after depression without a pre-incubation).

The result is that forskolin increases the EPSP amplitude in all three groups, with 127.8% (n = 5, ±15% SEM) increase in the control group with 1hr incubation, 36.16 % (n = 5, ±6.4% SEM) increase in the control group without incubation, and 56.29% (n = 5, ±7.8% SEM) increase in the after depression without incubation group (**Figure 6.1**).

The amplitude of EPSPs increases about 400% when 5-HT is applied briefly (~5min) (Dixon and Atwood, 1985) in non-depressed terminals. In this study, also in non-depressed terminals, forskolin only facilitates the synaptic activity by 127.8% with prior 1hr incubation and 36.16% without incubation. This is consistent with the previous finding that cAMP signaling pathway only mediates part of the 5-HT action. In addition, the higher percentage change (p = 0.008, Mann - Whitney Rank Sum Test) with 1hr incubation indicates that forskolin produces a slowly developed facilitation of EPSPs by increasing the intracellular concentration of cAMP as described by Dixon and Atwood (1989). Overall, the study of forskolin, PLC inhibitor in chapter 4, together with the previous studies, established a scheme that phosphatidylinositol and cAMP second messenger systems are both involved to produce the facilitating effect of 5-HT in both depressed and non-depressed crayfish opener NMJs.

Future studies and potential implications

In the future, like the study conducted by Dixon and Atwood (1989), a scheme can be tested by other approaches such as preventing cAMP breaking down by utilizing cAMP analogs that are longer lasting in biological systems, and applying compounds which activate PKC. Ca^{2+} is a key player of synaptic transmission (Katz and Miledi. 1968), thus it would be interesting as a future experiment to test whether the entry of external Ca^{2+} plays a role in enhancing transmission after depression which may also be a possible mechanism of the 5-HT effect (Cooper et al., 2002; Delaney et al., 1991; Dixon and Atwood 1989; Southard et al., 2000), but this mechanism has not been fully elucidated. One possible approach is to depress the terminal with 20Hz continuous stimulation, then increase the stimulating frequency to 30 or 40Hz to see whether the depression can be rescued as Ca^{2+} influx is related stimulation frequency.

There is some suggestion that synaptic vesicles can take up Ca^{2+} quickly by a vesicular Ca^{2+}/H^{+} antiporter which plays a contributing role to the rapid Ca^{2+} buffering within the presynaptic cytoplasmic face (Dunant et al., 2009). Blocking the H^{+} pump with BA indirectly inhibits the Ca^{2+}/H^{+} antiporter. This would lead to larger $[Ca^{2+}]_i$ close to the synaptic vesicles and should promote an increase in probability of vesicle fusion. However we have not observed an increase in EPSP amplitude after incubation with bafilomycin A1 when evoked transmission resumed. Our observations don't directly support this proposed phenomenon at either the crayfish or the *Drosophila* NMJs. Further investigations with imaging of a Ca^{2+} indictor with fast line scanning might help to resolve these differences. It might be of interest to examine the duration of the quantal responses during bafilomycin A1 incubation as there is a potential that timing of the fusion pore may show some differences. A study by Pawlu et al., (2004) revealed that raising $[Ca^{2+}]_i$ at the *Drosophila* NMJ resulted in a shorter fusion pore open time and limits transmitter release.

It would be interesting to use bafilomycin A1 and FM1-43 in transgenetically modified *Drosophila* that have synaptophluorin tagged to structures in the synaptic cleft to see if vesicles recycled but with no drop in pH during vesicle fusion for BA treated NMJs; thus, proving that bafilomycin A1 works as expected in blocking the vesicular H^{+} pump, although, Sturman et al., (2006) have demonstrated that the vesicles at the *Drosophila* NMJ are not acidified as much as one might expect in comparison to other NMJs. If this is the case then we would have expected that bafilomycin A1 would not have worked as efficiently at *Drosophila* NMJs as the crayfish in reducing the refilling of synaptic vesicles. Potentially the vesicles at the crayfish NMJ also do not acidify as much as mammalian preparations. Such a comparative study remains to be examined.

