DISSERTATION

SYNAPTOTAGMIN: A MULTIFUNCTIONAL PROTEIN IN THE SYNAPTIC VESICLE CYCLE

Submitted by

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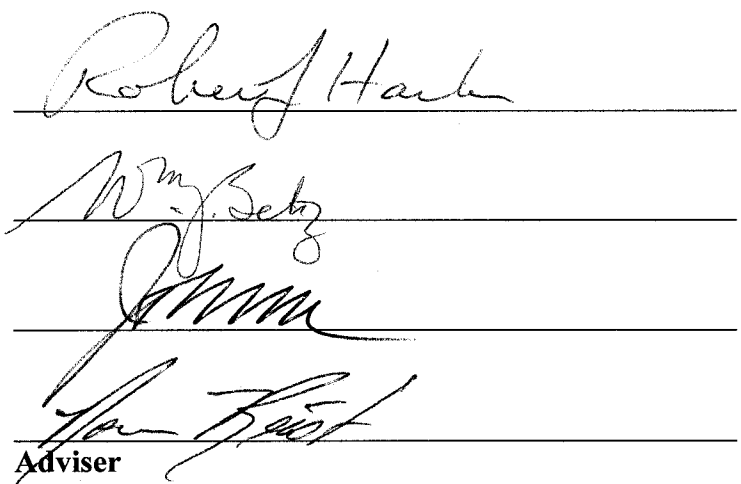
WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CARIN LOEWEN ENTITLED "SYNAPTOTAGMIN: A MUTLIFUNCTIONAL PROTEIN IN THE SYNAPTIC VESICLE CYCLE" BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSPOPHY.

Committee on graduate work



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ABSTRACT OF DISSERTATION

SYNAPTOTAGMIN: A MULTIFUNCTIONAL PROTEIN IN THE SYNAPTIC VESICLE CYCLE

Synaptotagmin is a synaptic vesicle protein whose cytosolic domain contains two C_2 domains, C_2A and C_2B. *In vitro*, synaptotagmin interacts with numerous presynaptic proteins. Genetic studies have demonstrated that synaptotagmin is critical for full synaptic transmission, and implicate synaptotagmin in synaptic vesicle docking, Ca^{2+}-sensing to trigger fusion and synaptic vesicle endocytosis. *In vitro* studies implicate synaptotagmin in synaptic vesicle priming. To further define synaptotagmin's role in the synaptic vesicle cycle, I examined three *Drosophila* synaptotagmin mutants at the third instar stage. The first was a *synaptotagmin null* (syt^{null}) mutant. The second harbored a mutation in the Ca^{2+}-binding motif of synaptotagmin's C_2B domain. The third harbored a mutation in the polylysine motif also located in the C_2B domain.

Although synaptic transmission is nearly abolished in syt^{null} mutants, the mutation does not cause gross morphological changes and synaptic arborizations develop normally. Indeed, with special care syt^{null} mutants can survive to adulthood. However, ultrastructural analysis revealed that synaptic vesicles, including docked vesicles, are severely decreased at active zones of syt^{null} neuromuscular junctions. syt^{null} terminals also accumulate large, membranous structures, possibly indicating a defect in endocytosis. These experiments permit syt^{null} third instars to serve as critical negative controls for synaptotagmin structure/function studies. Furthermore, they support a role for synaptotagmin in maintaining a population of synaptic vesicles in the nerve terminal,

as well as in synaptic vesicle docking, Ca^{2+}-sensing to trigger fusion, and synaptic vesicle endocytosis.

Previous work has shown that synaptic transmission is more severely disrupted in C_2B Ca^{2+}-binding motif mutants than it is in *syt*^{null} mutants. However, unlike *syt*^{null} mutants, synaptic ultrastructure is normal in C_2B Ca^{2+}-binding motif mutants. These mutants show normal levels of synaptic vesicles, including docked vesicles, and no accumulation of large membranous structures. These results indicate that the C_2B Ca^{2+}-binding motif is not involved in synaptotagmin's role in maintaining a population of synaptic vesicles at nerve terminals, in synaptic vesicle docking or in endocytosis. Instead, the near elimination of synaptic transmission observed in these mutants is likely due to the protein's inability to bind Ca^{2+} by its C_2B domain and undergo some critical Ca^{2+}-dependent interaction, strongly supporting a role for synaptotagmin in Ca^{2+}-sensing.

Previous work has shown that the polylysine motif in synaptotagmin's C_2B domain is also critical for full synaptic function. *In vitro*, this motif interacts with numerous presynaptic proteins. Thus, the defect in synaptic transmission recorded in C_2B polylysine motif mutants, could be due to a disruption of any of these interactions. Here I present data implicating this motif in synaptic vesicle recycling. I demonstrate that the motif is not involved in synaptic vesicle endocytosis, but does play a role prior to vesicle fusion. Furthermore, the C_2B polylysine motif mutants have a decreased probability of release. These results are consistent with the hypothesis that the polylysine motif is involved in synaptic vesicle priming.

In summary, these studies demonstrate that synaptotagmin is a multifunctional protein in the synaptic vesicle cycle. Synaptotagmin plays an important role in synaptic vesicle docking, priming, Ca^{2+}-sensing and endocytosis via distinct molecular interactions mediated by its various motifs.

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Chapter 1: Introduction

The primary way that neurons communicate with their targets is via the release of a chemical neurotransmitter from synaptic vesicles (Del Castillo and Katz, 1954). In neuromuscular synapses, for example, electrical action potentials travel along the axon to the presynaptic terminal where they signal voltage-gated calcium channels to open. Influx of calcium into the presynaptic terminal triggers the fusion of synaptic vesicles with the presynaptic membrane (Katz and Miledi, 1967; Heuser et al., 1979). After exocytosis, synaptic vesicle membrane and proteins are retrieved from the plasma membrane by endocytosis (Ceccarelli et al., 1972; Miller and Heuser, 1984). Re-internalized vesicles are refilled with neurotransmitter and readied for subsequent release. This is the synaptic vesicle cycle [reviewed by (Südhof, 2004)]. Many molecules thought to play a role in the synaptic vesicle cycle have been identified. However, the exact molecular mechanisms underlying the synaptic vesicle cycle are still unclear and are the subject of intense research. In this introduction I will attempt to summarize some of the current theories on both exo- and endocytosis and review why the protein synaptotagmin I is implicated in both of these processes. Finally, I will introduce the research I've completed that helps to clarify synaptotagmin's role in the synaptic vesicle cycle.

I. Exocytosis

A. The SNARE Hypothesis

Fusion of vesicles with target membranes is a fundamental cellular process, and virtually all cellular fusion events are mediated by a common molecular machinery, including the fusion of synaptic vesicles with the plasma membrane [reviewed by (Jahn et al., 2003)]. SNARE (Soluble NSF Attachment protein REceptor) proteins constitute a major component of this machinery (Söllner et al., 1993a; Söllner et al., 1993b). These proteins are targets of the clostridial neurotoxins (tetanus toxin and botulinum neurotoxin A-G), and their cleavage disrupts synaptic transmission [reviewed by (Schiavo et al., 2000)]. Insertion of SNAREs into reconstituted vesicles results in SNARE mediated fusion as evidenced by lipid mixing between the vesicles (Weber et al., 1998). Thus, SNAREs may comprise the minimal machinery for vesicle fusion.

SNAREs can be classified as v-SNAREs (v = vesicle) or t-SNAREs (t = target) depending on whether they are localized on vesicles or the target membrane [reviewed by (Rothman, 1994)]. The v-SNARE for synaptic vesicle exocytosis in *Drosophila* is n (neuronal)-synaptobrevin/VAMP (Vesicle Associated Membrane Protein). It is a small, C-terminal, transmembrane protein found on synaptic vesicles. SNAP-25 and syntaxin-1 are the two required t-SNARES. Syntaxin I is located on the plasma membrane, and like n-synaptobrevin/VMAP, syntaxin is also a C-terminal transmembrane protein. SNAP-25 is not a transmembrane protein, but is instead anchored to the plasma membrane via palmitoylated cysteine residues located in the middle of its sequence [reviewed by (Hughson, 1999)].

All SNAREs share a homologous region known as the SNARE motif, which contains eight heptad repeats typical for coiled coils (Weimbs et al., 1998; Bock et al., 2001). Free SNARE proteins are largely unstructured in solution (Fasshauer et al., 1997b; Fasshauer et al., 1997a; Fiebig et al., 1999; Margittai et al., 2001). However, when synaptobrevin/VAMP, syntaxin and SNAP-25 are combined, they spontaneously form a ternary complex (Söllner et al., 1993a) that is SDS resistant (Hayashi et al., 1994), thermally very stable and greatly increased in α-helical structure (Fasshauer et al., 1997a). The crystal structure of the ternary SNARE complex shows it to be a four-helix bundle structure in which four α-helices (one from synaptobrevin/VAMP, one from syntaxin and two from SNAP-25) make up a coiled coil (Sutton et al., 1998).

SNARE complex assembly and disassembly exhibit a profound hysteresis caused by a high energetic barrier between the folded and unfolded states (Fasshauer et al., 2002). Indeed, the fusion that occurs between SNAREs reconstituted into synthetic lipid bilayer vesicles occurs extremely slowly (Weber et al., 1998). This hysteresis suggests the presence of folding intermediates. Interestingly, the folding of syntaxin with SNAP-25 does not exhibit a hysteresis, and the presence of the syntaxin-SNAP-25 complex seems to speed up the formation of the SNARE complex (Fasshauer et al., 2002). Thus, at least one folding intermediate may be a complex of syntaxin and SNAP-25.

Syntaxin and SNAP-25 have been shown to exist as a heterodimer (Rickman et al., 2004a) and a heterotrimer (Fasshauer et al., 1997a; Margittai et al., 2001; Xiao et al., 2001). The structure of the heterodimer is unknown. In the heterotrimer, a second syntaxin molecule contributes a fourth helix where synaptobrevin/VAMP's helix would normally exist in the fully assembled SNARE complex. When synaptobrevin/VAMP is

added to the syntaxin-SNAP-25 complex, a helix from synaptobrevin/VAMP replaces the second syntaxin helix (Fasshauer et al., 1997a).

SNARE proteins in the *cis*-complex (when all three proteins are in the same membrane) are oriented in parallel, with their membrane proximal C-termini all at one end and their N-termini all at the other (Hanson et al., 1997b; Sutton et al., 1998; Lin and Scheller, 2000). This structure suggests that the *cis*-SNARE complex may form by the "zippering" together of *trans*-SNARE proteins (synaptobrevin/VAMP in the synaptic vesicle, and syntaxin and SNAP-25 in the plasma membrane) from their membrane distal N-termini to their membrane proximal C-termini [Fig. 1.1, and (Hanson et al., 1997a)].

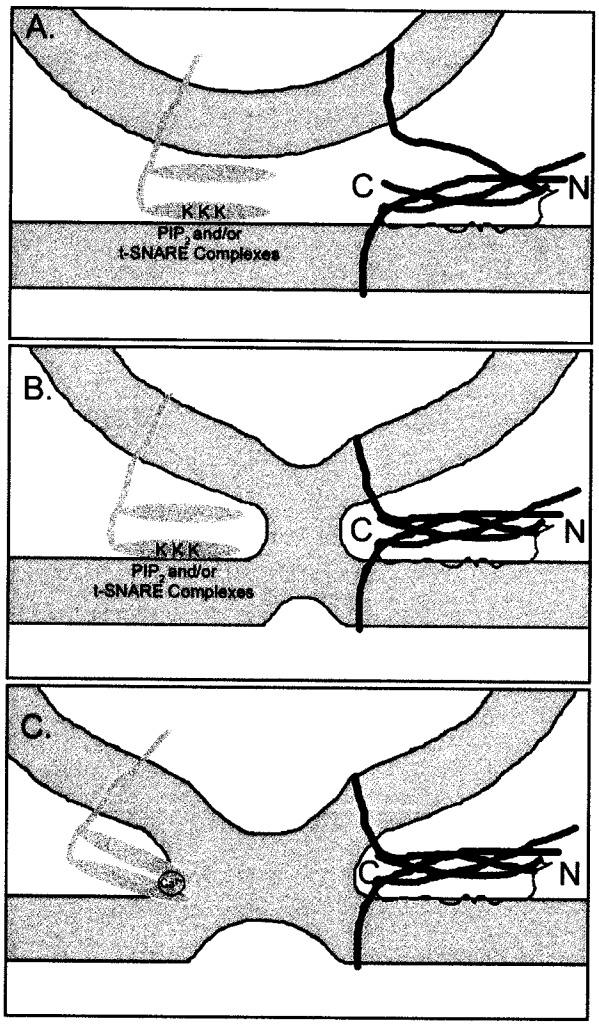

Figure 1.1 Model of synaptic vesicle fusion. (A) Prior to Ca^{2+} influx, the C_2B polylysine motif of synaptotagmin (syt, gold) interacts with PIP_2 and/or t-SNARE complexes in the plasma membrane, which may facilitate formation of *trans*-SNARE complexes. The N-terminus of synaptobrevin/VAMP (blue) in the synaptic vesicle interacts with the N-terminus of the syntaxin (red)/SNAP-25 (green) heterodimer to initiate formation of the *trans*-SNARE complex. (B) A *trans*-SNARE complex that is "zipped-up" to its transmembrane region. Syt may facilitate formation of "tight" *trans*-SNARE complexes. *trans*-SNARE complex formation may initiate partial fusion of the synaptic vesicle membrane with the plasma membrane, creating a semi-stable, hemi-fused intermediate. (C) Upon Ca^{2+} influx, syt's interaction with/penetration of the plasma membrane may destabilize the hemi-fused intermediate and cause subsequent fusion. Note: fusion likely involves the action of multiple syts and SNARE complexes. Only one of each is shown for simplicity.

Indeed, functional studies support this zippering hypothesis (Melia et al., 2002). Furthermore, *trans*-SNARE complexes have been shown to form prior to the Ca^{2+} influx that triggers fusion (Hua and Charlton, 1999; Xu et al., 1999). At least a portion of these *trans*-SNARE complexes exist in a partially "zipped-up" conformation, in which the N-terminus is "zipped" and thus protected from proteolytic neurotoxins, but the C-terminus is more unstructured and not protected (Hua and Charlton, 1999). Fully formed SNARE complexes are protected from cleavage by these proteolytic neurotoxins (Hayashi et al., 1994).

With synaptobrevin/VAMP in the synaptic vesicle and syntaxin and SNAP-25 in the plasma membrane, formation of the energetically favorable SNARE complex could provide the energy to overcome the repulsive forces between the lipid bilayers, bring the two membranes into close apposition with each other, and cause fusion (Hanson et al., 1997a; Fasshauer et al., 2002). However, the slow assembly rates of SNARE complexes (Weber et al., 1998; Fasshauer et al., 2002) seem incongruent with the very rapid membrane fusion seen in synaptic transmission, suggesting the critical involvement of accelerating factors.

One accelerating factor may be complexin proteins (McMahon et al., 1995), which bind to fully formed SNARE complexes (Pabst et al., 2000; Chen et al., 2002). Complexin proteins may stabilize *trans*-SNARE complexes by selectively binding complexes near the transmembrane region and helping bear the repulsive force between the synaptic vesicle membrane and the plasma membrane (Pabst et al., 2000; Chen et al., 2002). Knockout of both complexin proteins impairs, but does not abolish, synchronous, Ca^{2+}-triggered release (Reim et al., 2001). This result suggests that complexins may be

ancillary factors that promote synaptic transmission through stabilizing assembled SNARE complexes [reviewed by (Jahn et al., 2003; Südhof, 2004)].

Another hypothesis is that SNARE complex assembly itself does not result in fusion, but is instead important for steps preceding fusion [reviewed by (Jahn et al., 2003; Südhof, 2004)]. At least four lines of evidence support this hypothesis. First, the dependence of fusion on SNARES may be, at least partially, bypassed under certain conditions. Knockout of synaptobrevin/VAMP in *Drosophila* and mice abolishes or severely decreases evoked release, but spontaneous release is less effected (Broadie et al., 1995; Deitcher et al., 1998; Schoch et al., 2001). Similar results occur with SNAP-25 (Washbourne et al., 2002). Second, *in vivo* fusion requires the presence of other essential SNARE co-factors. For example, although some fusion events remain in the absence of synaptobrevin/VAMP (Broadie et al., 1995; Deitcher et al., 1998; Schoch et al., 2001) and SNAP-25 (Washbourne et al., 2002), they are completely abolished in Munc18 (a syntaxin chaperone) knockouts (Verhage et al., 2000). Third, SNARE complex assembly may be more important in promoting the Ca^{2+}-sensitivity of release than fusion itself (Coorssen et al., 1998; Tahara et al., 1998; Sorensen et al., 2002). Indeed, although SNARE complex formation itself is not Ca^{2+}-dependent (Hu et al., 2002), reducing the number of SNARE proteins in *Drosophila* reduces the Ca^{2+}-cooperativity of release (Stewart et al., 2000). Results from complexin knockout studies are consistent with this hypothesis, as they suggest that synaptotagmin (the putative Ca^{2+}-sensor for synchronous fusion) acts downstream of SNARE complex formation (Reim et al., 2001). Finally, experiments in chromaffin cells and synaptosomes have demonstrated that SNARE

complexes fully assemble during a priming step (Lonart and Südhof, 2000; Wei et al., 2000a) and thus help regulate the formation of readily-releasable vesicles.

In summary, evidence supports the hypothesis that SNARE complexes do form prior to fusion. At least a portion of these complexes exists in the *trans* conformation; and these complexes are only partially assembled. Formation of SNARE complexes prior to fusion may serve a priming function, regulating the formation of readily releasable vesicles and promoting Ca^{2+}-triggered, synchronous fusion. Although the energy provided by the formation of the energetically favorable SNARE complex is likely important for fusion *in vivo*, it is neither necessary nor sufficient. *In vivo*, spontaneous fusion can occur in the absence of synaptobrevin/VAMP and SNAP-25, and it seems to require the action of additional proteins.

One appealing model of fusion suggests that partially "zipped-up", *trans*-SNARE complex formation primes vesicles by creating a semi-stable, hemi-fusion intermediate. This fusion intermediate can progress to a full-blown fusion pore upon the action of some other mechanism, such as Ca^{2+}-triggered synaptotagmin membrane penetration [(Fig. 1.1, and reviewed by (Jahn et al., 2003; Südhof, 2004)].

B. Synaptic Vesicle Exocytosis is a Specialized Form of Membrane Fusion

Synaptic vesicle exocytosis is a particular form of membrane fusion that is characterized by: 1) specializations (active zones) restricting exocytosis to specific areas on the presynaptic membrane, 2) vesicles capable of fusing very quickly, and 3) Ca^{2+} regulation. As such, synaptic vesicle exocytosis must require factors in addition to the necessary SNARE proteins to confer these special characteristics.

1. Active Zones

Upon stimulation, synaptic vesicle exocytosis occurs at a specialized region in the nerve terminal called the active zone (Couteaux and Pecot-Dechavassine, 1970). Thin section electron micrographs illustrate that active zones in *Drosophila* (and other species) are characterized by electron dense deposits (active zone material) surrounded by clusters of synaptic vesicles (Osborne, 1975; Govind et al., 1980; Kosaka and Ikeda, 1983b). In *Drosophila*, the electron dense deposits are composed of a base that is capped by a meshwork of filaments. In tangential sections, the base often has a shape like a dog bone. The filaments appear to originate on either side of the base, pass over the base and radiate into the cytoplasm. In cross section, the base and capping filaments often appear T shaped. Thus, in *Drosophila* the electron dense material marking active zones is often referred to as a T bar (Atwood et al., 1993; Jia et al., 1993b; Koenig and Ikeda, 1999).

