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Molecular Mechanisms of Neuropeptide Secretion from Neurohypophysial Terminals: a Dissertation

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James M. McNally

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PROGRAM IN NEUROSCIENCE

Molecular Mechanisms of Neuropeptide Secretion from Neurohypophysial Terminals

A Dissertation Presented
By

James M. McNally

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**Anthony Carruthers, PhD,
Dean of the Graduate School of Biomedical Sciences**

PROGRAM IN NEUROSCIENCE

I would like to dedicate this work in the memory of my mother, Dianne McNally. Without her constant encouragement and support none of this would have been possible, and whose courage in the face of adversity constantly inspires me
I will miss you always

Acknowledgments

First and foremost, I would like to thank my advisor José Lemos for allowing me to perform my thesis research in his lab. I owe a large debit of gratitude to José for all of his support and guidance over the past few years.

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Abstract

A clear definition of the mechanisms involved in synaptic transmission is of paramount importance for the understanding of the processes governing synaptic efficacy. Despite decades of intense study, these mechanisms remain poorly understood. The work contained in this thesis examines several such mechanisms using the hypothalamic-neurohypophysial system (HNS), a classical preparation for the study of Ca^{2+}-dependent neuropeptide release.

The first portion of this thesis is comprised of my efforts to define the cellular machinery essential for the exocytosis of secretory granules isolated from peptidergic neurohypophysial terminals of the HNS. Here, using the planar lipid bilayer model system, I have been able to show that syntaxin alone in the target membrane is sufficient to elicit fusion of modified neurohypophysial secretory granules. Surprisingly, SNAP-25 does not appear to be necessary for this process. This suggests that syntaxin may be able to substitute for SNAP-25 to form functional non-cognate fusion complexes. Additionally, the coupling of amperometric detection with the planar lipid bilayer system has allowed me to confirm these results using native, unmodified secretory granules, and also provides some insight into the kinetics of release in this reconstituted system. This model system should provide a convenient means for the study of additional regulatory factors believed to be involved in secretory vesicle exocytosis.

The second and third sections of this thesis involve my examination of the role of presynaptic Ca^{2+} stores in neuropeptide secretion from isolated peptidergic neurohypophysial terminals (NHT). I initially examined the source of recently discovered ryanodine-sensitive Ca^{2+} stores in this system. Using Immuno-electron microscopy I have found that ryanodine receptor (RyR) labeling appears to co-localize with large dense core granules. Additionally, I have shown that a large conductance cation channel, with similarities to the RyR, found in the membrane of these granules has the same characteristic response to pharmacological agents specific for the RyR. Further, application of RyR agonists modulates basal neuropeptide release from NHT. These results

suggest that the large dense core granules of NHT serve as the source of a functional ryanodine-sensitive Ca^{2+} store.

Recent work has revealed that spark-like Ca^{2+} transients, termed syntillas, can be observed in NHT. These syntillas arise from ryanodine-sensitive intracellular stores. In other neuronal preparations, similar Ca^{2+} transients have been suggested to affect spontaneous transmitter release. However, such a role for syntillas had yet to be examined. To assess if syntillas could directly trigger spontaneous release from NHT, I used simultaneous Ca^{2+} imaging along with amperometric detection of release. Amperometry was adapted to this system via a novel method of false-transmitter loading. Using this approach I have found <u>no</u> apparent correlation between these two events, indicating that syntillas are unable to directly elicit spontaneous transmitter release.

As this finding did not rule out an indirect modulatory role of syntillas on release, I additionally present some preliminary studies examining the ability of ryanodine-sensitive Ca^{2+} release to modulate vesicular priming. Using immunocytochemistry, I have shown that RyR agonist treatment shifts the distribution of neuropeptides toward the plasma membrane in oxytocinergic NHT, but not in vasopressinergic NHT. RyR antagonists have the opposite affect, again only in oxytocinergic NHT. Further, I have found that application of RyR agonists result in a facilitation of elicited release in NHT using membrane capacitance recording. This facilitation appears to be due primarily to an increase in recruitment of vesicles to the readily-releasable pool. These findings suggest that ryanodine-sensitive Ca^{2+} stores may be involved in vesicular priming in NHTs.

Taken together, the work presented in this thesis provides some new and interesting insights into the underlying mechanisms and modulation of transmitter release in both the HNS and other CNS terminals.

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CHAPTER I: General Introduction

The Hypothalamic-Neurohypophysial System

The hypothalamic-neurohypophysial system (HNS), illustrated in figure 1.1, represents a classical preparation for the study of Ca^{2+} stimulated release (Douglas and Poisner, 1964). The anatomy and physiology of the HNS has been well characterized and is highly conserved throughout all mammalian species (Stopa et al., 1993). The main neuronal component of this system is comprised of the magnocellular neurons (MCN), which are responsible for the release of the neuropeptides oxytocin (OT) and vasopressin (AVP). The cell bodies of these neurons reside in both the supraoptic and paraventricular nucleus of the hypothalamus. The MCN send the bulk of their axonal extensions along the hypothalamic tract to the posterior lobe of the pituitary, also know as the neurohypophysis. Here the axons form terminal expansions, which appear to comprise the main site of neuropeptide release for MCN. These terminals reside next to capillary beds, and release neuropeptide into the perivascular space to be carried out into the bloodstream.

In this work, the HNS has been employed to address both the molecular basis of exocytosis, as well as the role of intracellular calcium in neuropeptide release. Due to the limited experimental accessibility of most small nerve terminals endocrine cells are generally employed as a model system to study the fusion and release of large dense core granules. However, extrapolation of findings from these cells to central nerve terminals can be problematic. The HNS avoids these issues, as MCN terminals are true central nerve terminals. These

neurohypophysial terminals (NHT) are large in size (up to 10 µm), can be easily isolated, and are able to survive for extended periods after isolation, making them ideally suited for such research. NHTs provide a greatly simplified model system with which to define mechanisms affecting neuropeptide secretion, as they containing relatively little intracellular machinery beyond LDCG, microvesicles and mitochondria (Lemos, 2002). Further purification of NHTs yields highly enriched preparations of LDCGs, which can be employed to examine the nature of the granules themselves (Lemos et al., 1989).

I. Molecular Mechanism of Exocytosis

Exocytosis is the process of cellular secretion in which substances contained in intracellular vesicles are discharged from the cell by fusion of the vesicular membrane with the outer cell membrane. A comprehensive understanding of factors involved in exocytosis is of paramount importance in the study of synaptic function. Extensive work has been performed in this area, yielding a vast array of protein and lipid components that may be involved in this process. However, how these components are able to come together to elicit this function remains somewhat of a mystery. While molecular biology is a powerful tool; genetic manipulations, such as deletion or enhancement of proteins thought to be involved in fusion and release, can only tell us so much about what role these factors play in this process. Truly defining their role necessitates a simple reconstituted system using defined components.
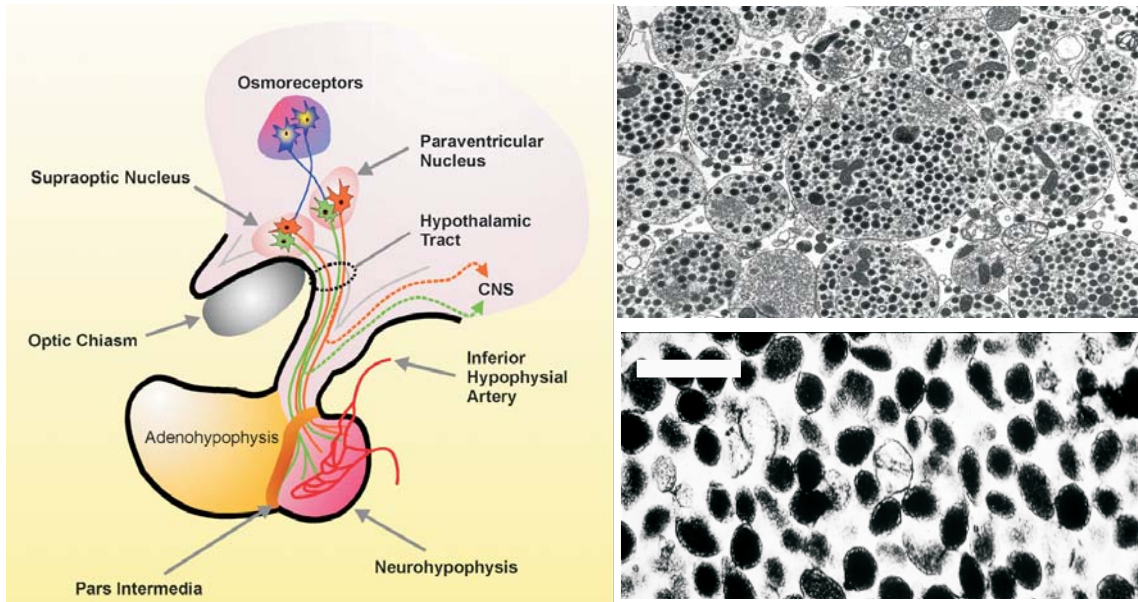
Figure 1.1: **The hypothalamic neurohypophysial system.** (**Left**) The main neuronal component of this system is comprised of magnocelluar neurons, which are responsible for the release of the neuropeptides oxytocin and vasopressin. The cell bodies of these neurons reside in the hypothalamus, and project their axons along the hypothalamic tract to the posterior lobe of the pituitary, the neurohypophysis. Terminal expansions of these axons reside next to capillaries beds, and release neuropeptide into the periventricular space, where it can be carried into the bloodstream. (**Top Right**) Electron micrograph of isolated neurohypophysial terminals. Terminals comprise the main site of peptide release in this system and contain little intracellular machinery beyond dense core granules. (**Bottom Right**) A highly purified preparation of isolated large dense core granules can be obtained via density gradient centrifugation of these terminals. (scale bar is 500 nm)

(**left:** figure adapted from Lemos, 2002; **Bottom Right:** Figure adapted from Lemos et al., 1989)

Specialized cells for neurotransmission, enzyme secretion, and hormone release utilize a highly regulated secretory process (see figure 1.2). Specifically in magnocellular neurons, neuropeptides are packaged into large dense core granules in the cell body and then transported down the axon to the nerve terminal. Once in the terminal granules are trafficked to the plasma membrane where they are docked and primed for release. These neuropeptide filled vesicles then fuse with the plasma membrane in a calcium-dependent manner and release their contents, allowing the neuropeptide to produce its downstream effect. The delivery and docking of secretory vesicles with the presynaptic membrane are topics of great interest; however the first section of this thesis will focus on the mechanism responsible for membrane fusion and release.

Core Fusion Machinery

In order for exocytosis to occur the vesicle membrane must be brought into close apposition with the plasma membrane. Extensive evidence confirms that SNARE (Soluable N-ethylmaleimide sensitive factor Attachment protein REceptor) proteins play a central role in this process (Jahn et al., 2003; Jahn, 2004; Sollner, 2004; Sudhof, 2004). Evidence of this role is best exemplified by research involving the SNAREs responsible for regulated neurotransmitter release in neurons and neuroendocrine cells. These SNARE proteins include syntaxin 1 and SNAP-25 located in the presynaptic membrane, and synaptobrevin-2/VAMP-2, in the membrane of secretory vesicles. Botulinum and
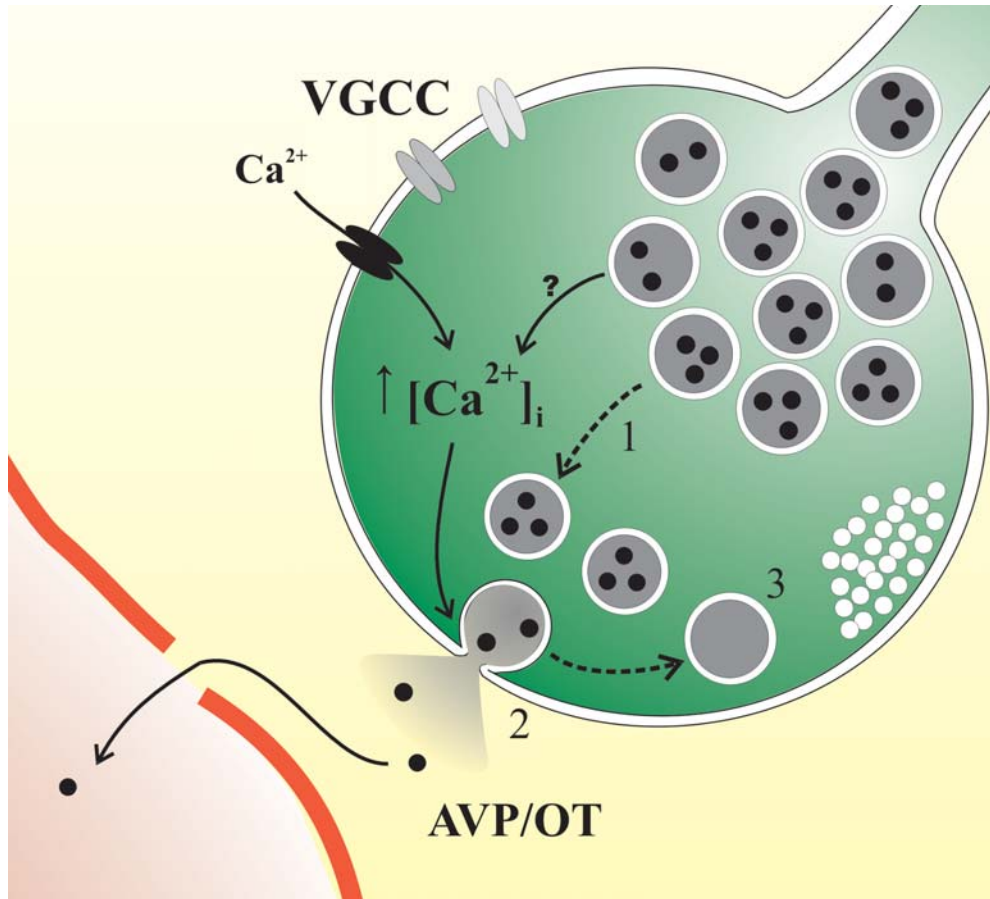
Figure 1.2: **The life cycle of Neurohypophysial dense core granules.** The neuropeptides oxytocin and vasopressin are packaged into LDCGs in the cell bodies of MCN and are transported to the nerve terminals. (**1**) Once in the nerve terminal LDCGs are trafficked to the plasma membrane, where they are docked and primed for fusion. (**2**) Primed LDCG can then undergo Ca^{2+}-triggered fusion, resulting in the release of neuropeptide into the bloodstream. This can process can proceed in two distinct ways. LDCG can undergo "kiss and run" fusion, where only a portion of the granule cargo is released, or full fusion resulting in complete collapse of the granule membrane into the plasma membrane, completely emptying the granule neuropeptide. If kiss and run fusion takes place, the granule can be recycled for further rounds of exocytosis, (**3**). If full fusion occurs, vesicular membrane proteins can then be taken back up via endocytosis for either degradation or recycling (not pictured).

tetanus neurotoxins, which block neurotransmitter release, specifically cleave these proteins showing that they play an essential role in this process (Montecucco and Schiavo, 1993).

All SNARE proteins posses a conserved region referred to as a SNARE motif. Association of the hydrophobic faces of SNARE motifs leads to the formation of a highly stable coiled-coil complex, the so called "SNARE fusion complex" (Sutton et al., 1998). The make-up of these motifs separates the SNAREs into two subfamilies, Q- and R-SNAREs, named after the amino acids found in the middle of the polar layer of the α-helical SNARE motif (Fasshauer et al., 1998). Biochemical evidence shows that stable SNARE fusion complex formation only occurs when the four-helical configuration contains three Q-SNARE motifs, one from syntaxin and two from SNAP-25, and one R-SNARE motif from VAMP 2. However, this may not be entirely the case. Other combinations of SNARE proteins are able to form complexes, although not as stable (Fasshauer et al., 1999). The physiological relevance of these complexes has yet to be determined.

All membrane fusion events of a secretory nature involve the formation of coiled-coil complex formation between SNAREs anchored in opposing membranes (Wojcik and Brose, 2007). Assembly of this complex is thought to act as a fusion catalyst by overcoming the energy barrier separating the vesicular and presynaptic membranes, leading to fusion (Lin and Scheller, 1997). Formation of this complex leads to a tight connection between the fusing

membranes (see figure 1.3). The mechanical force imparted on the membranes by this process leads to membrane deformation which facilitates fusion (Jahn and Scheller, 2006). Further, it has been found that syntaxin mutations alter neurotransmitter flux through fusion pores and alter pore conductance, revealing that SNAREs are an integral part of the fusion pore (Han et al., 2004)

This action of cognate SNAREs has been shown to be sufficient to drive fusion of liposomes, and of cells when SNAREs are translocated to the cell surface (Weber et al., 1998; Hu et al., 2003). However, fusion in such *in vitro* systems is extremely slow, requiring hours to reach saturation, much slower than the time timescale observed for native exocytotic events (Chow et al., 1996; Sabatini and Regehr, 1996). Additionally, this fusion proceeded independent of calcium. Mores recent studies have shown that inclusion of synaptotagmin in such reconstituted systems increases fusion efficiency in the presence of calcium (Tucker et al., 2004). However, these minimal systems still lack much of the regulatory mechanisms necessary to reconstruct native exocytosis.

Regulation of SNARE Mediated Fusion

Since exocytosis is a tightly regulated process, SNARE complex assembly alone does not instantaneously lead to membrane fusion. While the SNAREs appear to function as the core component of exocytosis, this process requires a host of other regulatory mechanisms. The proteins involved in these
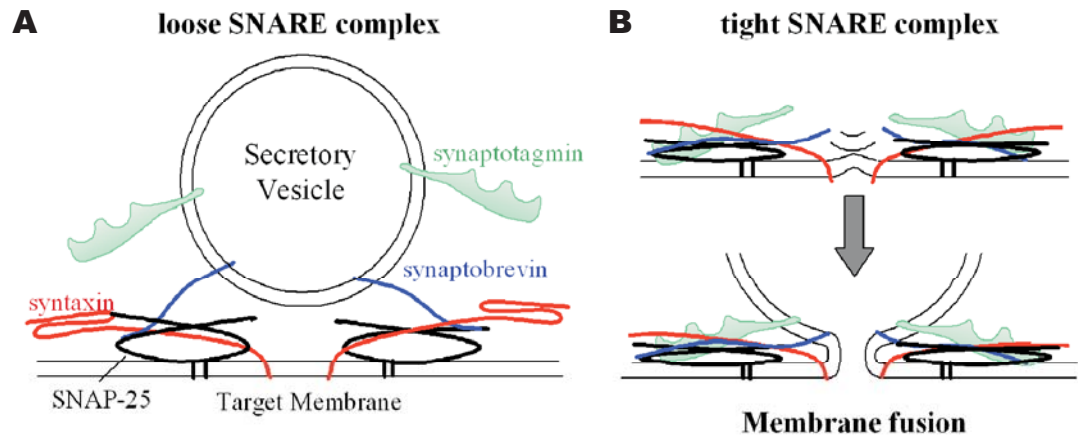
Figure 1.3: **The SNARE fusion complex.** (**A**) The SNARE fusion complex is formed by the transmembrane proteins synaptobrevin in the vesicular membrane, and syntaxin and SNAP-25 in the target membrane. Initially these proteins associate in a loose complex, where only the N-terminal portions of the SNARE motifs of these proteins associate. This association nucleates the formation of the coiled-coil SNARE fusion complex. Synaptotagmin, located in the vesicular membrane, contains several functional calcium binding domains and is believed to serve as a key component of the Ca^{2+}-trigger for fusion. (**B**) Upon stimulation with Ca^{2+}, synaptotagmin binds the SNARE complex and associates with acidic lipids in the plasma membrane. This allows the loosely associated SNAREs to further associate forming a tight SNARE complex. Thus, bringing the opposing vesicular and target membrane into close aposition, and slightly deforming the membranes. This process is believed to act as a catalyst for membrane fusion.

(Figure adapted from Woodbury, 2007)

mechanisms most likely determine the specificity of membrane fusion and provide for the calcium-dependence of transmitter release at the synapse.

Proteins in the sec1-Munc18 (SM) family, have been shown to play a role in modulating the association of SNARE proteins (Wojcik and Brose, 2007). Munc-18 has been shown to bind to syntaxin it in a closed conformation. In chromaffin cells, genetic deletion of Munc-18 results in a large reduction in the amount of LDCGs that are closely associated with the plasma membrane (Voets et al., 2001). This supports the idea that this protein plays a role in vesicle docking. Munc-18 has a high affinity for the closed conformation of syntaxin. In its closed form, the N-terminal regulatory domain binds and occludes the SNARE motif of syntaxin (Nicholson et al., 1998), thus keeping it from interacting with other SNAREs. Zilly et al (2006) have show that in drosophila that Munc18-1 can be displaced from syntaxin by synaptobrevin, but only in the presence of SNAP-25. Therefore, Munc-18 may aid in the formation of syntaxin/SNAP-25 heterodimers that are capable of associating with synaptobrevin, leading to fusion. Recent work shows that Munc-18 may also have a role in priming LDCGs for release in addition to its role in docking (Gulyas-Kovacs et al., 2007).

Munc-13 also appears to regulate SNARE complex assembly. It is believed act in a vesicle priming role, as Munc-13 knock-out neurons show a complete loss of readily releasable (primed) vesicles (Augustin et al., 1999). In chromaffin cells however, deletion of Munc-13 showed no affect on secretion, while overexpression of the protein significantly enhanced secretion (Stevens et

al., 2005). Use of Munc-13 mutants in this study showed that this enhancement of secretion (priming) was dependent upon munc-13's ability to bind syntaxin. As Munc-13 and Munc-18 were believed to bind to an overlapping domain of syntaxin 1, it was thought that Munc-13 may be responsible for displacing Munc-18 from syntaxin (Sassa et al., 1999). However, in vitro experiments show that purified Munc-13 cannot bind syntaxin (Basu et al., 2005), which would appear to indicate that other factors are required for this association. Evidence suggests that the syntaxin binding protein tomosyn may fill this role (Wojcik and Brose, 2007), and work in concert with Munc-13 to regulate the formation of the SNARE fusion complex.

The mechanism responsible for the calcium triggering of exocytosis has been studied intensively but has yet to be conclusively determined. Synaptotagmin and complexin are believed to play a significant role in this process. Complexins are small helical proteins that bind SNARE complexes and also appear to be necessary for Ca^{2+} dependent exocytosis. Several recent studies have shown that complexin is capable of blocking SNARE mediated membrane fusion (Giraudo et al., 2006; Schaub et al., 2006; Tang et al., 2006). Thus, complexin appears to act as a clamp on SNARE complexes, keeping them from driving fusion prior to activation by the proper signal.

The vesicular transmembrane protein Synaptotagmin 1 is believed to provide the fast Ca^{2+} trigger for exocytosis (Sudhof, 2004). This protein possesses two cytoplasmic calcium-binding C2 domains referred to as the C2A

and C2B domains (Bai and Chapman, 2004). These calcium binding domains complex with Ca^{2+} ions to interact with the negatively charged lipids in the plasma membrane. Synaptotagmin 1 has also been shown to bind SNARE complexes in a Ca^{2+} dependent fashion. Upon Ca^{2+} influx, synaptotagmin interacts with the complexin clamped SNARE fusion complex and the target membrane in such a manner that displaces complexin, and promotes membrane disruption which leads to exocytosis (Carr and Munson, 2007).

Other trafficking and tethering factors such as the members of the Rab-GTPase superfamily, CAPS, and the exocyst may be involved in tethering and docking LDCG at the proper sites on the plasma membrane to undergo fusion (Ghijsen and Leenders, 2005; Sugita, 2008). However, the contribution of SNARE to these processes have not been fully worked out (Jahn and Scheller, 2006). Although much has been elucidated, the complete understanding of the role of SNAREs and other regulatory factors in mediating exocytosis still remains elusive.

Development of a Planar lipid Bilayer Model Synapse

For exocytosis to occur in the synapse, fusion of the vesicular membrane and the presynaptic membrane must occur. The first step in *in vitro* reconstitution of this process necessitates the development of a relatively simple model system that can be used to detectably follow vesicle fusion. Generally this has been investigated using liposome fusion assays, where the fusion of two populations of

artificial liposomes can be monitored using fluorescence (Kreye et al., 2008). However, this technique looks at the fusion of a large population of liposomes, and does not allow visualization of individual fusion events.

Our model system, the artificial bilayer (black lipid membrane) has proven to be more than sufficient for the modeling of the presynaptic membrane (Figure 1.4). Artificial bilayers, first described by Mueller et al.(Mueller et al., 1962), are ideal in that their lipid composition can be controlled, as well as the environment on either side of the bilayer, making the system extremely adaptable. In addition, proteins and other factors can be reconstituted into bilayer, adding to the system's versatility. Further the fusion of a liposome to a planar lipid bilayer more closely mimics fusion of a secretory vesicle with the plasma membrane, than fusion in other *in vitro* model systems (Woodbury et al., 2007).

Initial work performed with this model system utilized artificial vesicles loaded with fluorescent dye as a means to detect fusion. Using a fluorescence microscope, observation of the bilayer showed that fluorescent vesicles fuse to the bilayer and in so doing release their fluorescent contents (Woodbury and Hall, 1988; Woodbury, 1990). This simple system lacked many of the important biological components involved in fusion, and the amount of vesicle fusion observed was very low, requiring the addition of an osmotic gradient across the bilayer to drive the fusion. Fusion was increased only when the vesicle (*cis*) side of the bilayer was made hyperosmotic, a condition which should induce vesicles to shrink.
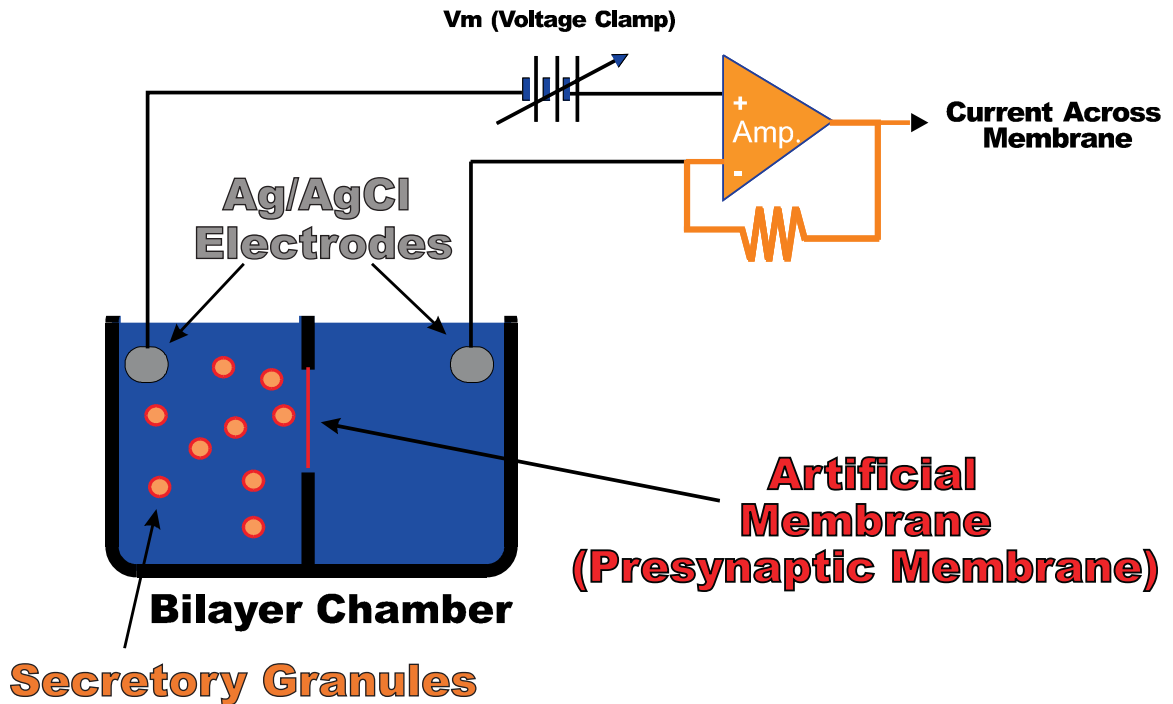
Figure 1.4: **The vertical planar lipid bilayer.** The vertical planar lipid bilayer apparatus consists of two chambers separated by a small apperature, upon which an artificial membrane can be formed. Bilayer conductance can be monitored with a standard voltage clamp amplifier. This system allows complete control of both the composition of the bilayer, as well as the solutions on either side. In our model system the artificial bilayer is used to represent the presynaptic membrane. Secretory granules can be added to one of the chambers, and their fusion with the planar bilayer can be monitored.

(Figure adapted from Woodbury, 1999)

Interestingly, addition of an ion channel to the vesicle membrane resulted in a large increase in number of vesicle fusions (Woodbury and Hall, 1988). This observation was quantitatively explained by increased entry of the osmotic agent through the vesicular channel. For vesicles in contact with the bilayer membrane, the increased vesicle osmolarity causes water to flow across both the bilayer and vesicle membranes inducing these vesicles to swell and eventually fuse with the bilayer (Woodbury and Hall, 1988).

The realization that the presence of ion channels in the vesicle membrane increases osmotic-driven fusion was integral in the development of an efficient and general means to induce and detect vesicle fusion. It has long been known that fusion of the vesicles containing ion channels with the artificial bilayer will result in the ion channel being incorporated into bilayer (Miller et al., 1976; Cohen, 1986). The addition of channels to the bilayer yields an increase in the bilayer conductance, which can be easily monitored with a standard voltage clamp amplifier. This provides a means of detecting fusion that allows for the monitoring of individual vesicle fusion events in real time.

The use of ion channel incorporation to follow vesicle fusion is often problematic due to the fact that the step-increase in membrane conductance resulting from ion channel incorporation is permanent and will eventually result in amplifier saturation and bilayer instability. In order to avoid this permanent increase in membrane conductance the antibiotic nystatin can be used as a marker channel. Low concentrations of nystatin are capable of forming ion

channels in membranes that contain the sterol, ergosterol (Woodbury and Miller, 1990; Kelly and Woodbury, 1996; Woodbury, 1999). Adding both nystatin and ergosterol to the vesicle membrane results in the presence of active nystatin channels in the vesicles. As figure 1.5 illustrates, when vesicles containing nystatin channels fuse with the planar bilayer, the active nystatin channel will be transferred into the planar bilayer, yielding an increase in membrane conductance. Because the planar bilayer is sterol free, the ergosterol associated with the active nystatin channel will dissipate, causing the channel to close resulting in a decay of the conductance increase caused by the channel. As previously shown (Woodbury and Miller, 1990; Woodbury, 1999), fusion of vesicles containing nystatin and ergosterol cause transient increases in bilayer conductance.

Using this nystatin/ergosterol technique, biological components can be incorporated into either the artificial bilayer or the vesicular membrane, and it can be determined if these components play a role in membrane fusion. Since fusion events can be recorded over a long period of time effects on the rate of fusion can also be assessed. This fusion detection system has the additional benefit that it provides its own internal control. As mentioned above vesicles containing ion channels will fuse with the bilayer in the presence of an osmotic gradient. This provides a means of determining if vesicles used were in fact competent to fuse with the bilayer regardless of additional components that may have been added.
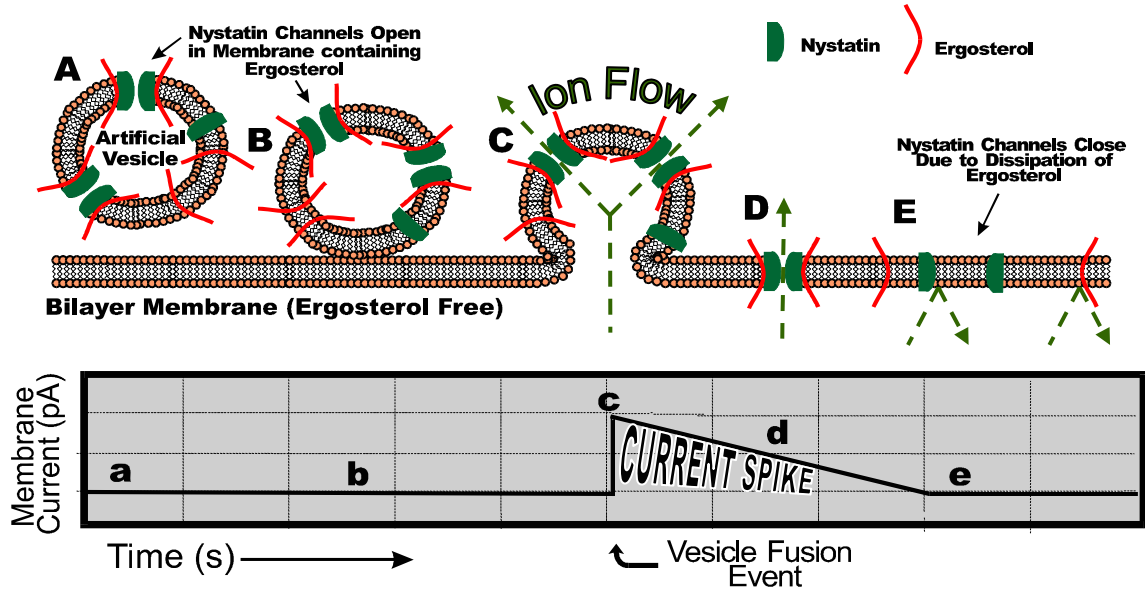
Figure 1.5: The nystatin/ergosterol technique. In order to visualize fusion of vesicles with the planar bilayer the pore forming antibiotic nystatin is added to the vesicular membrane along with ergosterol, which stabilizes these channels (**A-B**). When these vesicles fuse with an erogosterol free bilayer (**C**), the nystatin channels are inserted, increasing bilayer conductance. Shortly after fusion ergosterol diffuses away from the nystatin channels resulting in their closure (**D-E**). When measuring membrane current, fusion events are observed as rapid increases in conductance, signifying a fusion event, followed by a gradual decay as ergosterol diffuses away. (*Upper case letters correspond with lower case letters and shows what current trace looks like at each stage of vesicle fusion*)

(figure adapted from Woodbury, 1999)

Determination of the Minimal Components Required for Fusion

With present recombinant technology, large amounts of SNARE proteins, and other proteins believed to be involved in exocytosis, can be produced in expression systems and subsequently purified. These recombinant proteins can be easily reconstituted into both artificial vesicles and bilayers. This allows individual, as well as different subsets of proteins to be studied in a simplified and controlled environment.

Artificial vesicles can be modified in several different ways. Native vesicles can be isolated and "modified" by adding ergosterol and nystatin to their membranes, resulting in a vesicle that contains the native membrane proteins of the vesicle in addition to the nystatin marker channels that allow the detection of fusion. Also, as described above, purified recombinant proteins can be added to the vesicle membrane.

Chapter II of this thesis chronicles what we have learned about the fusion machinery involved in LDCG exocytosis using this model system. Part I of chapter V compares these findings to earlier research using this and other reconstituted systems, and comments on other areas of research where this model system should prove to be useful. Additionally, recent insights concerning transmitter release gained through the coupling of amperometric detection to the planar bilayer model system are presented (see Appendix I).

II. Intracellular Calcium and Secretion

As discussed in section I of this chapter, Ca^{2+} plays a central role in neurotransmitter release (Katz, 1969) While the influx of Ca^{2+} through voltage-gated Ca^{2+} channels (VGCC) is undoubtedly a very important source of Ca^{2+} affecting neuropeptide secretion, Ca^{2+} release from internal stores has increasingly been shown to play a role in this process (Berridge, 2006; Oheim et al., 2006). Therefore chapters III and IV of this thesis focus on the elucidation of the source of intracellular release, and the role this calcium release may play in basal neuropeptide release for NHT, respectively.

Ca^{2+} and Transmitter release

In neurons and secretory cells, depolarization induced opening of VGCC results in a rapid influx of extracellular Ca^{2+} into the cytosol. High concentrations of endogenous Ca^{2+} chelators heavily buffer this calcium entry resulting in discrete peaks of Ca^{2+}. These peaks termed "microdomains" (Simon and Llinas, 1985) result in high concentrations of Ca^{2+} being generated only in the immediate vicinity of the channel, dissipating rapidly distal to the site of Ca^{2+} entry. This keeps extracellular Ca^{2+} from reaching the deeper regions of cytosol (Pozzan et al., 1994). Ca^{2+} release from intracellular stores may provide a means of generating locally high concentrations of Ca^{2+} in such regions.

The spatial organization of secretory vesicles versus sites of Ca^{2+} entry in plays a central role in stimulus-secretion coupling. In synaptic nerve terminals

secretory vesicles are release only at specialized active release sites that include Ca^{2+} channels (Sheng et al., 1996). This close association yields extremely fast synchronous release, which is abruptly halted after stimulus due to strong intracellular Ca^{2+} buffering (Kits and Mansvelder, 2000).

In (neuro)endocrine cells, this does not appear to be the case. The LDCG of these cells do not appear to localize to specific active release sites. The bulk of these LDCG are thought to reside in a location much more distant from Ca^{2+} channels. This lack of spatial coupling of granules with Ca^{2+} channels leads to a long latency to release and release may continue after cessation of the depolarizing stimulus (Chow et al., 1996; Klingauf and Neher, 1997).

In chromaffin cells and NHT, Ca^{2+} entry through voltage-gated channels upon depolarization is only able to reach a limited fraction of LDCG (Horrigan and Bookman, 1994; Giovannucci and Stuenkel, 1997; Neher, 1998). This is believed to be due to the fact that, in (neuro)endocrine cells, LDCG are found to be in several different states of release readiness, referred to as vesicular release pools (Horrigan and Bookman, 1994; Giovannucci and Stuenkel, 1997; Neher, 1998; Kits and Mansvelder, 2000).

While there is still some controversy over the precise number and nature of these release pools, there is generally assumed to be three distinct pools. First, an immediately releasable pool, which is represented by a rapid burst of exocytosis of granules believed to be located near sites of Ca^{2+} influx and in a release-competent state. This pool is relatively small, rapidly depleted, and

released with a very short delay (~100ms). Second is a much slower sustained release of granules in the readily-releasable pool, which consists of granules that are also associated with the plasma membrane, but have a much longer delay between stimulus and release. There is some debate as to the make-up of this pool. These granules may represent a population of docked granules that do not share the same close proximity to VGCC as those in the immediately releasable pool (Giovannucci and Stuenkel, 1997). It is also possible that granules in this pool require additional priming steps to initiate fusion (Rettig and Neher, 2002). Finally, the third pool is the reserve vesicle pool, which is made up of all non-docked granules available for recruitment into the docked pools.

Beyond its ability to trigger exocytosis Ca^{2+} has been shown to function in numerous cellular pathways (Berridge, 2006), many of which are able to shape or tune synaptic efficacy (Chow et al., 1996). Ca^{2+} dependent processes appear to govern the kinetics of granule mobilization between the different vesicular release pools. Even modest increases in $[Ca^{2+}]_i$ can result in a shift in recruitment and passage of granules through this secretory pathway (Smith et al., 1998). Thus, it is possible that presynaptic Ca^{2+} stores could serve as a source of localized $[Ca^{2+}]_i$, able to modulate release by eliciting exocytosis, or changing the kinetics of granule movement between release pools.

Presynaptic Intracellular Calcium Stores

While a wealth of information regarding the role of intracellular calcium stores in postsynaptic regions, there is far less research on the topic of presynaptic stores. Less attention has been paid to these stores as they are quite difficult to study, due to the size and inaccessibility of most CNS presynaptic terminals. Ca^{2+} release from such stores is controlled by two types of receptor channels; the ryanodine receptor (RyR) and the inositiol 1,4,5-triphosphate (IP_3) receptor (Collin et al., 2005).

There are different isoforms of these receptors, several of which may coexist in a particular cell type. For instance there are three different subtypes of the RyR, expressed from three separate genes (for review see: Coronado et al., 1994; Fill and Copello, 2002). These different subtypes include the type 1 RyR (see figure 1.6), found mainly in skeletal muscle, the type 2 RyR, found in smooth muscle, and the type 3 RyR, found mainly in the brain. Such receptors may be differentially located in specific regions of a cell, providing differing sensitivities to Ca^{2+} at different sites, allowing Ca^{2+} to modulate distinct processes in the same cell.

In muscle, where RyR receptors were originally characterized, they have been shown to function in two different ways. Depolarization induced conformational changes in VGCCs lead to activation of type 1 RyR by a direct protein-protein interaction. Alternately in smooth muscle, type 2 RyR are activated by a mechanism known as Ca^{2+} induced Ca^{2+} release (CICR). Ca^{2+}
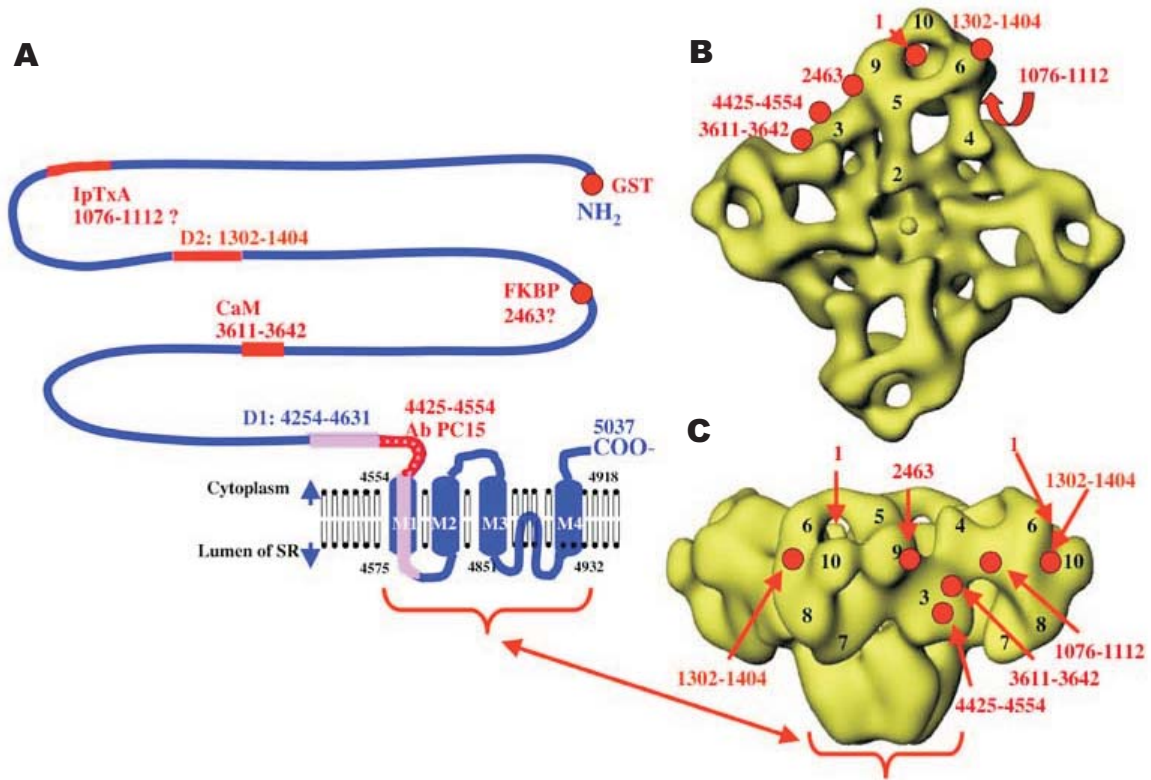
Figure 1.6: **Sequence and structural comparison of the ryanodine receptor** (**A**) This figure shows a schematic representation of the sequence of the type 1 RyR. The receptor is believed to have four transmembrane segments (M1-4) with a luminal loop between M3 and M4. This loop has been suggested to act as the pore forming domain of the receptor. The regions of the sequence highlighted in red are proposed ligand binding domains. The name of the ligand is also indicated in red, adjacent to the residue numbers. D1 and D2 are regions of high divergence between type 1 and 2 RyR. (**B and C**) Solid body representations of the type 1 RyR, cytoplasmic and side views respectively. The locations of the sequence-specific markers from **A** are indicated in red.