Improving our understanding in the fundamental properties related to regulation of synaptic transmission in one system will hopefully allow a better understanding in other systems even if they might vary somewhat in the processes. Both the crayfish and *Drosophila* NMJs have offered the filed a lot of insight in many of the process involved in synaptic function. The ability to also separate out pools of vesicles physiologically offers one more key piece to the puzzle regulating synaptic differentiation and function.

There are no direct clinical applications for humans tied to the findings in these studies; however, the knowledge of the potential mechanism in regulating synaptic function may provide indirect scientific benefits. The application of pharmacological agents in altering synaptic function used in these studies would likely be non-direct to specific neuronal tissues and the side effects could prove to be toxic to a whole organism at the concentrations used in these studies. The

5-HT recruitment of RP to the RRP at the crayfish NMJ might be unique to crayfish and a few other invertebrates. I am not aware of any reports in which RP vesicles can be recruited to a RRP in mammalian neuronal tissue. However, I am also not aware of studies directly examining this possibility in presynaptic neurons sensitive to 5-HT application in mammals. The serotonergic system in mammals is important for psychological and social wellbeing (Haleem, 2012; Noori et al., 2012). It would be of interest to indeed address the cellular regulation in modulation by 5-HT in synapses that are excited by application of 5-HT and better understand the mechanisms in the modulatory role of 5-HT with neuronal function and psychological status.

