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ABSTRACT OF DISSERTATION

Andrew Fredericks Moser Johnstone

The Graduate School

University of Kentucky

2006

PHYSIOLOGICAL AND ANATOMICAL ASSESSMENT OF
SYNAPSES AT THE CRAYFISH NEUROMUSCULAR JUNCTION

ABSTRACT OF DISSERTATION

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

By
Andrew Fredericks Moser Johnstone

Lexington, Kentucky

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

Lexington, Kentucky

2006

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ABSTRACT OF DISSERTATION

PHYSIOLOGICAL AND ANATOMICAL ASSESMENT OF SYNAPSES AT THE CRAYFISH NEUROMUSCULAR JUNCTION

The crayfish, *Procambarus clarkii*, has a multitude of ideal sites in which synaptic transmission may be studied. Its opener muscle, being innervated by a single excitatory neuron is a good model for studying the structure/function of neuromuscular junctions since the preparation is identifiable from animal to animal and the nerve terminals are visible using a vital dye. This allows ease in finding a suitable site to record from in each preparation and offers the ability to relocate it anatomically. Marking a recorded site and rebuilding it through electron microscopy gives good detail of synaptic structure for assesment.

In the first of these studies, low output sites known as stems (which lie between varicosities) were used to reduce n (number of release sites) in order to minimize synaptic complexity so individual quantal events could be analyzed by their unique parameters (area, peak, tau, rise time and latency). This was in attempt to uncover specific quantal signatures that could be traced back to the structure of the area recorded. It was found that even at stem regions synaptic structure is still complex having multiple synapses each of which could harbor a number of AZs. This gives insight as to how quantal analysis should be treated. Even low output synapses n must be treated at the AZ level.

Synaptic depression was studied at the crayfish extensor muscle. By depressing the phasic neuron and recording from the muscle it appears that

depression is a presynaptic phenomenon. The use of 5-HT gave insight to vesicular dynamics within the nerve terminal, by delaying depression and increasing maximum EPSP amplitude. TEM of phasic nerve terminals reveals no change in numbers of dock or RRP vesicles. Short term facilitation and vesicular dynamics were studied with the use of 5-HT and a neurotoxin TBOA, which blocks the glutamate transporter. In this study I showed differential mechanisms that control RRP and RP vesicles. By blocking glutamate reuptake, the RRP is depleted as shown by reduced EPSPs, but recovered with 5-HT application. The understanding of vesicle dynamics in any system has relevance for all chemical synapses.

KEY WORDS: Active zones, quantal analysis, synaptic ultrastructure, synaptic plasticity.

Andrew F. M. Johnstone

Dr. Robin L. Cooper (*Advisor*)

Dr. Brian C. Rymond (*Director of Graduate Studies*)

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November 30, 2006

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CHAPTER 1

General Background of synaptic transmission

Animals depend on the nervous system to transduce stimuli from the outside world and in turn react. This is done via precise connections within the nervous system by the functional cellular unit, the neuron. Most neurons use electrical signals, the action potential (AP), to transmit information along their axon. This AP is a large, short-lived change in the membrane potential (V_m) that travels along the distance of the plasma membrane and produces the same V_m at each point. AP's are the result of increased conductance (influx) of Na^+ (the rising phase) which causes depolarization of the membrane (Overton, 1902, Hodgkin, 1939). This is followed by a reduction in Na^+ conductance and a rise in outward conductance of K^+ (falling phase) which in turn repolarizes the cell. The onset of depolarization at nerve terminals induces the opening of voltage-gated Ca^{2+} channels, causing further depolarization. More important for synaptic transmission, is the fact that the rise in the intracellular calcium promotes synaptic vesicle fusion (del Castillo and Stark, 1952; Dodge and Rahamimoff, 1967). This releases transmitter into the synaptic cleft in which the transmitter binds to receptors on a target cell. The target cell may be another neuron, muscle or endocrine cell. The type of post-synaptic receptors and effects of the receptors on cellular functions are wide ranging. Depending on the receptor type, the activation may lead to excitation, in which membrane potential becomes more positive, or inhibition, in which the membrane potential becomes more negative. Such activation may come about by the receptor itself, being a ligand-

gated ion channel or by initiation of a cellular cascade to influence ion channels, IP3 for example (Berridge, 1998; Miyazaki, 1995). Receptors that mediate secondary cascades are referred to as metabotropic receptors, whereas receptors that act as a receptor mediated ion channel are termed ionotropic receptors.

Calcium is just one of many players in the synaptic vesicle fusion cycle (Sudhof, 2004) along with a multitude of proteins in the completion of vesicle fusion for transmitter release. The intracellular calcium concentration is very minute, compared to extracellular concentration, for example in the squid axon the $[Ca^{2+}]_i$ is about 0.1 μ M, while $[Ca^{2+}]_o$ is about 10 mM, (10,000 fold difference), thus it is a huge driving gradient for calcium to flow into the cell (Hodgkin, 1964). This calcium may differentially mobilize the readily releasable pool and the reserve pool of vesicles, (Rosenmund 1996; Kuromi 1998). Generally, it is accepted that the reserve pool is held in place in the cytoskeleton network that may be mobilized by a second messenger cascade, such as in response to serotonin (5-HT) as in crayfish, and then released in a calcium dependent manner (Dixon and Atwood, 1989; Dudel, 1965).

There are multiple proteins that are evolutionarily common between species that can sense this calcium concentration change and in turn, react to it. First, the vesicle is mobilized from the cytoskeleton, where it was held in place via synapsins. These synapsins are substrates for cAMP dependent protein kinase A (PKA) and Ca^{2+} /calmodulin dependent kinase. When the nerve terminal becomes depolarized, Ca^{2+} enters and in turn activates calmodulin kinase CaMK

which undergoes autophosphorylation and phosphorylates the synapsins. This phosphorylation breaks the vesicle free from the actin cytoskeleton and allows it to travel to the active zone (AZ), the cytoskeleton attachment point to the synapse, via the synapsins and the Rab protein. Recently, it has been shown in *Drosophila*, mammals and arthropods that the active zone complex and calcium channel location/clustering may be due to a coil-coil protein matrix known as the Bruchpilot matrix, which not only acts as a vesicle attractor, but also a vesicle guide to the optimal release site (Atwood, 2006; Kittel et al., 2006). The vesicle is then docked and primed to the AZ via various SNARE proteins, i.e. syntaxin and synaptobrevin. Once docked, an integral membrane protein on the synaptic vesicle, synaptotagmin, is able to sense the $[Ca^{2+}]_i$ in the presynaptic terminal. Synaptotagmin has the ability to bind four calcium ions and in turn undergo a conformational change that leads to complete fusion with the membrane and allows transmitter release. In addition, it was shown in *Drosophila* that mutations in the calcium binding domains of synaptotagmin had an effect in vesicle numbers and vesicle size, suggesting it may play a role in vesicle structure as well (Loewen et al, 2006). As of now, the exact mechanism of synaptic transmission is still incomplete although there are multiple models that are accepted. One of which being the quantal-vesicular-exocytotic hypothesis (Del Castillo and Katz, 1954; Fatt and Katz, 1952), in which a vesicle fuses with the pre-synaptic membrane and exocytoses its content. This however does not take into effect the fact that the vesicle becomes part of the membrane itself since ultrastructural and physiological analysis shows no evidence of the pre-synaptic

membrane enlarging. This phenomena is somewhat resolved by the “Kiss and Run” model, in which the vesicle fuses, releases transmitter and then pinches off, via NSF proteins and dynamin proteins, to be recycled either partially or fully empty (Ceccarelli et al., 1973; Aravanis et al., 2003). There are different paths that a vesicle may take in this model. One, a slower rate (i.e. when the cell is at rest) the vesicles are recruited from the reserve pool (RP) to the readily releasable pool (RRP), releases content and is then recruited to the endosome for refilling. The second way being under high stimulation rate, the vesicles take the same path from the RP, but after pinching off are directly re-recruited to the RRP to be filled by cytosolic transmitter. Both baths require the use of the vesicle monoamine transporter (VMAT) located on the vesicle membrane to pump free transmitter in the cytosol or within the endosome, specifically the catecholamines (dopamine, nor epinephrine, epinephrine) and 5-HT. In the case of glutamate, vesicles utilize a vacuolar-type H⁺-ATPase transporter specifically for glutamate (VGLUT) (Naito and Ueda, 1983; Ozkan and Ueda, 1998). In *Drosophila*, the number of VGLUTs present on the vesicle membrane has been shown to have an effect on the quantal size; over expression causes it to increase, while only one is necessary to fill the vesicle with glutamate, although the synaptic vesicles appear smaller (Daniels et al., 2004 and 2006).

Vertebrates and invertebrates handle the removal and recycling of transmitter in different manners so that vesicles may repackage and release again. For instance, in vertebrates the transmitter is either directly broken down in the synaptic cleft such as with acetylcholine via acetylcholinesterase (MacIntosh,

1961; Potter, 1970) or transported into supporting cells, specifically astrocytes (Huang and Bergles, 2004) and converted back into unmodified amino acids. For example, glutamate being converted to glutamine via glutamine synthase and then transported back into the nerve terminal. In invertebrates, such as the crayfish, glutamate is transported directly back into the terminal via the glutamate transporter found on the presynaptic terminal plasma membrane (Logsdon et. al., 2006). Another recent approach to synaptic transmission is the idea of porocytosis, which is still consistent with the hypothesis by Fatt and Katz (quantal-vesicular-exocytotic hypothesis), in which the vesicle forms a pore with the pre-synaptic membrane to release its contents (Kriebel, et al, 2001).

Calcium plays an important role, but so do many proteins that specifically bind and alter calcium levels such as the aforementioned Ca^{2+} /calmodulin-dependent protein kinase (CaMK), a protein that can function as a calcium memory device. Activation of CaMK occurs when intracellular calcium concentrations rise and remains activated when the intracellular concentrations are brought back down to basal levels. In short, with bursts of activity calcium flows in and activates the CaMK allowing it to phosphorylate more synapsins and allow more docking/fusion of the vesicles. During maintained stimulation the calcium levels increase more, thus CaMK would start to recruit more vesicles to maintain transmission though maintained electrical activity.

Other proteins serve as calcium buffers to modify the abundance of calcium in the nerve terminal cytoplasm. Calbindin, calretinin and parvalbumin are examples of such proteins (Wasserman et al., 1966; Rogers, 1987; Pechere,

1974). These proteins belong to the EF-hand calcium-binding proteins. Structurally these proteins are characterized by the presence of a conserved helix-loop-helix motifs (Schwaller et al., 2002), which bind Ca^{2+} ions with high affinity. These proteins can easily affect synaptic transmission via how tightly they bind calcium. If these proteins were in high abundance and had a very fast K_D then they may bind a majority of the calcium before being able to induce vesicle fusion. So, in short bursts, when calcium entry is intermittent, these proteins may bind up so much that no response is seen, while in a sustained stimulation, calcium may be able to build up to overcome the calcium binding proteins and synaptic transmission will take place. One could imagine the alterations in synaptic transmission if the fall K_D to Ca^{2+} binding proteins were slow as well as when the cytoplasmic calcium levels are low. The proteins with low binding affinity would allow free Ca^{2+} to increase more rapidly over time than ones with tighter binding, possibly inducing spontaneous transmitter release.

Not only do cytoplasmic proteins interact with calcium and alter its effects, membrane bound proteins are also an important participant in calcium handling. The sodium-calcium exchangers (NCX) are such membrane-bound proteins (Reuter and Seitz, 1968; Baker and Blaustein, 1968). This system plays in conjunction with the Ca^{2+} ATPase pump and functions using the concentration gradient of sodium. For every 3 Na^+ in, 1 Ca^{2+} is co-transported out, until it reaches a steady state with that of the passive Ca^{2+} influx (Caputo et al, 1989). During a short burst of activity and intermittent calcium influx, the NCX should be active along with other mechanisms to sequester and buffer Ca^{2+} . The

endoplasmic reticulum (ER) and mitochondria also play a role in regulating intracellular Ca^{2+} during neural activity (Tang and Zucker, 1997). With a sustained stimulation, the residual Ca^{2+} will build up and cause the other systems to become saturated. During this time the NCX will transport Ca^{2+} out at a very high rate, in an effort to keep $[\text{Ca}^{2+}]_i$ low. Depending on the species, the NCX or the ATP-ase clears the residual Ca^{2+} after sustained stimulation. For instance in the crayfish, the NCX plays a larger role in expelling Ca^{2+} , while in *Drosophila* (Lnenicka, 2003) it seems to be the opposite (Rumpal and Lnenicka, 2005).

Quantal Theory

In 1952, Katz, Fatt and del Castillo coined the term quanta, in their experiment showing that ACh can be released in packets and spontaneously as miniature endplate potentials (MEPPs or mini's). This phenomenon was given this name in relation to the physical limitations of the individual packets, referring to them as the smallest amount that can exist in the nerve terminal (Fatt and Katz, 1952) or in terms of synaptic transmission, release or failure of release. Del Castillo and Katz found that each packet of vesicle being released at specific sites (n), had a certain probability of release (p) for a given nerve impulse, thus with any given stimulation, release may or may not occur, accounting for the term "quantal." This phenomenon is important for studying synaptic transmission in that one is able to study a response from a single vesicle which gives insight to larger multi-quantal events in relation to excitatory postsynaptic potentials (EPSPs). Because it is estimated that the number of transmitter molecules are

basically constant within each vesicle or quanta, depending on chemical transmitter (e.g. 7000 for ACh, 4000 for glutamate) (Kuffler and Yoshikami, 1975, Villanueva et al., 1990), an EPSP is the result of one or many quanta being released simultaneously due to the influx of Ca^{2+} from the presynaptic action potential.

The opener muscle preparation from the first walking leg of the crayfish is advantageous to the study of synaptic transmission and quantal content, because it is quite durable. Unlike preparations from mammals, which require a very tightly regulated environment, the crayfish preparation is stable in a simple physiological saline solution within a wide range of temperatures for many hours. Mammalian neurons are very sensitive to pH fluctuation in the bathing medium, which usually has to be tightly regulated by CO_2 because of HCO_3^- associated buffering, whereas for the crustacean preparation, a HEPES or phosphate buffer works well. Another advantage of the crayfish opener muscle is that it is innervated by only one excitatory motor neuron, which branches to the separate fibers within the muscle, which is identifiable from preparation to preparation. The motor nerve terminals consist of a series of swellings (i.e. varicosities), where the majority of synapses exist. An additional advantage is the effect of synaptic transmission elicits graded post-synaptic potentials that are non-spiking in physiological saline. The extent of the graded depolarization regulates the degree of muscle contraction and tension. With low frequency stimulation of the excitatory motor neuron, the synapses produce a low occurrence of vesicular fusion with the presynaptic membrane. Thus, individual vesicular events can be

monitored. Investigation in the regulation of synaptic properties and action of neuromodulators requires precise measurements of quantal content to dissect pre- and post- synaptic components of transmission.

Determination of Quantal Parameters and Stems:

The most direct method for determining quantal content is to count the number of quanta released by the nerve impulse. This is possible at the crayfish neuromuscular junction (NMJ) by using low stimulation frequencies (1-3Hz). This data may then be calculated to estimate binomial parameters of transmitter released from the presynaptic nerve terminal (Johnson and Wernig, 1971; Wernig, 1972; Smith et al., 1991). Direct quantal counting becomes problematic in other preparations in normal physiological saline (e.g. frog, mouse, *Drosophila*, *C. elegans*, etc.) because it is not possible to determine directly all the quantal units evoked by a nerve impulse. This can be overcome by altering the specimen bathing solution, i.e. for frogs, adding Mg^{2+} to the saline (to block NMDA receptors at the NMJ) solution to higher concentrations than physiologically normal. This allows single events to be observed at low frequency stimulation.

The crayfish motor nerve terminal consists of a series of varicosities (swellings in the nerve terminal) along the terminal as it innervates the muscle. I used the “stem” region of the terminals, which lie between neighboring varicosities in some aspects of my studies with direct structure-function analysis of the nerve terminals. A single varicosity contains up to 50 or 60 AZ's, while it

has been previously reported that the recorded stem region contains fewer synapses with a reduced number of AZ's (4-9 synapses, 0-14 AZ's) (Florey and Cahill, 1982). This also required the precise determination of quantal parameters—in order to correlate structure with function. Difficulties arise in such structure/function analysis for the NMJ of vertebrates since it is estimated that more than one hundred quantal units may appear for each nerve impulse and once a threshold is reached an action potential is initiated, thus masking the graded quantal responses.

Measures of Transmission:

In the studies I conducted in assessing synaptic transmission I utilized the well-documented macro-patch recording technique (Dudel, 1981) and quantal analysis (Katz and Miledi 1967, 1968; Viele et al., 2003). This approach was used to ensure correct measurement of synaptic transmission along precise regions of the nerve terminals of crayfish. Quantal events at specific regions along the nerve terminal can then be recorded. The rise time, decay time, peak amplitude and the area of quantal event for discrete evoked events are able to be assessed and analyzed using multiple stochastic procedures to determine groupings of the individual synaptic quantal events within the defined region of the nerve terminal.

A specific vital dye, 4-Di-2-Asp, highlights nerve terminals in crayfish, but is ineffective in insects or salt water living crustaceans (i.e., lobsters or crabs). This vital staining allows the experimenter to visualize individual varicosities and

regions between the varicosities, known as stems (Cooper et al., 1995b). This vital dye technique replaces having to blindly place the electrode randomly along the terminal and aids in marking the area of the NMJ that was physiologically monitored by the loose patch electrode. By coating the rim of the electrode with polystyrene beads, some beads are left behind which demarks the precise region recorded along the nerve terminal. The particular beads used are fluorescent and electron dense, allowing one to see them with light microscopy as well as with transmission electron microscopy (TEM) in a precise area. By labeling the nerve terminal that was physiologically assessed, it is possible to fix the tissue and serially thin section the area to visualize the synaptic structures with TEM. In some cases the beads are lost, but are photographed before the loss. These photographs can be used to find a local anatomical marker to rebuild this site by finding that anatomical site through thick sectioning. The electron micrographs can then be used for reconstruction into a three-dimensional representation of the area recorded (Cooper et al., 1995b, 1996b). This procedure was previously used to identify low and high output synapses in relation to distribution of AZ's (Cooper et al., 1995b).

Quantal analysis and Binomial Instability:

Current interest in the field of synaptic transmission is in determining why some synapses are selectively utilized and recruited depending on demand. In addition, the precise pathway of vesicles and kinetics of recycling is currently intensely investigated. Variation in quantal response is also of current interest to

the field of synaptic physiology in relation to development, synaptic plasticity and pharmacological studies. Varied location of vesicular fusion on a synapse may be the reason for fluctuation in the measured quantal responses. This may result from the post-synaptic cell only receiving a fraction of the total transmitter within a single vesicle. In theory, different size or shape of the unitary postsynaptic current can occur from release over the center of the receptor array as compared to the edge of the synapse. This possibility assumes that the postsynaptic array of receptors is not saturated from the release of a single vesicle. On the other hand, if the postsynaptic receptor array is saturated by the release of a single vesicle then the currents should be uniform each time that site is activated. For these two scenarios, any fluctuation in the size or shape of the postsynaptic currents observed may be either due to varied sites releasing on the presynaptic face within a synapse, or that various synapses are being utilized. There are examples for both cases to account for the variation in the size or shape of single vesicular events in various preparations. It is also possible that both scenarios may co-exist since one does not exclude the other from occurring. The topic of quantal variation and evidence for pre- and post-synaptic contributions has been reviewed (Faber et al., 1998). In the past, one counted the event as a single or double, etc. quantal event and estimated the n and p to describe the synaptic sites. However this type of analysis does not consider the individual characteristics of the quanta which could possibly indicate a signature for a particular synapse. In an earlier study it has been possible to estimate n and p using characteristics of single vesicular events (Viele et al., 2003). In addition, I

have also estimated the changes in n and p (based of quantal signatures) as the frequency of stimulation is changed. This technical aspect is utilized throughout my research projects and some time has been taken in double checking automated programs that Mark Lancaster (Graduate student, Dept. of Statistics, University of KY) and Dr. Kert Viele (Dept. of Statistics, University of KY) have been working on in relation to these projects.

Determination of discrete quantal events at the crayfish NMJ is possible, thus allowing one to determine the parameters of synaptic transmission at a quantal level of discrete analysis. The quantal occurrences in the evoked releases are used to estimate best fits to known distributions such as binomial or Poisson. By obtaining the best fit distribution to the observed distribution, quantal parameters are derived. Typically, mean quantal content (m) is used as a representation of the average number of events per given stimulus, p , representing the probability of events at a release site and, n , standing for the number of release sites (Del Castillo & Katz, 1954). Studies have shown that estimating n and p can be very problematic in most preparations. More attention in searching for various components in the evoked responses may better estimate n and p —perhaps the size and shape of the individual quantal events and their distribution of particular quantal subsets. The goals of this study are to use modern statistics that incorporate differences in the sizes and shapes of quantal events to better estimate n and p . Previous methods of estimation show that n and p are very irregular, but they can be divided into sub-clusters that have distinct probabilities of occurrence. Thus, each n can have a particular p

assigned to it. This notion strays from convention in assuming a uniform p for each n .

Because n and p cannot be measured directly, multiple approaches have developed over the years to estimate n and p by studying the distribution of the recorded evoked events (McLachlan, 1975; Korn and Faber, 1998). Such methods as these have been shown to be problematic when the synaptic efficiency is low. When this is the case, the distribution of observed evoked events is best fit by a Poisson distribution (Zar, 1999) or a binomial distribution ($n=1$). The Poisson distribution usually results in a large estimation for n and a small estimation for p (release is random at a low probability from numerous possible sites). As for a binomial aspect with a distribution of $n=1$, only one site is functional and p changes as the parameters of release are altered.

Previous methodology shows current ways of estimating n and p to be somewhat inaccurate. Thus, more precise methods to better estimate n and p must be developed. One parameter(s) that is possible to study is the size and shape of the evoked and spontaneous synaptic currents or potentials. Using classical counting methods, two separate evoked events would be counted as the same single event regardless of the differences in their characteristics (area, amplitude, latency, time to peak, etc.). Because some events have different characteristics, it may be useful to study the differences to better understand how many sites or combinations of sites are utilized during synaptic transmission. Previous structure/function relationships (Cooper et al., 1995b, 1996a,b) at discrete areas of the motor nerve terminal showed that there can be a multitude

of synapses ($n=30\sim 40$), each with a multitude of active zones, which when compared to current physiological counting methods n and p may indicate that $n=1$. This is in spite of the fact that each evoked event may have had variation, which is shown by the physiological recordings while the structural data may indicate that multiple sites are active individually or simultaneously in regards to vesicle fusion and transmitter release. This is one advantage that I have by recording from the stem region, where structural evidence shows there to be a reduced number of synapses and active zones. This will result in reducing n (in the physiological measurements by recording from the stem region). Quantal measurements in this region would most likely estimate a low n and thus structural analysis for functional correlation may be best embarked upon within this locale since there will be fewer compounding variables. In relation to classical means of obtaining n and p via direct counting of quantal events (Del Castillo and Katz, 1954) and the determination of their distribution of occurrence underestimate the actual number of functional sites based on anatomical information. This is why stochastic techniques that can incorporate differences in quantal sizes and shapes of evoked events to better estimate n and p will be utilized. The physiological information will then be directly correlated, at the physiological sites, to the structural morphology of the nerve terminals as discussed in Chapter 2.

Additional Studies in Synaptic Transmission:

The nervous system must be able to adapt and translate multiple signals, usually many at one time. One way the CNS can enhance or depress or alter synaptic transmission rapidly without having to undergo morphological changes in synaptic structure is by modulating the communication. Such actions are known to occur by neuromodulators (i.e. naturally occurring chemicals that alter neuronal function) which can act rapidly or slowly and have short (seconds to minutes) or long lasting (minutes to hours) effects on neuronal function. This requires the system to be able to change its response quickly and efficiently via neuromodulation. Thus, neuromodulation may have many outcomes when it comes to the efficacy of the target it is affecting, these include but are not limited to: increase or decrease in synaptic transmission, desensitization of the neuron target to neurotransmitters or internalization/increased expression of postsynaptic receptors. The underlying mechanism of neuromodulation can be via a metabotropic receptor which induces various second messenger cascades (i.e. IP3 via 5-HT), which in turn affects the ionic channel of the target cell or by direct interaction with the ionic channels that allows it to become more or less sensitive (Dwivedi et al., 1998). Another likely scenario is that second messengers could cause an increase in synaptic transmission due to vesicle mobilization via phosphorylation of synapsins or, conversely, phosphorylation of mediator proteins involved directly with the docking machinery, such as t-SNARE, v-SNARES, NSF's or MUNC proteins (Southard et. al., 2000). Neuromodulation may also effect up and down regulation of receptors in the target cell. In vertebrates it was shown, a down regulation of 5-HT_{2c} receptors is

able to be induced by activity in the whole animal (Broocks et. al., 1999). A neuromodulator's effect can be long lasting and is not always, if ever, localized to only the target cell, thus their effects can be observed a great distance from its origin and can act on numerous neurons. This allows the nervous system to remain plastic, being able to fine tune its communication with target cells in order to reciprocate a specific response.

Not only can studying neuromodulation give insight into synaptic transmission, but also to study specific synaptic modification. In Chapter Four, a fellow colleague, Ms. Stephanie Logsdon and I investigate the regulation of synaptic vesicles during short-term facilitation and neuromodulation. When discussing, specifically short-term facilitation, it is easier to first understand the broader term "facilitation" and then discuss some of the categories into which it may break down. Facilitation, an example of synaptic plasticity, was first shown at the crustacean NMJ (Fatt and Katz, 1953) and later at the frog NMJ (del Castillo and Katz, 1954). When recording end-plate potentials at the NMJ during a train of rapid stimulation, the amplitudes of the EPSPs increase throughout the duration of the stimuli. This result is not only during the train of stimulation, but well after the train has ended. If a second stimulus is elicited seconds later after the train, an EPSP is seen that is larger than that from a stimulus observed at the beginning of the train, this is what is referred to as facilitation. Del Castillo and Katz later showed that this was due to an increase in the mean quantal content (m), caused by an increase in probability of release rather than an increase of the

number of release sites (del Castillo and Katz, 1954; Dudel and Kuffler, 1961 and Kuno, 1964).

Facilitation can be broadly categorized into long term facilitation (LTF) and short term facilitation (STF). LTF was first described at the glutamatergic synapses at the crayfish NMJ (Sherman and Atwood, 1971) and later in the vertebrate hippocampus (referred to as long term potentiation or LTP in vertebrates; Bliss and Lomo, 1971), which demonstrated an increase in synaptic efficacy after a high frequency train was administered. This increase in synaptic efficacy led to increased amplitudes of EPSPs following the train of stimuli for hours later. When referring to LTF in the invertebrate nervous system (specifically crustacean), the duration of the LTF ranges from minutes to hours (Atwood and Wojtowicz, 1986).

The underlying mechanisms of LTF were proposed by Katz and Miledi to be a residual calcium concentration in the nerve terminal from subsequent action potentials (Katz and Miledi, 1968). Because calcium increases the probability of transmitter release, a build up of residual calcium that has not had a chance to be buffered, exchanged (by the NCX) or chelated (Ca^{2+} binding proteins) would be available to induce more vesicular release. Later studies suggested that it is not primarily residual Ca^{2+} for LTF or the time lag that create the response, but the residual Ca^{2+} is still thought to be the major contributor for STF (Wojtowicz et. al., 1988). Also, it was proposed that an accumulation of sodium ions contributed to LTF (Atwood et. al., 1976). This was supported by intentionally increasing the internal concentration of Na^+ by poisoning the Na/K pump with ouabain. Later it

was found not to play a role in the direct facilitation, but did play a role in its duration (Nussinovitch and Rahamimoff, 1988). A post-synaptic response has also been noted to account for LTF, that was shown in the hippocampus, by AMPA receptors being inserted into the post-synaptic membrane thus making the target more sensitive to the transmitter (Shi et al., 1999).

Short term facilitation is like that of LTF since an increase in synaptic output, due to a subsequent stimuli, is enhanced but in this case for one to several seconds (Zucker, 1982). This example of facilitation was utilized to study the synaptic output at the crayfish opener muscle in Chapter 4 to decipher how vesicle pools were being regulated in the nerve terminal. STF can be induced by giving a train of stimuli, in which each subsequent EPSP amplitude is larger than the previous due to residual calcium building up in the nerve terminal. By using this method, one can use different EPSP peaks within the recording to measure a ratio to index the degree of facilitation (i.e. a train of ten pulses at 40 Hz, the 5th and 10th pulse can be measured to find if there is a substantial increase, decrease or no change in amplitude $(10^{\text{th}}/5^{\text{th}}) - 1$).

Studying vesicular dynamics in and among various pools of vesicles within the nerve terminal, STF and neuromodulation were used and discussed in Chapter 4. In this study a neurotoxin, DL-threo- β -benzyloxyaspartate (TBOA), which blocks the glutamate transporter on the nerve terminal plasma membrane was used. In postsynaptic targets, the excitatory synaptic potentials are incremental in relation to the numbers of packets of transmitter released below a threshold potential (del Castillo and Katz, 1954a). This is generally accepted by a

packet of neurotransmitter contained within a vesicle being released from the presynaptic nerve terminal into the synaptic cleft (Dudel and Kuffler, 1961; Kuffler and Yoshikami, 1975b). A number of studies have set out to address the question of whether all the vesicles are equally potent in eliciting quantal postsynaptic responses (McLachlan, 1978; Connor et al., 1997). One aspect to solve this issue is to know if vesicular packaging is equal for all vesicles. This can be partly addressed through examining which vesicle pools are recruited during electrical activity and in the presence of neuromodulators. Since it is known in a number of preparations that there are populations of vesicles within nerve terminals that behave differently in their fusion and recycling kinetics, the ability of these pools to be recruited during electrical activity of the nerve as well as in the presence of neuromodulators which have secondary actions on vesicular kinetics was examined. By addressing this in relation to presynaptic function, a better understanding of fluctuations in quantal responses during synaptic depression, facilitation and synaptic differentiation will hopefully be achieved. In this study, the known simplicity of the synaptic structure at the crayfish opener NMJ and its quantal nature of transmitter release to assess discrete synapses within the motor nerve terminals were used (Dudel and Kuffler, 1961; del Castillo and Katz, 1954a, b). The opener muscle in the crayfish walking leg, as used in this study, is non-spiking and graded postsynaptic potentials are directly related to the number of presynaptic vesicle fusion events (Dudel, 1981, 1989; Dudel et al., 1988; Dudel and Kuffler, 1961) as also shown at the frog NMJ when the

responses are below the threshold level for an action potential in the muscle (Kuffler and Yoshikami, 1975).