(Figure adapted from Wagenknecht and Samsó, 2002)

influx via VGCC raises $[Ca^{2+}]_i$ which results in activation of RyR leading to efflux of Ca^{2+} from intracellular stores. While Ca^{2+} release from IP_3 regulated stores is controlled through the activation of metabatropic receptors. Work in this thesis will focus on the effects of ryanodine sensitive presynaptic stores.

A large and varied number of pharmacological agents aid in the elucidation of cellular mechanisms involving ryanodine sensitive Ca^{2+} stores. The plant alkyloid ryanodine, after which the receptor was named, has been shown to bind with high affinity and specificity to RyRs, even at high micromolar concentrations (Waterhouse et al., 1987). This pharmacological agent has an interesting affect on the RyR as it induces long-lived sub conductance openings of the channel at low concentrations (nM–10 µM), while blocking the channel at higher concentrations (>100µM). Xanthines like caffeine at high concentrations (10 mM), have been shown to activate the RyR by potentiating it's sensitivity to Ca^{2+}. Caffeine additionally has inhibitory effects on the IP3 receptor, enabling resolution of the effects of the two stores (Ehrlich and Bezprozvanny, 1994). The physiological agonist, cyclic-ADP ribose (cADPR) has also been found to affect ryanodine-sensitive calcium stores (Fill and Copello, 2002).

Some evidence suggests that presynaptic ryanodine sensitive stores are capable of modulating neurotransmitter release in a number of neuronal preparations, while other studies argue against such a role (Bouchard et al., 2003; Collin et al., 2005; Berridge, 2006). Thus the relationship between intracellular calcium stores and release has yet to be conclusively determined. In

peripheral nerve terminals for instance, Ca^{2+} entry due to extended trains of stimuli have been shown to activate a CICR mechanism that amplifies the rise in $[Ca^{2+}]_i$ and enhances asynchronous neurotransmitter release (Narita et al., 1998; Narita et al., 2000).   This also appears to be the case in both hippocampal neurons (Emptage et al., 2001), and neocortical glutamatergic synapses (Simkus and Stricker, 2002) where these calcium stores appear to modulate the frequency of miniature inhibitory postsynaptic currents (mIPSC).   These studies imply that Ca^{2+} release from intracellular stores plays a role in setting the level of basal activity in these neurons.   However, this has not always been found to be the case.   In brainstem glycinergic synapses such an effect on mIPSCs was not observed, arguing against a significant role of presynaptic calcium stores (Lim et al., 2003).

Similar discrepancies are found in studies looking at the role of RyR sensitive stores in elicited release.   In photoreceptors and hair cells from the organ of Corti, RyR have been shown to modulate the amplitude of the rise in $[Ca^{2+}]_i$ induced by depolarizing stimuli (Kennedy and Meech, 2002; Krizaj et al., 2003; Lelli et al., 2003).   In hair cells, inhibiting ryanodine sensitive Ca^{2+} release, also inhibits neurotransmitter release, as measured with membrane capacitance recording (Lelli et al., 2003). Galant and Marty (2003) have demonstrated that ryanodine-sensitive intracellular calcium stores play a role in evoked neurotransmitter release at the basket cell-purkinje cell synapse.   While other

studies have suggested that ryanodine sensitive stores have no effect on transmitter release from Purkinje cell synapses (Carter et al., 2002).

Presynaptic calcium stores have also been suggested to be involved in eliciting changes in synaptic efficacy. Bath application of ryanodine has been suggested to block induction of a form of long term pontentiation (LTP) at mossy fiber-CA3 synapses (Lauri et al., 2003). Also, in CA3 pyramidal neuron synapses, long term depression (LTD) is dependent on release of Ca^{2+} from ryanodine sensitive stores (Unni et al., 2004). Finally, at Schaffer collateral-CA1 synapses a cADPR-mediated signaling pathway plays a key role in inducing LTD (Reyes-Harde et al., 1999).

Recent work has demonstrated that spontaneous intracellular Ca^{2+} transients can be seen in neuronal preparations (Emptage et al., 1999; Melamed-Book et al., 1999; Llano et al., 2000; De Crescenzo et al., 2004; Ouyang et al., 2005). These spontaneous Ca^{2+} signals appear similar to "Ca^{2+} sparks" from RyR in muscle. In cultured hipocampal neurons these calcium events have been suggested to effect spontaneous miniature synaptic currents (Emptage et al., 2001). Additionally in cerebellar interneurons, spontaneous Ca^{2+} transients, termed SCaTs, have been observed to mediate bursts of mIPSCs, and induce "maximinis", large amplitude mIPSCs that are believed to represent the synchronous release of several quanta (Llano et al., 2000; Bardo et al., 2002).

Ca^{2+} syntillas

In isolated NHT, transients Ca^{2+} release events have been observed using high speed calcium imaging (De Crescenzo et al., 2004). These events termed "Ca^{2+} "syntillas", (figure 1.7) persist in the absence of extracellular Ca^{2+}, and appear to emanate from a ryanodine-sensitive intracellular Ca^{2+} store. Furthermore, the frequency of syntillas can be modulated in a voltage dependent manner, a process termed voltage induced Ca^{2+} release or "VICaR" (De Crescenzo et al., 2006). This is presumably due to the direct coupling of type 1 RyR with L-type Ca^{2+} channels as the voltage dependence of syntillas is decreased upon application of nifedipine. In spite of all this information, the source of the released Ca^{2+} and a physiological role for this phenomenon has yet to be conclusively determined.

Vesicular Calcium Stores

LDCG and synaptic vesicles in many system, including NHT have been shown to contain a significant amount of Ca^{2+} (Nicaise et al., 1992; Thirion et al., 1995; Gerasimenko et al., 1996; Scheenen et al., 1998; Mundorf et al., 2000; Mitchell et al., 2001; Mahapatra et al., 2004). In chromaffin granules, LDCG contain anywhere between 20-40 mM Ca^{2+} (Mahapatra et al., 2004), this makes up ~60% of the total Ca^{2+} contained in the cell (Haigh et al., 1989). However, due to high luminal buffering only a small fraction of this Ca^{2+} is freely diffusible (Hutton, 1984). Thus, vesicular Ca^{2+} has generally been assumed to be non-
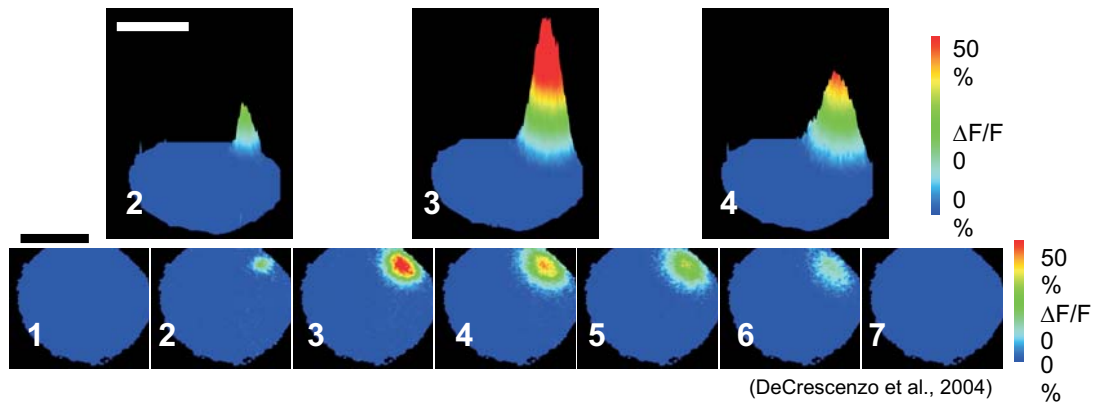
Figure 1.7: The Ca^{2+} syntilla. Figure illustrates a single Ca^{2+} syntilla from a patched murine neurohypophysial terminal in Ca^{2+}-free solution at a membrane potential of -80 mV. This syntilla was visualized with the fluorescent Ca^{2+} indicator fluo-3 (50 µM) using widefield optics and was recorded at 100 Hz (10 ms/image). Numbered images above were taken sequentially starting just before the onset of the syntilla. In these images warmer colors denote higher concentrations of Ca^{2+}. (Scale bar is 5 µm)

(figure adapted from DeCrescenzo et al., 2004)

functional and serve only as a means to expel Ca^{2+} from the cell (Pozzan et al., 1994).

Recent evidence, however, suggests that this may not be the case, and that vesicular Ca^{2+} ($[Ca^{2+}]_v$) may be a significant variable in regulated secretion. In insulin secreting ß-cells, ryanodine and cyclic-ADP ribose, causes a dramatic decrease in $[Ca^{2+}]_v$, showing $[Ca^{2+}]_v$ represents a dynamic store (Mitchell et al., 2001). Further, work in both ß-cells and chromaffin cells, suggests that the depletion of this $[Ca^{2+}]_v$ store markedly reduces secretion (Scheenen et al., 1998; Mundorf et al., 2000). More directly, LDCG isolated from prancreatic acinar cells were observed to release luminal Ca^{2+} in response to the application of both IP3 and cADPr (Gerasimenko et al., 1996). However, this finding remains controversial (Yule et al., 1997).

Could Ca^{2+} syntillas emanate from Vesicular Stores?

As mentioned earlier, the NHT represent a fairly simple system, that contains little intracellular machinery beyond LDCG (Lemos, 2002). Generally ryanodine-sensitive stores are believed to be located in the endoplasmic reticulum (ER) (Bouchard et al., 2003). However, there is little evidence that NHT contain ER (but see Broadwell and Cataldo, 1984). If the ER does not serve as the source of the Ca^{2+} released during syntillas, where does this calcium come from? RyR have also been found to be localized to mitochondrial membranes (Fill and Copello, 2002). Poisoning the mitochodria with cyanide,

however, does not have an affect on the appearance of syntillas (De Crescenzo et al., 2004). This leaves open the possibility that the LDCG could serve as the source of this Ca^{2+}.

Already in this system, there is a significant amount of circumstantial evidence pointing towards the granules serving as the source of ryanodine sensitive Ca^{2+} release. As mentioned above, LDCG in the NHT are known to contain a significant quantity of Ca^{2+} (Nicaise et al., 1992). Interestingly, the average number of Ca^{2+} ions released per syntilla (280,000 ; De Crescenzo et al., 2004) compares favorably to the amount of free Ca^{2+} contained in one NSG (Nicaise, Per. Comm.). Immunocytochemical labeling of RyR in the NHT yields a punctuate distribution throughout the terminal (De Crescenzo et al., 2004), similar to what would be expected for LDCG themselves. Also, a large conductance non-specific cation channel has been isolated from the NSG membrane (Lee et al., 1992; Yin et al., 2002), that possesses properties that are almost identical to the single channel properties of known mammalian RyR (Fill and Copello, 2002). These properties include a similar large non-specific cation conductances along with subconductance states, similar pH sensitivity, sensitivity to blockade by heptanol and octanol, and bimodal regulation by similar concentrations of free Ca^{2+}.

Since the Ca^{2+} syntillas in NHT emanate from ryanodine-sensitive stores (De Crescenzo et al., 2004), in order to show that NSG are the source of syntillas, localization of RyR to the membrane of these granules must be

demonstrated. Therefore chapter III of this thesis will attempt to determine if this is the case using immuno-electron microscopy. Additionally, the NSG channel described above will be tested to see if it is sensitive to the effects of agonists/antagonists specific for the RyR.

What is the physiological role of syntillas?

The evidence presented above suggests that spark-like Ca^{2+} release from presynaptic stores may play a role in modulating transmitter release in a number of neuronal systems. In muscle cells, the involvement of Ca^{2+} sparks in excitation-contraction coupling is well known (Cheng et al., 1996), making it tempting to speculate that Ca^{2+} syntillas of the NHT may play a role in depolarization-secretion coupling. Any mechanism that contributes to the rise in $[Ca^{2+}]_i$ near readily releasable LDCG could conceivably contribute to release. If LDCG can act as local Ca^{2+} stores, it would provide a source of Ca^{2+} strategically located to play a role in transmitter release and/or granule recruitment (figure 1.8).

In this system, ryanodine sensitive Ca^{2+} stores have already been shown to have a effect on the release of oxytocin, through a mechanism that involves the physiological RyR agonist cADPR (Jin et al., 2007). Additionally, in our laboratory a number of NHT population release experiments have been performed, assessing the effects of both ryanodine and caffeine on neuropeptide release (Velazquez-Marrero et al., 2002). Caffeine (20mM), a strong agonist of
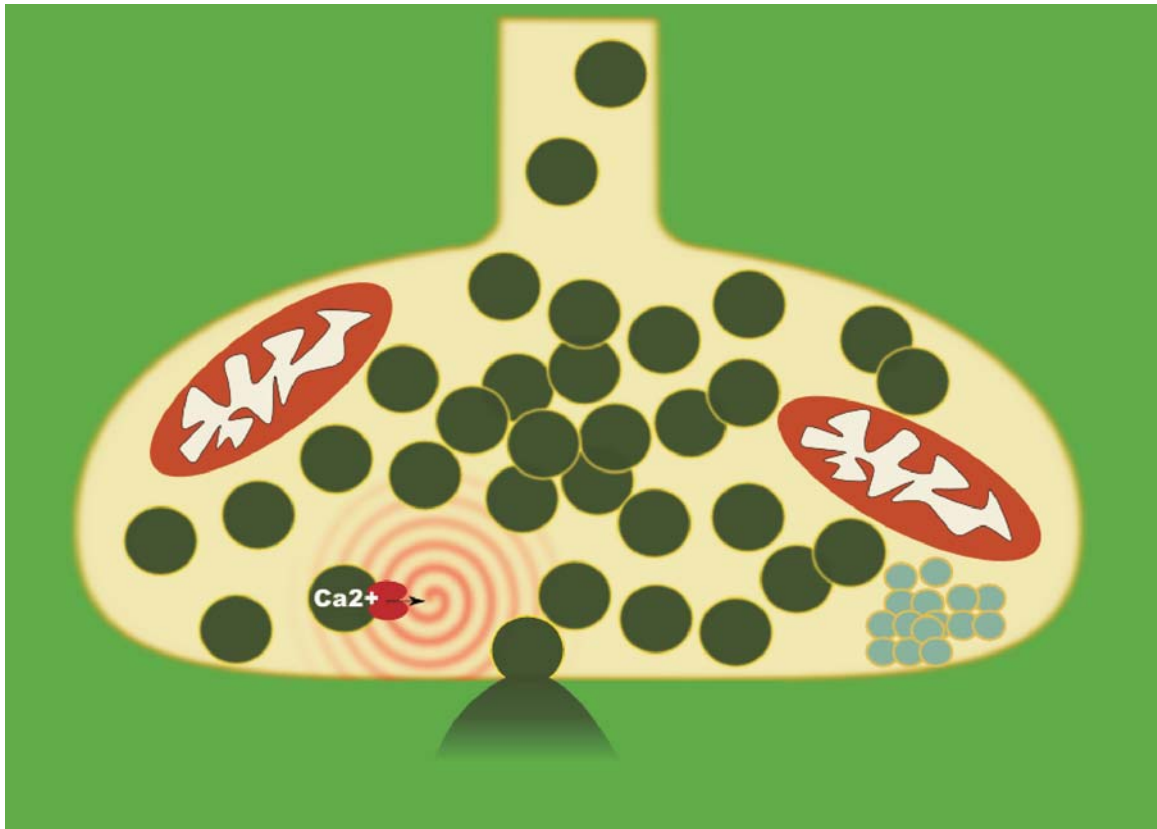
Figure 1.8: **What is the physiological role of Ca²⁺ syntillas?** LDCG exocytosis is known to be a calcium dependent process. As this is the case release of a significant amount of calcium precisely where exocytosis occurs could conceivably induce release. Thus, Ca²⁺ syntillas may be able to directly elicit LDCG exocytosis in neurohypophysial terminals.

the RyR, induces an increase in neuropeptide release from these terminals in the absence of extracellular Ca^{2+}. Ryanodine at 100 µM, a concentration at which ryanodine is known to inactivate RyR, is shown to attenuate this caffeine induced release. However, these experiments lack the spatial and temporal resolution required to directly assess the role of Ca^{2+} syntillas in release.

The evidence above makes it plausible that increases in $[Ca^{2+}]_I$, due to Ca^{2+} syntillas may play a role in the modulation of release either due to activation of RyR via depolarization, or via a CICR like mechanism. Such a process could play a major role in control of OT and AVP release. In chapter IV of this thesis amperometric detection is employed in NHT to directly assess the role of Ca^{2+} syntillas on spontaneous neuropeptide release. Defining the role of Ca^{2+} syntillas will provide a more complete understanding of the mechanisms behind transmitter release in this and other central nervous systems.

References:

Augustin I, Rosenmund C, Sudhof TC, Brose N (1999) Munc13-1 is essential for fusion competence of glutamatergic synaptic vesicles. Nature 400:457-461.

Bai J, Chapman ER (2004) The C2 domains of synaptotagmin--partners in exocytosis. Trends Biochem Sci 29:143-151.

Bardo S, Robertson B, Stephens GJ (2002) Presynaptic internal Ca2+ stores contribute to inhibitory neurotransmitter release onto mouse cerebellar Purkinje cells. Br J Pharmacol 137:529-537.

Basu J, Shen N, Dulubova I, Lu J, Guan R, Guryev O, Grishin NV, Rosenmund C, Rizo J (2005) A minimal domain responsible for Munc13 activity. Nat Struct Mol Biol 12:1017-1018.

Berridge MJ (2006) Calcium microdomains: organization and function. Cell Calcium 40:405-412.

Bouchard R, Pattarini R, Geiger JD (2003) Presence and functional significance of presynaptic ryanodine receptors. Prog Neurobiol 69:391-418.

Broadwell RD, Cataldo AM (1984) The neuronal endoplasmic reticulum: its cytochemistry and contribution to the endomembrane system. II. Axons and terminals. J Comp Neurol 230:231-248.

Carr CM, Munson M (2007) Tag team action at the synapse. EMBO Rep 8:834-838.

Carter AG, Vogt KE, Foster KA, Regehr WG (2002) Assessing the role of calcium-induced calcium release in short-term presynaptic plasticity at excitatory central synapses. J Neurosci 22:21-28.

Cheng H, Lederer MR, Xiao RP, Gomez AM, Zhou YY, Ziman B, Spurgeon H, Lakatta EG, Lederer WJ (1996) Excitation-contraction coupling in heart: new insights from Ca2+ sparks. Cell Calcium 20:129-140.

Chow RH, Klingauf J, Heinemann C, Zucker RS, Neher E (1996) Mechanisms determining the time course of secretion in neuroendocrine cells. Neuron 16:369-376.

Cohen F (1986) Fusion of Liposomes to Planar Bilayers. In: Ion Channel Reconsitution (Miller C, ed), pp 131-138. New York: Plenum Press.

Collin T, Marty A, Llano I (2005) Presynaptic calcium stores and synaptic transmission. Curr Opin Neurobiol 15:275-281.

Coronado R, Morrissette J, Sukhareva M, Vaughan DM (1994) Structure and function of ryanodine receptors. Am J Physiol 266:C1485-1504.

De Crescenzo V, ZhuGe R, Velazquez-Marrero C, Lifshitz LM, Custer E, Carmichael J, Lai FA, Tuft RA, Fogarty KE, Lemos JR, Walsh JV, Jr. (2004) Ca2+ syntillas, miniature Ca2+ release events in terminals of hypothalamic neurons, are increased in frequency by depolarization in the absence of Ca2+ influx. J Neurosci 24:1226-1235.

De Crescenzo V, Fogarty KE, Zhuge R, Tuft RA, Lifshitz LM, Carmichael J, Bellve KD, Baker SP, Zissimopoulos S, Lai FA, Lemos JR, Walsh JV, Jr.

(2006) Dihydropyridine receptors and type 1 ryanodine receptors constitute the molecular machinery for voltage-induced Ca2+ release in nerve terminals. J Neurosci 26:7565-7574.

Douglas WW, Poisner AM (1964) Calcium Movement in the Neurohypophysis of the Rat and Its Relation to the Release of Vasopressin. J Physiol 172:19-30.

Ehrlich BE, Bezprozvanny I (1994) Intracellular calcium release channels. Chin J Physiol 37:1-7.

Emptage N, Bliss TV, Fine A (1999) Single synaptic events evoke NMDA receptor-mediated release of calcium from internal stores in hippocampal dendritic spines. Neuron 22:115-124.

Emptage NJ, Reid CA, Fine A (2001) Calcium stores in hippocampal synaptic boutons mediate short-term plasticity, store-operated Ca2+ entry, and spontaneous transmitter release. Neuron 29:197-208.

Fasshauer D, Sutton RB, Brunger AT, Jahn R (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc Natl Acad Sci U S A 95:15781-15786.

Fasshauer D, Antonin W, Margittai M, Pabst S, Jahn R (1999) Mixed and non-cognate SNARE complexes. Characterization of assembly and biophysical properties. J Biol Chem 274:15440-15446.

Fill M, Copello JA (2002) Ryanodine receptor calcium release channels. Physiol Rev 82:893-922.

Galante M, Marty A (2003) Presynaptic ryanodine-sensitive calcium stores contribute to evoked neurotransmitter release at the basket cell-Purkinje cell synapse. J Neurosci 23:11229-11234.

Gerasimenko OV, Gerasimenko JV, Belan PV, Petersen OH (1996) Inositol trisphosphate and cyclic ADP-ribose-mediated release of Ca2+ from single isolated pancreatic zymogen granules. Cell 84:473-480.

Ghijsen WE, Leenders AG (2005) Differential signaling in presynaptic neurotransmitter release. Cell Mol Life Sci 62:937-954.

Giovannucci DR, Stuenkel EL (1997) Regulation of secretory granule recruitment and exocytosis at rat neurohypophysial nerve endings. J Physiol 498 (Pt 3):735-751.

Giraudo CG, Eng WS, Melia TJ, Rothman JE (2006) A clamping mechanism involved in SNARE-dependent exocytosis. Science 313:676-680.

Gulyas-Kovacs A, de Wit H, Milosevic I, Kochubey O, Toonen R, Klingauf J, Verhage M, Sorensen JB (2007) Munc18-1: sequential interactions with the fusion machinery stimulate vesicle docking and priming. J Neurosci 27:8676-8686.

Haigh JR, Parris R, Phillips JH (1989) Free concentrations of sodium, potassium and calcium in chromaffin granules. Biochem J 259:485-491.

Han X, Wang CT, Bai J, Chapman ER, Jackson MB (2004) Transmembrane segments of syntaxin line the fusion pore of Ca2+-triggered exocytosis. Science 304:289-292.

Horrigan FT, Bookman RJ (1994) Releasable pools and the kinetics of exocytosis in adrenal chromaffin cells. Neuron 13:1119-1129.

Hu C, Ahmed M, Melia TJ, Sollner TH, Mayer T, Rothman JE (2003) Fusion of cells by flipped SNAREs. Science 300:1745-1749.

Hutton JC (1984) Secretory granules. Experientia 40:1091-1098.

Jahn R (2004) Principles of exocytosis and membrane fusion. Ann N Y Acad Sci 1014:170-178.

Jahn R, Scheller RH (2006) SNAREs--engines for membrane fusion. Nat Rev Mol Cell Biol 7:631-643.

Jahn R, Lang T, Sudhof TC (2003) Membrane fusion. Cell 112:519-533.

Jin D, Liu HX, Hirai H, Torashima T, Nagai T, Lopatina O, Shnayder NA, Yamada K, Noda M, Seike T, Fujita K, Takasawa S, Yokoyama S, Koizumi K, Shiraishi Y, Tanaka S, Hashii M, Yoshihara T, Higashida K, Islam MS, Yamada N, Hayashi K, Noguchi N, Kato I, Okamoto H, Matsushima A, Salmina A, Munesue T, Shimizu N, Mochida S, Asano M, Higashida H (2007) CD38 is critical for social behaviour by regulating oxytocin secretion. Nature 446:41-45.

Katz B (1969) The Release of Neurotransmitter Substances. Liverpool: Liverpool University Press.

Kelly ML, Woodbury DJ (1996) Ion channels from synaptic vesicle membrane fragments reconstituted into lipid bilayers. Biophys J 70:2593-2599.

Kennedy HJ, Meech RW (2002) Fast Ca2+ signals at mouse inner hair cell synapse: a role for Ca2+-induced Ca2+ release. J Physiol 539:15-23.

Kits KS, Mansvelder HD (2000) Regulation of exocytosis in neuroendocrine cells: spatial organization of channels and vesicles, stimulus-secretion coupling, calcium buffers and modulation. Brain Res Brain Res Rev 33:78-94.

Klingauf J, Neher E (1997) Modeling buffered Ca2+ diffusion near the membrane: implications for secretion in neuroendocrine cells. Biophys J 72:674-690.

Kreye S, Malsam J, Sollner TH (2008) In Vitro Assays to Measure SNARE-Mediated Vesicle Fusion. Methods Mol Biol 440:37-50.

Krizaj D, Lai FA, Copenhagen DR (2003) Ryanodine stores and calcium regulation in the inner segments of salamander rods and cones. J Physiol 547:761-774.

Lauri SE, Bortolotto ZA, Nistico R, Bleakman D, Ornstein PL, Lodge D, Isaac JT, Collingridge GL (2003) A role for Ca2+ stores in kainate receptor-dependent synaptic facilitation and LTP at mossy fiber synapses in the hippocampus. Neuron 39:327-341.

Lee CJ, Dayanithi G, Nordmann JJ, Lemos JR (1992) Possible role during exocytosis of a Ca(2+)-activated channel in neurohypophysial granules. Neuron 8:335-342.

Lelli A, Perin P, Martini M, Ciubotaru CD, Prigioni I, Valli P, Rossi ML, Mammano F (2003) Presynaptic calcium stores modulate afferent release in vestibular hair cells. J Neurosci 23:6894-6903.

Lemos JR (2002) Magnocellular Neurons. In: Nature Encyclopedia of Life Sciences., pp 1-5. London: Nature Publishing Group.

Lemos JR, Ocorr KA, Nordmann JJ (1989) Possible role for ionic channels in neurosecretory granules of the rat neurohypophysis. Soc Gen Physiol Ser 44:333-347.

Lim R, Oleskevich S, Few AP, Leao RN, Walmsley B (2003) Glycinergic mIPSCs in mouse and rat brainstem auditory nuclei: modulation by ruthenium red and the role of calcium stores. J Physiol 546:691-699.

Lin RC, Scheller RH (1997) Structural organization of the synaptic exocytosis core complex. Neuron 19:1087-1094.

Llano I, Gonzalez J, Caputo C, Lai FA, Blayney LM, Tan YP, Marty A (2000) Presynaptic calcium stores underlie large-amplitude miniature IPSCs and spontaneous calcium transients. Nat Neurosci 3:1256-1265.

Mahapatra NR, Mahata M, Hazra PP, McDonough PM, O'Connor DT, Mahata SK (2004) A dynamic pool of calcium in catecholamine storage vesicles. Exploration in living cells by a novel vesicle-targeted chromogranin A-aequorin chimeric photoprotein. J Biol Chem 279:51107-51121.

Melamed-Book N, Kachalsky SG, Kaiserman I, Rahamimoff R (1999) Neuronal calcium sparks and intracellular calcium "noise". Proc Natl Acad Sci U S A 96:15217-15221.

Miller C, Arvan P, Telford JN, Racker E (1976) Ca++-induced fusion of proteoliposomes: dependence on transmembrane osmotic gradient. J Membr Biol 30:271-282.

Mitchell KJ, Pinton P, Varadi A, Tacchetti C, Ainscow EK, Pozzan T, Rizzuto R, Rutter GA (2001) Dense core secretory vesicles revealed as a dynamic Ca(2+) store in neuroendocrine cells with a vesicle-associated membrane protein aequorin chimaera. J Cell Biol 155:41-51.

Montecucco C, Schiavo G (1993) Tetanus and botulism neurotoxins: a new group of zinc proteases. Trends Biochem Sci 18:324-327.

Mueller P, Rudin DO, Tien HT, Wescott WC (1962) Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. Nature 194:979-980.

Mundorf ML, Troyer KP, Hochstetler SE, Near JA, Wightman RM (2000) Vesicular Ca(2+) participates in the catalysis of exocytosis. J Biol Chem 275:9136-9142.

Narita K, Akita T, Osanai M, Shirasaki T, Kijima H, Kuba K (1998) A Ca2+-induced Ca2+ release mechanism involved in asynchronous exocytosis at frog motor nerve terminals. J Gen Physiol 112:593-609.

Narita K, Akita T, Hachisuka J, Huang S, Ochi K, Kuba K (2000) Functional coupling of Ca(2+) channels to ryanodine receptors at presynaptic terminals. Amplification of exocytosis and plasticity. J Gen Physiol 115:519-532.

Neher E (1998) Vesicle pools and Ca2+ microdomains: new tools for understanding their roles in neurotransmitter release. Neuron 20:389-399.

Nicaise G, Maggio K, Thirion S, Horoyan M, Keicher E (1992) The calcium loading of secretory granules. A possible key event in stimulus-secretion coupling. Biol Cell 75:89-99.

Nicholson KL, Munson M, Miller RB, Filip TJ, Fairman R, Hughson FM (1998) Regulation of SNARE complex assembly by an N-terminal domain of the t-SNARE Sso1p. Nat Struct Biol 5:793-802.

Oheim M, Kirchhoff F, Stuhmer W (2006) Calcium microdomains in regulated exocytosis. Cell Calcium 40:423-439.

Ouyang K, Zheng H, Qin X, Zhang C, Yang D, Wang X, Wu C, Zhou Z, Cheng H (2005) Ca2+ sparks and secretion in dorsal root ganglion neurons. Proc Natl Acad Sci U S A 102:12259-12264.

Pozzan T, Rizzuto R, Volpe P, Meldolesi J (1994) Molecular and cellular physiology of intracellular calcium stores. Physiol Rev 74:595-636.

Rettig J, Neher E (2002) Emerging roles of presynaptic proteins in Ca++-triggered exocytosis. Science 298:781-785.

Reyes-Harde M, Empson R, Potter BV, Galione A, Stanton PK (1999) Evidence of a role for cyclic ADP-ribose in long-term synaptic depression in hippocampus. Proc Natl Acad Sci U S A 96:4061-4066.

Sabatini BL, Regehr WG (1996) Timing of neurotransmission at fast synapses in the mammalian brain. Nature 384:170-172.

Sassa T, Harada S, Ogawa H, Rand JB, Maruyama IN, Hosono R (1999) Regulation of the UNC-18-Caenorhabditis elegans syntaxin complex by UNC-13. J Neurosci 19:4772-4777.

Schaub JR, Lu X, Doneske B, Shin YK, McNew JA (2006) Hemifusion arrest by complexin is relieved by Ca2+-synaptotagmin I. Nat Struct Mol Biol 13:748-750.

Scheenen WJ, Wollheim CB, Pozzan T, Fasolato C (1998) Ca2+ depletion from granules inhibits exocytosis. A study with insulin-secreting cells. J Biol Chem 273:19002-19008.

Sheng ZH, Rettig J, Cook T, Catterall WA (1996) Calcium-dependent interaction of N-type calcium channels with the synaptic core complex. Nature 379:451-454.

Simkus CR, Stricker C (2002) The contribution of intracellular calcium stores to mEPSCs recorded in layer II neurones of rat barrel cortex. J Physiol 545:521-535.

Simon SM, Llinas RR (1985) Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. Biophys J 48:485-498.

Smith C, Moser T, Xu T, Neher E (1998) Cytosolic Ca2+ acts by two separate pathways to modulate the supply of release-competent vesicles in chromaffin cells. Neuron 20:1243-1253.

Sollner TH (2004) Intracellular and viral membrane fusion: a uniting mechanism. Curr Opin Cell Biol 16:429-435.

Stevens DR, Wu ZX, Matti U, Junge HJ, Schirra C, Becherer U, Wojcik SM, Brose N, Rettig J (2005) Identification of the minimal protein domain required for priming activity of Munc13-1. Curr Biol 15:2243-2248.

Stopa EG, LeBlanc VK, Hill DH, Anthony EL (1993) A general overview of the anatomy of the neurohypophysis. Ann N Y Acad Sci 689:6-15.

Sudhof TC (2004) The synaptic vesicle cycle. Annu Rev Neurosci 27:509-547.

Sugita S (2008) Mechanisms of exocytosis. Acta Physiol (Oxf) 192:185-193.

Sutton RB, Fasshauer D, Jahn R, Brunger AT (1998) Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. Nature 395:347-353.

Tang J, Maximov A, Shin OH, Dai H, Rizo J, Sudhof TC (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. Cell 126:1175-1187.

Thirion S, Stuenkel EL, Nicaise G (1995) Calcium loading of secretory granules in stimulated neurohypophysial nerve endings. Neuroscience 64:125-137.

Tucker WC, Weber T, Chapman ER (2004) Reconstitution of Ca2+-regulated membrane fusion by synaptotagmin and SNAREs. Science 304:435-438.

Unni VK, Zakharenko SS, Zablow L, DeCostanzo AJ, Siegelbaum SA (2004) Calcium release from presynaptic ryanodine-sensitive stores is required for long-term depression at hippocampal CA3-CA3 pyramidal neuron synapses. J Neurosci 24:9612-9622.

Velazquez-Marrero C, Custer E, De Crescenzo V, ZhuGe R, Fogarty KE, Walsh JV, Jr., Lemos JR (2002) Caffeine Stimulates Basal Neuropeptide Release from Nerve Terminals of the Neurohypophysis. J Neurosci 202:440.448.

Voets T, Toonen RF, Brian EC, de Wit H, Moser T, Rettig J, Sudhof TC, Neher E, Verhage M (2001) Munc18-1 promotes large dense-core vesicle docking. Neuron 31:581-591.

Waterhouse AL, Pessah IN, Francini AO, Casida JE (1987) Structural aspects of ryanodine action and selectivity. J Med Chem 30:710-716.

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. Cell 92:759-772.

Wojcik SM, Brose N (2007) Regulation of membrane fusion in synaptic excitation-secretion coupling: speed and accuracy matter. Neuron 55:11-24.

Woodbury DJ (1990) In: Proceedings of the Twelfth Annual International Conference of the IEEE Engineering in Medicine and Biology Society, pp 1747-1748.

Woodbury DJ (1999) Nystatin/ergosterol method for reconstituting ion channels into planar lipid bilayers. Methods Enzymol 294:319-339.

Woodbury DJ, Hall JE (1988) Vesicle-membrane fusion. Observation of simultaneous membrane incorporation and content release. Biophys J 54:345-349.

Woodbury DJ, Miller C (1990) Nystatin-induced liposome fusion. A versatile approach to ion channel reconstitution into planar bilayers. Biophys J 58:833-839.

Woodbury DJ, McNally JM, Lemos JR (2007) SNARE-Induced fusion of Liposomes to a Planar Bilayer. . In: Planar Lipid Bilayers (Leitmannova Liu A, ed): Elsevier Press.

Yin Y, Dayanithi G, Lemos JR (2002) Ca(2+)-regulated, neurosecretory granule channel involved in release from neurohypophysial terminals. J Physiol 539:409-418.

Yule DI, Ernst SA, Ohnishi H, Wojcikiewicz RJ (1997) Evidence that zymogen granules are not a physiologically relevant calcium pool. Defining the distribution of inositol 1,4,5-trisphosphate receptors in pancreatic acinar cells. J Biol Chem 272:9093-9098.

Zilly FE, Sorensen JB, Jahn R, Lang T (2006) Munc18-bound syntaxin readily forms SNARE complexes with synaptobrevin in native plasma membranes. PLoS Biol 4:e330.

CHAPTER II: Syntaxin 1A is Sufficient to Elicit Spontaneous Fusion of Modified Neurohypophysial Secretory Granule with a Planar Lipid Bilayer

PREFACE

Material presented in this chapter has been published in the following locations:

<u>Manuscripts and Book Chapters:</u>

McNally, J.M., D.J. Woodbury, and J.R. Lemos. (2004) Syntaxin 1A Drives Fusion of Large Dense-Core Neurosecretory Granules Into a Planar Lipid Bilayer. Cell Biochem Biophys. 41(1):11-24.

Woodbury, D.J., **J.M. McNally**, and J.R. Lemos. (2007) SNARE-induced fusion of vesicles to a Planar Bilayer. Chapter 10 *in* "Advances in planar lipid bilayers and liposomes", edited by A. Leitmannova-Liu. (Elsevier) Vol. 5:285-311.

<u>Abstracts:</u>

McNally, J.M., J.F. Henegan; D.J. Woodbury; J.R. Lemos. (2000) Syntaxin 1a Is Sufficient to Cause Spontaneous Fusion of Bovine Neurosecretory Granules to a Planar Lipid Bilayer. *Soc. Neurosci. Abs.* **26**: 128.16

McNally, J.M., D.J. Woodbury, J.R. Lemos. (2003) Syntaxin Mutants Modulate Fusion of Dense Core Granules to a Planar Lipid Bilayer. *Biophys.J.* **81***:A*950.

McNally, **J.M.**, D.J. Woodbury, & J.R. Lemos. (2003) Syntaxin 1a Is Sufficient For Spontaneous Fusion Of Both Neurohypophysial And Chromaffin Dense Core Granules To A Lipid Bilayer. *Mol. Biol. Cell* **14(12):** A123.

Abstract:

The SNARE complex, involved in vesicular trafficking and exocytosis, is composed of proteins in the vesicular membrane (v-SNAREs) that intertwine with proteins of the target membrane (t-SNAREs). Our results show that modified large dense core neurosecretory granules (NSG), isolated from the bovine neurohypophysis, spontaneously fuse with a planar lipid membrane containing only the t-SNARE syntaxin 1A. This provides evidence that syntaxin alone is able to form a functional fusion complex with native v-SNAREs of the NSG. The fusion was similar to constitutive, not regulated, exocytosis since changes in free $[Ca^{2+}]$ had no effect on the syntaxin-mediated fusion. Several deletion mutants of syntaxin 1A were also tested. The removal of the regulatory domain did not significantly reduce spontaneous fusion. However, a syntaxin deletion mutant consisting of only the trans-membrane domain was incapable of eliciting spontaneous fusion. Finally, a soluble form of syntaxin 1A (lacking its trans-membrane domain) was used to saturate the free syntaxin binding sites of modified NSGs. This treatment was shown to block spontaneous fusion of these granules to a bilayer containing full-length syntaxin 1A. This method provides an effective model system to study possible regulatory components affecting vesicle fusion.

Introduction:

Neurotransmitter release has been intensely studied but is not yet fully understood.   Recent work (Woodbury and Rognlien, 2000) has shown that small clear synaptic vesicles are able to fuse to a planar lipid bilayer containing only the t-SNARE syntaxin in the absence of any external driving force (i.e. fuse spontaneously). In this paper, we extend this finding to large dense core granules isolated from the bovine neurohypophysis. These Neurosecretory Granules (NSG) also fuse spontaneously to a planar lipid bilayer, containing only the protein syntaxin 1A, in the same Ca^{2+} independent fashion as synaptic vesicles. These results provide evidence that the model of the minimal components required for fusion proposed by Woodbury (Woodbury, 1999a), could in fact represent a general mechanism of membrane fusion. We have also examined the effects of several deletion mutants of syntaxin 1A on NSG fusion.

Neurotransmission is considered to be composed of at least three separate but connected steps. First, there is the packaging and delivery of vesicles containing neurotransmitter (NT) to the plasma membrane. Second, there is the fusion of these vesicles with the plasma membrane leading to NT release at the synapse. Third, there are regulatory components that control fusion so that a given NT is released only when the appropriate signal is given. This study focuses on the cellular components required for vesicle fusion.

Previous research has led to the identification of many cellular components that are believed to play either an essential or supportive role in the

fusion of vesicles and NT release. Among these there is increasing evidence that SNARE (soluble N-ethylmaleimide sensitive factor attachment protein receptor) proteins specialize in mediating intracellular fusion (Jahn et al., 2003). In fact, the SNARE hypothesis proposes that a general apparatus is used to bring about fusion, and that specificity is established by the pairing of proteins that together form the SNARE complex (Sollner et al., 1993; Sudhof, 1995). SNAREs have been found to be members of a family of proteins that appear to be distributed in a compartmentally specific fashion, with one set attached to the vesicle (v-SNAREs), and another set attached to the vesicle's target membrane (t-SNARES). A successful fusion event would only take place when complementary v-SNAREs and t-SNAREs come together.

In recent years, there has been a great amount of research performed on SNARE proteins in an attempt to determine their specific roles in vesicle fusion and NT release. It is presently believed that synaptobrevin (VAMP) acts as the v-SNARE in secretory vesicles that dock with the t-SNAREs, syntaxin and SNAP-25, in the presynaptic membrane (Schiavo et al., 1997; Zhong et al., 1997; Weber et al., 1998). These three SNAREs are able to form a stable trimeric complex. SNAP-25 contributes two α-helical domains, known as SNARE motifs, to this structure and syntaxin and synaptobrevin both contribute one. When the hydrophobic faces of the SNARE motifs interact, they are packed closely together to form the hydrophobic core of a coiled-coil (Weimbs et al., 1998). The interaction of these SNAREs may be controlled by NSF, α-SNAP, MUNC18,

synaptotagmin, and Ca^{2+}. The main role of NSF and α-SNAP appears to be the separation of v-SNAREs from t-SNAREs after fusion, in order to prepare for subsequent rounds of fusion (Ungermann et al., 1998). The N-terminal regulatory domain of syntaxin (Parlati et al., 1999) comprises a three helical bundle that binds and occludes its SNARE motif, when bound to Munc18 (Rizo and Sudhof, 2002).

The role of synaptotagmin may be to act as a calcium sensor when needed, by bringing both the vesicular and target membranes into close apposition, through a proteolipid interaction, allowing the SNAREs to interact (Hu et al., 2002). However, these results were obtained using a fusion assay that relies on detecting fluorescently tagged liposomes. Liposome fusion results in dequenching of the fluorescent tag leading to an increase in relative fluorescence that can be quantitated. Interactions seen with this method take place over very long time scales and accurate quantification of the amount of fluorescence due to specific liposome fusion is difficult.

We have previously demonstrated that it is possible to measure fusion of synaptic vesicles (SV) in real time with a simple planar lipid bilayer system (Kelly and Woodbury, 1996). These earlier experiments, however, lacked much of the cellular machinery believed to be involved in fusion, and thus required the addition of an external force (an osmotic gradient) to produce fusion. In more recent studies, it has been found that through the reconstitution of essential

cellular components, spontaneous vesicle fusion can be observed without using an external driving force (Woodbury and Rognlien, 2000).

This finding led to the development of the nystatin/ergosterol fusion technique (Woodbury and Miller, 1990). This method takes advantage of the properties of the antibiotic, nystatin. Low concentrations of nystatin can form ion channels in membranes that contain the sterol, ergosterol, but not in ergosterol-free membranes. Therefore, if the vesicle membrane contains ergosterol and the planar bilayer membrane does not, nystatin channels will be active in the vesicle membrane, but after vesicle fusion, they will close as the ergosterol disperses into the planar lipid bilayer. The addition of nystatin channels to the vesicle membrane provides us only with a means to detect fusion of these vesicles to a planar bilayer. A fusion event is evidenced in real time by an increase in bilayer conductance with a transient decay (see Fig. 2.1B) (Woodbury and Miller, 1990; Woodbury, 1999b). This study takes advantage of this technique to examine the role of the SNARE protein syntaxin 1A in the fusion of isolated bovine NSG with a planar lipid bilayer.

Materials and Methods:

Preparation of modified (Nys/Erg) Vesicles or Artificial Vesicles

Artificial vesicles were prepared as previously reported (Woodbury, 1999b). Initially a stock ergosterol (Sigma-Aldrich) solution was produced in chloroform (10 mg/ml). This stock solution was then used to make a stock lipid

solution (SLS). The SLS contained phosphatidylethanolamine (POPE), phosphatidylcholine (POPC), phosphatidylserine (POPS), and ergosterol (Erg.), at a weight ratio of 2:1:1:1.5 (POPE:POPC:POPS:Erg.). All phospholipids used were purchased dissolved in chloroform (10 mg/ml) from Avanti Polar Lipids, Inc. A Nystatin (Sigma-Aldrich) stock solution was also prepared by dissolving 2.5 mg of nystatin/ml in anhydrous methanol. The SLS and nystatin stock solutions were stored at -20ºC.