The active zone material (AZM) opposes the synaptic cleft and junctional folds, where the postsynaptic receptors are located [reviewed by (Rash et al., 1988)]. Recently, the use of electron microscope tomography has provided amazing details about the organization of the AZM at frog neuromuscular junctions (Harlow et al., 2001). This material was found to have three components: beams, ribs and pegs. The beam is an electron dense strand that runs parallel to the presynaptic membrane. Electron dense ribs tether synaptic vesicles on either side of the beam to the beam. Periodically pegs connect the ribs to the plasma membrane (Harlow et al., 2001). The molecules that make up the beams, ribs and pegs are unknown, but they probably include Munc13s, RIMs, piccolo, bassoon, ERCs and α-liprins. These are classes of large, non-membrane proteins that

bind to each other (and other presynaptic molecules) and probably form an extremely large, multi-component, single complex at the active zone [reviewed by (Südhof, 2004)].

Freeze-fracture electron micrographs from many organisms, including frog [reviewed by (Rash et al., 1988)], rat (Rash and Ellisman, 1974), and the housefly (Saint Marie and Carlson, 1982), have demonstrated that the active zone associated plasma membrane is studded by large-diameter intramembrane particles (IMPs). The exact organization of these particles in the plasma membrane differs among organisms. Nevertheless, some of these IMPs are proposed to be voltage-gated Ca^{2+} channels (Pumplin et al., 1981). Consistent with the theory that some IMPs are Ca^{2+} channels, Ca^{2+} channels are highly concentrated at active zones of frog neuromuscular junctions (Robitaille et al., 1990; Cohen et al., 1991; Torri Tarelli et al., 1991). This is also true in *Drosophila.* In *Drosophila, cacophony* encodes the $\alpha 1$ subunit of a primary, presynaptic, voltage-gated Ca^{2+} channel (Smith et al., 1996; Dellinger et al., 2000; Kawasaki et al., 2000; Kawasaki et al., 2002). Cacophony has been localized (at the light level) to active zones (Kawasaki et al., 2004). It is thought that Ca^{2+} probably acts at a very short distance from Ca^{2+} channels (Llinás et al., 1976; Pumplin et al., 1981; Smith et al., 1993) [reviewed by (Augustine et al., 2003)]. Thus, localizing voltage-gated Ca^{2+} channels specifically to the active zone helps limit synaptic vesicle exocytosis to active zones.

2. Speed of Exocytosis

Once voltage-gated Ca^{2+} channels open and intracellular Ca^{2+} levels rise, synaptic vesicle exocytosis occurs very rapidly, within 50-350 μs (Llinás et al., 1981; Heidelberger et al., 1994; Sabatini and Regehr, 1996). This short latency does not leave

time for multiple molecular rearrangements. Therefore, it is thought that synaptic vesicles first dock at active zones and then undergo some kind of priming/maturation reaction that makes them fusion competent, such that they are ready to quickly fuse at the arrival of the Ca^{2+} signal [reviewed by (Zheng and Bobich, 1998; Klenchin and Martin, 2000; Martin, 2002)].

The molecular rearrangements involved in synaptic vesicle priming are just beginning to be understood, due in large part to experiments on dense core granule (DCG) release from adrenal chromaffin cells. These experiments suggest that priming involves the formation of *trans*-SNARE complexes [reviewed by (Rettig and Neher, 2002; Martin, 2003)]. When DCG exocytosis is induced by increasing intracellular Ca^{2+} via Ca^{2+} uncaging, two phases of release are measured, a burst phase and a subsequent sustained release phase (Xu et al., 1998). As partially "zipped up" SNARE complexes exist prior to fusion (Hua and Charlton, 1999), the burst phase is thought to be due to the fusion of DCGs associated with these partially "zipped up" SNARE complexes. Thus, these DCGs are in a "primed" or ready-release state and require only elevated intracellular Ca^{2+} for fusion. The sustained release phase is thought be due to the fusion of DCGs that have to undergo slower steps of recruitment to the ready-release state. These steps would include the formation of *trans*-SNARE complexes (Xu et al., 1998). Supporting the hypothesis that the formation of a *trans*-SNARE complex underlies priming, overexpression of SNAP-25 harboring a mutation in its "zero layer" glutamine (a residue that becomes embedded within the four-helix bundle of the SNARE complex) reduces the sustained component in Ca^{2+} uncaging experiments (Wei et al., 2000b). As

the burst phase of release is unaffected by this mutation, these data suggest that priming does occur in these mutants, it is just slower.

Furthermore, the time course of the burst phase can be fit by two exponentials, the first with a faster time constant and the second with a slower time constant (Xu et al., 1998; Xu et al., 1999). Thus, *trans*-SNARE complexes may exist in two functionally distinct states. Experiments using proteolytic neurotoxins and Fab fragments (low molecular weight antibody fragments) suggest that these two states may correspond to two distinct *trans*-SNARE complex conformations, "loose" and "tight" (Xu et al., 1998; Xu et al., 1999). Removing the nine C-terminal amino acids in SNAP-25 or adding a Fab fragment against the C-terminus of SNAP-25 severely decreases or eliminates the fast component of the burst phase without greatly affecting the slow component of the burst phase (Xu et al., 1998; Xu et al., 1999). Thus, *trans*-SNARE complexes in the "tight" conformation may be fully "zipped" all the way to the C-terminus, whereas in the "loose" conformation, *trans*-SNARE complexes are not as fully "zipped." The two forms of release (and thus presumably the two forms of *trans*-SNARE complexes) have been shown to interconvert on the order of a few seconds (Voets et al., 1999).

Curiously, removal of synaptotagmin I also abolishes the fast component of the burst phase without affecting the slow component (Voets et al., 2001). As the Ca^{2+}-dependent interaction between synaptotagmin and SNAP-25 requires the C-terminus of SNAP-25 (Gerona et al., 2000; Zhang et al., 2002), this interaction may underlie the "tight" conformation. Fusion of DCGs, however, can occur directly from either the "loose" or "tight" conformation (as opposed to the need for a transition from the "loose"

to the "tight" conformation) because the slow component of the burst is unaffected by the SNAP-25 C-terminal alterations or the removal of synaptotagmin I.

Ca^{2+} uncaging experiments have also demonstrated that the amplitude of the burst phase increases when the Ca^{2+} concentration preceding the flash is elevated. Likewise, the amplitude of the burst phase is reduced when pre-flash Ca^{2+} concentrations are also reduced (Voets et al., 1999). As the burst phase is thought to represent the fusion of vesicles that have been primed via formation of *trans*-SNARE complexes, these results suggest that Ca^{2+} can stimulate priming (i.e., the formation of *trans*-SNARE complexes). However, Ca^{2+} also appears to stimulate vesicle docking (Koenig et al., 1993) via an interaction between synaptotagmin and SNAP-25/23 (Chieregatti et al., 2002; Chieregatti et al., 2004). Thus, whether the increase in the amplitude of the burst phase is due to increased Ca^{2+}-dependent priming or increased Ca^{2+}-dependent docking is still unclear.

In summary, according to the above model, priming involves the formation of "tight" and "loose" *trans*-SNARE complexes. Fusion can occur with either type of priming; however it is faster from the "tight" conformation. The molecular interactions underlying "tight" and "loose" SNARE complex conformations are not fully understood. Formation of the "tight" conformation appears to involve the C-terminus of SNAP-25 and synaptotagmin I, whereas neither are required for the type of priming that leads to the "loose" conformation and the release of DCGs during the slow phase of the burst component and during the sustained component.

According to the above model, priming should be affected by the availability of SNARE proteins to participate in SNARE complexes. Experiments on the proteins Munc18 and Munc13 show this to be the case. Syntaxin forms a tight complex with the

protein Munc18. In this complex, Munc18 holds syntaxin in a "closed" conformation such that its C-terminal helix cannot participate in the SNARE complex (Misura et al., 2000). Munc13 is postulated to be the protein that "opens" syntaxin and allows it participate in the SNARE complex [reviewed by (Rettig et al., 1996; Martin, 2003)]. Munc13 knockouts in *C. elegans*, *Drosophila* and mice severely decrease neurotransmitter release (Betz et al., 1998; Aravamudan et al., 1999; Augustin et al., 1999). Thus, Munc13 is thought to be an important molecule involved in priming. Indeed, overexpressing Munc13 in chromaffin cells increases release from both burst components, as well as from the sustained phase (Ashery et al., 2000).

3. Ca^{2+} Regulation

A critical component of synaptic vesicle exocytosis is its dependence on Ca^{2+} influx, which links neurotransmitter release to presynaptic action potentials. Numerous studies have attempted to quantify the intracellular Ca^{2+} concentrations required for fast, neurotransmitter release [reviewed by (Augustine et al., 2003)]. Early studies at goldfish bipolar ribbon synapses and squid giant terminals suggested that high presynaptic Ca^{2+} concentrations, on the order of 100-200 μM, were necessary for fast release [reviewed by (Augustine, 2001; Augustine et al., 2003)]. However, Ca^{2+} uncaging experiments at the calyx of Held demonstrate that much lower Ca^{2+} concentrations are required at this synapse (Bollmann et al., 2000; Schneggenburger and Neher, 2000). At calyx of Held synapses, release was undetectable when intracellular Ca^{2+} was <1 μM, resembled release observed during a normal action potential at >5 μM, and saturated at >20 μM [reviewed by (Augustine, 2001; Augustine et al., 2003; Südhof, 2004)]. Thus, the

intracellular Ca^{2+} concentration required for fast neurotransmitter release differs at different synapses. It has not been measured for neurotransmitter release at *Drosophila* neuromuscular junctions.

C. Synaptic Vesicle Pools

Synaptic vesicles are proposed to reside in distinct populations/pools within the nerve terminal [reviewed by (Rizzoli and Betz, 2005)]. Birks and MacIntosh (Birks and MacIntosh, 1961) were the first to propose two pools of vesicles in the cat cervical ganglion, a small, readily releasable pool (RRP) and a larger, non-readily releasable one [reserve pool (RP)]. Since then numerous studies have provided evidence for multiple pools of vesicles at various synapses from many organisms (Elmqvist and Quastel, 1965; Zimmermann and Denston, 1977; Zimmermann and Whittaker, 1977; Agoston et al., 1985; Liu and Tsien, 1995; Pieribone et al., 1995; Stevens and Tsujimoto, 1995; Kuromi and Kidokoro, 1998; Richards et al., 2000). At least four functionally distinct pools have been described, although the terminology for these pools has not been consistent. I will refer to these four pools as: 1) docked, 2) immediately releasable (IRP), 3) readily releasable (RRP), and 4) reserve (RP). The exact classification of some of these pools has been elusive, due in part to differences in how they behave in different organisms and in different synapses. I will limit my discussion of synaptic vesicle pools to cultured hippocampal synapses as well as to neuromuscular junctions in *Drosophila* and frog. [For a more thorough review see (Rizzoli and Betz, 2005)].

Application of hypertonic or hyperkalemic solutions to synapses causes a burst of neurotransmitter release that subsequently diminishes during the application. In cultured

hippocampal synapses, release has been shown to recover after a period of time following the removal of the solution. The burst of neurotransmitter release, its subsequent diminishment during solution application, and its recovery after solution removal is suggested to result from the release, depletion and subsequent refilling of a RRP of vesicles (Stevens and Tsujimoto, 1995; Rosenmund and Stevens, 1996). This RRP of vesicles appears to be the same pool of vesicles that is utilized when release is evoked by action potentials, and it can be depleted after two sec of 20 Hz stimulation (Rosenmund and Stevens, 1996). In these cultured hippocampal synapses, the RRP has been shown to correspond roughly to the vesicles that appear "docked" at active zones (Schikorski and Stevens, 2001).

Docked synaptic vesicles can be defined morphologically. Synaptic vesicles cluster around active zones, and some of the vesicles in this cluster are in direct contact with the presynaptic membrane and are embedded in the AZM (Couteaux and Pecot-Dechavassine, 1970; Landis, 1988; Landis et al., 1988; Hirokawa et al., 1989). The vesicles in direct contact with the presynaptic membrane represent the "morphologically" docked pool. "Physiologically" docked vesicles are those that are able to fuse with the presynaptic membrane at the Ca^{2+} signal. "Physiologically" docked vesicles are those that make up the RRP in hippocampal synapses. Since fusion is an event dependent on probability, not all morphologically docked vesicles will fuse in response to the Ca^{2+} signal (Schweizer et al., 1995). Indeed, in cultured hippocampal cells, about ~10% of the docked vesicles do not appear to fuse during stimulation of the RRP (Schikorski and Stevens, 2001). Thus, in a cultured hippocampal synapse, the RRP is a large subset

(~90%) of morphologically docked vesicles and has been estimated to be ~5.0 ± 3.0 quanta per synapse (Dobrunz and Stevens, 1997).

The difference between morphologically docked vesicles and physiologically docked vesicles may involve priming [reviewed by (Klenchin and Martin, 2000; Rettig and Neher, 2002; Martin, 2003)]. Results from Munc13 knockout experiments support this hypothesis. Hippocampal synapses from Munc13 knockout mice have dramatically decreased evoked release and a smaller RRP of vesicles as detected by hypertonic sucrose stimulation. However, the level of docked vesicles is unaffected (Augustin et al., 1999). Furthermore, overexpressing Munc13 in chromaffin cells leads to a threefold increase in secretion due to a larger number of vesicles released during both the burst and sustained phases (Ashery et al., 2000).

Although in cultured hippocampal synapses this primed pool is also the RRP and nearly equals the docked pool (Schikorski and Stevens, 2001), at neuromuscular junctions these pools are not equivalent. In neuromuscular junctions, the RRP is defined as those vesicles that maintain release during moderate stimulation, and it is much larger than the docked and primed pools [reviewed by (Rizzoli and Betz, 2005)]. In neuromuscular junctions, the primed pool is often referred to as the immediately releasable pool (IRP) (Delgado et al., 2000; Richards et al., 2003). The IRP is exhausted by a few stimuli (Delgado et al., 2000; Richards et al., 2003), and in *Drosophila* is estimated to be ~230 synaptic vesicles (Delgado et al., 2000). At low frequency stimulation the size of the IRP probably determines the amplitude of synaptic currents (Kuromi and Kidokoro, 1999). The synaptic vesicles in the IRP pool are in dynamic equilibrium with the entire vesicle population (Li and Schwarz, 1999). In neuromuscular

junctions from both frog (Richards et al., 2003) and *Drosophila* (Delgado et al., 2000), the RRP has been estimated to be between 10% and 20% of the total vesicle population. As docked vesicles (which include primed vesicles) make up only a few percent of the total vesicles at neuromuscular junctions (Reist et al., 1998), the RRP likely includes vesicles clustered around active zones as well as docked vesicles (which includes the IRP).

In *Drosophila*, early evidence for at least two pools of vesicles came from studies examining endocytosis in flies harboring a temperature sensitive mutation in the protein dynamin (*shibire*). Dynamin is a GTPase that participates in the pinching off of invaginated membrane in order to internalize it into the nerve terminal (Hinshaw and Schmid, 1995; Takei et al., 1995). At the non-permissive temperature, synaptic vesicle endocytosis is completely blocked in *shibire* mutants. Repeated stimulation of *shibire* nerve terminals eventually results in complete depletion of synaptic vesicles, and evoked release is abolished (Koenig et al., 1983). The flies become paralyzed. Upon return to the permissive temperature, synaptic vesicles reform from the plasma membrane and repopulate the terminal (Koenig et al., 1983). Two modes of endocytosis have been described in *shibire* mutants, at both synapses in retinula cells and neuromuscular junctions. One is a slow mode that emanates from sites away from active zones. The second is a faster mode that arises directly from active zones (discussed in more detail below). These two modes were suggested to replenish two distinct pools of synaptic vesicles (Koenig and Ikeda, 1996, 1999).

FM 1-43 labels two functionally and topographically distinct synaptic vesicle pools at *Drosophila* neuromuscular junctions [reviewed by (Kidokoro et al., 2004)]. FM

1-43 is a fluorescent probe that reversibly inserts into membranes and can be used to label endocytosed synaptic vesicles (Betz et al., 1992). Using different stimulation protocols, FM 1-43 appears to selectively label two distinct synaptic vesicle pools: 1) low frequency (≤10Hz) or high K^+ stimulation labels the readily releasable pool, located in the periphery of boutons; and 2) high frequency stimulation (30 Hz) also labels the reserve pool, located in the center of boutons (Kuromi and Kidokoro, 1998; Delgado et al., 2000; Kuromi and Kidokoro, 2000). Thus, the RRP appears to be preferentially utilized during low frequency stimulation, whereas vesicles in the reserve pool are recruited for exocytosis during high frequency stimulation (Kuromi and Kidokoro, 2000). The slow recycling pathway observed in electron micrographs that emanates from sites away from the active zone may serve to repopulate the RP, whereas the faster pathway that emanates from the active zone may serve to repopulate the RRP (Koenig and Ikeda, 1996, 1999).

Further evidence for multiple vesicle pools in *Drosophila* comes from electrophysiological studies. In *Drosophila* neuromuscular junctions, high frequency stimulation causes evoked junctional current (EJC) amplitudes to decrease over time (Delgado et al., 2000). The magnitude of the decrease is proportional to the frequency of the stimulation; larger decreases occur with faster stimulation rates. This phenomenon, which also occurs in other synapses and in other organisms, has long been considered to reflect the depletion of synaptic vesicles in nerve terminals that occurs when the rate of vesicle use is faster than the rate of vesicle replacement [reviewed by (Zucker and Regehr, 2002)]. In *Drosophila*, the time course of decline in amplitude can be fit with

two exponentials and a steady-state component, suggesting the presence of three synaptic vesicles pools. These pools include the IRP, the RRP and the RP (Delgado et al., 2000).

II. Endocytosis and Recycling

After exocytosis, synaptic vesicle membrane is endocytosed from the plasma membrane (Ceccarelli et al., 1972) and recycled to generate new synaptic vesicles [reviewed by (Murthy and De Camilli, 2003)]. Since the first description of endocytosis and recycling at synapses (Ceccarelli et al., 1973; Heuser and Reese, 1973), there has been debate over the mechanism and speed of these processes.

A. Mode of Recycling

Early on, electron micrographs of frog neuromuscular junctions suggested the presence of at least two modes of synaptic vesicle recycling (Ceccarelli et al., 1973; Heuser and Reese, 1973; Heuser et al., 1979; Miller and Heuser, 1984). One was a slower process that involved the complete collapse of synaptic vesicles into the plasma membrane, and subsequent clathrin mediated endocytosis [reviewed by (Murthy and De Camilli, 2003)]. The second was a faster process in which synaptic vesicles fuse with the plasma membrane, but do not fully collapse into it. Later evidence suggested that in the faster pathway, neurotransmitter is released via a narrow fusion pore that can quickly re-close after opening. Upon closure of the fusion pore, the vesicle is quickly recaptured in its entirety [reviewed by (Valtorta et al., 2001; Murthy and De Camilli, 2003)].

Two modes of endocytosis, similar to those described above, are thought to occur in *Drosophila*. The electron microscopy study in *shibire* mutants examining synaptic

vesicle reformation after depletion, uncovered two, apparently distinct, recycling pathways: one emanating from presynaptic membrane at sites away from the active zone, and one emanating from the active zone itself (Koenig and Ikeda, 1996, 1999). The active zone pathway functioned to replace a small population of synaptic vesicles surrounding the dense body, whereas the non-active zone pathway seemed to replenish a larger, reserve pool of synaptic vesicles located further from active zones. Endocytosis of vesicles via the non-active zone pathway occurred more slowly than the active zone pathway, and seemed to operate via multiple steps. In contrast, synaptic vesicles appeared to pinch off directly from the plasma membrane in the active zone pathway (Koenig and Ikeda, 1996). As the *shibire* mutation blocks all endocytosis and leads to complete synaptic vesicle depletion, both pathways must depend on the protein dynamin.