With physiological and pharmacological means we set out to address whether the uptake of synaptically released glutamate is important in the refilling of the readily releasable pool (RRP) of vesicles that rapidly recycles during maintained stimulation. In addition, we examined whether the RP of vesicles can be recruited, by the use of 5-HT, while the RRP remains reduced functionally or is rendered non-functional. The goal is also to provide further insight into the regulation of the RP and recycling of the RRP within nerve terminals in a model synaptic preparation.

As previously mentioned above, 5-HT at the crayfish NMJ greatly enhances transmitter release (Dudel, 1965a) presynaptically by increasing the probability of vesicular fusion (Southard et al., 2000) and is thought to do so by recruiting RP of vesicles to the RRP (Sparks and Cooper, 2004; Cooper et al., 2003). The two populations of vesicles can be differentiated by depleting the function of the RRP by preventing them from being repackaged during rapid recycling. Recruiting the RP with 5-HT while reducing the ability of the rapidly recycling pool from repackaging allows the dissection of the various pools within the terminal. In crayfish motor neurons, 5-HT mediates its rapid effect through the IP_3 second messenger system (Dixon and Atwood, 1989; Delaney and Tank, 1991). The action of STF in enhancing release was recently addressed by studying actions of 5-HT at the crayfish NMJ which suggested that different pools of vesicles might reside in the terminal due to alternate recycling mechanisms (Sparks and

Cooper, 2004). The recycling of vesicles is generally depicted as following two different routes for recycling: a rapid loop and a slower one that reprocess the vesicles within the endosome (Klingauf et al., 1998; Kuromi and Kidokoro, 1998; Palfrey and Artalejo, 1998; Richards et al., 2000; Stevens and Williams, 2000).

The rapid recycling process is promoted during repetitive electrical depolarization of the terminal to maintain output. A homeostasis can even exist to recycle vesicles and buffer Ca^{2+} so that a plateau in release is observed; however a slight alteration in the stimulation frequency or actions of a neuromodulator offset the balance in the various vesicle recycling paths (Sparks and Cooper, 2004). To address whether the RP can be recruited to the RRP during maintained electrical activity of the terminal, the RRP needed to be selectively targeted to be allowed to recycle but also tagged to distinguish release from those of the RP. This can be approached by depleting transmitter within the vesicles of the rapidly recycling pool while not altering the RP within a short window of time.

Rapid functioning synapses clear released transmitter in the synaptic cleft quickly, which is one mechanism used to avoid desensitization of postsynaptic receptors in order to detect subsequent evoked release within several milliseconds (Dudel and Schramm, 2003) and prevent spillover to neighboring synapses (DiGregorio et al., 2002; Huang and Bordey, 2004). The glutamate-ergic synapses within the vertebrate CNS clear glutamate by use of glutamate transporters. It is generally accepted that the major recycling path for glutamate is through astrocytes (Huang and Bergles, 2004). These supportive cells take up

the glutamate from the synaptic cleft via glutamate transporters. Within the cell glutamate is converted to glutamine which is then released out of the astrocyte. The transporters on the nerve terminal then take up glutamine which is converted back to glutamate and transported into the vesicles by specific vesicle associated transporters (Christensen and Fonnum, 1992; Takamori et al., 2000). Thus, for glutamate-ergic terminals the glutamate enters back into the terminal by two means: (1) Directly via presynaptic transporters, and (2) through the use of supportive cells by a glutamate-glutamine cycle.

Vertebrates are known to have multiple types of glutamate transporters. In mice, there appear to be five genes that encode six distinct glutamate transporters (Huang and Bergles, 2004). The motor nerve terminals at the crayfish NMJ are glutamate-ergic and since glia cells do not enfold with the nerve terminals on the muscle the variable of supportive cells is eliminated in the process of vesicle repackaging (Atwood and Cooper, 1995; Cooper et al., 1995a). Thus, the presynaptic nerve terminal is the likely means of clearing glutamate from the synaptic cleft. The reuptake process during normal synaptic transmission is expected to be rapid since these NMJ can desensitize quickly with exogenously applied glutamate. In the absence of glutamate the receptors resensitized to be responsive to a subsequent exposure (Dudel and Schramm, 2003). The presynaptic re-uptake at the crayfish NMJ is also supported by the fact that the blocker for vertebrate glutamate transporters, TBOA, can block the re-uptake of glutamate at the crayfish NMJ (Dudel and Schramm, 2003). Hence,

use was made of blocking glutamate re-uptake to assess vesicle dynamics within the nerve terminal during rapid release and recycling of the RRP of vesicles.

Through the use of TEM, one can address whether the recycling pool of vesicles is altered in numbers when the presynaptic transporters are compromised. I examined for differences in the dynamics of the RP and the number of docked vesicles anatomically with TEM to compare with physiological assessments and predictions of vesicle dynamics during blockade of the transporter as well as during recruitment of a RP by 5-HT. Vesicles and vesicle pools are easily identified in nerve terminals in TEM which provides insight into the state of the nerve terminal before and after pharmacological exposure. In most nerve terminals examined, at a resting state, a small number of vesicles are docked to the active zone accompanied by a close proximity RRP of vesicles and a RP of vesicles located a small distance away (Rizzoli and Betz, 2003). After TBOA induction, the functional responses of the RRP of vesicles are known to become depleted (Dudel and Schramm, 2003). Thus, it is of interest to know by TEM if there is a decrease in vesicles docked or ones in the RRP.

In Chapter 3, another preparation from the crayfish is utilized to study presynaptic depression and vesicular dynamics. Synaptic depression and potentiation are well recognized ongoing physiological phenomenon within the nervous system. However the detailed cellular mechanisms and modulation of the processes are not yet fully understood, although forms of plasticity such as this have been approached through the studies of mutations in *Drosophila*, specifically mutations that affect learning and memory. Mutations such as *dunce*

(a cAMP specific phosphodiesterase mutation) and *rutabaga* (an adenylylase mutation) disrupt different forms of plasticity (Chen et al., 1986; Levin et al., 1992). Although the recently discovered *akt* mutant which seems to be specifically critical for induction LTD, but not other forms of plasticity (i.e. STF), suggests differential roles in signal transduction control over plasticity (Guo and Zhong, 2006). Even though 200 different proteins are known to be present within the synaptic site, their role in potentiation and depression remain elusive (Sanes and Lichtman, 1999). Synaptic depression is generally defined as a use-dependent long-lasting decrease in synaptic strength (Linden and Conner, 1995). Long-term synaptic depression has been classically studied in the cerebellar slice. Two forms appear to be present. One form is called "heterosynaptic depression", and the other is referred to as "homosynaptic depression". The heterosynaptic depression is described as being when a different synaptic connection is activated than the one that actually depresses. Whereas the homosynaptic depression of synapses is in which the same site has had the previous activity. Some evidence points to a presynaptic component while other evidence points to a postsynaptic site. This inconclusiveness may reside in the experimental limitations in previous investigations. Since LTD is thought to play a major role in memory and behavior, one can hope that knowing how to modulate the processes in defined simple systems an overall understanding will prevail. In this study, a system that demonstrates homosynaptic depression via presynaptic mechanisms and that is modulated by 5-HT is presented.

Studies which involve CNS circuits within the animal or in culture have not allowed one to monitor electrical transmission at the actual sites of synaptic communication; instead cell bodies are recorded from to obtain an understanding of the electrophysiological process at the synaptic connections on a dendritic tree or in the presynaptic terminals. The advantage of examining synaptic depression at the crayfish NMJ is that the sites of synaptic contact can be studied directly. Modulation in the rate of synaptic depression and the recovery process opens the door to deciphering the mechanisms by which synaptic depression functions. For these purposes, we made use of the crayfish leg extensor preparation and exogenous application of 5-HT to the preparation. This preparation provides several experimental advantages since individual muscle fibers are innervated by both phasic and tonic motor neurons. Repetitive 5Hz 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, 1996). The presence of the tonic terminals alongside the phasic terminals allows one to assess whether the postsynaptic target is greatly modified during and after depression of the phasic motor neuron. Since 5-HT increases the number of vesicles that are released with evoked stimulation, it was postulated that synaptic depression would occur at an increased rate during evoked release in the presence of 5-HT. Likewise, after a nerve terminal is depressed in the absence of 5-HT, it was postulated that recovery of depression would be enhanced if 5-HT was made available following synaptic depression. To determine, by physiological means, if synaptic

depression in this preparation is occurring as a result of fewer vesicles being released or alterations of the function of postsynaptic receptors, focal macropatch recordings of postsynaptic currents and measures of single quanta from defined regions of the motor nerve terminal were performed. The glutamatergic receptors at the NMJ in crayfish are of a quisqualate subtype and demonstrate a quick recovery from desensitization (Dudel et al., 1993).

With the aid of TEM and serial sectioning we also set out to address, by anatomical means, if the RRP of vesicles that rapidly recycles during maintained stimulation is altered in depressed nerve terminals. In addition, since electrical activity is known to recruit vesicles from a reserve pool (RP) in crayfish motor nerve terminals (Sparks and Cooper, 2004) I examined the population in depressed terminals as compared to non-stimulated terminals. Since 5-HT appeared to be able to recruit vesicles in a depressed nerve terminal one would expect an even greater decrease in the RP. This condition was also examined anatomically in order to account for the physiological observations. My goal in these studies has been to provide further insight into the scheme in the operations of the RRP and the RP within nerve terminals in a model synaptic preparation within various states of function.

The extensor muscle of the crayfish walking leg is an ideal preparation to study synaptic depression. Depression is another form of synaptic plasticity, in which continuous stimulation results in the decrease in postsynaptic potentials. Again, the neuromodulator 5-HT was used to study vesicular dynamics in this system. Once depression had set in, 5-HT was added to the saline, which

resulted in the return of the postsynaptic potentials. Again, this is due to a separate pool of vesicles (the reserve pool) under a separate control mechanism than that of the readily releasable pool, via second messenger cascade elicited by the serotonin. TEM was used to verify changes in the RRP.

By using the opener preparation, the extensor preparation and TEM, synaptic plasticity and vesicular dynamics can be studied physiologically as well as visually. These experiments gave insight into how the nerve terminal is able to incorporate numerous signals that allow it to be plastic and in turn translate those signals into meaningful messages.

In summary, these projects all contribute to a better understanding of the structure/function relationship of the nerve terminal. Utilization of neuromodulators, such as 5-HT, in Chapters 3 and 4, gives insight into nerve terminal physiology which relates to vesicular dynamics. In addition, neurotoxins (TBOA, Chapter 4) that block specific pathways of transmitter reuptake and change synaptic plasticity (short term facilitation, Chapter 4 and synaptic depression, Chapter 3), were used to directly manipulate vesicular dynamics. This allowed other aspects of the system to be studied. These experiments gave further understanding into the functional aspect of nerve terminals. With the advantages of TEM, the synapses, AZs and in some cases, pools of vesicles were visualized in nerve terminals and rebuilt in a spatial orientation, which provided a better comprehension of structure and how it relates to function.

The specific aims of this study are:

1. Determine the degree of quantal groupings via the characteristics of singly evoked quantal currents obtained from various low-output stem regions, at different rates of stimulation. Is there a recruitment of particular types of single quantal responses within the distribution of events? If this is the case, then is this an indication that some sites release with different probabilities? If this is not the case, then the probabilities increase consistently or possibly remain random, in spite of possible assorted synaptic structure.
2. Use stochastic techniques to better estimate n and p , via size, decay time and shape of the quantal event. This data can then be used to compare and quantify the classical n and p procedures to demonstrate their inaccuracies. An algorithm to quantify evoked events has been written in R Basic by colleague Mark Lancaster from the Department of Statistics at the University of Kentucky.
3. Directly determine via serial reconstruction from electron micrographs the number of active zones, synapses, and synaptic complexity that are present in the labeled sites in which the quantal synaptic measures are obtained. Determine the synaptic structural and physiological relationship based on numbers of active zones and spacing of active zones within the defined recorded regions of the terminal. Determination in the relationship of vesicle populations around the various types of active zones.

In addition to addressing the structure and function of active zones, questions related to synaptic plasticity and vesicle dynamics were also addressed.

4. Using neuromodulators and neurotoxins to deduce possible mechanisms underlying synaptic plasticity. Synaptic plasticity, synaptic depression and facilitation were used as measures of synaptic function. Are vesicular pools regulated differently during these synaptic phenomena? If so, how are they regulated and/or manipulated during this synaptic plasticity?

CHAPTER 2

Structure/Function Assessment of Crayfish Neuromuscular Junctions:

Stem regions

INTRODUCTION

The release of transmitter at neuromuscular junctions of the opener muscle in crayfish has been shown to be quantal in nature. The tonic opener muscle offers the advantage of being able to record quantal events at specific visually identified release sites, thus allowing measurement of the physiological parameters of vesicle release which can be used to directly correlate to synaptic structure.

These experiments take advantage of a vital dye (4-Di-2-Asp) which labels nerve terminals allowing visualization of individual varicosities and areas between the varicosities, known as stems. Stems were chosen as the region to study because of its low synaptic output due to its reduction of release sites. By rebuilding the site recorded from via TEM, the number of synapses and AZs are revealed, thus the parameters of mean quantal content (m), n (number of release sites) and p (probability of release) may be more accurately assessed. By reducing n to a few possible release sites, p may be more accurately estimated from physiological quantal recordings if the parameters of a quantal event provide a synaptic signature. Such a quantal signature could come about by parameters of: area, rise time, peak, latency and tau decay. This allows more factors to assess discrete synapses and their probability of release than what is currently being approached in the field.

In this study, it is shown that even at a site such as the stem region, synaptic transmission is still complex and p for discrete sites may not be feasible to estimate, but this study has provided insight into how one should approach calculating n and p when the data becomes available for more ideal preparations.

General Background

Evoked neurotransmitter release is dependant on depolarization induced Ca^{2+} influx through specific voltage gated channels on the presynaptic terminal (Llinas et al., 1981; Augustine et al., 1985; Augustine 2001; Zucker et al., 1991; Sugimori et al., 1994). This Ca^{2+} influx induces vesicle fusion to the synapse through protein interactions, i.e. syntaxin, synaptobrevin, synaptotagmin and numerous others (Sudhof, 2004). This influx was shown to be essential for transmitter release by Katz and Miledi (1967, 1968) by showing that alteration in the in the $[\text{Ca}^{2+}]_o$ concentration in turn caused a shift in the amount of transmitter that was released from the nerve terminal. These channels have been shown to be adjacent to the sites of vesicular fusion and release of neurotransmitter (Pumplin et al., 1981). Freeze-fracture electron micrographs have revealed numerous groups of intramembraneous entities that are in close association with sites of vesicular release (Couteaux & Pecot-Dechavassine, 1970; Govind et al., 1994, Cooper et al., 1996). In addition, TEM by Heuser and Reese (1974) showed at the frog neuromuscular junction, vesicles in mid-fusion with the presynaptic terminal, revealing an omega-shape vesicle. These omega-shaped vesicles were aggregated near release sites that were identified by an electron

dense mass on the presynaptic terminal which may take a variety of forms from different types of synapses. These dense structures have a broad variety of shape and form from species to species. For example, the salamander retinal rod synapse shows an array of dense bars lined with ribbons of synaptic vesicles aligned for docking (Rao-Mirotznik et al., 1995). At the frog NMJ the AZ's are long dense bars, in which vesicles associate (Kriebel et al., 2000). At the crayfish excitatory neuromuscular junction, a dense body in the shape of a hemisphere is atop the presynaptic terminal (Jahromi and Atwood, 1974), while in mammals these dense structures align themselves in a organized array on the synapse that work as a entire unit with vesicular docking (Vrensen, 1980). Although the dense body is not completely understood nor its makeup revealed, it is a constant indicator from species to species as the site of vesicular accumulation. The summation of these structures, the dense body and intramembraneous particles are indicative of the synaptic active zone (Couteaux, 1970 and Couteaux and Pecot-Dechavessine, 1974).

Evidence supports that the structural makeup of the synapse, in relation to AZs in multiple vertebrate and invertebrate models, is most likely correlated with the amount of transmitter that is released (Rheuben, 1985; Walrond and Reese, 1985; Cooper et al., 1996b). One advantage of the invertebrate crustacean model for synaptic transmission is that physiological recordings can be directly associated with synaptic ultrastructure at vesicular release sites. Through TEM, ultrastructural components may be identified and quantified such as number and size of synapses, number of active zones, abundance of vesicular pools, i.e.

docked vesicles or those in readily release or reserve pools, although vesicular pools were difficult to make out during these experiments due to confounding circumstances (low magnification, thick formvar and relatively thick sections). Using the power of electron microscopy and the advantages of the crustacean preparation, the precise area that was physiologically assessed can be rebuilt in 3D. This gives insight for examining whether ultrastructure correlates to the physiological recorded quantal events such as synaptic area, number of AZs, and spacing of AZs on synapses (Cooper et al., 1995b, 1996b). Although it was previously shown how the spacing of active zones and their relative distances to calcium channels can affect the probability that a vesicle will fuse with the presynaptic membrane (Cooper et al., 1996b), it does not account for the quantal current variability seen when recording from a synaptic site. Because of this variability, one could assume that there are multiple sites that may be active within a recorded location. This suggests that there are groupings in the characteristics in the quantal recordings such as size, shape, rise times and decay constants.

Incorporation of known ultrastructure of the recorded synaptic site through electron microscopy, (i.e. number and location of active zones in relation to postsynaptic receptor array) provides a new and significant approach in order to better characterize quantal parameters. To better address this, directly choosing a site of synaptic transmission with a known low number of release sites would remove many of the confounding variables associated with recording from an entire varicosity along a nerve terminal since they contain numerous synapses.

The stem between varicosities is such an area that was made use of for this particular reason. The hope was by using this simplistic synaptic preparation a fundamental insight into how fluctuations in the postsynaptic potentials come about could be correlated to synaptic structure. This fundamental assessment is the basis of synaptic communication at these NMJs but also for synapses in the central nervous system of all animals.

Del Castillo and Katz (1954) showed transmitter released from the neuromuscular junction is quantal in nature, this is also true for the crayfish NMJ (Dudel, 1981; Cooper et al., 1995c). The concept of this quantal nature has been confirmed for many different synapses from multiple species, including mammalian CNS neurons (Redman, 1990; Hessler et al., 1993; Rosenmund et al., 1993). Characterization of transmission and its alteration by activity or neuromodulators demands measurement of quantal content of transmission (the number of quantal units generated by a nerve impulse). Assessing these characteristics is easily done in some systems as compared to others. The crayfish opener muscle is such a preparation that allows many of the problems associated with quantal currents and the analysis thereof to be kept at a minimum (Cooper 1995c).

This project utilized the advantages of the well documented macro-patch as well as the classical approach of quantal analysis (Katz and Miledi 1967, 1968) to investigate identified sites of synaptic transmission, specifically the low output stem region of the crayfish tonic neuron. Not only does this preparation offer the

ability to record quantal events directly at the release sites and to give insight to vesicle parameters such as latency and frequency, but also rise time, decay time, peak amplitudes and area of individual evoked events which gives multiple variables in which various stochastic methods are able to be used to examine quantal groupings of individual synaptic currents. Otherwise cable properties of potential decay need to be taken into consideration such as dendrites to a soma as in vertebrate CNS preparations.

Determination of Quantal Parameters:

The most direct technique to assess quantal content is to directly count the number of quanta per nerve impulse. This is feasible at the crayfish opener preparation at low frequency paradigms (1~3Hz). Once analyzed, it may be used to estimate binomial parameters for transmitter released (Johnson and Wernig, 1971; Wernig, 1972; Viele et al., 2003). In other preparations it is difficult or impossible to decipher all the quantal units caused by a nerve impulse, unless physiological changes are made to the preparation such as in the frog neuromuscular junction where the $[Mg^{2+}]_o$ is increased to reduce the number of multiple quantal units to keep below threshold of inducing an action potential in the muscle. This approach has the disadvantage in that the preparation is removed from normal physiological parameters.

Various statistical measures and derived parameters were used to quantify synaptic efficacy in novel ways for these crayfish terminals. One approach is to cluster the various quantal characteristics and determine if there are populations of quantal units. Initially the peak amplitude and the area of depolarization were

used to examine for quantal clustering by a mixed modeling (Viele et al., 2003). Basically the analysis examined for clusters in quantal characteristics. With this approach the change in the occurrence within a defined population or if a new population appeared was used for indexing the stimulus paradigm. In this past study n and p could be estimated for stimulation frequencies of 1Hz, 2Hz, and 3Hz. The findings showed that as the stimulation frequency increased, new sites are recruited (thus an increase in n) and the probability of release (p) increased for each site. It should be noted that in this past study the recordings were made over the varicosities of motor nerve terminals and with the same type of preparation as used in the current study. More recently, another type of analysis was performed to quantify characteristics of quantal responses under a defined stimulation condition (Viele et al., 2006). The method is called self-modeling regression, known in the statistics literature as SEMOR. This method uses transforms to determine differences in the individual quanta from an average quantal response for indexing quantal fluctuations. Such measures included peak amplitude, latency, area under the curve, and decay time for more powerful statistical examination. Unlike past analyses, this newer method is automated which removes personal biases in determining quantal occurrences (Lancaster et al., 2006). These methods allowed quantal responses recorded at the stem region of the terminals to be analyzed.

Thus, it is of general interest to more accurately study of synaptic transmission in chemical synapses. Understanding how the structure and the

function of nerve terminals relate would provide insight into how the nervous system communicates for all animals.

Methods

General

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

Identification and marking of stem regions

To identify low output stem area of the motor neuron, the vital dye 4-Di-2-Asp (Molecular Probes) was used. This dye highlights the neuron, which allows the focal macro-patch to be placed at a specific site—the stem (Cooper et al., 1995b). Using this approach also allows the specific site to be labeled for thin sectioning (~100-120 nm) by using fluorescent and electron dense beads either by preloading them around the lumen of the patch electrode or after a recording is made to label the site. If the electrode is pre-coated before recording then when the patch electrode is removed, a ring of beads is left behind, marking the

site (Figure 2.1). This method was previously used to rebuild complex synapses at high and low output varicosities to identify the number of active zones between them (Cooper et al., 1995b, 1996b). In some cases, the beads did not always remain on the preparation to the end of embedding, but photographs taken of beads before they were lost were used to identify the area anatomically. For instance, if a site was recorded from in close proximity to an axon bifurcation, then thick sectioning would allow locating that area. Then thin sections could be made at that region.

Physiology

To elicit an evoked response, the excitatory axon was selectively stimulated by placing a branch of the leg nerve (from the merus segment) into a suction electrode connected to a Grass stimulator (Dudel and Kuffler, 1961). The stimulations were provided to the excitatory nerve. The selection of stimulation frequencies depended on the experimental paradigm as stated in the results.

Synaptic field potentials were measured with focal macropatch electrodes to assess presynaptic vesicular events. The varicosities on the living terminals were visualized using the vital fluorescent dye 4-Di-2-ASP (Molecular Probes) (Magrassi et al., 1987; Cooper et al., 1995a). It was demonstrated in an earlier report that 4-Di-2-ASP had no effect on transmission with the concentration (5 μ M) used in this study (Cooper et al., 1995a). The synaptic potentials were obtained using the loose patch technique by lightly placing a 10-20 μ m fire-

Figure 2.1

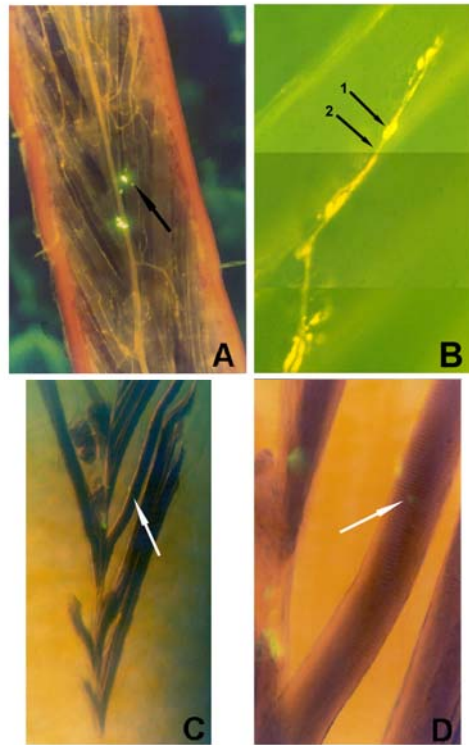


Figure 2.1: Stem preparation: **A:** Opener muscle stained with 4-di-2-Asp. Polystyrene beads present from rim of focal electrode and area specifically recorded (arrow). **B:** High magnification of muscle fiber stained with 4-di-2-Asp showing nerve terminal innervation with varicosities (1) and stem regions between varicosities (2). **C:** Low magnification of dissected, fixed and trimmed opener muscle showing the region recorded from with a focal electrode (white arrow). **D:** High magnification of C, region recorded (white arrow).

field Excitatory Postsynaptic Potentials (fEPSPs) polished glass electrode directly over a spatially isolated varicosity along the nerve terminal. The fEPSPs and field miniature excitatory postsynaptic potentials (fmEPSPs) can readily be recorded (Cooper et al., 1995b, 1996a; del Castillo and Katz, 1954a). Electrical signals were recorded on-line to a Power Mac 9500 or to a Dell Latitude D600 computer via a MacLab/4s or a PowerLab/4s interface respectively.

In addition, to direct quantal variation, their characteristics were measured as described below.

Quantal Analysis

Quantal analysis was carried out with the assistance of a statistical program written in R language (Lancaster et. al, 2006). This program has the ability to collect single event accounting for DC shift and to create a baseline using the tails of traces that have a low occurrence of events, to measure different parameters of quantal events: Area under the event, rise time, peak amplitude, tau decay and latency. When analyzing each run of traces, in some cases the noise was so great the program was not able to delineate parameters needed to find the spike (a relatively large negative potential, a relatively large positive potential and a next local minimum potential) to accurately measure the parameters needed. If this was the case, the location and number of points that corresponded to the spike were removed from all the traces and replaced with an artificial spike with the same number of points, but larger and more prominent.

Statistical Analysis

To test whether there were similarities or differences between the quantal events at each stimulation frequency (1Hz – 3Hz), the well documented ANOVA statistical test was used. A Tukey test followed the ANOVA test to determine if certain groups were similar or different (i.e. specifically 1Hz to 2Hz or 2Hz to 3 Hz, etc.). Since in some cases there was no significant difference between the entire set of points within a particular frequency, sub groups of each frequency were binned into 4 subgroups by dividing the range and subjected to ANOVA and Tukey tests.

Transmission Electron Microscopy (TEM)

All preparations were fixed in a 2.5% gluteraldehyde, 0.5% formaldehyde buffered solution (0.1 M sodium cacodylate, 0.022%wt CaCl₂, 4%wt sucrose, and adjusted to pH of 7.4) for one hour with two changes and post fixed with a 2% osmium tetroxide buffered solution and embedded in Eponate 812. The samples were serially thin sectioned (120nm) on a Reichert Ultracut microtome and post stained with uranyl acetate and lead citrate. Sections were then viewed on a Philips FEI Tecnai, Bio Twin 12 model transmission electron microscope at 80 or 100 kV.

Preparation of Formvar Coated Grids

In order to ensure that grid bars from commonly used EM grids would not interfere with the site of interest, slotted nickel grids (1X2 mm), were coated with 1% formvar (Electron Microscopy Sciences, EMS). These grids were used to

capture sections prior to coating. Formvar dissolved in Di-ethylene chloride was used to coat non-charged, pre-cleaned slides using a film-casting device (EMS). Once coated, the slide was quickly dried using a heat lamp. A razor blade was then used to scrape the edges of the coated slide. Warm/moist air was then blown on the edge of the slide to loosen the formvar from the slide. The slide was then dipped at $\sim 35^\circ$ angle into distilled water, which allows the formvar to completely separate from the slide leaving it floating on the water's surface.

The detached formvar was then collected using a domino rack (EMS), a plastic coated platform with holes that are slightly larger than an EM grid (3mm in diameter). This allows the holes to be covered by the formvar. The domino racks were then slowly dried at room temperature in clean glass Petrie dishes over night.

The resin block with the embedded tissue was then trimmed to a block face of $\sim 0.25 \text{ mm}^2$, cut with a diatome (3mm diamond knife) at a 6° cutting angle. Serial sections were then collected in three section increments. These sections were then collected from the diamond knife boat with a slotted grid (held in the slot by water cohesion) held by forceps and gently placed on the domino rack coated with formvar. Once a domino was full, it was placed back into the Petrie dish and placed in an oven ($\sim 37^\circ$) to evaporate any remaining water and to promote the formvar to adhere to the nickel grid.

Once dry, grids (with sections) were removed from the domino rack using a 2.5mm acu-punch biopsy tool (Acuderm Inc.). The grids were then placed in a

Hiroki staining flat and stained in a Hiroki staining dish (EMS) using the staining procedure described in the transmission electron microscopy methods.

Measurements for synapses and AZ distribution.

Each electron micrograph that had a synapse was cataloged. In some cases complete serial sections of synapses were obtained. The presynaptic terminals were classified as excitatory by the shape of the vesicles as well as if presynaptic inhibition was present. Inhibitory terminals reveal oblique shaped vesicles (Tse et al., 1991) and were not further used for quantification. The appearance of dense bodies associated with synapses within the presynaptic terminal was used to define an AZ. Within the crayfish NMJ, AZs are places where vesicles cluster in association to fusion with the presynaptic membrane (Cooper et al., 1996b). These dense bodies are thought to serve as cytoskeleton attachment points to deliver tethered synaptic vesicles possible from reserve pools. The presence of presynaptic inhibition was evident in two of the three reconstructed stems. Presynaptic inhibition is a phenomenon in which the inhibitory motor neuron has synapses on excitatory neurons; thus, a neuron synapsing onto another neuron, with an AZ present, would indicated it as the inhibitor (Figure 2.2), since no presynaptic excitation from the exciter to the inhibitor has ever been established.