To prepare a batch of vesicles 200 µl of SLS and 5 µl of the nystatin stock were added to a glass test tube, and dried under a stream of nitrogen. After complete solvent evaporation 200 µl of 150 mM KCl, 8 mM HEPES (pH 7.2) was added making the final nystatin concentration 63 µg/ml. The mixture was then vortexed for ~5 min and sonicated in a water bath sonicator (RT) for several 30 sec. bursts until the mixture became translucent. These vesicles were then tested by the planar lipid membrane method to determine if the vesicles were capable of fusion. If not used immediately, vesicles were flash frozen with a dry ice-ethanol bath and stored in a -20ºC freezer. Vesicles could be stored for several days before use. Due to alterations from freezing and thawing, additional sonication (~15-30 sec) was necessary for stored vesicle mixture before use.

Purification of Native Bovine Neurosecretory Granules

The NSG of bovine posterior pituitary glands were obtained by differential centrifugation as previously reported by Nordman et al (Nordmann et al., 1979).

Bovine posterior pituitary glands were dissected within 10-15 minutes of slaughter, and stored in warm (37ºC) homogenizing solution (HS). This HS contained 0.3 M sucrose, and 10 mM Tris-HEPES and 1 mM EGTA at pH 7.0. The glands were washed several times with Listerine and HS, finely minced with scissors, and then homogenized (at 1,000 rpm, 5 times up and down) after adding 2-3 ml of cold HS (from this point on all solutions were kept ice cold). The homogenate was then sequentially centrifuged with a SS-34 rotor in a Sorval RC-5B centrifuge. The homogenate was first centrifuged for 5 minutes at 3,000 rpm (800 x g), the supernatant was then taken and centrifuged another 10 minutes at 5,000 rpm (3,000 x g), the supernatant was then taken and was centrifuged an additional 10 minutes at 8,000 rpm (8,000 x g). The final supernatant was then taken, HS solution was added (almost filling the centrifuge tube), and centrifuged for 20 minutes at 16,000 rpm (27,000 x g). The resulting pellet was resuspended thoroughly using a micro-homogenizer in 1 ml HS. The homogenate was then added to an isosmotic Percoll (Pharmacia) solution almost filling the centrifuge tube and mixed well. The mixture was then centrifuged for 45 minutes at 16,000 rpm (27,000 x g). The portion between the upper white band and lower white band was then collected. Enough homogenizing solution for further separation was then added to the collected material, which was then centrifuged for an addition 40 minutes in order to remove the leftover percoll. After completion, the white band above the clear sticky pellet of percoll at the bottom of the tube was collected. The collected material was then brought to a total protein

concentration of 1 mg/ml, aliquoted and stored at –80ºC. Previous work has demonstrated the purity of this preparation to be between 90% - 95%, with mitochondria being the major contaminant (Lemos et al., 1989).

Modification of Native Vesicles

Native bovine NSG were modified following the procedure previously reported by Woodbury (Woodbury, 1999b). Briefly, the procedure is as follows. Nystatin/Ergosterol vesicles (200-250 µl) were made as described above and a 39 µl aliquot of purified bovine NSG was added to the batch. The vesicle solution was then subjected to three sequential freeze, thaw, and sonicate cycles to intermix the membranes of the two vesicle populations. This intermixing of membranes forms vesicles that possess both the native membrane proteins of the NSG and nystatin channels, which allow the detection of fusion of these vesicles. These modified neurosecretory granules (mNSG) were stored frozen until a short time before use. For syntaxin 1A-11 or mock treatment, the mNSG preparation was aliquoted into two 80 µl volumes and the remainder kept as the untreated fraction. 20 µl of purified syntaxin 1A-11 (0.6 mg/ml) was added to one aliquot and an equal volume of thrombin cleavage buffer (the buffer containing purified syntaxin) was added to the other. These aliquots were then allowed to stand at room temperature for one hour, after which they were flash frozen until use.

Formation of Bilayers

Planar lipid bilayers were formed as previously described (Woodbury, 1999b; Yin et al., 2002). First a decane solution (50µl) was produced containing 75% POPE and 25% POPC (10 mg/ml). Bilayers were formed on a 150-250 µm diameter hole in a polystyrene cup; the hole was positioned between the two chambers of the bilayer apparatus. The chambers of the bilayer apparatus were filled with 150 mM KCl, 8 mM HEPES, pH 7.2 [1 ml in trans chamber and 0.7 ml in the cis chamber]. Bilayers were formed by coating the tip of a micro-pipette with the decane solution, immersing the pipette into the chamber, blowing a small bubble at the pipette tip, and then drawing this bubble across the hole in the cup. The current across the bilayer was then monitored using a standard patch clamp amplifier (HEKA EPC9). Data was monitored and stored using both PClamp6 (Axon Instruments) and/or Pulse (HEKA).

Reconstitution of Syntaxin into a Bilayer

Syntaxin 1A-1, 1A-8, 1A-11 and 1A-12 (purified from E. coli as described in Woodbury and Rognlien (2000), buffered in thrombin cleavage buffer: 2.5 mM $CaCl_2 \bullet 2H_2O$, 50 mM Tris, 150 mM NaCl (pH 8.0)) were reconstituted into bilayers by brushing < 0.5 µl of the protein solution on the bilayer in the same fashion that was used in order to create the bilayer. Briefly, the tip of a micro-pipette was coated with the protein solution, immersed into the chamber, and a small bubble formed at the pipette tip. This bubble was then drawn across the hole in the cup.

This "brushing" process was repeated three times in order to ensure incorporation of the protein into the bilayer. After each brushing, the bilayer was allowed to recover its initial electrical properties prior to the next brushing step or recording.

Fusion of Vesicles with a Bilayer

For vesicle fusion experiments, after bilayer formation, 20 µl of the vesicles to be tested were added to the cis chamber of the bilayer apparatus with stirring. It was initially observed if these vesicles were capable of fusing with the planar bilayer "spontaneously", or in the absence of an external driving force. Then, after the addition of an osmotic gradient, we looked at the vesicles ability to undergo "induced" fusion, or fusion driven by an external driving force. In order to determine the number of fusion events that took place during each condition, the current through the membrane was observed at a fixed transmembrane holding potential of +60 mV. A fusion event was defined as a sudden increase in membrane conductance greater than 3 times the RMS noise of the trace, followed by a slow decay (Woodbury, 1999b). When an osmotic gradient was required to induce fusion, the KCl concentration of the cis chamber was raised to ~ 410 mM through the addition of 70 µl of 3 M KCl to the chamber and subsequent removal of the same volume. The addition of an osmotic gradient to induce fusion serves as a positive control to ensure that vesicles used were intact, as all vesicles used, regardless of protein content, should be able to

undergo induced fusion with the planar bilayer. Only experiments where induced fusion was observed were considered in these results.

Results:

Effects of Syntaxin 1A-1

In each experiment, spontaneous fusion of mNSGs, prepared with nystatin/ergosterol as outlined above, was studied for 5 or 10 minutes. This was followed by the addition of a salt concentration gradient across the bilayer, providing a driving force to induce fusion of the vesicles. This last step confirms that sufficient numbers of vesicles were delivered to the bilayer.

The results of our fusion experiments show that mNSGs, presumably containing native v-SNAREs, are not able to spontaneously fuse to a protein-free bilayer (Figure 2.1 Panel A, n = 5). However, Spontaneous fusion was observed between mNSG and a bilayer containing syntaxin 1A-1 (Figure 2.1 Panel B, n = 8). Conforming to previous results (Woodbury and Rognlien, 2000), artificial (protein-free) vesicles (AVs) were not able to spontaneously fuse to a protein-free bilayer (n = 10) or a bilayer containing syntaxin 1A-1 (n = 10) (Figure 2.2 Panels A and B, respectively). These findings show that the spontaneous fusion observed in figure 2.1 panel B is not due to syntaxin 1A-1 alone or to the native membrane protein of the mNSG alone. Since both syntaxin 1A-1 and the proteins of mNSG are necessary to induce spontaneous fusion, this fusion must be the result of a specific interaction between such proteins. These data
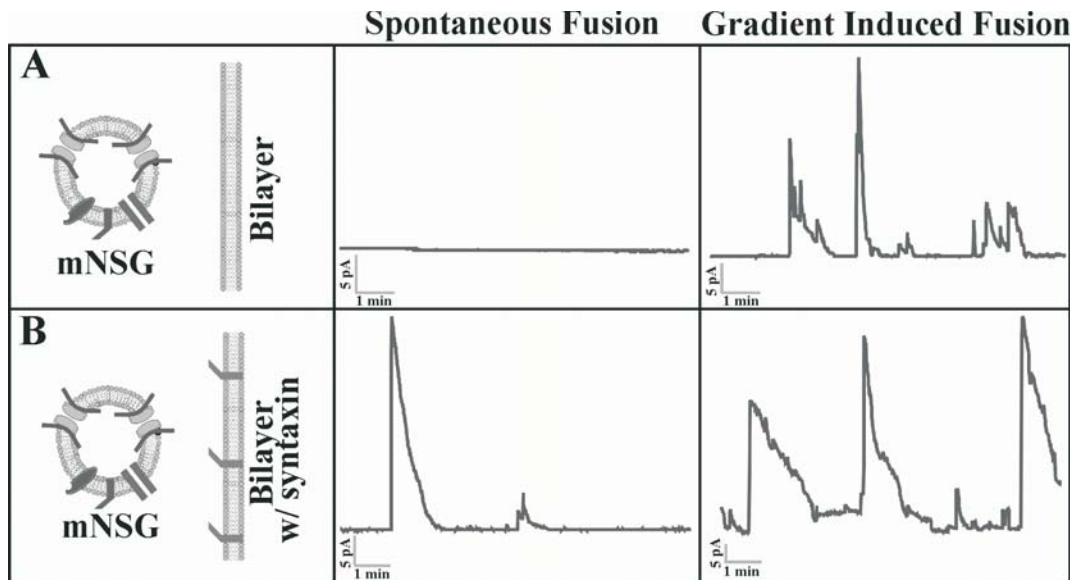
Figure 2.1: Fusion of mNSGs with a planar lipid bilayer. (**A**) Native NSG membrane proteins alone are unable to induce spontaneous fusion of granules. In this experiment, modified neurosecretory granules (mNSG; see Methods) were exposed to a protein free bilayer for a period of ten minutes, and the current across the membrane was recorded at a holding potential of 60 mV. As a positive control, an osmotic gradient was formed across the bilayer, and fusion activity monitored (right side). (**B**) Vesicular fusion occurs spontaneously between mNSG and a bilayer containing the t-SNARE syntaxin 1A-1 (left side). In this experiment, mNSG were exposed to a bilayer containing syntaxin. The experiment was performed in the same manner as described in A.
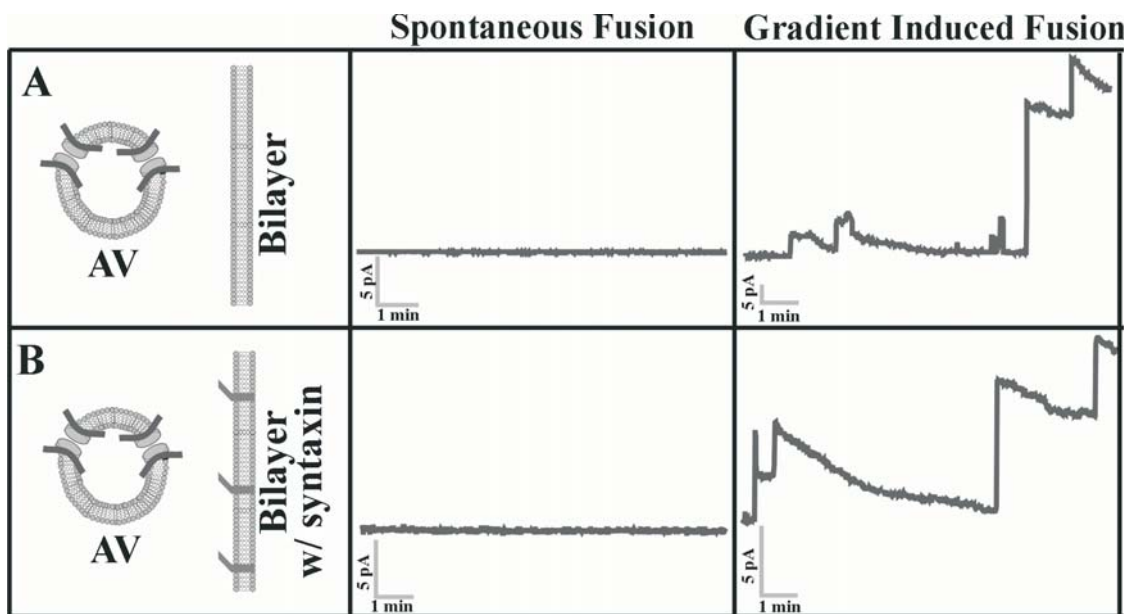
Figure 2.2: **Protein-free (artificial) vesicles do not fuse spontaneously with protein-free or syntaxin-containing membranes.** However, an osmotic gradient will still induce fusion of these vesicles. (**A**) Artificial vesicles were exposed to a protein-free artificial lipid membrane for a period of ten minutes and the current across the membrane was recorded. As a positive control an osmotic gradient was formed across the bilayer, and fusion activity monitored (right side). (**B**) To determine if the spontaneous fusion observed in Fig. 1B was strictly a result of the addition of syntaxin to the bilayer, or if it was, in fact, evidence of a protein-protein interaction between the native NSG membrane proteins in the mNSG and the t-SNARE syntaxin, artificial vesicles were exposed to a bilayer containing syntaxin 1A-1. This experiment was performed in the same manner as described above. The results show that the presence of syntaxin alone is insufficient to induce spontaneous fusion of artificial vesicles without the native membrane proteins from NSGs.

supports the hypothesis that the t-SNARE syntaxin is sufficient for the formation of a stable fusion complex with the native membrane proteins of bovine NSG.

Effects of Syntaxin 1A Deletion Mutants

To determine the effects of the putative regulatory domain of syntaxin on the observed spontaneous fusion, we used deletion mutants of our syntaxin 1A-1 construct (see Figure 2.5A). Syntaxin 1A-8 and syntaxin 1A-12 were incorporated into the lipid bilayer and then mNSGs were allowed to fuse. The average fusion rate (ratio of spontaneous to induced fusions or S/I fusion) obtained with each mutant was then compared to that of the full-length syntaxin 1A-1. In order to compare the results of these experiments with those of the preceding section, the

spontaneous fusion rate in each case was normalized to the induced fusion rate for that particular experiment. This rate ratio provides us with a way to measure differences in the rate of protein-mediated fusion irrespective of variations in individual experiments, such as the number of vesicles added to the chamber, and their rate of delivery to the bilayer.

The first deletion mutant we tested, syntaxin 1A-8 (n = 5), lacked the N-terminal regulatory portion of the protein. There was no significant difference ($p > 0.05$) in the observed spontaneous fusion rate in this case compared to that of full-length syntaxin 1A-1 (Figure 2.3A & 2.5B, respectively, Syntaxin 1A-1: S/I
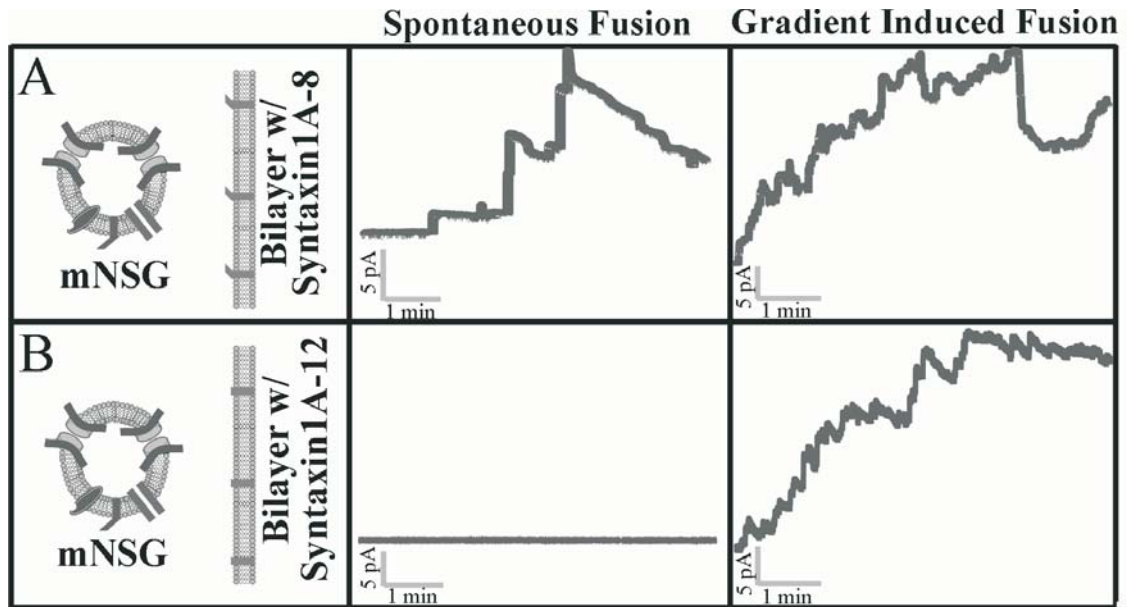
Figure 2.3: The effects of syntaxin 1A deletion mutants on mNSG fusion.
(**A**) mNSGs were allowed to spontaneously fuse to a planar lipid bilayer containing the deletion mutant syntaxin 1A-8 (see Fig. 5A) for a period of five minutes. During this time fusion activity was recorded by monitoring the current across the bilayer (left side). The positive control (osmotically-induced fusion) is shown on the right side. (**B**) Inability of deletion mutant syntaxin 1A-12 (see Fig. 5A) to elicit spontaneous fusion of mNSGs. This experiment was performed in the same manner as described above.

Fusion Rate (fusions/min) was 0.262±0.045 SEM; Syntaxin 1A-8: S/I Fusion was 0.301±0.085 SEM).

Previous work has suggested that SNARE transmembrane segments are able to drive membrane fusion (Langosch et al., 2001). Therefore a second deletion mutant, Syntaxin 1A-12, consisting of just the trans-membrane portion of syntaxin, was tested (n = 7). Both the regulatory N-terminal domain and the domains believed to be involved in SNARE fusion complex formation were removed. When tested with the nystatin/ergosterol method, syntaxin 1A-12 was unable to elicit significant spontaneous fusion (Fig 2.3B: 0.014 S/I Fusion ± 0.01 SEM). Figure 2.5B clearly shows that there is a significant ($p = < 0.01$) difference between the spontaneous fusion rate seen with syntaxin 1A-12 vs. syntaxin 1A-1.

The final deletion mutant examined in this study was syntaxin 1A-11, which lacked the trans-membrane domain of the protein. This freely soluble form of syntaxin was used to pre-treat a standard mNSG preparation prior to observing its ability to fuse to a syntaxin 1A-1 containing bilayer (see Materials and Methods). We reasoned that if a specific protein-protein interaction, between the syntaxin 1A-1 in the bilayer and native proteins in the mNSGs, is in fact responsible for the spontaneous fusion seen, pre-treatment of mNSGs with syntaxin 1A-11 should result in saturation of all syntaxin binding sites. Such pre-treatment of mNSG should produce a dominant-negative effect on their ability to undergo spontaneous fusion to a syntaxin 1A-1 bilayer. This is precisely the result observed in figure 2.4 and summarized in figure 2.5C (n = 4). Prior
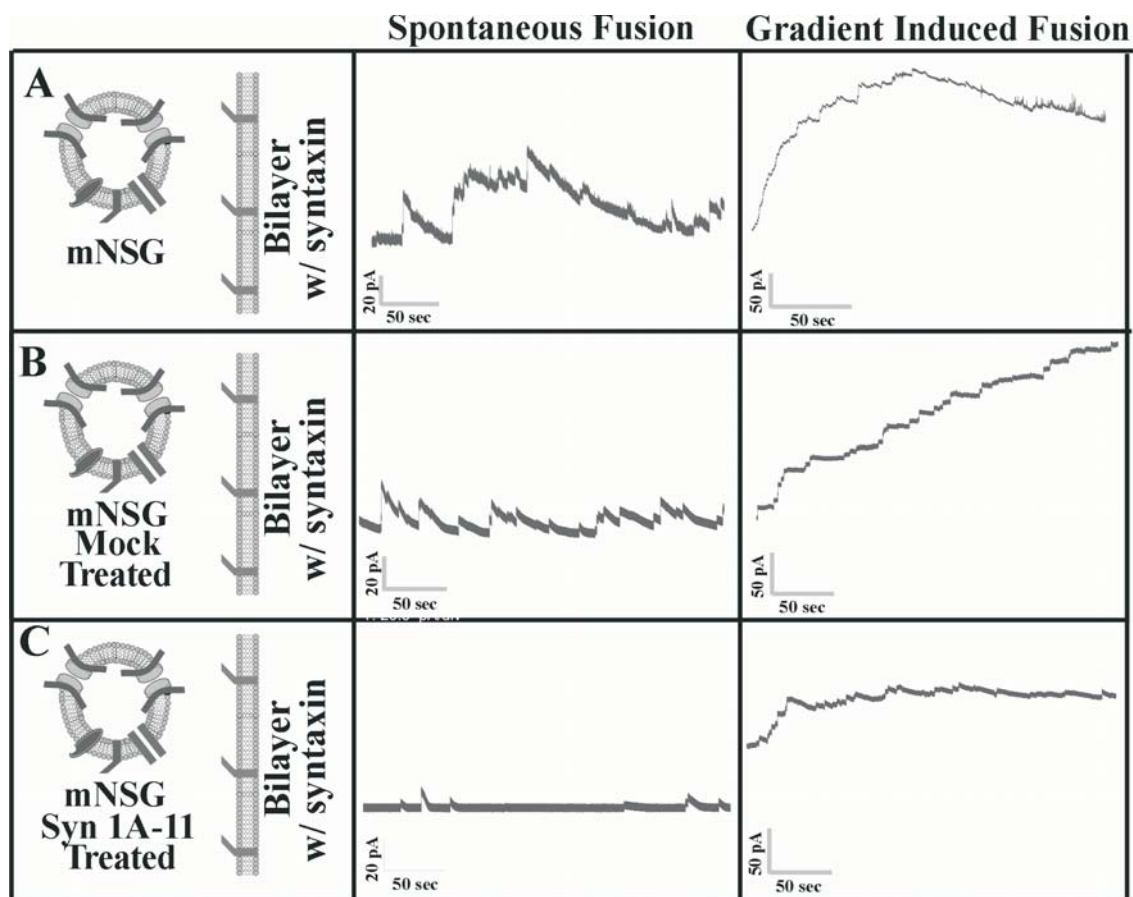
Figure 2.4: **Preincubation of mNSG with the soluble form of syntaxin (1A-11) leads to a substantial decrease in the ratio of spontaneous to induced fusion events seen.** (**A**) Untreated mNSGs were allowed to spontaneously fuse with a planar bilayer containing syntaxin 1A-1 for a period of 5 minutes (left side). A gradient was then formed across the bilayer inducing the mNSG to fuse for 5 minutes as a control (right side). (**B**) Spontaneous and induced fusion of mNSGs receiving a mock treatment (one hour incubation of 80 µl mNSG with 20 µl thrombin cleavage buffer at RT). (**C**) Spontaneous and induced fusion of mNSGs treated with syntaxin 1A-11 (20 µl of purified syntaxin 1A-11 in same buffer as in B).
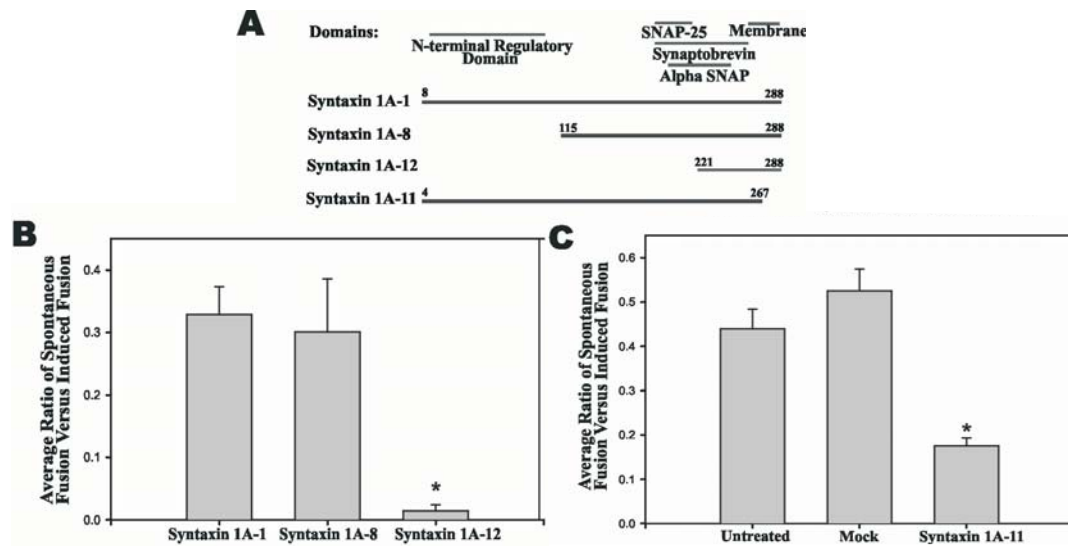
Figure 2.5: (**A**) **Schematic of syntaxin deletion mutants used in this study.** At the top are indicated the regions corresponding to the N terminal regulatory domain, the SNARE binding domains, and the transmembrane region. The lower part of the figure shows the full-length syntaxin (1A-1) and the deletion mutants (1A-8, 1A-11, and 1A-12) used. The bar for each syntaxin deletion mutant represents the portion of the full-length syntaxin that the mutant contains. (**B**) Graph comparing the ratio of spontaneous to induced fusion rates between full-length syntaxin 1A-1 (n = 8) and the two deletion mutants, syntaxin 1A-8 (n = 5) and 1A-12 (n = 7). Comparison of the syntaxin 1A-1 fusion rate to that of the two deletion mutants via one way ANOVA shows that the fusion rate attained with syntaxin 1A-12 is significantly different (p < 0.05), while no significant difference is seen for syntaxin 1A-8. (**C**) Graph comparing the ratio of spontaneous to induced fusion rates between untreated mNSGs, Mock treated mNSGs (see fig. 4B), and Syntaxin 1A-11 treated mNSGs allowed to fuse to a bilayer containing syntaxin 1A-1. Analysis of these results by one-way ANOVA shows that there is not a significant difference between the untreated and mock treated mNSGs' ability to fuse to a bilayer containing Syntaxin 1A-1. However, syntaxin 1A-11 treatment of mNSGs does significantly (p < 0.05) reduce their rate of fusion to a syntaxin 1A-1 bilayer compared to untreated mNSGs. (*Significant difference: p < 0.05)

incubation of mNSGs with syntaxin 1A-11, results in a significant ($p = < 0.05$)

reduction in fusion rate (0.175 S/I Fusion ± 0.018 SEM) with a syntaxin 1A-1

bilayer when compared to the fusion rate of those same vesicles prior to

treatment (0.440 S/I Fusion ± 0.044 SEM). This treatment appears to have little

effect on the induced fusion rate of these granules and the effect is not seen with

mNSG treated with a mock solution consisting of syntaxin 1A-11 buffer alone

(0.525 S/I Fusion ± 0.049 SEM).

Effects of Ca^{2+} on Spontaneous Fusion

Since it can be assumed that a protein-protein interaction was taking place

between the mNSGs and syntaxin 1A-1 containing bilayer, and given that NT

release is a Ca^{2+} dependent process, we attempted to determine if the presence

of Ca^{2+} has any effect on spontaneous mNSG fusion. The experiment (Figure

2.6) began with no free Ca^{2+} in the bath solution. The free Ca^{2+} concentration

was then increased to 10 μM, the concentration that has been shown to elicit

maximal NSG release in native rat neurohypophysial terminals (Lee et al., 1992).

Subsequently, the calcium concentration of the bath solution was then brought

back to zero through the addition of sufficient EDTA (see Materials and

Methods). The result of this experiment (Figure 2.6) shows that neither the

presence nor absence of Ca^{2+} has any effect on this spontaneous fusion. A

paired t-test analysis of the number of fusion events occurring in the initial Ca

free condition versus those occurring with 10 µM free Ca indicates that there is
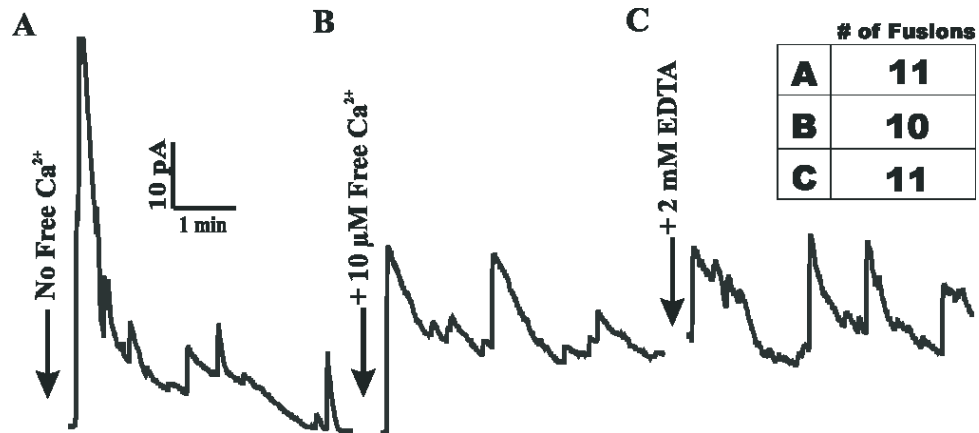
Figure 2.6: **The effects of free Ca^{2+} on spontaneous fusion.** This representative experiment began with a bath solution with sufficient EDTA to lower free Ca^{2+} to <1nM. (**A**) mNSGs were allowed to spontaneously fuse with a syntaxin containing bilayer for a five minute period. (**B**) The concentration of free Ca^{2+} was increased to 10 μM and the spontaneous fusion of the mNSG recorded. (**C**) 2mM EDTA was added to the chamber solution again lowering free Ca^{2+} to <1nM. The fusion of mNSGs was again observed for a five-minute period. These data (see box) show that free Ca^{2+} has no effect on the number (#) of mNSG fusions with a bilayer containing syntaxin and suggest that the missing Ca^{2+} regulating components are not mandatory for the fusion process *per se*.

no significant (p = 0.37) difference between the number of fusion events occurring in these two conditions (n = 6). These findings demonstrate that Ca^{2+} is not essential to the syntaxin dependent fusion process but may act as a modulatory agent that works in conjunction with other components. This leads us to believe that specific elements involved in the regulation of neuropeptide release by Ca^{2+} are missing from this model system.

Disscusion:

There is mounting evidence that specific SNARE proteins can form the basic machine that drives membrane fusion (Jahn et al., 2003). However, possible alternate forms of the fusion machine and its regulation remain controversial. Binding assays have identified numerous proteins that interact with different SNARE proteins or with the SNARE complex. With respect to fusion, these proteins may be critical components, regulators, or merely sticky but unrelated components. Genetic knock-out studies have shown that some of these proteins are critical for life, while others do not have a readily apparent phenotype. One definitive way to determine if a particular mix of SNARE proteins is sufficient to drive fusion is to reconstitute the components into an in vitro system. This was first done by Weber et al. (Weber et al., 1998) using a liposome-liposome fusion assay and placing syntaxin and SNAP-25 (t-SNAREs) in one population of vesicles and synaptobrevin (v-SNARE) in another (see also (Hu et al., 2002)). This first defined set of minimal conditions was expanded by

Woodbury and Rognlien (Woodbury and Rognlien, 2000) who used a hybrid system to show that biologically derived vesicles (small clear synaptic vesicles) could fuse with target membranes containing only syntaxin (but no SNAP-25).

Here we have shown that syntaxin fusion also works with large dense-core neurosecretory granules. The observed spontaneous fusion appears to result from a protein-protein interaction, because fusion was not observed without the addition of both syntaxin 1A and the native membrane proteins of bovine NSG. Furthermore, pretreatment of mNSGs with a soluble form of syntaxin inhibits spontaneous fusion.

The addition of nystatin/ergosterol channels and artificial lipids to large dense-core granules are not required to observe this result. Recent work in our lab has shown syntaxin 1A is also sufficient to induce spontaneous fusion of large dense core granules isolated from bovine chromaffin cells with a planar lipid bilayer (McNally et al., 2003). This was shown with both the nystatin/ergosterol technique used above and via amperometric recording of the fusion of unmodified secretory granules.

It is intriguing that SNAP-25 (also a t-SNARE) is not required in the bilayer to induce fusion of the native secretory vesicles. Biochemical studies have shown that SNAP-25 is a major contributor to the coiled-coil helix that is believed to form between SNARE proteins during membrane fusion (Poirier et al., 1998; Weis and Scheller, 1998). One possible reason SNAP-25 was not required in our system is that some SNAP-25 may have co-purified with our native bovine

NSG. This explanation is unlikely since SNAP-25 is a t-SNARE and it has been shown that v- and t-SNAREs must be present on opposite membranes in order for fusion to occur (Nichols et al., 1997). Another possibility is that a different protein from the NSG membrane may be able to take the place of SNAP-25. Proteins such as synaptotagmin and synaptophysin, that are believed to play a regulatory role in the SNARE fusion complex, may be able to substitute for SNAP-25 in the formation of a stable fusion complex. In addition, it has been suggested (Misura et al., 2001) that syntaxin itself might be able to take the place of SNAP-25 in fusion complex formation. Self-association of the core region of syntaxin, involved in fusion complex formation, is a well-defined phenomenon. We favor the hypothesis that syntaxin aggregates to form a t-SNARE complex that is competent to induce fusion of native secretory vesicles. If this is the case, it is yet to be determined if syntaxin complexes form in solution or after inserting into the bilayer membrane.

We have also shown that syntaxin fusion requires much, but not all of the syntaxin molecule. At the C-terminus, both the membrane spanning region and the SNARE motif are necessary (and sufficient) for fusion. Removal of either region eliminates fusion. The SNARE motif is the minimal interacting region between SNARE proteins. For the SNARE fusion complex it includes domains from syntaxin, Synaptobrevin/VAMP, and separate N- and C-terminal domains of SNAP-25. These interacting domains are around 60-80 amino acids in length with at least one heptad repeat. Individual SNARE proteins are largely

unstructured, however, with SNARE complex formation there is an increase in α-helical content (Poirier et al., 1998). The first and fourth residue of each repeat is hydrophobic, making one face of the α-helix hydrophobic (Weis and Scheller, 1998). In this study, we observed no spontaneous fusion when the SNARE motif was removed (syntaxin 1A-12) and the addition of a syntaxin without its transmembrane domain (syntaxin 1A-11) blocked, not enhanced, fusion. These data support the conclusion that syntaxin fusion is driven by specific interactions between SNARE proteins in the mNSG membrane and the SNARE motif of the membrane-bound syntaxin.

The N-terminal domain of syntaxin function as a regulatory domain by controling the availability of the C-terminal region of the protein, involved in SNARE complex assembly (Nicholson et al., 1998). Previously it has been shown that removal of the regulatory domain from syntaxin makes SNARE-driven fusion of phospholipid vesicles more rapid and efficient (Parlati et al., 1999) or slower and less efficient (Rognlien and Woodbury, 2003). In contrast to these studies we have found that the N-terminal regulatory domain of syntaxin was not required for mNSG fusion, and its presence or absence did not alter the rate of this fusion. There are two possible explanations for this finding. First, the syntaxin that we are using may constitutively be in the open conformation. The second, and more likely, possibility is that the system lacks the additional regulatory components, such as munc18 (Nicholson et al., 1998; Rizo and

Sudhof, 2002; Jahn et al., 2003), necessary to hold syntaxin in the closed conformation.

The results presented here are consistent with those obtained using synaptic vesicles isolated from the electric organ of Torpedo californica (Woodbury and Rognlien, 2000). Furthermore, they extend syntaxin fusion to large dense-core granules, suggesting that this fusion machine may have general application throughout many biological systems. The fact that calcium is not required for fusion in these and other reconstituted systems ((Weber et al., 1998; Parlati et al., 1999; Misura et al., 2001) but see (Hu et al., 2002)) suggests that the reconstituted proteins form a fundamental fusion machine. This machine, or a variant with homologous proteins, may also be responsible for constitutive fusion necessary for unregulated exocytosis. Indeed, SNARE proteins appear to be involved in a general fusion apparatus that is remarkably conserved from yeast to humans. The results presented here help to expand our understanding of the role of SNARE proteins in this molecular mechanism, which drives membrane fusion and neurotransmitter release. Ultimately, information gleaned from these and related experiments may lead to the building of an artificial synapse made completely of isolated or cloned biological components (Woodbury, 1999a). An artificial synapse would provide a powerful method with which to identify and study the different regulatory components affecting membrane fusion.

References:

Hu K, Carroll J, Fedorovich S, Rickman C, Sukhodub A, Davletov B (2002) Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion. Nature 415:646-650.

Jahn R, Lang T, Sudhof TC (2003) Membrane fusion. Cell 112:519-533.

Kelly ML, Woodbury DJ (1996) Ion channels from synaptic vesicle membrane fragments reconstituted into lipid bilayers. Biophys J 70:2593-2599.

Langosch D, Crane JM, Brosig B, Hellwig A, Tamm LK, Reed J (2001) Peptide mimics of SNARE transmembrane segments drive membrane fusion depending on their conformational plasticity. J Mol Biol 311:709-721.

Lee CJ, Dayanithi G, Nordmann JJ, Lemos JR (1992) Possible role during exocytosis of a Ca(2+)-activated channel in neurohypophysial granules. Neuron 8:335-342.

Lemos JR, Ocorr KA, Nordmann JJ (1989) Possible role for ionic channels in neurosecretory granules of the rat neurohypophysis. Soc Gen Physiol Ser 44:333-347.

McNally JM, Woodbury DJ, Lemos JR (2003) Syntaxin Mutants Modulate Fusion of Dense Core Granules to a Planar Lipid Bilayer. Biophys J 81:A950.

Misura KM, Scheller RH, Weis WI (2001) Self-association of the H3 region of syntaxin 1A. Implications for intermediates in SNARE complex assembly. J Biol Chem 276:13273-13282.

Nichols BJ, Ungermann C, Pelham HR, Wickner WT, Haas A (1997) Homotypic vacuolar fusion mediated by t- and v-SNAREs. Nature 387:199-202.

Nicholson KL, Munson M, Miller RB, Filip TJ, Fairman R, Hughson FM (1998) Regulation of SNARE complex assembly by an N-terminal domain of the t-SNARE Sso1p. Nat Struct Biol 5:793-802.

Nordmann JJ, Louis F, Morris SJ (1979) Purification of two structurally and morphologically distinct populations of rat neurohypophysial secretory granules. Neuroscience 4:1367-1379.

Parlati F, Weber T, McNew JA, Westermann B, Sollner TH, Rothman JE (1999) Rapid and efficient fusion of phospholipid vesicles by the alpha-helical core of a SNARE complex in the absence of an N-terminal regulatory domain. Proc Natl Acad Sci U S A 96:12565-12570.

Poirier MA, Xiao W, Macosko JC, Chan C, Shin YK, Bennett MK (1998) The synaptic SNARE complex is a parallel four-stranded helical bundle. Nat Struct Biol 5:765-769.

Rizo J, Sudhof TC (2002) Snares and Munc18 in synaptic vesicle fusion. Nat Rev Neurosci 3:641-653.

Rognlien K, Woodbury DJ (2003) Reconstituting SNARE Proteins into BLMs. In: Planar Lipid Bilayers (BLMs) and Their Applications (Tein HT, Ottova-Leitmannova A, eds), pp 479-488.

Schiavo G, Stenbeck G, Rothman JE, Sollner TH (1997) Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. Proc Natl Acad Sci U S A 94:997-1001.

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature 362:318-324.

Sudhof TC (1995) The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature 375:645-653.

Ungermann C, Nichols BJ, Pelham HR, Wickner W (1998) A vacuolar v-t-SNARE complex, the predominant form in vivo and on isolated vacuoles, is disassembled and activated for docking and fusion. J Cell Biol 140:61-69.

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. Cell 92:759-772.

Weimbs T, Mostov K, Low SH, Hofmann K (1998) A model for structural similarity between different SNARE complexes based on sequence relationships. Trends Cell Biol 8:260-262.

Weis WI, Scheller RH (1998) Membrane fusion. SNARE the rod, coil the complex. Nature 395:328-329.

Woodbury DJ (1999a) Building a bilayer model of the neuromuscular synapse. Cell Biochem Biophys 30:303-329.

Woodbury DJ (1999b) Nystatin/ergosterol method for reconstituting ion channels into planar lipid bilayers. Methods Enzymol 294:319-339.

Woodbury DJ, Miller C (1990) Nystatin-induced liposome fusion. A versatile approach to ion channel reconstitution into planar bilayers. Biophys J 58:833-839.

Woodbury DJ, Rognlien K (2000) The t-SNARE syntaxin is sufficient for spontaneous fusion of synaptic vesicles to planar membranes. Cell Biol Int 24:809-818.

Yin Y, Dayanithi G, Lemos JR (2002) Ca(2+)-regulated, neurosecretory granule channel involved in release from neurohypophysial terminals. J Physiol 539:409-418.

Zhong P, Chen YA, Tam D, Chung D, Scheller RH, Miljanich GP (1997) An alpha-helical minimal binding domain within the H3 domain of syntaxin is required for SNAP-25 binding. Biochemistry 36:4317-4326.

CHAPTER III: Functional Ryanodine Receptors are Found in Neurohypophysial Secretory Granule Membranes

PREFACE

Material presented in this chapter has been or will be published in the following locations

<u>Manuscripts:</u>

J.M. McNally, C. Velazquez-Marrero, E.E. Custer, S.D. Kraner*, M .Muschol*, L. Obaid*, B.M. Salzberg*, and J.R. Lemos. (2008) Functional Ryanodine Receptors are Found in the Membranes of Neurohypophysial Secretory Granules. (In Preparation)

<u>Abstracts:</u>

J.M. McNally, C. Velazquez-Marrero, E.E. Custer, D.J. Woodbury, J.R. Lemos. (2006) Ryanodine Stimulates Both a Neurosecretory Granule Channel and Neuropeptide Release from Nerve Terminals of the Neurohypophysis. Biophys. J. 90:1083A.

Abstract:

Recently, highly localized Ca^{2+} release events, e.g. Ca^{2+} sparks previously described in muscle cells, have been observed in neuronal preparations. In murine neurohypophysial terminals, these Ca^{2+} release events, termed Ca^{2+} syntillas, appear to emanate from a ryanodine sensitive intracellular Ca^{2+} pool, and their frequency in modulated by depolarizing stimuli. As traditional sources of ryanodine sensitive Ca^{2+} release have been ruled out in neurohypophysial terminals, we tested the hypothesis that large dense core granules, which are known to contain a significant amount of calcium, represent the source of the syntillas. Previous work has shown that a large conductance non-specific cation channel, present in the granule membrane, may play a role in exocytosis. This channel displays several characteristics, including multiple conductances and Ca^{2+}-dependence, that are identical to those observed in ryanodine receptors (RyR). Here we present evidence that the granule channel is pharmacologically affected in the same characteristic manner as the RyR: it is activated in the presence of the RyR agonist ryanodine (at low concentrations), and blocked by the RyR antagonist ruthenium red. Furthermore, neuropeptide release experiments show that the RyR agonists ryanodine, caffeine, and imperatoxin A induce neuropeptide release from neurohypophysial terminals. Additionally, immunogold labeled electron micrographs show that ryanodine receptors are localized specifically to large dense core granules. This evidence suggests that RyR-dependent Ca^{2+} release events could represent mobilization of Ca^{2+} from granular stores. Thus, localized Ca^{2+} release at the precise location of exocytosis could provide Ca^{2+} amplification and either affect or modulate neuropeptide hormone release physiologically.