1. The Slow Recycling Pathway

The slow recycling pathway observed in *Drosophila* is likely the classic synaptic vesicle recycling pathway discovered by Heuser and Reese (Heuser and Reese, 1973; Heuser et al., 1974, 1976; Heuser et al., 1979; Heuser and Reese, 1981). This pathway is similar to the pathway of constitutive and/or ligand-stimulated recycling in that it utilizes clathrin-mediated endocytosis [reviewed by (Mellman, 1996)]. In this pathway, after synaptic vesicle fusion, vesicle membrane fully collapses into the plasma membrane and is subsequently re-internalized via clathrin and accessory proteins [reviewed by (Brodin et al., 2000; Slepnev and De Camilli, 2000)]. In the constitutive and/or ligand-stimulated recycling pathway, clathrin-coated endocytic vesicles typically fuse with early/sorting endosomes. However, synaptic vesicle recycling may bypass the endosome. Instead,

endocytic vesicles may mature directly into synaptic vesicles (Takei et al., 1996; Murthy and Stevens, 1998). Thus, although classic synaptic vesicle recycling is similar to constitutive and/or ligand-stimulated recycling, there are important differences.

Synaptic vesicle recycling via this classical pathway is certainly utilized at *Drosophila* neuromuscular junctions (Zhang, 2003). In *Drosophila*, mutations in classical pathway accessory proteins, such as dynamin (Kosaka and Ikeda, 1983a, b), AP-2 (González-Gaitán and Jäckle, 1997), AP-180 (Zhang et al., 1998a), endophilin (Guichet et al., 2002; Rikhy et al., 2002; Verstreken et al., 2002; Fabian-Fine et al., 2003) and synaptojanin (Verstreken et al., 2003) all lead to synaptic vesicle loss or changes in synaptic vesicle morphology, as well as impaired synaptic transmission. Furthermore, clathrin is recruited to the nerve terminal plasma membrane from the cytosol when *Drosophila shibire* mutants are stimulated by high K^+ at the non-permissive temperature (Kuromi et al., 2004).

Further supporting the hypothesis that classic synaptic vesicle recycling occurs at *Drosophila* neuromuscular junctions, extensive evidence demonstrates that at least a portion of synaptic vesicle recycling at *Drosophila* neuromuscular junctions utilizes endosomes. First, cisternal structures of around 150 nm have been identified in wild-type *Drosophila* terminals (Koenig et al., 1993; Wucherpfennig et al., 2003). Electron micrographs show that these cisternae label with an endosomal marker (Wucherpfennig et al., 2003). Fluorescence microscopy shows that most synaptic boutons in *Drosophila* contain at least one structure that is labeled by this marker. These fluorescently labeled endosomal structures disappear during synaptic vesicle depletion and reappear when synaptic vesicles reform (Wucherpfennig et al., 2003). Thus, these endosomal structures

can turn into synaptic vesicles. Second, in addition to small, clear, synaptic vesicles, a second, morphologically distinct type of vesicle is seen in *Drosophila* nerve terminals. This second type of vesicle is ~70 nm in diameter and has been suggested to be a recycling intermediate (Kosaka and Ikeda, 1983b; Fergestad et al., 1999; Wucherpfennig et al., 2003). When endocytosis is first allowed to resume in *shibire* mutants after synaptic vesicle depletion, nascent endocytic vesicles in the form of collared pits accumulate at the plasma membrane (Kosaka and Ikeda, 1983a, b). Because the collared pits that form in *shibire* mutants have a similar diameter to the ~70 nm vesicles seen in wild-type terminals, the ~70 nm structures in wild-type terminals may be newly endocytosed vesicles (Wucherpfennig et al., 2003). In non-neuronal cells, the small GTPase Rab5 mediates the fusion of endocytic vesicles with endosomes (Bucci et al., 1992; Horiuchi et al., 1997). In *Drosophila*, inactivating Rab5 leads to an increase in ~70 nm diameter vesicles and a concomitant decrease in the ~150 nm cisternae (Wucherpfennig et al., 2003). This phenotype suggests that newly endocytosed ~70 nm vesicles subsequently fuse with cisternae/endosomes during the recycling process. Finally, the endosomal system is important for synaptic transmission, because *Drosophila* mutants with inactive Rab5 show decreased neurotransmitter release, whereas mutants over-expressing Rab5 show increased release (Wucherpfennig et al., 2003).

2. The Fast Recycling Pathway

Although it is well documented that *Drosophila* neuromuscular junctions utilize the classic fuse-and-collapse recycling pathway, ultrastructural evidence indicates that a second, faster pathway also functions at *Drosophila* retinula synapses and neuromuscular

junctions (Koenig and Ikeda, 1996, 1999). Although the mechanism of this fast retrieval pathway is less clear, in *Drosophila* all endocytosis is dynamin dependent (Koenig et al., 1983), so any fast pathway must utilize a fuse-pinch-linger type of mechanism (fuse-pinch-linger is described below).

The majority of evidence for a faster type of vesicle retrieval comes mainly from electrophysiological measurements of secretory granule release. Although secretory granules also undergo complete collapse into the plasma membrane, on occasion they fuse transiently with the plasma membrane via a narrow fusion pore. This narrow fusion pore can stay open for several seconds, which is plenty of time for the full contents of the vesicle to be released [reviewed by (Artalejo et al., 1998; Palfrey and Artalejo, 1998)]. Recently, imaging studies have also demonstrated that secretory granules in PC12 cells sometimes do not collapse into the plasma membrane after fusion. Rather, after exocytosis, these vesicles are retrieved intact and remain in place (Holroyd et al., 2002; Taraska et al., 2003). A portion of this intact retrieval occurs via a mechanism that utilizes the protein dynamin (Holroyd et al., 2002). Thus, this pathway seems to be mediated by regulation of the fission pore rather than just a reversal of the fusion pore.

The dynamin-dependent, fast retrieval of an intact vesicle that remains in place after exocytosis, has been called fuse-pinch-linger [reviewed by (Ryan, 2003)]. A second, even faster mode of retrieval, called kiss-and-run, has also been proposed. Kiss-and-run is thought to be a simple reversal of the fusion pore; therefore it would not be dependent on dynamin [reviewed by (Ryan, 2003)]. Under standard conditions, kiss-and-run (as measured by electrophysiological recordings) occurs very rarely in both secretory granules and synaptic-like microvesicles (Ales et al., 1999; Klyachko and Jackson, 2002).

In *Drosophila*, all synaptic vesicle endocytosis depends on dynamin (Koenig et al., 1983). Thus, in *Drosophila* a kiss-and-run mechanism of retrieval does not occur.

Additional evidence for a fast recycling pathway comes from imaging studies examining the rate of uptake or release of endocytic tracers into or out of synaptic vesicles during stimulation. The bulk of these studies have been conducted on hippocampal synapses where synaptic vesicles have been shown to recycle intact, without going through an endosomal intermediate (Murthy and Stevens, 1998). These studies suggest the existence of a fusion pore that is so transient, fluorescent dye does not have time to fully escape from labeled vesicles into the extracellular fluid or be taken up from the extracellular fluid into endocytosed vesicles (Klingauf et al., 1998; Stevens and Williams, 2000; Verstreken et al., 2002; Aravanis et al., 2003b; Aravanis et al., 2003a).

Theoretically, fluorescent dye can escape from synaptic vesicles either via departitioning into the extracellular fluid or via lateral diffusion into the plasma membrane. The time constant of lateral diffusion is predicted to be ~10 ms (Zenisek et al., 2002). Thus, if tracers were able to diffuse laterally out of the vesicles into the plasma membrane, fusion and recapture that did not permit dye release would have to occur with a very rapid time course via a kiss-and-run mechanism. However, optical studies on dye release from vesicles show that this speed is not achieved (Aravanis et al., 2003a). The fastest time constant reported for endocytosis of a single vesicle at hippocampal synapses is ~ 420-860 ms (measured via optical recordings) (Gandhi and Stevens, 2003) and 56 ms for a single vesicle in the calyx of Held (measured via capacitance recordings) (Sun et al., 2002), [see however, Yamashita et al., (2005)]. Thus, it appears as though lateral diffusion does not occur. It may be that the presence of a

proteinaceous fusion pore restricts lateral diffusion (Han et al., 2004). Consistent with this hypothesis, strong evidence supports that syntaxin lines the fusion pore (Han et al., 2004; Han and Jackson, 2005).

One study provides functional evidence supporting the theory that *Drosophila* utilize a fast endocytic pathway (Verstreken et al., 2002). Endophilin is an accessory protein involved in clathrin mediated endocytosis [reviewed by (Huttner and Schmidt, 2002)]. Nerve terminals in *Drosophila* endophilin mutants are severely depleted of synaptic vesicles (Guichet et al., 2002; Verstreken et al., 2002). However, a small cluster of synaptic vesicles persists at active zones in the endophilin knockouts (Verstreken et al., 2002). This cluster of vesicles is enough to maintain neurotransmitter release at control levels during low frequency stimulation (Guichet et al., 2002; Verstreken et al., 2002), and persistent neurotransmitter release (albeit at levels well below controls) during high frequency stimulation (Verstreken et al., 2002).

In order to maintain release during high frequency stimulation, this small population of synaptic vesicles at the active zone must undergo numerous rounds of endocytosis. This population of synaptic vesicles is resistant to loading with FM 1-43, suggesting that these vesicles are retrieved from the membrane too quickly to allow the dye to enter them (Verstreken et al., 2002). Although the ability of endophilin mutants to maintain neurotransmitter release at control levels during high frequency stimulation is severely impaired, synaptic transmission is normal in endophilin mutants during low frequency stimulation (Verstreken et al., 2002). Yet the terminals of these mutants contain only a small population of synaptic vesicles surrounding active zones (Guichet et al., 2002; Verstreken et al., 2002). Together, these data support the hypothesis that the

active zone population is capable of sustaining low frequency release and is internalized via a fuse-pinch-linger mechanism; whereas the non-active zone pathway maintains neurotransmitter release during high frequency stimulation and is retrieved via the classic, fuse-and-collapse mechanism of endocytosis.

Data from *shibire* flies is consistent with this. After nerve terminals are depleted and synaptic vesicles are allowed to reform in *shibire* mutants, the active zone population of synaptic vesicles reforms first (Koenig and Ikeda, 1996, 1999). Evoked release recovers at approximately the same time as the active zone population, before the reserve pool has had a chance to reform. During this time, enough transmitter is available to sustain neurotransmitter release during 20Hz stimulation (Koenig and Ikeda, 1999).

A faster recycling pathway utilized by the RRP and a slower recycling pathway utilized by the RP is also suggested to occur at frog neuromuscular junctions. After depletion of the RRP (~20% of all vesicles) by high frequency stimulation, it refills via synaptic vesicles recaptured directly from the plasma membrane (not vesicles mobilized from the RP) in about one min. The RP refills much more slowly (with a half-time of ~eight min) from vesicles that bud from cisternae and surface membrane infoldings (Richards et al., 2003). Furthermore, during low frequency stimulation, neurotransmitter release is, at least partially, maintained by recycled synaptic vesicles (Richards et al., 2003). The endocytic mechanism used to maintain the RRP at the frog neuromuscular junction seems to involve direct recapture from the plasma membrane. No evidence of a transient fusion pore that opens and closes too fast for dye uptake or release has been found at this synapse (Wu and Betz, 1996; Rizzoli and Betz, 2004).

B. Speed of Endocytosis

As discussed above, the speed of endocytosis may depend on the mode of synaptic vesicle recycling utilized. Nonetheless, numerous studies have attempted to measure the maximum rate of endocytosis in various synapses, and I will address the results of some of these studies here.

The maximum internalization rate of a single vesicle has either been measured directly or can be roughly estimated from data, in a number of different synapses. In hippocampal synapses, optical measurements suggest that a single vesicle can internalize with a time constant of ~420-860 ms (Gandhi and Stevens, 2003). *Drosophila* neuromuscular junctions can maintain release of up to ~1000 quanta/sec (Delgado et al., 2000). Assuming ~550 active zones (Atwood et al., 1993), this gives a maximum recycling rate of ~550 ms/vesicle/active zone. As this rate includes endocytosis as well as all the other steps involved in synaptic vesicle recycling, the actual rate of internalization must be faster than 550 ms/vesicle. In frog neuromuscular junctions, FM 1-43 uptake experiments suggest that a single synaptic vesicle can endocytose with a time constant of ~300 ms (Wu and Betz, 1996). Capacitance measurements at goldfish retinal bipolar cells suggest that the time constant of endocytosis for a single vesicle can be as fast as ~60 ms (von Gersdorff and Matthews, 1994). Capacitance measurements at calyx of Held synapses have demonstrated that endocytosis of a single vesicle can occur with a similarly fast time constant (56 ms). However, at these same synapses when more than a single vesicle is released, the endocytic time constant for a single vesicle becomes ~800 ms (Sun et al., 2002). Recently, the extremely fast endocytic time constant measured at calyx of Held synapses has been called into question (Yamashita et al., 2005). This

extremely fast time constant may be an artifact of capacitance recordings because it seems to be unrelated to synaptic transmission; it is also observed when transmitter release is abolished by treating the cells with botulinum neurotoxin E (Yamashita et al., 2005). Thus, the time constant of ~800 ms may reflect a more accurate measurement for the maximum rate of single vesicle endocytosis in calyx of Held synapses.

In summary, the time constant of endocytosis for a single synaptic vesicle is estimated to be less than one second at many synapses. Constitutive and/or ligand-stimulated clathrin-mediated recycling is thought to take several minutes. The slow nature of this process has been an argument against synaptic vesicle recycling utilizing clathrin-mediated endocytosis. However, clathrin-mediated endocytosis of synaptic vesicles has been shown to require the presence of numerous accessory factors [reviewed by (Brodin et al., 2000; Slepnev and De Camilli, 2000)]; and synaptic vesicle recycling may bypass the need to fuse with early/sorting endosomes (Takei et al., 1996; Murthy and Stevens, 1998). Both of these factors may speed up the clathrin-mediated recycling of synaptic vesicles. Furthermore, in a frog nerve terminal that synapses onto muscle, enough clathrin material is available to form ~30,000 clathrin coated structures (Torri-Tarelli et al., 1987). The synaptic vesicle recycle time has been estimated to be between 75-90 sec in this preparation [reviewed by (Betz and Wu, 1995)]. Thus, if all synaptic vesicle endocytosis occurred via clathrin, ~300-400 synaptic vesicles could recycle per second. Assuming ~300 active zones per terminal, this gives an endocytic rate of ≤1 vesicle/sec/active zone. Thus, clathrin-mediated endocytosis could also be the mechanism that underlies very fast synaptic vesicle endocytosis.

One consistent finding among various synapses is that the time required to complete synaptic vesicle internalization increases with the amount of membrane awaiting endocytosis (von Gersdorff and Matthews, 1994; Ryan et al., 1996a; Wu and Betz, 1996; Klingauf et al., 1998; Stevens and Wesseling, 1999; Pyle et al., 2000; Richards et al., 2000; Sankaranarayanan and Ryan, 2000; Sun et al., 2002; Richards et al., 2003). For example, at frog neuromuscular junctions, refilling the RRP (~20% of the entire vesicle pool) once it has been depleted takes ~60 sec; whereas refilling the RP occurs with a half-time of ~8 min (Richards et al., 2003). In hippocampal synapses, when release is evoked by 10 action potentials at 10 Hz, the time course of endocytosis for the population of vesicles remaining fused to the plasma membrane after the stimulation stops is ~four sec. When release is evoked by 600 action potentials at 10 Hz, the time course of endocytosis for the population of vesicles remaining fused after the stimulation stops is ~90 sec (Sankaranarayanan and Ryan, 2000).

Because the time required to complete synaptic vesicle internalization increases with the amount of membrane awaiting endocytosis, it has been suggested that the machinery responsible for endocytosis operates at a fixed rate but with a limited capacity. Thus, the reason that the time constant increases with increased release is because the endocytic machinery becomes saturated (Sankaranarayanan and Ryan, 2000). At frog neuromuscular junctions, a maximum of ~30,000 coated structures can exist at any one time (Torri-Tarelli et al., 1987). Thus, clathrin may be the limiting factor in synaptic vesicle endocytosis. If endocytosis operates at a fixed rate but with a limited capacity, the same initial velocity of retrieval (number of vesicles per second) should be seen following multiple or single vesicle fusions. One study examining endocytosis at rat

cultured hippocampal synapses provides evidence to support this model. When release was evoked by 10 Hz stimulation, and the time constant of endocytosis was measured for the population of vesicles that remained fused after the stimulation stopped, endocytosis behaved as a saturable process and the initial velocity of internalization remained nearly constant (Sankaranarayanan and Ryan, 2000). The maximal endocytic rate was calculated to be between 0.33 and 1 vesicle per second (Sankaranarayanan and Ryan, 2000). However, another study using hippocampal synapses suggests that internalization of a single vesicle can occur with at least three distinct rates: 1) fast (420-860 ms), 2) slow (8-21 sec), and 3) stranded (vesicle membrane remains on the surface until a subsequent nerve impulse triggers its retrieval) (Gandhi and Stevens, 2003). Unlike the previous study, in this study endocytosis was measured after only a single vesicle was induced to fuse. Thus, when a train of action potentials induces synaptic vesicle fusion, subsequent endocytosis appears to occur at a fixed rate with a limited capacity. However, when only a single synaptic vesicle is evoked, there may be multiple modes of internalization.

III. Synaptotagmin

Synaptotagmin I is a transmembrane protein that makes up 7-8% of total synaptic vesicle protein (Matthew et al., 1981; Chapman and Jahn, 1994). Synaptotagmin is known to play a role in the synaptic vesicle cycle. Genetic studies in *C. elegans* (Nonet et al., 1993), mice (Geppert et al., 1994; Fernández-Chacón et al., 2001; Voets et al., 2001; Shin et al., 2003; Stevens and Sullivan, 2003; Nishiki and Augustine, 2004a, b) and *Drosophila* (DiAntonio et al., 1993; Littleton et al., 1993b; Broadie et al., 1994;

DiAntonio and Schwarz, 1994; Littleton et al., 1994; Littleton et al., 2001a; Loewen et al., 2001; Mackler and Reist, 2001; Mackler et al., 2002; Robinson et al., 2002; Yoshihara and Littleton, 2002) have shown that synaptotagmin is required for efficient synaptic transmission. Although synaptic transmission persists in syt^{null} mutants (DiAntonio et al., 1993; Broadie et al., 1994; Geppert et al., 1994; Loewen et al., 2001; Voets et al., 2001; Mackler et al., 2002; Yoshihara and Littleton, 2002; Shin et al., 2003; Nishiki and Augustine, 2004b), it is severely altered. However, synaptotagmin's exact role in the synaptic vesicle cycle is unclear. It has been proposed to function in docking synaptic vesicles at active zones, priming synaptic vesicles for fusion, sensing the Ca^{2+} required for fusion, stabilizing the fusion pore, and endocytosis.

A. Protein Structure

Clues to synaptotagmin's role in the synaptic vesicle cycle may be found in its protein structure. Synaptotagmins constitute a large family of membrane-trafficking proteins [reviewed by (Südhof, 2002)]. There are at least 7 synaptotagmin isoforms in *Drosophila* (Adolfsen et al., 2004) and 15 in mammals [reviewed by (Südhof, 2002)]. Synaptotagmin I, the most conserved synaptotagmin (Dai et al., 2004), contains six domains [Fig. 1.2, and (Perin et al., 1991a)]: 1) an intravesicular amino-terminal domain that is glycosylated (IV), 2) a single transmembrane region (TMR), 3) a sequence that separates the transmembrane region from the two C_2 domains, 4 & 5) two C_2 domains, (C_2A and C_2B - repeats that are homologous to the regulatory C_2 region of protein kinase C), and 6) a conserved, carboxyl-terminal sequence following the C_2B domain

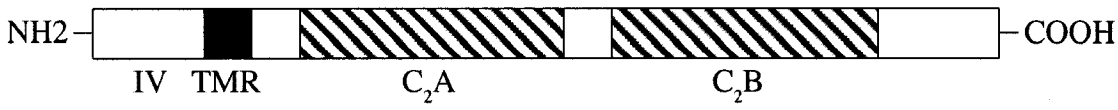

Figure 1.2 Domain structure of Synaptotagmin. TMR, transmembrane region. IV, intravesicular domain. C_2A and C_2B, two C_2 domains.