Synapses at the crayfish NMJ do not have a grid of AZ on the synapses but show synaptic variation such that some synapses may only possess a single

Figure 2.2

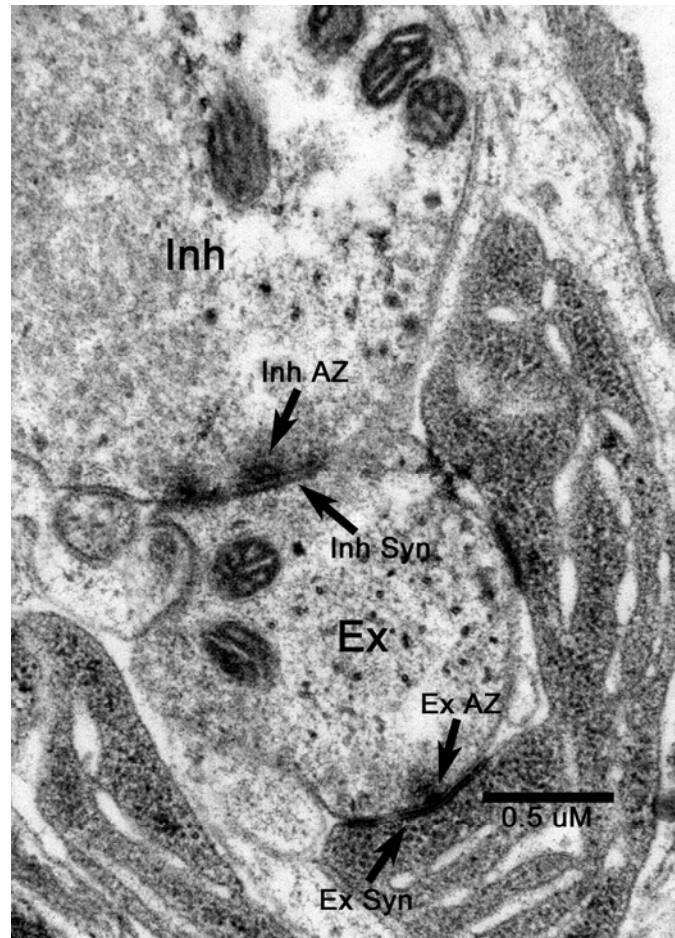


Figure 2.2: Identification of the excitator stem for 3D reconstruction (section from stem 2). Electron micrograph of the excitator stem (Ex) innervated by the inhibitor neuron (Inh) showing presynaptic inhibition. The inhibitor synapse (Inh Syn) reveals an AZ (inh AZ) as well as the exciter synapse (Ex Syn and Ex AZ). Scale bar = 0.5μM.

AZ while others might have multiple AZs at varying distances from one another (Cooper et al., 1996b). In the crayfish opener preparation, the dense bodies of the excitatory terminals are viewed as hemispheres of about 60nm in diameter sitting with the cross-section of a hemisphere facing the synapse (Atwood and Cooper, 1994a). This is documented by the occasional on face view in parallel sectioning of synapses (Cooper et al., 1996b). Thus, in 100-120nm cross-section thickness a dense body can be contained within a single section. If the dense body is seen within two sections then sections either side of these sections were also used for analysis. Care for stereological errors in measuring objects in TEM from 2D images of 3D tissue were implemented as previously described (Atwood and Cooper, 1996a; Feuerverger et al., 2000; Kim et al., 2000).

RESULTS

Quantal measures & characteristics

Five opener stem preparations were recorded for quantal analysis, three of which were rebuilt through electron microscopy. The macropatch technique was used to record quantal events from the stem region between two varicosities of the exciter neuron. To study quantal variation using such a low output region of innervation a low frequency stimulation paradigm was used. Each stem was recorded for 1998 runs at 1Hz, 2Hz and 3Hz, which gives rise to different responses from the recording. Figure 2.3 shows what types of responses can be seen during a typical recording. Each trace has two things in common, one an artifact (arrow one), due to the stimulus traveling through the saline bath and two,

a spike (arrow two) which represents the field response of the presynaptic action potential (i.e. spike) recorded under the patch electrode traveling down the neuron. After the spike, the majority of trial runs show a failure (A), but some traces show single (B) or multiple events (C). Since the interest is in characteristic shapes of single events for this study, these multiple events were not analyzed, but are included in the mean quantal content. Another phenomenon that may occur is spontaneous events known as mini's (Fig 2.3 D). These are events that occur randomly and are not due to the evoked stimuli, these too were not analyzed and not included in the mean quantal content. The single evoked events were analyzed using the R quantal analysis program (see methods). This allowed relatively quick computation of mean quantal content, area of each event, as well as peak amplitude, latency, tau decay and time to peak amplitude (Figure 2.4). Table 2.1 shows the mean quantal content of each stem at 1Hz to 3Hz broken down into single events (demarcated a "1"), multiple events (demarcated as numbers, each representing the number of event during that run), total number of traces analyzed (N) and mean quantal content (m).

It has been shown previously, as stimulation frequency goes up so does the mean quantal content (Viele et al, 2003). This was not the case for the stem recordings, as m was more erratic and random. In some cases m would begin high (1Hz stimulation) descend to lower level (2Hz stimulation) and then raise again (3Hz stimulation). This occurred for both stems 1 and 3. While in stems 2, 4 and 5 m would begin low (1 Hz stimulation), raise (2 Hz stimulation), and then decrease (3 Hz stimulation) again. To determine if there was any

Table 2.1

STEM 1			
	1Hz	2Hz	3Hz
0	1701	1904	1847
1	163	92	124
2	3	2	4
N	1867	1998	1998
<i>m</i>	.091	.048	.066
STEM 2			
	1Hz	2Hz	3Hz
0	1841	1829	1924
1	150	168	72
2	3	1	1
3	2	0	1
N	1996	1998	1998
<i>m</i>	.081	.086	.043
STEM 3			
	1Hz	2Hz	3Hz
0	1865	1946	1936
1	125	52	54
2	4	0	0
3	1	0	0
8	1	0	0
N	1996	1998	1990
<i>m</i>	.072	.026	.027
STEM 4			
	1Hz	2Hz	3Hz
0	1823	1808	1860
1	170	187	129
2	5	3	8
3	0	0	1
N	1998	1998	1998
<i>m</i>	.090	.097	.074
STEM 5			
	1Hz	2Hz	3Hz
0	1866	1859	1859
1	85	117	127
2	14	18	3
3	2	3	5
4	0	1	3
5	1	0	0
N	1968	1998	1997
<i>m</i>	.063	.088	.080

Table 2.1. *Quantal analysis of the five stems that were recorded. Each stem was analyzed using the automated firing program to analyze single events (1's). The raw data was also visually inspected to find all multiple firings as well (doublets, triplets, etc.) so mean quantal content could be assessed. The first column states the number of discrete events (0, failures; 1, single events; 2 double events; etc..) that occurred per stimulus trial. The remaining columns state the observed number of occurrences during each frequency (1-3Hz), 1998 trial for each frequency. **Row n:** Because some traces are problematic to analyze, for a multitude of reasons, they must be thrown out (not counted), N represents the number of useable traces for each frequency. The mean quantal content (m) for each condition is shown for each of the total number of trials.*

Figure 2.3

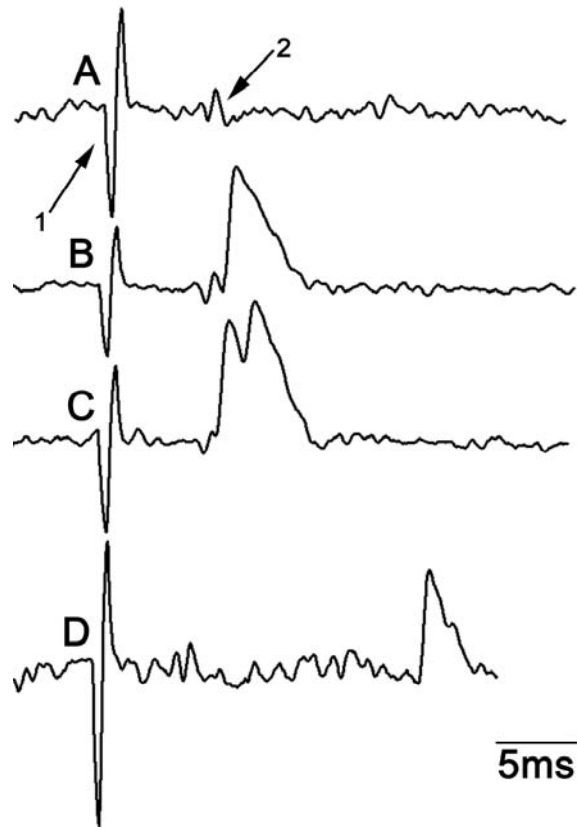


Figure 2.3: Quantal Analysis of Stems: **A:** Trace representing a failure, recorded as 0, not analyzed. 1: Artifact. 2: Spike. **B:** Trace representing a single evoked event (occurring directly after the spike), recorded as a single event **C:** Trace representing a multiple event (in this case a doublet), recorded as two events. **D:** Trace representing a spontaneous event, but a failure in evoking an event, not analyzed.

Figure 2.4

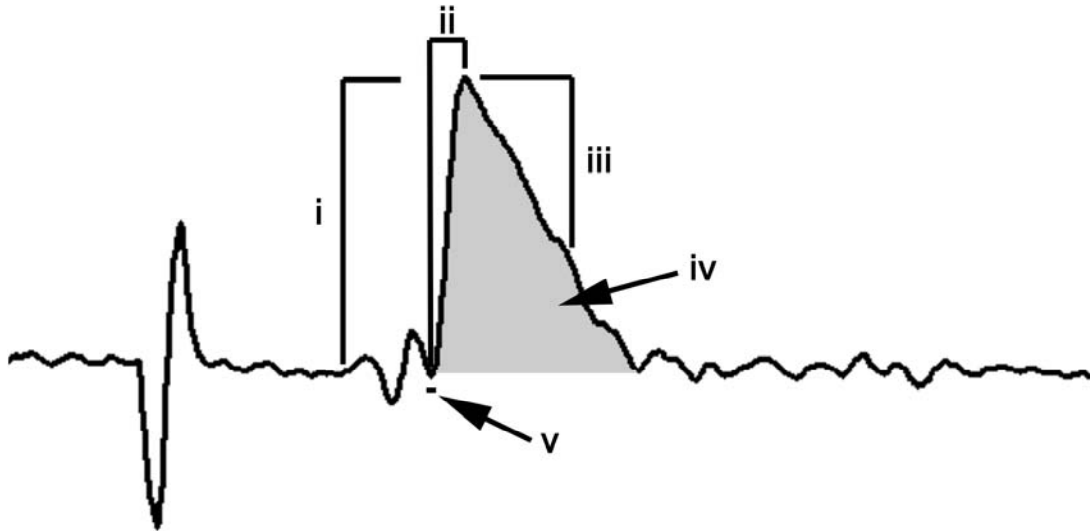


Figure 2.4: Parameters that are measured from single quantal events. **i:** Peak amplitude. **ii:** Time to reach peak amplitude. **iii:** Tau decay time. **iv:** (grey) Area of the event. **v:** Latency from the spike.

Figure 2.5A

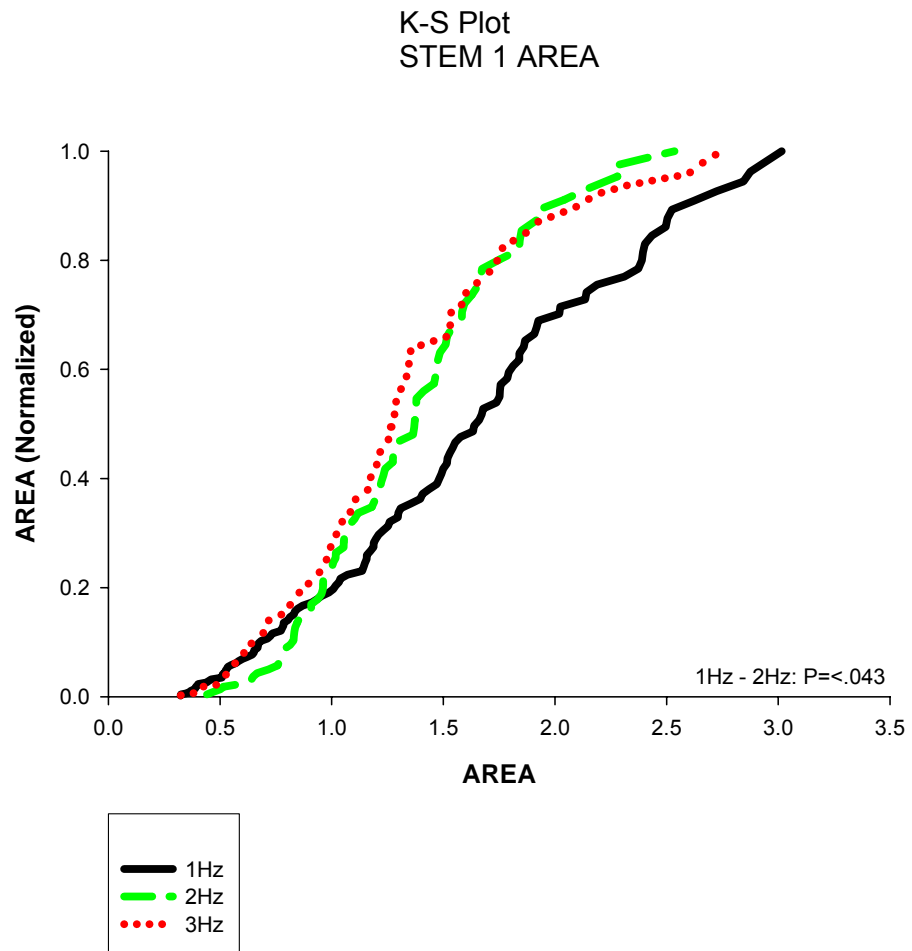


Figure 2.5B

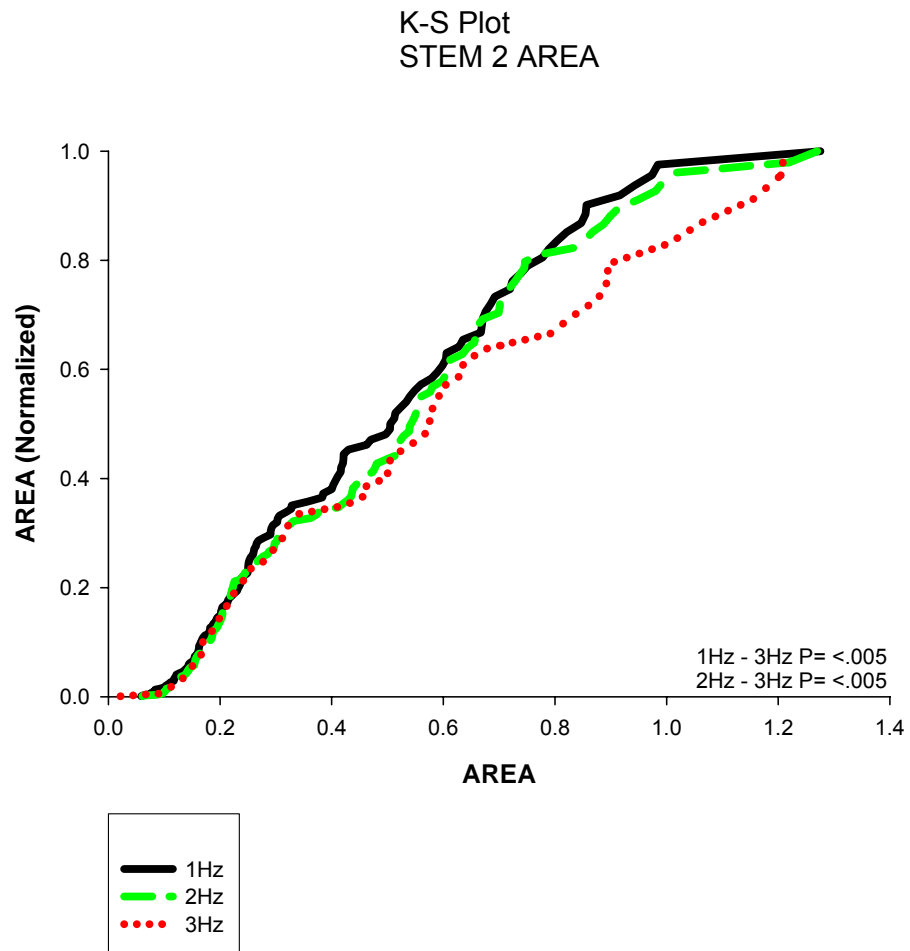


Figure 2.5C

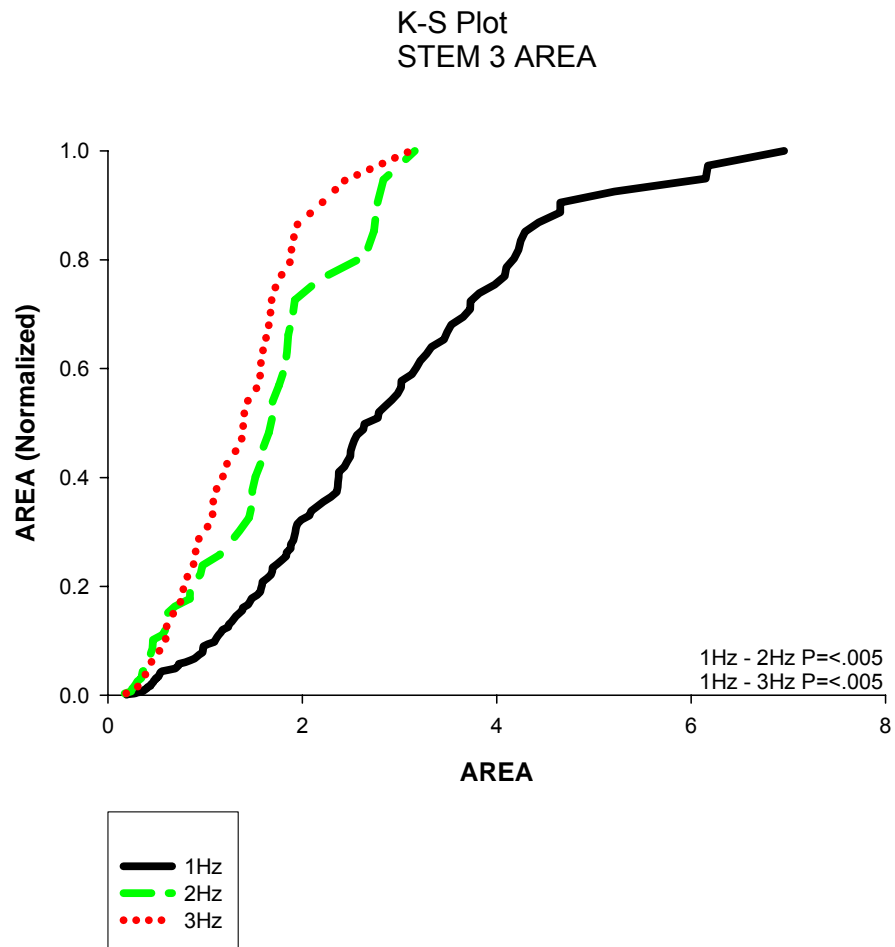


Figure 2.5D

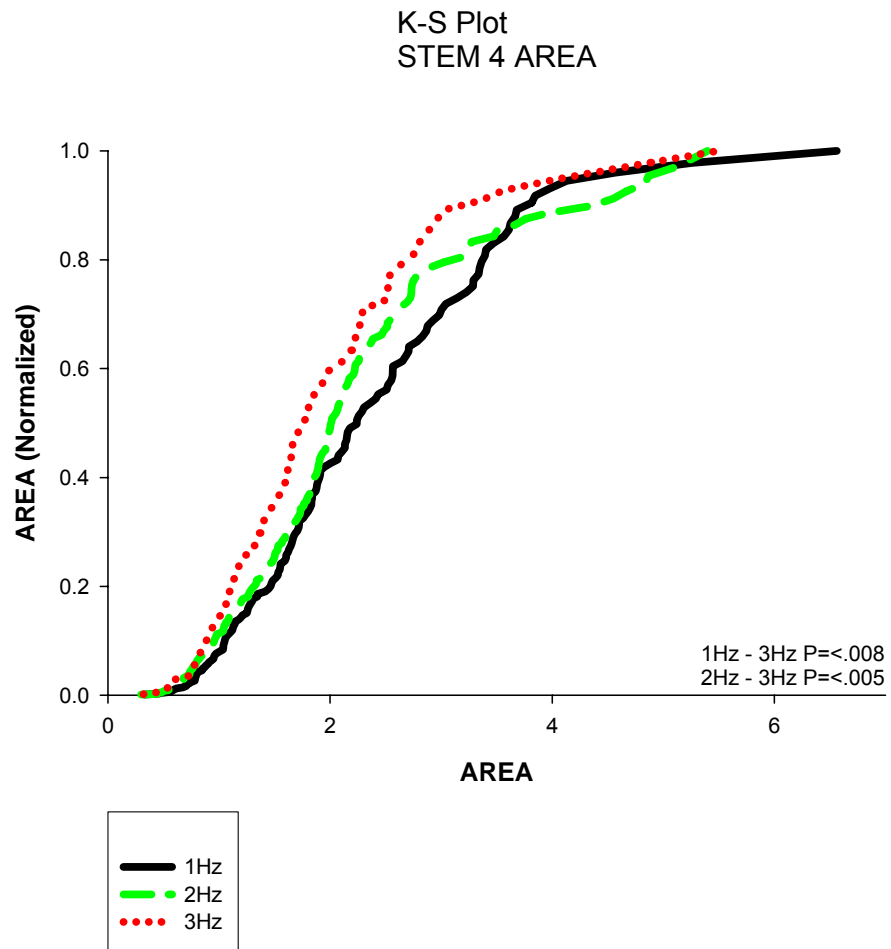
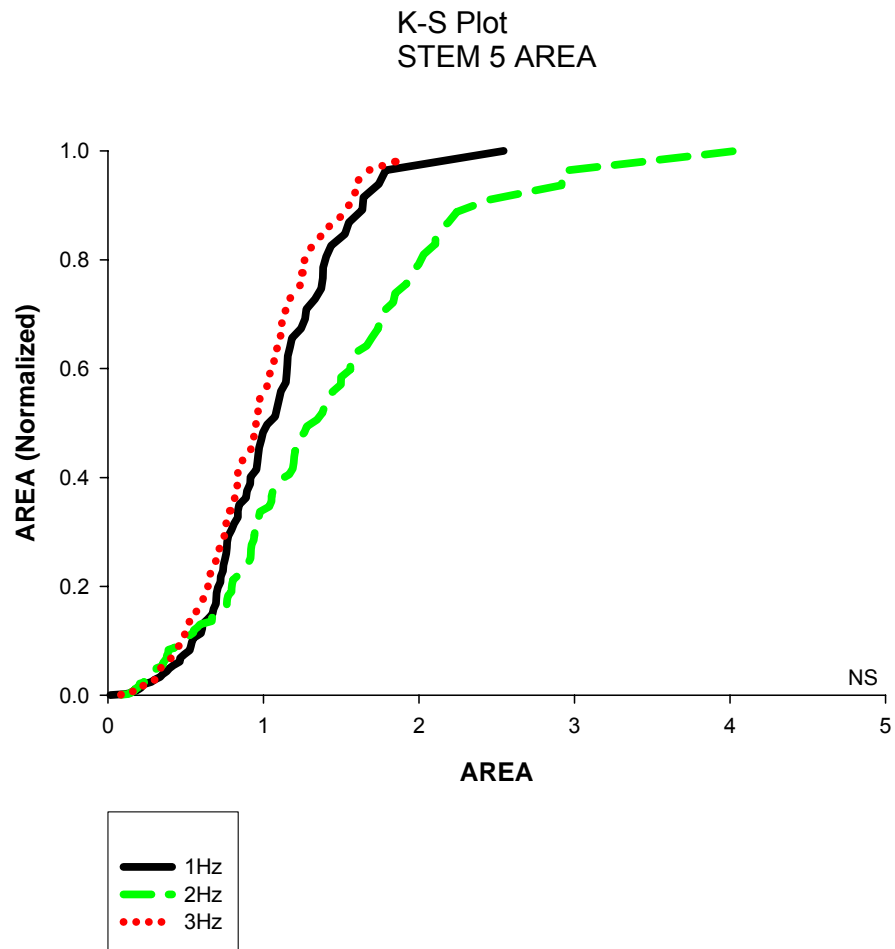


Figure 2.5E



Figures 2.5 A–E: K-S results or Stems. Each stem was subjected to a K-S test, in which each stem area was rank summed, and normalized to one. This was compared to actual area (x-axis). Significance between groups (1Hz – 2 Hz, 1Hz – 3Hz, 2Hz – 3Hz) is labeled at the bottom right hand corner of each graph. If there was no significance, it is labeled as NS.

significant difference between groups, each stem's area was subjected to a Kolmogorov-Smirnov test (Figure 2.5 A-E). This test attempts to determine if two sets of data differ significantly, with the assumption that there is no difference in distribution of the data. This is done by calculating a D value that indicates the most significant difference between the groups. In the stems tested significant differences were found between most of the groups from each stem.

To determine if quantal groupings were related to stimulation frequency, the occurrences within the distribution as a whole for the quantal area measure were examined with a Kolmogorov-Smirnov test. To examine subsets of the distributions an ANOVA test was used for each of the measured quantal parameters. Figures 2.6 A-E shows area along with statistical significance at the bottom right hand corner of each respective graph. All the stems, except stem 5, showed at least one significant difference between groups. Stem one showed a significant difference between the 1Hz – 2 Hz group ($P=0.043$), Stem two showed significance between the 1Hz – 3Hz ($P<0.005$) and 2Hz – 3Hz ($P<0.005$) groups. Stem three, showed significance between the 1Hz – 2Hz ($P=0.005$) and 1Hz – 3Hz ($P<0.005$) groups. Stem four showed significance between the 1Hz – 3Hz ($P<0.008$) and 2Hz to 3Hz ($P<0.005$) groups. Stem five showed no significant difference between any of the groups. Although there was no constant significant trend between each individual stem, there were differences. Thus, it was of interest to indicate if there were differences within those groups for each parameter (area, peak, time to peak, latency and tau). Therefore, each stem was subjected to ANOVA and Tukey's test. The distributions were