Introduction:

It is well known that hormone secretion necessitates the influx of extracellular calcium through voltage gated calcium channels. However, an increasing amount of evidence makes it clear that Ca^{2+} release from internal stores may provide another important source of Ca^{2+} for this process (Salzberg et al., 1997; Collin et al., 2005; Berridge, 2006). The hypothalamic-neurohypophysial system, responsible for the secretion of the neuropeptides oxytocin and vasopressin, represents a classical model system for the study of Ca^{2+} stimulated release (Douglas and Poisner, 1964). Isolated neurohypophysial nerve terminals comprise the main site of hormone release in this system. As these terminals are specialized secretory structures, they contain relatively little intracellular machinery beyond large dense core granules, microvesicles, and mitochondria. This provides a greatly simplified model system with which to define mechanisms involved in secretion (Lemos et al., 1989).

Recently it has been demonstrated that highly localized Ca^{2+} transients, termed Ca^{2+} syntillas, can be observed in isolated neurohypophysial terminals (NHT). These syntillas are observed even in the absence of extracellular Ca^{2+} and appear to emanate from a ryanodine-sensitive intracellular Ca^{2+} store (De Crescenzo et al., 2004). Further, while these syntillas are observed spontaneously, depolarization induces an increase in their frequency without affecting their amplitude (De Crescenzo et al., 2006). In spite of all this

information, the source of the released Ca^{2+} and a physiological role for this phenomenon has yet to be defined.

Large dense core granules, as well as synaptic vesicles, contain a substantial amount of Ca^{2+} (Nicaise et al., 1992; Thirion et al., 1995; Gerasimenko et al., 1996; Scheenen et al., 1998; Mundorf et al., 2000; Mitchell et al., 2001; Gerasimenko et al., 2006). In chromaffin granules, for instance, the intragranular Ca^{2+} makes up 60% of the total Ca^{2+} contained in the cell (Haigh et al., 1989). While this has been well known for many years, the precise role of this vesicular pool of Ca^{2+} is still quite controversial. Although secretory vesicles contain this high percentage of total internal Ca^{2+}, they contain a smaller amount of free Ca^{2+} than other organelles such as the endoplasmic reticulum and Golgi, due to the high buffering capacity of vesicles (Hutton, 1984). This has led to the widespread assumption that the vesicular Ca^{2+} pool is immobile and serves only as a means by which the cell removes intracellular Ca^{2+} (Nordmann and Zyzek, 1982; Pozzan et al., 1994).

Recently, however, it has been demonstrated that vesicular Ca^{2+} is a significant regulatory component in several secretory systems (Nicaise et al., 1992; Pozzan et al., 1994; Thirion et al., 1995; Gerasimenko et al., 1996; Martinez et al., 1996; Scheenen et al., 1998; Mundorf et al., 2000; Mitchell et al., 2001; Mitchell et al., 2003; Mahapatra et al., 2004; Gerasimenko et al., 2006). Work with insulin secreting ß-cells has shown that the secretory granules of these cells represent a ryanodine-sensitive dynamic Ca^{2+} store. By expressing a

synaptobrevin-aqueorin chimera in these cells, Mitchell et al. (2001) were able to demonstrate that ryanodine and cyclic-ADP ribose (cADPr), another RyR agonist, causes a dramatic decrease in vesicular Ca^{2+}. A similar effect has been shown in pancreatic acinar cells (Gerasimenko et al., 2006). Furthermore, isolated large dense core granules from this system have been shown to release Ca^{2+} following treatment with IP3 and cADPr (Gerasimenko et al., 1996). Work by Scheenen et al. (1998) with ß-cells has shown, at the single cell level, that depletion of vesicular Ca^{2+} markedly reduces secretion. This effect on secretion following vesicular Ca^{2+} depletion has also been demonstrated in chromaffin cells (Mundorf et al., 2000).

Neurohypophysial secretory granules (NSG) , large dense core granules isolated from NHT, are also known to contain a significant amount of Ca^{2+} (Thirion et al., 1995). However, the physiological role of this calcium is presently not understood. Interestingly, the calculated number of Ca^{2+} ions released per syntilla (280,000; De Crescenzo el al., 2004) compares favorably to the amount of free Ca^{2+} contained in one NSG (G. Nicaise, Per. Comm.).

Ion channels have been reported to be present in the membranes of secretory vesicles in many systems (Woodbury, 1995), but the physiological function of these channels has yet to be elucidated. Specifically in NSG, a large multi-conductance non-specific cation channel has been characterized that appears to be involved in secretion (Lee et al., 1992; Yin et al., 2002). Interestingly, this channel possesses properties that are almost identical to the

known single channel properties of mammalian RyR (Ma et al., 1988; Coronado et al., 1994; Fill and Copello, 2002). Both of these channels share similar multiple conductances and are regulated in a bimodal fashion by Ca^{2+}, with peak channel activity occurring at a free calcium concentration of 10 µM.

Since Ca^{2+} syntillas in neurohypophysial terminals emanate from ryanodine-sensitive stores, the localization of RyR to the membrane of these granules would argue that NSGs are the origin of syntillas. Here we use immunogold labeling of neurohypophysial terminals to examine whether RyRs co-localize with NSG. Furthermore, single channel analysis of the large conductance NSG channel is employed to determine if this channel is sensitive to pharmacological agents specific for the RyR. RyR agonists and antagonists were also tested to determine if mobilization of this ryanodine-sensitive calcium store is capable of affecting hormone release from isolated neurohypophysial terminals. This diversified approach has allowed us to determine that the NSGs are involved in ryanodine-sensitive calcium release and can modulate secretion from these central nervous system terminals.

Materials and Methods:

Immuno-Electron microscopy

Freshly dissected mouse posterior pituitary glands were prepared in the following manner. Tissue blocks were prepared by fixing glands for 4 hours at 4°C in a 200mM sodium cacodylate buffer containing 16% purified monomeric

paraformaldehyde, and 8% purified monomeric glutaraldehyde (pH 7.4). After fixation the tissue was transferred directly into a 100 mM sodium cacodylate buffer (pH 7.4) to wash. The tissue was then quickly rinsed with ice cold dH$_2$O and transferred to 50% EtOH at 4°C. After 10 minutes of incubation, the EtOH was increased to 70%, then 80% and finally 90% EtOH, after 10 minutes of incubation at each concentration. Then the tissue was placed in a 2:1 mixture of EtOH and LR White and allowed to incubate at room temperature for 30 minutes. Then the mixture was replaced with pure LR White and allowed to stand for an additional hour; then changed to fresh resin and left at 4°C overnight in a firmly sealed vial. The following morning, the tissue was embedded in gelatin capsules, overfilled, and cured at 58°C for ~30 hours.

For immunostaining, the fixed and embedded glands were cut into 80 nm thick sections (Leica Ultracut S microtome), and mounted of 200 mesh thin bar nickel grids. After drying, the grids were blocked for one hour at room temperature with 1% ovalbumin and 0.2% cold water fish skin gelatin in PBS (pH 7.4) to prevent non-specific binding. After removing excess solution, the grid was then incubated overnight at 4°C with primary antibody (described below) diluted in PBS (pH 7.4). After washing with Tris buffered PBS (pH 7.4) for 20 minutes, the grids were then incubated with 10nm colloidal Au anti-rabbit for one hour at room temperature. The grids were then wash two times with Tris buffered saline and then one with dH$_2$O. Finally the labeled grids were stained briefly (3

minutes) in aqueous unranyl acetate solution, washed again with dH_2O and allowed to air dry. Labeled Grids were observed in a JEOL100 CX microscope,

Fixed neurohypophysial sections were labeled with Pan-RyR specific rabbit polyclonal antisera, raised against a peptide representing the carboxy-terminal 16 amino acids of the RyR, a sequence that is conserved in all mammalian RyR subtypes (Tunwell et al., 1996). In addition antibodies specific for type 1 and type 2 RyR were employed. These antibodies, a generous gift from F. Anthony Lai (Wales Heart Research Institute, Cardiff University, UK), were generated using the following epitopes unique to RyR1 and RyR2: RREGPRGPHLVGPSRC for RyR1 (Mitchell et al., 2003) and KAALDFSDAREKKKPKKDSSLSAV for RyR2 (Tunwell et al., 1996). Representative images are shown for slices labeled with each of the primary antibodies used.

Isolated Bovine Neurosecretory Granules

The NSG of bovine posterior pituitary glands were obtained as previously reported (McNally et al., 2004). Briefly, bovine neurohypophyses were collected and stored in warm (37°C) isoosmotic homogenizing solution (HS): 300 mM sucrose, 10 mM Tris-HEPES and 1 mM EGTA (pH 7.0) to prevent release. The glands were then washed, finely minced with scissors, and homogenized in ice cold HS. The homogenate was first sequentially centrifuged and then NSG were separated on a percoll density gradient, yielding an extremely pure preparation of

NSG (Lemos et al., 1989). The purified NSG were then aliquoted, rapidly frozen

and stored at –80° for later use.

Single Channel Analysis in Planar Lipid Bilayer

Bilayers were formed as previously described (Yin et al., 2002; McNally et

al., 2004). Briefly, phosphatidylethanolamine (PE) and phosphatidylserine (PS)

(Avanti Polar Lipids, 10 mg/ml in chloroform) were mixed at a 3:1 ratio (w:w), and

then dried under a stream of argon. The lipids were then resuspended in decane

(Sigma) and used for formation of lipid bilayers. A 150-250 μm aperture in a

polystyrene cup (Sarstedt) was positioned between the two chambers of the

bilayer apparatus. The chambers of the bilayer apparatus were then filled with

50 mM KCL, 10 mM HEPES, 2mM EGTA (pH 7.3) [1 ml trans and 0.7 ml cis].

Bilayers were then formed across the aperture. After bilayer formation, a

250mM/150mM KCl gradient (cis:trans) was established across the bilayer.

Purified bovine NSG and 10 μM free Ca^{2+} were added to the cis chamber, which

corresponded to the cytoplasmic side of the channel. The Trans chamber

remained at zero Ca^{2+} to ensure that channels without the proper orientation

would remain inactive. After incorporation of a channel into the bilayer, chamber

stirring was stopped to avoid further channel incorporation. The current across

the bilayer was then be monitored using a standard patch clamp amplifier (EPC9,

HEKA). Data was collected and stored in a computer and analyzed with a single

channel analysis program (TAC X4.1, Bruxton).

Population Release Assay

After decapitation, the neurohypophyses from Swiss Webster mice were harvested and homogenized in an isoosmotic solution of 270 mM sucrose, 1 mM EGTA, 10 mM HEPES (pH 6.8). The homogenate was then loaded onto filters and perfused, using a peristaltic pump, with normal Locke's saline (mM): 140 NaCl, 5 KCl, 10 glucose, 1 $MgCl_2$, 2.2 $CaCl_2$, 10 HEPES, (pH 7.2) preheated to 37^oC, unless otherwise noted. Hormone release was determined by collecting the perfusate using a standard fraction collector. This perfusate was then frozen and stored at -4^oC until quantitative analysis of hormone content could be performed using an Enzyme Linked Immuno-Specific Assay (ELISA, Assay Designs, Ann Arbor, MI). Drugs were applied by addition to the perfusing solution.

Results:

Localization of RyR via ImmunoEM

Murine NHTs have been shown to posses all three subtypes of the RyR (Salzberg et al., 1997; De Crescenzo et al., 2004; De Crescenzo et al., 2006). Interestingly, immunocytochemical labeling indicates that the distribution of the RyR labeling appears similar to that of known vesicular markers. However, due to the small size of the NSG (~200 nm), it is not possible to determine if RyR are colocalized with NSG using immunocytochemistry. Therefore, Immunogold EM was employed to resolve this relationship. Fixed neurohypophysial slices were

labeled with primary antisera specific for either type 1, type 2 RyR or an antisera specific for all types of RyR (Gracious gift of F. Anthony Lai, Cardiff University, UK). Antibody labeling of the slices was visualized by conjugating the secondary antibody to immunogold beads (10 nm). Electron micrographs of these labeled slices (figure 3.1) show that immunogold bead decoration was specifically localized to large dense core granules (NSGs).

Ryanodine Receptor Agonists/Antagonist Affect NSG Channel Activity

A large multiple-conductance non-specific cation channel that is bimodally regulated by Ca^{2+} has been shown to reside in the NSG membrane (Lee et al., 1992; Yin et al., 2002). The characteristics of this channel are remarkably similar to those of the mammalian RyR (Ma et al., 1988; Coronado et al., 1994; Fill and Copello, 2002). Combined with the EM data above, this suggests that the NSG channel may in fact be a RyR. Therefore, the NSG channel was examined to determine if it had the same characteristic response as the RyR to activation by ryanodine, and blockade by ruthenium red (Coronado et al., 1994; Fill and Copello, 2002). .

Using planar lipid bilayer recording, NSG channels were isolated from highly purified bovine secretory granules (see Methods). For these experiments the free Ca^{2+} concentration was kept at a constant 10 µM in the *cis* chamber of the bilayer apparatus, a Ca^{2+} concentration known to elicit peak activity for both the NSG channels (Lee et al., 1992) and the RyR (Coronado et al., 1994). After

Pan RyR

Type 1 RyR

Type 2 RyR
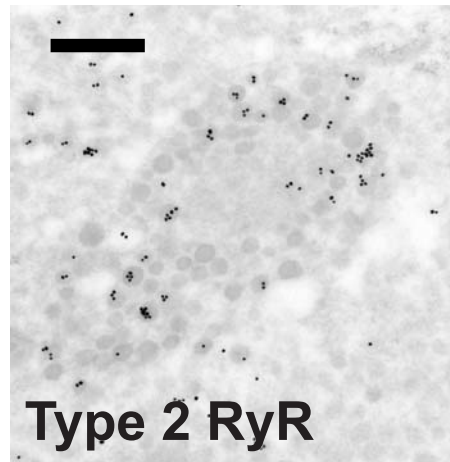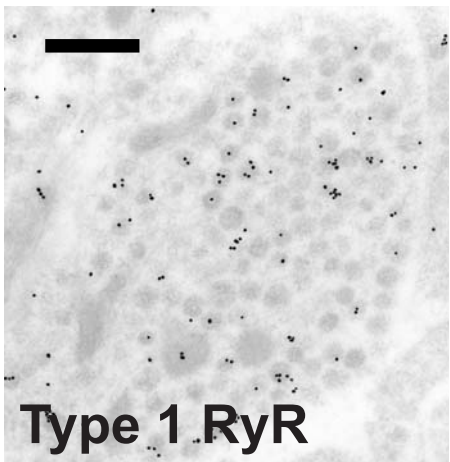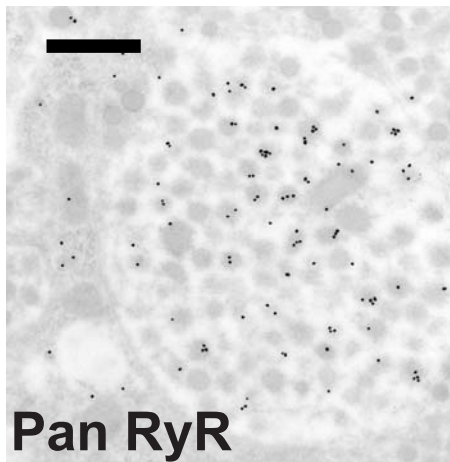
Figure 3.1: RyR labeling is localized to Neurosecretory Granules. Electron micrographs of neurohypophysial slices labeled with Immunogold beads conjugated to pan-RyR primary antibodies and both RyR type 1 and type 2 specific antibodies (*Top, middle, and bottom images respectively*). In all three cases the immunogold labeling decorates the NSG of the neurohypophysial terminals. (Scale Bars are equivalent to 500 nm).

incorporation into the bilayer, the activity of the NSG channel was recorded for several minutes, providing a means to assess its basal activity (Figure 3.2 top).

Ryanodine (5-10 µM), a specific agonist of the RyR, was then added to the *cis* well with stirring, and channel activity was again recorded (Figure 3.2 bottom). In these experiments application of ryanodine led to a statistically significant (p<0.05) increase in the probability of the channel residing in its subconductance state (O_1), from 0.23 ± 0.08 under basal conditions to 0.85 ± 0.1 in the presence of ryanodine (n=3, see Figure 3.2 right). Furthermore, the open probability of the channel (p_o) increased from 0.64 ± 0.24 under control conditions to 0.86 ± 0.09 in the presence of ryanodine. While this is not a statistically significant increase in p_o (p=0.15), an increase in p_o was observed in all isolated NSG channels tested (3/3). Thus, the NSG channel responded to ryanodine in the same characteristic manner as a native RyR.

The effects of the RyR antagonist ruthenium red on the NSG channel were also examined using the same protocol described above. Following the addition of ruthenium red (5 µM) to the *cis* chamber, there was a statistically significant (p<0.05) reduction in the p_o from 0.59 ± 0.09 under basal conditions to 0.08 ± 0.04 with ruthenium red (n=4). This effect is shown in figure 3.3, where the channel activity of this NSG channel ceases several minutes after application of ruthenium red.

NSG Channel Alone

NSG Channel +10 µM Ryanodine

5 pA

2 sec

HP = -10 mV
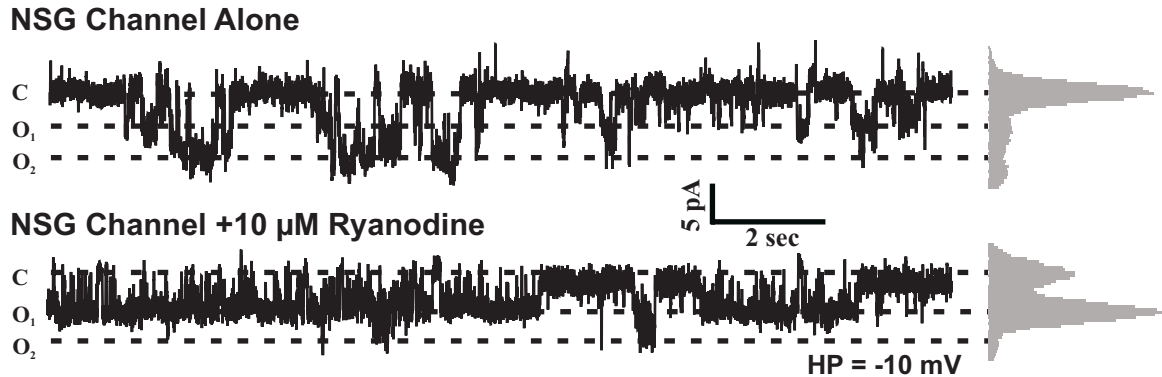
Figure 3.2: **The NSG Channel Responds to Agonist Concentrations of Ryanodine in the Same Characteristic Manner as RyRs.** Single channel recording showing the effects of RyR agonist ryanodine (10 µM) on the NSG Channel isolated in a PE:PS (3:1) planar lipid bilayer. The bilayer apparatus contained 250 mM KCl, 10 mM HEPES, and 10 µM free Ca^{2+} in the *cis* well and 150 mM KCl, 10 mM HEPES in the *trans* well. (**Top**) A large conductance cation channel, isolated from purified NSG, was recorded at a steady holding potential (−10 mV) for several minutes. (**Bottom**) After the addition of ryanodine (10 µM) to the *cis* chamber of the bilayer apparatus, the same channel was recorded. As shown by the all points amplitude histogram (**right**), ryanodine causes an increase in the channel's open probability, but at a decreased conductance. Under control conditions the NSG channel remained in the closed state (C) 84% of the time and was in open states one and two (O_1, O_2) 10% and 6% of the time respectively. After application of ryanodine the channel remains in the closed state only 31% on the time, and is in open state one 66% of the time and open state two only 3 % of the time.

NSG Channel Alone

NSG Channel + 5µM Ruthenium Red
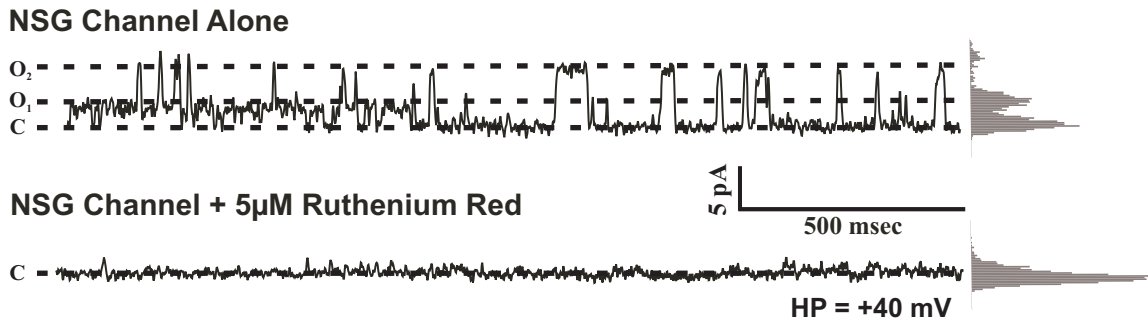
Figure 3.3: Ruthenium red inhibits NSG channel openings. The bilayer apparatus contained 150 mM KCl, 10 mM HEPES, and 10 µM free Ca^{2+} in the *cis* well and 150 mM KCl, 10 mM HEPES in the *trans* well. (**Top**) An NSG channel isolated in a planar bilayer was recorded at a steady holding potential of +40 mV for several minutes. (**Bottom**) After the addition of the RyR antagonist ruthenium red (5 µM), the activity of the same channel was greatly reduced. This is further illustrated by the all points amplitude histogram (right). Under control conditions the NSG channel remained in the closed state (C) 57% of the time and was in open states one and two (O_1, O_2) 36% and 7% of the time respectively. After application of ruthenium red (5 µM) channel openings were rapidly inhibited.

RyR Agonist Application Leads to Secretion

Previous work has suggested that the NSG channel described above was involved in neuropeptide secretion (Lee et al., 1992). Since we now know that this channel is sensitive to agonist levels of ryanodine, we wanted to determine whether or not mobilization of Ca^{2+} from ryanodine sensitive stores could play a role in neuropeptide secretion from neurohypophysial terminals. Therefore, we examined the effects of agonist concentrations of ryanodine on hormone release from a population of isolated NHT. For these experiments NHT were perfused with a Normal Locke's solution containing no added free Ca^{2+} and 2 mM EGTA, to rule out extracellular Ca^{2+} entry into the terminal as a cause of any observed affect. Addition of ryanodine (100 nM) to the perfusate resulted in an increase in basal hormone release (Figure 3.4 left, n = 3). Imperatoxin A (100 nM), a scorpion toxin that serves as a highly specific agonist of the RyR (Gurrola et al., 1999), is also able to elicit an increase in basal release (Figure 3.4 right, n = 3).

Discussion:

The idea that NSGs posses a dynamic store of Ca^{2+} is a very attractive concept, in the sense that it provides a source of Ca^{2+} in the precise location where secretion occurs. Further, the proximity of docked NSGs to voltage-gated Ca^{2+} channels enables this process to act as an elegant Ca^{2+}-amplifier in the event that Ca^{2+}-entry alone is insufficient to affect NSG exocytosis. The simplicity of neurohypophysial terminals provides an excellent system to examine such
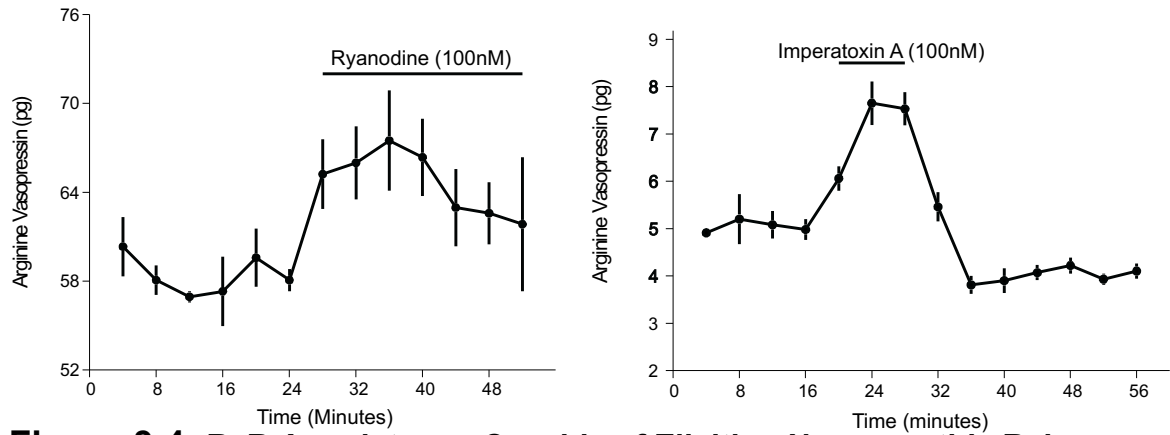
Figure 3.4: RyR Agonists are Capable of Eliciting Neuropeptide Release in the Absence of Extracellular Calcium. These peptide release experiments show the release of vasopressin from isolated neurohypophysial terminals in response to the application of RyR agonists. Terminals were perfused with calcium free Normal Locke's solution where calcium was replaced with 200 μM EGTA. Fractions of perfusate were collected and assayed for vasopressin content via ELISA. (**Top**) Application of ryanodine at agonist concentrations (100 nM) resulted in a 16% increase in peptide release above baseline. (**Bottom**) Application of imperatoxin A, a scorpion toxin that has been shown to be a highly specific agonist of the type 1 RyR, also resulted in a 60% increase in peptide release.

phenomena. The experiments here provide evidence that intracellular Ca^{2+} release events in neurohypophysial terminals emanate from ryanodine-sensitive Ca^{2+} stores that are identified as the NSGs, and, that mobilization of Ca^{2+} from vesicular stores serves to modulate neuropeptide secretion.

From our experimental results, the RyR appears to reside exclusively in the NSG membrane. Two lines of evidence suggest that this is the case. First, Immunogold labeled electron micrographs show that RyR are localized specifically to NSGs in neurohypophysial terminals (Fig 3.1). Secondly, a large multi-conductance cation channel, similar in nature to the RyR, was isolated from the membrane of NSG, and shown to be affected by specific ryanodine receptor agonists and antagonists in the same characteristic fashion as the RyR itself (see Fig 3.2 & 3.3), suggesting that these two channels are one and the same.

Earlier work from our group suggested that the NSG cation channel might in fact be synaptophysin, due to effects that the synaptophysin specific SY-38 anitbody had on channel activity (Yin et al., 2002). However, more recent studies characterizing the channel activity of purified synaptophysin (Gincel and Shoshan-Barmatz, 2002) describe a channel that is quite different from the NSG channel. Interestingly, this discrepancy could be due to the fact the c-terminal region of synaptophysin, the site of SY-38 antibody binding, exhibits significant homology with a region of the type1 RyR. This particular region is thought to contain one of the calcium binding domains of the RyR (Coronado et al., 1994), which might explain the effects the SY-38 antibody had on the calcium

dependence of the NSG channel (Yin et al., 2002). However, additional experimentation is required to determine the validity of this hypothesis.

If syntillas represent Ca^{2+} release from vesicular stores, then it would make sense that localized Ca^{2+} release in the precise location where exocytosis is occurring must play a role in modulating release. Ryanodine-sensitive intracellular Ca^{2+} release has been shown to have a facilitatory effect on transmitter release in a number of different neuronal systems (Llano et al., 2000; Bardo et al., 2002; Galante and Marty, 2003; Conti et al., 2004; Ouyang et al., 2005). This evidence has led to some speculation that syntillas may function in such a manner in terminals (Berridge, 2006; Oheim et al., 2006) despite the fact that this has yet to be proven. While in contrast, other systems show that ryanodine sensitive calcium release plays little or no role in transmitter release (Carter et al., 2002; Lim et al., 2003). Specifically in the neurohypophysis, however, ryanodine-sensitive calcium release has been shown to play a physiologically relevant role in oxytocin secretion (Jin et al., 2007).

Our hormone release results indicate that in NHT, ryanodine-sensitive stores play a facility role in secretion. Mobilization of these Ca^{2+} stores results in an increase in basal release. How exactly this facilitation is conferred on release still remains unknown. In chromaffin cells simultaneous calcium imaging and amperometric recording was performed to determine the relationship between syntillas and spontaneous quantal release events (ZhuGe et al., 2006). Surprisingly, in this study no correlation was observed between the two events,

arguing against a direct role of syntillas in eliciting secretion. This however does not rule out an indirect modulatory role, as the relationship between syntillas and release has yet to fully examined in the NHT. These studies are currently in progress (McNally et al., 2006, and see Chapter IV).

Beyond a direct interaction with the Ca^{2+} sensing mechanism involved in secretion, Ca^{2+} has also been shown to regulate a number of other intracellular processes (Berridge, 2006). As demonstrated by Llinas et al. (1991), a Ca^{2+} dependent process is involved in releasing vesicles tethered to the actin cytoskeleton. Recently, evanescent wave microscopy studies of chromaffin cells have revealed that Ca^{2+} domains from voltage-gated calcium channels result in the mobilization of granules toward sites of Ca^{2+} influx (Becherer et al., 2003).

There is precedence to suggest such a role for syntillas in the hypothalamic neurohypophysial system. Ludwig and Leng (2006), have shown that intracellular calcium release has a priming effect on dendritic neuropeptide secretion from magnocellular neurons. This priming involves the relocation of secretory granules from reserve pools to the readily releasable pool. While this phenomenon has yet to be demonstrated in the NHT itself, it is possible that syntillas could play a role in vesicle mobility and the recruitment of vesicles to docked pools on the plasma membrane.

In conclusion, the above work suggests that voltage-dependent Ca^{2+} transients, Ca^{2+} syntillas, may reflect release from ryanodine-sensitive vesicular stores. Such increases in intracellular Ca^{2+} that occur in these terminals may

play a role in modulating peptide hormone secretion by either Ca^{2+}- induced Ca^{2+}-release or by activation of RyR via depolarization. Such a process could play a major role in the control of oxytocin and vasopressin release from NHT. However, further work is required to specifically define the mechanism behind this modulation.

References:

Bardo S, Robertson B, Stephens GJ (2002) Presynaptic internal Ca2+ stores contribute to inhibitory neurotransmitter release onto mouse cerebellar Purkinje cells. Br J Pharmacol 137:529-537.

Becherer U, Moser T, Stuhmer W, Oheim M (2003) Calcium regulates exocytosis at the level of single vesicles. Nat Neurosci 6:846-853.

Berridge MJ (2006) Calcium microdomains: organization and function. Cell Calcium 40:405-412.

Carter AG, Vogt KE, Foster KA, Regehr WG (2002) Assessing the role of calcium-induced calcium release in short-term presynaptic plasticity at excitatory central synapses. J Neurosci 22:21-28.

Collin T, Marty A, Llano I (2005) Presynaptic calcium stores and synaptic transmission. Curr Opin Neurobiol 15:275-281.

Conti R, Tan YP, Llano I (2004) Action potential-evoked and ryanodine-sensitive spontaneous Ca2+ transients at the presynaptic terminal of a developing CNS inhibitory synapse. J Neurosci 24:6946-6957.

Coronado R, Morrissette J, Sukhareva M, Vaughan DM (1994) Structure and function of ryanodine receptors. Am J Physiol 266:C1485-1504.

De Crescenzo V, ZhuGe R, Velazquez-Marrero C, Lifshitz LM, Custer E, Carmichael J, Lai FA, Tuft RA, Fogarty KE, Lemos JR, Walsh JV, Jr. (2004) Ca2+ syntillas, miniature Ca2+ release events in terminals of hypothalamic neurons, are increased in frequency by depolarization in the absence of Ca2+ influx. J Neurosci 24:1226-1235.

De Crescenzo V, Fogarty KE, Zhuge R, Tuft RA, Lifshitz LM, Carmichael J, Bellve KD, Baker SP, Zissimopoulos S, Lai FA, Lemos JR, Walsh JV, Jr. (2006) Dihydropyridine receptors and type 1 ryanodine receptors constitute the molecular machinery for voltage-induced Ca2+ release in nerve terminals. J Neurosci 26:7565-7574.

Douglas WW, Poisner AM (1964) Stimulus-Secretion Coupling in a Neurosecretory Organ: the Role of Calcium in the Release of Vasopressin from the Neurohypophysis. J Physiol 172:1-18.

Fill M, Copello JA (2002) Ryanodine receptor calcium release channels. Physiol Rev 82:893-922.

Galante M, Marty A (2003) Presynaptic ryanodine-sensitive calcium stores contribute to evoked neurotransmitter release at the basket cell-Purkinje cell synapse. J Neurosci 23:11229-11234.

Gerasimenko JV, Sherwood M, Tepikin AV, Petersen OH, Gerasimenko OV (2006) NAADP, cADPR and IP3 all release Ca2+ from the endoplasmic reticulum and an acidic store in the secretory granule area. J Cell Sci 119:226-238.

Gerasimenko OV, Gerasimenko JV, Belan PV, Petersen OH (1996) Inositol trisphosphate and cyclic ADP-ribose-mediated release of Ca2+ from single isolated pancreatic zymogen granules. Cell 84:473-480.

Gincel D, Shoshan-Barmatz V (2002) The synaptic vesicle protein synaptophysin: purification and characterization of its channel activity. Biophys J 83:3223-3229.

Gurrola GB, Arevalo C, Sreekumar R, Lokuta AJ, Walker JW, Valdivia HH (1999) Activation of ryanodine receptors by imperatoxin A and a peptide segment of the II-III loop of the dihydropyridine receptor. J Biol Chem 274:7879-7886.

Haigh JR, Parris R, Phillips JH (1989) Free concentrations of sodium, potassium and calcium in chromaffin granules. Biochem J 259:485-491.

Hutton JC (1984) Secretory granules. Experientia 40:1091-1098.

Jin D, Liu HX, Hirai H, Torashima T, Nagai T, Lopatina O, Shnayder NA, Yamada K, Noda M, Seike T, Fujita K, Takasawa S, Yokoyama S, Koizumi K, Shiraishi Y, Tanaka S, Hashii M, Yoshihara T, Higashida K, Islam MS, Yamada N, Hayashi K, Noguchi N, Kato I, Okamoto H, Matsushima A, Salmina A, Munesue T, Shimizu N, Mochida S, Asano M, Higashida H (2007) CD38 is critical for social behaviour by regulating oxytocin secretion. Nature 446:41-45.

Lee CJ, Dayanithi G, Nordmann JJ, Lemos JR (1992) Possible role during exocytosis of a Ca(2+)-activated channel in neurohypophysial granules. Neuron 8:335-342.

Lemos JR, Ocorr KA, Nordmann JJ (1989) Possible role for ionic channels in neurosecretory granules of the rat neurohypophysis. Soc Gen Physiol Ser 44:333-347.

Lim R, Oleskevich S, Few AP, Leao RN, Walmsley B (2003) Glycinergic mIPSCs in mouse and rat brainstem auditory nuclei: modulation by ruthenium red and the role of calcium stores. J Physiol 546:691-699.

Llano I, Gonzalez J, Caputo C, Lai FA, Blayney LM, Tan YP, Marty A (2000) Presynaptic calcium stores underlie large-amplitude miniature IPSCs and spontaneous calcium transients. Nat Neurosci 3:1256-1265.

Llinas R, Gruner JA, Sugimori M, McGuinness TL, Greengard P (1991) Regulation by synapsin I and Ca(2+)-calmodulin-dependent protein kinase II of the transmitter release in squid giant synapse. J Physiol 436:257-282.

Ludwig M, Leng G (2006) Dendritic peptide release and peptide-dependent behaviours. Nat Rev Neurosci 7:126-136.

Ma J, Fill M, Knudson CM, Campbell KP, Coronado R (1988) Ryanodine receptor of skeletal muscle is a gap junction-type channel. Science 242:99-102.

Mahapatra NR, Mahata M, Hazra PP, McDonough PM, O'Connor DT, Mahata SK (2004) A dynamic pool of calcium in catecholamine storage vesicles. Exploration in living cells by a novel vesicle-targeted chromogranin A-aequorin chimeric photoprotein. J Biol Chem 279:51107-51121.

Martinez JR, Willis S, Puente S, Wells J, Helmke R, Zhang GH (1996) Evidence for a Ca2+ pool associated with secretory granules in rat submandibular acinar cells. Biochem J 320 (Pt 2):627-634.

McNally JM, Woodbury DJ, Lemos JR (2004) Syntaxin 1A Drives Fusion of Large Dense-Core Neurosecretory Granules Into a Planar Lipid Bilayer. Cell Biochem Biophys 41:11-24.

McNally JM, De Crescenzo V, Walsh JV, Jr., Lemos JR (2006) Are Calcium Syntillas Directly involved in Eliciting Neuropeptide Release from Nerve Terminals of the Neurohyopophysis? Soc Neurosci Abs 32:337.311.

Mitchell KJ, Lai FA, Rutter GA (2003) Ryanodine receptor type I and nicotinic acid adenine dinucleotide phosphate receptors mediate Ca2+ release from insulin-containing vesicles in living pancreatic beta-cells (MIN6). J Biol Chem 278:11057-11064.

Mitchell KJ, Pinton P, Varadi A, Tacchetti C, Ainscow EK, Pozzan T, Rizzuto R, Rutter GA (2001) Dense core secretory vesicles revealed as a dynamic Ca(2+) store in neuroendocrine cells with a vesicle-associated membrane protein aequorin chimaera. J Cell Biol 155:41-51.

Mundorf ML, Troyer KP, Hochstetler SE, Near JA, Wightman RM (2000) Vesicular Ca(2+) participates in the catalysis of exocytosis. J Biol Chem 275:9136-9142.

Nicaise G, Maggio K, Thirion S, Horoyan M, Keicher E (1992) The calcium loading of secretory granules. A possible key event in stimulus-secretion coupling. Biol Cell 75:89-99.

Nordmann JJ, Zyzek E (1982) Calcium efflux from the rat neurohypophysis. J Physiol 325:281-299.

Oheim M, Kirchhoff F, Stuhmer W (2006) Calcium microdomains in regulated exocytosis. Cell Calcium 40:423-439.

Ouyang K, Zheng H, Qin X, Zhang C, Yang D, Wang X, Wu C, Zhou Z, Cheng H (2005) Ca2+ sparks and secretion in dorsal root ganglion neurons. Proc Natl Acad Sci U S A 102:12259-12264.

Pozzan T, Rizzuto R, Volpe P, Meldolesi J (1994) Molecular and cellular physiology of intracellular calcium stores. Physiol Rev 74:595-636.

Salzberg BM, Kraner SD, Muschol M, Obaid AL (1997) Calcium Release from Intraterminal Stores Plays a Direct Role in Release from Peptidergic Nerve Terminals in Mammals: Evidence from Light Scattering in Mouse Neurohypophysis. J Gen Physiol 110:16a.

Scheenen WJ, Wollheim CB, Pozzan T, Fasolato C (1998) Ca2+ depletion from granules inhibits exocytosis. A study with insulin-secreting cells. J Biol Chem 273:19002-19008.

Thirion S, Stuenkel EL, Nicaise G (1995) Calcium loading of secretory granules in stimulated neurohypophysial nerve endings. Neuroscience 64:125-137.

Tunwell RE, Wickenden C, Bertrand BM, Shevchenko VI, Walsh MB, Allen PD, Lai FA (1996) The human cardiac muscle ryanodine receptor-calcium release channel: identification, primary structure and topological analysis. Biochem J 318 (Pt 2):477-487.

Woodbury DJ (1995) Evaluation of the evidence for ion channels in synaptic vesicles. Mol Membr Biol 12:165-171.

Yin Y, Dayanithi G, Lemos JR (2002) Ca(2+)-regulated, neurosecretory granule channel involved in release from neurohypophysial terminals. J Physiol 539:409-418.

ZhuGe R, DeCrescenzo V, Sorrentino V, Lai FA, Tuft RA, Lifshitz LM, Lemos JR, Smith C, Fogarty KE, Walsh JV, Jr. (2006) Syntillas release Ca2+ at a site different from the microdomain where exocytosis occurs in mouse chromaffin cells. Biophys J 90:2027-2037.

CHAPTER IV: Single Calcium Syntillas do not Trigger Exocytosis from Nerve Terminals of the Neurohypophysis.

PREFACE

Material presented in this chapter has been or will be published in the following locations:

Manuscripts:

McNally, J.M., V. DeCrescenzo, K.Fogarty, R. Tuft, J.V. Walsh, and J.R. Lemos. (2008). Single Calcium Syntillas do not Trigger Exocytosis from Nerve Terminals of the Neurohypophysis. (*in preparation*)

Abstracts

McNally, J.M., DeCrescenzo, V., Walsh, J.V. and Lemos, J.R. (2006) Are calcium syntillas directly involved in eliciting neuropeptide release from nerve terminals of the neurohypophysis? *Soc. Neurosci. Abs.* 32: 337.11.

McNally, J.M., DeCrescenzo, V., Walsh, J.V. and Lemos, J.R. (2007) Single calcium syntillas do not trigger exocytosis from nerve terminals of the neurohypophysis. *WCNH Abs.* 51

Abstract:

Recently, highly localized Ca^{2+} release events, similar to Ca^{2+} sparks in muscle, have been observed in neuronal preparations. Specifically, in murine neurohypophysial terminals, the Ca^{2+} in these events, termed Ca^{2+} syntillas, emanate from a ryanodine-sensitive intracellular Ca^{2+} pool and increase in frequency with depolarization in the absence of Ca^{2+} influx. Despite this knowledge of the nature of these calcium release events, their physiological role in this system has yet to be defined. Since calcium has been shown to induce peptide release from these nerve terminals, localized Ca^{2+} release events which occur in the precise location of the final exocytotic event(s), may directly trigger exocytosis. It has already been shown that pharmalogical agents that modulate the activity of the ryanodine receptor also modulate the rate of basal exocytotic release as well as the rate of syntillas. However, in order to directly establish the validity of this hypothesis, a means of visualizing individual release events which has heretofore been unavailable for these CNS terminals, was required. To address this question, peptidergic neurohypophysial terminals were loaded with the false transmitter dopamine, allowing amperometric recording of exocytotic events. Use of this technique confirms earlier findings that modulation of ryanodine receptor activity can affect the rate of basal secretion, at the single terminal level. However, simultaneous amperometric recording of secretion along with high speed imaging of Ca^{2+} syntillas has now established that these two events do <u>not</u> display any observable temporal or spatial correlation. Syntillas occuring in close proximity to the carbon fiber do <u>not</u> produce amperometric events, and, conversely, amperometric events appear in the absence of syntillas. This confirms similar findings in chromaffin cells. While these results indicate that syntillas do <u>not</u> play a direct role in eliciting release, they do not rule out indirect modulatory effects of syntillas on secretion.

Introduction:

Calcium serves as an integral intracellular messenger, known to be involved in many essential physiological processes, making the spatial and temporal organization and control of $[Ca^{2+}]_i$ a complex and important process. In excitable exocytotic cells, the release of neurotransmitters and hormones are tightly coupled to elevations in the free $[Ca^{2+}]_i$ (Katz, 1969). While the influx of Ca^{2+} through voltage-gated Ca^{2+} channels (VGCC) is undoubtedly a very important source of Ca^{2+} affecting exocytotic release, Ca^{2+} release from internal stores evoked by depolarization or Ca^{2+}-induced Ca^{2+} release (CICR) may provide another important source of Ca^{2+} for this process (Berridge, 2006).

The high concentration of immobile Ca^{2+} buffers in the cytosol of most cells results in very tight control of $[Ca^{2+}]_i$. Quantitative modeling has demonstrated this, showing that the influx of Ca^{2+} through voltage gated calcium channels results in discrete peaks of Ca^{2+}, or "microdomains", in the immediate vicinity of Ca^{2+} channels (Simon and Llinas, 1985). If this is the case, then upon stimulation, Ca^{2+} entering through VGCC would reach the deeper regions of cytosol very slowly if at all (Pozzan et al., 1994). Therefore, Ca^{2+} release from rapidly mobilizable stores, via depolarization, CICR, or another second messenger, may provide a means of generating high localized concentrations of Ca^{2+}, distal to sites of Ca^{2+} influx.