The crystal structure of synaptotagmin I's isolated C_2A domain (Sutton et al., 1995) and of synaptotagmin III's double C_2 domains (C_2AB) (Sutton et al., 1999) show that both C_2 domains form a stable, eight-stranded, β-sandwich with flexible loops emerging from the top and bottom (Fig. 1.3A). The β-sandwich is formed by two, four-stranded, anti-parallel, β-sheets. Both C_2 domains bind Ca^{2+} ions exclusively at the tip of the sandwich in a cuplike cavity formed by the top loops connecting β2 with β3 and β6 with β7. In each domain, the bound Ca^{2+} ions are partially coordinated by five, highly conserved, acidic residues that reside in the two flexible loops [Fig. 1.3B, and (Sutton et al., 1995; Shao et al., 1996; Ubach et al., 1998; Sutton et al., 1999; Fernandez et al., 2001)]. NMR studies show that the C_2A domain binds three Ca^{2+} ions (Ubach et al., 1998), and that the C_2B domain binds only two, due to its lack of a serine side chain in loop three (Fernandez et al., 2001).

Ca^{2+}-binding to synaptotagmin does not cause a major conformational change, but instead changes synaptotagmin's electrostatic potential (Shao et al., 1998). Thus it is thought that Ca^{2+} binding to synaptotagmin acts as an "electrostatic switch" for neurotransmitter release by regulating synaptotagmin's molecular interactions with presynaptic molecules (Shao et al., 1997; Shao et al., 1998; Ubach et al., 1998; Zhang et al., 1998b; Sutton et al., 1999). Which molecules interact with Ca^{2+}-bound

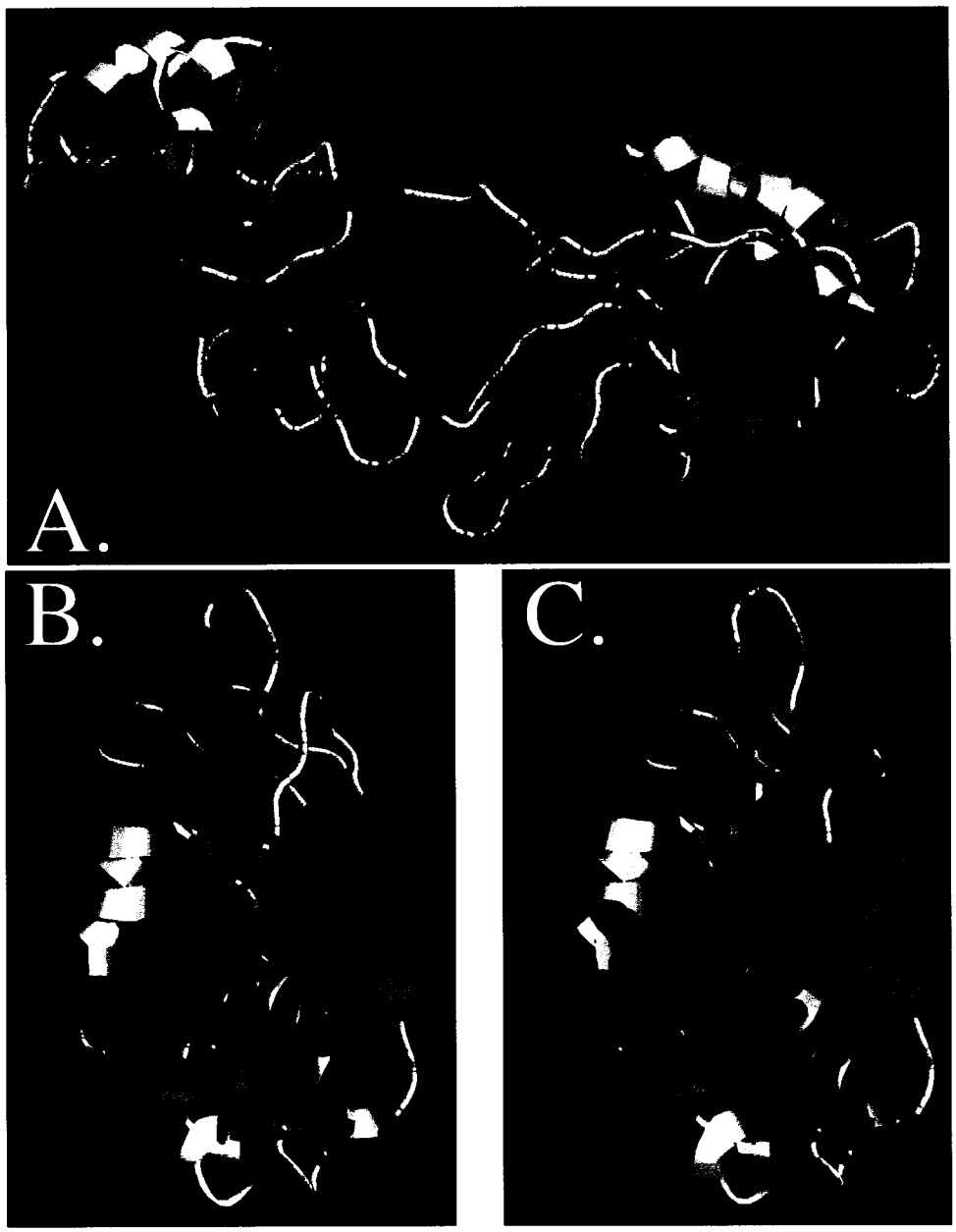

Figure 1.3 (A) 2-D projection of 3-D structure of the cytoplasmic C_2A and C_2B domains of rat synaptotagmin III. Green circles represent bound Ca^{2+} ions. The C_2A domain binds three Ca^{2+} ions, but only two are shown. The C_2B domain binds two Ca^{2+} ions, but only one is shown. (B) 2-D projection of the C_2B domain of rat synaptotagmin I. The five acidic residues that mediate Ca^{2+}-binding are shown in red and purple. Red aspartates were mutated to asparagines in C_2B Ca^{2+}-binding motif mutants. (C) Same projection as in B, but polylysine motif residues are shown. Purple lysines were mutated to glutamines in C_2B polylysine motif mutants. Cyan residues are also lysines. Green residue is an arginine. Generated from PDF files [1DQV, (A); 1tjx, (B) and (C)] using the public domain RasMol program (http://www.umass.edu/microbio/rasmol/).

synaptotagmin and how these interactions participate in synaptic vesicle fusion remain a major unanswered questions in synaptic transmission.

In addition to the Ca^{2+}-binding motif in the C_2A and C_2B domains, synaptotagmin I also contains a polylysine motif in the fourth strand of the C_2B β-sandwich (Fig. 1.3C). This motif imparts a patch of positively charged residues on the C_2B domain. Despite the fact that the entire synaptotagmin sequence is only ~50-65% conserved, the polylysine motif is virtually identical in all synaptotagmin isoforms among various species (Rickman et al., 2004c). Such a high level of conservation suggests that the motif plays an important role in synaptotagmin function. Specific lysine residues within this region are critical for synaptic transmission. When three lysines (379, 380 and 384) in *Drosophila* synaptotagmin I are mutated to glutamines, evoked transmitter release is decreased by ~40% (Mackler and Reist, 2001).

Although the function of the polylysine motif in synaptic transmission is unknown, numerous biochemical interactions have been mapped to this motif. The first two of these lysines are critical for synaptotagmin's *in vitro* binding to the 'synprint' (see page 63) region of N-and P/Q type Ca^{2+} channels, AP-2, other molecules of synaptotagmin (Chapman et al., 1998), and PIP_2-containing membranes (Bai et al., 2004a). All three lysines have been implicated in synaptotagmin's *in vitro* binding to members of the inositol high polyphosphate series (Fukuda et al., 1995), and syntaxin/SNAP-25 heterodimers (Rickman et al., 2004c). The polylysine motif has also been shown to bind to β-SNAP (Schiavo et al., 1995). As this motif is implicated in numerous *in vitro* interactions, the decrease in evoked transmitter release seen in

polylysine motif mutants (Mackler and Reist, 2001) could be caused by a disruption of any or all of these interactions.

B. Synaptotagmin's Role in Ca^{2+}-Sensing

Synaptotagmin was first proposed to function as a Ca^{2+}-sensor for neurotransmitter release when it was found to bind Ca^{2+} at physiological levels in a complex with negatively charged phospholipids (Brose et al., 1992). Since then, numerous biochemical studies have confirmed this interaction (Davletov and Südhof, 1993; Chapman and Jahn, 1994; Li et al., 1995a; Chae et al., 1998; Chapman and Davis, 1998; Zhang et al., 1998b; Davis et al., 1999; Bai et al., 2000; Earles et al., 2001; Fernandez et al., 2001; Bai et al., 2002; Mackler et al., 2002). In addition to binding phospholipids, synaptotagmin also undergoes Ca^{2+}-stimulated interactions with: syntaxin (Chapman et al., 1995; Li et al., 1995b; Schiavo et al., 1997; Shao et al., 1997; Davis et al., 1999; Earles et al., 2001; Robinson et al., 2002; Shin et al., 2003; Bai et al., 2004b), snap-25 (Schiavo et al., 1997; Gerona et al., 2000; Earles et al., 2001; Zhang et al., 2002; Shin et al., 2003; Bai et al., 2004b), t-SNARE heterodimers (Gerona et al., 2000; Tucker et al., 2003; Bai et al., 2004b), complete SNARE complexes (Davis et al., 1999; Earles et al., 2001; Zhang et al., 2002; Bai et al., 2004b), other molecules of synaptotagmin (Wu et al., 2003), and PIP_2 containing membranes (Schiavo et al., 1996). Thus, synaptotagmin has the potential to link the Ca^{2+} influx triggered by an action potential with molecular rearrangements between itself on the synaptic vesicle and proteins and/or phospholipids in the plasma membrane.

At least two kinetically distinct components of neurotransmitter release have been identified at both neuromuscular junctions and central synapses (Miledi and Orkand, 1966; Meiri and Rahamimoff, 1971; Barrett and Stevens, 1972; Goda and Stevens, 1994; Atluri and Regehr, 1998). In the first component, the rate of neurotransmitter release is fast, and release occurs nearly "synchronously" with the stimulus. This fast, synchronous release component comprises the majority of evoked release. In the second component, the rate of neurotransmitter release is slower and occurs "asynchronously" with the stimulus. This slower, asynchronous component of release is triggered by a mechanism that exhibits higher Ca^{2+} affinity than the mechanism that triggers the fast, synchronous component of release. These two distinct components of release led to the hypothesis that at least two Ca^{2+}-sensors mediate neurotransmitter release. One is thought to be a low affinity, fast sensor that mediates the majority of evoked release, the synchronous component. The second is thought to be a high affinity, slow sensor that mediates the slow, asynchronous component of release.

Synaptotagmin I is proposed to be the low affinity, fast Ca^{2+}-sensor. syt^{null} mutations in mice (Geppert et al., 1994; Voets et al., 2001; Shin et al., 2003; Nishiki and Augustine, 2004b) and *Drosophila* (Broadie et al., 1994; Loewen et al., 2001; Mackler et al., 2002; Yoshihara and Littleton, 2002) appear to selectively abolish, or at least severely decrease, the fast, synchronous component of release.

Strong evidence supports the hypothesis that synaptotagmin I is the main Ca^{2+}-sensors for fast, synchronous release. In *Drosophila*, when two of the Ca^{2+}-binding acidic residues in synaptotagmin's C_2B domain (D416, D418, Fig. 1.3B) are mutated to asparagines (syt^{DN}) and the mutant protein is expressed as a transgene in the

synaptotagmin null (syt^{null}) background (*-/-;P[syt^{DN}]*), the apparent Ca^{2+}-affinity of synaptic transmission is reduced and Ca^{2+}-triggered, synchronous neurotransmitter release is almost entirely eliminated (>95% decrease) (Mackler et al., 2002). These experiments demonstrate that synaptotagmin's ability to bind Ca^{2+} by the C_2B domain is critical for synaptic transmission. In addition, a point mutation in the C_2A domain of rat synaptotagmin, R233Q, decreases the apparent Ca^{2+} affinity of synaptotagmin for phospholipids by ~twofold and also lowers the Ca^{2+} sensitivity of neurotransmitter release by ~twofold (Fernández-Chacón et al., 2001). These experiments demonstrate that Ca^{2+}-dependent phospholipid binding by the C_2A domain is also critical for synaptic transmission. Together these studies strongly support the hypothesis that synaptotagmin is the main Ca^{2+}-sensor for fast, synchronous neurotransmitter release.

Although syt^{null} mutations appear to severely decrease the fast, synchronous component of release, studies in mouse cultured hippocampal synapses and *Drosophila* embryo neuromuscular junctions show that the slow, asynchronous component of release is increased in syt^{null} mutants. Indeed, in syt^{null} mutants the slow, asynchronous component appears to provide an absolute level of neurotransmitter release similar to that recorded in wild-type synapses (Yoshihara and Littleton, 2002; Shin et al., 2003; Nishiki and Augustine, 2004b). Thus, synaptotagmin may act as a catalyst for fast, synchronous release. In this case, the increase in asynchronous release seen syt^{null} mutants would be mediated by the high affinity, slow Ca^{2+}-sensor. In this model, when synaptotagmin is present, few vesicles are released by the high affinity, slow sensor because these vesicles instead get released by synaptotagmin, the low affinity, fast sensor. Consequently, asynchronous release is increased in syt^{null} mutants because vesicles are not released by

synaptotagmin, so they are available to be released by the second, high affinity, slow sensor.

One possible molecular mechanism underlying synaptotagmin's ability to catalyze fast, synchronous release could involve synaptic vesicle priming via formation of *trans*-SNARE complexes. In synaptotagmin knockouts, the fast component of the burst phase of DCG release is selectively abolished (Voets et al., 2001). This has lead to the hypothesis that synaptotagmin facilitates formation of "tight" *trans*-SNARE complexes. Formation of "tight" *trans*-SNARE complexes may speed up membrane fusion. For example, "tight" *trans*-SNARE complexes could from a semi-stable, hemi-fusion intermediate [Fig. 1.1, and reviewed by (Jahn et al., 2003)]. This intermediate might progress to a full fusion more rapidly than fusion from a priming step that didn't include this hemi-fused intermediate. Furthermore, synaptotagmin may also speed up fusion via its Ca^{2+}-dependent membrane penetration [Fig. 1.1, and (Chae et al., 1998; Chapman and Davis, 1998; Davis et al., 1999)]. Thus, synaptotagmin's ability to catalyze fast, synchronous release may be explained by its ability to promote "tight" *trans*-SNARE complexes and undergo Ca^{2+}-dependent membrane penetration. In the absence of synaptotagmin, vesicle priming may only result in the formation of "loose" *trans*-SNARE complexes. As DCG fusion can occur via either "tight" or "loose" *trans*-SNARE complexes, the asynchronous release recorded in syt^{null} mutants may be release that is mediated by these "loose" *trans*-SNARE complexes.

Whether asynchronous release is increased in *Drosophila* syt^{null} third instars has not been examined. Indeed, distinguishing between evoked asynchronous release and non-evoked spontaneous release may be difficult at this developmental stage, as

spontaneous release is increased ~threefold in *syt*^{null} larvae compared to controls (Loewen et al., 2001). An increase in spontaneous release has also been reported for *syt*^{null} embryos (Broadie et al., 1994); although another study on *syt*^{null} embryos did not see such an increase (Yoshihara and Littleton, 2002). The increased rate of spontaneous fusion seen in some preparations has lead to the hypothesis that in addition to facilitating evoked release, synaptotagmin may also function as a "brake" for spontaneous release [reviewed by (Popov and Poo, 1993)]. Alternatively, an increase in spontaneous release may be a compensatory response to the severe decrease in evoked neurotransmitter releases that occurs in *syt*^{null} mutants.

If synaptotagmin I is the Ca^{2+}-sensor for fast, synchronous release, what it the Ca^{2+}-sensor for slow, asynchronous release? One appealing hypothesis is that other synaptotagmin isoforms with different Ca^{2+}-binding properties function as Ca^{2+}-sensors for the slow component of release [reviewed by (Südhof, 2004)]. Indeed, the synaptotagmin gene family has at least 15 members in vertebrates [reviewed by (Südhof, 2004)] and seven in *Drosophila* (Adolfsen et al., 2004). Many of these have different Ca^{2+}-binding properties (Sugita et al., 2001; Sugita et al., 2002; Shin et al., 2004). Supporting the hypothesis that other isoforms of synaptotagmin mediate Ca^{2+}-dependent asynchronous release, when synaptotagmin peptide fragments are incubated with permeablized PC12 cells, fragments exhibiting higher Ca^{2+} affinities potently inhibit release at Ca^{2+} concentrations were the C_2 domain of synaptotagmin 1 is inactive (Sugita et al., 2001; Sugita et al., 2002).

Whether or not other isoforms of synaptotagmin mediate Ca^{2+}-dependent asynchronous release in *Drosophila* is still unknown. In *Drosophila,* synaptotagmin I is

reported to be the only synaptotagmin isoform found in *Drosophila* presynaptic nerve terminals (Adolfsen et al., 2004). This same study demonstrated that synaptotagmin IV is located only in the postsynaptic cell. However, this analysis was done at the light level, based on the location of fluorescent signals. Unfortunately, the resolution limits of light microscopy make distinguishing between pre- and postsynaptic localization difficult. Thus, it remains possible that synaptotagmin IV is found in both the pre- and postsynaptic cell. Furthermore, whether or not the severe decrease in synaptic transmission recorded in *Drosophila* syt^{null} mutants can be rescued by expression of the synaptotagmin IV isoform is unclear. One study reports that this isoform can rescue evoked transmitter release (Robinson et al., 2002) and another reports that it cannot (Adolfsen et al., 2004). Thus, whether or not other synaptotagmin isoforms mediate asynchronous release is still unclear.

Another intriguing possibility is that the assembled SNARE complex itself may function in Ca^{2+}-sensing, as it contains several potential Ca^{2+}-binding sites (Fasshauer et al., 1998a). These binding sites could form a slow Ca^{2+}-sensor that synaptotagmin could convert into a fast one, potentially by one or more of synaptotagmin's numerous molecular interactions (Voets et al., 2001).

In summary, strong evidence supports that synaptotagmin I is the main Ca^{2+}-sensor for fast, synchronous release. However, whether synaptotagmin acts solely as a catalyst for fast, synchronous release or additionally as a brake for neurotransmitter release has not been clearly resolved. As Ca^{2+}-dependent, asynchronous release persists in syt^{null} mutants, at least one other Ca^{2+}-sensor must exist. Two appealing hypotheses are that Ca^{2+}-sensing for asynchronous release is mediated by other isoforms of

synaptotagmin with differing Ca^{2+} sensitivities, or that it is mediate by SNARE complexes themselves. However, evidence supporting either of these hypotheses is lacking.

C. Synaptotagmin's Molecular Interactions

As the main Ca^{2+}-sensor for neurotransmitter release, synaptotagmin must link Ca^{2+} influx to synaptic vesicle fusion. However, how Ca^{2+}-bound synaptotagmin mediates fusion is still a major unanswered question in synaptic transmission. Synaptotagmin's ability to undergo Ca^{2+}-stimulated molecular interactions is thought to be a critical component of its Ca^{2+}-sensing role. Thus, it is important to identify synaptotagmin's interacting molecules, understand how Ca^{2+} affects the interactions, and map the binding sites in both synaptotagmin and its partners. In the next section, I will review what is currently known regarding synaptotagmin and its molecular interactions in the context of how these interactions may mediate synaptic vesicle fusion.