Figure 2.6A

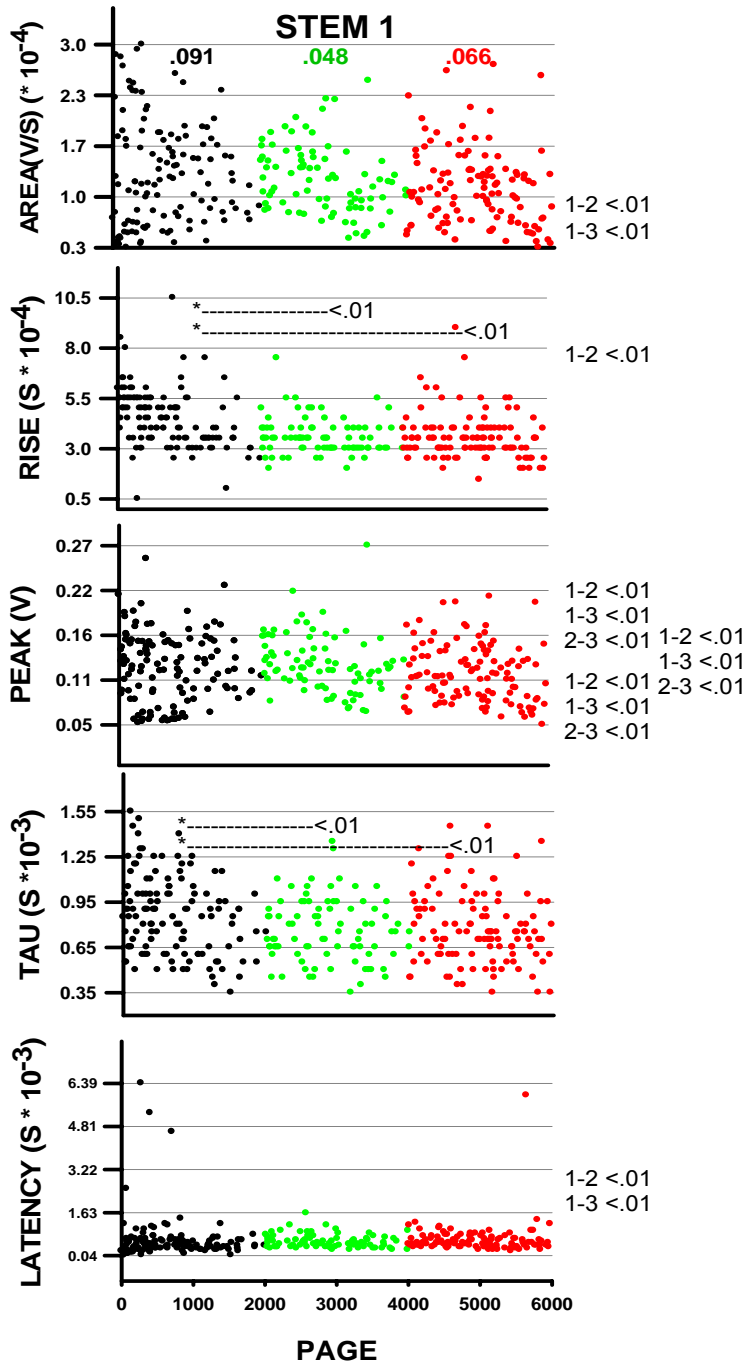


Figure 2.6B

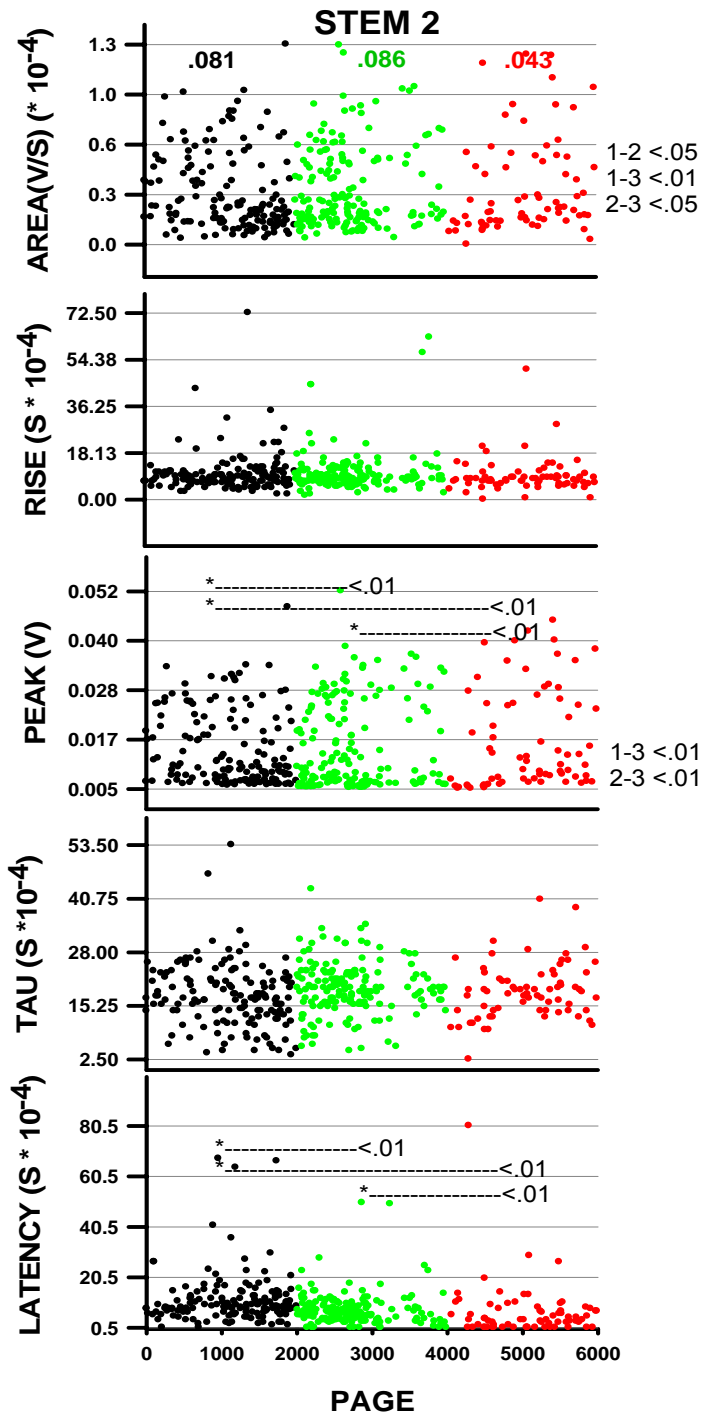


Figure 2.6C

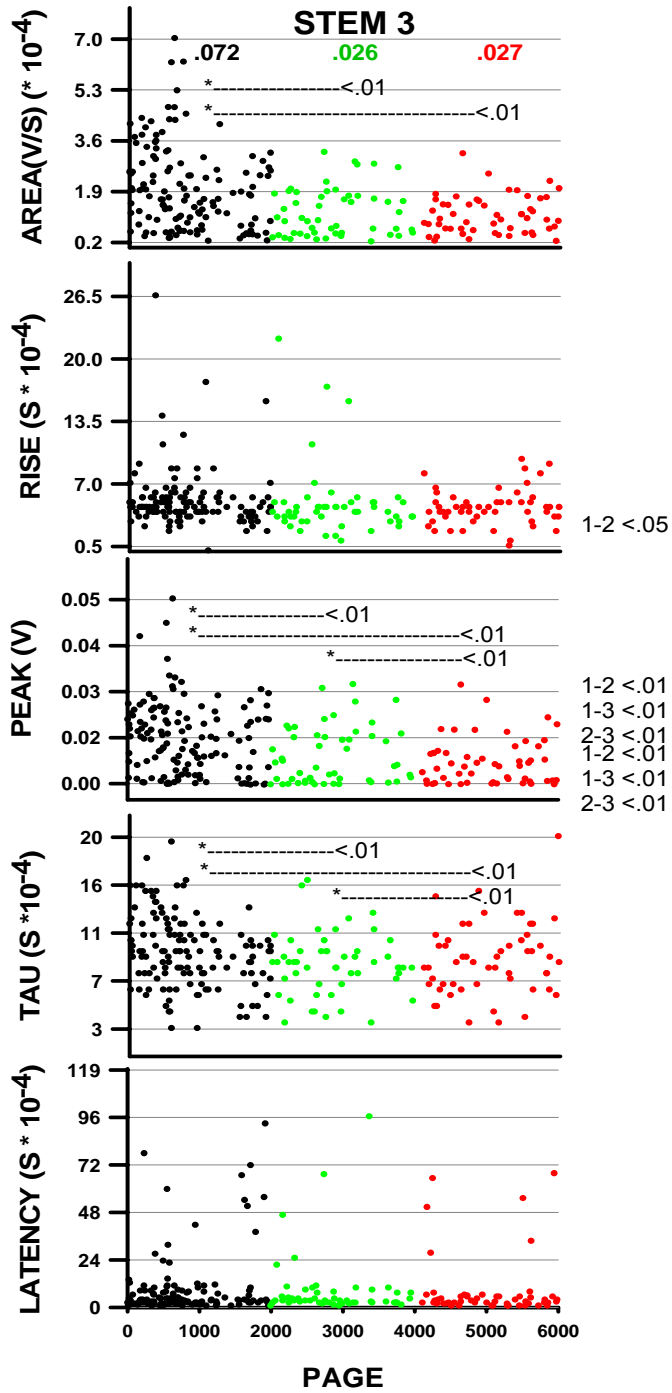


Figure 2.6D

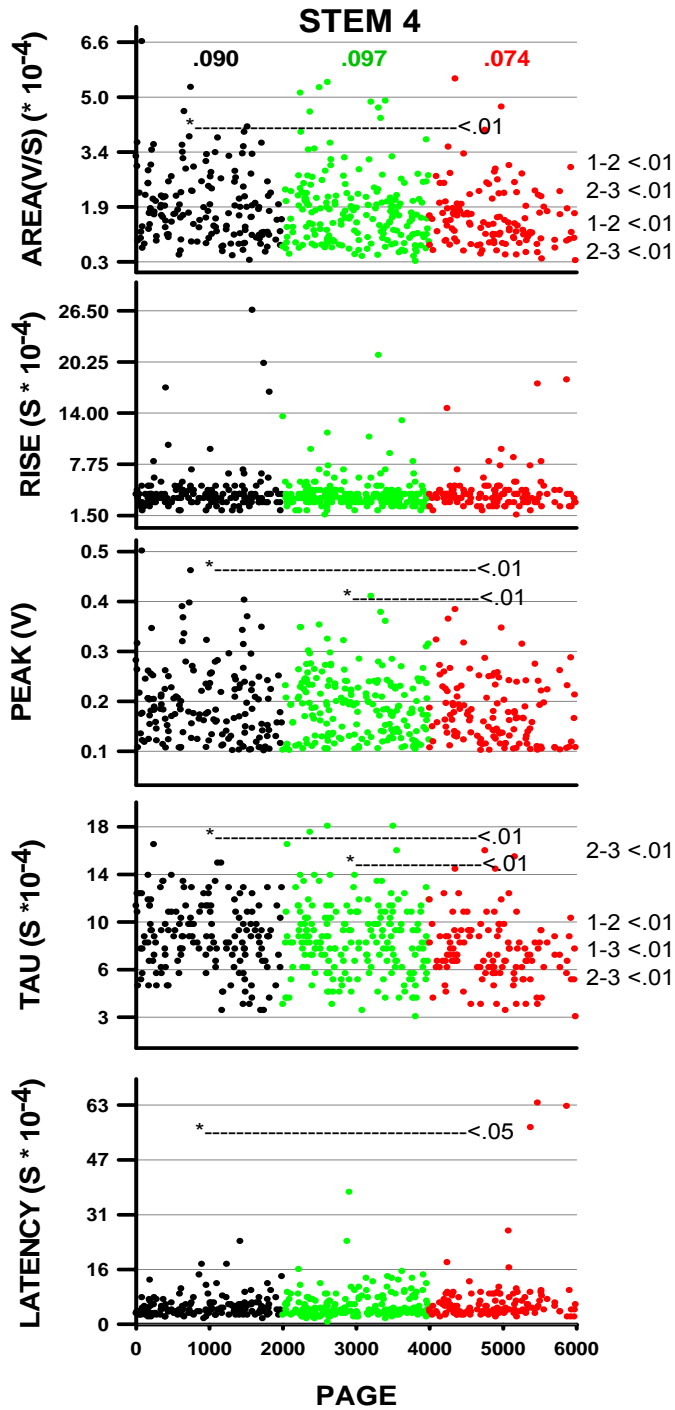
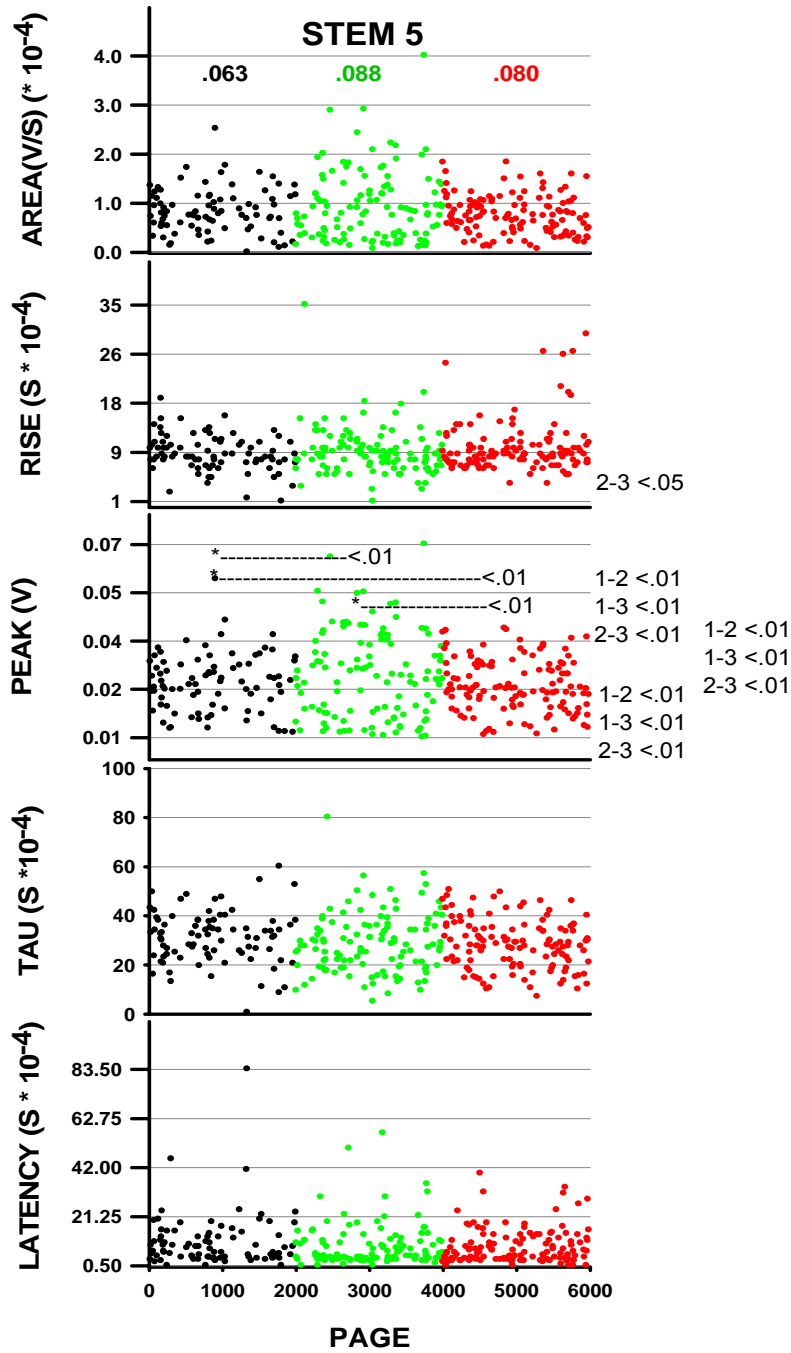


Figure 2.6E



Figures 2.6 A - E: Graphical and statistical analysis of quantal characteristics for each stem that was recorded. For each stem, the area, rise, peak, decay and latency were calculated using the automated R program. In each separate graph of individual stems, the data for each frequency is shown as a scatter plot, **1Hz: black, 2Hz: Green, 3Hz: Red.** **Mean quantal content** for each frequency is indicated in the same color as its respective scatter plot. **Y-axis grid lines** indicate the range of the four bins that were used to analyze each frequency group. Each frequency's variance, as well as the individual bins were analyzed by ANOVA statistical analysis to test for significant differences between the three groups, and then analyzed by Tukey, a multiple comparison test, to test for significant differences within those three groups. Tukey values for whole groups (i.e. all of 1 Hz to all of 2 Hz), if significant, are labeled at the top of each graph. The asterisks represent the first group being compared with the second group, indicated by the end of the dashed in which the significance is stated. Tukey values for individual bins, if significant, are stated to the right of each graph and respectable bin. The numbers indicate which groups are significant (i.e. 1-2 to the right of bin 1 would indicate groups 1 Hz, bin 1 to 2 Hz, bin 1). Note that not all groups or bins were significant or testable, thus not all graphs have labeled values.

compared for each of the four subsets within the range to test for differences within each subset due to stimulation. The four subsets within the range were chose for each quantal parameter by dividing the full range into four evenly spaced subsets.

The results of the ANOVA test and Tukey's test for the 1 Hz data is shown in black, the 2 Hz in green and the 3 Hz in red. The *m* is shown in the same respective color above each group in the top "area" graph. ANOVA statistical analysis was run on the three stimulation frequency groups to test for significance between them then followed by a post hoc Tukeys test to determine significance between individual groups (i.e. 1Hz to 2Hz, 1Hz to 3Hz, etc.). If the results between the frequency groups were significantly different, a recorded at the top of the graph for each parameter (area, rise, peak, tau and latency) is shown with the asterisks representing the first group connected to a series of dashes that end at the next group to which it was compared. Stem one was significantly different in rise time between the 1Hz – 2Hz groups ($P < 0.01$) and 1 Hz – 3Hz ($P < 0.01$) groups, as was the same for the Tau groups ($P < 0.01$). Stem two showed a significant difference between peak amplitudes and latencies between all three stimulation groups ($P < 0.01$). Stem 3 had a difference in area between 1Hz – 2Hz ($P < 0.01$) and 1Hz – 3Hz ($P < 0.01$) groups as well in the peak amplitudes and tau decays ($P < 0.01$). Stem 4 had significant differences in areas between the 1Hz – 3Hz ($P < 0.01$), in peak amplitudes 1Hz – 3 Hz and 2Hz – 3Hz ($P < 0.01$), in Tau decays 1Hz – 3Hz and 2Hz – 3Hz ($P < 0.01$), and in latencies,

1Hz – 3Hz ($P < 0.05$). Finally, stem five showed only differences in the peak amplitudes, but in all three stimulation frequency groups ($P < 0.01$).

Structural analysis

The next part of this study was to rebuild the recorded region to reveal the actual number of synapses, AZ's as well as measure synaptic area and distance between AZs. Figure 2.7 shows a 3-dimensional representation of stem 1 rebuilt from electron micrographs. Each color represents a different synapse and each synapse is represented 2-dimensionally to the sides of the stem. The lines represent the synaptic midlines from the sections while the ovals represent relative AZ location, while perforated represent sections that were unable to be salvaged.

When considering the synaptic area, measurement of error was considered since stereological issues are relevant. A 3-D object represented in 2-D planes presents special requirements to deal with potential error in making measurements. When dealing with the synaptic ends via TEM, the last section portrays a blunt end, while a synapse from sections obtained in parallel to the plane are known to be curved on their edges. The process of correcting the error measures are shown in Figure 2.8 and accounted for in Table 2.2. In addition if a section was lost that occurred at the end of a synapse, the last section with a synapse present was treated as the edge of the synapse.

Not only was synaptic area error calculated for but also AZ distance error. Because the section is ~100-120nm in thickness, and an AZ is 60 nm in

Table 2.2

Stem	n Sections	% Capture	Syn	Syn Area	Syn Area (um2) w/ Err	Diff	AZ	AZ Dis (u^2)	
1	75	83	1	14.67	14.76	0.09	0		
			2	8.21	8.4	0.19	1		
								2	0.578
			3	6.55	6.84	0.29	1		
								2	0.549
			4	4.03	4.104	0.07	1		
								2	4.104
			5	11.36	11.49	0.13	1		
								2	0.602
			2	72	78	1	3.48	3.64	0.16
							2	0.485	
2	0.019	0.0428				0.02	0		
3	0.918	0.983				0.06	1		
								2	1.281
4	4.53	4.64				0.11	1		
								2	0.362
								3	0.632
5	0.0263	0.0736				0.05	1		
6	7.61	7.726				0.12	1		
7	7.87	7.96	0.09	1					
8	2.34	2.379	0.04	1					
9	2.31	2.379	0.07	1					
					2	0.399			
3	106	70	1	5.22	5.88	0.66	0		
			2	8.68	9.19	0.51	1		
								2	0.6
								3	0.73
			3	0.752	0.8518	0.1			
4	1.154	1.295	0.14						

TABLE 2.2: *Measurements made from stem reconstructions. **Stem:** indicates which stem is being measured. **N Sections:** total number of thin sections made to reconstruct stem region. **%Capture:** percent of sections that were able to be saved and analyzed. **Syn:** Number of synapses on each reconstructed stem. **Syn Area:** The area (μm^2) each of the synapses cover on the reconstructed stem. **Syn Area w/ Err:** The possible total area of the synapse, taking into account the error of the section thickness (μm^2) (Fig 2.5). **Diff:** The difference in observed area and area accounting for error. **AZ:** The number of active zones per synapse, if present. **AZ Dis:** The observed distance between active zones on each synapse (+/- 60nm error).*

Figure 2.7

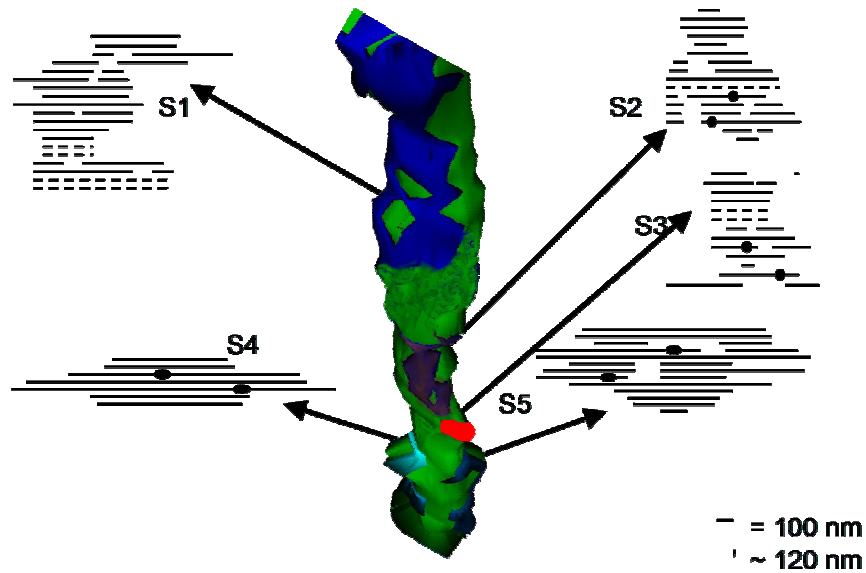


Figure 2.7. Reconstruction of Stem 1 from electron micrographs. Five synapses were found on STEM 1 (S1 – S5), each represented by a different color as well as two-dimensional representation (line diagrams). Each line represents the synaptic midline for that section. Active zone location is indicated by an oval for each synapse.

Figure 2.8

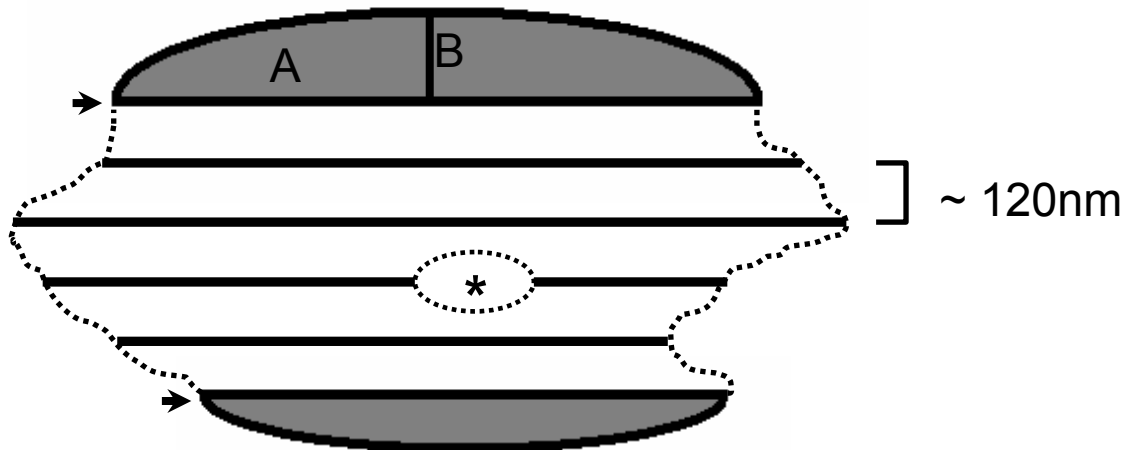


Fig. 2.8: Diagram depicting synapse (with no AZ's) with parameters used to measure synaptic area. **Arrowheads:** representing the first and last sections of the synapse. **Area in gray:** Error measurement for the edge of the synapse using the ellipse formula divided by 2: $((\pi \{ \pi \} * A * B) / 2)$. **Dotted line:** Hypothetical edges of synapse. **Asterisk:** Hypothetical perforation contained within one section. **Bracket:** measurement between the edges of two adjacent sections, ~120nm.

diameter, it is possible for an AZ to be located within any part of the section thickness, thus there is a degree of error to be accounted for with consideration for section thickness (Figure 2.9). The maximum distance and minimum distance was calculated and included in the overall analysis of AZ distance (Table 2.2).

Table 2.2 shows all the calculations of the rebuilt stems in which electrophysiological recordings were made. In the table, the stem number is listed in the first column. Each stem was not completely rebuilt because some sections are lost during thin sectioning. The number of total sections accounted for are listed in the second column, while the third column gives the percentage of those sections that were actually accounted for and analyzed. Synapses are numbered for each stem and the synaptic area is reported with the error in the proceeding columns. The difference is calculated in the following column. Each stem had a range of synapses (1-9) and had a range of AZ's (0 -14) in which the distance error between them were accounted for in the remaining columns. The largest synaptic area recorded was 2.379 um^2 (including error) while the smallest was recorded was 0.0428 um^2 (including error), these two extremes were also found within the same stems suggesting uniqueness in individual stems. The distance between AZ's varied between stems. For stem 1, the minimum AZ distance was 0.6 um^2 and maximum was 0.73 um^2 . For stem 2, the minimum distance was 0.549 um^2 , and minimum was 4.104 um^2 . Stem 3, minimum 0.399 um^2 to a maximum of 1.281 um^2 .

DISCUSSION

Varicosity and stem comparison

Figure 2.9:

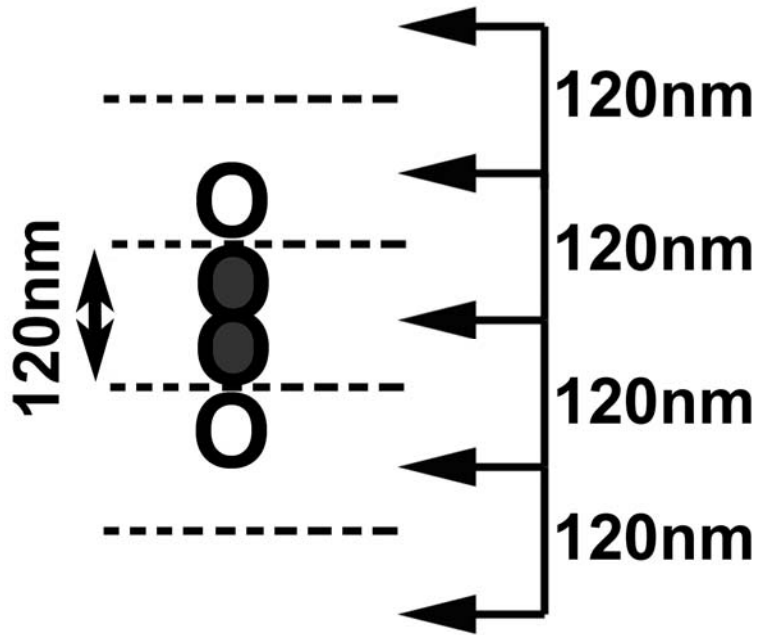


Fig. 2.9: Diagram depicting synapse made of 5 sections (arrows) with 2 AZ's (gray circles) showing the potential range the AZ may be located in a ~120nm section (empty circles). The minimum distance is 0nm, while the maximum distance is 120nm (closest AZ edge). Dotted lines represent section midpoints where the center of the AZs are assumed to lie.

In this study it was attempted to correlate structure and function of nerve terminals and to increase the accuracy of quantal analysis, by utilizing a nerve terminal region that has low synaptic efficacy (i.e. the stem region). The stem region is located between varicosities and has been shown by this study and others to have fewer synapses and fewer AZ than the varicosities. Florey and Cahill (1982) reconstructed the nerve terminals in the crayfish *Astacus leptodactylus*, and found the varicosities had about 10 synaptic sites and the stem regions had about ~2-3. While with the crayfish *Procambarus clarkii*, varicosities contained up to 50-60 synapses (Cooper et al., 1995). In this current study it was found that the stem regions contained 4-9 synapses (in a length of 10µm of the stem) and contained anywhere from 0-14 AZs (a synapse with no active zone is known as a blank synapse). This indicates species differences in nerve terminal innervations. The stem regions were specifically used in my study to intentionally reduce the number of release sites (n) to remove confounding variables such as large number of synapses, each of which could contain a multitude of AZ's (Cooper et. al, 1996). Such synaptic complexity causes difficulty in delineating the number of release sites (n) and/or the probability (p) of each release sites from the quantal recordings. Classically, m was calculated by counting the number of observed events, all events being considered equal, over the number of stimulation trials. This study provides direct structural comparisons to physiological responses obtained from defined synaptic regions, while considering the different parameters of each singly evoke event, which greatly enhances our understanding of synaptic physiology.

It had previously been reported that the varicosities on the opener muscle of the crayfish contained up to 50 synapses, each of which could contain 0 to 5 AZ's (Cooper et al., 1996). Between these AZ's the calcium distribution was accounted for in which AZ's $\leq 200\text{nm}$ was modeled to possibly influence each others' probability of release (Cooper et al, 1996). In varicosities, it was shown that as stimulation frequency increased the mean quantal content also increased. This suggested that new synaptic sites might be recruited as the stimulation frequency increased (Viele et al., 2003), but because of the large number of potential release sites, to make a prediction of which quantal signature came from which synapse was impractical. Thus, the stem region of the neuron was targeted for this study in hopes of fewer synapses with less complexity for characterizing single quantal events.

When analyzing data from the stem region it was found that it does not respond in the same manner as the varicosities of previous studies. The m was consistently erratic and in most cases the parameters measured for each quantal response (area, rise, peak, time to peak and latency), remained mostly consistent, indicating that not much fluctuation was occurring in the quantal responses at differing stimulation frequencies (1Hz-3Hz). The five preparations in which the quantal parameter correlations were made to structure is suggestive that the number of synapses is not related to possible quantal signatures. The possible variables that give rise to quantal variation may be too confounding to determine possible quantal signatures for a particular AZ or synapse even in this synaptically reduced preparation.

The stem region functions synaptically different from the varicosity in relation to m and correlation to stimulation frequency. Why this region responds differently is not known. The first obvious difference between the varicosity and the stem region is size. Varicosities can be up to 5 μm in diameter, while the stem region ranges from 0.5 μm to 1.5 μm . Just this size difference may alter the way the neuron handles calcium. Because calcium is buffered in multiple ways in varicosities such as ER, mitochondria, buffering proteins, and removal methods (PCMAS and NCX) (Scheuss et al, 2006), the calcium may be removed quickly so as to not affect neighboring synapses and AZ's. But what is the distribution of these systems in the stem region, a region of low innervation that most likely does not play a major role for the excitation of the muscle? In the stem region, event variability does not seem to increase as drastically with low stimulation (1Hz-3Hz) as it does in the varicose region, which could be partly due to a difference in calcium handling (Viele et al., 2003). If the abundance of these calcium handling systems are fewer in number in this region then calcium would be allowed to build up (Atwood et al., 1997 Review). In such a small volume, such as in the stem region, it may be possible that calcium affects each site equally, thus recruitment of new synapses or AZ's would be masked, regardless of stimulation frequency. This could be tested by using calcium indicators such as fura-2 and the use of confocal microscopy (Tang et. al., 2000; Cooper et al., 1995a). If calcium sparks stayed persistent in the stem region while it remained dynamic in the varicosity, it would suggest that calcium may be a major factor in how the stem's synaptic regions react to calcium influx.

As previously shown in the opener motor neuron the amount of calcium in the nerve terminal increases with stimulation frequency (Cooper et al., 1995a), this is one of the bases of synaptic plasticity. Sodium also increases with stimulation frequency (Winslow et al., 2002), which has been shown to have an effect, but not directly in LTF (Atwood and Tse, 1988). Thus, another factor that may come into play in the response of the stem to increased frequency are the likely higher $[Ca^{2+}]_i$ and $[Na^+]_i$ during stimulation and potentially differential decay of the response as compared to the increased volume of the varicosity.

An additional observation from the data recorded from the stem is that in some runs, the mean quantal content decreased overall from 1Hz to 3 Hz (stems 1, 2 and 4). Because this is at low frequency stimulation it is possible that the stem region is more susceptible to low frequency depression (LFD) than the varicose region (Takeda and Kennedy, 1965). This is a phenomenon, in which the neuron depresses in response to very low frequencies (0.0016 Hz-0.2Hz), much lower than this study, but cannot be ruled out since this study was done at a region other than the varicosity and has not previously been investigated for LFD. The overall mechanism of LFD is unknown, but is recognized to be a presynaptic phenomenon and phosphorylation-dependent, as shown through kinase inhibition (Silverman-Gavrila et al., 2005). If this is the case, then second messenger cascades must be considered to be involved. Considering that such activation of second-messengers could have a greater impact in the stem with a smaller reserve pool of vesicles as compared to the varicosity might be worth future consideration.

Synapses in the stem were observed without AZ's, albeit there were missing sections, suggesting that silent synapses may be present at the stem region of the neuron. Silent synapses lack AZ's and are thought to be inactive at rest, but as shown in hippocampal neurons, it can be activated if long term synaptic plasticity is induced by repetitive stimulation through actin polymerization (Yao et al, 2006). Thus, they play a role in synaptic plasticity. Since it is possible they exist at the stem region, they may be a factor in the response recorded from the stem region, but because they were identified as silent even after the increase in the frequency (1Hz – 3Hz), it is possible that they stayed silent and played no role in the stems recorded in the stimulation paradigm used in this study.