Recently highly localized intracellular Ca^{2+} release events have been observed in neuronal preparations (Emptage et al., 2001; Galante and Marty,

2003; De Crescenzo et al., 2004; Ouyang et al., 2005; Yao et al., 2006). In isolated nerve terminals of magnocellular neurons, these transient events appear similar in nature to Ca^{2+} sparks of heart, striated, and smooth muscle. These events, termed "syntillas", are observed even after the removal of extracellular Ca^{2+} and appear to be derived from a ryanodine-sensitive intracellular Ca^{2+} pool (De Crescenzo et al., 2004). The ryanodine receptors responsible for syntillas have been found to interact with dihydropyridine receptors, conferring a voltage dependence on these events (De Crescenzo et al., 2006). In addition, although syntillas are observed at the resting membrane potential, their frequency is increased with depolarization without affecting their amplitude, a process defined as voltage-induced calcium release (VICaR). Notwithstanding all this information, neither a source for the released Ca^{2+} or a physiological role for this phenomenon has yet to be conclusively determined (but see Chapter III).

As observed in frog peripheral motor nerve terminals, Ca^{2+} entry due to extended trains of stimuli activate a CICR mechanism that amplifies the rise in $[Ca^{2+}]_i$ and enhances asynchronous neurotransmitter release (Narita et al., 1998; Narita et al., 2000). A similar mechanism has also been proposed to influence spontaneous multi-vesicular release events (large amplitude miniature postsynaptic currents) via Ca^{2+} mobilization from a presynaptic ryanodine-sensitive store at inhibitory synapses onto cerebellar Purkinje cells (Llano et al., 2000). Therefore, it is possible that Ca^{2+} release events, such as syntillas, may be responsible for driving spontaneous basal release.

In muscle, the involvement of Ca^{2+} sparks in excitation-contraction coupling is well known (Cheng et al., 1996), making it tempting to speculate that these neuronal Ca^{2+} syntillas may play a role in stimulus-secretion coupling. Galante and Marty (2003) have recently demonstrated that such ryanodine-sensitive intracellular calcium stores play a role in evoked neurotransmitter release at the basket cell-Purkinje cell synapse.

Increases in intracellular Ca^{2+} not only initiate exocytosis of both secretory vesicles and large dense core vesicles (LDCV), but also serve to modulate secretory responses (Chow et al., 1994). The spatial organization of secretory vesicles versus sites of Ca^{2+} entry in nerve terminals plays a central role in stimulus-secretion coupling. In synaptic terminals Ca^{2+} channels and synaptic vesicles are thought to be localized in close proximity. The release machinery of classic synaptic terminals is organized into active release sites that include Ca^{2+} channels (Sheng et al., 1996). This close association yields extremely fast release, which is abruptly halted due to strong intracellular Ca^{2+} buffering (Kits and Mansvelder, 2000).

In neuroendocrine systems, such as neurohypophysial terminals (NHT), this is not the case. In these systems release occurs much more slowly and necessitates repetitive stimulus patterns. This is generally believed to be due to the prepatory mobilization and priming of LDCV for release (Seward et al., 1995; Giovannucci and Stuenkel, 1997; Ludwig et al., 2002). Although the mechanisms underlying this have yet to be fully defined, it has been shown that

LDCVs of these cells do not appear to localize to specific active release sites (Morris and Pow, 1991). The bulk of these LDCVs are thought to reside in a location much more distant from Ca^{2+} channels. In chromaffin cells, this lack of spatial coupling of LDCVs with Ca^{2+} channels has been shown to result in significant delay (~10ms) in release and may lead to asynchronous release after cessation of the depolarizing stimulus (Chow et al., 1994). Taken together, these facts make intracellular calcium release events, such as syntillas, particularly interesting in NHT, as they may provide an additional means of driving the calcium-dependent process of release.

Syntillas have also been observed in chromaffin cells, which are another secretory cell type (ZhuGe et al., 2006). Surprisingly, in this system syntillas do not trigger spontaneous release events, suggesting that the calcium liberated by syntillas must be localized to a distinct calcium microdomain separate from those involved in exocytosis. However, syntillas observed in this system differ from those characterized in NHT. Chromaffin cells possess a different subset of ryanodine receptors compared to NHT, and their syntillas lack the voltage-dependence seen in NHT. This has lead to speculation that syntillas may function in a different physiological role in nerve terminals.

In order to directly address this question, we have developed a means by which individual exocytotic events can be observed in peptidergic NHT. Here we report that, while release of intracellular calcium from ryanodine sensitive stores may affect the rate of basal release from isolated NHT, there is no evidence to

support a direct interaction between the intracellular calcium released by syntillas and neuropeptide secretion. This may suggest that, as in chromaffin cells (ZhuGe et al., 2006), syntillas occur in calcium microdomains separate from sites of exocytosis.

Materials and Methods:

Isolation of Murine NHT and electrophysiology:

NHT from Swiss Webster mice were prepared as previously described (Cazalis et al., 1987; Wang et al., 1999; De Crescenzo et al., 2004). After decapitation, the neurohypophysis was harvested and dissociated via homogenization performed at 37°C in an isoosmotic solution of 270 mM sucrose, 1 mM EGTA, 10 mM HEPES (pH 7). The dissociated terminals were then plated in the bottom of a perfusion chamber, and perfused with Normal Locke's saline (NL) (mM): 140 NaCl, 5 KCl, 10 glucose, 1 $MgCl_2$, 2.2 $CaCl_2$, 10 HEPES, (pH 7.3). For experiments in zero Ca^{2+} NL, 200 µM EGTA was substituted for calcium. NHT were identified by their smooth appearance, lack of nucleus, and high content of dark vesicles.

False Transmitter Loading and Amperometric Recording

NHT were loaded with the oxidizable transmitter, dopamine (DA), via its addition to the intracellular pipette solution. After patching a terminal in either the whole cell or perforated patch configuration, DA (15 mM) was dialyzed into the

terminal and is trafficked into acidic compartments, including the LDCVs (Kim et al., 2000).   A 5 µm diameter carbon fiber electrode (CFE), voltage-clamped at 700 mV, was placed in close apposition to the plasma membrane of the terminal. When loaded LDCVs undergo exocytosis, DA released from NHT contacts the carbon fiber, where its subsequent oxidation results in a detectable current flow through the electrode. Successful loading usually required ~5 minutes, and was determined by the appearance of spontaneous amperometric events at a resting membrane potential of -80 mV (see Fig. 4.1).

Experiments examining spontaneous amperometric events alone were performed in the perforated patch configuration using a pipette solution of (mM): 130 Cs-glutamate, 1 $MgCl_2$, 10 HEPES, 20 TEA-Cl, 15 DA (pH 7.3) β escin was used as a perforating agent for these experiments (Fan and Palade, 1998; Sarantopoulos et al., 2004), as it forms holes in the membrane large enough to allow entry of DA into the terminal while allowing for a longer recording time than can be attained using the standard whole cell configuration. Agonists and antagonists were applied to NHT through addition of the drug to the bath. Ananlysis of individual amperometric spikes was performed using an Igor pro (Wavemetrics) macro, quanta analysis, written by Eugene Mosharov of David Sulzer's lab (available at www.sulzerlab.org). Amperometric events were detected using a peak threshold cutoff of 2.5 times the root mean square of trace noise. Calculation of the total amperometric charge was performed using Origin

7 (Originlab Corp.). Amperometric charge was calculated by integrating baseline subtraceted amperometric current traces.

Imaging of transient Ca^{2+} Release events

Fluorescence imaging of intracellular Ca^{2+} transients was performed in the same manner as described previously (De Crescenzo et al., 2004; De Crescenzo et al., 2006; ZhuGe et al., 2006). NHT were patched in the whole cell configuration using a pipette solution of (mM): 135 KCl, 2 MgCl2, 30 HEPES, 4 Mg-ATP, 0.3 Na-GTP, 0.05 K-fluo3, and 15 DA (pH 7.2). Addition of the fluorescent calcium indicator dye K$_5$-fluo-3 (50 µM) to pipette solution allowed for imaging of intracellular calcium release events.

High-speed calcium imaging was performed using a custom built inverted wide field microscope equipped with a Nikon 100x (1.3 NA) oil immersion objective and high-resolution CCD camera, providing 133 nm of specimen per image pixel. Imaging was performed at a rate of 50 Hz (10ms exposure) for 4 seconds at a time. The total amount of calcium released per syntilla was quantified by measuring its "signal mass" as previously described (Sun et al., 1998; ZhuGe et al., 2000; De Crescenzo et al., 2004).

Membrane Capacitance Recording

Dissociated murine NHT were recorded from using standard whole cell configuration, using a pipette solution of (mM): (pH 7.3). NH terminals were

patched with 5-10 MΩ glass microelectrodes coated with sigmacote to reduce

pipette capacitance. Capacitance recording was conducted in a bath solution of

Locke's saline described above.   Membrane capacitance was monitored using a

software lock-in amplifier (SLIA) integrated with a computer controlled patch-

clamp amplifier (Pulse, EPC-10, HEKA)(Gillis, 2000). Changes in membrane

capacitance, directly related to secretory activity, were followed using the

"sine+dc" method (Lindau and Neher, 1988; Chen and Gillis, 2000; Gillis, 2000).

To measure changes in membrane capacitance, a 15 mV 1500 hZ sine wave

was superimposed onto the clamped DC holding potential (-80 mV).

Results:

DA Loading of NHTs

It has been shown that application of RyR agonists (Caffeine, Ryanodine,

Imperatoxin A) can elicit peptide release from populations of isolated NHT in the

absence of extracellular calcium (Velazquez-Marrero et al., 2002). While these

results are quite intriguing, population release assays do not provide either the

temporal or spatial resolution necessary for direct comparison of release with

syntillas. Accomplishing this goal required the development of a means by which

quantal exocytotic events could be observed in real time. The electrochemical

detection technique of amperometry has been successfully employed for this

purpose in other systems. However, as NHT do not package readily oxidizable

material into their large dense core granules, it is not feasible to use this technique to monitor endogenous peptide release.

Previous work has shown that cultured secretory cells are capable of packaging and releasing exogenous transmitters, provided there is a sufficiently high concentration of that transmitter present in the cytosol (Zhou and Misler, 1996; Kim et al., 2000). In order to develop a practical means of accomplishing this in NHTs, the exogenous transmitter DA was loaded by its addition into the whole-cell patch pipette solution. This method has been employed to allow differentiated neurons to secrete false transmitters (Dan et al., 1994).

As displayed in figure 4.1B, no amperometric activity was observed in NHTs prior to DA loading. However, several minutes after whole-cell break-in, allowing DA to dialyze into the terminal cytosol, the same terminal shows numerous spontaneous current spikes appear. These events appear similar to amperometric release events recorded under similar conditions from chromaffin cells, a system that releases a native oxidizable transmitter (compare to Figure 4.1A).

Close examination of NHT amperometric events (Table 1, and Figure 4.1C) shows that they possess similar kinetic parameters to those observed in chromaffin cells, with the exception of their size. This is not surprising as one would not expect that the amount of false transmitter loaded into a vesicle could come close to approaching the concentration present in a system where amperometry can be used under native conditions. Additionally, the

A Chromaffin Cell

5 pA

5 s

B NHT

1 pA

10 s

Before Loading

After Loading

C

10 pA

10 ms

2 pA

10 ms

0.5 pA

10 ms
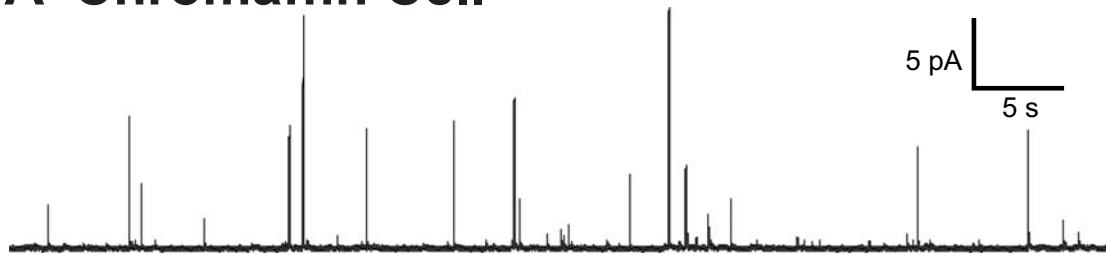
Chromaffin Cell

NHT
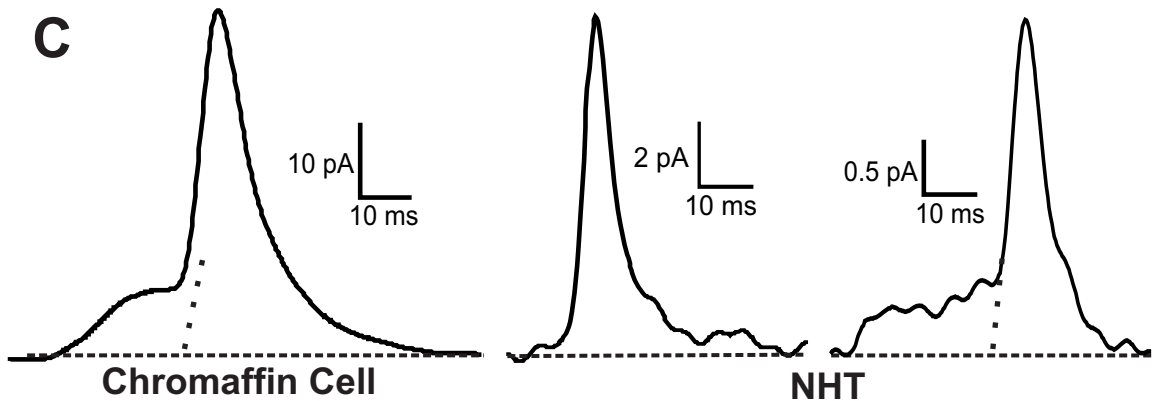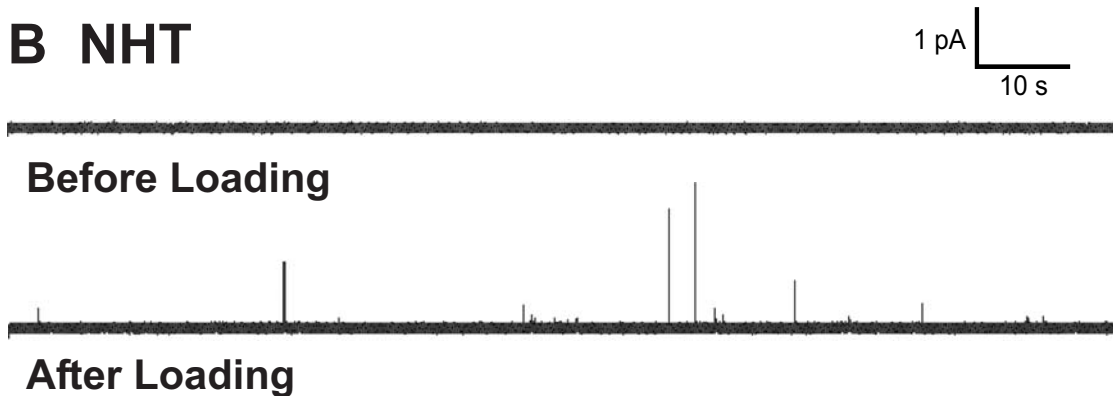
Figure 4.1: False-transmitter loading of whole-cell patched NHT, allows detection of spontaneous amperometric events similar to those seen in chromaffin cells. (**A**) Amperometric recording from an isolated murine adrenal chromaffin cell showing spontaneous release events.   (**B**) Amperometric recording performed immediately after whole-cell patch-clamp of a NHT shows that no amperometric activity is detectable in a non-DA loaded terminal. After several minutes, allowing DA to dialyze into the terminal from the patch pipette, spontaneous amperometric release events can be observed. (**C**) Amperometric events recorded from both chromaffin cells and false-transmitter loaded terminals appear very similar in nature, with the exception of their size. Prespike feet, a hallmark of amperometric recording of quantal release events, can be seen in a number of events (see right) recorded from loaded NHTs.

Table 1. *Comparison of amperometric events recorded from chromaffin cells to events from false transmitter loaded NHT*

	Chromaffin Cells	NHT
Area (pC)	0.138 ± 0.232	0.024 ± 0.027
$t_{1/2}$ (ms)	20.76 ± 14.22	7.57 ± 3.60
I_{max} (pA)	6.84 ± 0.146	2.68 ± 3.51
Rise Time (pA/ms)	1.27 ± 2.92	0.70 ± 1.01
Decay (ms): τ_1	29.97 ± 47.73	11.70 ± 23.08
τ_2	88.34 ± 116.73	28.92 ± 56.06

Data is the mean ± SD of pooled values of amperometric events recorded in NL. Chromaffin Cells, N = 4, n = 512. NHT, N = 32, n = 1375. Event decay fit with a double exponential.

amperometric events observed were quite variable in amplitude (see I_{max}, table 1). This is consistent with the findings of previous false transmitter-loading studies, and is predicted to be due to considerable diversity in vesicle loading (Kim et al., 2000).

Interestingly, small current steps preceded some of the amperometric spikes observed in false transmitter-loaded NHT which appear similar in nature to the prespike "foot" observed during native catecholamine release events from chromaffin cells (figure 4.1C). The prespike foot is believed to correspond to the slow release of transmitter through a fusion pore prior to pore widening and complete collapse of the vesicle into the plasma membrane (Chow et al., 1992). Such prespike feet have also been described in other false transmitter loaded systems (Zhou and Misler, 1996). The appearance of this hallmark of native amperometric release recorded from false transmitter-loaded terminals yields further evidence that these amperometric events represent exocytotic release.

Comparison of Amperometry with Capacitance Recording

To ensure that events detected via amperometry do in fact represent exocytotic release, membrane capacitance recordings were performed in concert with amperometric detection. When depolarizing stimuli of different durations were applied to an individual terminal (Figure 4.2 A-D), the amount of amperometric activity increased as the duration of the depolarizing stimulus increased. This response mirrored that seen using membrane capacitance to

Figure 4.2: **Amperometric activity in false transmitter loaded terminals correlates with exocytotic activity observed using membrane capacitance recording.** (**A-D**) Simultaneous amperometric and membrane capacitance recordings taken from a single false transmitter loaded terminal. The terminal was given a depolarizing stimulus that increased in duration from **A** to **D**. Both the amount of amperometric activity and the evoked change in membrane capacitance increased with longer durations of depolarizing stimuli. (**E**) Plotting the total amperometric charge versus the evoked change in capacitance (delta Cm) reveals a strong linear correlation. Data obtained from this terminal was representative of the correlation seen between amperometric and membrane capacitance recordings taken from individual terminals (solid black line, $r^2 = 0.99$). Dashed lines 1-4 represent linear fits of amperometric and capacitance data from four other individual terminals ($r^2 = 0.89$, 0.97, 0.99, and 0.99 respectively).

assay exocytotic activity. Interestingly, with longer duration stimuli, asynchronous exocytotic activity was apparent in both the amperometric and membrane capacitance records (Figure 4.2 C&D). It is very telling that as soon as amperometric activity subsides, the increase in membrane capacitance also stops. Plotting the magnitude of the change in membrane capacitance versus the total amount of amperometric activity (amperometric charge) shows a strong linear correlation between these two measures of release in this and other false transmitter-loaded terminals (Figure 4.2E). Slight variations in the slope of this correlation were observed from terminal to terminal possibly representing slight differences in false transmitter loading efficiency, or differing placement of the CFE altering the percent of total exocytosis detected amperometrically.

Ryanodine-Sensitive Stores can Affect Basal Exocytosis from NHT

Amperometric recording of spontaneous release events from artificial transmitter-loaded terminals provides a means to determine the rate of spontaneous release. Figure 4.3A shows traces of amperometric currents recorded from such a terminal held at a resting membrane potential of -80 mV. Bath addition of ryanodine at RyR agonist levels (5 µM) results in a rapid increase in the observed rate of spontaneous release. This significant ($p < 0.05$) increase in release rate is, on average, 2.42 times the basal rate (figure 4.3B). This result confirms, at the isolated terminal level, the findings of previous NHT
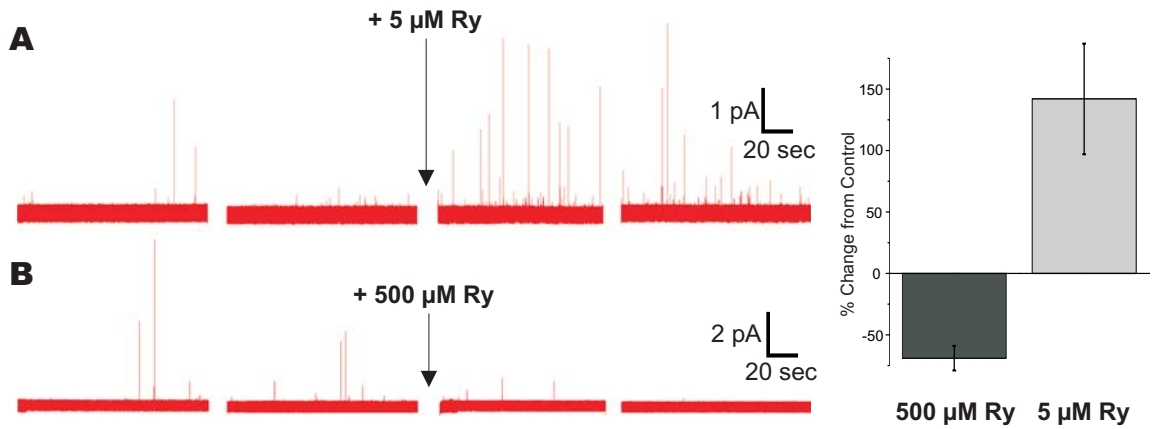
Figure 4.3: **Ryanodine affects the basal rate of exocytotic activity from nerve terminals as measured by amperometry.** Amperometric traces provide an example of the affects of ryanodine on spontaneous exocytotic release. Each trace represents sequential two minute amperometric records from false transmitter-loaded terminal perforated-patched with ß-escin, and held at a resting membrane potential of -80mV in Normal Locke's. (**A**) Addition of RyR agonist levels of ryanodine (5 µM) results in an increase in the basal release rate. (**B**) Conversely, addition of ryanodine at a RyR antagonistic concentration (500 µM) to the bath between the second and third amperometric trace results in a reduction of the basal release rate. (**C**) Bar graph summarizing the effects of ryanodine on release across terminals. Agonist levels of ryanodine causes a 2.42 ± 0.45 fold increase in the rate of spontaneous release, when compared to the rate under basal conditions (n=5 terminals). Antagonist levels of ryanodine decreases spontaneous release to 0.31 ± 0.1 times the basal rate (n = 7 terminals). To rule out any vehicle effect on the release rate, NL alone was added to the bath in the same manner that antagonist ryanodine was added. This vehicle treatment appeared to slightly, but not significantly ($p > 0.05$), raise the spontaneous release rate 1.4 fold (n=3), thus we may be underestimating the affect of antagonist ryanodine on basal release.

population release studies, which have shown that peptide release is increased by RyR agonists (Velazquez-Marrero et al., 2002).

Addition of ryanodine at RyR antagonist levels (500 µM) results in a significant ($p < 0.05$) decrease in the spontaneous release rate to 0.31 times the basal rate. Previous work (De Crescenzo et al., 2004) has shown that antagonist levels of ryanodine reduce the frequency of syntillas to a similar degree. Taken together these two results spark interest, yet are indirect and leave open the question as to what may be the relationship between these two events.

There is No Correlation Between Syntillas and Spontaneous Release Events

As the frequency of both syntillas and exocytosis are modulated by the same concentrations of ryanodine, is it possible that the calcium released during a syntilla could directly elicit release? Spatiotemporal modeling of syntillas demonstrates that the average syntilla is capable of raising intracellular calcium to supramicromolar concentrations for tens of milliseconds hundreds of nanometers away from the site of calcium release (De Crescenzo et al., 2004). In NHT, a free calcium concentration of 10 µM is though to be maximal for triggering exocytotic release (Lee et al., 1992). Therefore, if a syntilla were to occur in close proximity to an exocytotic release site, it could conceivably drive the exocytotic event.

To look at this relationship in a more straightforward manner, a combination of high speed calcium imaging and amperometric detection of release were utilized. We observed a large number of syntillas in false

transmitter-loaded terminals in calcium-free media (99 syntillas observed from 19 different terminals), of these syntillas, 19 occurred proximal to the CFE. These syntillas were recorded from 10 different terminals. The magnitude of each of these syntillas was determined using the "signal mass" approach (Sun et al., 1998; ZhuGe et al., 2000), which yields the amount of calcium ions released (see figure 4.5).

The rate at which exocytosis occurs depends upon the kinetics of the fusion machinery, which for large dense core granules has been determine to be on the order of a few ms (Heinemann et al., 1994). In addition, the detection of exocytotic release with amperometry results in a delay of few milliseconds after granule fusion (Chow et al., 1996). Given these rates, if the calcium released by a syntilla was directly responsible for eliciting exocytosis, one would expect a exocytotic release event within 10's of ms after appearance of the syntilla. However, in <u>no</u> case did we see any evidence of such an immediate correlation between the observed syntilla and release; no matter the magnitude of the syntilla (see figures 4.4 and 4.5).

Another possibility is that spontaneous exocytotic events could trigger syntillas. In no case did any spontaneous release event, observed in false transmitter-loaded terminals during Ca^{2+} imaging, show any evidence of correlation with syntillas (Figure 4.6, n=16). Additionally, extended amperometric traces were recorded, monitoring spontaneous exocytotic activity well before and after 14 of the syntillas occurring at the CFE. Even on this larger time scale no
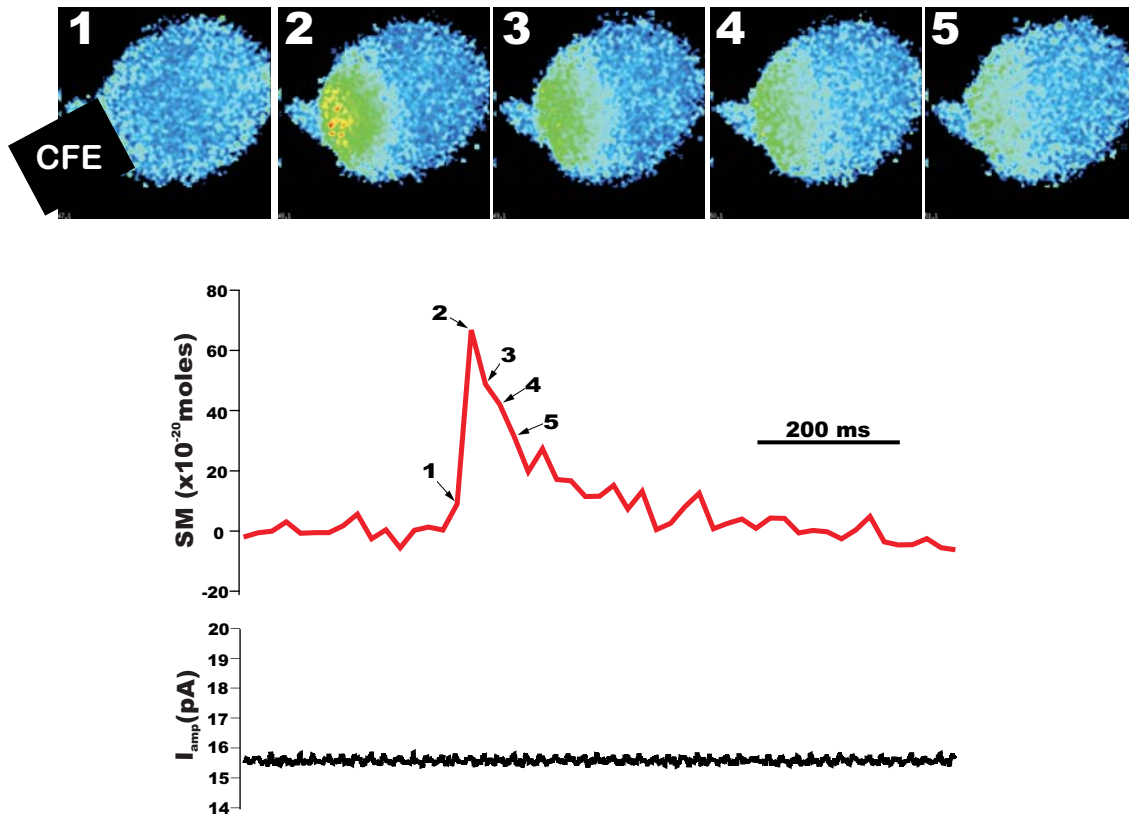
Figure 4.4: **Calcium syntillas do not trigger spontaneous release events.** In false transmitter-loaded terminals, calcium imaging of syntillas and amperometric recording of exocytotic events can be performed simultaneously. (**Top**) Leftmost image shows positioning of the carbon fiber electrode (CFE) relative to a whole-cell patched terminal loaded with the false transmitter, dopamine, and the calcium indicator dye, fluo-3. Images 1-5 represent the image frames taken immediately prior to the appearance of the syntilla and the following four frames, respectively. The syntilla is observed to occur precisely in the area where the CFE is positioned. (**Bottom**) Concurrent records of fluorescence in the region of the terminal where the syntilla occurs (red trace) and amperometric current (**black trace**) show that there are no amperometric events immediately correlated with this syntilla, despite the fact that this syntilla represents the release of ~ 400,000 calcium ions (S.M. = 66.4×10^{-20} mol) in the precise area where the CFE is located. The numbers on the fluorescence trace correspond to the numbered images above.
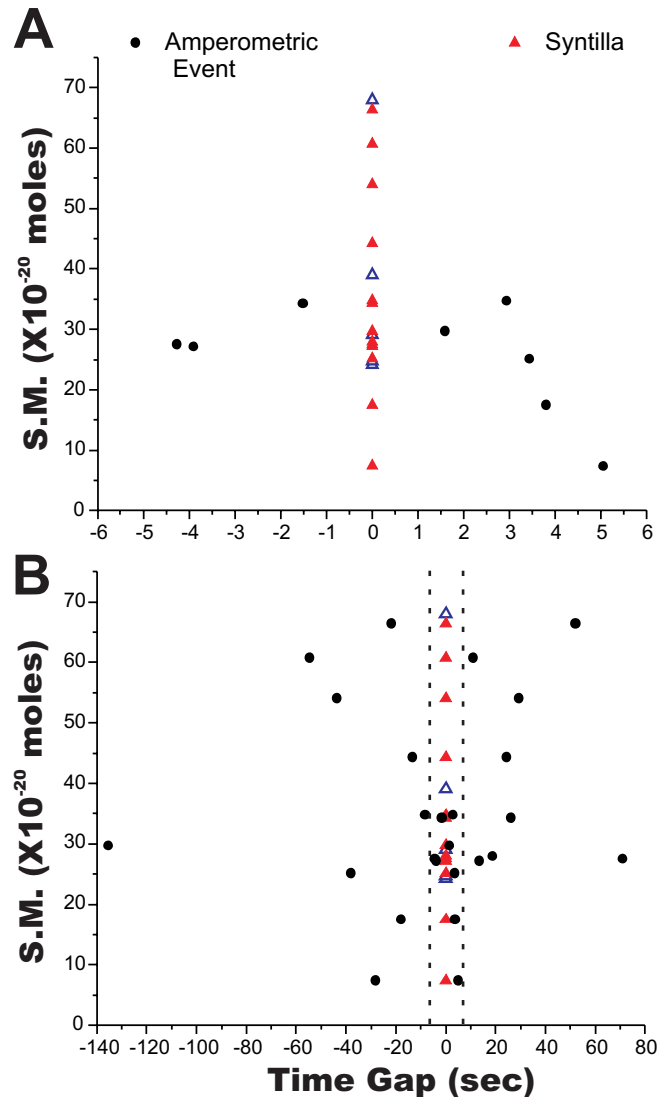
Figure 4.5: **There is no temporal correlation between syntillas and spontaneous release events.** Syntillas observed occurring in the region of the terminal were a CFE electrode had been placed were plotted based on their signal mass (S.M.). Red triangles represent syntillas occurring near the CFE where long duration amperometric recording was performed before and after the syntilla. For these syntillas, the closest spontaneous amperometric events occurring both before and after the syntilla (black circles) have been plotted. Blue triangles represent syntillas where amperometric recording was performed only during calcium imaging. (**A**) Plot of syntillas versus amperometric events shows that there is no apparent temporal correlation on the scale that one would predict would be required if syntillas were directly responsible for eliciting exocytosis (see Results). (**B**) Expanding the time scale of the previous graph to include the nearest preceding and following amperometric event (red triangles only) still does not provide and any evidence of temporal correlation.
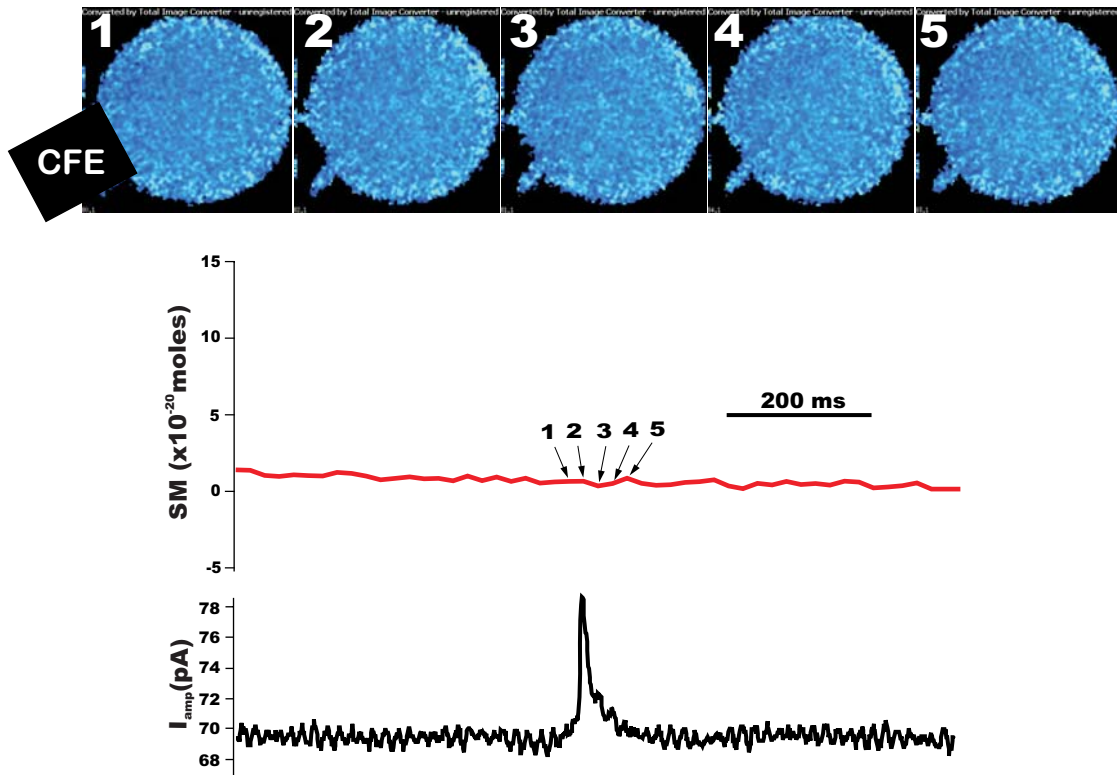
Figure 4.6: **Spontaneous amperometric release events do not trigger Calcium syntillas.** As in figure 4.3, calcium imaging of syntillas and amperometric recording of exocytotic events were performed simultaneously in false transmitter-loaded terminals. (**Top**) Leftmost image shows positioning of CFE relative to a whole-cell patch-clamped terminal. Images 1-5 represent the image frames taken during the occurrence of the spontaneous false-transmitter release event. (**Bottom**) Concurrent records of fluorescence in the region of terminal where the CFE was positioned (red trace) and amperometric current (**black trace**) shows that there are no calcium release events occurring near the CFE when the spontaneous release event is detected. The numbers on the fluorescence trace correspond to the temporal position of the numbered images above.

discernable correlation between syntillas and exocytosis was observed (figure 4.5B).

Discussion:

In NHT liberation of calcium from ryanodine-sensitive internal stores has been shown to be capable of altering the rate of basal exocytotic release (Velazquez-Marrero et al., 2002; Jin et al., 2007). Adaptation of amperometric detection to NHT has allowed us to examine this at the single-terminal level. However, simultaneous observation of both individual syntillas and spontaneous release events provided <u>no</u> evidence for a direct correlation of the two events (Figure 4.5). This is similar to earlier findings in chromaffin cells (ZhuGe et al., 2006).

Looking strictly at the rates of these two processes yields further evidence that syntillas are not required for spontaneous exocytotic release. As discussed earlier, in the presence of extracellular calcium the rate of basal release, as observed with amperometry, is approximately five events per minute. Since amperometry shows approximately 10% of the secretory activity for a terminal, the rate of basal release for the entire terminal would be ~50 release events per minute. This is roughly twice the frequency of syntillas in the presence of extracellular calcium, ~27 per minute. (De Crescenzo et al., 2004).

Spatiotemporal modeling of syntillas by DeCresenzo et al. (2004) has shown that an average syntilla (~250,000 Ca ions) occurring at the plasma

membrane will raise intracellular Ca^{2+} to 10 μM, the concentration believed to be required for neuropeptide secretion (Lee et al., 1992), 500 nm from the site of release. This effective distance is reduced as the source of the syntillas is moved away from the plasma membrane. Therefore, the fact that syntillas are not capable of directly eliciting spontaneous exocytotic events leads us to theorize that the calcium released by syntillas must be localized to a microdomain separate from where exocytosis occurs.

It is possible that multiple syntillas could work in concert to elicit an exocytotic event in NHT. Similar calcium release events observed in dorsal root ganglion neuronal somata have been suggested to be involved in secretion, with a spark-secretion coupling probability of 11.4 calcium sparks per exocytotic event (Ouyang et al., 2005). However, such a relationship has not been observed in our system. Only in one instance did we observe multiple syntillas in close proximity to the CFE within several milliseconds of each other and in this case no correlated exocytotic event was detected.

Calcium has been shown to modulate a number of intracellular processes. In superior cervical ganglion neurons for instance, calcium "glows" are observed along with calcium sparks. These two distinct types of intracellular calcium release events display different kinetics, with glows persisting over many seconds (Yao et al., 2006). The physiological role of these two events is unknown, yet the fact that two distinct mechanisms of intracellular calcium release exist, may suggest that intracellular calcium release has the ability to

distinctly modulate multiple physiological processes in concert, such as membrane excitability (Burdyga and Wray, 2005), and vesicular trafficking (Llinas et al., 1991; Neher, 2006; Hosoi et al., 2007; Shakiryanova et al., 2007), as well as exocytosis. Syntillas, which raise intracellular calcium to supramicromolar concentrations in very small regions, may function in such a manner by acting on calcium sensitive targets in one domain, while leaving targets in another domain unaffected.

If the physiological role of syntillas is not to directly trigger exocytosis, then why does modulation of calcium release from ryanodine-sensitive stores alter the basal rate of exocytosis? Regardless, these findings do not rule out an indirect modulatory role of syntillas. Future work, examining evoked release in this system is required to answer this question (see Appendix II).

Ryanodine-sensitive calcium release may be functionally modulating neurotransmitter release via a CICR-like mechanism. If this is the case, application of RyR agonists would make the RyR more likely to open. Upon opening of VGCCs and influx of extracellular calcium, syntillas are triggered by both voltage and calcium influx. The increased probability of opening RyRs would then allow ryanodine-sensitive calcium stores to contribute more calcium to the cytosol, thereby potentiating release.

A second possibility is that syntillas modulate the trafficking of granules between release pools. It has been suggested that the mobilization and recruitment of LDCVs to the plasma membrane is a calcium-dependent process

(Llinas et al., 1991; Becherer et al., 2003; Shakiryanova et al., 2005; Neher, 2006). Recently, Shakiryanova et al. (2007) have shown that RyR-mediated calcium release is sufficient to trigger the mobilization of LDCVs at the *Drosophila* neuromuscular junction, via activation of CaMKII. Interestingly, it appears that CaMKII is more sensitive to calcium released from internal stores than to extracellular calcium in this study. This may suggest that the calcium sources for both triggering exocytosis and mobilization of LDCVs are distinct.

The ability of intracellular calcium to mobilize LDCVs has already been observed in the cell bodies and dendrites of the hypothalamic neurohypophysial system, where intracellular calcium release has been demonstrated to have a priming affect on neuropeptide release (Ludwig and Leng, 2006). EM studies show that this priming effect involves the relocation of LDCVs closer to the plasma membrane (Tobin et al., 2004). These results suggest that the release of ryanodine-sensitive calcium via syntillas may lead to an increase in the trafficking of LDCVs from reserve pools to the plasma membrane, thereby increasing the size of the readily-releasable pool.

Previous work has demonstrated that pharmacologically increasing LDCV mobility enhances neuropeptide release (Ng et al., 2002; Ng et al., 2003). While not being directly responsible for eliciting exocytosis, increased trafficking of granules to the plasma membrane by syntillas, could indirectly cause an increase in the release rate. This is may simply be due to the fact that more granules are

in close proximity to the plasma membrane where they have a much higher probability of being released (Morris and Pow, 1991).

While syntillas do not appear to be directly involved in eliciting neuropeptide release, our findings suggest that they have a modulatory role in the process. The localization of syntillas to distinct microdomains separate from the sites of exocytosis confirms the exquisite control of intracellular calcium in terminals as it allows this second messenger to control multiple processes independent of one another. Syntillas may prove to be a factor in mobilizing or priming vesicles for release. This represents a functional mechanism for plasticity in NHT and, perhaps, other CNS terminals.

References:

Becherer U, Moser T, Stuhmer W, Oheim M (2003) Calcium regulates exocytosis at the level of single vesicles. Nat Neurosci 6:846-853.

Berridge MJ (2006) Calcium microdomains: organization and function. Cell Calcium 40:405-412.

Burdyga T, Wray S (2005) Action potential refractory period in ureter smooth muscle is set by Ca sparks and BK channels. Nature 436:559-562.

Cazalis M, Dayanithi G, Nordmann JJ (1987) Hormone release from isolated nerve endings of the rat neurohypophysis. J Physiol 390:55-70.

Chen P, Gillis KD (2000) The noise of membrane capacitance measurements in the whole-cell recording configuration. Biophys J 79:2162-2170.

Cheng H, Lederer MR, Xiao RP, Gomez AM, Zhou YY, Ziman B, Spurgeon H, Lakatta EG, Lederer WJ (1996) Excitation-contraction coupling in heart: new insights from Ca2+ sparks. Cell Calcium 20:129-140.

Chow RH, von Ruden L, Neher E (1992) Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells. Nature 356:60-63.

Chow RH, Klingauf J, Neher E (1994) Time course of Ca2+ concentration triggering exocytosis in neuroendocrine cells. Proc Natl Acad Sci U S A 91:12765-12769.

Chow RH, Klingauf J, Heinemann C, Zucker RS, Neher E (1996) Mechanisms determining the time course of secretion in neuroendocrine cells. Neuron 16:369-376.

Dan Y, Song HJ, Poo MM (1994) Evoked neuronal secretion of false transmitters. Neuron 13:909-917.

De Crescenzo V, ZhuGe R, Velazquez-Marrero C, Lifshitz LM, Custer E, Carmichael J, Lai FA, Tuft RA, Fogarty KE, Lemos JR, Walsh JV, Jr. (2004) Ca2+ syntillas, miniature Ca2+ release events in terminals of hypothalamic neurons, are increased in frequency by depolarization in the absence of Ca2+ influx. J Neurosci 24:1226-1235.

De Crescenzo V, Fogarty KE, Zhuge R, Tuft RA, Lifshitz LM, Carmichael J, Bellve KD, Baker SP, Zissimopoulos S, Lai FA, Lemos JR, Walsh JV, Jr. (2006) Dihydropyridine receptors and type 1 ryanodine receptors constitute the molecular machinery for voltage-induced Ca2+ release in nerve terminals. J Neurosci 26:7565-7574.