1. Synaptotagmin's Interaction with Phospholipids

Synaptotagmin's interaction with negatively charged phospholipids was the first of many Ca^{2+}-dependent interactions attributed to this protein (Brose et al., 1992). Ca^{2+}-dependent phospholipid binding is the most characteristic property of C_2 domains [reviewed by (Rizo and Südhof, 1998)]. This capability was first mapped to synaptotagmin's C_2A domain (Davletov and Südhof, 1993; Chapman and Jahn, 1994). The binding between C_2A and negatively charged phospholipids is highly electrostatic (Chae et al., 1998; Chapman and Davis, 1998; Zhang et al., 1998b). Basic residues that

reside in loop one and three, surrounding the Ca^{2+}-binding pocket, mediate synaptotagmin's interaction with negatively charged phospholipids (Chae et al., 1998; Zhang et al., 1998b; Fernández-Chacón et al., 2001). The negatively charged phospholipid headgroups interact with these positively charged residues and with the bound Ca^{2+} ions, thereby filling Ca^{2+}'s empty coordination sites (Ubach et al., 1998; Zhang et al., 1998b; Fernández-Chacón et al., 2001). Completion of Ca^{2+}'s coordination sites by negatively charged phospholipids greatly increases synaptotagmin's affinity for Ca^{2+}, which is intrinsically quite low (Ubach et al., 1998; Zhang et al., 1998b; Fernández-Chacón et al., 2001). Thus, disrupting Ca^{2+}-binding by C_2A abolishes its phospholipid binding (Bai et al., 2000). *In vitro* studies show that a hydrophobic residue in loop three actually inserts into the plasma membrane in the presence of Ca^{2+} and may contribute to phospholipid binding (Chae et al., 1998; Chapman and Davis, 1998; Davis et al., 1999).

The Ca^{2+}-binding pocket in C_2B is very similar to that of C_2A. It is also surrounded by basic residues and contains hydrophobic side chains similar to those found in C_2A's Ca^{2+}-binding pocket (Ubach et al., 1998; Sutton et al., 1999; Fernandez et al., 2001). Thus, it would be surprising if the C_2B domain did not also undergo Ca^{2+}-dependent phospholipid binding; and indeed, in *Drosophila* it does (Mackler et al., 2002). However, whether or not the isolated C_2B domain undergoes Ca^{2+}-dependent phospholipid binding in rat is still debated. While some experiments demonstrate no binding (Schiavo et al., 1996; Bai et al., 2000; Earles et al., 2001; Wu et al., 2003), others do show Ca^{2+}-dependent binding, although to a lesser extent than the isolated C_2A domain (Brose et al., 1992; Fernandez et al., 2001). Reduced phospholipid binding by the isolated C_2B domain may be because this domain only binds two Ca^{2+} ions, whereas

the C_2A domain binds three (Ubach et al., 1998; Fernandez et al., 2001). Recombinant synaptotagmin purified by standard procedures has been shown to contain bacterial contaminants (Ubach et al., 1998). These contaminants may inhibit phospholipid binding by C_2B and explain why some studies have not shown binding. One study reports that properly purified C_2B domains from rat do exhibit Ca^{2+}-dependent phospholipid binding (Fernandez et al., 2001); however, another reports that they do not (Wu et al., 2003). Finally, some studies show that both C_2 domains are necessary for high affinity, Ca^{2+}-dependent phospholipid binding (Earles et al., 2001; Bai et al., 2002). In *Drosophila*, both of the isolated C_2 domains, as well as the entire cytoplasmic domain, bind phospholipids in the presence of Ca^{2+} equally well (personal communication, Ian Robinson).

Role of Phospholipid Binding in the Synaptic Vesicle Cycle

The Ca^{2+}-dependent interaction between synaptotagmin and negatively charged phospholipids is likely critical for synaptic transmission. Disruption of phospholipid binding by a point mutation in the C_2A domain (Fernández-Chacón et al., 2001) or by mutations in the C_2B domain (Mackler et al., 2002) significantly disrupts neurotransmitter release. However, although these synaptic transmission defects correlate well with disrupted phospholipid binding, they may also be due to other disrupted Ca^{2+}-dependent interactions that have not yet been tested in these mutants.

It is likely that synaptotagmin's electrostatic interaction with phospholipids via acidic residues and Ca^{2+}, as well as its direct penetration of phospholipid membranes by a hydrophobic residue in both C_2 domains, directly influences the lipid transition states

during fusion. This by itself may be sufficient to trigger fusion pore opening (Cevc and Richardsen, 1999). As discussed above, studies on the small, soluble protein complexin suggest that synaptotagmin may act to trigger fusion after SNARE complexes have formed [reviewed by (Jahn et al., 2003)]. The formation of *tr*ans-SNARE complexes that are stabilized by complexin proteins, may create a meta-stable, hemi-fusion intermediate. Ca^{2+}-triggered phospholipid binding by synaptotagmin, and the subsequent insertion of two hydrophobic residues of synaptotagmin into the plasma membrane, may destabilize the fusion intermediate and trigger fusion pore opening [Fig. 1.1, and reviewed by (Jahn et al., 2003)].

2. Synaptotagmin's Interactions with SNARES

Biochemical experiments have shown that synaptotagmin does not bind synaptobrevin/VAMP directly (Schiavo et al., 1997). However, many *in vitro* studies using recombinant proteins demonstrate that synaptotagmin is able to interact directly with both syntaxin (Bennett et al., 1992; Chapman et al., 1995; Li et al., 1995b; Kee and Scheller, 1996; Schiavo et al., 1997; Shao et al., 1997; Davis et al., 1999; Earles et al., 2001; Robinson et al., 2002; Shin et al., 2003; Bai et al., 2004b), [see however, Gerona et al., (2000)] and SNAP-25 (Schiavo et al., 1997; Gerona et al., 2000; Earles et al., 2001; Zhang et al., 2002; Shin et al., 2003; Bai et al., 2004b).

Syntaxin and SNAP-25 are known to form complexes (Chapman et al., 1994) consisting of either heterodimers (Rickman et al., 2004a) or heterotrimers [see page 3, and (Fasshauer et al., 1997a; Margittai et al., 2001; Xiao et al., 2001)]. Structural studies indicate that formation of the SNARE complex may begin with the assembly of a

syntaxin/SNAP-25 complex, to which synaptobrevin/VAMP subsequently binds (Fasshauer et al., 1997a; Sutton et al., 1998; Fiebig et al., 1999; Fasshauer et al., 2002). Synaptotagmin also binds this syntaxin/SNAP-25 complex, which I will refer to as a t-SNARE complex (Gerona et al., 2000; Rickman and Davletov, 2003; Tucker et al., 2003; Bai et al., 2004b; Rickman et al., 2004c). Indeed, the ability of synaptotagmin to bind t-SNARE complexes is synaptotagmin's most conserved activity, even more conserved than calcium/phospholipid binding. Many synaptotagmin isoforms that do not bind Ca^{2+} still bind t-SNARE complexes through a Ca^{2+}-independent mechanism (Rickman et al., 2004b). Finally, in addition to binding t-SNARE complexes, synaptotagmin also binds to fully assembled SNARE complexes (Schiavo et al., 1997; Davis et al., 1999; Earles et al., 2001; Zhang et al., 2002; Bai et al., 2004b).

Is Synaptotagmin's binding to SNARES Ca^{2+}-dependent?

Syntaxin

In the studies listed above, Ca^{2+} promoted the interaction between synaptotagmin and syntaxin, with two exceptions (Bennett et al., 1992; Kee and Scheller, 1996). Bennett et al. (1992) did not look at the effects of Ca^{2+}. Kee and Scheller (1996) found that Ca^{2+} *inhibited* the interaction of GST-C_2AB or GST-C_2B with soluble syntaxin. However, the interaction became Ca^{2+}-dependent when it was tested using GST-syntaxin and soluble C_2AB. It also was Ca^{2+}-dependent when GST-C_2A was used. Kee and Scheller (1996) suggested that the reason the interaction between syntaxin and synaptotagmin is Ca^{2+}-independent when synaptotagmin (C_2AB or C_2B) is fused to GST, and Ca^{2+}-dependent when syntaxin is fused to GST, may be due to steric hindrance by

the GST moiety of the closer C_2A domain, whose interaction with syntaxin is Ca^{2+}-dependent. With GST-C_2AB, steric hindrance of the C_2A domain might cause the C_2B domain and its Ca^{2+}-independent interaction to dominate the interaction. Similarly, Robinson et al. (2002) claim that the Ca^{2+}-dependent interaction they show between GST-C_2A and soluble syntaxin becomes Ca^{2+}-independent when GST-C_2AB is used.

Thus, the Ca^{2+}-dependence of the interaction between synaptotagmin and syntaxin seems to depend on which protein is attached to GST and which C_2 domain of synaptotagmin is tested. With C_2A, the interaction is always Ca^{2+}-dependent. When C_2AB or C_2B is fused to GST, the interaction is Ca^{2+}-independent. However, the interaction between C_2AB and syntaxin becomes Ca^{2+}-dependent when C_2AB is removed from GST. Thus, the interaction between syntaxin and synaptotagmin is likely to be Ca^{2+}-dependent *in vivo* where neither protein is fused to GST.

SNAP-25

Ca^{2+} has been shown to stimulate the interaction between synaptotagmin and SNAP-25 (Schiavo et al., 1997; Gerona et al., 2000; Earles et al., 2001; Zhang et al., 2002; Shin et al., 2003; Bai et al., 2004b). However, all but two (Earles et al., 2001; Bai et al., 2004b) of these studies also demonstrate lower levels of binding without Ca^{2+} [see however, Rickman and Davletov, (2003)]. In the binding studies done by Earles et al. (2001), a recombinant synaptotagmin harboring a mutation in the C_2B polylysine motif was used. If this motif provides Ca^{2+}-independent binding [as it does with t-SNARE complexes (Rickman et al., 2004c)], it would not be seen in these mutants. However, the only experiment that examined binding between purified, native, soluble synaptotagmin

and SNAP-25 showed no Ca^{2+}-independent binding (Rickman and Davletov, 2003). Thus *in vivo*, the interaction between synaptotagmin and SNAP-25 is likely mainly a Ca^{2+}-dependent one.

t-SNARE Complexes

Results from studies on the interaction between synaptotagmin and t-SNARE complexes also support the idea that the Ca^{2+}-dependence of the interaction can change depending on whether synaptotagmin is attached to GST and which domain of synaptotagmin is tested (Tucker et al., 2003). Tucker et al. (2003) show that, like with syntaxin, the interaction between C_2A and t-SNARE complexes is always Ca^{2+}-dependent. Also, the interaction between GST-C_2AB or GST-C_2B and t-SNARE complexes is Ca^{2+}-independent. However, when C_2AB is removed from GST, Ca^{2+} stimulates the amount of t-SNARE complex binding considerably, although Ca^{2+}-independent binding still does occur. The authors suggest that either the GST moiety itself or the immobilization of synaptotagmin onto beads leads to a Ca^{2+}-independent interaction between t-SNARE complexes and C_2B or C_2AB. However, this argument does not completely hold up. When C_2B is removed from GST, its binding to t-SNARE complexes remains Ca^{2+}-independent, although the amount of binding is less than that of C_2AB (Chapman et al., 1996; Tucker et al., 2003). Also, the only experiment that examined binding with purified, native, soluble synaptotagmin shows that it binds t-SNARE complexes without Ca^{2+} (Rickman and Davletov, 2003). Synaptotagmin's Ca^{2+}-independent interaction with t-SNARE complexes has been mapped to the polylysine motif in synaptotagmin's C_2B domain (Rickman et al., 2004c). Thus, it appears as if

synaptotagmin can bind to t-SNARE complexes in the absence of Ca^{2+}. However, the addition of Ca^{2+} stimulates binding.

Full SNARE Complexes

Only in the presence of Ca^{2+}, is native synaptotagmin coimmunoprecipitated from rat brain with native SNARE complexes by an anti-synaptobrevin antibody (Earles et al., 2001). Also, Ca^{2+} stimulates the coimmunoprecipitation of recombinant rat synaptotagmin with mini-SNARE complexes [the H3-containing minimal core of the SNARE complex that is solely composed of the four helix bundle and lacks all other domains (Fasshauer et al., 1998b)] by an anti-synaptobrevin antibody (Davis et al., 1999; Littleton et al., 2001b). In Davis et al. (1999), the authors state that the Ca^{2+}-dependence of synaptotagmin's interaction with SNAREs is lost a few days after purification. Unfortunately, the experiments by Littleton et al. (2001) and Davis et al. (1999) use a recombinant synaptotagmin with a G374D mutation. This mutation results in a C_2B domain that is largely unfolded (Ubach et al., 2001). As discussed above, synaptotagmin has also been shown to undergo Ca^{2+}-independent binding with t-SNARE complexes (Schiavo et al., 1997; Gerona et al., 2000; Littleton et al., 2001b; Rickman and Davletov, 2003; Rickman et al., 2004c) via the polylysine motif in the C_2B domain (Rickman et al., 2004c). Consequently, if the C_2B domain provides Ca^{2+}-independent binding of full SNARE complexes, it may not be seen in experiments using the G374D clone. So, synaptotagmin undergoes a Ca^{2+}-dependent interaction with SNARE complexes, but whether it also undergoes Ca^{2+}-independent binding is less clear.

In summary, synaptotagmin's interaction with isolated and complexed SNARES is complicated and difficult to study *in vitro* because reaction conditions greatly affect the results. Many studies that have demonstrated a Ca^{2+}-stimulated interaction between synaptotagmin and syntaxin (Chapman et al., 1995; Li et al., 1995b; Schiavo et al., 1997; Shin et al., 2003), synaptotagmin and SNAP-25 (Schiavo et al., 1997; Gerona et al., 2000; Zhang et al., 2002; Shin et al., 2003), and synaptotagmin and t-SNARE or full SNARE complexes (Gerona et al., 2000; Littleton et al., 2001b; Shin et al., 2003; Tucker et al., 2003), also show some binding in the absence of Ca^{2+}. [See however, Rickman and Davletov, (2003) and Rickman et al., (2004c), who show Ca^{2+}-independent binding only to t-SNARE heterodimers and not individual SNARES; Davis et al., (1999), who show only Ca^{2+}-dependent binding to syntaxin and SNARE complexes, but who use recombinant synaptotagmin harboring mutations in the C_2B domain; and Earles et al., (2001) and Bai et al., (2004b), who show only Ca^{2+}-dependent binding to individual SNARES and SNARE complexes.] Thus, it seems likely that synaptotagmin probably does bind SNAREs, especially t-SNARE complexes, in the absence of Ca^{2+}. However, the amount of binding may be minimal, and the addition of Ca^{2+} greatly promotes binding. If true, then the amount of protein used in binding experiments as well as the sensitivity of protein detection becomes critical; otherwise, lower levels of Ca^{2+}-independent binding might be missed. Also, studies that detect Ca^{2+}-independent binding, need to determine if Ca^{2+}-promotes further binding.

Regions and Residues that Participate in Synaptotagmin-SNARE Binding

Syntaxin

Syntaxin has been shown to bind to synaptotagmin's isolated C_2A domain (Li et al., 1995b; Kee and Scheller, 1996; Shao et al., 1997; Davis et al., 1999; Robinson et al., 2002; Shin et al., 2002). However, because the addition of the C_2B domain can change the Ca^{2+}-dependence of this interaction (Kee and Scheller, 1996; Robinson et al., 2002; Shin et al., 2003), the C_2B domain might also play a role in binding. Indeed, the isolated C_2A domain has been shown to be insufficient for high-affinity binding to syntaxin (Davis et al., 1999). For high-affinity binding, both the C_2A domain and more than half of the C_2B domain are necessary (Davis et al., 1999). Ca^{2+}-binding residues in the C_2A domain (Shao et al., 1997; Davis et al., 1999), as well as basic residues surrounding the Ca^{2+}-binding pocket in C_2A (Shao et al., 1997) are involved in syntaxin binding. However, the Ca^{2+}-binding motifs in both C_2 domains must be disrupted to completely knock-out Ca^{2+}-dependent syntaxin binding (Earles et al., 2001). Synaptotagmin has been shown to bind within the H3/transmembrane domain of syntaxin (Chapman et al., 1995; Kee and Scheller, 1996; Davis et al., 1999).

SNAP-25

The region of synaptotagmin that binds to SNAP-25 has not been clearly established, although both C_2 domains seem to be involved (Earles et al., 2001). The C_2B domain can undergo Ca^{2+}-independent binding to SNAP-25 (Schiavo et al., 1997; Shin et al., 2003), [see however, Rickman and Davletov, (2003)], whereas the C_2A

domain shows only Ca^{2+}-dependent binding (Gerona et al., 2000; Shin et al., 2003), [see however, Earles et al., (2001), who show no binding by C_2A alone]. GST-C_2AB undergoes Ca^{2+}-independent binding that is stimulated by Ca^{2+} (Zhang et al., 1998a; Gerona et al., 2000; Shin et al., 2003). Ca^{2+}-stimulated binding is decreased by mutations in the Ca^{2+}-binding motif of either C_2A or C_2B. It is abolished by simultaneous mutations in the Ca^{2+}-binding motif of both C_2 domains (Earles et al., 2001). Synaptotagmin binds to a series of aspartates in the C-terminus of SNAP-25 (Gerona et al., 2000; Zhang et al., 2002). These residues reside on the surface of SNAP-25 when it is in a helical conformation. Neutralization of these aspartates significantly decreases Ca^{2+}-stimulated synaptotagmin binding and Ca^{2+}-dependent exocytosis (Zhang et al., 2002).

t-SNARE Complexes

Synaptotagmin's interaction with t-SNARE complexes may also involve both C_2 domains. Although Tucker et al. (2003) show that GST-C_2AB and GST-C_2B bind t-SNARE complexes nearly equally well, when removed from GST, C2AB binds better than C_2B alone. Ca^{2+} stimulates the interaction between C_2AB and t-SNARE complexes whereas the interaction between C_2B and t-SNARE complexes remains Ca^{2+}-independent. Although the Ca^{2+}-dependent interaction between synaptotagmin and t-SNARE complexes has been shown to involve both C_2 domains, the Ca^{2+}-independent interaction (Schiavo et al., 1997; Gerona et al., 2000; Rickman and Davletov, 2003; Tucker et al., 2003; Rickman et al., 2004c) has been mapped to the polylysine motif in synaptotagmin's C_2B domain (Rickman et al., 2004c).

Role of Synaptotagmin-SNARE Binding in the Synaptic Vesicle Cycle

Numerous studies indicate that synaptotagmin's interaction with SNARES is likely important for neurotransmitter release. Synaptotagmin fragments can inhibit neurotransmitter release from cracked PC12 cells (Desai et al., 2000; Earles et al., 2001; Tucker et al., 2003); and the inhibitory activity of the fragments is correlated with their Ca^{2+}-dependent ability to bind SNAP-25, syntaxin (Earles et al., 2001) and t-SNARE complexes (Tucker et al., 2003). Likewise, the ability of SNAP-25 to bind synaptotagmin is correlated with its ability to mediate release from cracked PC12 cells (Zhang et al., 2002). Expression of synaptotagmin's cytoplasmic domain in chromaffin cells also inhibits neurotransmitter release. The ability of synaptotagmin to inhibit neurotransmitter release from chromaffin cells is correlated with its Ca^{2+}-independent ability to bind t-SNARE complexes (Rickman et al., 2004c). Synaptotagmin stimulates membrane fusion mediated by neuronal SNAREs reconstituted into vesicles (Mahal et al., 2002), and under certain conditions, this stimulation is also Ca^{2+}-dependent (Tucker et al., 2004). Finally, lengthening the linker region between synaptotagmin's two C_2 domains disrupts synaptotagmin's Ca^{2+}-dependent interaction with t-SNARE complexes. This mutation also abrogates the inhibitory activity of syt fragments in PC12 cell secretion assays and destabilizes fusion pores (Bai et al., 2004b). As the linker mutation did not have an affect on synaptotagmin's Ca^{2+}-dependent membrane binding (including PIP_2 containing membranes), these data provide strong evidence that synaptotagmin's interaction with SNARES is a critical component of its ability to mediate fusion.