Another dynamic to the stem regions is that they are surrounded by much less subsynaptic reticulum. This brings a post synaptic component into play as well for the responsiveness in this region. With a varicosity, the entire surface is surrounded by subsynaptic reticulum with synapses occurring on all regions. For instance, if a varicosity (treated as a sphere) is $5\mu\text{m}$ in diameter the surface area associated with it is $78.5\ \mu\text{m}^2$ ($4\ \pi\ r^2$). While a stem (treated as a cylinder, with a length of $20\mu\text{m}$) the surface area is $31.8\ \mu\text{m}^2$ ($(2\ \pi\ r^2) + (2\ \pi\ r)\ h$). This is less than half the surface area of the varicosity, thus, more synaptic glutamate receptors (quisqualate-type) could be found surrounding the varicosity in the synaptic regions as compared to the stems synaptic regions, giving another possible factor into the differing response from the stimulation. Due to this difference in size means the distribution of glutamate receptors, in synaptic

regions, could easily be different at the stem region as compared to the varicosity. If one were to consider the postsynaptic receptor array being consistent in packing density, the exact location where a vesicle fuses with the synapse affects the amount of transmitter received by a specific area of the postsynaptic receptor array. Suppose the vesicle fuses on an edge of the presynaptic synaptic surface (Uteshev and Pennefather, 1997), the result would be a different size or shape of a recorded postsynaptic current (due to the entire quantal package not being deposited over the receptors) compared to a vesicle that released directly over the center of the array. In addition, one could also consider that a single quanta might saturate the postsynaptic array, thus, release from a synaptic edge would alter the quantal shape as would a subsequent central release. An edge release would not have a concentrated release over the receptors and would lose some glutamate due to spillover resulting in a smaller event, while a subsequent vesicular release could result in a blunt peak, due to the glutamate receptors not being completely reset from the first release.

The degree of subsynaptic reticulum could also alter the degree of amplification of the membrane potential due to differences in series resistance. Such a factor has not been addressed previously for regional differences along these types of varicose-stem terminals but has been investigated for a vertebrate NMJ in regards to the enfoldings in the plasma membrane of the muscle and degree of activation of voltage gated ion channels (Martin, 1994).

Synapses were found to have a range of AZ's from 0 to 3 at the stem region, showing that the stem region is still fairly complex and not as simplistic as

originally thought in studying structure and function of synapses in relation to correlating a quantal signature. Although, in all the stems reconstructed, the AZs were not in close enough proximity to imply that their calcium influx domains would impact the neighboring AZ according to previous computational models (Cooper et al, 1996). This alludes to the possibility that when calcium enters the cell in the low frequency stimulation paradigm each AZ acts independent of the others. However the buffering of calcium in the stem region needs to be considered and this has not been addressed specifically for the stem region in the previous computational assessment (Cooper et al, 1996). This leads to the possibility that the calcium build up in the stem region would also mask the calcium cloud distribution between AZ's and possibly whole synapses. Of course, it would have been ideal if a single synapse with a single AZ was obtained in the reconstructions as then it would have been easy to know which synapse the quantal responses had originated. In addition, mechanisms for variations, if present, in the quantal responses would have been able to be addressed with more substantive possibilities.

Since this study was to more accurately estimate n and p from physiological and anatomical data, it is important to discuss what n actually refers to. Some investigators refer to n as an entire varicosity (Korn et al., 1981), while others count each synapse as n , yet some suggest each AZ (Redman, 1990, Faber, 1991). Since in this preparation each AZ can dock up to 6 to 7 vesicles, as seen in freeze fractures as well as TEM sections obtained parallel to the synapse face (Cooper et al., 1995, 1996 a,b; Cooper et al., 1996b) one could

consider each docking site as an n . Thus, a single AZ would have an n of 6-7 but each n in this case would have an equal probability of release so possibly a functional $n=1$ could be assigned to an AZ. However, if a release occurs at one site its probability of a second release would be reduced for a period of time. This would alter the probability of neighboring sites within a given time period. If one considers each docking site as an n , then even a single AZ is complex to study in reference to p considering that the release at one site would be dependent on its own site's history as well as a neighboring site's history. Thus, the assumption that each site is independent would not be valid. This level of statistical investigation has not been tackled in the synaptic field properly. This is likely because of a lack of experimental measurements to address the issue.

Obviously there is a wide range of defining n . In this study, stems were recorded, which are located between varicosities and quantal events are able to be recorded thus, n does not necessarily mean individual varicosities, but could still account for synapses and AZ's. In addition, because a quantal event is the result of a single vesicle being released (Katz and Fatt, 1952), it would be logical to say that n is due to the area a vesicle fuses. Because a synapse can harbor a multitude of AZ's, even in low output terminal areas, such as the stem region, one can not account for which AZ is "active" at a given time, thus we can not define which synapse accounts for each n .

This study has attempted to use a preparation which reduced the number of synapses and AZs in order to examine the structure and function relationship. A goal was to also re-asses defining n and p as well as identifying active

synapses by electrophysiological measures. Through TEM, numbers of synapses and AZ were to be revealed to relate to the characteristic parameters of quantal events. Thus, it might have been possible to analyze a specific quantum signature and relate it to synaptic structure. Analysis from this study, shows that synaptic structure is complex even at a low output sites, such as the stem region of nerve terminal, to draw direct correlations on quantal signatures to vesicle fusion sites. Because even the stem region of the tonic opener neuron is too complex, n cannot be determined (as of yet) from recorded quantal responses. Although, the study has offered insight into analysis of n and p as well as measures of quantal fluctuation in these simpler synaptic preparations, the hunt continues to find still yet preparations with single synapses containing a single AZ to define quantal variation and structural/functional relationships.

Future directions in this research area will be to find a terminal containing fewer synapses, to further reduce the confounding variables found at complex synaptic regions. One preparation might be the European crayfish (*Astacus leptodactylus*) which was studied by Cahill and Florey in 1982. They showed an even more reduced number of synapses than was found in the stem regions of *Procambarus clarkii* (as low as 2-3), which means by sheer chance a recorded site may only contain one synapse and one AZ. This of course comes with a disadvantage. *Procambarus* has been studied as a model organism for synaptic transmission for over 60 years, which makes it a virtual playground for studying and researching the different parameters of synaptic transmission. Unfortunately, the amount of synaptic transmission data on *Astacus* is

considerably less. Nonetheless, this maybe an excellent followup or cross comparison for structure and function assessment.

Another possible future direction would be to study this question *in vitro*. Using neuron cultures, it may be possible to manipulate cultures to have only one synapse/AZ system. Nerve cord explant cultures have been previously prepared from *Procambarus clarkii* in which phasic and tonic motor axons have been stimulated to innervate muscle (Egid and Lnenicka, 1993; Rumpal and Lnenicka, 2003). This would be difficult to follow as each study would need to be serially reconstructed by TEM to ensure synaptic structure. This also has its own disadvantages in that it is removed from physiological parameters, but could possible allude to a synaptic structure and function.

There are additional parameters that need to be accounted for in order to consider structure and function of nerve terminals. The fact that structure and function alone have limitations since many biochemical factors play a role in the overall function of synaptic transmission; thus, synaptic structure alone can not be considered in isolation. It is known that biochemical properties (i.e. second messenger cascades, calcium binding, phosphorylation events etc.) that affect m , in which n is compromised, would impact p at any given site (Dixon and Atwood, 1989; Sudhof, 2004; Xia and Storm, 2005, Yao et al., 2006)

Thus, in conclusion, a study which can account for structural complexity issues and biochemical pathways of synapses is of interest to the field of synaptic transmission.

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Chapter 3

Presynaptic Depression in Phasic Motor Nerve Terminals and Influence of 5-HT on Docked Vesicles

INTRODUCTION

In this study the overall goal was to decipher the mechanisms of presynaptic depression and how it is altered by the neuromodulator 5-HT. This a peripheral project to the main structure/function project that was addressed with the stem region in Chapter two. In Chapter two, simplistic synapses were utilized to automatically reduce the number of release sites so that the quantal theory could be reviewed in a very reductionist approach. In the case of the extensor preparation, the synapses are much more complex, having more active zones and having a much higher output. This preparation also gives the advantage of having the phasic nerve terminal that is easily fatigued, unlike the opener preparation that is only innervated by a fatigue resistant tonic motor neuron.

The phasic nerve innervation of the extensor is also non-spiking, thus producing graded postsynaptic potentials. This has applications to vertebrates in that it is analogous to the dendritic innervation of the central synapses, which also have graded potentials. Because the crayfish NMJs offer analysis of synaptic transmission at a quantal level, it becomes obvious why one would want to study synaptic depression in this type of system. Considering that synaptic depression is thought to play a major role in learning, memory and behavior any further understanding of the mechanisms behind synaptic depression will be useful to a wide variety of issues. Neuromodulation by 5-HT allows easy

manipulation of the system that can be physiological monitored and visually assessed by TEM, that gives insight into what mechanisms are controlling the this presynaptic phenomenon.

This study was a project that started in the late 1990's by an undergraduate (University of KY) at the time, Scott Kellie, to study the post synaptic physiological parameters in the extensor preparations to best work out paradigms to study synaptic depression. I picked this project back up because of my interest in synaptic plasticity and to fine tune my TEM training for the main structure/function project. Acknowledgments should also be given to another undergraduate, Dexter Reener, who did a one month internship in the laboratory as well two high school science teachers (Lexington, KY), Ms. Heidi Anderson and Ms. Berry Hart. The high school teachers were supported by NSF for a research internship for high school science teachers. They helped with extensor preparations and vesicle analysis from TEM micrographs.

METHODS

Animals

Mid-sized crayfish (*Procambarus clarkii*), measuring 6-10 cm in body length were obtained from Atchafalaya Biological Supply Co. (Raceland, LA). Animals were housed in an aquatic facility within the laboratory in individual tanks, and were fed fish food pellets every three days. Only male crayfish in their intermolt stage were used.

Anatomy

The extensor muscle of the first walking leg was prepared by the standard dissection (Bradacs et al., 1997). The tissue was pinned out in a Slygard dish for viewing with a Nikon, Optiphot-2 upright fluorescent microscope using a 40X (0.55 NA) Nikon water immersion objective. Dissected preparations were maintained in crayfish saline, a modified Van Harreveld's solution (205mM NaCl; 5.3mMKCl; 13.5mM CaCl₂ 2H₂O; 2.45mM MgCl₂ 6H₂O; 0.5mM HEPES adjusted to pH 7.4) at 14⁰C.

Physiology

Evoked Post-Synaptic Potentials (EPSPs)

Intracellular muscle recordings were made with a 3M KCl-containing microelectrode placed in a muscle fiber in the opener muscle. A Grass S-88 stimulator and stimulus isolation unit with leads to a standard suction electrode set-up were used to stimulate the phasic excitatory nerve. EPSPs were measured when stimulating at 0.5Hz and 5Hz frequencies. Selective stimulation of the excitatory axon was carried out by using a 'macro-patch' electrode with an inner diameter of 15 to 20 μ m placed directly on the phasic axon (Bradacs et al., 1997). The axon type is easily identified after staining because 4-Di-2-ASP stains the tonic axon more brightly, due to the larger number of mitochondria within it (Atwood and Nguyen, 1995).

After depression, a period of ten minutes was allowed to elapse before repeating the 0.5 Hz stimulation for 5 events followed by a continuous 5Hz stimulation. In some of the preparations, 5-HT (100 nM) was added to the bath

during this time period. In other preparations, 5-HT (100 nM) was applied before the initial depression was induced to examine if the rate of depression is altered by 5-HT. To assess if the properties of the muscle had changed after synaptic depression occurred in the phasic motor neuron, the EPSPs of the tonic motor neuron were monitored within the same muscle fiber.

Responses were recorded with a standard intracellular electrode amplifier (AxoClamp 2A, Axon Instruments). Electrical signals were recorded onto VHS tape and on-line to a Power Mac 9500 via a MacLab/4s interface. EPSPs were recorded at 10 kHz. All events were appropriately scaled to known test pulses applied through the electrode and directly measured on an oscilloscope. The corrected scale was then adjusted with MacLab Scope software (version 3.5.4).

Application of neuromodulators

To apply exogenous compounds to the preparation, the bathing medium was rapidly exchanged with saline containing 5-HT (Sigma). Solutions were made fresh before each experiment from a frozen standard (10 mM in saline).

Transmission Electron Microscopy (TEM)

All preparations were fixed in a 2.5% gluteraldehyde, 0.5% formaldehyde buffered solution (0.1 M sodium cacodylate, 0.022%wt. CaCl_2 , 4%wt. sucrose, and adjusted to pH of 7.4) for one hour with two changes and post fixed with a 2% Osmium Tetroxide buffered solution and embedded in Eponate 812. The samples were serially thin sectioned on a Reichert ultracut E microtome, picked

up on slotted grids that were laid on formvar. The grids were post stained with uranyl acetate and lead citrate. Sections were then viewed on a Phillips FEI Tecnai, Bio Twin 12 model transmission electron microscope at 80kV.

Measurements for vesicle location and distribution.

Each transmission electron micrograph that contained a synapse was cataloged. In some cases complete serial sections of synapses were obtained. The presynaptic terminals were classified as excitatory by the shape of the vesicles. Inhibitory terminals reveal oblique shaped vesicles (Tse et al., 1991) and were not further used for quantification. The appearance of dense bodies associated with synapses within the presynaptic terminal was used to define an active zone (AZ). Within the crayfish NMJ, AZs are areas where vesicles cluster in association to fusion with the presynaptic membrane (Cooper et al., 1996b). These dense bodies are thought to serve as cytoskeleton attachment points to deliver tethered synaptic vesicles possible from reserve pools.

Synapses at the crayfish NMJ do not have a grid of AZ on the synapses but show synaptic variation such that some synapses may only possess a single AZ while others might have multiple AZs at varying distances from one another (Cooper et al., 1996b). We sought to accurately measure docked, RRP and RP by serial sectioning the synapses around single AZs by insuring that the counts were not skewed by vesicles associated by neighboring AZ. Thus, three sections to either side of an AZ of interest were viewed and only sections within one section to either side of the section containing an AZ were used for anatomical

measures of vesicle pools. In the crayfish opener preparation, the dense bodies of the excitatory terminals are viewed as hemispheres of about 60nm in diameter sitting with the cross-section of a hemisphere facing the synapse (Atwood and Cooper, 1994a). This is documented by the occasional on face view in parallel sectioning of synapses (Cooper et al., 1996b). Thus, in 75nm cross-section thickness a dense body can be contained within a single section. If the dense body is seen within two sections then sections either side of these sections were also used for analysis (see results section). Care for stereological errors in measuring objects in TEM from 2-D images of 3-D tissue was implemented as previously described in Chapter 2.

Statistical analysis

Numerical data were represented as a mean with standard error of the mean (SEM). When the basic assumption of parametric Student's-*t* test were valid it was used otherwise, the non-parametric Wilcoxon rank sum test was used.

RESULTS

The phenotype of the extensor muscle fibers has previously been mapped for regional differences from proximal to distal and lateral to medial (Bradacs et al., 1997). It has also been previously established that all the fibers are innervated by both the phasic and tonic nerve terminals. Since the centrally located muscle fibers show pronounced tonic and phasic EPSPs, this region was

used throughout this study to investigate the depression of the phasic terminals and the effect of 5-HT (Figure 3.1). The axons of the phasic or the tonic nerve were selectively stimulated by placing a focal suction electrode on their large axons in the proximal region of the extensor to elicit the EPSPs (Figure 3.1). The EPSPs produced by the tonic nerve are not detectable with a single stimulus, but when a train of pulses are given, to induce short-term facilitation, they become enhanced (Figure 3.1).

The properties of synaptic depression and the influence of 5-HT

When the phasic nerve is stimulated at a low frequency (0.5 Hz) the EPSPs do not facilitate, however at a higher frequency (5 Hz) there is a pronounced facilitation which over time leads to a depressed response. A representative profile in the amplitude of the EPSPs with a stimulation paradigm of an initial 0.5 Hz followed by a 5 Hz for a prolonged duration to induce depression is shown in Figure 3.2. After the responses were fully depressed the stimulation was stopped. At this point in time, the bathing medium was either switched three times with normal saline or one containing 5-HT (100 nM) and left to recover for ten minutes before repeating the stimulation paradigm of 0.5Hz and 5Hz, however only 5 events at 0.5 Hz were recorded before increasing to 5 Hz. To index differences among the treatment groups four time parameters were measured (shown in Figure 3.2). The time from the initiation of the 5Hz stimulation to the peak of the facilitated response (i), time to the onset of decay

Figure 3.1

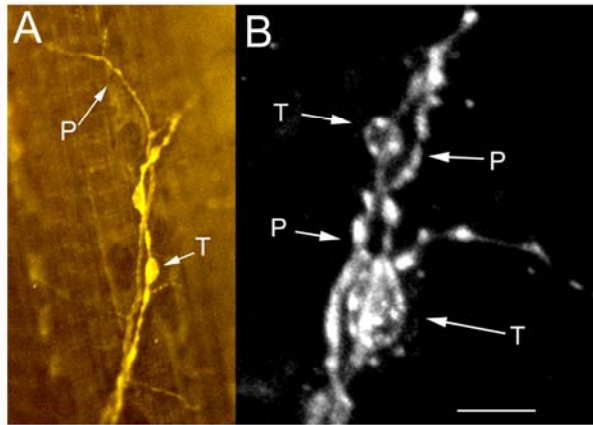
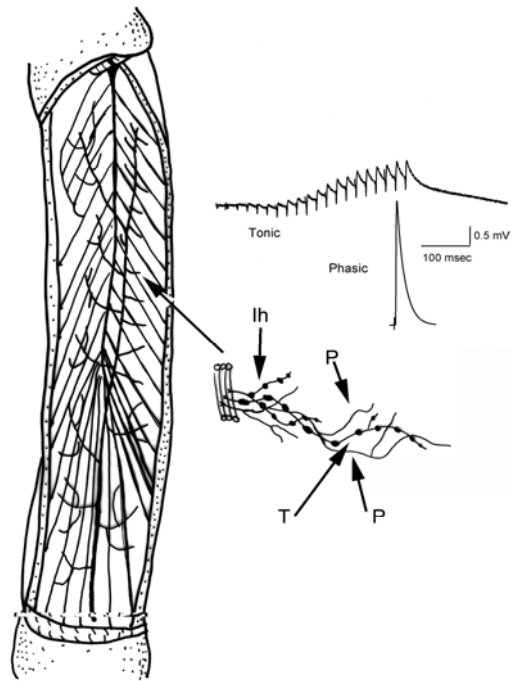


Figure 3.1: Top Left: Diagram depicting the extensor muscle preparation and tonic (T, largest), phasic (P) and inhibitor (Ih, smallest) innervation (insert). Each muscle fiber of the extensor is innervated by each (T, P and Ih). **Top:** The low output NMJ must be facilitated to detect the the EPSPs (Tonic), whereas the high output produces a large response with a single stimulus (Phasic). **Bottom: A:** Phasic and tonic extensor innervation stained with 4-Di-2-Asp. **B:** Anti-synaptotagmin antibody staining of a depressed phasic NMJ showing punctuate staining of vesicle pools in both tonic and phasic NMJs (scale bar 10um).

Figure 3.2

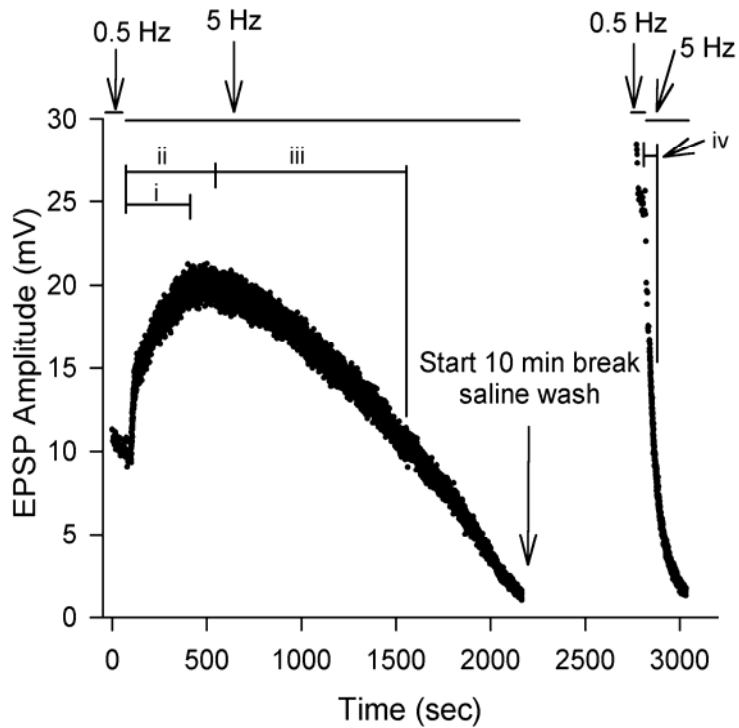


Fig. 3.2: EPSP amplitude (mV) under separate stimulation parameters. *i-iii:* Stimulation increase from 0.5 Hz to 5 Hz, which quickly starts to depress the phasic nerve. *iv:* After the phasic terminal is depressed, a 10 minute saline wash is carried out; once the stimulation returns, depression occurs more quickly due to the previous stimulation.

(ii), the time from the onset of the decay to the time in which the EPSP amplitude decayed to 50% of the maximum response (iii) were measured for the first stimulation phase. A second decay response was obtained from the peak of the recovery response to 50% of its amplitude during the second stimulation paradigm (iv).

To determine if 5-HT would result in an enhanced recovery after synaptic depression occurred, assuming that the depression was primarily a presynaptic phenomenon, the preparation was bathed in 5-HT (100nM) during the 10 minutes resting period. Since previous studies (Southard et al., 2000; He et al., 1999; Strawn et al., 2000) indicated that 5-HT promoted vesicle docking and increased the probability of release, we predicted that a more pronounced recovery would occur. In addition, one would assume that if 5-HT bathed the preparation during the entire stimulation paradigm, that depression would be offset during the high frequency stimulation. The influence of 5-HT is compared to preparations that were exposed only to saline with the same stimulation paradigms (n=5 in all three conditions).

The amplitudes of the EPSPs were monitored at various time points throughout the entire paradigm for the three experimental conditions. Since each preparation varied in the initial EPSP amplitudes a percent change was used for comparisons. The percent difference was determined in the amplitude of the EPSPs during the initial 0.5 Hz stimulation to the 0.5 Hz stimulation after a ten minute rest. Likewise a percent difference in the EPSP amplitude from the 0.5 Hz

initial stimulation to the maximum facilitated response within the first stimulation trial. The results of these percent differences draws one to determine that 5-HT produced a larger peak response as compared to saline exposure during the first stimulation phase ($p < 0.05$). The exposure of 5-HT after the initial depression did not produce an enhanced recovery, as expected, from the depressed state. Exposure to 5-HT throughout the entire stimulation conditions also produced a reduction in recovery from depression as compared to only saline exposure (Figure 3.3A). However, exposure to 5-HT did produce a larger peak response during the initial facilitation during the 5Hz stimulation ($p < 0.05$, Figure 3.3A-far right column).

The differences in the variables recorded to reach a maximum response, the onset of decay and the decay rate for the three experimental conditions are shown in Figure 3B (the various time points are shown in Figure 3.2). When the preparations were bathed in 5-HT before starting the routine stimulation paradigm, the time to a peak response ($p < 0.05$) and the time to the onset of depression was prolonged ($p < 0.05$, Figure 3.3B) as compared to saline only exposure. Exposure to 5-HT did cause the preparation to decay faster once decay was initiated, as seen by the time it took to decay to 50% the maximum EPSP amplitude. During the second stimulation paradigm, the preparations also exposed to 5-HT throughout the entire period depressed faster than those only exposed to saline or 5-HT after the initial depression.

Nerve terminal profiles

Figure 3.3

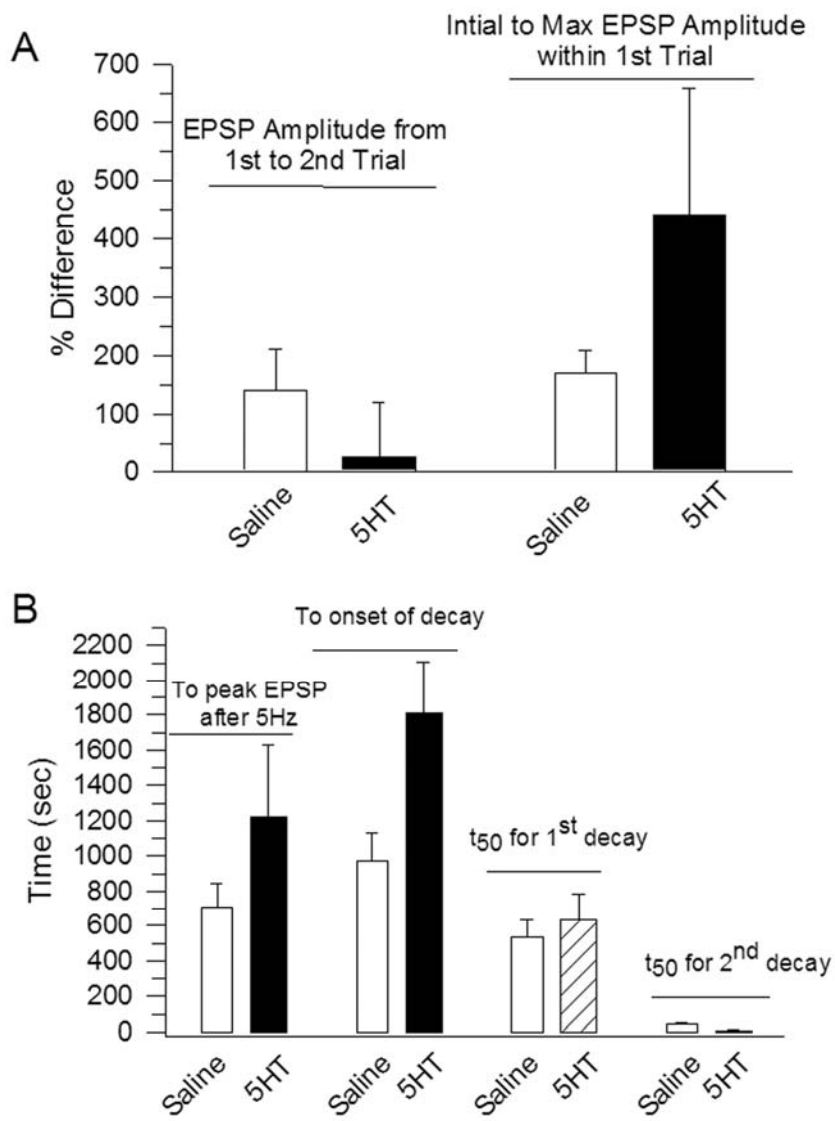


Figure 3.3 A: EPSP amplitude percent difference from 1st to 2nd trial. The addition of 5-HT greatly reduces the EPSP percentage between trials, but increases the max EPSP with in the 1st trial, suggesting two vesicle pools are active, but under separate control mechanisms. **B:** Time to peak EPSP, decay and time to 50% decay for the 1st and 2nd trials in saline and in presence of 5-HT. 5-HT increases the time to peak EPSP amplitude and the onset of decay, but lowers the time for 50% decay for the 1st and 2nd trials, suggesting that 5-HT causes an initial increase in transmitter release, but a slower recovery rate after depression onset (Figure 3.2).

To place the focal macropatch electrode directly over the phasic nerve terminal in order to record synaptic currents, the terminals were visualized with the vital dye 4-Di-2-ASP (Fig. 3.1B). The phasic terminals are easily identified by their filiform morphology as compared to the tonic terminals which contain varicosities. The synaptic locations where vesicles cluster are observed by immunocytochemical staining with the antibody to synaptotagmin (Fig. 3.1B, Cooper et al., 1995a). Patches are present within the large varicosities which are related to the synaptic structure observed in these terminals (Bradacs et al., 1997; King et al., 1996; Msghina and Atwood, 1998). It is also obvious that there are patches along the length of the phasic terminals which also correlate to the synaptic domains (Musghina et al., 1999). Some preparations were immediately fixed after induction of synaptic depression and stained with anti-synaptotagmin. There were no differences in punctate patterns that could be detected between depressed terminals and non-depressed phasic terminals.

The phasic nerve terminals were visualized by TEM so that the readily RRP and docked vesicles could be quantified (Figure 3.4 A and B). Using the method described (see Methods), docked and RRP vesicles were quantified from three samples of each experimental paradigm. The control group (denoted as "Control" in Table 3.1), was stimulated at 5Hz until depressed. The first experimental group was stimulated at 5Hz until depression set in and then 5-HT was added (1uM) (denoted as D→5-HT on Table 3.1). The second experimental group was stimulated at 5Hz in the presense of 5-HT until depression set in (denoted as 5-HT in Table 3.1). No difference was observed in the distribution of

Table 3.1

Preparation	Order	RRP	Docked
Control 1	-1	13	5
	0	15	5
	+1	11	4
Mean		13	5
Control 2	-1	13	3
	0	17	4
	+1	11	2
Mean		14	3
Control 3	1	23	5
	0	21	7
	+1	16	6
Mean		20	6
Cumulative Mean:		16	5
Preparation	Order	RRP	Docked
D→5HT 1	-1	14	4
	0	12	5
	+1	10	14
Mean		12	8
D→5HT 2	-1	7	1
	0	23	4
	+1	10	5
Mean		13	3
D→5HT 3	1	24	4
	0	23	4
	+1	18	4
Mean		22	4
Cumulative Mean:		16	5
Preparation	Order	RRP	Docked
5HT 1	-1	9	2
	0	13	2
	+1	17	2
Mean		13	2
5HT 2	-1	10	2
	0	10	1
	+1	8	2
Mean		9	2
5HT 3	-1	10	2
	0	11	2
	+1	11	3
Mean		11	2
Cumulative Mean:		11	2

Table 3.1: Vesicle counting data: Using the method described, docked and readily releasable pool (RRP) vesicles were quantified from three samples of each parameter. 1. Controls: Stimulation at 5Hz until depression was observed. 2. Depression to 5-HT (D→5HT): Stimulation at 5Hz until depression was observed, after which 5-HT (1uM) was added to the preparation. 3. 5-HT on preparation whole time (5-HT): Preparations were in the presence of 5-HT (1uM) during the whole stimulation until depression was observed (onset of depressed delayed but once started depression was quick). Preliminary data between the controls and D→5-HT showed little to no difference as compared to the 5-HT exposure group in which there was a 31% decrease in the RRP and a 60% decrease in docked vesicles.

Figure 3.4.