Emptage NJ, Reid CA, Fine A (2001) Calcium stores in hippocampal synaptic boutons mediate short-term plasticity, store-operated Ca2+ entry, and spontaneous transmitter release. Neuron 29:197-208.

Fan JS, Palade P (1998) Perforated patch recording with beta-escin. Pflugers Arch 436:1021-1023.

Galante M, Marty A (2003) Presynaptic ryanodine-sensitive calcium stores contribute to evoked neurotransmitter release at the basket cell-Purkinje cell synapse. J Neurosci 23:11229-11234.

Gillis KD (2000) Admittance-based measurement of membrane capacitance using the EPC-9 patch-clamp amplifier. Pflugers Arch 439:655-664.

Giovannucci DR, Stuenkel EL (1997) Regulation of secretory granule recruitment and exocytosis at rat neurohypophysial nerve endings. J Physiol 498 (Pt 3):735-751.

Heinemann C, Chow RH, Neher E, Zucker RS (1994) Kinetics of the secretory response in bovine chromaffin cells following flash photolysis of caged Ca2+. Biophys J 67:2546-2557.

Hosoi N, Sakaba T, Neher E (2007) Quantitative analysis of calcium-dependent vesicle recruitment and its functional role at the calyx of Held synapse. J Neurosci 27:14286-14298.

Jin D, Liu HX, Hirai H, Torashima T, Nagai T, Lopatina O, Shnayder NA, Yamada K, Noda M, Seike T, Fujita K, Takasawa S, Yokoyama S, Koizumi K, Shiraishi Y, Tanaka S, Hashii M, Yoshihara T, Higashida K, Islam MS, Yamada N, Hayashi K, Noguchi N, Kato I, Okamoto H, Matsushima A, Salmina A, Munesue T, Shimizu N, Mochida S, Asano M, Higashida H (2007) CD38 is critical for social behaviour by regulating oxytocin secretion. Nature 446:41-45.

Katz B (1969) The Release of Neurotransmitter Substances. Liverpool: Liverpool University Press.

Kim KT, Koh DS, Hille B (2000) Loading of oxidizable transmitters into secretory vesicles permits carbon-fiber amperometry. J Neurosci 20:RC101.

Kits KS, Mansvelder HD (2000) Regulation of exocytosis in neuroendocrine cells: spatial organization of channels and vesicles, stimulus-secretion coupling, calcium buffers and modulation. Brain Res Brain Res Rev 33:78-94.

Lee CJ, Dayanithi G, Nordmann JJ, Lemos JR (1992) Possible role during exocytosis of a Ca(2+)-activated channel in neurohypophysial granules. Neuron 8:335-342.

Lindau M, Neher E (1988) Patch-clamp techniques for time-resolved capacitance measurements in single cells. Pflugers Arch 411:137-146.

Llano I, Gonzalez J, Caputo C, Lai FA, Blayney LM, Tan YP, Marty A (2000) Presynaptic calcium stores underlie large-amplitude miniature IPSCs and spontaneous calcium transients. Nat Neurosci 3:1256-1265.

Llinas R, Gruner JA, Sugimori M, McGuinness TL, Greengard P (1991) Regulation by synapsin I and Ca(2+)-calmodulin-dependent protein kinase II of the transmitter release in squid giant synapse. J Physiol 436:257-282.

Ludwig M, Leng G (2006) Dendritic peptide release and peptide-dependent behaviours. Nat Rev Neurosci 7:126-136.

Ludwig M, Sabatier N, Bull PM, Landgraf R, Dayanithi G, Leng G (2002) Intracellular calcium stores regulate activity-dependent neuropeptide release from dendrites. Nature 418:85-89.

Morris JF, Pow DV (1991) Widespread release of peptides in the central nervous system: quantitation of tannic acid-captured exocytoses. Anat Rec 231:437-445.

Narita K, Akita T, Osanai M, Shirasaki T, Kijima H, Kuba K (1998) A Ca2+-induced Ca2+ release mechanism involved in asynchronous exocytosis at frog motor nerve terminals. J Gen Physiol 112:593-609.

Narita K, Akita T, Hachisuka J, Huang S, Ochi K, Kuba K (2000) Functional coupling of Ca(2+) channels to ryanodine receptors at presynaptic terminals. Amplification of exocytosis and plasticity. J Gen Physiol 115:519-532.

Neher E (2006) A comparison between exocytic control mechanisms in adrenal chromaffin cells and a glutamatergic synapse. Pflugers Arch 453:261-268.

Ng YK, Lu X, Levitan ES (2002) Physical mobilization of secretory vesicles facilitates neuropeptide release by nerve growth factor-differentiated PC12 cells. J Physiol 542:395-402.

Ng YK, Lu X, Gulacsi A, Han W, Saxton MJ, Levitan ES (2003) Unexpected mobility variation among individual secretory vesicles produces an apparent refractory neuropeptide pool. Biophys J 84:4127-4134.

Ouyang K, Zheng H, Qin X, Zhang C, Yang D, Wang X, Wu C, Zhou Z, Cheng H (2005) Ca2+ sparks and secretion in dorsal root ganglion neurons. Proc Natl Acad Sci U S A 102:12259-12264.

Pozzan T, Rizzuto R, Volpe P, Meldolesi J (1994) Molecular and cellular physiology of intracellular calcium stores. Physiol Rev 74:595-636.

Sarantopoulos C, McCallum JB, Kwok WM, Hogan Q (2004) Beta-escin diminishes voltage-gated calcium current rundown in perforated patch-clamp recordings from rat primary afferent neurons. J Neurosci Methods 139:61-68.

Seward EP, Chernevskaya NI, Nowycky MC (1995) Exocytosis in peptidergic nerve terminals exhibits two calcium-sensitive phases during pulsatile calcium entry. J Neurosci 15:3390-3399.

Shakiryanova D, Tully A, Hewes RS, Deitcher DL, Levitan ES (2005) Activity-dependent liberation of synaptic neuropeptide vesicles. Nat Neurosci 8:173-178.

Shakiryanova D, Klose MK, Zhou Y, Gu T, Deitcher DL, Atwood HL, Hewes RS, Levitan ES (2007) Presynaptic ryanodine receptor-activated calmodulin kinase II increases vesicle mobility and potentiates neuropeptide release. J Neurosci 27:7799-7806.

Sheng ZH, Rettig J, Cook T, Catterall WA (1996) Calcium-dependent interaction of N-type calcium channels with the synaptic core complex. Nature 379:451-454.

Simon SM, Llinas RR (1985) Compartmentalization of the submembrane calcium activity during calcium influx and its significance in transmitter release. Biophys J 48:485-498.

Sun XP, Callamaras N, Marchant JS, Parker I (1998) A continuum of InsP3-mediated elementary Ca2+ signalling events in Xenopus oocytes. J Physiol 509 (Pt 1):67-80.

Tobin VA, Hurst G, Norrie L, Dal Rio FP, Bull PM, Ludwig M (2004) Thapsigargin-induced mobilization of dendritic dense-cored vesicles in rat supraoptic neurons. Eur J Neurosci 19:2909-2912.

Velazquez-Marrero C, Custer E, De Crescenzo V, ZhuGe R, Fogarty KE, Walsh JV, Jr., Lemos JR (2002) Caffeine Stimulates Basal Neuropeptide Release from Nerve Terminals of the Neurohypophysis. J Neurosci 202:440.448.

Wang G, Dayanithi G, Newcomb R, Lemos JR (1999) An R-type Ca(2+) current in neurohypophysial terminals preferentially regulates oxytocin secretion. J Neurosci 19:9235-9241.

Yao LJ, Wang G, Ou-Yang KF, Wei CL, Wang XH, Wang SR, Yao W, Huang HP, Luo JH, Wu CH, Liu J, Zhou Z, Cheng HP (2006) Ca2+ sparks and Ca2+ glows in superior cervical ganglion neurons. Acta Pharmacol Sin 27:848-852.

Zhou Z, Misler S (1996) Amperometric detection of quantal secretion from patch-clamped rat pancreatic beta-cells. J Biol Chem 271:270-277.

ZhuGe R, Fogarty KE, Tuft RA, Lifshitz LM, Sayar K, Walsh JV, Jr. (2000) Dynamics of signaling between Ca(2+) sparks and Ca(2+)- activated K(+) channels studied with a novel image-based method for direct intracellular measurement of ryanodine receptor Ca(2+) current. J Gen Physiol 116:845-864.

ZhuGe R, DeCrescenzo V, Sorrentino V, Lai FA, Tuft RA, Lifshitz LM, Lemos JR, Smith C, Fogarty KE, Walsh JV, Jr. (2006) Syntillas release Ca2+ at a site different from the microdomain where exocytosis occurs in mouse chromaffin cells. Biophys J 90:2027-2037.

CHAPTER V: General Discussion

I. The Mechanism of Exocytosis

Chapter II of this thesis, along with previous studies, shows that with reconstitution of the essential components of membrane fusion, spontaneous fusion can be observed in a planar lipid bilayer model system (Woodbury and Rognlien, 2000; Rognlien and Woodbury, 2003; McNally et al., 2004). Using the nystatin/ergosterol method, described earlier, it has been determined that the addition of simply the SNARE protein syntaxin 1 to the bilayer results in the ability of modified secretory vesicles to fuse in the absence of an osmotic driving force. As shown in Chapter II (see figure 2.1), modified neurosecretory granules, presumably containing native SNAREs, are unable to spontaneous fuse to a protein-free bilayer. However, with the addition of syntaxin to the bilayer spontaneous fusion was observed. Protein-free vesicles, containing only nystatin, ergosterol, and purified lipids, do **not** spontaneously fuse to a bilayer regardless of the presence of syntaxin. This finding has also been extended to large dense core granules isolated from bovine chromaffin cells (McNally et al., 2003 and see Appendix I), and small clear synaptic vesicles isolate from the *Torpedo* electric organ (Woodbury and Rognlien, 2000; Rognlien and Woodbury, 2003). The fact that this result is the same for both large dense core granules and small clear synaptic vesicles illustrates the remarkable conservation of the process of SNARE driven fusion.

One concern in these experiments is the possibility that impurities are reconstituted into the planar bilayer along with recombinant syntaxin, and that

these impurities might affect vesicular fusion. To test this hypothesis, syntaxin was prepared five different ways, such that each preparation contained different concentrations of syntaxin and impurities. Each syntaxin preparation was tested for its ability to support fusion of modified synaptic vesicles. We observed a strong correlation between the amount of syntaxin in the preparation and the observed fusion rate of modified synaptic vesicles (Merino et al., 2003). Samples with low amounts of syntaxin and a generally higher concentration of contaminates did not support fusion. This indicates that syntaxin alone, and not a contaminant, is responsible for eliciting spontaneous fusion of synaptic vesicles. Further in appendix I of this thesis amperometric detection is combined with our planar lipid bilayer system, allowing us to confirm our results, using unmodified LDCG.

Also of concern is the method with which syntaxin was incorporated into the bilayer. The first technique involves wetting a pipette tip with a solution containing the purified protein, and simply "brushing" the protein into a preformed bilayer. This technique is quick and easy, but provides no means to control the amount of protein incorporated into the bilayer. To addresses this concern, purified protein can also be added directly to the lipid solution used to form the bilayer or added to the solution surrounding the bilayer and allowed to spontaneously insert.

Treatment of modified neurosecretory granules with a soluble form of syntaxin, lacking the transmembrane domain, significantly inhibits spontaneous

fusion (McNally et al., 2004 and see Chapter II). This dominant negative experiment provides evidence that a protein-protein interaction is in fact responsible for the spontaneous fusion observed, and that treatment with soluble syntaxin saturates all syntaxin binding sites on the granule membrane, leading to an inhibition of fusion. It is presently unclear what individual factors in the vesicular membrane function in concert with syntaxin to promote this spontaneous vesicle fusion. This is due to the fact that the modified vesicles posses the full complement of native membrane proteins present in vesicles. Further experiments where artificial vesicles containing individual recombinant proteins, such as the vesicular SNARE VAMP, need to be performed. However, treatment of modified neurosecretory granules with botulinum toxin B, which specifically cleaves VAMP, inhibits spontaneous fusion with a syntaxin containing bilayer by ~75% when compared to untreated granules (McNally et al., 2003). This finding supports our belief that VAMP must be involved in eliciting spontaneous fusion.

Membrane Fusion without SNAP-25

It is interesting to note that **no** SNAP-25 is required in the bilayer to achieve spontaneous vesicle fusion. This conflicts with earlier findings in the first reconstituted SNARE fusion system, which relied on liposome-liposome fusion. Using this system Weber et al. (1998) demonstrated that all three SNAREs were necessary and sufficient for vesicle-vesicle (v-v) fusion. Many reports have

confirmed that v-v fusion requires all three SNAREs and that SNARE-driven v-v fusion is Ca^{2+} activated when synaptotagmin is added to the system (Hu et al., 2002; Tucker et al., 2004), leaving at issue our earlier observation that SNAP-25 was not necessary. However, several groups have recently developed vesicle-bilayer (v-B) fusion systems. In contrast to the results with v-v fusion, these studies show that SNAP-25 does <u>not</u> enhance v-B fusion and that syntaxin and synaptobrevin are sufficient (Bowen et al., 2004; Liu et al., 2005), consistent with our results.

The topological differences between v-v and v-B fusion may provide clues for the role of SNAP-25. In v-v fusion high curvature may lead to a greater release of energy as two vesicles fuse, but the activation energy for fusion may remain high. In v-B fusion, the fusion event may release less energy, but the activation energy may be less than in v-v fusion. A higher activation energy for v-v fusion would be expected since the outer monolayer of vesicles has positive curvature and the fusion intermediate hemifused state, requires lipids with negative curvature. In v-B fusion, such lipids could be recruited from the bilayer membrane, thus lowering the activation energy for fusion. Assuming the activation energy for v-v fusion is higher than v-B fusion the experimental data suggest that SNAP-25 may serve to increase the force SNAREs provide to drive fusion.

Our findings also support an ever-increasing amount of research that shows that the SNARE proteins are not the source of specificity as had been

originally proposed by the SNARE hypothesis (Sollner et al., 1993). SNARE proteins are notoriously promiscuous (Jahn and Scheller, 2006); therefore it is our belief that in our experiments the Q-SNARE motifs of syntaxin are able to substitute for those thought to be provided by SNAP-25 (see figure 5.1). This is supported by other in *vitro* experiments (Fasshauer et al., 1999; Yang et al., 1999), that have shown it is possible to assemble non-cognate SNARE complexes. This is most likely due to high conservation of the SNARE motif. Even in *in vivo* systems SNAREs have been shown to participate in multiple SNARE interactions (von Mollard et al., 1997; Tsui and Banfield, 2000; Liu and Barlowe, 2002) .

It is also of note that in genetic experiments SNAP-25 and synaptobrevin only appear to be required for rapid vesicle fusion. Knocking-out SNAP-25 severely inhibits evoked release, but spontaneous release persists (Washbourne et al., 2002). Similar findings are observed in the absence of synaptobrevin (Deitcher et al., 1998; Schoch et al., 2001). This would appear to indicate that there is significant redundancy in the system that governs membrane fusion and supports the notion that SNARE motif exchange is widely tolerated in the formation of the SNARE fusion complex.

If it is not the SNAREs that govern the specificity of membrane fusion then what ensures that secretory vesicles fuse when and where they are supposed to in native synapses? SNAREs have been found to be much more selective *in vivo*, than in reconstituted systems (Scales et al., 2000). This is most likely due
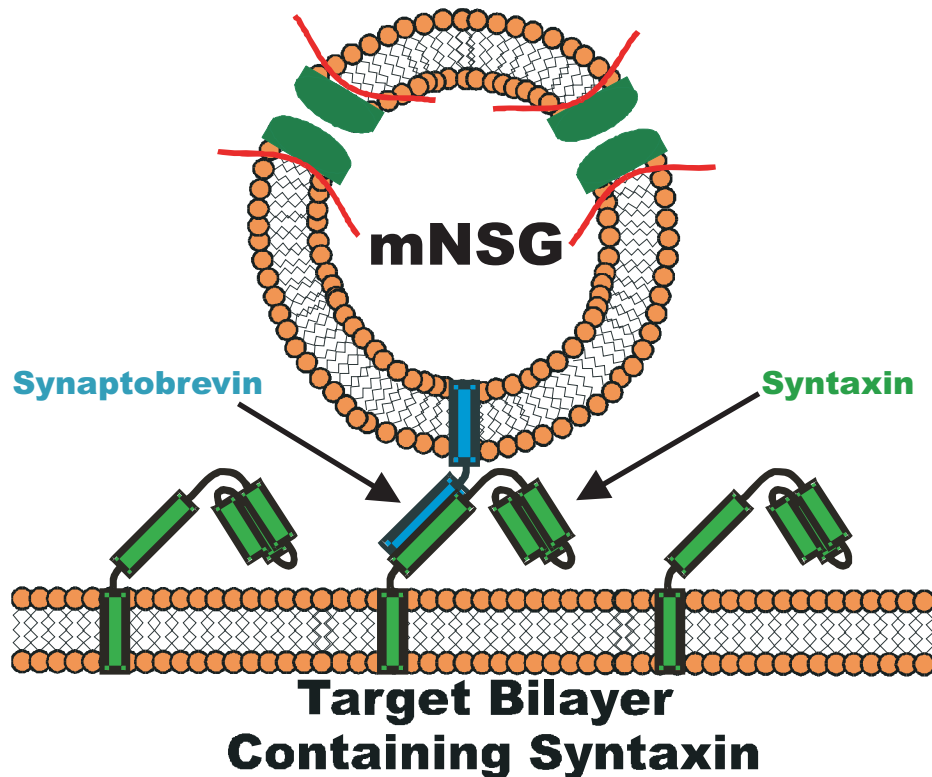
Figure 5.1: **Spontaneous fusion of mNSG with a planar bilayer containing syntaxin 1A alone.** Previous studies have determined that the minimal configuration of proteins required to drive membrane fusion consist synaptobrevin in the vesicular membrane, and syntaxin 1 and SNAP-25 in the target membrane. Surprisingly, we have shown that mNSG are capable of fusing spontaneously with a planar lipid bilayer containing **only** the SNARE protein syntaxin 1A, and no SNAP-25. This finding has been confirmed for the fusion of both LDCG and small synaptic vesicles, and more recently in other reconstituted systems assaying fusion of liposomes with a planar bilayer.

to the fact that *in vivo* SNAREs exist in their natural environment and are allowed to interact with a host of regulatory proteins that are able to mediate membrane specificity and dictate the triggering of fusion.

Regulatory Components and Calcium Dependent Fusion

After determining the minimal configuration of proteins required to achieve membrane fusion, regulatory components can be added to the system piecewise, in order to get a full understanding of their roles in the synapse. Syntaxin contains an N-terminal regulatory domain that is thought to bind and occlude the SNARE motif of the protein (Nicholson et al., 1998). Deletion mutants have been used in our studies to look at the effects of this regulatory domain on the spontaneous fusion described above. In experiments using modified neurosecretory granules (McNally et al., 2004 and see Chapter II), full-length syntaxin-1a and syntaxin 1a-8, which lacks much of the regulatory domain, both were equally effective in eliciting spontaneous fusion. This result indicates that the regulatory domain of syntaxin is not playing a role in influencing fusion in this model system. SNAREs have been found to be largely unstructured *in vitro*, therefore this model system may lack the proper regulatory factors required to allow the regulatory domain of syntaxin to function. Syntaxin 1a-12, which lacks the entire regulatory domain and the SNARE motif of syntaxin, is unable to elicit spontaneous fusion. Similar experiments, using modified small synaptic vesicles

(Rognlien and Woodbury, 2003) yield the same result for syntaxin 1a-12. Interestingly, syntaxin 1a-7 and 1a-9, deletion mutants which like syntaxin 1a-8 lack portions of the regulatory domain, show a seven-fold reduction in spontaneous fusion when compared to the full length version of the protein. The reason for this difference between neurosecretory granule fusion and that of synaptic vesicles remains unclear.

Calcium is not effective in modulating spontaneous fusion of modified vesicles with a bilayer containing syntaxin (Woodbury and Rognlien, 2000; Rognlien and Woodbury, 2003; McNally et al., 2004). This is interesting because synaptotagmin, the protein believed to confer calcium dependence on fusion, is present in the modified vesicles used in these experiments. Work with a different reconstituted systems, has shown that the presence of synaptotagmin enhances vesicle-vesicle fusion presumably through its interaction with SNARE proteins (Hu et al., 2002). However, the reconstituted target vesicle membrane contained syntaxin and SNAP-25, unlike our own. Since synaptotagmin has been shown to interact with SNAP-25 it is possible that this is the reason that no calcium dependence can be demonstrated. Regardless, it appears that as presently constituted our model system does not posses all of the components required for the calcium regulation of vesicular fusion.

II. Intracellular Calcium Stores and Secretion

Chapters III and IV of this thesis provide insight into both the location of ryanodine sensitive calcium stores in NHT as well as it's physiological role in these terminals. Chapter III suggests that syntillas arise from a ryanodine sensitive vesicular store. Using immuno-electron microscopy we have shown that at least both types 1 and 2 RyR labeling colocalizes with NHT dense core granules. Further, previous studies had indicated that a large conductance non-specific cation channel that has characteristics similar to that of a RyR is present in the membrane of NHT dense core granules (Lee et al., 1992; Yin et al., 2002). Here we have shown that specific agonists and antagonists of the RyR have the same characteristic effects on this channel's activity.

Recent work by Jin et al. (2007), has shown that ryanodine sensitive Ca^{2+} release is capable of modulating oxytocin secretion from NHT. In this study, genetic deletion of the transmembrane receptor CD38, which catalyzes the formation of the physiological RyR agonist cyclicADP ribose, disrupts both depolarization-induced oxytocin secretion and Ca^{2+} elevation. Similarly, in this thesis and previous studies (Velazquez-Marrero et al., 2002), release of Ca^{2+} from these stores is able to increase basal neuropeptide release form NHT in the absence of extracellular calcium, suggesting that ryanodine-sensitive Ca^{2+} stores may have a role in modulating release in this system.

Despite the seemingly strategic localization of this Ca^{2+}, Chapter IV shows that syntillas are not responsible for directly eliciting individual spontaneous

transmitter release events. In order to determine this, a method allowing transmitter release from NHT on the same temporal scale as Ca^{2+} imaging and in a spatially defined manner was required. Therefore, amperometric detection was adapted to this system using a novel false-transmitter loading approach. Using this technique we confirmed earlier population release results showing that ryanodine application at both agonist and antagonist concentrations were able to affect the basal level of transmitter release.

These findings are similar to those of earlier studies of presynaptic Ca^{2+} transients in other neuronal systems. As discussed in chapter I, in cultured hippocampal neurons, as well as cerebellar interneurons, spontaneous Ca^{2+} transients have been shown to modulate the frequency of spontaneous miniature inhibitory postsynaptic currents (Llano et al., 2000; Emptage et al., 2001). However, in these systems the precise localization of these Ca^{2+} transients was difficult to determine due to the small size of presynaptic terminals. This leaves the presynaptic localization of these events in question. Additionally, transmitter release activity was measured by recording postsynaptic currents, thus these studies were unable to rule out a postsynaptic effect on transmitter release.

This is not the case with NHTs. The coupling of amperometric detection with our system allows direct assessment of the correlation of Ca^{2+} transients to transmitter release. Additionally, the fact that this NHT preparation is strictly presynaptic leaves no doubt that the Ca^{2+} transients observed in this system are localized to presynaptic sites. Here we have shown that combined amperometric

detection along with high-speed Ca^{2+} imaging in NHT showed <u>no</u> direct correlation between Ca^{2+} syntillas and spontaneous transmitter release. These findings do not, however, rule out an indirect modulatory role of syntillas on neuropeptide release in this system.

NHT vs. Chromaffin Cell Syntillas

Earlier work by Zhuge et al. (2006) has shown that Ca^{2+} syntillas can also be observed in mouse chromaffin cells, another system commonly used to study LDCG secretion. In this study, as in our own, amperometric detection was employed to directly assess the ability of syntillas to directly elicit spontaneous transmitter release, and similarly showed no correlation between these two events. Therefore, it appears that in both chromaffin cells and NHT Ca^{2+} syntillas yield Ca^{2+} domains separate from those occurring with the influx of Ca^{2+} through voltage gated calcium channels (VGCC), and do not appear to co-localize with LDCG that are primed for release.

Even though the main findings of both this work and our own are essentially identical, there are some important discrepancies that need to be addressed. Such differences are not wholly unexpected as the body of research in this field shows not all neuronal preparations show the same role of presynaptic stores in release (Bouchard et al., 2003; Collin et al., 2005; Berridge, 2006). In particular, chromaffin cells show no change in the rate of spontaneous release events with application of antagonist concentrations of RyR, unlike the

NHT. Interestingly, while there is no change in the basal release rate, there was a significant increase in the size of basal release events (ZhuGe et al., 2006). This effect was not observed in NHT. However, as NHT necessitated false transmitter loading to detect these events such analysis may not be useful, as such loading may not be even throughout all LDCG (Kim et al., 2000).

There are several possible reasons for these differences. Most notably, these are two completely different secretory systems which do not possess the same subset of RyR subtypes (De Crescenzo et al., 2006; ZhuGe et al., 2006). Differential expression of different subtypes of intracellular Ca^{2+} release channels has been suggested to allow Ca^{2+} to act as a signaling agent in a number of different pathways (Berridge, 2006). This is strongly supported by the fact that no voltage dependence has been observed for syntillas in chromaffin cells, unlike in NHT, and antagonist concentrations of ryanodine virtually abolish syntillas in chromaffin cell, whereas they block only half of the syntillas in NHT. Also, as mentioned previously in chapter IV, these experiments were not conducted in precisely the same fashion. In NHT, the effect of ryanodine on basal release was assessed on individual terminals, by first determining the rate of release under control conditions, then in the presence of the drug. In chromaffin cells this was assessed by determining the average rate of release of a population of chromaffin cells under control conditions versus ryanodine treated conditions.

In both NHT and chromaffin cells, the fact that the simultaneous measurement of syntillas and amperometric activity was performed with no

extracellular calcium may have played a role in the lack of correlation between these two events. It was necessary to conduct these experiments in this manner to ensure that Ca^{2+} events observed were truly emanating from an intracellular source. However, this lack of extracellular Ca^{2+} may have reduced the basal free calcium concentration, resulting in syntillas not being able to raise the intracellular calcium high enough to elicit release. This may be supported by the fact that treatment with both agonist and antagonist concentrations of ryanodine results in a robust effect on basal secretion in the presence of physiological levels of Ca^{2+} (see figure 4.3). Thus, these experiments should be repeated in physiological concentrations of Ca^{2+} to see if syntillas then have any effect.

Another possible reason for the lack of correlation between syntillas and spontaneous release events in these two systems could be that multiple syntillas are required in order to elicit exocytosis. Recently, such a relationship has been proposed to be involved in somatic transmitter release from dorsal root ganglion neurons (Ouyang et al., 2005). In these studies, the Ca^{2+} spark-secretion coupling probability was estimated to be 6-11 sparks per vesicle. This does not appear to be the case in our system, as several syntillas were observed to occur proximal to the carbon fiber electrode, within 10 ms of one another, and yet no correlated release was observed.

Possible role of Syntillas

If syntillas are not involved in directly eliciting spontaneous neuropeptide release in the NHT, then what possible role could such events play in these terminals? As discussed in chapter I of this thesis, Ca^{2+} is not only responsible for eliciting transmitter release, but also serves to shape and tune synaptic activity. Therefore, syntillas may be involved in an earlier step in elicited exocytosis (see figure 5.2). The recruitment of secretory vesicles from reserve to release pools has been shown to be a calcium-dependent process (Llinas et al., 1991; Hosoi et al., 2007). Additionally such processes as actin remodeling, which has been suggested to play a role in vesicular trafficking, have been shown to be calcium dependent (Malacombe et al., 2006).

Recent studies investigating dendritic neuropeptide release in magnocelluar neurons, the very neurons from which NHT arise, has found that release of Ca^{2+} from intracellular stores is capable of priming LDCG for later activity-dependent release (Ludwig et al., 2002; Ludwig and Leng, 2006). Upon activation of this priming mechanism LDCG are seen to be trafficked to the plasma membrane, presumably from reserve pools to sites of release. This priming has been shown to persist for a prolonged period, and may provide a means of changing the nature of interactions between these neurons and their neighbors (Ludwig et al., 2002). As such a mechanism has already been observed in the dendrites of magnocellular neurons, could a similar mechanism also exist in their terminals? This has yet to be fully investigated, but preliminary
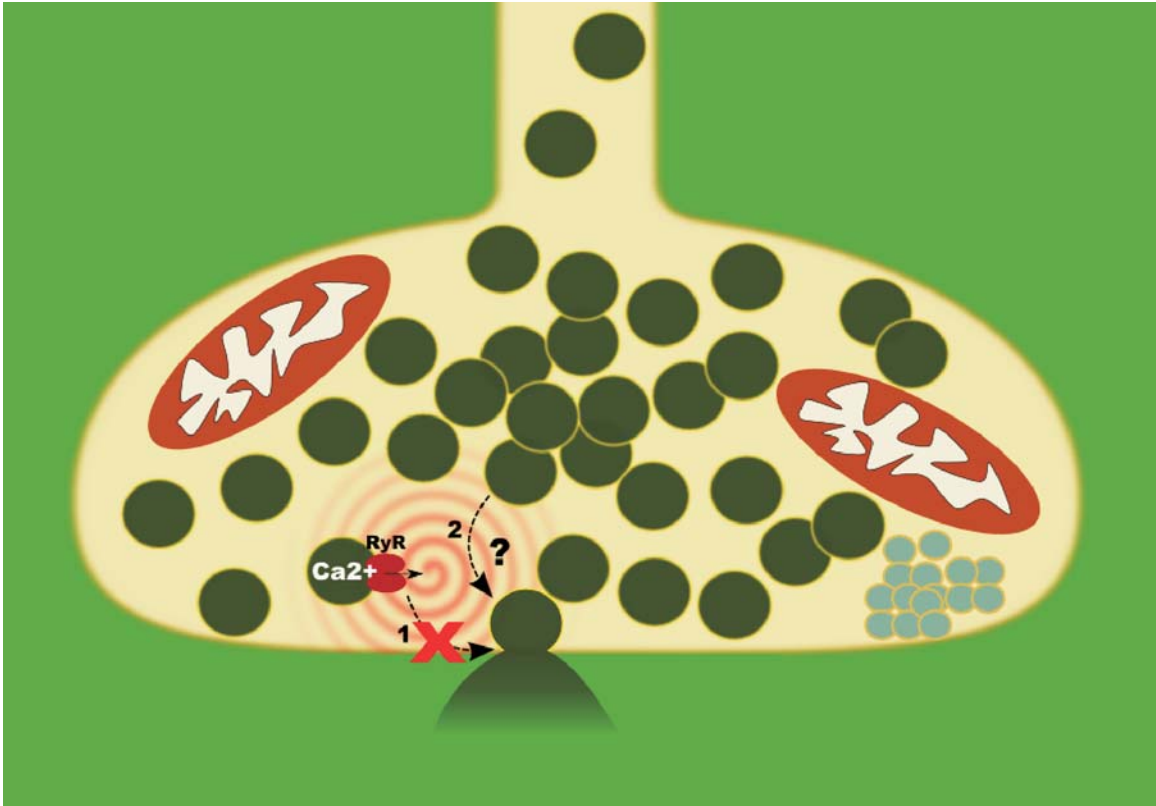
Figure 5.2: What do syntillas do? (**1**) Chapter IV of this thesis reveals that syntillas do not appear to be able to directly elicit spontaneous transmitter release in NHT. However, this does not rule out the involvement of syntillas in modulating earlier steps of secretion that precede exocytosis. (**2**) Evidence exists that intracellular Ca^{2+} stores are capable of priming LDCG for release from dendrites in this very system. Therefore, it may be possible that syntillas are involved in the trafficking of LDCG to the plasma membrane of NHT for release.

work demonstrating that this may in fact be the case is presented in appendix II of this thesis (see figure AII.6).

Physiological stimulation of neuropeptide secretion in this system results from high frequency bursts of action potentials that last a number of seconds (Cazalis et al., 1985). In order for neuropeptide release to persist for such a long duration, NHT would presumably require a mechanism capable of replenishing LDCGs in an activity-dependent manner. The ability of syntillas to be upregulated in a voltage-dependent manner, and presumable via calcium-induced calcium release, would appear to make them an ideal candidate to be responsible for such a role. Thus, providing an activity-dependent intracellular calcium release mechanism that could upregulate recruitment of secretory granules from reserve pools to active release pools during long bursts of stimuli.

While the above possibilities focus on aspects of transmitter regulation prior to elicited release, it is also possible that syntillas may affect neuropeptide release after formation of the fusion pore. As discussed above, in chromaffin cells application of antagonist ryanodine concentrations leads to a decrease in the quantal size of release events (ZhuGe et al., 2006), possibly resulting in an inhibition of secretion from these cells. While this may be due to syntillas recruiting a different population of LDCG for release (Grabner et al., 2005), release of calcium from LDCG via syntillas could play a role in influencing the state of LDCG cargo, which could have affects on the rate of release.

LDCG contain extremely high concentrations of cargo (Michael et al., 2006). This cargo is stored in a crystalin-state in order to keep it less osmotically active. During "kiss and run" fusion granules may only release a small fraction of their contents, as granule cargo may dissolve slowly into the extracellular solution. Therefore, the solubility of LDCG cargo could play a significant role in determining the rate of release.

Divalent cations tighten the crosslinking between the negatively charged peptides in LDCG, stabilizing this matrix. The efflux of vesicular Ca^{2+} resultant from syntillas could destabilize this matrix and thus alter the rate of release from LDCG. In the NHT, earlier studies have theorized that release of LDCG calcium was required prior to granule fusion (Nicaise et al., 1992). Also, this could explain the decrease in release seen in chromaffin cells when blocking syntillas with antagonist concentrations of ryanodine. Other studies in chromaffin cells have shown a stimulus-dependent increase in the size of individual quantal release events (Pothos et al., 2002). Interestingly, the mechanism behind this increase in quantal size appears to be independent of extracellular Ca^{2+}, leaving open the possibility the vesicular Ca^{2+} release via syntillas could be responsible.

General Conclusion

This thesis has presented a number of interesting new insights into the molecular mechanisms responsible for the release of transmitter at NHT.

Initially, work using the planar lipid bilayer model synapse is presented showing that membrane fusion between isolated neurohypophysial LDCG and a planar bilayer containing only the SNARE protein syntaxin 1A can occur spontaneously. This is quite unexpected as previous work had indicated that SNAP-25 was required as well. In appendix I, this result was confirmed using amperometric detection to monitor membrane fusion and ensuing transmitter release in our planar bilayer model system. This allowed us to address some of the concerns regarding this system, and further, allowed detailed examination of reconstituted transmitter release. In the future, it is our goal to use this system to fully reconstruct Ca^{2+}-dependent membrane fusion (see A1.6).

Additional work examined the role of ryanodine sensitive stores in NHT. Here we have presented evidence localizing these stores to LDCG. Then using combined amperometry and Ca^{2+} imaging we examined the relationship between syntillas, Ca^{2+} release events from ryanodine sensitive stores, and transmitter release. Quite unexpectedly, syntillas which would appear to be localized precisely where one would expect them to elicit release, do not. In Appendix II we provided preliminary evidence suggesting that these events may function in a LDCG priming role. While the ultimate physiological role Ca^{2+} syntillas has yet to be fully elucidated, this thesis has provided some answers as to the nature of these events, as well as stimulating new questions. These results will hopefully aid in elucidation of the processes behind transmitter release from NHT as well as other CNS terminals.

References:

Berridge MJ (2006) Calcium microdomains: organization and function. Cell
 Calcium 40:405-412.

Bouchard R, Pattarini R, Geiger JD (2003) Presence and functional significance
 of presynaptic ryanodine receptors. Prog Neurobiol 69:391-418.

Bowen ME, Weninger K, Brunger AT, Chu S (2004) Single molecule observation
 of liposome-bilayer fusion thermally induced by soluble N-ethyl maleimide
 sensitive-factor attachment protein receptors (SNAREs). Biophys J
 87:3569-3584.

Cazalis M, Dayanithi G, Nordmann JJ (1985) The role of patterned burst and
 interburst interval on the excitation-coupling mechanism in the isolated rat
 neural lobe. J Physiol 369:45-60.

Collin T, Marty A, Llano I (2005) Presynaptic calcium stores and synaptic
 transmission. Curr Opin Neurobiol 15:275-281.

De Crescenzo V, Fogarty KE, Zhuge R, Tuft RA, Lifshitz LM, Carmichael J,
 Bellve KD, Baker SP, Zissimopoulos S, Lai FA, Lemos JR, Walsh JV, Jr.
 (2006) Dihydropyridine receptors and type 1 ryanodine receptors
 constitute the molecular machinery for voltage-induced Ca2+ release in
 nerve terminals. J Neurosci 26:7565-7574.

Deitcher DL, Ueda A, Stewart BA, Burgess RW, Kidokoro Y, Schwarz TL (1998)
 Distinct requirements for evoked and spontaneous release of
 neurotransmitter are revealed by mutations in the Drosophila gene
 neuronal-synaptobrevin. J Neurosci 18:2028-2039.

Emptage NJ, Reid CA, Fine A (2001) Calcium stores in hippocampal synaptic
 boutons mediate short-term plasticity, store-operated Ca2+ entry, and
 spontaneous transmitter release. Neuron 29:197-208.

Fasshauer D, Antonin W, Margittai M, Pabst S, Jahn R (1999) Mixed and non-
 cognate SNARE complexes. Characterization of assembly and biophysical
 properties. J Biol Chem 274:15440-15446.

Grabner CP, Price SD, Lysakowski A, Fox AP (2005) Mouse chromaffin cells
 have two populations of dense core vesicles. J Neurophysiol 94:2093-
 2104.

Hosoi N, Sakaba T, Neher E (2007) Quantitative analysis of calcium-dependent
 vesicle recruitment and its functional role at the calyx of Held synapse. J
 Neurosci 27:14286-14298.

Hu K, Carroll J, Fedorovich S, Rickman C, Sukhodub A, Davletov B (2002)
 Vesicular restriction of synaptobrevin suggests a role for calcium in
 membrane fusion. Nature 415:646-650.

Jahn R, Scheller RH (2006) SNAREs--engines for membrane fusion. Nat Rev
 Mol Cell Biol 7:631-643.

Jin D, Liu HX, Hirai H, Torashima T, Nagai T, Lopatina O, Shnayder NA, Yamada
 K, Noda M, Seike T, Fujita K, Takasawa S, Yokoyama S, Koizumi K,

Shiraishi Y, Tanaka S, Hashii M, Yoshihara T, Higashida K, Islam MS, Yamada N, Hayashi K, Noguchi N, Kato I, Okamoto H, Matsushima A, Salmina A, Munesue T, Shimizu N, Mochida S, Asano M, Higashida H (2007) CD38 is critical for social behaviour by regulating oxytocin secretion. Nature 446:41-45.

Kim KT, Koh DS, Hille B (2000) Loading of oxidizable transmitters into secretory vesicles permits carbon-fiber amperometry. J Neurosci 20:RC101.

Lee CJ, Dayanithi G, Nordmann JJ, Lemos JR (1992) Possible role during exocytosis of a Ca(2+)-activated channel in neurohypophysial granules. Neuron 8:335-342.

Liu T, Tucker WC, Bhalla A, Chapman ER, Weisshaar JC (2005) SNARE-Driven, 25-Millisecond Vesicle Fusion In Vitro. Biophys J 89:2458-2472.

Liu Y, Barlowe C (2002) Analysis of Sec22p in endoplasmic reticulum/Golgi transport reveals cellular redundancy in SNARE protein function. Mol Biol Cell 13:3314-3324.

Llano I, Gonzalez J, Caputo C, Lai FA, Blayney LM, Tan YP, Marty A (2000) Presynaptic calcium stores underlie large-amplitude miniature IPSCs and spontaneous calcium transients. Nat Neurosci 3:1256-1265.

Llinas R, Gruner JA, Sugimori M, McGuinness TL, Greengard P (1991) Regulation by synapsin I and Ca(2+)-calmodulin-dependent protein kinase II of the transmitter release in squid giant synapse. J Physiol 436:257-282.

Ludwig M, Leng G (2006) Dendritic peptide release and peptide-dependent behaviours. Nat Rev Neurosci 7:126-136.

Ludwig M, Sabatier N, Bull PM, Landgraf R, Dayanithi G, Leng G (2002) Intracellular calcium stores regulate activity-dependent neuropeptide release from dendrites. Nature 418:85-89.

Malacombe M, Bader MF, Gasman S (2006) Exocytosis in neuroendocrine cells: new tasks for actin. Biochim Biophys Acta 1763:1175-1183.

McNally JM, Woodbury DJ, Lemos JR (2003) Syntaxin 1A is Sufficient for Spontaneous Fusion of Both Neurohypophysial and Chromaffin Dense Core Granules to a Planar Lipid Bilayer. In: 43rd Annual Meeting of the American Society for Cell Biology. San Francisco.

McNally JM, Woodbury DJ, Lemos JR (2004) Syntaxin 1A Drives Fusion of Large Dense-Core Neurosecretory Granules Into a Planar Lipid Bilayer. Cell Biochem Biophys 41:11-24.

Merino S, Corless A, Adams D, Woodbury DJ (2003) Alternate Purification Methods Provide Evidence that Syntaxin, Not Contaminants, Drives Fusion. In: 47th Biophysical Society Annual Meeting. San Antonio.

Michael DJ, Cai H, Xiong W, Ouyang J, Chow RH (2006) Mechanisms of peptide hormone secretion. Trends Endocrinol Metab 17:408-415.

Nicaise G, Maggio K, Thirion S, Horoyan M, Keicher E (1992) The calcium loading of secretory granules. A possible key event in stimulus-secretion coupling. Biol Cell 75:89-99.

Nicholson KL, Munson M, Miller RB, Filip TJ, Fairman R, Hughson FM (1998) Regulation of SNARE complex assembly by an N-terminal domain of the t-SNARE Sso1p. Nat Struct Biol 5:793-802.

Ouyang K, Zheng H, Qin X, Zhang C, Yang D, Wang X, Wu C, Zhou Z, Cheng H (2005) Ca2+ sparks and secretion in dorsal root ganglion neurons. Proc Natl Acad Sci U S A 102:12259-12264.

Pothos EN, Mosharov E, Liu KP, Setlik W, Haburcak M, Baldini G, Gershon MD, Tamir H, Sulzer D (2002) Stimulation-dependent regulation of the pH, volume and quantal size of bovine and rodent secretory vesicles. J Physiol 542:453-476.

Rognlien KT, Woodbury DJ (2003) Reconstituting SNARE proteins into BLMs. In: Planar Lipid Bilayers (BLMs) and Their Applications (Tein HT, and Ottova-Leitmannova, A., ed), pp 479-488: Elsevier Science B.V.

Scales SJ, Chen YA, Yoo BY, Patel SM, Doung YC, Scheller RH (2000) SNAREs contribute to the specificity of membrane fusion. Neuron 26:457-464.

Schoch S, Deak F, Konigstorfer A, Mozhayeva M, Sara Y, Sudhof TC, Kavalali ET (2001) SNARE function analyzed in synaptobrevin/VAMP knockout mice. Science 294:1117-1122.

Sollner T, Whiteheart SW, Brunner M, Erdjument-Bromage H, Geromanos S, Tempst P, Rothman JE (1993) SNAP receptors implicated in vesicle targeting and fusion. Nature 362:318-324.

Tsui MM, Banfield DK (2000) Yeast Golgi SNARE interactions are promiscuous. J Cell Sci 113 (Pt 1):145-152.

Tucker WC, Weber T, Chapman ER (2004) Reconstitution of Ca2+-regulated membrane fusion by synaptotagmin and SNAREs. Science 304:435-438.