How an interaction between synaptotagmin and SNAREs mediates fusion is unknown. Ca^{2+}-binding to synaptotagmin could initiate an interaction with SNAREs,

release already bound SNAREs, or rearrange existing SNARE interactions. The Ca^{2+}-independent interaction between synaptotagmin and t-SNARE complexes has been suggested to play a role in synaptic vesicle docking and/or priming at release sites (Fig. 1.1). The interaction may position syntaxin and SNAP-25 in close proximity to synaptobrevin/VAMP on the vesicle, thereby increasing the probability of SNARE complex formation (Hu et al., 2002; Rickman et al., 2004c).

3. Synaptotagmin's Interactions with Other Molecules of Synaptotagmin

Synaptotagmin has been shown to undergo two modes of oligomerization: 1) Ca^{2+}-dependent (Chapman et al., 1996; Sugita et al., 1996; Desai et al., 2000; Fukuda et al., 2000a; Fukuda and Mikoshiba, 2000; Littleton et al., 2001b; Wu et al., 2003) and, 2) Ca^{2+}-independent (Brose et al., 1992; Bai et al., 2000; Fukuda and Mikoshiba, 2000; von Poser et al., 2000). Ca^{2+}-independent oligomerization is mediated by a series of cysteine residues in synaptotagmin's transmembrane region (Bai et al., 2000; von Poser et al., 2000; Fukuda et al., 2001). Ca^{2+}-dependent oligomerization has been mapped to the C_2B polylysine motif (Chapman et al., 1998; Wu et al., 2003).

Recombinant synaptotagmin has been shown to bind bacterial contaminates via its C_2B polylysine motif when it is purified using standard techniques (Ubach et al., 2001). Thus, interactions mapped to the polylysine motif using recombinant protein purified by standard techniques may reflect artifacts of bacterial contamination rather than true binding partners. Indeed, synaptotagmin's ability to undergo Ca^{2+}-dependent oligomerization was called into question when it was demonstrated that properly purified recombinant synaptotagmin does not oligomerize in response to Ca^{2+} (Ubach et al.,

2001). However, it was subsequently demonstrated that synaptotagmin does undergo Ca^{2+}-dependent oligomerization, but only in the presence of planer lipids. Again, this interaction was mapped to the C_2B polylysine motif (Wu et al., 2003).

Role of Synaptotagmin Oligomerization in the Synaptic Vesicle Cycle

Ca^{2+}-dependent oligomerization may be important for synaptic transmission. The ability of synaptotagmin fragments to inhibit release from cracked PC12 cells is correlated with their ability to oligomerize (Desai et al., 2000). However, the inhibitory activity of these fragments may be mediated by interactions other than oligomerization, as these fragments have been shown to bind other molecules, such as AP-2 (Chapman et al., 1998), PIP_2 (Bai et al., 2004a), and t-SNARE complexes (Rickman et al., 2004c). The strongest evidence for the importance of Ca^{2+}-dependent oligomerization in synaptic transmission comes from a *Drosophila* synaptotagmin mutant (syt^{AD3}/T11). This mutation decreases Ca^{2+}-stimulated oligomerization (Littleton et al., 2001b) and also decreases neurotransmitter release (Littleton et al., 1994; Littleton et al., 2001b). When the AD3 mutation is engineered into rat synaptotagmin I and the recombinant protein is used in *in vitro* binding assays, Ca^{2+}-dependent phospholipid, syntaxin and SNARE complex binding, as well as Ca^{2+}-independent AP-2 and Ca^{2+} channel binding remained intact (Littleton et al., 2001b). Thus, these experiments more strongly support the role of Ca^{2+}-dependent oligomerization *in vivo*. However, these experiments cannot rule out that the synaptic transmission defect in syt^{AD3}/T11 mutants is due to some other disrupted interaction, especially since the mutant synaptotagmin allele T11 is completely uncharacterized.

Ca^{2+}-triggered synaptotagmin oligomerization has been suggested to assemble and cluster SNARE complexes into a collar-like fusion pore (Littleton et al., 2001b) and/or stabilize fusion pores (Desai et al., 2000; Wu et al., 2003). However, these theories lack supporting data. Oligomerization of synaptotagmin isoforms with different Ca^{2+}-binding features may create a variety of Ca^{2+}-sensors characterized by distinct Ca^{2+} sensitivities (Osborne et al., 1999). This may explain why the intracellular Ca^{2+} concentration required for fast neurotransmitter release differs at different synapses [reviewed by (Augustine et al., 2003)]. Recently oligomerization has been suggested to mediate synaptotagmin's ability to bind AP-2, thereby implicating oligomerization in endocytosis (Grass et al., 2004). These experiments used a fusion protein that consisted of GST, a hexacysteine-containing peptide linker and synaptotagmin's C_2B domain. Properly purified, this fusion protein does not bind to AP-2. However, when oxidizing conditions force the protein to oligomerize via the cysteine residues, the protein becomes able to bind AP-2 (Grass et al., 2004). As this form of oligomerization is highly un-physiological, and the same result occurs with only a 12 amino acid peptide corresponding to synaptotagmin's C_2B polylysine motif, the results seem more likely to reflect an artifact of the *in vitro* binding conditions rather than an important *in vivo* interaction.

In summary, the role of Ca^{2+}-dependent oligomerization *in vivo* is completely unclear; although it is proposed to stabilize fusion pores, create a variety of Ca^{2+}-sensors, or provide a binding site for AP-2. Also unclear is the role of Ca^{2+}-independent oligomerization *in vivo*; although it has been implicated in the regulation (inhibition) of coated pit assembly (von Poser et al., 2000).

4. Synaptotagmin's Interaction with AP-2

In vitro studies have demonstrated that the C_2B domain of synaptotagmin is a high affinity receptor for AP-2 (Zhang et al., 1994). This interaction is conserved in *Drosophila* (Littleton et al., 2001b). The interaction between AP-2 and synaptotagmin is mapped to synaptotagmin's C_2B polylysine motif (Chapman et al., 1998). However, as discussed above, it is currently unclear whether this *in vitro* interaction utilizing recombinant synaptotagmin involves the polylysine motif directly or is instead mediated by bacterial contaminants that bind to the polylysine motif after standard purification techniques (Ubach et al., 2001). Indeed, a recent study demonstrated that properly purified GST-synaptotagmin does not bind AP-2 (Grass et al., 2004). However, if the GST-C_2B protein is incubated first with Ca^{2+} and negatively charged liposomes, and then the liposomes are disrupted by 1% Triton X-100, subsequent binding of AP-2 is restored (Grass et al., 2004).

Grass et al. (2004) suggest that Ca^{2+}-dependent oligomerization of synaptotagmin (in the presence of lipid membranes) reveals an AP-2 binding site. However, the authors do not demonstrate that their C_2B peptide is oligomerized when it is binding AP-2. Indeed, two lines of evidence argue against oligomerization being the cause of AP-2 binding in these experiments. First, properly purified GST-synaptotagmin has been shown not to undergo Ca^{2+}-dependent oligomerization when it is incubated with phosphatidylserine containing micelles (Wu et al., 2003). It only oligomerizes in the presence of planar membranes (Wu et al., 2003). Second, increased oligomerization of synaptotagmin is correlated with decreased, not increased, endocytosis (Llinás et al., 2004). Thus, it is more likely that prior incubation of synaptotagmin's C_2B domain with

negatively charged liposomes just allows phosphatidylserine to substitute for the bacterial contaminants. In this case, the restored AP-2 binding would also be an artifact of the assay rather than an important *in vivo* interaction.

Although the binding of recombinant synaptotagmin to AP-2 is questionable, experiments utilizing native synaptotagmin also support its interaction with AP-2. Synaptosomal membranes (containing native synaptotagmin) bind AP-2 from rat brain cytosol. When these membranes are incubated with anti-synaptotagmin antibodies prior to the addition of rat brain cytosol, less AP-2 binds (Haucke and De Camilli, 1999), suggesting a direct interaction between native synaptotagmin in the membrane and soluble AP-2 in the cytosol. Anti-AP-2 antibodies coimmunoprecipitate synaptotagmin and anti-synaptotagmin antibodies coimmunoprecipitate AP-2 from rat brain cytosol (Haucke and De Camilli, 1999). Although coimmunoprecipitation studies do not demonstrate a direct interaction, experiments with recombinant proteins have shown a direct interaction (Haucke et al., 2000). The major synaptotagmin binding site in AP-2 is mapped to a charged region in its μ subunit. When two residues in this region (Tyr344 and Lys354) are mutated to alanines, the μ subunit's ability to bind synaptotagmin's C_2B domain fused to GST is decreased (Haucke et al., 2000). Although this binding interaction is subject to the same criticism as other *in vitro* binding experiments using recombinant synaptotagmin, namely, bacterial contaminants leading to binding artifacts, it is interesting that this same mutation also abolishes the μ subunit's ability to bind to synaptosomal membranes (which would not contain the bacterial contaminants). This result suggests a direct interaction between the two proteins and implicates synaptotagmin in the recruitment of AP-2 to membranes.

Role of AP-2 Binding in the Synaptic Vesicle Cycle

Since AP-2 is an adaptor protein essential for clathrin mediated endocytosis (Kirchhausen, 1999), the interaction between synaptotagmin and AP-2 may mediate an endocytic function for synaptotagmin. Synaptotagmin was first proposed to function in endocytosis when *C. elegans* mutants lacking synaptotagmin showed impaired, but not eliminated, synaptic function (Nonet et al., 1993). Consistent with an endocytic deficit, the nerve terminals are severely depleted of synaptic vesicles in *C. elegans* and *Drosophila* first instar synaptotagmin I null mutants [(Jorgensen et al., 1995; Reist et al., 1998), see also Chapter 3].

Three functional studies implicate synaptotagmin in endocytosis. First, fluorescein-assisted light inactivation (FALI) of synaptotagmin I after exocytosis but before endocytosis, completely blocks internalization of n-synaptobrevin and FM 4-64 (Poskanzer et al., 2003). Since FALI does not degrade the protein, detection of the exact proteins affected by FALI cannot be determined. However, if FALI were disrupting a protein other than synaptotagmin, this endocytic protein must be so close to synaptotagmin that it is likely a directly interacting protein. Such a scenario would also implicate synaptotagmin function in synaptic vesicle endocytosis.

Second, repeated trains of 150 stimuli at 200 Hz induce a large, and sometimes complete, reduction in neurotransmitter release from squid giant terminals. When antibodies to synaptotagmin's C_2B domain are injected into the squid giant terminals, recovery from this reduction is abolished, whereas recovery is unaffected in control terminals. Vesicular density at the active zone, and the number of clathrin-coated vesicles is also reduced in anti-C_2B injected terminals (Llinás et al., 2004). As the

amplitude and latency of the postsynaptic response to an initial train of high frequency stimulation was not modified by injection of the antibody, even after a 2 h period following injection, Llinás et al. (2004) conclude that the anti-C_2B antibody disrupts endocytosis in the squid terminals. Consequently, the severe decrease in evoked transmitter release after repeated trials of high frequency stimulation results from the inability to recover from the synaptic vesicle depletion induced by the high frequency stimulation trials. However, as the terminals only show an ~35% decrease in vesicular density one would not expect synaptic transmission to be so severely decreased in these terminals. For example, *endophilin null* mutations in *Drosophila* reduce synaptic vesicle density nearly 8-fold compared to controls, yet these mutants have normal synaptic transmission during low frequency stimulation (Verstreken et al., 2002).

The third functional study involving synaptotagmin and endocytosis demonstrates that endocytosis persists at hippocampal synapses from *syt*^{null} mice, but it is kinetically >twofold slower (Nicholson-Tomishima and Ryan, 2004). The results of this study are most easily reconciled with current theories on AP-2's role in endocytosis [reviewed by (Schwarz, 2004)]. AP-2 also binds phosphoinositides as well as transmembrane proteins containing a YXXϕ motif [reviewed by (Murthy and De Camilli, 2003)]. Thus, AP-2's interaction with synaptotagmin is not expected to be a requisite step for AP-2 recruitment to the membrane [reviewed by (Schwarz, 2004)]. Consistent with this, the recycling pathway in *syt*^{null} *Drosophila* embryos is capable of robustly re-supplying the readily releasable pool; synaptic vesicle fusion induced by high K^+ can exceed 10 Hz and continue unabated for over an hour in these mutants (Yoshihara and Littleton, 2002).

Thus, it is unclear why both the antibody injection and FALI experiments show a complete block of endocytosis.

5. Synaptotagmin's Interaction with PIP_2 Containing Membranes

Synaptotagmin undergoes a Ca^{2+}-independent and a Ca^{2+}-stimulated interaction with PIP_2 containing membranes (Schiavo et al., 1996; Bai et al., 2004a). The Ca^{2+}-independent interaction has been mapped to the polylysine motif in the C_2B domain with properly purified synaptotagmin, so it is not an artifact of bacterial contamination (Bai et al., 2004a).

Role of Synaptotagmin-PIP_2 Binding in the Synaptic Vesicle Cycle

PIP_2 is implicated in numerous steps of synaptic vesicle endocytosis, such as clathrin coat recruitment and invagination, fission of endocytic pits, and clathrin uncoating [reviewed by (Cremona and De Camilli, 2001)]. In addition, numerous studies have implicated PIP_2 in dense core vesicle exocytosis, including docking and/or fusion reactions [reviewed by (Cremona and De Camilli, 2001)]. However, whether PIP_2 actually plays a role in synaptic vesicle exocytosis is less clear.

Recently, knockout studies demonstrated that mice with decreased levels of PIP_2 show impaired synaptic transmission at hippocampal synapses (Di Paolo et al., 2004). The mutant synapses exhibited endocytic delays that are consistent with a role for PIP_2 in clathrin dependent endocytosis. For example, after 90 sec of stimulation by 90 mM KCl, mutant terminals showed decreased numbers of coated vesicles and increased numbers of endosome like structures. However, the PIP_2 mutants also exhibited synaptic

transmission defects that could be explained by a docking and/or priming defect, implicating PIP_2 in these steps as well. For example, during stimulation at 20 Hz, mutant terminals showed increased synaptic depression that was apparent by the third shock. The PIP_2 knockouts released fewer vesicles during high sucrose stimulation and exhibited a decreased frequency of spontaneous release. Although the overall number of synaptic vesicles in nerve terminals, including docked vesicles, was not specifically analyzed in these studies, the smaller RRP in PIP_2 knockouts does not seem to be secondary to synaptic vesicle depletion; electron micrographs of unstimulated terminals showed no obvious differences between mutant and controls (Di Paolo et al., 2004). Thus, synaptotagmin's interaction with PIP_2 containing membranes may link synaptotagmin to synaptic vesicle endocytosis and/or refilling of the RRP.

Recent *in vitro* studies have implicated synaptotagmin's Ca^{2+}-independent interaction with PIP_2 containing membranes in docking synaptic vesicles at active zones and/or priming them for fusion [Fig. 1.1, and (Bai et al., 2004a)]. Because PIP_2 is predominantly located in the plasmamembrane (Holz et al., 2000; Micheva et al., 2001), synaptotagmin's Ca^{2+}-independent preference for binding to PIP_2 containing membranes facilitates a *trans* interaction between synaptotagmin in the synaptic vesicle membrane and the plasma membrane. *In vitro*, this *trans* interaction increases the rate of synaptotagmin's Ca^{2+}-dependent penetration into lipid membranes (Bai et al., 2004a), a step thought to be important for fusion.

6. Synaptotagmin's Interaction with the Inositol High Polyphosphate Series

Synaptotagmin undergoes Ca^{2+}-independent interactions with members of the Inositol High Polyphosphate Series (IHPS) (Fukuda et al., 1994; Niinobe et al., 1994). This interaction has also been mapped to the polylysine motif in C_2B (Fukuda et al., 1995).

Role of IHPS in the Synaptic Vesicle Cycle

Injection of IP_4, IP_5 or IP_6 into squid giant terminals blocks neurotransmitter release during high frequency stimulation (Llinás et al., 1994), implicating the IHPS in synaptic transmission. However, what role, if any, synaptotagmin binding to the IHPS plays *in vivo* is unknown.

7. Synaptotagmin's Interaction with β-SNAP

The soluble protein NSF binds to *cis*-SNARE complexes via SNAPs (Soluble NSF Attachment Proteins). Three different isoforms of SNAP have been identified (α, β, γ). Although α- and γ-SNAP are found in all cells, β-SNAP is expressed only in neurons (Whiteheart et al., 1993). After SNAP and NSF bind to *cis*-SNARE complexes, the complexes are disassembled via the hydrolysis of ATP by NSF [reviewed by (Li and Chin, 2003)].

Synaptotagmin from crude brain extracts has been shown to bind specifically to GST-β-SNAP. As this interaction is blocked by members of the IHPS, it is likely via the polylysine motif (Schiavo et al., 1995). β-SNAP/synaptotagmin complexes can recruit NSF. However, unlike NSF bound to SNARE complexes via α-SNAP, NSF/β-

SNAP/synaptotagmin complexes are stable upon the addition of Mg^{2+}-ATP (Schiavo et al., 1995). Thus, the function of the synaptotagmin/β-SNAP interaction is unknown.

8. Synaptotagmin's Interaction with Ca^{2+} Channels

Vertebrate synaptotagmin coimmunoprecipitates with N-type (Bennett et al., 1992; Leveque et al., 1992; Yoshida et al., 1992) and P/Q-type (Martin-Moutot et al., 1996) Ca^{2+}-channels. The interaction between synaptotagmin and Ca^{2+} channels involves a synaptic protein interaction (synprint) site in the II-III loop of the α 1A and α1B subunits of these Ca^{2+} channels (Sheng et al., 1994; Rettig et al., 1996; Sheng et al., 1996; Kim and Catterall, 1997; Sheng et al., 1997) and the C_2B polylysine motif of synaptotagmin (Chapman et al., 1998).

Role of Ca^{2+}-channel binding in the Synaptic Vesicle Cycle

Synaptotagmin's interaction with Ca^{2+}-channels has been suggested to link synaptic vesicles close to the site of Ca^{2+} entry or modulate Ca^{2+} channel activity [reviewed by (Zamponi, 2003)]. However, two lines of reasoning question whether synaptotagmin's *in vitro* interaction with Ca^{2+}-channels is actually important *in vivo*. First, invertebrate Ca^{2+} channels do not contain a synprint site (Spafford et al., 2003), yet are certainly capable of neurotransmitter release. Second, the interaction between Ca^{2+}-channels and synaptotagmin is mapped to areas on both proteins that undergo numerous interactions *in vitro*. The synprint site also binds syntaxin and SNAP-25 [reviewed by (Catterall, 1999)] and the polylysine motif in C_2B binds t-SNARE complexes, other

molecules of synaptotagmin, AP-2, PIP_2 and the IHPS (see above). *In vitro*, synaptotagmin's C_2B polylysine motif is a very sticky site.

D. Synaptotagmin's Role in Synaptic Vesicle Docking

Neurotransmitter release occurs preferentially at active zones, where synaptic vesicles cluster. Some of the vesicles in the cluster are morphologically docked, directly apposed to the plasma membrane. Proteins in the synaptic vesicle membrane and proteins in the plasma membrane are thought to mediate docking. Without docking factors, the synaptic vesicle membrane would be repelled by the plasma membrane. Currently it is unclear which molecules mediate recruitment of synaptic vesicles to the plasma membrane. Morphologically docked vesicles remain in the absence of synaptobrevin/VAMP (Hunt et al., 1994; Broadie et al., 1995) and syntaxin (Broadie et al., 1995), indicating that other proteins must mediate this step. Synaptotagmin I is a likely candidate. Evidence for synaptotagmin's role in docking comes from an ultrastructural study demonstrating that syt^{null} mutations in *Drosophila* first instars severely decrease morphologically docked vesicles at central synapses (Reist et al., 1998). In this study, overall synaptic vesicle populations were also decreased (~50% of controls). However, the docked vesicle population was most severely affected (decreased to ~25% of controls) (Reist et al., 1998) Similar results are seen in *Drosophila* syt^{null} third instars (see Chapeter 3).