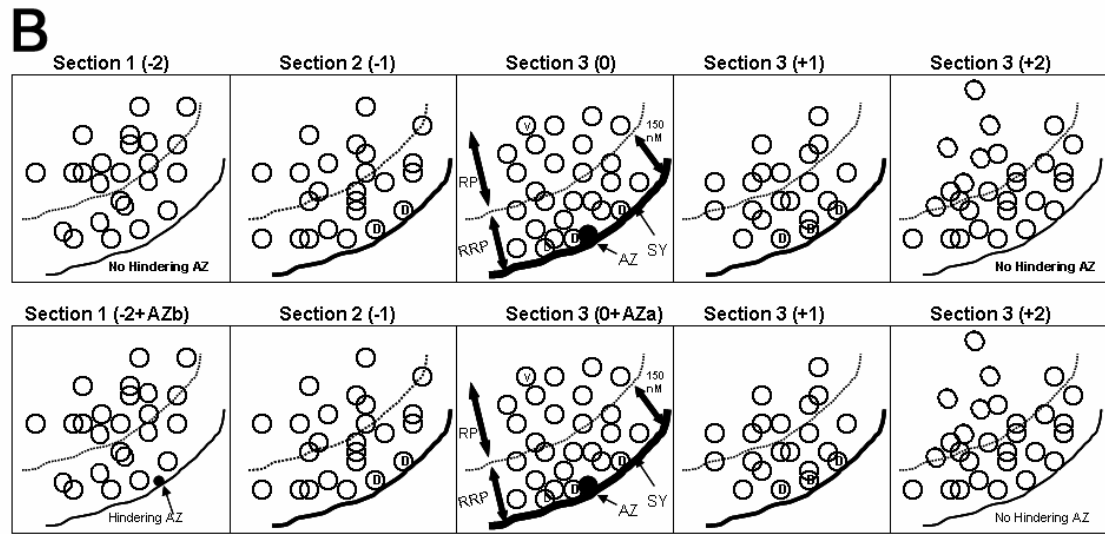
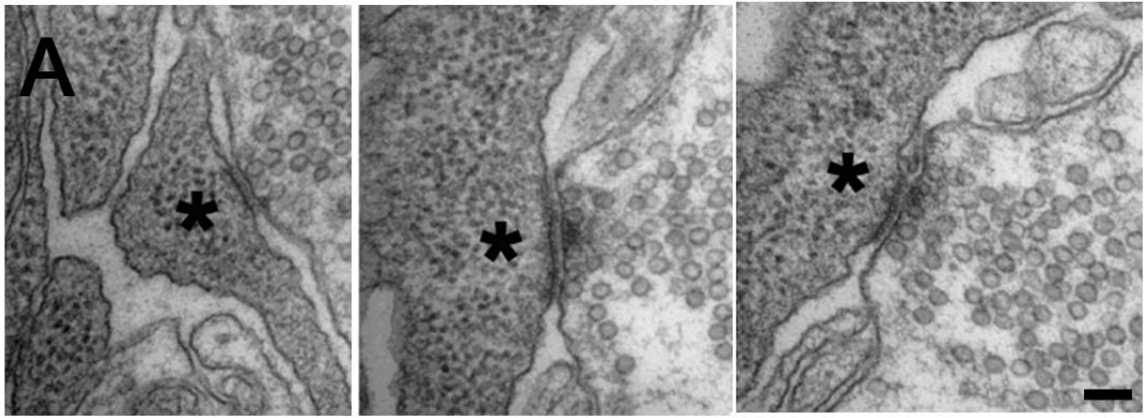


Figure 3.4: The readily releasable pool and docked vesicles were quantified visually by serial TEM (* *Denotes same position for each section*). **A.** An example TEM serial sections that would be used for vesicle quantification, in this case, a control (no 5-HT). **B: (upper panel)** Diagram depicting a serial sections that would be used. An AZ (demarked as section “0”), a section in front (demarked “-1”) and behind (demarked “+1”) were used in the quantification. Sections -2 and +2 were also inspected to insure there were no neighboring AZ’s. Vesicles that were in direct contact with the synapse were counted as docked, while any vesicles within 150nm of the synapse were counted as the RRP. Any vesicles beyond that was considered to be the RP and not counted. **B: (lower panel)** Diagram depicting serial sections that would not be used due to neighboring active zones (AZ).

RRP and docked vesicles due to the D→5-HT paradigm. However a 31% decrease in the RRP and a 60% decrease in docked vesicles was observed in the 5-HT treatment group (Table 3.1).

DISCUSSION

In this study it was demonstrated that within a presynaptic phasic motor nerve terminal of the crayfish extensor NMJ the RP and RRP of vesicles were identifiable by physiological and neuromodulatory means. This was not necessarily by examining recycling (as in Chapter 4 of this dissertation), but by their active status in the nerve terminal. This was approached by depressing the phasic nerve terminal with low stimulation frequency (5 Hz) in the presence or the addition of 5-HT while monitoring EPSPs with intracellular muscle recordings. Given the results, I suggest that two vesicle pools are at work. One that is active during a low stimulation frequency which can be depleted rapidly and another that can be recruited by neuromodulators which prolong the onset of depression as seen by the increase in the time taken to reach a maximum EPSP amplitude, but can be quickly depleted and remain depleted, once depression has set in. Since the muscle could still be stimulated by a tonic motor neuron while the phasic terminals are depressed this demonstrates that the muscle is still responsive but synaptic depression is due to purely phenomena associated with the phasic NMJ. To investigate the potential association of docked and RRP vesicle pools in relation to synaptic depression the phasic nerve terminals were examined with TEM; however, there were no observable differences in the

proportion of RRP and docked vesicles was observed for depressed terminals (Figure 3.4 and Table 3.1).

One form of depression, long term depression (LTD), has offered many insights into the mechanisms involved in synaptic depression. LTD was first described in 1978 in pyramidal cells from the CA 1 region of the hippocampus (Dunwiddie and Lynch, 1978) and has since been studied in a number of other systems such as the dentate gyrus (Poschel and Manahan-Vaughan, 2006) and the cerebellum (Zhang and Linden, 2006). Although LTD occurs in these systems, it does so by different mechanism and each has a specific cellular pathway for induction and maintenance of depression. LTD has now been shown to involve both presynaptic and postsynaptic components depending on the synaptic model used. For instance, LTD was blocked by adding an NMDA antagonist to the hippocampus preparation (Dudek and Bear, 1992; Mulkey and Malenka, 1992), but had no effect in other areas in other brain locations. Thus, in these preparations, LTD seems to be linked to metabotropic glutamate receptors (Maren and Fanselow, 1995) and to be mostly a postsynaptic phenomenon.

There are postsynaptic mechanisms that account for synaptic depression in other systems. For example, in the rodent visual cortex and in neurons of the hippocampus depression has been shown to be due to internalization of AMPA receptors in the postsynaptic targets (Malenka and Bear, 2004). The several possible postsynaptic factors in the crustacean NMJ used in our study, such as receptor desensitization, receptor internalization, or spread of local mediators

to neighboring synapses, all of which do not appear to be credible to account for the results obtained. Since the time to half decay of desensitization is on the order of 15 ms for glutamate receptors at the crayfish NMJ, then stimulation of 5Hz (i.e., 200 msec delay), as we used, should be sufficient time to remove any desensitization from each evoked stimuli. In addition, since facilitation occurs when increasing the stimulation from 0.5 Hz to 5 Hz and that it takes on the order of 20 to 40 minutes of continuous 5Hz stimulation to induce the onset of depression, which then occurs at a rapid rate, also argues against glutamate receptor desensitization to account for a mechanism of this homosynaptic depression. Local mediated influence of synapses does not appear to occur in this system since the synapses of the phasic and tonic motor neurons are in close association at many points along their innervation of a single muscle fiber, it would be likely that if there are cytoplasmic signals within the muscle they would also alter the responsiveness of the glutamate receptors at the tonic NMJ. Since no alteration in the amplitudes of the EPSPs or in the short-term facilitation is observed after depression of the phasic nerve terminals, suggests against diffusible factors that might be associated with the stimulated postsynaptic sites and also suggests against a retrograde signal from the muscle to the nerve terminal unless a selective mechanism to target only the phasic terminals in the midst of the tonic terminals could exist.

In order to fully address postsynaptic receptivity, the difference in the fEPSPs from spontaneous releases prior and during depression needs to be examined. In other words, if the nerve terminal is depressed but spontaneous

release is observed it would indicate that the postsynaptic receptors are not desensitized. This would fully show that depression in this system is clearly presynaptic in origin. In addition, one might be able to determine if RRP vesicles are docked but can't fuse despite an influx of calcium. This can be addressed by depressing the phasic neuron in saline, if the addition of 5-HT caused a faster recovery then it would indicate a differential vesicle pool system, or at least one that is not targeted by electrical activity alone. A decreased recovery time would indicate that RRP is responsive to the electrical activity, while the RP is responsive to neuromodulation that allows its vesicles to be released and recruited to the RRP. It may also be possible to quickly deplete the RRP of the phasic neuron by exposing the nerve terminals with high osmolarity sucrose (Van der Kloot, 1987). This again could be subjected to 5-HT in which recovery can be studied. It is very probable that the evoked pool of vesicles and those induced by the brief hyperosmotic treatment prior to depression as well as afterwards are not the same, however, the postsynaptic receptors arrays are the same in both conditions.

It has been shown that the vesicles are more sensitive to fusion at phasic NMJ of this preparation for an equal calcium exposure (Miller et al. 2005) which likely accounts for the higher mean quantal content at phasic as compared to tonic NMJs (Msghina et al., 1998, 1999). Further studies in this preparation have shown the calcium binding protein frequenin to be higher in phasic terminals than tonic nerve terminals (Jeromin et al., 1999). It was also shown that the amount of calcium is also greater in phasic terminals than tonic terminals during

depolarization (Msghina et al., 1999). These results indicate that in combination with the amount of frequenin, available calcium is quite different between the tonic and phasic nerve terminal. In addition, ultrastructural analysis of the phasic and tonic motor nerve terminals reveal a difference in size and number as well as make-up of mitochondria in phasic and tonic nerve terminals (King et al., 1996). A section through the phasic nerve terminal contains a single unbranched mitochondria, while the tonic terminal contains multiple large multibranched mitochondria. These morphological and anatomical differences one could predict that there are differences in the amount of available ATP in each respective nerve terminal. Thus, the phasic terminal could be more prone to ATP depletion due to fewer mitochondria, influencing synaptic mechanisms (i.e. vesicle recruitment, recycling, etc.). These factors alone or combined may account for the mechanisms of the presynaptic depression in the phasic nerve terminal. Another possibility is that autoinhibition of the presynaptic terminal could account for some of the synaptic depression. The working models speculated today for autoinhibition of glutamatergic synapses involve kainate autoreceptors. Recent studies in the hippocampus show presynaptic kainate receptors decrease the influx of calcium reducing evoked transmission (Kamiya and Ozawa, 2000) and this type of autoinhibition also involves the adenylyl cyclase/protein kinase A path (Negrete-Diaz et al., 2006). More recently it was demonstrated in vertebrate CNS that kainate receptors exist pre- and post-synaptically and given that there are five different subunits of kainate receptors a wider variety of responses might be possible (Park et al., 2006). In fact, for cortical neurons the kainate induce

depression is now assumed to occur due to release of calcium from internal stores to produce LTD (Park et al., 2006). Such a possibility could occur at the crayfish NMJ but in this preparation the working model is that if a increase in calcium release occurred from internal stores that transmission would increase (Dropic et al., 2005). Recent evidence (Bhatt and Cooper 2005; Bhatt et al., 2006) suggests presynaptic kainate receptors at the *Drosophila* NMJ which is analogous to the crayfish NMJ in many ways. Conversely, in Schaffer collateral synapses (mouse) presynaptic kainate receptors enhance the probability of vesicular fusion (Sun and Dobrunz, 2006). A similar situation in variation and density of presynaptic autoreceptor subtypes controlling the net response might be present in crayfish motor nerve terminals. In fact, Schramm and Dudel (1997) reported that activation of glutamatergic autoreceptors at the crayfish NMJ could result in either depression or excitation of transmission which they accounted for by varying proportions of receptor subtypes.

This present study demonstrates for glutamatergic phasic NMJs that the number of docked vesicles are not different between terminals with or without exposure to 5-HT; thus, the calcium (which is likely required for priming) most likely is not affected by autoreception. But, this could easily be confirmed by examining calcium influx, via calcium indicators to identify alterations in calcium influx during synaptic depression. Also, this study shows a possible alternative action to that of the mossy fiber, in which glutamate activates autoinhibition of the MF (via PKA), since equal numbers of docked vesicle numbers of control and experimental groups show the same number of docked vesicles. Thus, an

alternative mechanism could be in play at the crayfish phasic nerve terminal, possibly due to the eluded reduction of ATP and/or the recycling of empty vesicles since. Comparative studies can also help in understanding the mechanisms at play, for example it is known that vesicles can recycle empty as demonstrated at the *Drosophila* NMJ (Kidokoro et al., 2004).

The 5-HT receptor subtype at the crayfish NMJ appears to share some characteristics with the vertebrate 5HT-2 family (Tabor and Cooper, 2001). These receptors undergo up- and down-regulation in their functional sensitivity (Cooper et al., 2002) suggesting that they regulate synaptic function differently in acute and chronic behavioral states which might be associated with alterations in systemic 5-HT levels (Yeh et al., 1999; Listerman et al., 2000; Dornberg et al., 2000). Thus, this gives a possible mechanism as to how the phasic nerve terminal reacts to neuromodulation from 5-HT, by up or down regulating the 5-HT receptors, it may be more or less receptive to neuromodulation.

A feasible mechanism to account for the induced presynaptic depression in the crayfish phasic motor nerve terminals is a rundown in available ATP. In biochemical assays it has been shown that ATP promotes NSF binding with α -SNAP and that ATP is required for the undocking via clathrin coated recycling (Rothman, 1994). A possible experiment to determine if the mechanisms responsible for the rundown is related to energy required for the vesicular docking and recycling (i.e., ATP dependent processes), could include a rapid release of caged ATP within depressed terminals (Tsien and Zucker, 1986).

The potential mechanism of action for 5-HT is that of phosphorylation of synaptically relevant proteins such as Munc-18-1 or synaptophysin or even NSF. These scenarios are possible considering that phosphorylation of the first two proteins has been shown, in vitro, to inhibit their ability to interact with the SNARE proteins, and that they may regulate the interaction of syntaxin for Munc-18-1 and synaptobrevin for synaptophysin. Other proposed mechanisms of action are that 5-HT may lead to phosphorylation of synapsins (which was a Nobel Prize worthy discovery by Paul Greengard in 2000) to increase the pool of free vesicles for docking or possibly actions on calcium influx and release from internal stores (Yang and Zucker, 1999). To address these proposed mechanisms further studies should be conducted. Results from such studies will allow one to determine the effects on both the presynaptic and postsynaptic components to further understand potential mechanisms responsible for the development of synaptic depression.

CHAPTER 4

The regulation of synaptic vesicle pools within motor nerve terminals during short-term facilitation and neuromodulation. (used with permission)

INTRODUCTION

In postsynaptic targets, the excitatory synaptic potentials are incremental in relation to the numbers of packets of transmitter released below a threshold potential (del Castillo and Katz, 1954a). This is generally accepted by a packet of neurotransmitter contained within a vesicle being released from the presynaptic nerve terminal into the synaptic cleft (Dudel and Kuffler, 1961; Kuffler and Yoshikami, 1975b). A number of studies have set out to address if all the vesicles are equally potent in eliciting quantal postsynaptic responses (McLachlan, 1978; Parsons et al., 1999). In order to resolve some of the issues in vesicular packaging one needs to determine if the vesicle pools are equally recruited during electrical activity and in the presence of neuromodulators. Since it is known in a number of preparations that there are populations of vesicles within nerve terminals that behave differently in their fusion and recycling kinetics, we examined the ability of these pools to be recruited during electrical activity of the nerve as well as in the presence of neuromodulators which have secondary actions on vesicular kinetics. By addressing this in relation to presynaptic function, a better understanding in fluctuations in quantal responses during synaptic depression, facilitation and synaptic differentiation will hopefully be achieved. In this study, we make use of the known simplicity of the synaptic

structure at the crayfish opener neuromuscular junction (NMJ) and its quantal nature of transmitter release to assess discrete synapses within the motor nerve terminals (Dudel and Kuffler, 1961; del Castillo and Katz, 1954a,b). The opener muscle in the crayfish walking leg, as used in this study, is non-spiking and graded postsynaptic potentials are directly related to the number of presynaptic vesicle fusion events (Dudel, 1981, 1988, 1989; Dudel and Kuffler, 1961;) as also shown at the frog NMJ when the responses are below the threshold level for an action potential in the muscle (Kuffler and Yoshikami, 1975).

With physiological and pharmacological means we set out to address if the uptake of synaptically released glutamate is important in the refilling of the readily releasable pool (RRP) of vesicles that rapidly recycles during maintained stimulation. In addition, we examined if reserve pool (RP) of vesicles can be recruited, by the use of serotonin (5-HT), while the RRP remains reduced functionally or is rendered non-functional. The goal is also to provide further insight into the regulation of the RP and recycling of the RRP within nerve terminals in a model synaptic preparation.

Since 5-HT at the crayfish NMJ greatly enhances transmitter release (Dudel, 1965a) presynaptically at NMJs by increasing the probability of vesicular fusion (Southard et al., 2000) and is thought to do so by recruiting RP of vesicles to the RRP (Sparks and Cooper, 2004; Cooper et al., 2003). The two populations of vesicles can be differentiated by depleting the function of the RRP by preventing them from being repackaged during rapid recycling. Recruiting the RP with 5-HT while reducing the ability of the rapidly recycling pool from repackaging allows

the dissection of the various pools within the terminal. In crayfish motor neurons, 5-HT mediates its rapid effect through the IP_3 second messenger system (Dixon and Atwood, 1989; Delaney et al., 1991). The action of short-term facilitation (STF) in enhancing release was recently addressed from actions of 5-HT at the crayfish NMJ which suggested that different pools of vesicles might reside in the terminal due to alternate recycling mechanisms (Sparks and Cooper, 2004). The recycling of vesicles is generally depicted as following two different routes for recycling: a rapid loop and a slower one that reprocess the vesicles within the endoplasmic reticulum (Klingauf et al., 1998; Kuromi and Kidokoro, 1998; Palfrey and Artalejo, 1998; Parsons et al., 1999; Richards et al., 2000; Stevens and Williams, 2000).

The rapid recycling process is promoted during repetitive electrical depolarization of the terminal to maintain output. A homeostasis can even exist to recycle vesicles and buffer calcium so that a plateau in release is observed, however a slight alteration in the stimulation frequency or actions of a neuromodulator offset the balance in the various vesicle recycling paths (Sparks and Cooper, 2004). To address if the RP can be recruited to the RRP during maintained electrical activity of the terminal the RRP needed to be selectively targeted to be allowed to recycle however tagged to distinguish release from those of the RP. This can be approached by depleted transmitter within the vesicles of the rapidly recycling pool while not altering the RP within a short window of time.

Rapid functioning synapses clear released transmitter in the synaptic cleft quickly which is one mechanism to avoid desensitization of postsynaptic receptors in order to detect subsequent evoked release within several milliseconds (Dudel and Schramm, 2003) and prevent spillover to neighboring synapses (DiGregorio et al., 2002; Huang and Bordey, 2004). The glutamate-ergic synapses within the vertebrate CNS clear glutamate by use of glutamate transporters. It is generally accepted that the major recycling path for glutamate is through astrocytes (Huang and Bergles, 2004). These supportive cells take up the glutamate from the synaptic cleft via glutamate transporters. Within the cell glutamate is converted to glutamine which is then released out of the astrocyte. The transporters on the nerve terminal then take up glutamine which is converted back to glutamate and transported into the vesicles by specific vesicle associated transporters (Christensen and Fonnum, 1992; Takamori et al., 2000). Thus, for glutamate-ergic terminals the glutamate enters back into the terminal by two means: (1) Directly via presynaptic transporters, and (2) through the use of supportive cells by a glutamate-glutamine cycle.

Vertebrates are known to have multiple types of glutamate transporters. In mice, there appears to be five genes that encode six distinct glutamate transporters (Huang and Bergles, 2004). The motor nerve terminals at the crayfish NMJ are glutamate-ergic and since glia cells do not enfold with the nerve terminals on the muscle the variable of supportive cells is eliminated in the process of vesicle repackaging (Atwood and Cooper, 1995; Cooper et al., 1995a). Thus, the presynaptic nerve terminal is the likely means to clear

glutamate from the synaptic cleft. The reuptake process during normal synaptic transmission is expected to be rapid since these NMJ can desensitize quickly with exogenously applied glutamate. In the absence of glutamate the receptors resensitized to be responsive to a subsequent exposure (Dudel and Schramm, 2003). The presynaptic re-uptake at the crayfish NMJ is also supported by the fact that the blocker for vertebrate glutamate transporters, DL-threo- β -benzyloxyaspartate (TBOA), can block the re-uptake of glutamate at the crayfish NMJ (Dudel and Schramm, 2003). Hence, we made use of blocking glutamate re-uptake to assess vesicle dynamics within the nerve terminal during rapid release and recycling of the RRP of vesicles.

Through the use of TEM, one can address whether the recycling pool of vesicles is altered in numbers when the presynaptic transporters are compromised. We examined for differences in the dynamics of the RP and the number of docked vesicles anatomically with TEM to compare with physiological assessments and predictions of vesicle dynamics during blockade of the transporter as well as during recruitment of a RP by 5-HT. Vesicles and vesicle pools are easily identified in nerve terminals in TEM which provides insight to the state of the nerve terminal before and after pharmacological exposure. In most nerve terminals examined, at a resting state, a small number of vesicles are docked to the active zone accompanied by a close proximity RRP of vesicles and a RP of vesicles located a small distance away (Rizzoli and Betz, 2003). After TBOA induction, the functional responses of the RRP of vesicles are known to

become depleted (Dudel and Schramm, 2003). Thus, it is of interest to know by TEM if there is a decrease in vesicles docked or ones in the RRP.

In this study an undergraduate, Stephanie Logsdon (University of KY), conducted the electrophysiology experiments and analysis. My role was to process the tissues for TEM, section and image, and finally to analyze the data obtained from the TEM.

METHODS

General

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

Physiology

To elicit an evoked response, the excitatory axon was selectively stimulated by placing a branch of the leg nerve (from the merus segment) into a suction electrode connected to a Grass stimulator (Dudel and Kuffler, 1961). The short term facilitation (STF) was induced by providing a train of ten pulses at 40Hz, at

three second intervals, to the excitatory nerve. The selection of stimulation frequencies was chosen to be similar to earlier studies using the same preparation (Crider and Cooper, 1999, 2000; Cooper and Ruffner, 1998). In addition the distal muscle fibers were always used in this study, as illustrated in Figure 4.1, since it is easy to follow these muscle bundles throughout fixation and processing for electron microscopy in order to obtain physiological and anatomical correlation of similar type synapses (Cooper et al., 1996; Mykles et al., 2002). Continuous stimulation at 20 Hz and 50 Hz was also applied to the excitatory nerve to compare the effects of TBOA.

Intracellular EPSP recordings were performed by standard procedures (Crider and Cooper, 2000; Cooper et al., 1995a). The 5-HT (1 μ M) containing saline was made in crayfish saline from frozen stock of 1 mM 5-HT. When exposing the preparation to 5-HT, the entire bathing medium was rapidly exchanged (less than 30 seconds). All chemicals were obtained from Sigma Chemical (St Louis, MO, USA). Electrical signals were recorded on-line to a Power Mac 9500 or to a Dell Latitude D600 computer via a MacLab/4s or a PowerLab/4s interface respectively.

field Excitatory Postsynaptic Potentials (fEPSPs)

In addition, synaptic field potentials were also measured with focal macropatch electrodes to assess presynaptic vesicular events. The varicosities on the living terminals were visualized using the vital fluorescent dye 4-Di-2-ASP (Molecular Probes) (Magrassi et al., 1987; Cooper et al., 1995a). It was

Figure 4.1

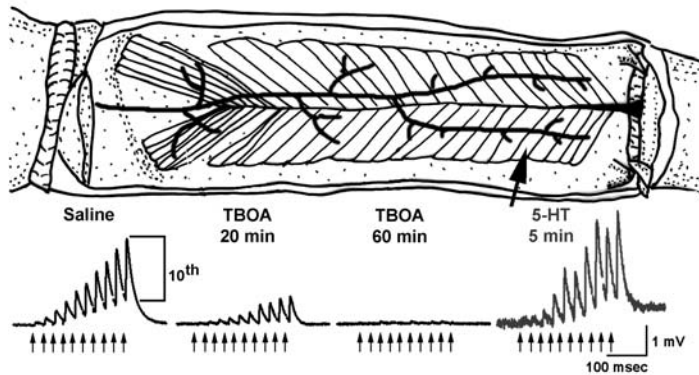


Figure 4.1: The effects of TBOA on synaptic transmission. A schematic of the opener muscle in the crayfish walking leg with associated excitatory postsynaptic potentials (EPSPs). Recordings were made in distal muscle fibers (arrow). Representative EPSP responses to a train of ten stimulation pulses given at 40 Hz before and during exposure to TBOA ($10\mu\text{M}$) followed by TBOA and 5-HT (lower traces). The amplitude of the EPSPs is measured from the trough preceding the EPSP of interest to the peak response, as shown for the tenth pulse during saline exposure. As the preparation is exposed to TBOA the EPSP amplitudes decrease over time. After the 10th pulse in the train is reduced by at least a tenth of the initial amplitude the preparation is exposed to 5-HT ($1\mu\text{M}$). 5-HT increases the amplitude of the EPSPs. The arrows in the traces of EPSPs represent the 10 stimulation pulses.

demonstrated in an earlier report that 4-Di-2-ASP had no effect on transmission within the concentration (5 μ M) used in this study (Cooper et al., 1995a). The synaptic potentials were obtained using the loose patch technique by lightly placing a 10-20 μ m fire-polished glass electrode directly over a spatially isolated varicosity along the nerve terminal. The evoked field excitatory postsynaptic potentials (fEPSPs) and field miniature excitatory postsynaptic potentials (fmEPSPs) can readily be recorded (Cooper et al., 1995b, 1996a; del Castillo and Katz, 1954a).

In addition to the direct quantal measures, the area of the evoked and spontaneous events was measured over time in each preparation for comparison within a preparation to determine if the area of the quantal units were altered. The area of the evoked and spontaneous events was determined by the Simpson's mathematical method after the baseline of each trace was controlled for DC offset (Viele et al., 2003). The DC offset was adjusted by setting the average of the baseline to zero. The noise that was added over time in the measures was corrected for by subtraction of the average noise determined within baseline recording. This approach adjusted for the noise independent of the duration measured for each event. The area analysis was managed by converting the traces stored as Scope files to rich text files. The rich text files were then used in conjunction with subroutines written in "R basic". This software is freeware and maintained by CRAN (Comprehensive R Archive Network) and downloadable from <http://cran.r-project.org>. The computational assessment computed evoked events as any deflection above noise from baseline within the

20msec window following the action potential spike and was recorded as a result of the nerve terminal depolarization. The output files were then imported in Sigma Plot (version 8.0) for graphical purposes and quantifying the measures.

Analysis

The STF for train stimulation was indexed by the ratio in the amplitudes of the 10th and the 5th EPSP within a train (Crider and Cooper, 2000). The numeral one was subtracted from the ratio to ensure that if there is no facilitation occurring, the facilitation index (FI) will then be zero. The EPSP amplitudes were measured by the difference from the trough preceding the event to the peak amplitude of the event. Using the 1st EPSP amplitude within a train to calculate FI can lead to erroneously high values when the 1st EPSP is small as reported in earlier studies. Thus, measures of the 5th EPSPs to the 10th EPSPs are more reliable for indexing the effect of TBOA. To index a 50% decline in EPSP amplitude, ten responses were measured from a baseline to the peak at various time intervals. Each ten pulses were averaged and graphed in order to ascertain the time point of a 50% decline in amplitude. The times for train stimulation, 20 Hz continuous stimulation, and 50 Hz continuous stimulation were compared. The mean time for a 50% decay was used for statistical comparisons. The induction of depression was stopped when the amplitude of the 10th EPSP in the train reached 1/10th the initial amplitude. Percent change after addition of 5-HT was also calculated and compared among the three stimulation types. An average of ten consecutive responses was used at varying time intervals to ascertain the effects of TBOA

and 5-HT. The average EPSP amplitude directly before 5-HT addition and the largest average EPSP amplitude produced by 5-HT were used to calculate the percent change. The relative change was measured from the depressed EPSP amplitude to the peak response to 5-HT. All exposures to 5-HT occurred after depression of the EPSP amplitude reached 1/10th of their initial value. This was the standard used for all the preparations. Of course each preparation took a different amount of time to reach the 1/10th EPSP amplitude. The number of stimuli given to reach a 50% reduction in the EPSP amplitude was also used for indexing purposes. The three groups (40Hz train stimulation, 20Hz continuous stimulation, and 50Hz continuous stimulation) were compared using a one-way ANOVA with follow up t-tests when the assumptions were valid, and by a Kruskal-Wallis test when the assumptions were not met. R was used for these procedures.

Transmission Electron Microscopy (TEM)

All preparations were fixed in a 2.5% gluteraldehyde, 0.5% formaldehyde buffered solution (0.1 M sodium cacodylate, 0.022%wt CaCl₂, 4%wt sucrose, and adjusted to pH of 7.4) for one hour with two changes and post fixed with a 2% osmium tetroxide buffered solution and embedded in Eponate 812. The samples were serially thin sectioned on a Reichert ultracut microtome and post stained with uranyl acetate and lead citrate. Sections were then viewed on a Philips FEI Tecnai, Bio Twin 12 model transmission electron microscope at 80kV.

Measurements for vesicle location and distribution.

Each transmission electron micrograph that revealed a synapse was cataloged. In some cases complete serial sections of synapses were obtained. The presynaptic terminals were classified as excitatory by the shape of the vesicles. Inhibitory terminals reveal oblique shaped vesicles (Tse et al., 1991) and were not further used for quantification. The appearance of dense bodies associated with synapses within the presynaptic terminal were used to define an active zone (AZ). Within the crayfish NMJ, AZs are places where vesicles cluster in association to fusion with the presynaptic membrane (Cooper et al., 1996b). These dense bodies are thought to serve as cytoskeleton attachment points to deliver tethered synaptic vesicles possible from reserve pools.

Synapses at the crayfish NMJ do not have a grid of AZ on the synapses but show synaptic variation such that some synapses may only possess a single AZ while others might have multiple AZs at varying distances from one another (Cooper et al., 1996b). We sought to accurately measure docked and RRP by serial sectioning the synapses around single AZs by insuring that the counts were not skewed by vesicles associated by near neighboring AZ. Thus, three sections to either side of an AZ of interest were viewed and only sections within one section to either side of the section containing an AZ were used for anatomical measures of vesicle pools. In the crayfish opener preparation, the dense bodies of the excitatory terminals are viewed as hemispheres of about 35 to 40 nm in diameter sitting with the cross-section of a hemisphere facing the synapse (Atwood and Cooper, 1994a). This is documented by the occasional on

face view in parallel sectioning of synapses (Cooper et al., 1996b). Thus, in 75nm cross-section thickness a dense body can be contained within a single section. If the dense body is seen within two sections then sections either side of these sections were also used for analysis. Care for stereological errors in measuring objects in TEM from 2D images of 3D tissue were implemented as previously described (Atwood and Cooper, 1996a; Feuerverger et al., 2000; Kim et al., 2000).