Velazquez-Marrero C, Custer E, De Crescenzo V, ZhuGe R, Fogarty KE, Walsh JV, Jr., Lemos JR (2002) Caffeine Stimulates Basal Neuropeptide Release from Nerve Terminals of the Neurohypophysis. J Neurosci 202:440.448.

von Mollard GF, Nothwehr SF, Stevens TH (1997) The yeast v-SNARE Vti1p mediates two vesicle transport pathways through interactions with the t-SNAREs Sed5p and Pep12p. J Cell Biol 137:1511-1524.

Washbourne P, Thompson PM, Carta M, Costa ET, Mathews JR, Lopez-Bendito G, Molnar Z, Becher MW, Valenzuela CF, Partridge LD, Wilson MC (2002) Genetic ablation of the t-SNARE SNAP-25 distinguishes mechanisms of neuroexocytosis. Nat Neurosci 5:19-26.

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. Cell 92:759-772.

Woodbury DJ, Rognlien K (2000) The t-SNARE syntaxin is sufficient for spontaneous fusion of synaptic vesicles to planar membranes. Cell Biol Int 24:809-818.

Yang B, Gonzalez L, Jr., Prekeris R, Steegmaier M, Advani RJ, Scheller RH (1999) SNARE interactions are not selective. Implications for membrane fusion specificity. J Biol Chem 274:5649-5653.

Yin Y, Dayanithi G, Lemos JR (2002) Ca(2+)-regulated, neurosecretory granule channel involved in release from neurohypophysial terminals. J Physiol 539:409-418.

ZhuGe R, DeCrescenzo V, Sorrentino V, Lai FA, Tuft RA, Lifshitz LM, Lemos JR, Smith C, Fogarty KE, Walsh JV, Jr. (2006) Syntillas release Ca2+ at a site different from the microdomain where exocytosis occurs in mouse chromaffin cells. Biophys J 90:2027-2037.

APPENDIX I: Amperometric Detection of Release from a Planar Lipid Bilayer Model System

PREFACE

Material presented in this chapter has been published in the following locations:

<u>Book Chapters:</u>

Woodbury, D.J., **J.M. McNally**, and J.R. Lemos. (2007) SNARE-induced fusion of vesicles to a Planar Bilayer. Chapter 10 *in* "Advances in planar lipid bilayers and liposomes", edited by A. Leitmannova-Liu. (Elsevier) Vol. 5:285-311.

<u>Abstracts:</u>

J.M. McNally, D.J. Woodbury, & J.R. Lemos. (2003) Syntaxin 1a Is Sufficient For Spontaneous Fusion Of Both Neurohypophysial And Chromaffin Dense Core Granules To A Lipid Bilayer. *Mol. Biol. Cell* 14(12): A123.

J.M. McNally, D.J. Woodbury, & J.R. Lemos. (2007) Amperometric Detection of Spontaneous Fusion of Chromaffin Dense Core Granules to a Lipid Bilayer. *J. Gen. Physiol.* 130: 9a

J.M. McNally, D.J. Woodbury, & J.R. Lemos. (2008) Spontaneous fusion of chromaffin dense core granules to a lipid bilayer detected by amperometry. *Biophys. J.* 92: 1266

Abstract:

The SNARE hypothesis proposes that a general apparatus (composed of syntaxin, SNAP-25 & synaptobrevin) drives exocytosis of both small synaptic vesicles and large dense core granules (LDCG). We have previously demonstrated that LDCG from bovine neurohypophysial terminals are able to spontaneously fuse with a planar lipid bilayer containing <u>only</u> the t-SNARE syntaxin 1A (See Chapter II). In these experiments, native LDCG were <u>modified</u> to contain nystatin channels, allowing each fusion event to be visualized by monitoring bilayer conductance. Here, we confirm these results with <u>native</u> chromaffin cell LDCGs using **amperometry**. After establishing that chromaffin cell LDCGs behave in the same manner as neurohypophysial LDCGs when modified to contain nystatin channels, amperometric detection was coupled with our planar lipid bilayer model system to successfully detect release of native LDCGs. These events displayed characteristics similar to those of catecholamine release events from chromaffin cells. This approach also allowed for analysis of differences between spontaneous and osmotically-driven fusion events. Interestingly, small current steps preceded some of the observed amperometric spikes which appear similar in nature to the pre-spike "<u>foot</u>" observed during native release events from chromaffin cells. The pre-spike foot is attributed to the slow release of transmitter through a fusion pore prior to pore widening and complete collapse of the vesicle into the plasma membrane. The appearance of pre-spike feet preceding amperometric events seen in our extremely simple model system implies that all of the components required to form a fully functional fusion pore are present. In conclusion, this method provides an effective <u>model system</u> to study possible components involved in vesicular exocytosis, as well as shedding some light on the long held mystery of the composition of the fusion pore itself.

Introduction:

Exocytosis is the process of cellular secretion in which substances contained in intracellular vesicles are discharged from the cell by fusion of the vesicular membrane with the plasma membrane. A comprehensive understanding of the factors involved in this process is of paramount importance in the study of synaptic function. Extensive work has been performed in this area, yielding a vast array of protein and lipid components that may be involved in this process (Jahn et al., 2003). However, how these components are able to come together to elicit this function remains somewhat of a mystery.

While molecular biology is a powerful tool; genetic manipulations, such as deletion or enhancement of proteins thought to be involved in fusion and release, can only tell us so much about what role these factors play in this process. Truly defining the role regulatory proteins play in this process necessitates a simple reconstituted system using defined components. Therefore, it has been our goal to develop a "model synapse", by reconstituting each of the steps of calcium-dependent membrane fusion.

For exocytosis to occur in the synapse, fusion of the vesicular membrane and the presynaptic membrane must occur. Thus, the first step in the *in vitro* reconstitution of this process necessitates the development of a relatively simple model system that can be used to detectably follow vesicle fusion. While the liposome-liposome fusion assay has been commonly employed to study this process (Kreye et al., 2008), this system lacks the capability to look at individual

fusion events. Additionally, the membrane curvature involved in liposome-liposome fusion does not correlate well to that seen under native secretory vesicle-plasma membrane fusion.

It is for these reasons that we have chosen to use the planar lipid bilayer, as the basis for our model synapse (Woodbury, 1999a). Planar lipid bilayers, first described by Mueller et al.(1962), are ideal in that their lipid and protein composition can be controlled, as well as the environment on either side of the bilayer, making the system extremely adaptable. In this *in vitro* system the planar lipid bilayer is used to represent the plasma membrane, to which the fusion of individual secretory vesicles can be monitored in real time. Visualization of vesicle fusion necessitates the addition of both the pore-forming antibiotic, nystatin, and ergosterol to the vesicle membrane (Woodbury and Miller, 1990). When these nystatin/ergosterol vesicles fuse with the planar bilayer, the nystatin pores are incorporated into the bilayer. This incorporation can be observed by monitoring bilayer conductance. Since the planar bilayer is ergosterol free, nystatin pores, which are stabilized by ergosterol, collapse shortly after their incorporation into the bilayer. This provides the ability to observe numerous membrane fusion events without amplifier saturation.

As discussed in chapter II of this thesis, use of this model system has provided interesting insights into the ability of SNARE proteins to catalyze the process of membrane fusion. In contrast to liposome-liposome reconstitution studies, SNAP-25 is not required as a t-SNARE for this process (Woodbury and

Rognlien, 2000; McNally et al., 2004). The presence of the SNARE protein syntaxin in the target bilayer by itself is sufficient to elicit membrane fusion with synaptic vesicles containing native membrane proteins. This finding has also been observed in other vesicle-planar bilayer reconstituted systems (Liu et al., 2005).

While *in vitro* membrane fusion assays provide an elegant means of following membrane fusion, they provide little information about vesicular content release. This has been previously addressed in the planar lipid bilayer system by loading liposomes with fluorescent dye, allowing visualization of liposome fusion and content release (Woodbury and Hall, 1988). However, these experiments lacked much of the cellular machinery believed to be involved in the exocytosis, and required addition of an osmotic gradient to drive fusion.

Therefore, we have incorporated amperometric detection into our artificial synapse. This approach is commonly used to follow catecholamine release from chromaffin cells (Chow et al., 1992). Amperometric recording from isolated chromaffin cells allows the detection of individual exocytotic events, as their large dense core granules contain a high concentration of readily oxidizable catecholamine. Therefore we surmised that if we could adapt this technique to our planar bilayer model system, fusion of purified chromaffin granules with the planar bilayer could be detected in much the same way. Amperometric detection will provide means to detect release far superior to the fluorescence technique

described above in that it requires far less equipment, and provides more telling data about the kinetics of vesicular content release.

Here we confirm that modified chromaffin granules (mCG), like modified neurohypophysial granules, are able to undergo spontaneous fusion with an artificial bilayer containing only the SNARE protein syntaxin 1A. Then we confirm this finding using amperometry to detect release of native chromaffin granules, which also spontaneously fuse to a bilayer containing only syntaxin. Further, analysis of amperometric release events recorded from the bilayer allows us to gain some insight into the nature of reconstituted fusion.

Materials and Methods:

Purification of Native Bovine Chromaffin Granules

Bovine CGs were purified from calf adrenal glands via differential centrifugation. The adrenal glands were dissected within 10-15 minutes of slaughter, and stored in warm (37ºC) homogenizing solution (HS). This HS contained 0.3 M sucrose, and 10 mM Tris-HEPES and 1 mM EGTA at pH 7.0. In the laboratory, the adrenal medulla was dissected from gland. This tissue was then washed several times with Listerine and HS, finely minced with scissors, and then homogenized (at 1,000 rpm, 5 times up and down) after adding 2-3 ml of cold HS (from this point on all solutions were kept ice cold). The homogenate was then sequentially centrifuged with a SS-34 rotor in a Sorval RC-5B centrifuge. The homogenate was first centrifuged for 5 minutes at 3,000 rpm

(800 x g), the supernatant was then taken and centrifuged another 10 minutes at 5,000 rpm (3,000 x g), the supernatant was then taken and was centrifuged an additional 10 minutes at 8,000 rpm (8,000 x g). The final supernatant was then taken, HS solution was added (almost filling the centrifuge tube), and centrifuged for 20 minutes at 16,000 rpm (27,000 x g). The resulting pellet was taken and resuspended thoroughly using a micro-homogenizer in 1 ml HS, which was then added to an isosmotic Percoll (Pharmacia) solution almost filling the centrifuge tube and mixed well. The mixture was then centrifuged for 45 minutes at 16,000 rpm (27,000 x g). The portion between the upper white band and lower white band was then collected. Enough homogenizing solution for further separation was then added to the collected material, which was then centrifuged for an addition 40 minutes in order to remove the leftover percoll. After completion, the white band above the clear sticky pellet of percoll at the bottom of the tube was collected. The collected material was then brought to a total protein concentration of 1 mg/ml, aliquoted and stored at -80°C.

Modification of Native Vesicles

Native bovine NSG were modified following the procedure previously reported by Woodbury (Woodbury, 1999b). Briefly, nystatin/ergosterol modified vesicles (200-250 µl) were made as described in the methods section of Chapter II. An aliquot of purified bovine CG was added to this vesicle preparation, and the solution was then subjected to three sequential freeze, thaw, and sonicate

cycles to intermix the membranes of the two vesicle populations. These modified

chromaffin granules (mNSG) were stored frozen until a short time before use.

Formation of Bilayers

Planar lipid bilayers were formed as previously described (Woodbury,

1999b; Yin et al., 2002; McNally et al., 2004). First a decane solution (50µl) was

produced containing 75% phosphatidylethanolamine and 25%

phosphatidylcholine. All phospholipids used were purchased dissolved in

chloroform (10 mg/ml) from Avanti Polar Lipids, Inc. Bilayers were formed on a

150-250 µm diameter hole in a polystyrene cup; the hole was positioned between

the two chambers of the bilayer apparatus. The chambers of the bilayer

apparatus were filled with 150 mM KCl, 8 mM HEPES, pH 7.2 [1 ml in trans

chamber and 0.7 ml in the cis chamber]. Bilayers were formed by coating the tip

of a micro-pipette with the decane solution, immersing the pipette into the

chamber, blowing a small bubble at the pipette tip, and then drawing this bubble

across the hole in the cup. The current across the bilayer was then monitored

using a standard patch clamp amplifier (HEKA EPC9). Data was monitored and

stored using Pulse acquisition software (HEKA).

Reconstitution of Syntaxin into a Bilayer

Syntaxin 1A-1 was expressed and purified from E. coli as described in

Woodbury and Rognlien (2000), buffered in thrombin cleavage buffer: 2.5 mM

$CaCl_2 \cdot 2H_2O$, 50 mM Tris, 150 mM NaCl (pH 8.0). Syntaxin was reconstituted into bilayers by brushing < 0.5 µl of the protein solution on the bilayer in the same fashion that was used in order to create the bilayer. Briefly, the tip of a micro-pipette was coated with the protein solution, immersed into the chamber, and a small bubble formed at the pipette tip. This bubble was then drawn across the hole in the cup. This "brushing" process was repeated three times in order to ensure incorporation of the protein into the bilayer. After each brushing, the bilayer was allowed to recover its initial electrical properties prior to the next brushing step or recording.

Fusion of mCGs with a Bilayer

After bilayer formation, 20 µl of mCGs were added to the cis chamber of the bilayer apparatus with stirring. It was initially observed if these vesicles were capable of fusing with the planar bilayer "spontaneously" (e.g. in the absence of an external driving force). Then, after the addition of an osmotic gradient, we looked at the vesicles ability to undergo "induced" fusion, or fusion driven by the addition of an osmotic gradient. In order to determine the number of fusion events that took place during each condition, the current through the membrane was observed at a fixed transmembrane holding potential (+60 mV). A sudden increase in membrane conductance followed by decay, results from incorporation of nystatin/ergosterol channels into the membrane and is indicative of fusion of a single vesicle (Woodbury, 1999b). When an osmotic gradient was required to

induce fusion, the KCl concentration of the cis chamber was raised to ~ 410 mM through the addition of 70 µl of 3 M KCl to the chamber and subsequent removal of the same volume. Since the addition of an osmotic gradient to induce fusion was used as positive control, only experiments where induced fusion was observed were considered in the results. All vesicles used, regardless of protein content, should be able to undergo induced fusion with the planar bilayer.

Amperometric Detection of Release from a Planar Lipid Bilayer

In order to record amperometric event from a planar bilayer, our bilayer apparatus was modified slightly to accommodate the addition of a micromanipulator and carbon fiber electrode (CFE). A dual patch clamp amplifier (HEKA EPC9/2) was used to monitor both membrane conductance and amperometric current simultaneously. After formation of a bilayer, as described above, a large diameter (30 µm) CFE (World Precision Instruments) was positioned in close apposition to the *trans* side of the bilayer (see figure A1.2A). Proper electrode positioning was determined by monitoring the bilayer capacitance, which would increase slightly when in contact with the CFE. After positioning, the CFE was then held at a potential capable of oxidizing catecholamines (700 mV). After addition of an aliquot (20 µL) of purified chromaffin granules to the *cis* chamber of the bilayer setup, with stirring, the amperometric current was digitally recorded. Release events were observed as

spikes in the current trace (see Fig. A1.2B) resulting from the oxidation of released catecholamine as it contacted the carbon fiber.

Results:

Modified Chromaffin Granules Fuse Spontaneously with a Planar Bilayer Containing Syntaxin-1A

To confirm that chromaffin granules would perform in the same manner as neurohypophysial secretory granules (see chapter II) in our planar lipid bilayer system, mCGs were prepared using nystatin and ergosterol as outlined above. These mCGs were assayed for spontaneous fusion for a period of 10 minutes. This was followed by the addition of an osmotic gradient (KCl: 410 mM *cis*: 150 *trans*) across the bilayer, providing a driving force to induce vesicle fusion. This last step confirms that sufficient numbers of mCGs were delivered to the bilayer.

The results of these fusion experiments show that mCGs behave in the same manner as modified neurohypophysial secretory granules. mCGs, which presumably contain native v-SNAREs, are not able to spontaneously fuse to a protein-free bilayer (Figure A1.1A). However, Spontaneous fusion was observed between mNSG and a bilayer containing only syntaxin 1A-1 (Figure A1.1B).
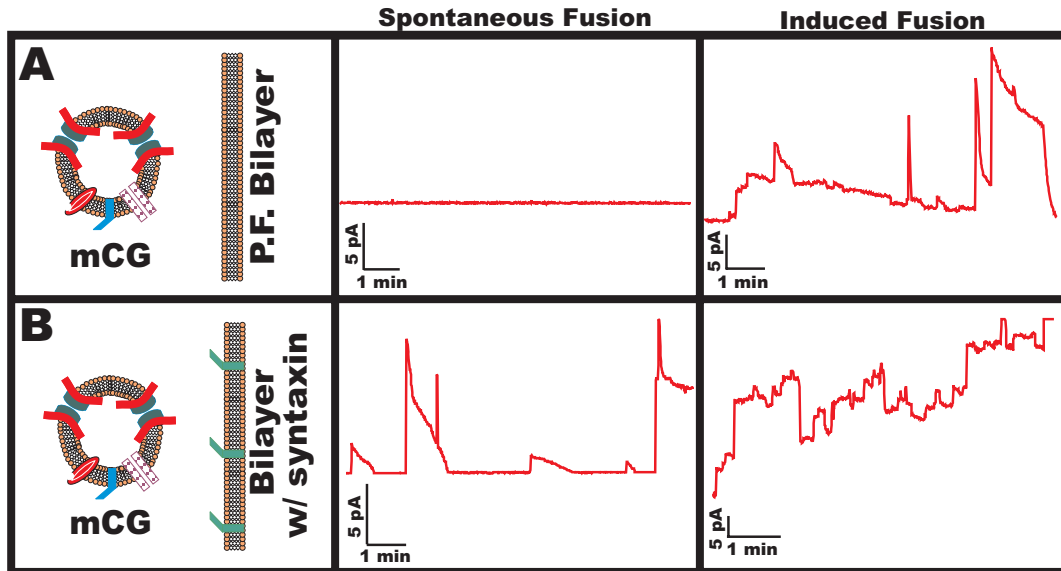
Figure AI.1: **Fusion of modified CGs with a planar lipid bilayer.** (**A**) Native CG membrane proteins alone are unable to induce spontaneous fusion of granules. In this experiment, modified CGs were exposed to a protein-free bilayer for a period of ten minutes, and the current across the membrane was recorded at a holding potential of +60 mV. As a positive control, an osmotic gradient was formed across the bilayer, and fusion activity monitored (right side). (**B**) Vesicular fusion occurs spontaneously between modified CGs and a bilayer containing the t-SNARE syntaxin 1A-1 (left side). The experiment was performed in the same manner described above.

Amperometric Detection of Spontaneous Fusion of Native Chromaffin Granules

with a Planar Bilayer Containing Syntaxin-1A

After formation of a planar lipid bilayer, the tip of a CFE was placed in close apposition to the *trans* side, and placed at a potential of 700 mV (figure AI.2A). After adding native CGs to the opposite side of the bilayer, the current across the CFE was monitored. Amperometric events were observed as upward deflection in the current trace (figure AI.2B).
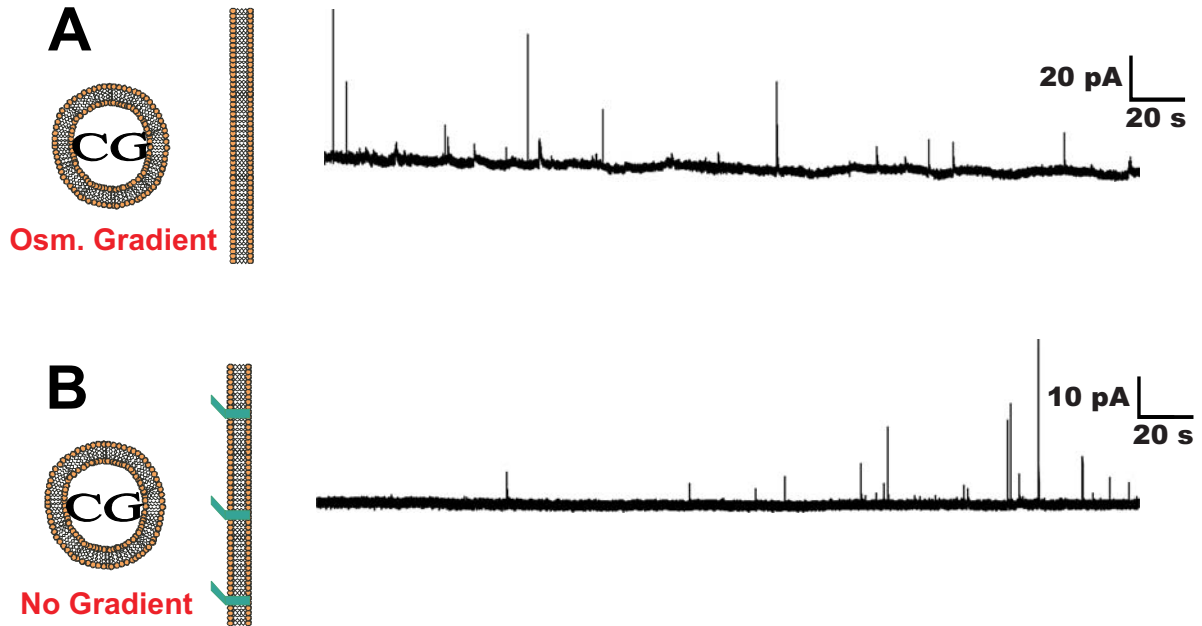
Then fusion of native chromaffin granules was assayed for 10 minutes. Amperometric detection of native CG fusion with a protein-free bilayer was observed (AI.3A). However, this fusion required the presence of an osmotic gradient across the bilayer. Addition of the t-SNARE syntaxin 1A to the bilayer enables native chromaffin granules to fuse spontaneously, in the absence of any outside driving force (AI.3B). This confirms our previous findings using the nystatin/ergosterol technique, but now using unmodified CGs.

Analysis of Amperometric Events Recorded from a Planar Bilayer

In order to compare the amperometric events we have observed in the planar bilayer to events occurring *in situ*, we recorded a number of amperometric events from isolated mouse chromaffin cells (provided by R. Zhuge, UMass). Both sets of amperometric events were analyzed in the same fashion and the means of the pooled values are displayed in table AI.1. Most strikingly, events recorded from the planar bilayer are much larger than chromaffin cell events, as
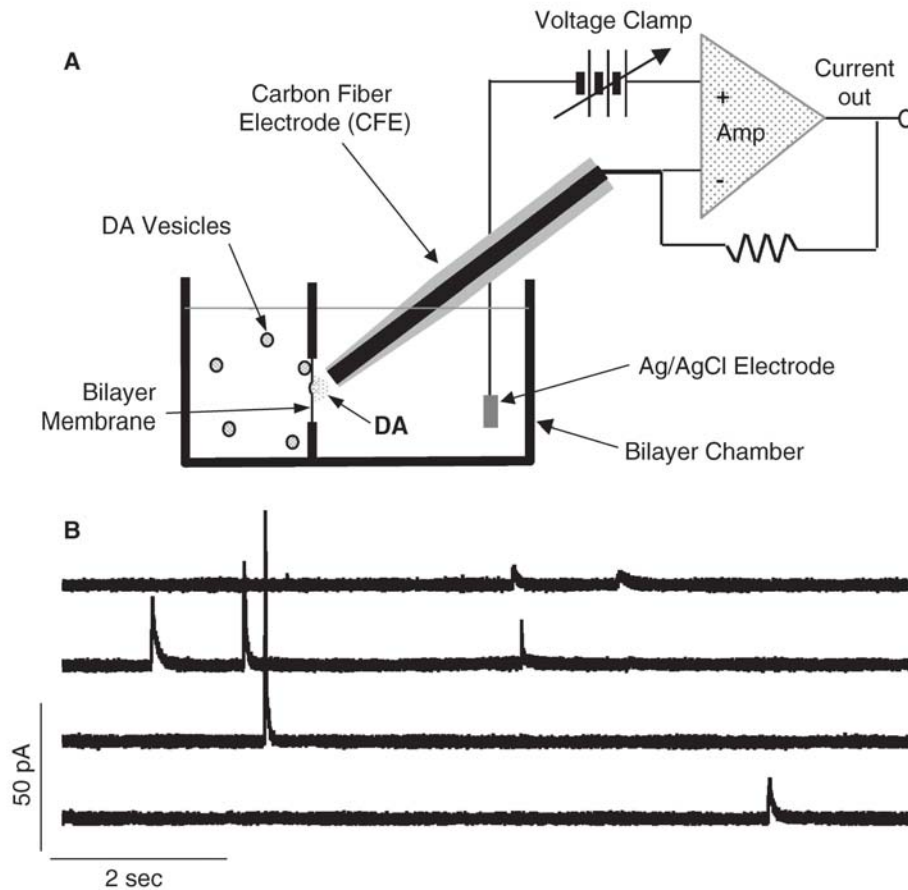
Figure AI.2: **Configuration of a planar bilayer system for detection of vesicular fusion and release via amperometry.** (**A**) A carbon fiber electrode (CFE) is placed in close apposition to the *trans* side of the planar bilayer. Vesicles containing an oxidizable transmitter are added on the opposite (*cis*) side. Release following vesicle fusion is detected by the CFE as a small current generated by the oxidiation of the released substance. (**B**) Amperometric current traces showing the detection of spontaneous catecholamine release from native chromaffin granules fusing to a planar lipid bilayer. CFE holding potential = 700 mV. (Figure was originally published in Woodbury et al., 2007)

Figure AI.3: **Native chromaffin granules can fuse spontaneously with a bilayer containing syntaxin 1A.** (**A**) Native chromaffin granule fusion with a protein-free bilayer can be detected amperometrically. However, fusion necessitates the presence of an osmotic gradient across the bilayer. (**B**) Addition of the t-SNARE syntaxin 1A to the bilayer enables native chromaffin granules to fuse spontaneously, in the absence of any outside driving force.

Table AI.1 *Comparison of amperometric events recorded from chromaffin cells to events from a planar bilayer*
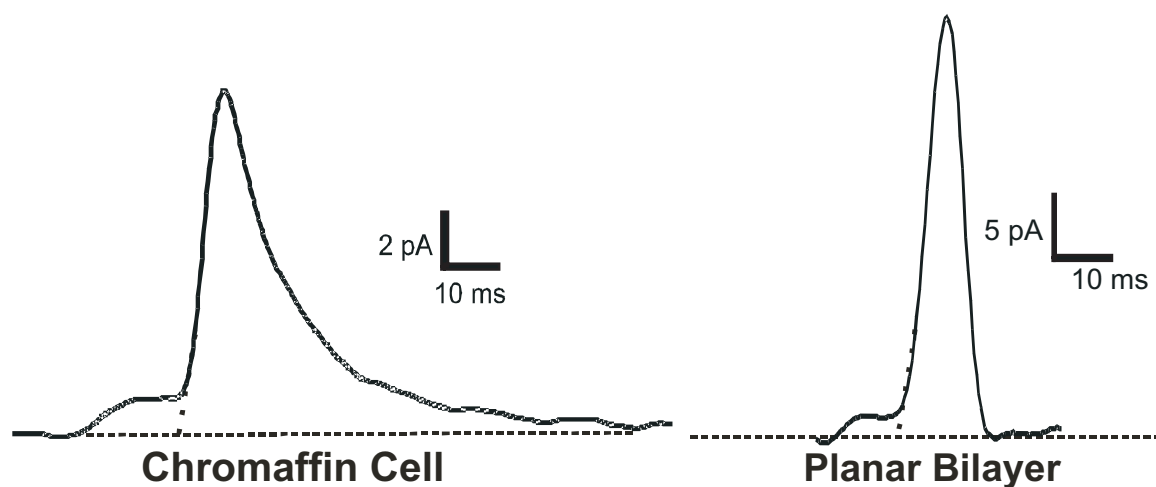
	Chromaffin Cells	Planar Bilayer
Q (pC)	0.138 ± 0.232	1.493 ± 1.68
$t_{1/2}$ (ms)	20.76 ± 14.22	20.93 ± 32.86
I_{max} (pA)	6.84 ± 0.146	41.78 ± 40.13
Rise Time (pA/ms)	1.27 ± 2.92	9.85 ± 9.99
Decay (ms): τ_1	29.97 ± 47.73	47.61 ± 74.40
τ_2	88.34 ± 116.73	-

Data is the mean ± SD of pooled values of amperometric events recorded in NL. Chromaffin Cells, N = 4, n = 512. NHT, N = 32, n = 1375. Event decay fit with either a double or single exponential.

shown by both the average I_{max} and Q values. This large difference may be in part due to the differing methods used to record from the bilayer and the chromaffin cells. A much larger CFE was used on the bilayer (30 μm vs. 10 μm diameter), resulting in higher background noise. This increased noise may have occluded smaller events in the bilayer. Despite the large difference in size, the $t_{1/2}$ value is similar for both. It also appears from the rise time and decay data that the amperometric events recorded from the bilayer occur more slowly than those from the chromaffin cell. The decay of planar bilayer amperometric events were best fit with single exponentials, while those of chromaffin cells were best fit with double exponentials.

Interestingly, a number of amperometric events recorded from the planar bilayer exhibit prespike feet, like those observed in chromaffin cell events (Fig. AI.4). These feet are believed to represent the slow release of transmitter through a fusion pore prior to pore widening and complete collapse of the vesicle into the plasma membrane. The appearance of feet leads us to surmise that our simple system possesses all of the components required to form a fully functional fusion pore. As shown in table AI.2, feet observed in planar bilayer amperometric events are much larger than those seen in native chromaffin cell amperometric events. This is to be expected as the events themselves are much larger. However, the duration of these feet is quite similar.

Comparing amperometric events recorded from a protein-free bilayer from those recorded from a bilayer containing syntaxin 1A indicates that those

Figure AI.4: **Comparison of native amperometric events with events recorded from a planar lipid bilayer.** The Current trace on the left shows an individual amperometric spike recorded from a murine chromaffin cell. While on the right a current trace from an amperometric spike recorded from a planar lipid bilayer is shown. This amperometric spike is the result of the fusion of an isolated bovine chromaffin granule with the planar bilayer. Interestingly, a number of amperometric events recorded from the planar bilayer exhibit prespike feet, like those observed in native chromaffin cell amperometric events.

Table AI.2 *Comparison of prespike feet recorded from chromaffin cells to those from a planar bilayer*
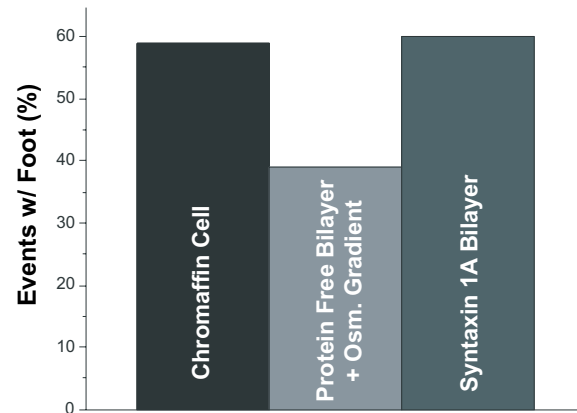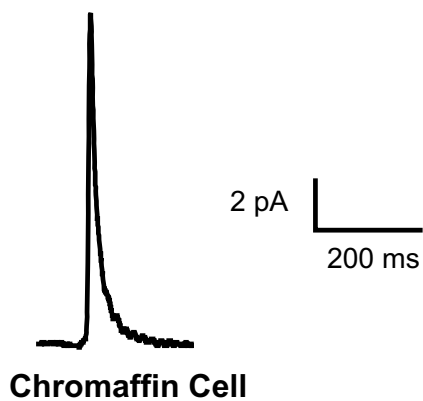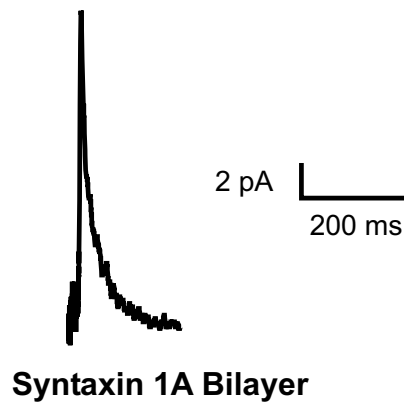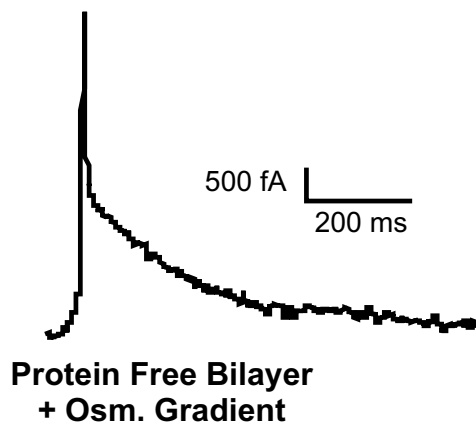
	Chromaffin Cells	Planar Bilayer
Q_{foot} (pC)	0.008 ± 0.027	0.130 ± 0.358
t_{foot} (ms)	11.47 ± 17.51	8.18 ± 44.28
I_{foot} (pA)	0.43 ± 0.98	2.63 ± 5.14

Data is the mean ± SD of pooled values from amperometric events recorded in NL. Chromaffin Cells, N = 4, n = 305. NHT, N = 32, n = 61.

recorded from a syntaxin containing bilayer are much more similar in nature to events recorded from a chromaffin cell. This is exhibited in figure AI.5 the averaged events from each situation plotted above, where the duration of the chromaffin cell and syntaxin bilayer averaged event is quite similar, while that from a protein-free bilayer occurs over a much longer period of time. This is also illustrated by the τ's, which are quite similar for the chromaffin cell and syntaxin bilayer events (30 ms, and 39 ms respectively), but quite a bit longer for those from the protein-free bilayer (152 ms). Interestingly, 59% of amperometric events recorded from chromaffin cells have detectable feet, while only 39% of events recorded from a protein-free bilayer display feet. Addition of syntaxin to the bilayer results in the percentage of events with feet rising to 60% which is quite comparable to that of chromaffin cells.

Discussion:

The incorporation of amperometric detection into our planar lipid bilayer model system provides a means to visualize exocytosis not available in other reconstituted systems, and does not necessitate the addition of exogenous components to the vesicular membrane. The planar lipid bilayer model system provides an experimental system in which individual membrane fusion events can be monitored in real-time and, allows kinetic analysis of these release events. Taken together, this data illustrates the usefulness of this model system

**Protein Free Bilayer
+ Osm. Gradient**

500 fA

200 ms

Syntaxin 1A Bilayer

2 pA

200 ms

Chromaffin Cell

2 pA

200 ms

Figure AI.5: Comparison of amperometric events recorded from a protein free bilayer to those recorded from a bilayer containing syntaxin 1A Amperometric events recorded from a syntaxin containing bilayer are more similar in nature to events recorded from a chromaffin cell. This is exhibited by the averaged events from each situation plotted above, where the duration of the chromaffin cell and syntaxin bilayer averaged event is quite similar, while that from a protein-free bilayer occurs over a much longer period of time. This is also illustrated by the τ's, which are quite close for the chromaffin cell and syntaxin bilayer events (30 ms, and 39 ms respectively), but quite a bit longer for those from the protein-free bilayer (152 ms). Also, as illustrated in the bar graph above, 59% of amperometric events recorded from chromaffin cells have detectable feet, while only 39% of events recorded from a protein-free bilayer display feet. Addition of syntaxin to the bilayer results in the percentage of events with feet rising to 60% which is quite comparable to that of chromaffin cells.

as a means with which to study components involved the process of membrane fusion.

This work addresses several major criticisms of the nystatin/ergosterol technique. First, the addition of the artificial components nystatin, and ergosterol, along with purified lipids to our modified secretory granules might influence their ability to fuse with the bilayer. Our ability to amperometrically detect spontaneous CG fusion with only a syntaxin-containing bilayer confirms earlier results obtained using the nystatin/ergosterol technique (Woodbury and Rognlien, 2000; McNally et al., 2004; Woodbury et al., 2007). Since amperometric detection of release provides the same result using completely native granules, the additional components used in the nystatin/ergosterol technique play no significant role.

Additionally it could be argued that using the nystatin/ergosterol technique, nystatin channel transfer may take place between granules and the artificial bilayer without full membrane fusion. Here we show that catecholamine release can be detected using amperometry which shows that the interaction of syntaxin with native membrane proteins does it fact result in full membrane fusion, providing further proof that there must be some form of protein-protein interaction occurring between components in the native chromaffin granule membrane, and the t-SNARE syntaxin 1A. This suggests that SNAP-25 may not be required as a t-SNARE, as previously believed (Weber et al., 1998), for formation of a functional fusion complex.
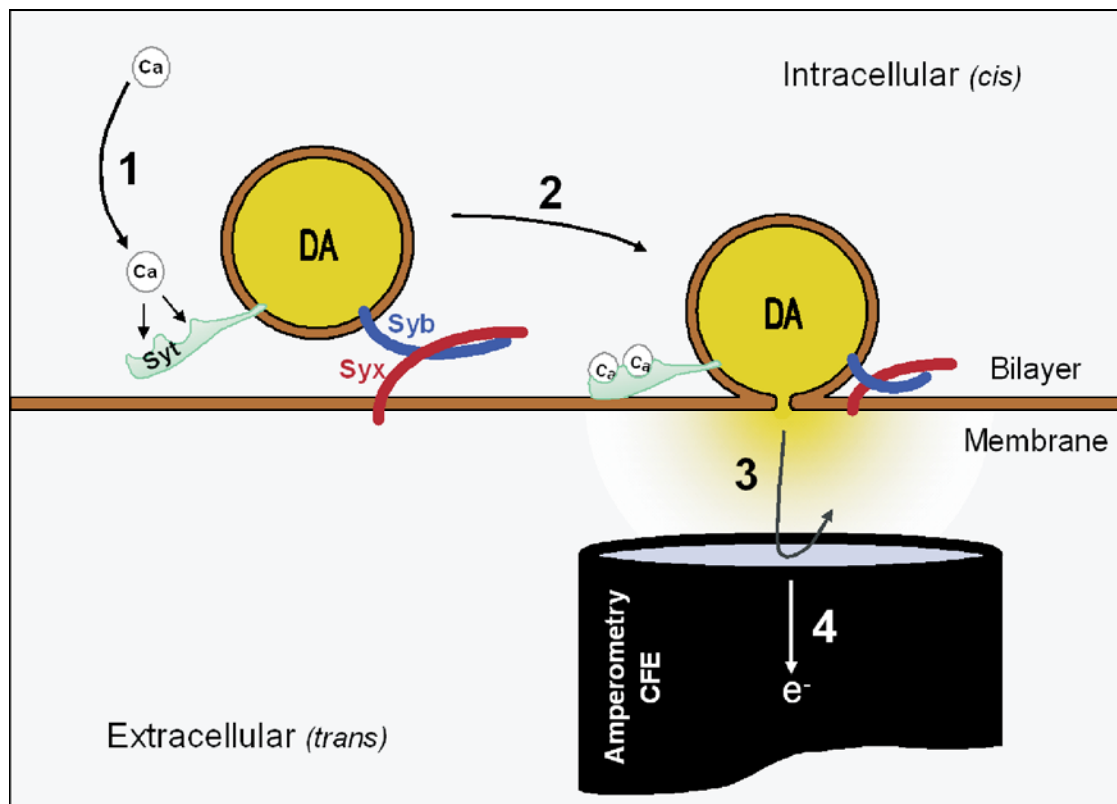
Figure AI.6: **The planar lipid bilayer model synapse.** The addition of amperometry to planar lipid bilayer system completes our reconstituted synapse model. This system provides a means with which to observe individual membrane fusion events as well as transmitter release in real time. It is our ultimate goal to use this system to reconstitute each of the steps (**1-3**) of calcium dependent membrane fusion. A model synapse as such would prove to be invaluable for studying the role that other regulatory proteins play in this process.

Another important finding is the appearance of "feet" in some of the amperometrically recorded release events (see figure A1.2). In previous amperometric work feet are indicative of the formation of a fusion pore prior to total membrane fusion between a vesicle and a cell membrane. The appearance of feet in amperometric spikes seen in our extremely simple model system implies that all of the components required to form a fusion pore are present. In addition, while it is known that atypical SNARE motif combinations, like those forming in our model system, form *in vitro*, it has not been determined if these less stable SNARE combinations are physiologically relevant (Fasshauer et al., 1999). These amperometric results would argue that, at least in this case, an atypical SNARE combination is in fact relevant, given the fact that pore formation and vesicular content release can be seen.

The planar lipid bilayer system offers a simple and unique means with which to study the process of exocytosis and model the synapse. Work in this system and others have convincingly shown that SNAREs function as the minimal core components of the fusion process. However, SNAREs alone do not appear to be able to confer any specificity or temporal regulation to vesicular fusion and release, suggesting that this system is a model of constitutive exocytosis, which is also thought to be driven by SNARE proteins.

This model system allows us to address several important questions facing those who study the synapse. Studies with earlier reconstituted systems, such as the liposome fusion assay, have not been able to demonstrate fast

kinetics of SNARE mediated fusion. The artificial bilayer system allows for the monitoring of individual vesicle fusion events in real time. Also, the successful addition of amperometry to our systems allows us to monitor the kinetics of reconstituted release with a temporal capability far greater than that allowed using the liposome fusion assay. Additionally, amperometric detection of release should allow for determination of the physiological relevance of non-cognate SNARE complexes seen in earlier *in vitro* work. Overall, this system provides a powerful tool that can be used to study the numerous proteins and environmental parameters thought to play a role in exocytosis.

References:

Chow RH, von Ruden L, Neher E (1992) Delay in vesicle fusion revealed by electrochemical monitoring of single secretory events in adrenal chromaffin cells. Nature 356:60-63.

Fasshauer D, Antonin W, Margittai M, Pabst S, Jahn R (1999) Mixed and non-cognate SNARE complexes. Characterization of assembly and biophysical properties. J Biol Chem 274:15440-15446.

Jahn R, Lang T, Sudhof TC (2003) Membrane fusion. Cell 112:519-533.

Kreye S, Malsam J, Sollner TH (2008) In Vitro Assays to Measure SNARE-Mediated Vesicle Fusion. Methods Mol Biol 440:37-50.

Liu T, Tucker WC, Bhalla A, Chapman ER, Weisshaar JC (2005) SNARE-driven, 25-millisecond vesicle fusion in vitro. Biophys J 89:2458-2472.

McNally JM, Woodbury DJ, Lemos JR (2004) Syntaxin 1A Drives Fusion of Large Dense-Core Neurosecretory Granules Into a Planar Lipid Bilayer. Cell Biochem Biophys 41:11-24.

Mueller P, Rudin DO, Tien HT, Wescott WC (1962) Reconstitution of cell membrane structure in vitro and its transformation into an excitable system. Nature 194:979-980.

Weber T, Zemelman BV, McNew JA, Westermann B, Gmachl M, Parlati F, Sollner TH, Rothman JE (1998) SNAREpins: minimal machinery for membrane fusion. Cell 92:759-772.

Woodbury DJ (1999a) Building a bilayer model of the neuromuscular synapse. Cell Biochem Biophys 30:303-329.

Woodbury DJ (1999b) Nystatin/ergosterol method for reconstituting ion channels into planar lipid bilayers. Methods Enzymol 294:319-339.

Woodbury DJ, Hall JE (1988) Vesicle-membrane fusion. Observation of simultaneous membrane incorporation and content release. Biophys J 54:345-349.

Woodbury DJ, Miller C (1990) Nystatin-induced liposome fusion. A versatile approach to ion channel reconstitution into planar bilayers. Biophys J 58:833-839.

Woodbury DJ, Rognlien K (2000) The t-SNARE syntaxin is sufficient for spontaneous fusion of synaptic vesicles to planar membranes. Cell Biol Int 24:809-818.

Woodbury DJ, McNally JM, Lemos JR (2007) SNARE-Induced fusion of Liposomes to a Planar Bilayer. . In: Planar Lipid Bilayers (Leitmannova Liu A, ed): Elsevier Press.

Yin Y, Dayanithi G, Lemos JR (2002) Ca(2+)-regulated, neurosecretory granule channel involved in release from neurohypophysial terminals. J Physiol 539:409-418.