Synaptotagmin's interaction with SNAP-25, which is likely to be predominantly Ca^{2+}-dependent, has been proposed to dock synaptic vesicles at active zones (Schiavo et al., 1997; Chieregatti et al., 2002; Chieregatti et al., 2004). In support of this theory, Ca^{2+}

stimulated granule cell docking and induces the formation of SNAP-25–synaptotagmin complexes in neuroendocrine cells (Chieregatti et al., 2002). This docking was dependent on SNAP-25; it decreased when SNAP-25 was cleaved by botulinum neurotoxin E. Granule cell docking was also disrupted by addition of both GST-C_2AB peptides and anti-synaptotagmin antibodies. However, granule cell docking was not disrupted by addition of GST-C_2B peptides (Chieregatti et al., 2002). Consistent with this hypothesis, Ca^{2+} can stimulate synaptic vesicle docking at *Drosophila* adult coxal neuromuscular junctions. Increasing extracellular Ca^{2+} increased both the number of active zones possessing docked vesicles, as well as the number of docked vesicles per site (Koenig et al., 1993).

Alternatively, the highly conserved WHXL motif in synaptotagmin's C-terminus may mediate synaptic vesicle docking (Fukuda et al., 2000b). The WHXL motif has been shown to mediate the association between synaptotagmin's cytosolic domain and the plasma membrane in PC12 cells. Injection of a peptide containing the WHXL sequence into squid giant terminals blocked synaptic transmission and decreased morphologically docked vesicles (Fukuda et al., 2000b).

If the WHXL motif mediates synaptic vesicle docking, then synaptotagmin's docking interaction is mapped to the C_2B domain. However, Chieregatti et al. (2002) found no effect of GST-C_2B on granule cell docking. Thus, although the studies by Chieregatti et al. (2002 and 2003) and Fukuda et al. (2000) implicate synaptotagmin in docking, they propose different molecular mechanisms.

IV. Rationale and Specific Aims

A. Rationale

Synaptotagmin's full role in the synaptic vesicle cycle remains unclear. Synaptotagmin's structure contains multiple protein motifs, and biochemical studies demonstrate that synaptotagmin undergoes interactions with numerous presynaptic molecules. Some of these interactions implicate synaptotagmin in docking synaptic vesicles at release sites, priming vesicles for fusion, the fusion process itself, as well as endocytosis.

The overarching goal of my graduate research was to characterize further synaptotagmin's *in vivo* role in the synaptic vesicle cycle. I accomplished this goal through my analysis of *Drosophila* syt^{null} mutants, and analysis of two site-directed synaptotagmin mutants, one with a mutation in synaptotagmin's C_2B Ca^{2+}-binding motif (Fig. 1.3B), and one with a mutation in synaptotagmin's C_2B polylysine motif (Fig. 1.3C).

Dr. Jennifer Mackler generated both site-directed mutants while she was a graduate student in Dr. Noreen Reist's lab. The mutations were generated in *Drosophila synaptotagmin* cDNA. The mutant cDNA was inserted into a P-element and injected into *Drosophila* embryos. Transformants with P-element insertion on the third chromosome were selected and crossed into a syt^{null} genetic background. Thus, the only source of synaptotagmin in the mutant larvae is the mutant synaptotagmin. I used transgenic wild-type larvae as the positive controls for the transgenic mutants. In these controls, wild-type synaptotagmin was inserted into the third chromosome via a P-element and

transformants were crossed into the *syt*^{null} background. *syt*^{null} larvae were used as negative controls.

B. Specific Aim 1: Characterization of syt^{null} *third instars*

My first specific aim was to characterize *Drosophila syt*^{null} third instars. Chapter 2 describes work completed by Dr. Mackler and me on this characterization. In *Drosophila*, the *syt*^{null} mutation results in severe synaptic transmission defects (DiAntonio et al., 1993; Broadie et al., 1994; Loewen et al., 2001; Mackler et al., 2002; Yoshihara and Littleton, 2002). These mutants were reported to die at the late embryo or early first instar stage. In Chapter 2, I report that *Drosophila syt*^{null} mutants can survive to adulthood under special culturing conditions. Increased survival allowed electrophysiological (by Dr. Mackler) and morphological (by me) characterization of *Drosophila syt*^{null} third instar neuromuscular junctions. This analysis permits these larvae to serve as critical negative controls for future synaptotagmin structure/function studies, as this is the *Drosophila* stage most amenable to electrophysiological recordings. Indeed, all the experiments performed on the polylysine motif mutants and C_2B Ca^{2+}-binding motif mutants reported in Chapters 3 and 4 were performed on larvae at this stage.

C. Specific Aim 2: Characterization of the Synaptic Ultrastructure in syt^{null} *Mutants and* C_2B *Ca^{2+}-Binding Motif Mutants*

Synaptic transmission is severely disrupted in *Drosophila syt*^{null} third instars (Loewen et al., 2001) and even more severely disrupted in *Drosophila* third instars

harboring mutations in synaptotagmin's C_2B Ca^{2+}-binding motif [Fig. 1.3B, and (Mackler et al., 2002)]. These results suggest that synaptotagmin is the major Ca^{2+} sensor for neurotransmitter release and that the severe decrease in neurotransmitter release recorded in the C_2B Ca^{2+}-binding motif mutants is a direct consequence of synaptotagmin's inability to bind Ca^{2+}. However, in *Drosophila* first instar central synapses, synaptotagmin is implicated in both synaptic vesicle docking and maintenance of synaptic vesicle pools (Reist et al., 1998). Thus, the severe decrease in synaptic transmission recorded in C_2B Ca^{2+}-binding motif mutants may also be explained by altered synaptic vesicle distribution. An apparent decrease in both synaptic transmission and Ca^{2+} affinity would also be observed in C_2B Ca^{2+}-binding motif mutants if synaptic vesicles were no longer located appropriately close to the site of Ca^{2+} influx.

My second specific aim was to determine if synaptic vesicle distribution is altered in synaptotagmin C_2B Ca^{2+}-binding motif mutants. To make this determination, I completed an ultrastructural analysis of neuromuscular junctions from C_2B Ca^{2+}-binding motif mutants at the third instar stage. The number of vesicles in the vicinity of active zones, including docked vesicles, was determined for both small, clear, synaptic vesicles as well as for two populations of larger diameter vesicles.

The ultrastructural phenotype of neuromuscular junctions from syt^{null} mutants was unknown; yet much of the research on synaptotagmin function in *Drosophila* is performed at this synapse. Thus I included syt^{null} third instar neuromuscular junctions in my ultrastructural analysis as well.

D. Specific Aim 3: Further Define the Role of the Polylysine Motif in the Synaptic Vesicle Cycle

Synaptic transmission is disrupted in *Drosophila* larvae harboring mutations in synaptotagmin's C_2B polylysine motif [Fig. 1.3C, and (Mackler and Reist, 2001)]. The *in vitro* molecular binding partners of synaptotagmin's C_2B polylysine motif implicate this motif in oligomerization of synaptotagmin, synaptic vesicle docking and/or priming, and endocytosis. However, the *in vivo* role of the polylysine motif in the synaptic vesicle cycle is completely unknown. Thus, my third specific aim was to further define the role of the polylysine motif in the synaptic vesicle cycle.

E. Summary

In the following three chapters, I report the results of my analysis on three *Drosophila* synaptotagmin mutants. Together, these results support that synaptotagmin is a multi-functional protein playing multiple roles in the synaptic vesicle cycle. My data implicate synaptotagmin in synaptic vesicle endocytosis, docking, priming, and the Ca^{2+}-sensing to trigger fusion. Through the analysis of specific site-directed mutations in the synaptotagmin protein, my research continues the work of mapping synaptotagmin's roles to specific motifs within the protein. This work is a critical component of understanding the molecular mechanisms that mediate the function of synaptotagmin in the synaptic vesicle cycle.

Chapter 2: *Drosophila* synaptotagmin I Null Mutants Survive to Early Adulthood

Abstract

Synaptotagmin is a synaptic vesicle protein required for efficient neurotransmitter release, yet its exact role in the synaptic vesicle cycle is unclear. *Drosophila* presents an ideal organism for studies aimed at determining the *in vivo* functions of proteins. However, synaptotagmin studies have been limited by the early (embryonic or first instar) lethality previously reported for *Drosophila* synaptotagmin I null (syt^{null}) mutants. Here we report a new culturing technique that enhances survival of severely uncoordinated mutants thereby permitting *Drosophila* syt^{null} mutants to survive through early adulthood. We examined synapses in syt^{null} third instar larvae by electrophysiology and found that they exhibit severely decreased and asynchronous evoked neurotransmitter release, as well as an increased rate of spontaneous neurotransmitter release, as previously seen in first instar syt^{null} larvae. The ability to examine severe synaptotagmin mutants as third instar larvae, a stage where electrophysiological and morphological analyses are more easily accomplished, will facilitate structure/function studies.

Introduction

The secretion of neurotransmitter and subsequent recycling of membrane and proteins during synaptic transmission is an area of intense study. Many key proteins have been identified and mechanisms underlying the synaptic vesicle cycle are beginning to be elucidated. Synaptotagmin is a transmembrane protein found on synaptic vesicles and is required for efficient synaptic transmission. Synaptotagmin I knockouts (syt^{null}) have been generated in mice (Geppert et al., 1994), *Drosophila* (DiAntonio et al., 1993; Littleton et al., 1993b) and *C. elegans* (Nonet et al., 1993). In all three organisms, syt^{null} mutants exhibited decreased evoked transmitter release (Broadie et al., 1994; Geppert et al., 1994; Jorgensen et al., 1995).

Synaptotagmin contains several distinct domains, including two Ca^{2+}-binding C_2 domains. These two C_2 domains have a number of Ca^{2+}-independent and Ca^{2+}-dependent interactions with other synaptic molecules, including: syntaxin (Chapman et al., 1995; Li et al., 1995b; Kee and Scheller, 1996), SNAP-25 (Schiavo et al., 1997), SNARE complexes (Davis et al., 1999; Gerona et al., 2000; Leveque et al., 2000), AP-2 (Zhang et al., 1994), β-SNAP (Schiavo et al., 1995), high inositol polyphosphates (Fukuda et al., 1994) and other molecules of synaptotagmin (Chapman et al., 1996; Damer and Creutz, 1996), [see however, Ubach et al., (2001)]. These biochemical interactions, coupled with the severe disruption of synaptic transmission seen in syt^{null} mutants, have led to a number of hypotheses regarding synaptotagmin's function. Synaptotagmin is proposed to mediate several steps in the vesicle cycle, including: 1) docking of synaptic vesicles at active zones, 2) Ca^{2+}-sensing to trigger exocytosis, and 3) recycling of synaptic vesicles from the plasma membrane following fusion.

Mutagenesis studies have identified specific amino acids that are critical for synaptotagmin to interact with other synaptic molecules. These *in vitro* biochemical interactions must also be tested *in vivo*. *Drosophila* provides an ideal system for such experiments (Sentry and Kaiser, 1995; Perrimon, 1998; Mackler and Reist, 2001). The relative ease of generating transgenic lines in *Drosophila* facilitates *in vivo* investigation of the effects of site-directed synaptotagmin mutations on behavior, synaptic transmission, and synaptic morphology. A severe limitation, however, has been the early stage of lethality reported in *Drosophila* syt^{null} mutants, which die as late embryos or first instar larvae (DiAntonio et al., 1993; Littleton et al., 1993b). Due to the small size of first instar larvae (~450 μm total length), electrophysiological recordings at this stage are technically difficult.

Here we report a new culturing technique that enables *Drosophila* syt^{null} mutants to survive to early adulthood. The syt^{null} mutants then die shortly after eclosion. We have analyzed the electrophysiological phenotype of syt^{null} third instar larvae; these larvae can now serve as an important negative control in functional studies examining site-directed synaptotagmin mutants in *Drosophila*. In addition, our culturing technique may make larval viability, and therefore analysis, possible for other mutant *Drosophila* lines exhibiting severely uncoordinated behavior.

Methods

Drosophila Genetics and Husbandry: syt^{AD4} is a *synaptotagmin* allele that contains a stop codon at amino acid 32 (DiAntonio and Schwarz, 1994). Due to probable second site mutations on this chromosome, we also used a syt^{AD4} line that had been outcrossed

to a second chromosome bearing the P element, *P[elavGal4 w^+]*. No synaptotagmin I protein is expressed in these mutants (DiAntonio and Schwarz, 1994) and thus the *syt^{AD4}* allele is referred to as *syt^{null}* throughout. In the cross, *yw; syt^{null}/CyO y^+* to *yw; syt^{null} P[elavGal4 w^+]/CyO y^+*, *syt^{null}* larvae were selected on the basis of being *y*. In the cross, *yw; syt^{null}/CKG19* to *yw; syt^{null} P[elavGal4 w^+]/CKG19*, *syt^{null}* larvae were selected on the basis of being *GFP-* (Casso et al., 2000). Both the *y* and *GFP- syt^{null}* larvae have the genotype *yw; syt^{null}/syt^{null} P[elavGal4 w^+]*. Flies were allowed to mate for 48 hours on molasses plates containing a dab of yeast paste. *syt^{null}* larvae were selected within 24 hours of hatching and were placed on molasses feeding plates in humidity chambers stored at 25°C or 19°C. High humidity was rigorously maintained by keeping thoroughly moistened Kimwipes within each Petri dish chamber. To promote survival, larvae were placed with their anterior end in fresh drops of liquid that consisted of water with a small amount of yeast dissolved in it. Larvae were generally transferred to fresh drops of this yeast solution one to two times a day. Care was taken that their trachea remained exposed to the air. Pupae were also kept in high humidity until eclosion.

Immunoblotting: Six wild-type and six *syt^{null}* third instar larvae were dissected in HL3 saline [5 mM KCl, 70 mM NaCl, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM HEPES, 1.5 mM $CaCl_2$, 115 mM sucrose, 5 mM trehalose, pH 7.2; (Stewart et al., 1994)] and their (CNSs) were removed. The CNSs were solubilized in 20 μl of buffer (5% SDS, 10% glycerol in 0.1M Tris buffer), boiled 10 minutes, and briefly centrifuged. Total protein

concentration was measured using the NanoOrange Protein Quantitation Kit (Molecular Probes). Dithiothreitol was subsequently added to a final concentration of 50 mg/ml. Samples were boiled for 5 minutes, then ~30 μg of total protein was loaded into each lane. Samples were electrophoresed as previously described (Mackler and Reist, 2001). Primary antibodies used were an anti-synaptotagmin antiserum, Dsyt-2 (Littleton et al., 1993a) diluted 1:1000 and an anti-actin monoclonal antibody, MAB 1501 (Chemicon International, Temecula, CA), diluted 1:20,000. Equal protein loading was confirmed by Coomassie brilliant blue staining of the polyacrylamide gel (not shown) and anti-actin staining of the blot.

Immunohistochemistry: Third instar larvae were dissected in HL3 saline, fixed for 30 minutes in 4% paraformaldehyde in phosphate buffered saline (PBS), rinsed in PBS containing 0.5% Triton X-100 (PBST), incubated overnight at 4°C in Dsyt-2 or fluorescein-conjugated anti-HRP (Jackson Immunoresearch Laboratories) diluted 1:200 in PBST containing 10% normal goat serum (PBST-NGS), and washed in PBST. Preparations labeled with Dsyt-2 were incubated 1 hour in fluorescein-conjugated goat-anti-rabbit IgG (Jackson Immunoresearch Laboratories) diluted 1:200 in PBST-NGS and washed in PBST. Preparations were mounted in Citifluor AF-1 (Ted Pella, Inc.) prior to imaging. Z series of neuromuscular junctions were collected on an Olympus F-IX70 LSM confocal microscope using Fluoview software. Anti-HRP antibodies bind to neuronal membranes in *Drosophila.* This label allowed visualization of synaptic arborizations. For bouton counts, the public domain Image J program (http://rsb.info.nih.gov/ij/) was used to view each series of images and boutons were

counted in a blind manner. Subsequently, the muscle length and width (for muscles 6 and 7 combined) were measured using an ocular micrometer to calculate the inner muscle surface area as an estimate of muscle fiber size (Schuster et al., 1996). Student's t tests were performed using SAS software 6.12 (SAS Institute, Cary, NC). Adobe Photoshop was used for figure preparations.

Electrophysiology: Electrophysiology experiments were performed at room temperature in HL3 saline on muscle fiber six from abdominal segments three and four of third instar larvae. Fibers were impaled with 10-20 MΩ electrodes filled with a solution of 3 parts 2M potassium citrate to one part 3M potassium chloride. EJPs were generated by stimulating segmental nerves with 1 ms pulses of 30-50 nA at 0.05 Hz. Stimulation protocols and analyses were accomplished as previously described (Mackler and Reist, 2001). Intracellular recordings of spontaneous mEJPs were collected and analyzed as previously described (Mackler and Reist, 2001); however, due to the high frequency of events in syt^{null} larvae, only 20 seconds of mEJP data per muscle fiber were included in the analysis for both wild-type and syt^{null} larvae. Student's t tests were performed on all data using Microsoft Excel software.

Results

Drosophila syt^{null} mutants survive to adulthood

We found that the survival of *Drosophila syt^{null}* mutants depends critically on the humidity of their environment and the consistency of their food. If provided with a yeast solution (see methods), ~58% of syt^{null} first instar larvae survived to third instar. If the

yeast paste is too thick, larvae died. Approximately 92% of third instar larvae pupated and, in high humidity, ~59% of pupae eclosed. Adults could survive for several days at 18°C, although the majority died within 24 hours. syt^{null} mutants were mostly inactive. When larvae moved, they were slow and quite uncoordinated. When adults walked, they were extremely uncoordinated and fell often. If they landed on their backs, some could right themselves, but with considerable difficulty. We never observed adults to fly.

We confirmed that the syt^{null} mutants were not expressing synaptotagmin I protein. Western blot analysis with an anti-synaptotagmin antibody was performed on the central nervous system (CNS) of third instar larvae. Synaptotagmin I was present in the CNS from wild-type larvae, but was not present in the CNS from syt^{null} mutants (Fig. 2.1a). We also examined synaptotagmin expression at the synapse used for the electrophysiology experiments, the larval neuromuscular junction (Fig. 2.2). Bright labeling of neuromuscular junctions was seen in wild-type larvae (Fig. 2.2a), but no labeling was seen in the syt^{null} mutants (Fig. 2.2b). The confocal image acquisition settings were identical for the wild-type larva in figure 2.2a and the syt^{null} larva in figure 2.2b. The region shown in figure 2.2b was imaged again with the laser intensity increased until the background was clearly visible (Fig. 2.2c) to demonstrate the complete absence of immunolabeling in the syt^{null} larva.

Synaptic growth proceeds in syt^{null} larvae

The size and complexity of synaptic arborizations at neuromuscular junctions grow dramatically from late embryonic stages through third instar. There is a ~10 fold increase in the number of synaptic boutons during this period (Schuster et al., 1996).