RESULTS

The crayfish opener muscle in the walking leg is known to have regional variation in the amplitudes of the EPSPs across the muscle fiber in spite of being innervated by a single excitatory motor neuron (Mykles et al., 2002). Thus, in these experiments the distal muscle fibers were always used as illustrated in **Figure 1**. The relatively low output nature of the tonic-like motor nerve of the opener muscle produces a small amplitude EPSP when a single stimulus is provided. Since the postsynaptic responses are graded and the NMJ in this preparation can have pronounced facilitation (Crider and Cooper, 2000; Zucker, 1974; Zucker and Lara-Estrella, 1983), a short train of 10 pulses at 40Hz produces a significant increase in the EPSP amplitudes. The larger amplitude of the 10th response in the train provides an easy measure to examine changes over time in synaptic efficacy by observing an increasing or decreasing amplitude. When the preparation is exposed to saline containing TBOA (10 μ M)

all the EPSPs within the train decrease in amplitude over time. With exposure to 5-HT (1 μ M), after TBOA treatment, the responses show a pronounced enhancement in all cases and an even increase in amplitude greater than during the initial observation in saline (Figure 1, lower traces). In this experimental paradigm the preparation is continuously stimulated with the pulse train of 10 stimuli every 3 seconds during the TBOA exposure.

The time domain for running down the glutamate loaded vesicle pool varied among preparations. Thus, in order to compare among preparations an index of the time for the 10th EPSP within the train to decrease to an average 50% its initial average value was used. A representative analysis is shown in Figure 4.2. All EPSPs were collected for the 50Hz and 20Hz continuous stimulation paradigm, however analysis of the EPSP amplitude was only performed on sections of the fully observed file where changes in amplitude occurred. The average time to 50% reduction among all the preparations for each stimulation condition (40 Hz train, 20 Hz & 50Hz continuous stimulation) are presented in Figure 4.3A, while the average number of stimuli to a 50% reduction is presented in Figure 4.3B. The data in Figure 4.3B were far more amenable to ANOVA analysis than the data in Figure 4.3A because the data in Figure 4.3B could be transformed by a cube root transformation to achieve approximate normality, an assumption of ANOVA (the cube root transformation was confirmed by a Box-Cox transformation analysis as well. Without the transformation, the

Figure 4.2

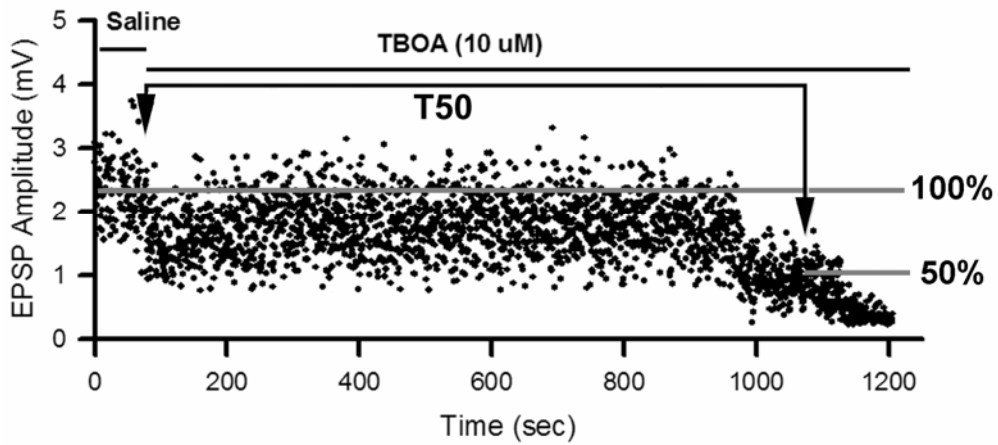


Figure 4.2: The presence of TBOA resulted in a reduction of evoked transmission. The 10th EPSP amplitude, during 40Hz-10pulse trains every 3 seconds, is shown decreasing over time after exposure. The time to 50% reduction in the 10th EPSP after exposure to TBOA was determined as indicated on the graph for each preparation. The same approach was used for the continuous stimulation of 20Hz and 50Hz paradigms.

variances of the three groups are quite unequal). We reject the hypothesis of equal treatment means ($p=0.015$) and used follow up t-tests which indicate the 40Hz train stimulation group is different from the other two groups ($p=0.008$ for comparing 40Hz to 20Hz, and $p=0.024$ for comparing 40Hz to 50Hz). No suitable transformation could be found for the time data in Figure 4.3A, which was then analyzed using the less sensitive Kruskal-Wallis nonparametric test. The only significant result achieved ($p=0.042$) for these data were the pair wise comparison between the 20Hz and 50Hz groups. Considering these are pair wise comparisons, we view this result as indicative, not conclusive, and would hope larger sample sizes would shed more light on this issue.

Since the 50Hz continuous stimulation rapidly depressed the EPSP amplitudes as compared to the 20Hz stimulation, one would expect that the reserve pool of vesicles would be reduced in the 50Hz stimulated preparations as compared to the 20Hz stimulated preparations. Since 5-HT is thought to mobilize the reserve pool of vesicles to the synapse at the crayfish NMJs (He et al., 1999; Southard et al., 2000; Cooper et al., 2002, 2003), we postulated that the 50Hz stimulated preparations would not show much, if any alteration, in the synaptic responses when exposed to 5-HT ($1\mu\text{M}$) as compared to the 20Hz or the preparations stimulated with an intermittent 40Hz trains. The results were surprising in that the 50Hz depressed synapses showed a pronounced enhancement upon exposure to 5-HT ($1\mu\text{M}$) comparable to the depressed terminals stimulated with the intermittent 40Hz trains and the continuous 20Hz

Figure 4.3

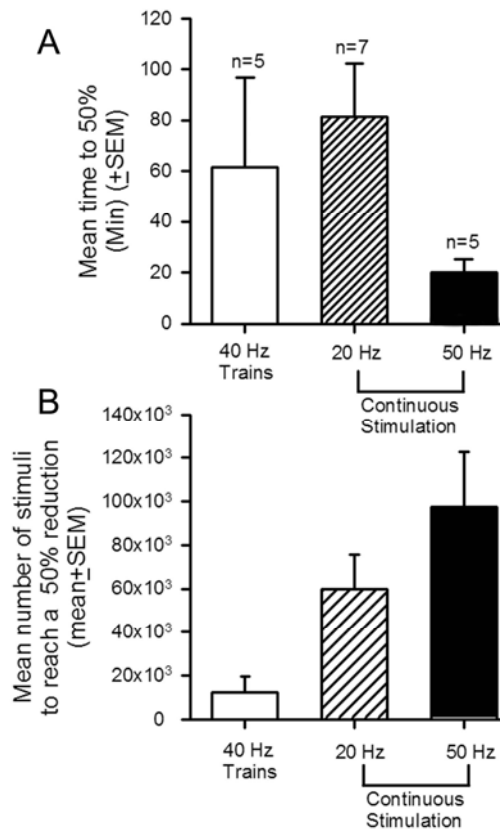


Figure 4.3: The time and number of stimuli to run down the EPSP amplitudes by 50% in the presence of TBOA. **(A)** shows the mean run down time to 50% the initial value for the different stimulation paradigms in the presence of TBOA, while **(B)** shows the number of stimuli. The data in (B) are more amenable to an ANOVA, where we reject the hypothesis of equal means ($p=0.015$). Follow up t -tests indicate the 40Hz train stimulation group is different from the other two groups ($p=0.008$ for comparing 40Hz to 20Hz, and $p=0.024$ for comparing 40Hz (Figure 4.4). The results of 5-HT exposure on electrically depressed nerve terminals is important because this suggests that that RP is not depleted. In Addition, this implies that the presynaptic sites are not blocked with empty

Figure 4.4

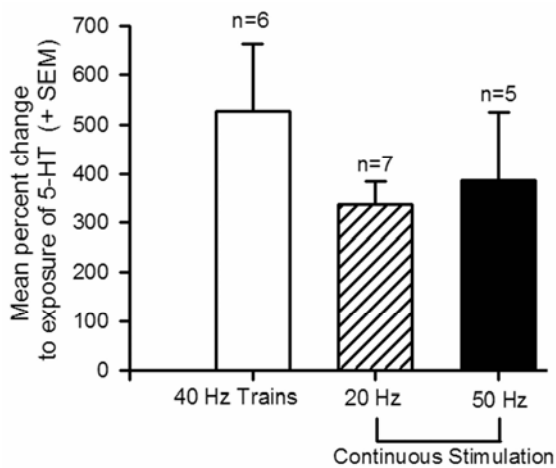


Figure 4.4: The enhancement with 5-HT of evoked transmission after TBOA treatment. After the synaptic responses were reduced to at least a tenth of their initial amplitude they were exposed to 5-HT. The average percent change to exposure of 5-HT and TBOA from the depressed state during TBOA alone exposure is shown. All depressed preparations were enhanced drastically by 5-HT ($p < 0.05$, Wilcoxon rank sum test). Thus, vesicles in the RP that are fully loaded with glutamate are able to be recruited into action.

to 50Hz). The data in figure (A) require less sensitive nonparametric methods, where the only indicative result was the pair wise comparison looking at the difference between the 20Hz and 50Hz groups ($p=0.042$). vesicles recycling due to the electrically stimulated depression. There must be ample docking and fusion space on depressed synapses for such a prominent enhancement of vesicle fusion upon 5-HT exposure.

One such mechanistic possibility for synaptic depression is that the glutamate receptors postsynaptically are internalized as shown for the CNS in vertebrates during the induction of long-term depression (LTD) (Wang and Linden, 2000; Matsuda et al., 2000). In this case, the size or area of the quantal depolarized responses should become smaller over the induction of the depression. This would be the case not only for the evoked quantal events but also for the events that arise due to spontaneous release, assuming that spontaneous events occur from the same synapses that were evoked electrically. To test for such a possibility the individual quantal events were monitored by a focal macropatch electrode placed directly over visually discrete varicosities on the nerve terminal before and during the induction of depression by TBOA exposure (Figure 4.5A). Single stimuli may evoke 1 or more quantal responses (a singlet, doublet, etc.), or none at all (a failure) (Figure 4.5B). Since we were interested in knowing if the size or area of the quantal responses varied during depression, the area (deflection off baseline) of the quantals were monitored over time (Figure 4.5C). The area measures were also monitored during stimuli that

Figure 4.5

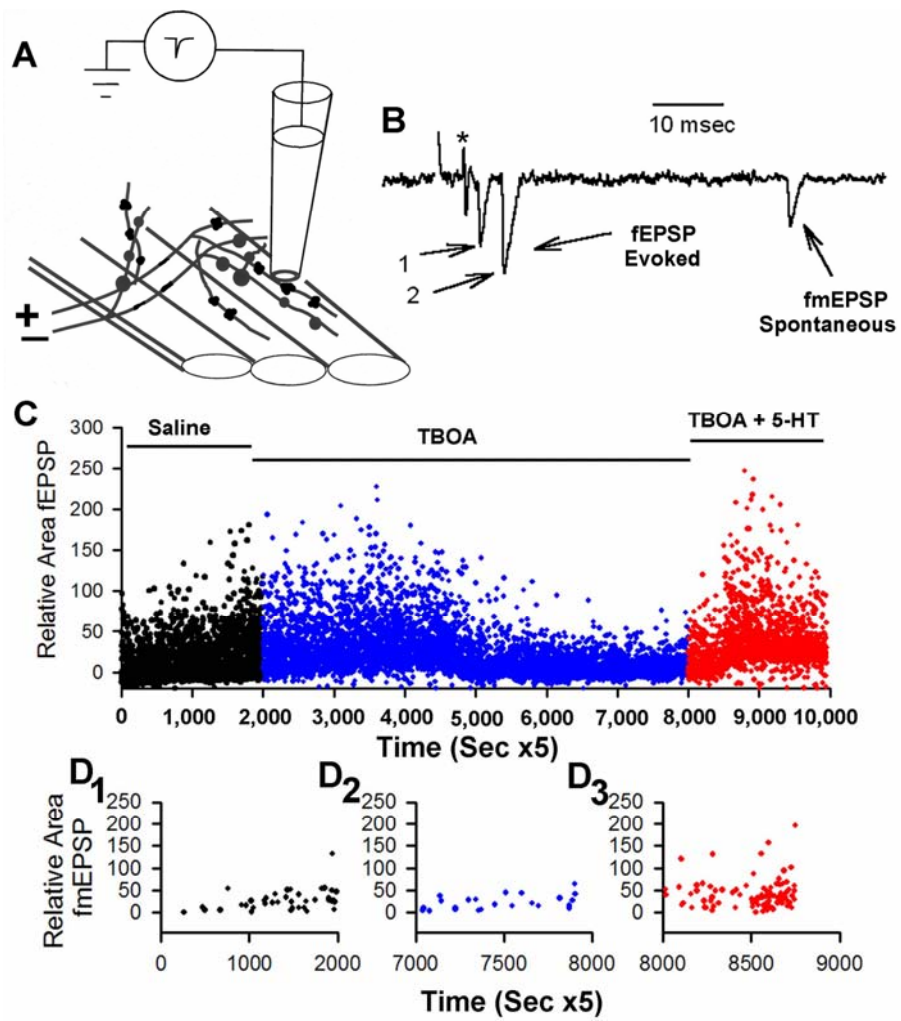


Figure 4.5: TBOA is shown to reduce the size of the quantal responses via presynaptic mechanisms. Recordings of individual evoked quantal events and spontaneous events were obtained by use of a focal macropatch electrode placed over visualized varicosities (**A**). The quantal responses from evoked release and spontaneous release were monitored (**B**). The area of the evoked field excitatory postsynaptic potentials (fEPSPs) decrease over time with TBOA (depleted vesicles). After synaptic depression occurs the exposure of 5-HT in the presence of TBOA recruited new populations of vesicles from a RP. When the area of the evoked and spontaneous (i.e., field miniature postsynaptic potentials, fmEPSPs) are plotted, it was observed that the spontaneous quantal events still occur when evoked transmission is depressed (**C**). The fmEPSPs during saline (**D₁**), depressed fEPSPs (**D₂**) as well as during 5-HT and TBOA exposure (**D₃**) indicate that postsynaptic sensitivity is not altered but the type of vesicle from the rapidly recycling pool or a RP varies. During the 5-HT exposure the evoked and spontaneous events are mixed in size and area suggesting the ability to recruit new populations of vesicles from a RP in TBOA depressed terminals. did not produce an event but were deemed to be a failure in evoking a response.

Since these NMJs of the opener muscle are very low in synaptic efficacy this is expected and had been reported previously (Cooper et al., 1996a,b). The normal

distribution in the area of the noise for baseline recordings (or failures) centers at zero. This is the prevalent measure during the late phase of exposure to TBOA with only a few evoked events being observed. Upon exposure of the terminal to 5-HT (1 μ M), after synaptic depression is maintained, the quantal responses appear rapidly (Figure 4.5C). Fewer failures in evoking a response were evident after the treatment with 5-HT. The responses of the macropatch recordings parallel the results obtained with the intracellular recordings which represent an ensemble average of the discrete regions of the nerve terminals that were monitored with the focal macropatch electrode.

It is apparent that the postsynaptic receptors are not rapidly down-regulated or internalized or even for that matter desensitized during the synaptic depression since quantal responses rapidly arise with exposure 5-HT. Additional evidence supports this notion from the monitoring of spontaneous events prior and during depression. The range in the area measures of the minis (i.e., spontaneous events) are not different (Figure 4.5D₁ and D₂). Given the minis might occur from all synapses present and not just the recruited synapses during the stimulation one would expect a significant portion of the minis to be reduced in size if the postsynaptic receptor array was compromised during depression. Also the minis showed a similar range during the 5-HT exposure which also suggest the same population of synapses are in use (Figure 5.5D₃). The results indicate that the depressed terminals have small quanta and after 5-HT treatment a mix of very small and normal sized quanta are present. This suggests that

some depressed vesicles are indeed mixing with recruited RP vesicles (due to 5-HT) which were fully packaged with glutamate.

To obtain structural evidence for the physiological measures, serial sections of the motor nerve terminals were reconstructed in preparations that were only stimulated in saline and depressed as compared to preparations depressed with exposure to TBOA. Three control and three TBOA treated preparations were examined. The fixation was rapid (<5 sec) after stimulation was ceased. The time delay was to remove the ground wire and exchange the saline for fix solution in the recording dish. The area surrounding an active zone (AZ) on crustacean NMJ synapses have a high incidence of docked vesicles and clustering as compared to regions of the synapse devoid of AZs (Atwood and Cooper, 1995, 1996a,b). For this reason, serial sections were obtained on either side of the section containing an AZ and all three sections were used for the analysis of the number of docked and RRP (within 150nm of the presynaptic face) of vesicles. A electron micrograph and a schematic of the analysis is shown in **Figure 4.6**. To insure that a neighboring AZ was not present on 2 sections removed from the section containing the AZ of interest, 4 to 5 serial sections were viewed on either side of the AZ in which the measures of docked and RP vesicles were being made. This extra precaution is necessary since vesicles are observed docked about 100nm distant from an AZ (Cooper et al., 1995a, 1996b). Thus, we are confident that the second section removed from the AZ of interest was not close to a subsequent section containing an AZ. The structural analysis for the number of docked and RRP vesicles are presented in **Table 4.1**. The

Table 4.1

Prep.	O	RRP	D
Control 1			
SY1	-1	19	5
	0	19	3
	+1	17	6
SY2	-1	20	2
	0	22	4
	+1	20	3
Control 2			
SY1	-1	11	6
	0	13	3
	0	28	5
	+1	14	5
SY2	-1	15	4
	0	18	4
	+1	15	3
Control 3			
SY1	-1	26	4
	0	20	3
	+1	11	3
SY2	-1	19	2
	0	21	4
	+1	17	4
Overall Mean		18	3

Prep.	O	RRP	D
TBOA 1			
SY1	-1	18	3
	0	12	3
	0	13	5
	+1	13	2
SY2	-1	16	3
	0	20	4
	+1	19	3
TBOA 2			
SY1	-1	12	4
	0	19	2
	+1	30	4
SY2	-1	15	4
	0	22	4
	+1	19	0
TBOA 3			
SY1	-1	23	5
	0	17	3
	+1	11	3
SY2	-1	16	3
	0	20	3
	0	22	3
	+1	11	4
Overall Mean		17	3

Table 4.1: Synaptic structural characteristics measured from nerve terminals that were monitored physiologically. A total of 12 synapses (two synapses from each preparation) were fully examined from three control and three TBOA treated preparations. The sections with defined excitatory synapses that revealed an active zone (AZ) were used as a reference point. These are synapses that contain a dense body in the inner face on the presynaptic side. Two serial sections to either side of a section containing a synapse of interest with an AZ were viewed for analysis. If no other AZ was observed on the same synapse over the 4 or 5 serial sections then one section on either side of the one containing the AZ was used for morphological measures of the vesicle pools. In some sections the dense body of the AZ was observed in two sections. The RRP and RP vesicle pools are defined in **O**: Order, **RRP** (readily releasable pool), **D**: Number of Docked vesicles.

Figure 4.6

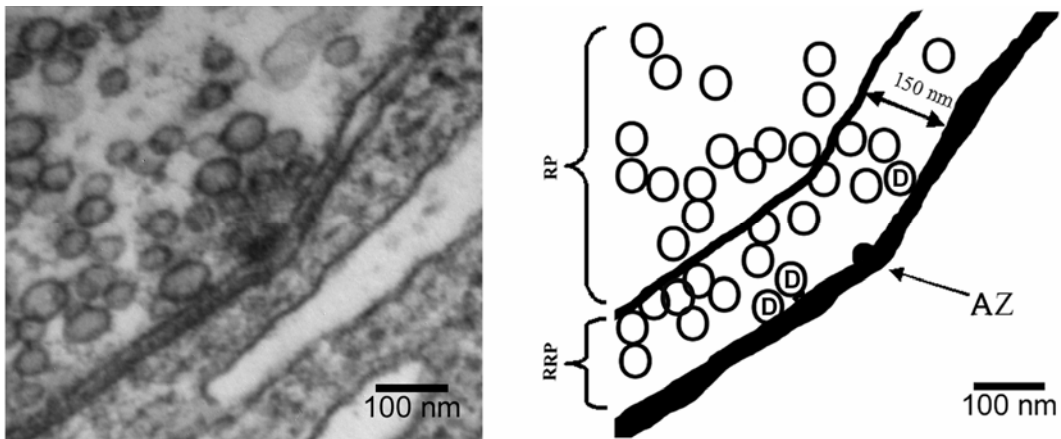


Figure 4.6: The docked and readily releasable vesicle pools were determined by transmission electron microscopy. A representative synapse showing vesicles docked and ones neighboring the presynaptic face (A). The vesicles and synaptic surface are traced from the photomicrographs for determining the number of docked (D) and readily releasable pool (RRP) of vesicles (B). The RRP are vesicles that are not docked but within 150 nm of the presynaptic face. If half or more of a bordering vesicle is within the 150 nm line it is considered to reside in the RRP. Only vesicles touching the synaptic face are considered docked. Only vesicles along the length of synapses were analyzed.

lateral extent of the area measured (along the cell membrane) for the vesicle docking was only on synaptic surfaces. There did not appear to be any effect of the TBOA treatment in the number of vesicles in RRP or in the number of docked vesicles. Sometimes an AZ is seen in two serial sections due to being randomly sectioned (Atwood and Cooper, 1996a). In these cases the section order in the table reads 0 & 0 for the sections containing an AZ and -1 or +1 for the section on either side of the one(s) containing an AZ. The splitting of an AZ was present in one preparation a control and in two TBOA treated preparations. A total of six synapses in each condition were examined. Two different synapses in each tissue sample were analyzed in detail as described.

DISCUSSION

In this study we have demonstrated that within a presynaptic motor nerve terminal of the crayfish NMJ the RP and the RRP of vesicles are distinguishable, by physiological and pharmacological means, in their recycling paths during electrical activity of the terminals. This was accomplished by the use of TBOA, a presynaptic reuptake inhibitor of glutamate transporters, and use of the neuromodulator 5-HT to recruit the RP. This synaptic transmission was assessed by intracellular recordings and quantal analysis from recordings of discrete regions on the nerve terminal obtained by focal macropatch electrodes. During exposure to TBOA and continuous stimulation the evoked single quantal events as well as the spontaneous single events were reduced in size over time. Upon recruiting the RP of vesicles, by 5-HT during maintained high frequency

stimulation in the presence of TBOA, the occurrence in normal single events was increased on average along with the smaller size range observed during the TBOA treatment. The wide variation in size and area of the traces for single events after 5-HT induction was compared to the distributions before and during TBOA treatments. Thus, some vesicles did not have reduced glutamate content (i.e., RP) while others did (i.e., ones rapidly recycling) which suggests that the RP was immune to the treatment of TBOA within the short exposure period but that the rapidly recycling vesicles could not package enough glutamate within their short recycling period.

In a recent study addressing the recruitment of synapses during electrical stimulation alone or in conjunction with 5-HT it was postulated that to maintain a plateau in the amplitude of the graded EPSPs in this preparation during continuous stimulation a steady state of influx, buffering, and efflux of calcium ions is occurring (Sparks and Cooper, 2004). The efflux is probably via the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. A similar model for the actions of electrical stimulation and for 5-HT on vesicle pools was proposed as shown in Figure 4.7. However in the study herein, we address the role of the glutamate transporter in order to further distinguish the rapid recycling vesicle path and the RP path which is enhanced by 5-HT. In the absence of 5-HT and without electrical stimulation few spontaneous releases occur resulting in vesicles to recycle via a slow process (path 1 to 2, Figure 4.7A). In the presence of high frequency electrical stimulation, as we used, a vesicle that was just recaptured from the presynaptic membrane may also recycle through an endosome (ES) intermediate to enter

Figure 4.7

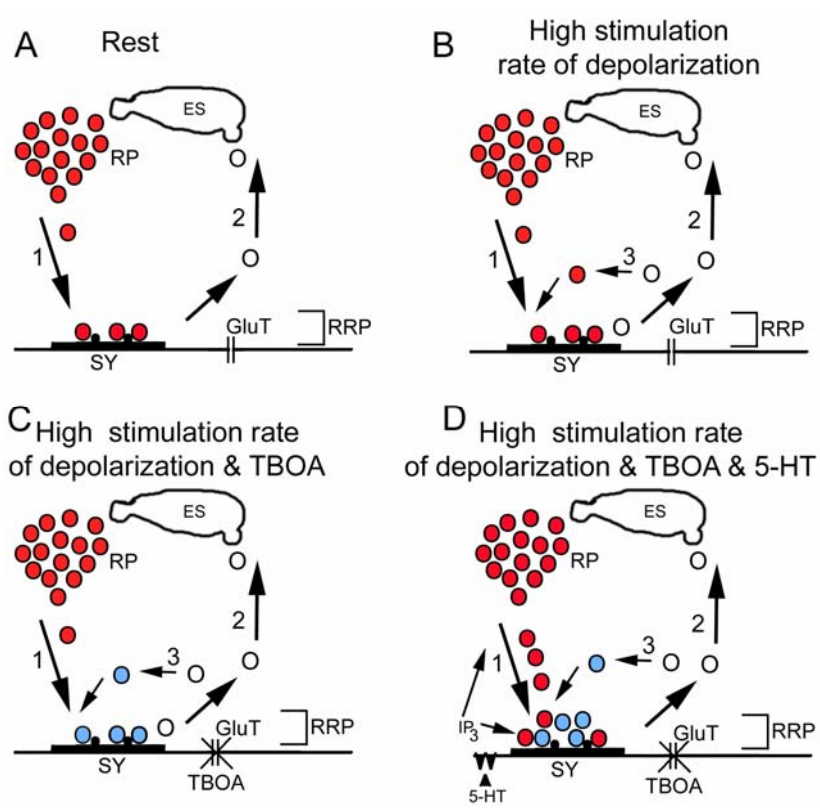


Figure 4.7: A schematic representation of the vesicle pathways within the presynaptic motor nerve terminal. **(A)** In the absence of electrical stimulation **(A)** of the nerve terminal few vesicles will spontaneously be released at slow rate; thus, a slow recycling path (path 1 to 2) will be utilized. Upon electrical stimulation **(B)** path 3 will be recruited into action. Vesicles may be recruited from the reserve vesicle pool (RP, path 1) to the readily releasable vesicle pool (RRP) for docking and fusion with the presynaptic membrane as well during electrical stimulation. The vesicles may then recycle through a slow process and intermediate endosomal (ES) stage (path 2), as well as path 3 that is relatively rapid in recharging the vesicles with transmitter before the vesicle ends up back at the RRV pool. Exposure to TBOA depletes the cytoplasmic glutamate for the rapid recycling vesicles to fill by blocking the glutamate transporter (GluT) **(C)**. The blue vesicles represent the partially filled ones. However, after inducing synaptic depression with the presence of TBOA, the depressed synapses can be recruited by exposure of the terminals to 5-HT. During electrical stimulation the nerve terminal is primed by promoting path 1. The additional activation of the second messenger IP3 can recruit vesicles from RP as well as enhance priming and docking of vesicles at the synapses within the RRP by phosphorylating synaptically relevant proteins. When combining electrical activity, incubation with TBOA and exposure to 5-HT paths 1, 2 and 3 are actively producing a mixed response of partly and fully packaged vesicles **(D)**.

and reside in a reserve pool of vesicles (RP) (path 1 to 2, Figure 7B). As discussed earlier the endosome mediated path is somewhat of an out dated model and supportive evidence in this system is lacking in these motor nerve terminals (Sparks and Cooper, 2004). Mounting evidence supports the notion that that path 3 (Figure 7B) is utilized during repetitive rapid transmission (De Camilli and Takei, 1996; De Camilli et al., 2001; Aravanis et al., 2003). When TBOA is present which is blocking the glutamate transporter (GluT) then the readily recycling pools are not fully packaged with glutamate prior to being released (Figure 4.7C). An additional path in the rapidly recycling path (path 3) induced for electrical activity could be supplemented by vesicles entering from the RP (path 1) by the recruitment induced by 5-HT through IP₃ (Figure 7D). This could be mechanistically explained by promoting vesicles to be released from synapsins and enhance docking by phosphorylating key docking-related proteins by IP₃. (Cooper et al., 2002; He et al., 1999). 5-HT involves an IP₃ cascade in crayfish motor axons (Dixon and Atwood, 1989; Delaney et al., 1991) and various protein kinases are also known to be activated. The types of kinases that can be activated by 5-HT in mobilizing RP vesicles is worthy of future investigations within this system. For example, in *Aplysia* PKC and PKA have been shown to be utilized during 5-HT-induced facilitation (Braha et al., 1990; Chang et al., 2003; Ghirardi et al., 1992; Manseau et al., 2001). It was also shown in *Aplysia* that 5-HT-induced activation of ApCdc42 is dependent on the PI3 kinase and PLC pathways. These then recruit the downstream effectors neuronal Wiskott-Aldrich syndrome protein (N-WASP) and p21-Cdc42/Rac-activated kinase (PAK). These

can regulate the reorganization of the presynaptic actin network (Udo et al., 2005). Recent studies have also revealed that apCAM (*Aplysia* cell adhesion molecule) is associated with structural changes during LTF. Data suggests that the down-regulation of TM-apCAM is required to promote both EPSP enhancement in pre-existing synapses and varicosity formation in nonsynaptic regions (Han et al., 2004). In postnatal rat hippocampal neuronal cultures it was shown that CaM kinase controls vesicle mobilization at low stimulus frequency, while MAP kinase/calcineurin were both active at low and high stimulation frequencies (Chi et al., 2003). Even though 5-HT has not been linked in these neurons to these kinases, the possibility could exist in other preparations such as the crayfish NMJ.