APPENDIX II: Indirect Modulation of Secretion from Neurohypophysial Terminals by Ryanodine Sensitive Intracellular Calcium Stores

PREFACE

Material presented in this chapter has been published in the following locations:

Abstracts:

J.M. McNally, V. DeCrescenzo., J.V. Walsh, & J.R. Lemos. (2006) Are calcium syntillas directly involved in eliciting neuropeptide release from nerve terminals of the neurohypophysis? *Soc. Neurosci. Abs.* 32: 337.11.

J.M. McNally, V. DeCrescenzo., J.V. Walsh, & J.R. Lemos. (2007) Single calcium syntillas do not trigger exocytosis from nerve terminals of the neurohypophysis. *WCNH Abs.* 51.

Abstract:

Recent work has suggested that presynaptic ryanodine-sensitive calcium stores may play a role in shaping spontaneous neurotransmitter release. In both neurohypophysial nerve terminals and chromaffin cells, simultaneous recording of both ryanodine-sensitive calcium transients and secretion has provided no evidence for a direct link between these two events. While these results indicate that syntillas do <u>not</u> play a direct role in eliciting release from neurohypophysial terminals, they do not rule out an indirect modulatory role in release. In the dendrites of magnocellular neurons, intracellular calcium release has been shown to function in the priming of large dense core granules for neuropeptide release. However, such a mechanism has yet to be described in the nerve terminals. Here we used immunocytochemical labeling for the neuropeptides oxytocin- and vassopressin-filled large dense core granules, to follow changes in their disribution in isolated neurohypophysial terminals, with activation or inhibition of ryanodine-sensitive calcium release. These experiments revealed that application of ryanodine receptor agonists leads to a shift of oxytocin, but not vassopressin label to the plasma membrane, signifying that such a priming mechanism may exist in these terminals. This is further confirmed using membrane capacitance recording, which shows that depolarization-induced release is potentiated in the presence of agonist levels of ryanodine. This potentiation appears favor a later phase of release wich suggests an increase in size of the readily-releasable pool.

Introduction:

Magnocellular neurons of the hypothalamic neurohypophysial system are responsible for the secretion of the neuropeptides oxytocin (OT) and vasopressin (AVP), and represent a classical system used to study Ca^{2+}-dependent release (Douglas and Poisner, 1964). High speed Ca^{2+} imaging has shown that spontaneous Ca^{2+} transients, termed Ca^{2+} syntillas, are localized specifically to the presynaptic neurohypophysial terminals (NHT) of these neurons (De Crescenzo et al., 2004). These syntillas were shown to arise from a ryanodine-sensitive intracellular store, as they appear in the absence of extracellular Ca^{2+} and their frequency is affected by pharmacological agents specific for the ryanodine receptor (RyR). However, the physiological role of these Ca^{2+} release events has yet to be defined.

Recently, the contribution of intracellular calcium stores to the function of presynaptic terminals has become a topic of great interest (Bouchard et al., 2003; Collin et al., 2005; Berridge, 2006). In a number of neuronal preparations presynaptic intracellular Ca^{2+} stores have been found to play a role in transmitter release (Bouchard et al., 2003). Specifically, in both cultured hippocampal neurons, and cerebellar interneurons, Ca^{2+} transients similar to syntillas have been suggested to modulate spontaneous transmitter release.

Jin et al. (2007) have shown that Ca^{2+} release from ryanodine sensitive stores can alter OT secretion from NHT via a mechanism that necessitates the physiological RyR agonist cyclic-ADP ribose. Such a role of ryanodine sensitive

stores in NHT is also demonstrated in Chapter III of this thesis and in earlier studies (Velazquez-Marrero et al., 2002), where application of RyR agonists and antagonists were shown to alter the level of basal neuropeptide release from a population of NHT. Further, in chapter IV of this thesis, amperometric detection was used to confirm this finding at the single terminal level.

Despite this, simultaneous Ca^{2+} imaging and amperometric detection has found that syntillas do not appear to directly elicit release in NHT. This finding was also observed in chromaffin cells, in which syntilla-like events have been observed (ZhuGe et al., 2006). These findings, however, do not rule out the ability of syntillas to act on earlier stages of neuropeptide secretion.

Recent work by Ludwig et al. (2002) has shown that mobilization of intracellular Ca^{2+} can induce OT release from dendrites of magnocellular neurons without increasing the electrical activity of the cell body, and without inducing secretion from the nerve terminals. Additionally, release of this intracellular Ca^{2+} can also prime the releasable pool of LDCG in these dendrites. This priming action makes dendritic OT available for release in response to subsequent spike activity (figure AII.1)

While this LDCG priming mechanism has been characterized in the dendrites of magnocellular neurons, it has yet to be examined if such a mechanism exists in the terminals of these neurons. Here we present preliminary evidence testing this hypothesis. First, immunocytochemistry is employed to determine if the distribution of neuropeptide in isolated NHT is
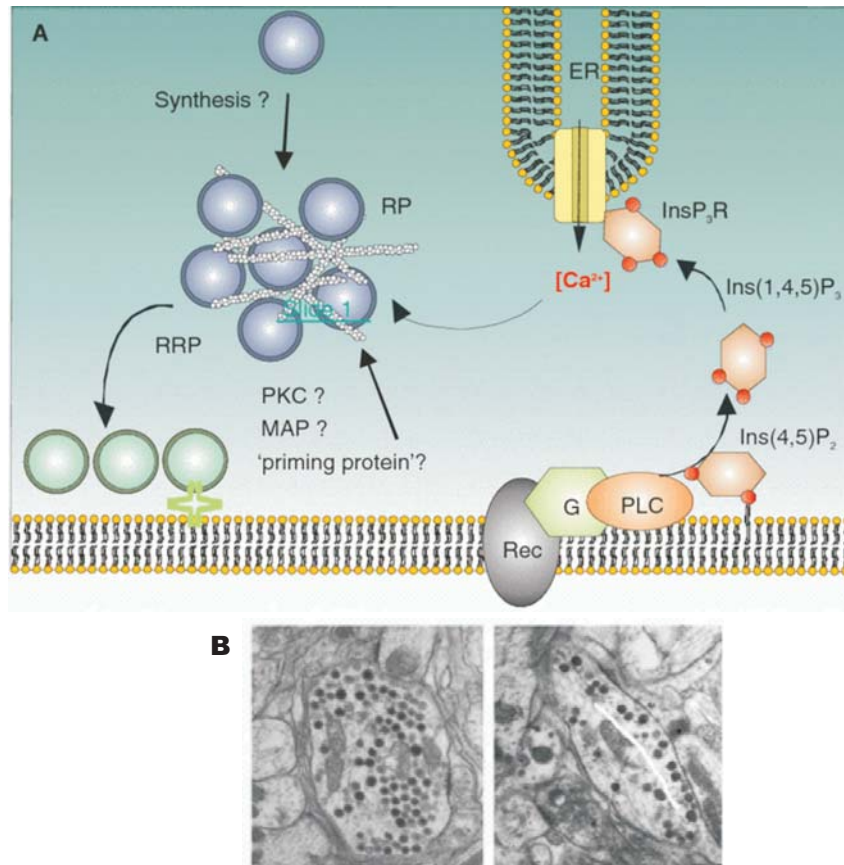
Figure AII.1: **Proposed mechanism for LDCG priming in the dendrites of magnocellular oxytocin neurons.** (**A**) Oxytocin binds to its receptor, leading to the activation of a phospholipase C (PLC) pathway. This results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-triphosphate (IP$_3$). IP$_3$ triggers the release of calcium from intracellular stores, which leads to the activation protein kinase C (PKC) and other factors. This results in "priming" by stimulating the movement of LDCG from the reserve pool (RP) to the readily releasable pool (RRP). This priming mechanism may include actions on protein processing, vesicle tethering, and/or vesicle transport. (**B**) Electron micrographs of dendrites of magnocellular neuons show the distribution of LDCG both before (left) and after (right) activation of this priming mechanism. LDCGs are seen to move closer to the plasma membrane after priming.
(Figure originally published in Leng et al., 2007)

affected by agonists and antagonists of the RyR. Then, membrane capacitance recording of individual NHT is used to determine the effects of agonist concentrations of ryanodine on elicited release. Additionally, this technique is used to assess the effects of ryanodine on the size of the readily-releasable pool.

Materials and Methods:

Isolation of Murine NHT

NHT from Swiss Webster mice were prepared as previously described (Cazalis et al., 1987; Wang et al., 1999; De Crescenzo et al., 2004). After decapitation, the neurohypophysis was harvested and dissociated via homogenization performed at 37° in an isoosmotic solution of 270 mM sucrose, 1 mM EGTA, 10 mM HEPES (pH 7). The dissociated terminals were then plated, and perfused with Normal Locke's (NL) saline (in mM): 140 NaCl, 5 KCl, 10 glucose, 1 $MgCl_2$, 2.2 $CaCl_2$, 10 HEPES, (pH 7.3). For experiments using Ca^{2+} free NL, 2.2 $CaCl_2$ was replaced by 200 μM EGTA. NHT were identified by their smooth appearance, lack of nuclei, and high content of dark vesicles.

Immunocytochemistry

After dissociation, NHT were plated in a few drops of low Ca^{2+} NL (NL with only 3 μM free Ca^{2+}) in a glass bottom culture dish (MatTek) and allowed to stand for 5-10 minutes to allow terminal adhesion. The dish was then washed with low Ca^{2+} NL to clean out debris, and then washed with NL. At this point the dishes were incubated for 15-30 minutes in either NL alone, or in the presence of

ryanodine (5 µM) or 8-Br-cADPR (400 nM). Upon completion of this incubation,

terminals were immediately fixed in 4% paraformaldehyde for 45 minutes, and

subsequently washed with phosphate-buffered saline (PBS). The terminals were

then labeled with primary antibodies specific for OT (Mouse anti-neurophysin I

(P-38) received from Hal Gainer, NIH) and AVP (goat anti-neurophysin II (V-15)

Santa Cruz Biotechnology). These primary antibodies were added at 1/300 and

1/100 dilutions, respectively, in PBS with 10% Donkey serum, and 0.1% Trition-X

100. Terminals were labeled overnight at 4°C. The following day, dishes were

washed extensively with PBS and then labeled with secondary antibodies

conjugated to fluorescent markers. AlexaFluor 350 labeled donkey anti-goat IgG

(Molecular Probes) for AVP labeling, and AlexaFluor 488 labeled donkey anti-

rabbit IgG (Molecular Probes) for OT labeling were used at a 1/200 dilution in

PBS with 10% Donkey serum, and 0.1% Trition-X 100. After labeling for 3-5

hours, dishes were washed with PBS then terminals were mounted in Prolong

Anti-fade medium and allowed to dry overnight.

After labeling and mounting was complete dishes were viewed using

standard wide field microscopy (63x objective). A z-stack of images was

generated for each individual terminal by taking images at .275 µm increments

along the z-axis. Standard DIC images were taken, along with images at each

emission wavelength for the fluorescent labels used, yielding three separate z-

stacks for each terminal. These images stacks were then deconvolved using a

high stringency deconvolution fast iterative algorithm (Axiovision). Analysis of

these images involved calculating the total amount of AVP and OT labeling in the membrane-defined region (within 450 nm of the plasma membrane). Using deconvolved images of both OT and AVP labeled terminals taken under like conditions, this value was calculated (see figure AI.2B) by first determining the total fluorescent signal (SI units) of the entire terminal. Then the inner fluorescence was found by determining the fluorescence of a circle whose diameter was 0.9 µm less than the total diameter of the terminal. Subtracting the inner fluorescence value for the total leaves the total fluorescence from the membrane-defined region. This value was calculated for the average of the 3 z-axis slices determined to be at the center of the terminal. Only terminals with a diameter greater than 3 µm were used in this analysis.

Membrane Capacitance Recording

Dissociated murine NHT were patched in the perforated-patch configuration, using a pipette solution of (in mM): 135 Cs-Gluconate, 12 CsCl, 10 HEPES, 1 $MgCl_2$, 4 NaCl (pH 7.3 with NaOH). Terminals were patched with 5-10 MΩ glass microelectrodes coated with sigmacote (Sigma-Aldrich) to reduce pipette capacitance. Recording were conducted in a bath solution of Locke's saline described above. Membrane capacitance was monitored using a software lock-in amplifier integrated with a computer controlled patch-clamp amplifier (Pulse, EPC-10, HEKA) (Gillis, 2000). Changes in membrane capacitance, directly related to secretory activity, were followed using the "sine+dc" method

(Lindau and Neher, 1988; Chen and Gillis, 2000; Gillis, 2000). To measure changes in membrane capacitance, a 15 mV 1500 Hz sine wave was superimposed onto the clamped DC holding potential (-80 mV).

Results:

RyR Modulation Affects the Distribution of OT, but not AVP Labeling

Immunocytochemical labeling was used to determine if presynaptic ryanodine sensitive Ca^{2+} stores have an effect on the distribution of LDCGs in NHTs. NHT isolated from four mice were plated into two dishes containing Ca^{2+} free NL, and incubated at 37° for 30 minutes in either in the presence or absence of an agonist concentration (5 μM) of ryanodine. After incubation NHT were immediately fixed and labeled with primary antibodies raised specific for both OT and AVP.   This labeling was visualized with secondary antibodies conjugated to fluorescent markers (see Methods).

To determine if ryanodine treatment resulted in an increase in the amount of LDCGs near the plasma membrane of NHT; we determined the total amount of neuropeptide labeling in the "membrane-defined region" (see Methods). For this study, we classified the membrane-defined region as being within 450 nm from the plasma membrane. Using this method of analysis we have shown that under control conditions, 17% ± 3 of neuropeptide labeling is present in the membrane-defined region of OT terminals (n = 9), and 13% ± 2 for AVP terminals (n = 8). For NHT treated with ryanodine, 28% ± 3 of labeling in OT terminals (n =

17) is in the membrane-defined region, a significant ($p < 0.05$) increase compared to control. AVP terminals (n = 14), however, show no change, with 15% ± 2 of AVP labeling in the membrane-defined region (figure AII.2).

A similar experiment was also performed using the RyR antagonist 8-bromo-cyclic adenosine diphosphate ribose (8-br-cADPR). This experiment was conducted in the same manner described above, except terminals were incubated with and without the drug for a period of 15 minutes. Under control conditions OT terminals (n = 13) were found to have 20% ± 2 of neuropeptide labeling in the membrane-defined region, and 10% ± 2 for AVP terminals (n = 13). For 8-br-cADPR treated NHT, 13% ± 2 of labeling in OT terminals (n = 8) is found in the membrane-defined region, a significant ($p < 0.05$) decrease. Whereas AVP terminals (n = 6), again, show no change, with 12% ± 2 of AVP labeling in the membrane-defined region (figure AII.3).

Ryanodine-Sensitive Calcium Stores Modulate Elicited Exocytosis

To assess the role of ryanodine sensitive calcium stores on elicited release, membrane capacitance recording was performed on a number of individual NHT under both control conditions (NL), and in the presence of agonist concentrations of ryanodine (NL + 1 µM ryanodine). Patched terminals were first given a series of five stimulus pulses at 2 minute intervals. After this, ryanodine was perfused into the dish and the same pattern of stimulus was repeated. For each of these pulses the change in membrane capacitance was recorded.
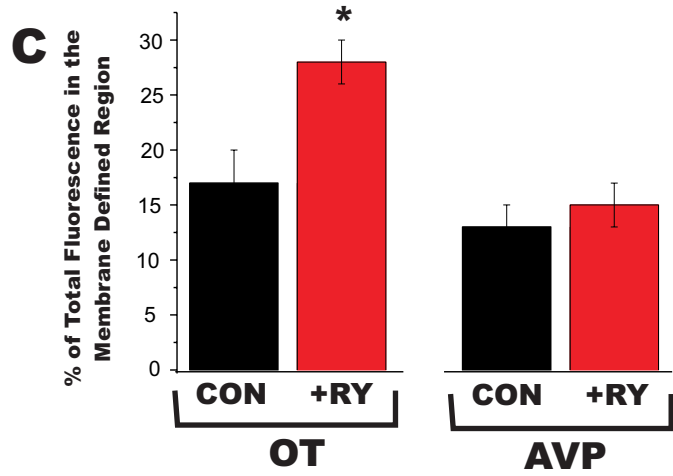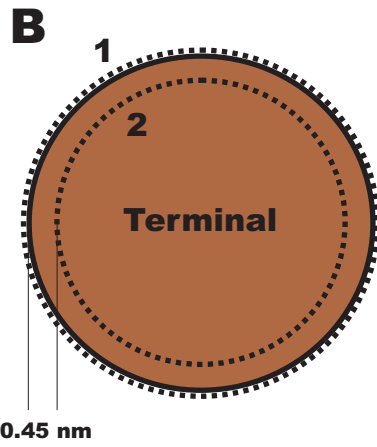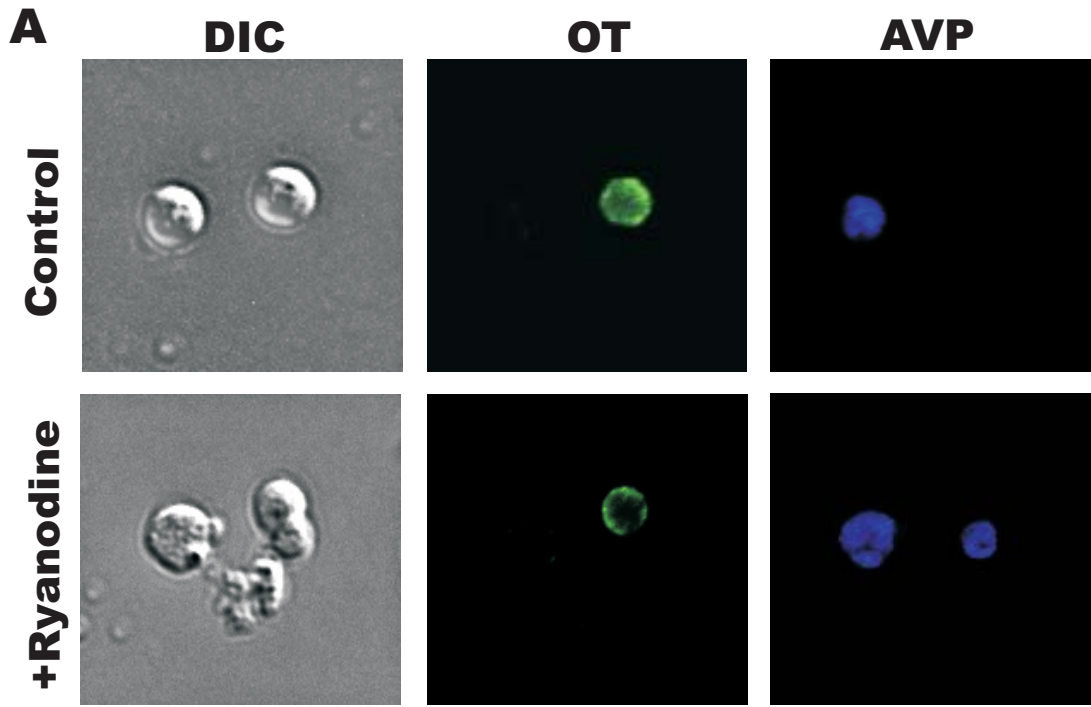
Figure AII.2: Ryanodine causes a shift of neuropeptide labeling towards the plasma membrane in OT terminals, but not AVP terminals. (**A**) Images show deconvolved fluorescence images of mouse NHTs labeled with primary antibodies against both OT and AVP. Control NHT were collected and allowed to incubate for 30 minutes in Ca^{2+}-free NL at 37°. Ryanodine treated NHT were incubated in Ca^{2+}-free NL with 5 µM ryanodine. After incubation both sets of NHT were fixed and labeled. (**B**) The total fluorescence in the membrane-defined region was determined by calculating the total fluorescence signal for each terminal (1), then determining the total fluorescence for a circle 0.9 nm smaller than the diameter of the terminal (2). Subtracting the inner fluorescence from the total (1-2) leaves the total fluorescence in the membrane-defined region, or within 450 nm of the plasma membrane. (**C**) Bar graphs illustrate the affect of ryanodine on membrane associated neuropeptide labeling in both OT and AVP terminals. OT terminals treated with ryanodine show a significant increase in fluorescence in the membrane-defined region, while no change is observed in AVP terminals.

A

B
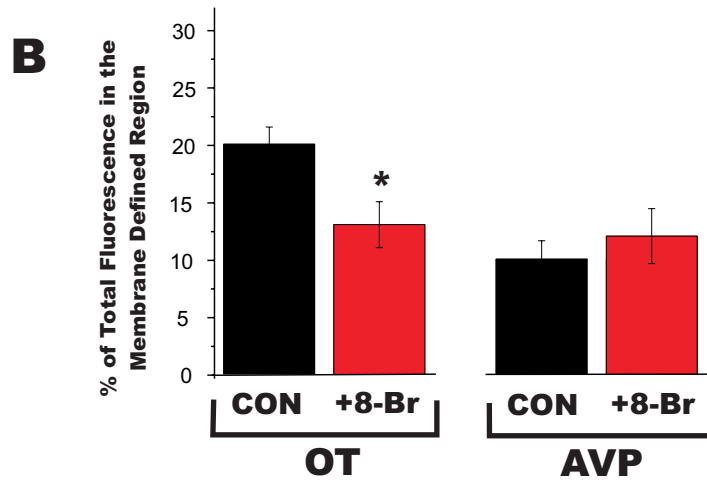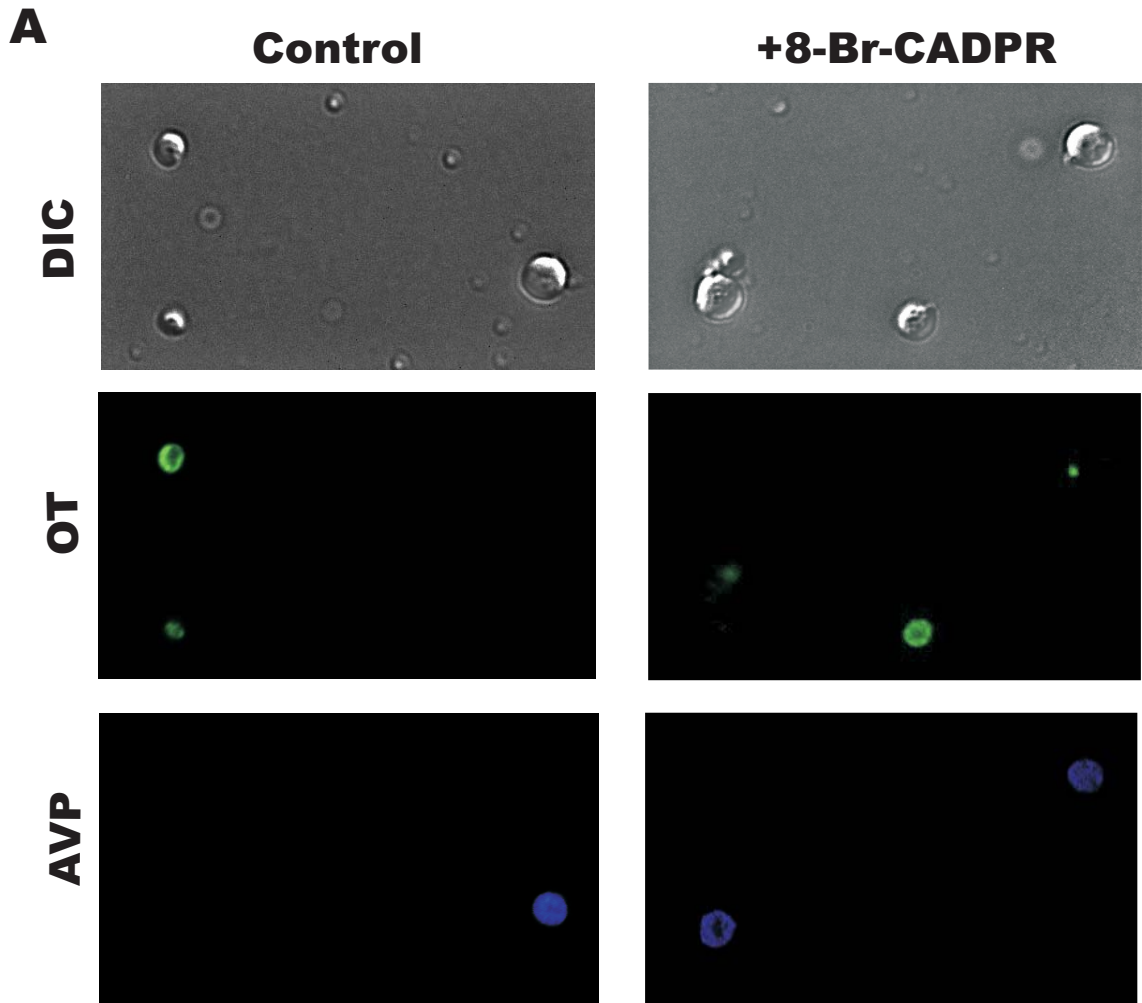
Figure AII.3: 8-Br-cADPR decreases the amount of neuropeptide labeling near the plasma membrane in OT terminals, but not AVP terminals. (**A**) Images show deconvolved fluorescence images of mouse NHTs labeled with primary antibodies against both OT and AVP. Control NHT were collected and allowed to incubate for 15 minutes in Ca^{2+}-free NL at 37º. 8-Br-cADPR-treated NHT were incubated in Ca^{2+}-free NL with 400 nM 8-Br-cADPR. After incubation both sets NHT were fixed and labeled. (**B**) Bar graphs illustrate the affect of 8-Br-cADPR on membrane associated neuropeptide labeling in both OT and AVP terminals. OT terminals treated with 8-Bromo-cADPR show a significant decrease in fluorescence in the membrane-defined region, while no change is observed in AVP terminals.

Capacitance traces obtain in each condition were averaged, and then compared to determine what affect the drug had on exocytotic activity. Initially a simple 500 ms step depolarizing pulse (-80 mV to 0 mV) was employed (Figure AII.4). In all terminals tested using this stimulus pulse, application of ryanodine led to a significant ($p < 0.05$) potentiation (41% ± 8.5) of the evoked change in capacitance (n = 7).

Application of a long duration (5-10 s) repetitive stimulus pattern was used to assess the size of the different vesicular release pools present in NHT, and to see if the size of these pools could change upon application of pharmacological agents affecting the ryanodine receptor. For this stimulus pattern, 80 ms steps from -80 mV to 0 mV were applied at a rate of 4 Hz. As above, in all terminals tested ryanodine application resulted in a potentiation (85.1% ± 35.1) of the change in membrane capacitance evoked by this pulse (n = 5). Three of five terminals tested yielded a biphasic capacitance response (Figure AII.5), similar to what has been previously observed in NHT of the rat (Seward et al., 1995; Giovannucci and Stuenkel, 1997). Interestingly, this biphasic capacitance response consisted of an "early phase" which lasted ~2 seconds, after which a second "late phase" of release would develop. In these terminals (n = 3), the potentiation of the evoked capacitance change by ryanodine heavily favored this later phase (130.0% ± 47.5) of release over the early phase (41.2 ± 23.4).
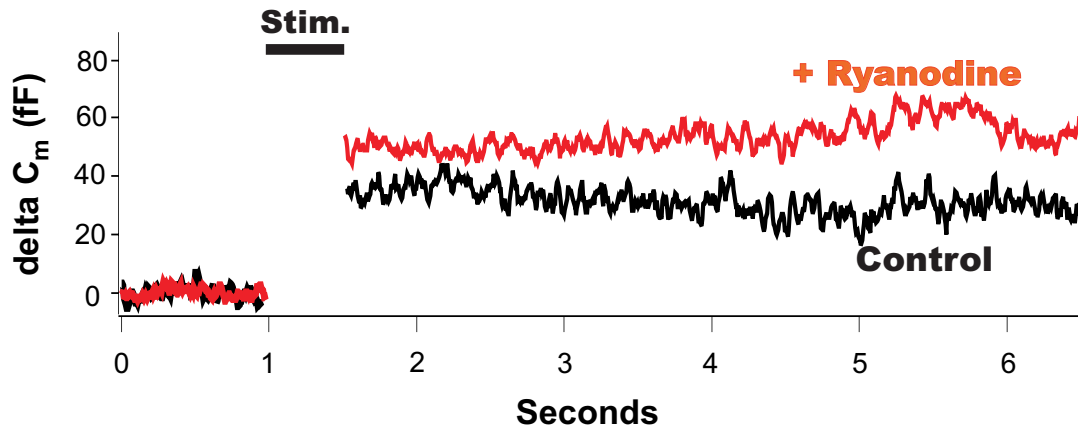
Figure AII.4: Ryanodine potentiates the amount of release elicited by a simple step depolarization. The averaged traces shown above are of membrane capacitance recordings performed on an isolated NHT. The NHT was stimulated with the same 500 ms step depolarization (-80 mV to 0 mV) both before and after bath perfusion with 1 µM ryanodine. The addition of ryanodine to the bath led to a significant potentiation of the change in membrane capacitance evoked by the stimulus.
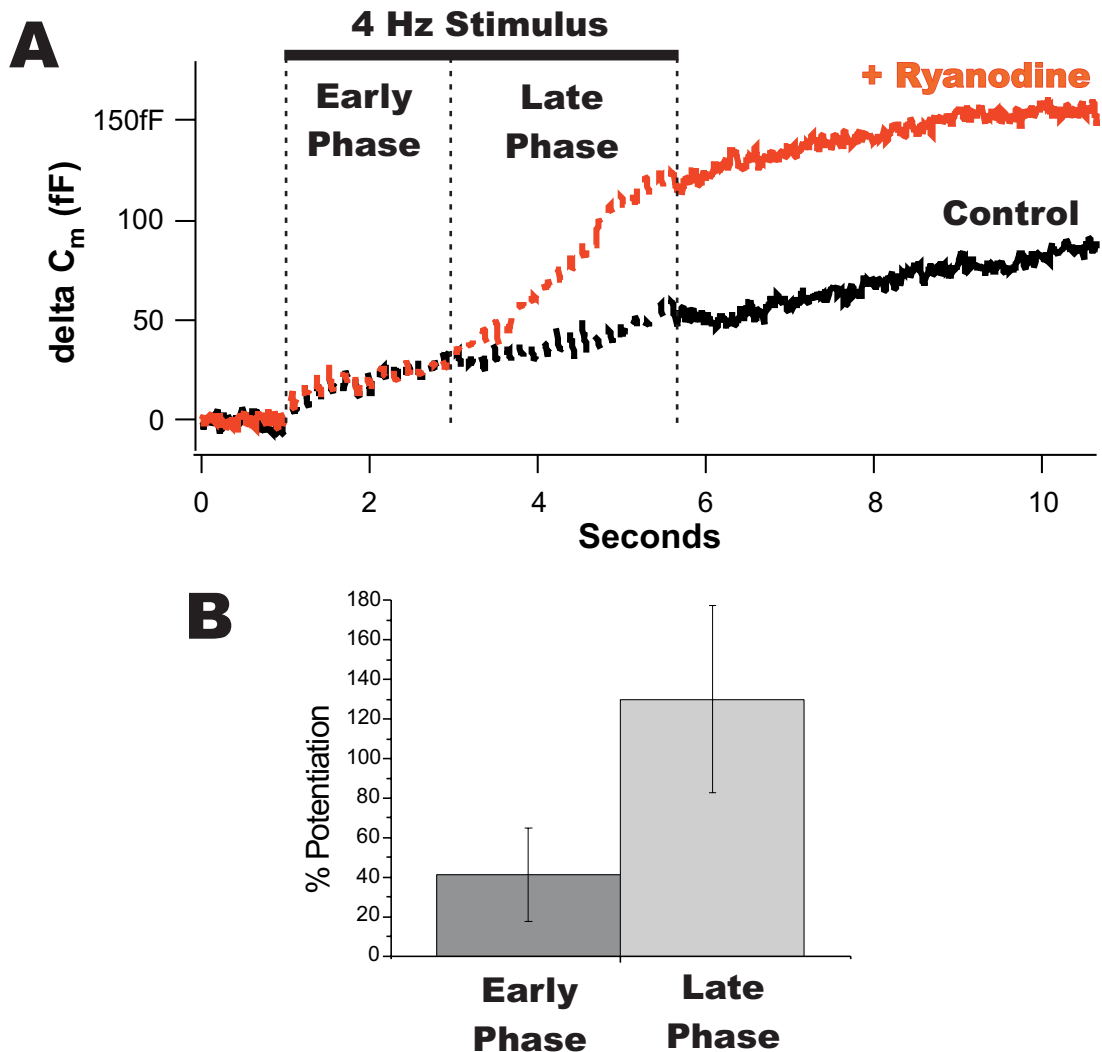
Figure AII.5: **Capacitance recording combined with the application of a long duration stimulus.** A repetitive long duration stimulus (80 ms steps from -80 mV to 0 mV, applied at 4 Hz) was used to asses the size of vesicular pools in perforated patched NHTs. In all cases, application of agonist concentrations of ryanodine (1 μM) yielded a potentiation of the capacitance response to the same repetitive stimuli. (**A**) A number of the NHT tested showed a biphasic response to this stimulus. These two phases, an early phase and a late phase, may represent distinct vesicular release pools. (**B**) Interestingly, the potentiation elicited by ryanodine appeared to strongly favor the late asynchronous phase of release. Bar graph displays this affect across a number of individual terminals (n = 3).

Discussion:

Presynaptic intacellular Ca^{2+} stores have been suggested to modulated transmitter release in a number of neuronal systems. However, how this Ca^{2+} is capable functioning in this role is still unclear. To address this issue in NHT we used immunocytochemical techniques to show that ryanodine-sensitive Ca^{2+} stores are capable of shifting the distribution of OT, but not of AVP, toward the plasma membrane. Additionally, membrane capacitance analysis is used to examine the role of ryanodine-sensitive Ca^{2+} stores in eliciting exocytosis from NHT. The results of these experiments indicate that ryanodine sensitive Ca^{2+} release can facilitate elicited exocytosis, possibly by influencing the size of vesicular release pools.

In the dendrites of magnocellular neurons, the ability of intracellular Ca^{2+} release to prime OT LDCG was demonstrated using electron microscopy (Leng et al., 2008). This study showed that in electron micrographs, after activation of LDCG priming, a higher percentage of granules are found near the dendritic membrane (within 500 nm). The results of our immunocytochemical analysis of neuropeptide distribution in NHT correlate well with these findings. As in the dendrites, release of Ca^{2+} from intracellular stores appears capable of recruiting OT LDCG to the plasma membrane. Thus, a similar priming mechanism may also be in place in the NHT.

As discussed in chapter I of this thesis, NHT are believed to posses three distinct pools of LDCG, believed to be comprised of granules in different states of

release-readiness (Seward et al., 1995; Hsu and Jackson, 1996; Giovannucci and Stuenkel, 1997). These pools include the immediately releasable pool (IRP), comprised of LDCG located near sites of Ca^{2+} influx, the readily releasable pool (RRP), granules further from these sites but near the PM, and the reserve pool (RP) of granules with a more cytosolic localization (see figure AII.6). In the membrane capacitance experiments presented above we show that not only does the application of a RyR agonist have a facilitory affect on elicited exocytosis from NHT (Figure AII.4), but also that this effect may alter the size of the RRP.

Several terminals tested using long duration repetitive stimuli show two distinct phases of release (Figure AII.5). As suggested by previous work (Giovannucci and Stuenkel, 1997), it is possible that these phases represent two distinct vesicular release pools, with the early phase representing the immediately releasable pool, while the late phase represents the readily releasable pool. Our findings show that in these NHTs, ryanodine potentiation of release strongly favored the later phase of release, indicating that ryanodine sensitive Ca^{2+} release increases the recruitment of LDCG to the RRP.

The remaining terminals showed a monophasic capacitance response, where this later phase of release was either not present, or overlapped the initial phase of release. This finding is interesting because, as we showed earlier, ryanodine sensitive Ca^{2+} release only affects neuropeptide distribution of OT terminals only, and had no apparent affect on AVP terminals. Thus the
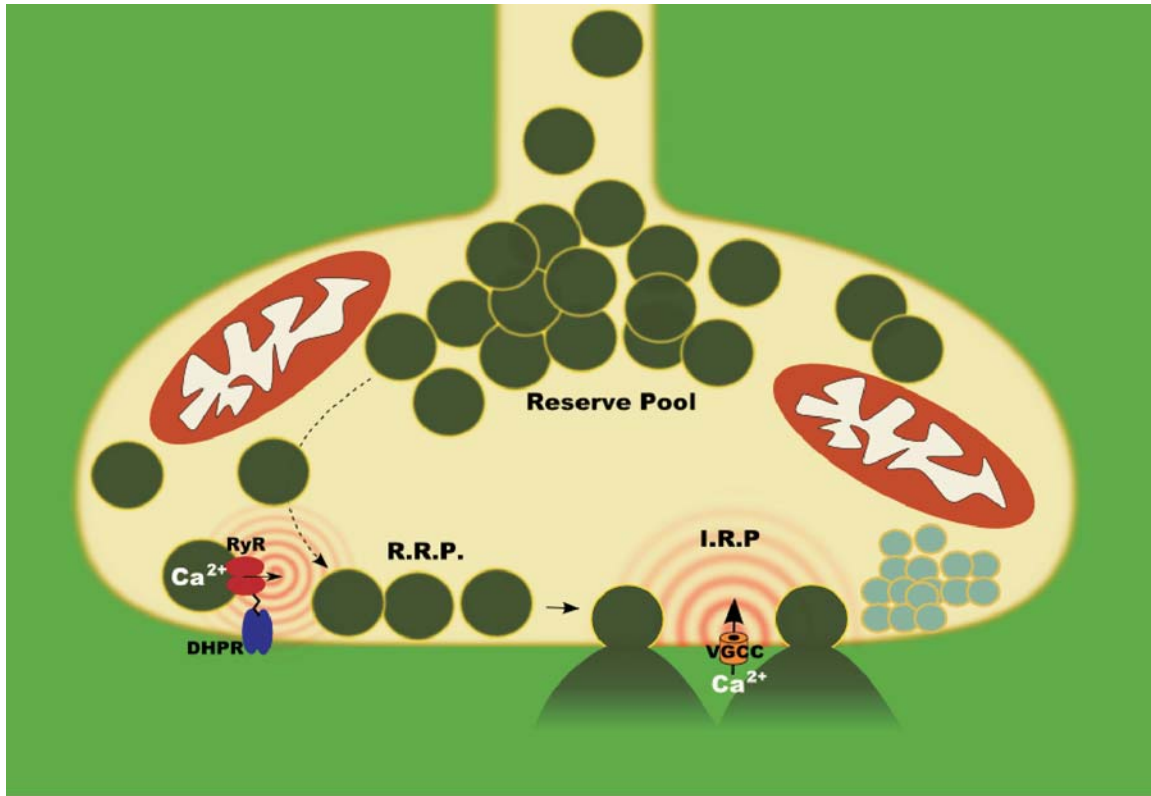
Figure AII.6: **Syntillas may be involved in recruiting LDCG to the readily releasable pool in NHT.**   Evidence presented here point to the ability of ryanodine-sensitive Ca^{2+} stores to functionally modulate the size of vesicular release pools in NHT.   The voltage dependence of syntillas, through their coupling to L-type Ca^{2+} channels (DHPR) would appear to make them ideal candidates to drive such a mechanism. Such a mechanism could serve as an activity-dependent means to recruit LDCG from reserve pools in the cytosol, to the readily-releasable pool adjacent to the plasma membrane.

difference in capacitance response pattern between these two groups may represent a difference between OT and AVP NHT. However, these terminals were not assayed for their peptide content, so it is not possible to determine if this was the case.

These preliminary studies provide evidence that, as in the dendrites of magnocellular neurons, release of Ca^{2+} from intracellular stores result in the priming of LDCG in NHT, leading to a facilitation of elicited release. This evidence, while indirect, hints at a role of syntillas in such a mechanism. However, further studies are required to determine definitively if this is the case. Such a mechanism, responsible for the recruitment of secretory vesicles in an activity-dependent fashion, could provide a means of shaping and tuning synaptic efficacy in this and other CNS systems.

References:

Berridge MJ (2006) Calcium microdomains: organization and function. Cell Calcium 40:405-412.

Bouchard R, Pattarini R, Geiger JD (2003) Presence and functional significance of presynaptic ryanodine receptors. Prog Neurobiol 69:391-418.

Cazalis M, Dayanithi G, Nordmann JJ (1987) Hormone release from isolated nerve endings of the rat neurohypophysis. J Physiol 390:55-70.

Chen P, Gillis KD (2000) The noise of membrane capacitance measurements in the whole-cell recording configuration. Biophys J 79:2162-2170.

Collin T, Marty A, Llano I (2005) Presynaptic calcium stores and synaptic transmission. Curr Opin Neurobiol 15:275-281.

De Crescenzo V, ZhuGe R, Velazquez-Marrero C, Lifshitz LM, Custer E, Carmichael J, Lai FA, Tuft RA, Fogarty KE, Lemos JR, Walsh JV, Jr. (2004) Ca2+ syntillas, miniature Ca2+ release events in terminals of hypothalamic neurons, are increased in frequency by depolarization in the absence of Ca2+ influx. J Neurosci 24:1226-1235.

Douglas WW, Poisner AM (1964) Calcium Movement in the Neurohypophysis of the Rat and Its Relation to the Release of Vasopressin. J Physiol 172:19-30.

Gillis KD (2000) Admittance-based measurement of membrane capacitance using the EPC-9 patch-clamp amplifier. Pflugers Arch 439:655-664.

Giovannucci DR, Stuenkel EL (1997) Regulation of secretory granule recruitment and exocytosis at rat neurohypophysial nerve endings. J Physiol 498 (Pt 3):735-751.

Hsu SF, Jackson MB (1996) Rapid exocytosis and endocytosis in nerve terminals of the rat posterior pituitary. J Physiol 494 (Pt 2):539-553.

Jin D, Liu HX, Hirai H, Torashima T, Nagai T, Lopatina O, Shnayder NA, Yamada K, Noda M, Seike T, Fujita K, Takasawa S, Yokoyama S, Koizumi K, Shiraishi Y, Tanaka S, Hashii M, Yoshihara T, Higashida K, Islam MS, Yamada N, Hayashi K, Noguchi N, Kato I, Okamoto H, Matsushima A, Salmina A, Munesue T, Shimizu N, Mochida S, Asano M, Higashida H (2007) CD38 is critical for social behaviour by regulating oxytocin secretion. Nature 446:41-45.

Leng G, Caquineau C, Ludwig M (2008) Priming in oxytocin cells and in gonadotrophs. Neurochem Res 33:668-677.

Lindau M, Neher E (1988) Patch-clamp techniques for time-resolved capacitance measurements in single cells. Pflugers Arch 411:137-146.

Ludwig M, Sabatier N, Bull PM, Landgraf R, Dayanithi G, Leng G (2002) Intracellular calcium stores regulate activity-dependent neuropeptide release from dendrites. Nature 418:85-89.

Seward EP, Chernevskaya NI, Nowycky MC (1995) Exocytosis in peptidergic nerve terminals exhibits two calcium-sensitive phases during pulsatile calcium entry. J Neurosci 15:3390-3399.

Velazquez-Marrero C, Custer E, De Crescenzo V, ZhuGe R, Fogarty KE, Walsh JV, Jr., Lemos JR (2002) Caffeine Stimulates Basal Neuropeptide Release from Nerve Terminals of the Neurohypophysis. J Neurosci 202:440.448.

Wang G, Dayanithi G, Newcomb R, Lemos JR (1999) An R-type Ca(2+) current in neurohypophysial terminals preferentially regulates oxytocin secretion. J Neurosci 19:9235-9241.

ZhuGe R, DeCrescenzo V, Sorrentino V, Lai FA, Tuft RA, Lifshitz LM, Lemos JR, Smith C, Fogarty KE, Walsh JV, Jr. (2006) Syntillas release Ca2+ at a site different from the microdomain where exocytosis occurs in mouse chromaffin cells. Biophys J 90:2027-2037.