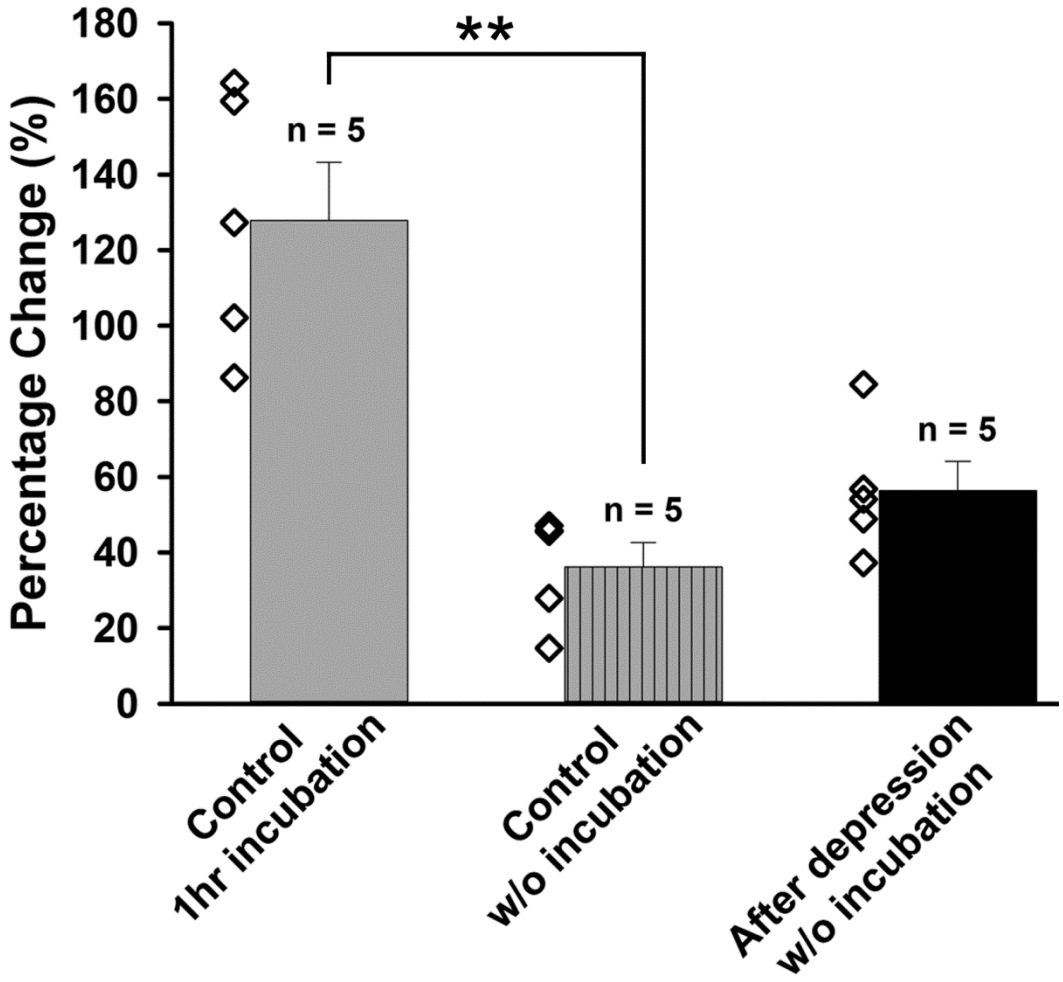

Figure 6.1: Percentage EPSP amplitude change in control with 1hr incubation, control without incubation and after depression without incubation groups. There is a 127.8% (*n* = 5, ±15% SEM) increase in control group with 1hr incubation, 36.16 % (*n* = 5, ±6.4% SEM) increase in control group without incubation, and 56.29% (*n* = 5, ±7.8% SEM) increase in after depression without incubation group, five preparations for each group.

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VITA

Author's Name – Wenhui Wu

Birthplace – Yellow Mountain City, Anhui Province, China

Education

2008 fall- 2013 August:
PhD in Biology, University of Kentucky, Lexington, KY, USA

2002 fall-2007 fall:
M.D. in Chinese and Western Medicine, School of Chinese and Western Medicine, Anhui College of Traditional Chinese Medicine, Hefei, China

Teaching Experiences

2008 fall-2013 spring:
Graduate Teaching Assistant in Biology, University of Kentucky, Lexington, KY, USA

Peer Reviewed Publications

1. Wu WH, and Cooper RL (2013) Physiological separation of vesicle pools in low- and high-output nerve terminals. **Neuroscience Research** 75:275–282.

2. Wu WH, Cooper RL (2012a) Serotonin and synaptic transmission at invertebrate neuromuscular junctions. **Exp Neurobiol**.21(3):101-112

3. Wu WH and Cooper RL (2012b) The regulation and packaging of synaptic vesicles as related to recruitment within glutamatergic synapses. **Neuroscience** 225:185–198.

4. Jones G, Jones D, Fang F, Xu Y, New D, **Wu WH** (2011) Juvenile hormone action through a defined enhancer motif to modulate ecdysteroid-activation of natural core promoters. **Comp. Biochem. Physiol.B** doi:10.1016/j.cbpb.2011.11.008. http://www.ncbi.nlm.nih.gov/pubmed/22142799

5. Wu WH and Cooper RL (2010) Physiological recordings of phasic and tonic neuromuscular junctions on the crayfish leg extensor muscle. Journal of

Visualized Experiments (JoVE) Jove 45, doi:10.3791/2319. http://www.jove.com/index/details.stp?id=2319

Manuscript Reviews

Reviewed manuscripts from the following journals or publishers

Journal of Visualized Experiments (JoVE) (2011)
Journal of Comparative Biochemistry and Physiology (2011)
The Journal of Physiology-London (2009)

Poster Presentations

1. Wu,W.-H. and Cooper, R.L. (2012). The regulation and packaging of synaptic vesicles related to recruitment within glutamatergic synapses. The Kentucky Academy of Science annual meeting. Oct.19-20, 2012, Eastern Kentucky University, Richmond, Kentucky.

2. Kong, W.-K., **Wu,W.-H.** and Cooper, R.L. (2012). The action of stimulating adenylyl cyclase within motor nerve terminals in the regulation of synaptic vesicles. The Kentucky Academy of Science annual meeting. Oct.19-20, 2012, Eastern Kentucky University, Richmond, Kentucky.

3. **Wu,W.-H.** and Cooper, R.L. (2012) The regulation and packaging of synaptic vesicles related to recruitment within glutamatergic synapses. Annual meeting of Society for Neuroscience. New Orleans, LA., USA.