At 50Hz stimulation the vesicle population must continually recycle in order to maintain the degree of output we observed over time. With a limited number of vesicles present within the terminal it is very likely that the rapid vesicle recycling path (Figure 4.7B) is quite active. We have shown with serial electron microscopy of terminals stimulated and exposed to TBOA that vesicles are present in similar quantities in the docking state and slightly less in the RRP as compared to control terminals. With the recycling time frame present during the 20Hz continuous stimulation vesicles probably have a sufficient time to transport some cytoplasmic glutamate into the vesicles via the glutamate transporters on vesicles for a while longer as compared to the 50Hz stimulation. However both stimulation paradigms produce depression. Perhaps the 50Hz recruits the RP vesicles and when mobilized they deplete rapidly in the presence of TBOA, while

during the 20Hz a slower but continuous recruitment occurs of the RP thus providing a longer period before the onset of depression. This is supported by the larger number of stimuli needed to obtain a 50% depression during 50Hz stimulation. The results suggest that there is not enough cytoplasmic glutamate for the rapid recycling vesicle to fill when TBOA is applied to block the extracellular reuptake (Figure 4.7C). However with the application of 5-HT, in the presence of TBOA and rapid stimulation, the vesicles that are fully prepackaged with transmitter are recruited from the RP since their quantal responses are just as large as quantal events prior to exposure of TBOA (Figure 6C and 7D). The evoked as well as spontaneous events observed during 5-HT application are of a mixed population in area of the voltage response which is expected since partially filled vesicles and ones filled prior to stimulation and exposure to TBOA are being recruited by 5-HT. This model of vesicle recruitment from a RP by actions of 5-HT supports previous findings on vesicular populations and kinetics at the crayfish NMJ (Cooper et al., 2003; Djokaj et al., 2001; Dudel, 1965a,b; Southard et al., 2000; Sparks and Cooper, 2004; Tabor and Cooper, 2002). The paths may be targeted independently by cellular processes, such as those by 5-HT-triggered IP3-induced phosphorylation and direct electrical depolarization for calcium-induced cellular responses (Wang and Zucker, 1998). In depressed NMJs of crayfish, release has been shown to be promoted by 5-HT through the use of FM1-43 vesicle dye studies and electrophysiologic studies, also suggesting that different pools and paths are present (Wang and Zucker, 1998). The approach with FM1-43 loading of vesicles during long-term facilitation

has revealed a non-uniform loading of synapses at the lobster NMJ, suggesting differential synaptic function (Kapitsky et al., 2005).

There is variation in quantal responses in the absence of TBOA, however the mean in the area of the single fEPSP is reduced with exposure to TBOA. The decrease in the mean area for single fEPSPs also supports the notion that TBOA is reducing the packaging within the RRP but not altering the RP within the short time frame of exposure used in this study. As for the general reasons for variation in quantal events in non-pharmacological treated preparations, there are a number of factors which are due to presynaptic as well as postsynaptic properties (see review- Faber et al., 1998). There are numerous studies which address the mechanisms for quantal variation and non-linear addition of evoked synaptic potentials (del Castillo and Katz, 1954a,b; Martin, 1955; Ginsborg, 1973; reviews by McLachlan, 1978; Faber et al., 1998; Pawlu et al., 2004). The most plausible explanation for the increased variation over time and reduce postsynaptic response during exposure to TBOA reported in this study is that a reduced glutamate reuptake occurs within the presynaptic terminal for use by the rapidly recycling vesicles.

Mechanisms that are proposed to account for quantal fluctuation can be tested in these relative 'simple synapses' of the crayfish NMJ since one can monitor unitary postsynaptic events. It is known that desensitization is not responsible for quantal fluctuation, since very low probability of release is induced, resulting in evoked events interspersed among many failures and that addition of 5-HT promotes even a greater number events after TBOA induced

depression occurs. In addition, the peak amplitudes do not show a flattened peak, which would be indicative of receptor saturation. Resensitization is rapid in this preparation (Dudel et al., 1992). These glutamate-ergic ligand gated receptor are a quisqualate type with rapid sodium conductance (Shinozake and Shibuya, 1974). There does not appear to be a wide variation in size among clear core synaptic vesicles in crayfish motor nerve terminals, but one needs to consider stereological problems in assessing vesicle dimensions (Atwood and Cooper, 1996a; Feuerwerker et al., 2000; Kim et al., 2000). Also, the size is not informative to the extent of the packaging content. With monitoring single vesicular induced fEPSPs from a spatially isolated varicosity using the focal recording technique, one at least knows that the variation is not due to electrotonic spread from distant sites on the muscle, as is the problem with intracellular monitoring of the postsynaptic cell.

One might argue that TBOA is blocking the glutamate receptors postsynaptically and resulting in reduced quantal sizes. Given that the 5-HT exposure occurs in the presence of TBOA at the same concentration as the previous TBOA exposure and the quantal sizes increase on average, it is not likely that TBOA is blocking the glutamate receptors. The potential mechanisms described in this report may well apply to other synapses within the CNS and NMJ of other preparations in which only inference can be made as to the structural and physiological responses because of the lack in the ability to know which nerve terminals are being used or where quantal events are occurring.

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Chapter Five

OVERALL DISCUSSION

One of the specific aims of this study was to determine if quantal characteristics, based on set parameters, could be grouped and possibly be correlated with synaptic ultrastructure from low output stem regions of the crayfish tonic opener muscle (Chapter 2). To address this goal, new techniques in analyzing quantal recordings, via an automated stochastic program were developed. Another aim was to investigate ways of differentiating synaptic vesicle pools and their physiological significance in synaptic plasticity through neurotoxins and neuromodulators (Chapters 3 and 4). These studies required rebuilding synaptic terminals by TEM to reveal number of synapses, AZs and vesicle locations. The results of these studies revealed that even a low output region of the nerve terminal, such as the stem region, contained a complex synaptic structure in regards to containing multiple AZs with varied spacing distances and synaptic size. In respect to mean quantal content and correlation to stimulation frequency the stem regions were variable in some showing depression while yet others facilitated. Thus, they behaved differently than what had previously been described for varicosities. The parameters to characterize single quantal events did not reveal any obvious sub-clusters in the distributions that could be used as potential synaptic signatures. Thus, particular shaped quantal units could not be assigned distinctive values for probability of release as originally hoped for in these low output stem regions. The studies on synaptic depression and vesicles recycling resulted in clearly defining physiological

distinctive vesicles pools. Such an understanding of the reserve pool had not been physiological defined previously by the use of neuromodulators.

The anatomical and physiological analysis of the stem regions in nerve terminals from this study allows one to now quantitatively compare the stem and varicosity regions. Such comparisons are important in understanding regional properties in the nerve terminal which could account for a number of physiological and morphological observed differences. For example, the developmental and maintained terminal structure of repeating varicosity and stem for the entire length of the terminal is not understood in regards to the regulation of this pattern throughout development of the animal. Why is it that the high output phasic nerve terminals on other muscles of the crayfish do not show such morphological differences of swellings along the nerve terminal as tonic, low output terminals? In addition the postsynaptic responses in the degree of sub-synaptic enfolding vary for the stem and varicose regions in tonic terminals but such differences are not as pronounced in the fine filiform terminals of the phasic terminal (King et al., 1996; Cooper et al., 1995b, Bradacs et al., 1997).

Even though the stem region revealed a reduced number of synapses and AZs, physiological quantal responses for different stimulation frequencies were consistent with very little variation, suggesting that the p for the stem region is more or less equal for each site, which is not the case for varicosities. This implies that a single varicosity, as well as single synapses can not be labeled as a single n , but that the individual AZs should be potentially considered as discrete n 's with each having a variable p value to account for the quantal

variation measured. However, since the stem region did not show quantal sub-clustering one approach would be to define the number of AZs as n with each having an equal p . On the other hand, each AZ site might have a different probability of release but because of variation in quantal responses, due to many possibilities of pre and/or postsynaptic factors, synaptic or AZ signatures based on shapes of quantal responses could not be defined. Until one is able to obtain synaptic preparations with a single AZ will the field be able to define if truly each fusion site of vesicles around a single AZ is equal in probability and so that an AZ can be considered as a single n or if multiple n 's need to be assigned for a given AZ with variable p . It would logically appear that even with a single AZ when 1 vesicle fuses that particular site would then have a lower probability of a subsequent release due to the fact the either another vesicle could not fuse onto the previous vesicle membrane that just fused (complete exocytosis vesicle fusion model), or that if vesicle kinetics operated by the kiss and run model that the vesicle will have to move out of the way before another vesicle could dock. In either case, that particular site would have a lower p for the subsequent fusion event for a defined amount of time. In such a case the neighboring docked vesicles would then be altered in their probability of release since. Thus each site of vesicle fusion has a dynamic probability of release. Given the condition that each n is independent for binomial distribution in determining n and p , a binomial model would not be suitable for characterization of a single AZ with multiple docked vesicles. Since each site would have a p that did not appear random, because of dependence on neighboring sites, and that the AZ as a whole could

be induced to have a relative high probability of release, depending on environmental conditions, the individual sites would not on average be low in probability of fusion. Thus, a Poisson distribution of quantal parameters would not be valid for characterization of release even for a single AZ. The classical approach of assuming uniform p models have been in debate for some time, since the variation in quantal measures are observed in a number of preparations that can not be fully explained by electrical cable properties of the tissue being recorded from nor obvious synaptic structural differences. Approaches to date for non-uniform fusion events have not considered the morphology of a simple single AZ such as for a circular ring of vesicles around a circular AZ on a synapse. Specific attention in characterizing quantal parameters is required and potential quantal variability for a single AZ should be considered before one can describe the n and p parameters for a single AZ. Such stochastic analysis would require each fusion site to have a variable p depending on its recent history, however an initial recruitment of the AZ and assignment of p might be considered to be uniform for each site. An earlier attempt in considering such non-uniform probabilities was approached by Dityatev et al., 2003 with principle component analysis. Non-uniform p has been considered for multiple synapses within a terminal as well as for different boutons/varicosities for a given terminal. Past studies are concerned more on the issue of recruitment of synapses not docking sites around a given AZ. However, the non-uniform stochastic approaches could be considered for a single AZ with variable p 's for each docked vesicle. The proximity of AZs and potential overlapping calcium domains has been considered

and postulated to cause uneven probability of vesicular fusion but without an assignment of variable p_s based on location around the AZs (Cooper et al., 1996b).

The potential attributes of the findings presented in Chapter 2, in regards to the lack of quantal sub-clusters and variable m with stimulation frequency as compared to varicosities, could imply a less stable pool of vesicles for recycling or even differences in calcium handling. There are many multifaceted biochemical differences that could explain the differential effects in the results of quantal variability and synaptic efficacy in relation to stimulation frequency in stems as compared to varicosities. Perhaps the known biochemical differences among phasic and tonic terminals may account for some possible differences in the stem and varicosity regions. It is apparent that the findings on my study have opened many new questions to be examined.

In Chapter 3, synaptic depression was studied at the crayfish extensor muscle and perturbation of vesicular kinetics for addressing the roles of the distinct vesicular pools and actions of the neuromodulator 5-HT. This preparation differs from the opener preparation used in Chapter 2 in that the muscle is innervated by two excitatory neurons, a phasic (high output- fatigable) and tonic (low output, fatigue resistant) neuron. It was found that stimulation of only the phasic neuron (5Hz), and recording intracellularly that EPSPs rapidly increased and then quickly depressed in amplitude. Following a 10 min break after depressing the terminal, when only exposed to saline, the initial EPSP amplitude was larger than the baseline responses prior to inducing depression,

however the rate of depression was greatly enhanced. Further studies with 5-HT and the rate of depression/recovery suggested new possibilities for the role of 5-HT within intact behaving animals. Application of 5-HT prolonged the time to the onset of depression, but once depression started it was more rapid than for terminals only exposed to saline. When a second series of stimulation pulses was presented after a 10 min rest, while exposed to 5-HT, the second induced depression was more rapid than the second depression series for preparations only exposed to saline. This provided insight as to how the nerve terminal controls its vesicle populations in relation to a use dependent need. In saline, the RRP appeared to be the main source of vesicles. Obviously this can only occur if the recycling was rapid for this pool as compared to the RP.

Additional information was gained in this study in the mechanisms of depression. It was considered in a study by Sakurai, 1990 that synaptic depression could be accounted for by a lack of receptivity postsynaptically due to internalization of receptors, however since exposure of 5-HT after depression resulted in a larger EPSPs than without exposure to 5-HT one might suggest that the mechanism of depression is a presynaptic phenomena in this NMJ preparation.

Given the observed results I suggest that the RP vesicles are recruited by actions of 5-HT. I postulate that a lack of ATP is likely a reason for the RRP vesicles to show fatigue in maintaining the high rate of recycling, but this is without definitive proof. It was of interest to see if these vesicle populations changed in the various experimental paradigms, thus TEM was used to visualize

the RRP and RP. It was found that between the control (Saline) and the two experimental groups (saline to 5HT and 5-HT only) the number of vesicles docked and in the RRP did not change a great deal, suggesting that RRP vesicles are either being recycled empty or that there is an energy conservation mechanism in play. This could explain why the same numbers of vesicles are present in saline and depressed terminals. Recently an energy conservation mechanism was possibly observed in the gold fish ON-type bipolar cells in which the RRP was reduced at night as compared to day as shown by Ca^{2+} currents and TEM (Hull et al., 2006). This explanation could also account why the RRP at the crayfish phasic neuron does not change during synaptic depression, in that the number of vesicles remain constant but the available ATP to recycle them is reduced in order to conserve energy. As far as I am aware, such modulation by 5-HT of the vesicle pools in relation to off setting synaptic depression is a novel finding in the field of synaptic transmission. I would hope that investigators of synaptic transmission in the CNS of vertebrates would examine if such possibilities exist for 5-HT modulation in relation to function of neuronal circuits.

In Chapter 4, synaptic vesicle pools were again studied, but at the tonic neuron of the opener muscle, during STF. The target of this study was the glutamate transporter on the presynaptic nerve terminal. The glutamate transporter is the mechanism in which the nerve terminal removes glutamate from the synaptic cleft for repackaging into the synaptic vesicles. To study its role in vesicular dynamics TBOA was utilized which blocks the transporters ability to transport glutamate (Dudel and Schramm, 2003). This in effect removes a

large portion of available glutamate in the nerve terminal, thus the RRP becomes depleted of glutamate to transport into the vesicles. This was shown as a run down effect in recorded EPSPs and fEPSPs. In addition, when 5-HT was introduced to the system, both EPSPs and fEPSPs returned to the same relative amplitudes. This suggested that two vesicle populations were present and under different regulational mechanisms. The RRP needing glutamate that is re-transported into the cytosol and the RP which are prepackaged vesicles were under neuromodulators, which in effect recruits the RP to the RRP. It was again of interest to visualize these two vesicle populations by TEM, so that vesicle numbers could be assessed under control and experimental scenarios. TEM micrographs revealed that there appeared to be no difference in RRP or docked vesicles. Which brings up the same questions that were brought up in Chapter 3. Are vesicles allowed to recycle empty or are they immobilized due do an energy conservation method?

Because the nerve terminal is not a static system, these three projects can be pulled together in order to see part of the big picture of the dynamics of synaptic transmission. Different conditions alter how the structure of the nerve terminal reacts to stimuli. The distribution of synapses and their AZ's alone is extremely plastic, not even considering the vesicle pools. In cultured hippocampal neurons, it has been shown that actin is important in activating immature boutons with immature synapses (silent synapses) during repetitive stimulation to induce long term potentiation (LTP) (Jun et al., 2006). Not only is the synaptic formation plastic during synaptic activity, but the AZ its self in

relation to the presynaptic proteins. Recently, cultured hippocampal neurons were treated with either control saline or saline containing an increase in K^+ , in order to see if the distribution of AZ associated proteins (Bassoon, Piccolo and Rim 1) was altered. It was found by high K^+ stimulation, that the AZ proteins Bassoon and Piccolo remained unaltered but, Rim 1 was decreased at the synapse but was increased in distances 34-200nm (the RP) from the presynaptic membrane (Tao-Cheng, 2006). This is interesting considering Rim 1 is required for the release of transmitter through the interaction of Munc 13-1 (Junge et al., 2004) and synaptic plasticity (Calakos et al., 2004; Kaeser and Sudhof, 2005). This experiment only put the cultured neurons in high K^+ for two minutes before fixation, suggesting that this modification of Rim 1 is relatively rapid which gives insight to the speed of synaptic remodeling.

Not only is the synapse and AZ dynamic, but the vesicle pools available are as well. Since the vesicle pools are divided into separate pools physiologically (the RP and the RRP) (Schluter et al., 2006) it makes sense for the cell to be able to utilize them differently under different conditions. The studies herein have shown their contribution to synaptic plasticity under normal and neuromodulatory effects. The exact mechanism and limitations of plasticity in relation to the vesicle pools are still unknown. It was found in hippocampal pyramidal neurons that induced depolarization, via high $[K^+]_o$, altered sensory adaptation (a situation in which a neuron is less responsive to a prolonged or repeated stimuli), decreased EPSC (currents) and reduced RRP size. This was also shown to be the case after several hours of repetitive stimulation (10Hz)

showing that the RRP is needed for adaptation (Moulder et al., 2006). Upon ultrastructural analysis, the number of docked or total number of RRP vesicles was unchanged compared to control, but when α -latrotoxin (spider toxin that induces vesicle fusion) was introduced to adapted neurons, vesicles were released. This led to the idea that the adaptation was due to a change in how a vesicle primes to the AZ (Moulder et al, 2006). When a vesicle is primed it requires an influx of calcium to fuse with the presynaptic membrane, in the adapted neuron the vesicle is less likely to fuse, because the neuron has become accustomed to the stimulus. This was reversed by application of α -latrotoxin, which has the ability to evoke vesicles that are docked in an adapted scenario by allowing Ca^{2+} to enter the cell causing the vesicle to fuse in a Munc13-1 independent manner (Augustin et al., 1999).

These experiments suggest a sort negative feedback mechanism at play. When a nerve terminal is undergoing adaptation, it allows vesicles to still be in place on the synapse, but alters the machinery needed to cause exocytosis, thus adaptation is observed presynaptically. The question remains, what is the mechanism of this feedback signal to not release each time a stimulus is given under adaptive situations? This brings in the idea of second messenger cascades that can alter synaptic function and thus give rise to synaptic plasticity. It was shown in Munc13-1 mutants that neither calcium ionophores or sucrose shock would allow release of docked vesicles, but could under α -latrotoxin application (Augustin et al., 1999). This implies that Munc13-1 may be a downstream target of a second messenger cascade to block release even in the

presence of Ca^{2+} (i.e. CAM Kinase). But then what is the case for synaptic facilitation and the way the RRP reacts? In the case of neurons that elicit synaptic facilitation, perhaps Munc13-1 is not a target for adapting to sustained or repetitive stimuli, but permitted to stay responsive to an influx of Ca^{2+} .

It is known that second messengers play a role in synaptic plasticity. Dixon and Atwood showed in 1989 that 5-HT in the crayfish caused an IP3 cascade to be activated and they suggested that it may cause an increase in intracellular Ca^{2+} (Dixon and Atwood, 1989). Later this was shown not to be the case by Delaney et al., (1991). However high temporal resolution of regions right at synaptic sites was not available at this time, so possibly a local Ca^{2+} alteration might be a mechanism to account for 5-HT's action. It is known that exposing the crayfish NMJ to $1\mu\text{M}$ of 5-HT will result in spontaneous vesicular fusion events. Thus, it is likely that 5-HT mediates IP3 that could directly result in Ca^{2+} release from internal stores (i.e., ER) through IP3 receptors on the ER. This is now a mechanism that is well established in other systems (Petersen and Cancela, 1999; Mattson et al., 2000; Berridge, 1997, 2005; Berridge et al., 2000) and recently been shown by pharmacological studies of caffeine and ryanodine to be feasible at the crayfish NMJ (Dropic et al., 2005).

This internal release of Ca^{2+} could activate calmodulin (binds four Ca^{2+} ions) which in turn activates CaM-Kinase (CaM-K) and in its fully active form can phosphorylate proteins to either activate or deactivate them. In this case CaM-K could phosphorylate in the integral vesicle membrane protein synapsin, which mobilizes it from the RP to the RRP, increasing synaptic output. In neurons of

Aplysia, 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 so that they could be docked to the presynaptic membrane.

Another example of second messengers affecting synaptic plasticity is that of cyclic AMP (cAMP). It was shown in hippocampal slices that cAMP induces LTP by up regulating the number of active boutons (shown by the vesicular dye FM1-43) via increased protein synthesis and activation of NMDA and AMPA receptors on the postsynaptic cell (Li et al., 1999). cAMP works by activating Protein Kinase A (PKA), which then has the ability to phosphorylate transcriptional factors such as CREB which may then up regulate certain proteins synthesis that are involved in synaptic transmission (i.e. AZ proteins). This possibly increases the number of vesicles that are released at a single AZ (Geppert et al., 1997), causes synthesis of new synaptic connections (Bolshakov, et al., 1997) or activates previously silent synapses (Yao, et al., 2006). It is also suggested that the cAMP and calmodulin pathways work in concert to achieve optimal gene up regulation (Dash et al., 1991).

Considering the whole organism and its behavior can defiantly impact what occurs at a cellular level, one has to keep this in mind in regqrd to synaptic function. It has been previously shown that the same neuron in crayfish studied at different periods of the year show different synaptic properties and morphology (Lnenicka and Zhao, 1991). In crayfish collected during the summer season, the phasic neuron when stimulated has a small initial EPSP, facilitates, is fatigue

resistant and has a greater capacity of transmitter release (the typical laboratory response of a tonic neuron). Morphologically, the summer phasic neuron terminal has more varicosities. In contrast, the winter crayfish phasic neuron elicits a large initial EPSP, but fatigues quickly (the typical laboratory preparation response of the phasic neuron), and presents fewer varicosities. This is possibly due to an energy conservation method that allows crayfish to alter their synaptic physiology depending on the demands the environment presents.

This situation obviously must come into play considering that all the crayfish studied in these experiments were taken from their native environment and set in an isolated tank in a laboratory setting separated from natural environmental cues. How does this cause quantal differences in vivo? In some cases, the crayfish were used soon after removal from their natural environment and others after they had been removed for, in some cases, for over a year. This obviously can have a tremendous impact on how a neuron's synaptic properties react to stimuli considering the crayfish has been docile for an extended amount of time. Most likely, housed in a storage tank has altering effects on different individuals, but being removed from seasonal changes and environmental cues would mean that the crayfish does not have input on how active it should be, thus giving an altered response from that directly taken from its natural habitat.

A second overall problem with studying synaptic transmission of the opener muscle is simply the method in which it is obtained. The walking legs are obtained by inducing the crayfish to autotomize them by crushing the ischiopodite (proximal leg segment closest to the body). This causes stress in the crayfish

which have an open circulatory system, thus any stress related neuromodulators (i.e. 5-HT) would be released and bath the opener muscle before autotomy, thus affecting neuronal function before experimentation. This was reduced to a minimum, by not handling the crayfish for an extended amount of time and obtaining the walking leg in a rapid manner. It was previously shown that NMJ's of gravid females and crayfish exposed to novel stress were less responsive to exogenous treatment of 5-HT (Page and Cooper, 2004), showing an alteration in neuronal function due to stress. Thus, generally males were used for my experimentations.

Not only is the opener muscle exposed to internal stores of neuromodulators before autotomy, but through autotomy the neurons that innervate the extensor and opener are removed from their cell body which originates in the first thoracic ganglion (Keim, 1915; Vogt, 2002). Although this showed no effect on synaptic depression in the *in situ* studies done here and has been shown to have no effect on depression in *in vivo* studies of the crayfish abdominal muscle (Cooper et al., 1998), subtle effects could exist with out the cell body for maintenance of synapses (protein turn over, essential vesicle or AZ proteins, etc.)

Some neuronal modifications that occur over the animal's life may not be accounted for in animals caught from the wild and held in the laboratory for the short observation period of a few weeks. For instance, it is unknown if the crayfish was extremely active before its capture or if it were in a hostile environment that would cause extreme stress. Also, being put into a laboratory

holding situation means that it does not receive natural light cycles. Even though it is attempted to simulate natural light conditions in the lab, natural circadian rhythms are sure to be affected.

Obviously, the crayfish offers a multitude of systems to study synaptic transmission, but as with all systems, crayfish have their limitations. Working with the systems available to us, the field of synaptic transmission continues to progress and even the crayfish will likely add to future discoveries in mechanisms of synaptic transmission. It is unfortunate that the crayfish does not serve as a genetic model as well, so specific proteins could be manipulated and observed. But, the crayfish may utilize genetic achievements from other systems such as yeast, *C. elegans* and *Drosophila*. Recently, a Nobel Prize worthy genetic tool (Fire and Mello, 2006), RNAi has proven to be a strong genetic tool for silencing genes in a multitude of systems but with different affinities. For example, RNAi in *Drosophila* must be genetically inserted into the genome to function, while in *C. elegans*, RNAi is readily absorbed through the gut. This useful tool may also work in the crayfish to down regulate certain proteins involved in synaptic transmission because of its accessible nervous system. It may be possible to incorporate RNAi into the open circulatory system for a long term study or, because of their size, the neurons may be directly injected with RNAi.

The ability to pull together biochemical, nuclear, genetic and structural factors in a complete neuronal function is of utmost importance to understand the nervous system as a whole. These experiments extend the knowledge base of

nerve terminal structure and function, and future experiments in this physiological model organism will indeed give further insight.

Appendix A: Crayfish Nerve/Muscle Fixation and staining

BUFFER SOLUTION A: (Store at 4°C)

- 1) 16.7 ml of 0.6 sodium cacodylate (.6mol/L)(.1L)(214g/mol)=12.84g into 100ml H₂O)
- 2) 0.022g CaCl (optional for Ca²⁺ testing)
- 3) 4.0g Sucrose
- 4) adjust to pH: 7.4 via HCl
- 5) adjust to 100ml with H₂O

FIXING SOLUTION: (Store at 4°C)

- 1) 6.25 ml of 8% glutaraldehyde
- 2) 0.625 ml of 16% formaldehyde or (0.1g of paraformaldehyde)
- 3) 13.0 ml of .01M Buffer Solution

Glutaraldehyde Fixation:

- 1) 2.5 % Glutaraldehyde and 0.5% formaldehyde dissolved in the buffer solution A adjusted to pH 7.4 via HCl.
- 2) Fix for 2 hrs.
 - O/N fixation in fridge may be acceptable.
- 3) Rinse 2hrs (4—8 changes) in buffer solution.

Post-Fix:

(Use a 2% OsO₄ in buffer A, OsO₄ comes in 4% ampules 10 ml, so cut down w/ buffer A)

- 1) Post-Fix 1 hour in 2% OsO₄ w/buffer.
- 2) Rinse in Buffer A for 15 min X 3
- 3) During Rinses, fix resin

Resin: (These measurements must be very accurate!)

Add: 4.25g EMBED 812

6g Araldite

Mix all w/ Stir Bar—fast rotation

11.25g DDSA

~10 min later...

Add: .47g Accelerator (DMP-30) and continue stirring.

-(NOTE: this makes a ¼ batch of resin, if doing many samples adjust accordingly)

EtOH Dehydrations (7 minutes each)

50%...70%...80%...90%...100%...ABSOLUTE 100%

2 X w/ Propylene Oxide (PO) (if using polystyrene beads, finish with ABSOLUTE 100%)

Infiltration:

In a small sample vile:

Add: 10ml PO

10ml Resin

Rotate O/N (remove PO)

-(Note: If infiltrating larger muscles may want to do additional PO/resin changes.
75% PO, 100% EtOH for 18hrs.)

Embedding:

1. Resin Recipe from above.
2. Mix for 30 Min (high rotation, it will get thick).
3. 2 resin changes one hour apart.
4. Place in resin trays.
5. Polymerize in oven @ 60°C for 48 hrs.
6. Remove and allow to harden ~ 2-4 hrs.

Post-Stain: ALWAYS SPIN DOWN FOR 10MIN, MAX SPEED.

First Stain: Uranyl acetate (dissolved in 70% EtOH)

1. Add UA to polypropylene centrifuge tube.
2. Add 70% EtOH, raise to 12-13 ml
3. label & Date tube (store in the dark)

Second stain: Lead citrate

1. Pre-Boil dddH₂O (to remove CO₂)
2. .04g Lead citrate to centrifuge tube.
3. Add pre boiled H₂O (cooled) to centrifuge tube to 10 ml.
4. Dissolve via rigorous vortexing.
5. Spin Down to remove impurities
6. Label & Date (store in the dark)

Procedure for Post-Stain

- A. Place parafilm in clean dish (lock down w/ forceps).
- B. Add on drop UA for each sample on parafilm.
- C. Do the same in a separate dish with PbCit.
- D. Lay grids upside down on drop (remember which side the sample is on).
- E. Allow to stain for 10 min in UA.
- F. Rinse well with dddH₂O—Carefully blot with filter paper.
- G. Transfer to drops of PbCit.
- H. Stain for 5 min.
- I. Rinse well with dddH₂O—Carefully blot with filter paper.
- J. Allow to fully dry on filter paper.
- K. Place in grid box

Appendix B: Inserting an Artificial Spike

For use with the “S” program written in “R” language found at: <http://www.r-project> and used with the analysis program in Chapter 2 of this dissertation (www.ms.uky.edu/~viele/epsps/epsps.html).

Inserting an artificial spike may be desirable if the spike is buried within the noise, the spike wanders within the trials (due to a change in conduction velocity), or is not prominent enough to be detected by the algorithm. The following steps will allow the user to insert an artificial spike in the area of all the traces that the recorded spike is found in the original data set.

The original data set is in a single column which needs to be converted to multiple columns, each column representing a single trace. These columns will then need to be transposed so that multiple points (representing the original spike) may be replaced by points that the algorithm may pick up. This new data set will in turn, need to be converted back to a single column so that the traces=epsps function can be run.

1. **matrix(output05g.txt,ncol=1000)** – This will take the column of 2,560,000 data points and convert them to 1000 columns each containing a single trace (2560 data points).
2. **matrix(output05g.txt,ncol=2560,byrow=T)** – This will transpose all the columns to rows so that all the points in each trace are in the same column e.g. there will be 2560 columns each containing 1000 points (each row now represents a single trace). This file will then have to be saved as a text file that can be imported into a spreadsheet.
3. **write(output05g.txt,file="c:/epsps/output05g1.txt",ncolumns=2560)** – This will save the matrix written in step 2 to a text file on your hard drive. When the file is imported into a spread sheet, locate the columns that correspond to the original spike found in the trace and replace the columns with values the algorithm may pick up (i.e. -.03, +.03, -.02 (a spike buried in the noise) to -1, +1, -.025 (an artificial spike that will be well out of the noise), refer to figure four). This will now need to be saved to your hard drive i.e. “c:/epsps/output05g2.txt”. This will then be imported back to R.
4. **output05g2=scan("c:/epsps/output05g2.txt")** – This will import your new spreadsheet with the artificial spike so it may be converted back into a single column to run in the traces=epsps function.

5. **matrix(output05g2,ncol=1)** – This will convert the new spreadsheet to a matrix and convert it into a single column containing all 2,560,000 points that can be run in the epsp.r program. This again needs to be saved as a text file so it can be read by the epsp.r program.

6. **write(output05g2,file="c:/epsps/output05g3.txt")** – This will save the new single column to your hard drive to be run by the epsp.r program. Now import this file instead of your original file in section 4.1.

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