4. **Wu, W.-H**. and Cooper, R.L. Packaging and physiological separation of the RRP and RP of vesicles within various types of presynaptic terminals. Spring Neuroscience Day, March 29, 2012, University of Kentucky, Lexington, Kentucky.

5. **Wu, W.-H** and Cooper, R.L. Packaging and physiological separation of the RRP and RP of vesicles within various types of presynaptic terminals. 26th Meeting of the Ohio Physiological Society, University of Cincinnati, October 6–7, 2011, Cincinnati, Ohio.

6. **Wu, W.-H,** and Cooper, R.L. Packaging and physiological separation of the RRP and RP of vesicles within various types of presynaptic terminals. Annual meeting of Society for Neuroscience. November 12-16, 2011, Washington, DC.

7. **Wu, W.-H,** Cooper, R.L. Packaging and physiological separation of the RRP and RP of vesicles within various types of presynaptic terminals. Annual meeting. Society for Neuroscience Bluegrass Chapter. March 31, 2011, Univ. of KY.

8. **Wu, W.-H,** Cooper, R.L. The regulation of synaptic vesicles within crayfish NMJ. Society for Neuroscience Bluegrass chapter. March 17, 2010, Univ. of KY.

9. **Wu, W.-H,** Hill, Jessica, Cooper, Robin L. "Influence of nicotine on physiology, development and behavior of Drosophila melanogaster". Society for Neuroscience Bluegrass chapter. March 18, 2009, Univ. of KY.

10. **Wu, W.-H,** Hill, Jessica, Cooper, Robin L. "Influence of nicotine on physiology, development and behavior of Drosophila melanogaster". The Society for Integrative & Comparative Biology 2009 Annual Meeting. Jan 5th, 2009, Boston, MA.

11. **Wu, W.-H,** Hill, Jessica, Cooper, Robin L. "Influence of nicotine on physiology, development and behavior of Drosophila melanogaster". The Kentucky Academy of Science 2008 Annual Meeting. Nov 1st, 2008, Lexington, KY.

Undergraduate Poster Presentations That I Mentored

1. Robinson, A.D., **Wu, W.-H**, Cooper R.L., 2011. Ephaptic transmission between motor neurons. Annual meeting of Society for Neuroscience. Washington, DC, USA.

2. Robinson, A.D., **Wu, W.-H**, Cooper R.L. Ephaptic transmission between motor neurons. Annual meeting. Society for Neuroscience Bluegrass Chapter. March 31, 2011, Univ. of KY.

3. Robinson, A.D., **Wu, W.-H**, Cooper R.L., 2011. Ephaptic transmission between motor neurons. Univ. of KY, Showcase of Scholars (9th annual undergraduate research event).

4. Armbruster, J., **Wu, W.-H.** and Cooper, R.L., 2010. The regulation of synaptic vesicles pool in nerve terminals. 5th Annual Showcase of Undergraduate Scholars, University of Kentucky, Lexington, Kentucky.

Affiliations

1. Judge for Fayette County Public School Science Fair-Bluegrass Chapter of the Society for Neuroscience (Feb 12, 2011).

2. Judge for 2009 & 2010 Central KY Regional Science and Engineering Fair (CKRSEF). University of Kentucky

3. The local middle school (Morton, 2009). Presenting hands on demonstrations of sensory physiology on the students and on invertebrate animals. (contact Ms. Jacobs-Science teacher).

4. Provided an entire day (March 16, 2009) to teaching all the 8th graders (4 classes) at Beaumont Middle School (Lexington, KY) differences in innate and learned behaviors. This was done with lecture and hands on activities. (Contact person: Mr. Patrick Goff, Science teacher at Beaumont Middle School).

5. Committee member of the Undergraduate Student Association, School of Chinese and Western Medicine from Dec 2003 to Dec 2004.

6. Member of the Society for Integrative & Comparative Biology.

Main Honors Received

1. Second place Scholarship for Academic Excellence, School of Chinese and Western Medicine, 2005-2006

2. Specialized scholarship for Academic Excellence, School of Chinese and Western Medicine, 2004-2005

3. Third place Scholarship for Academic Excellence, School of Chinese and Western Medicine, 2003-2004