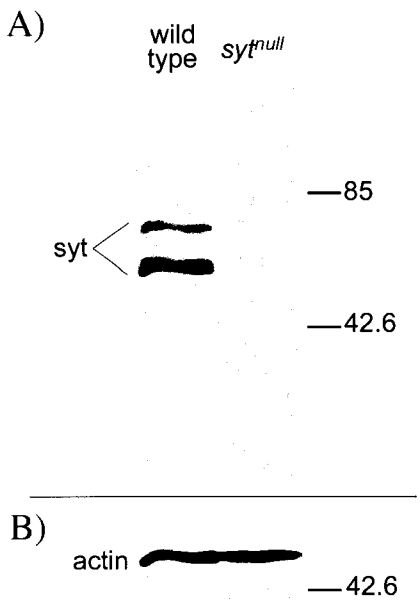

Figure 2.1 Synaptotagmin I is present in CNS from wild-type but not from *syt*^{null} larvae. **(A)** Western blot of CNS from wild-type and *syt*^{null} larvae probed with the anti-synaptotagmin I antibody, Dsyt-2. The faint upper band present in both the wild-type and *syt*^{null} lanes represents nonspecific labeling that is not consistently seen. **(B)** Same blot as in (A) probed with the anti-actin antibody MAB 1501 to demonstrate equal loading of total protein in the two lanes. Lacation of molecular weight markers of 85 kD and 42.6 kD are indicated

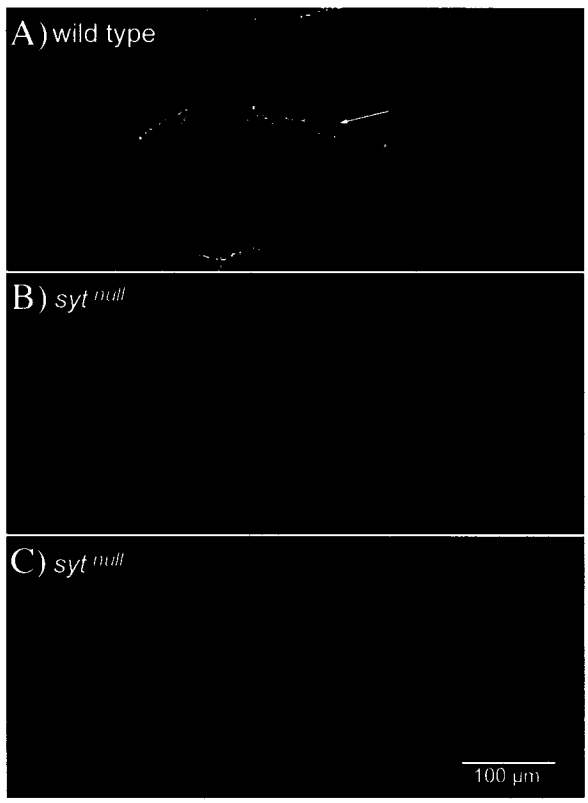

Figure 2.2 Synaptotagmin I is present at neuromuscular junctions in wild-type but not in *syt*^{null} larvae. **(A)** Confocal image of a wild-type third instar larva labeled with the anti-synaptotagmin I antibody, Dsyt-2. Arrow indicates the synaptic arborization on muscle fibers six and seven of abdominal segment three. **(B)** Confocal image of a *syt*^{null} thrid instar larva labeled and imaged as in (A). **(C)** Same *syt*^{null} larva as in (b) but imaged at a higher laser intensity so that background labeling is clearly visible.

To determine whether synaptic arborizations grow throughout larval development in the syt^{null} mutants, we counted the number of boutons in wild-type and syt^{null} third instar larvae. Only boutons in the arborizations on muscles six and seven from segment three and four were counted since these include the junctions we used for electrophysiological analyses. Wild-type larvae had 113 ± 12 boutons per arborization (mean ± SEM, n=13). syt^{null} larvae had 101 ± 6 boutons per arborization (mean ± SEM, n=14, p>0.3, Student's t test). This tally, however, does not take into account that the syt^{null} mutant larvae are smaller than the wild type controls. Since bouton number may be influenced by muscle fiber size in *Drosophila* larvae, we also normalized our counts relative to a surface area measurement (Schuster et al., 1996). The number of boutons/1 x 10^{-5} μm^2 was 158 ± 14 (mean ± SEM, n=13) in wild-type larvae and 180 ± 10 (mean ± SEM, n=14) in syt^{null} larvae (p>0.2, Student's t test). Neither of these measures was statistically significantly different. Thus, the syt^{null} mutation does not seem to cause any gross abnormalities in the growth of synaptic arborizations during larval development.

Synaptic transmission is severely impaired

To assess the physiological deficits in syt^{null} third instar larvae, we examined both evoked (Fig. 2.3) and spontaneous (Fig. 2.4) neurotransmitter release at larval neuromuscular junctions. All recordings were made in muscle fiber six of abdominal segments three and four. Evoked transmitter release was reduced by 94% in syt^{null} larvae compared to wild-type larvae. The mean excitatory junctional potential (EJP) amplitude in wild-type larvae was 42.7 ± 1.9 mV (mean ± SEM, n = 12 fibers) and in

syt^{null} larvae was 2.6 ± 0.3 mV (mean ± SEM, n = 14, p << 0.001, Student's t test, Fig. 2.3b). This decrease was not due to current leakage from muscle fibers; the mean input resistance of *syt*^{null} muscle fibers (10.0 ± 1.1 MOhms, mean ± SEM) was at least as high as wild-type fibers (7.4 ± 1.0 MOhms, mean ± SEM, p < 0.02, Student's t test). In addition, the evoked neurotransmitter release was asynchronous with the stimulus in *syt*^{null} larvae but synchronous in wild-type larvae (Fig. 2.3a).

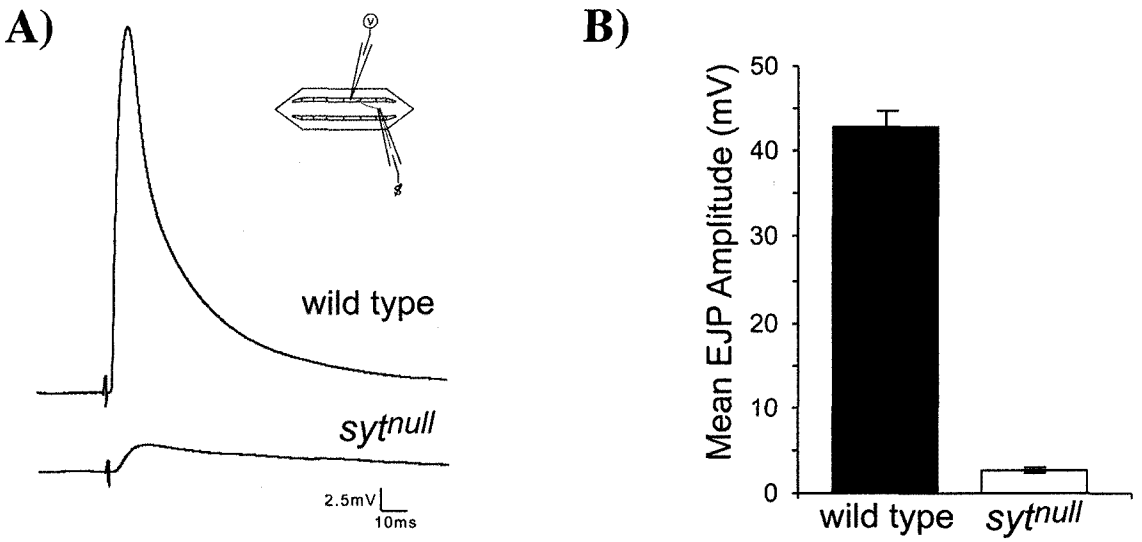

Figure 2.3 Evoked neurotransmitter release is asychronous and the amplitude is decreased in *syt*^{null} larvae. **(A)** Representative EJPs recorded from muscle fiber six in wild-type and *syt*^{null} larvae. Each trace represents the average of 30 individual traces from a single muscle fiber. The inset illustrates the stimulating and recording electrodes on a third instar larval preparation. **(B)** The mean EJP amplitude in wild-type larvae was 42.7 +/- 1.9 mV (mean +/- SEM, n=12 fibers) and in *syt*^{null} larvae was 2.6 +/- 0.3 mV (mean +/- SEM, n=14, P<0.001, Student's *t*-test).

The frequency of spontaneous transmitter release was increased in the absence of synaptotagmin I (Fig. 2.4). We measured the mean frequency of miniature excitatory junctional potentials (mEJP) in third instar larvae. The mean mEJP frequency in *syt*^{null}

larvae (9.3 ± 0.9 Hz, mean ± SEM, n = 12) was threefold higher than wild-type larvae (3.1 ± 0.8 Hz, mean ± SEM, n = 11, p << 0.001, Student's t test, Fig. 2.4b). These electrophysiological data concur with previous studies conducted on syt^{null} first instar larvae (Broadie et al., 1994). Thus, throughout larval development syt^{null} mutants exhibit decreased EJP amplitude, asynchronous neurotransmitter release and an increased mEJP frequency.

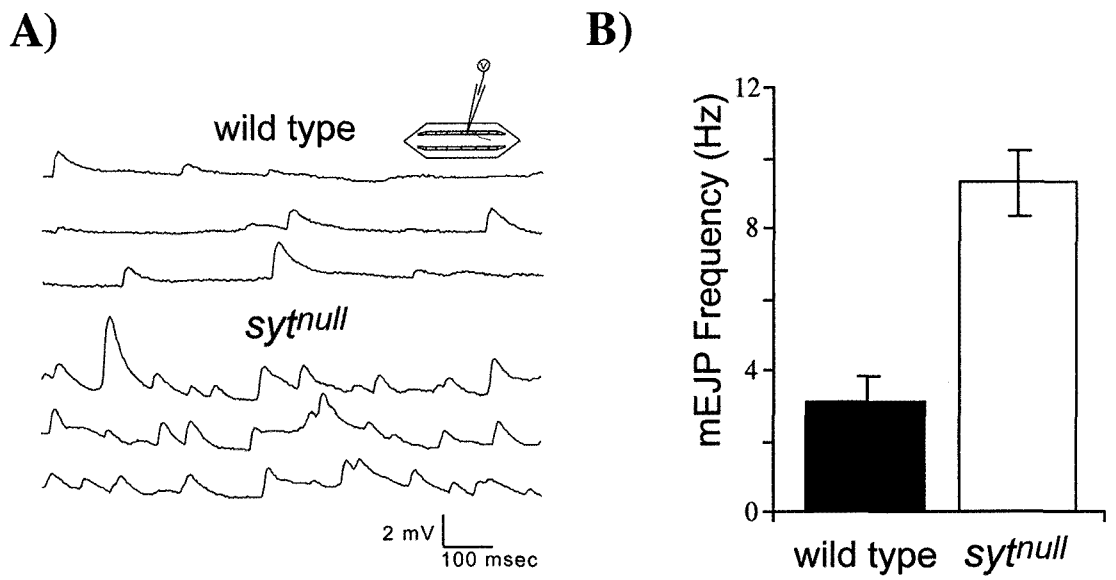

Figure 2.4 Spontaneous neurotransmitter release frequency is increased in syt^{null} larvae. **(A)** Representative traces showing mEJPs recorded from muscle fiber six in both wild-type and syt^{null} larvae. The inset illustrates the recording electrode on a third instar larval preparation. **(B)** The mean mEJP frequency in wild-type larvae was 3.1 +/- 0.8 Hz (mean +/- SEM, n = 11) and in syt^{null} larvae was 9.3 +/- 0.9 Hz (mean +/- SEM, n = 12, P < 0.001, Student's *t*-test)

Discussion

Here we report that *Drosophila* expressing no synaptotagmin I protein can survive to the adult stage. Previous investigators have found that syt^{null} mutations are not lethal

in C. elegans (Nonet et al., 1993), but are lethal in Drosophila (DiAntonio et al., 1993; Littleton et al., 1993b) and mice (Geppert et al., 1994). *syt*^{null} mice die within 48 hours of birth and *syt*^{null} *Drosophila* have been reported to die as late embryos or first instar larvae. Our findings may help explain the difference in lethality among *C. elegans*, mice, and *Drosophila syt*^{null} mutants. Using a *Drosophila syt*^{null} mutant line that was outcrossed to remove probable second site mutations, the majority of our *syt*^{null} embryos hatch, as similarly seen by DiAntonio et al., 1993. Since the subsequent survival of *Drosophila syt*^{null} larvae is critically dependent on the thickness of their food, perhaps *Drosophila* and mice have an increased complexity of feeding behavior, compared to *C. elegans*, which results in the early lethality of these mutants. Without synaptotagmin I protein, *Drosophila* may not be coordinated enough to survive in competition with heterozygous siblings. However, when separated from these competing siblings and raised in liquid food, *Drosophila syt*^{null} larvae are able to survive. Thus, the early lethality previously reported for these mutants is not a direct result of the lack of synaptotagmin I protein, but appears to be secondary to severely uncoordinated behavior that prevents feeding under standard culture conditions.

Our electrophysiological analysis of third instar larvae demonstrates that throughout larval development, the *syt*^{null} mutation results in a severe decrease in the amplitude of evoked transmitter release, asynchronous evoked release and an increase in the spontaneous release frequency. This dramatic alteration of electrophysiological properties is not a result of a failure to develop synaptic arborizations or synaptic boutons. The small amount of evoked neurotransmitter release that remains in the *syt*^{null} mutants could be mediated by either a synaptotagmin independent mechanism or by

another synaptotagmin isoform, such as synaptotagmin IV, which is expressed throughout the *Drosophila* nervous system (Littleton et al., 1999).

Given that the average EJP amplitude in third instar syt^{null} mutants is at most only ~6% of controls, it is remarkable that these null mutants can survive. Mutations in other presynaptic proteins that lead to severe deficiencies in synaptic transmission are often embryonic lethal. A null mutation in *Drosophila* neuronal-synaptobrevin (n-syb) results in no evoked synaptic currents in response to a 0.3 Hz stimulus, although these mutants do exhibit "wiggly movements" that seem to have a neural origin (Deitcher et al., 1998). In *Drosophila unc-*13^{null} mutants, the average excitatory junctional current (EJC) is reduced to less than 1% of controls. These mutants lack muscular peristalsis and neurally coordinated movement (Aravamudan et al., 1999). The *P[syx]/P[syx]* mutation in *Drosophila* syntaxin-1A decreases syntaxin-1A levels to ~30% of wild-type and reduces EJC amplitude to ~20% of wild-type controls (Schulze et al., 1995). All three mutants, *n-syb*^{null}, *unc-*13^{null} *and P[syx]/P[syx]* die in late embryonic stages. Several mutations of the *stoned (stn)* gene products result in decreased EJC amplitudes that range from ~23 – 68% of controls (Fergestad et al., 1999). Only one of these mutants which has an EJC amplitude of ~58% of control, can survive past the embryonic stage. Thus, the level of evoked transmitter release is not directly correlated to the ability of *Drosophila* embryos to hatch and survive.

The discovery that *Drosophila* syt^{null} mutants can survive is important for experiments aimed at determining the functional significance of synaptotagmin during the synaptic vesicle cycle. Due to the difficulty of using first instar larvae, only a very few investigators are able to assess the electrophysiological consequences of severe

synaptotagmin mutations, such as the syt^{null} mutants (Broadie et al., 1994). Other investigators have studied the effects of severe synaptotagmin mutations in the presence of small amounts of the wild type protein (DiAntonio and Schwarz, 1994) or in the presence of unidentified weak alleles of *synaptotagmin* (Littleton et al., 1994; Littleton et al., 2001b) that permitted survival to third instar. The effect of specific synaptotagmin mutations on synaptic function can now be determined directly in third instar larvae without the addition of extraneous sources of synaptotagmin.

The assessment of the physiological deficits in syt^{null} third instar larvae presented here now permits these larvae to serve as a critical negative control for synaptotagmin structure/function studies. Recently it has been reported that mutations in a series of conserved lysine residues on the second C_2 domain (C_2B) of synaptotagmin ($^{K}379,380,384^{Q}$) disrupt synaptic transmission in *Drosophila* (Mackler and Reist, 2001). These lysine residues have been shown to mediate synaptotagmin binding to the clathrin adaptor protein AP-2 (Zhang et al., 1994), neuronal calcium channels (Sheng et al., 1997), high inositol polyphosphates (Fukuda et al., 1994) and other molecules of synaptotagmin (Chapman et al., 1998). *Drosophila* expressing this mutant form of synaptotagmin exhibited a ~36% decrease in evoked transmitter release relative to the transgenic wild-type control. Although the disruption in synaptic transmission was readily apparent, without the syt^{null} mutant negative control it was not possible to ascertain how much synaptotagmin function was provided by this $syt^{K}379,380,384^{Q}$ mutation. The electrophysiological characterization of the syt^{null} mutants we present here, now allows us to report that $syt^{K}379,380,384^{Q}$ mutants maintain ~60% of

synaptotagmin function. This result indicates that while this C_2B polylysine domain is required to attain complete synaptotagmin function, other important domains within synaptotagmin must remain functional in this mutant.

Finally, this new fly culturing technique may be useful to groups creating mutations in other proteins of the *Drosophila* neuromuscular axis. It is likely that some of these mutations also produce flies that are too uncoordinated to eat and survive under standard culturing conditions. However, they may be able to survive under these modified culturing conditions. The ability to promote survival to third instar is an advantage for functional studies, since this stage is significantly more amenable to morphological and electrophysiological manipulations.

Additional unpublished results and discussion

In vitro, the presence of recombinant synaptotagmin has been shown to speed up the formation of SNARE complexes from individual recombinant SNARE proteins. Ca^{2+} had no effect on this stimulation. However, Ca^{2+} did lead to the eventual formation of SNARE complex dimers, but only when recombinant synaptotagmin was also present (Littleton et al., 2001b). In addition, the amount of SNARE complexes were shown by Western blotting to be severely reduced in the synaptotagmin mutants syt^{AD3}/T11 (Littleton et al., 2001b). Thus, it was suggested that synaptotagmin facilitates SNARE complex formation *in vivo* (Littleton et al., 2001b).

However, as the mutant synaptotagmin allele T11 is uncharacterized and *in vitro* results can be misleading, the hypothesis that synaptotagmin facilitates SNARE complex formation *in vivo* needs further testing. Using the new *Drosophila* culturing conditions

described above, I was able to grow *syt*^{null} mutants to the adult stage. I examined the levels of SNARE complexes in wild-type and *syt*^{null} adult fly heads by Western blotting (see appendix A for methods). If synaptotagmin does facilitate SNARE complex formation *in vivo*, *syt*^{null} mutants should show decreased levels of SNARE complexes compared to wild-type flies. However, unlike the results reported by Littleton et. al. (2001), I did not see any decrease in SNARE complex levels in *syt*^{null} mutants (Fig. 2.5). Thus, my results refute the hypothesis that synaptotagmin facilitates SNARE complex formation *in vivo*.

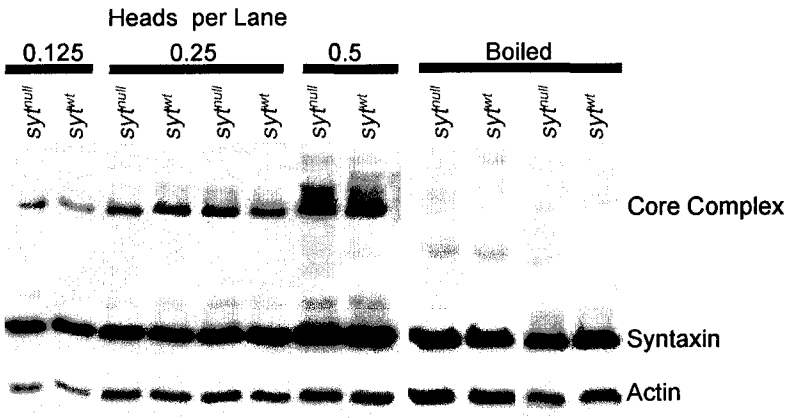

Figure 2.5 Core complex levels are not decreased in *syt*^{null} mutants. Western blot of wild-type and *syt*^{null} adult fly heads probed for core complex levels with an anti-syntaxin antibody. Boiled lanes demonstrate that boiling samples prior to loading disassembles SNARE complexes. The antibody labels two bands, one is 73 kD core complexes, and the other is 35 kD syntaxin monomers. The same blot was also probed with an anti-actin antibody to demonstrate protein loading.

Compared to the levels of SNARE monomers, SNARE complex levels are relatively low, even in wild-type flies. Furthermore, alterations in SNARE complex abundance are not general features of synaptic transmission defects. Indeed, SNARE complex levels have been examined in numerous *Drosophila* synaptic transmission mutants: *comatose, no action potential, paralyzed, seizure, shibire, and slowpoke*, as well

as some *Drosophila* neural signaling mutants: *Shaker, Hyperkinetic,* and *ether-a-go-go. shibire* and *comatose* mutants were the only two that showed appreciable alteration in the abundance of SNARE complexes (decreased in *shibire*, and increased in *comatose)* (Tolar and Pallanck, 1998). Thus, the result that syt^{null} flies have wild-type levels of SNARE complexes is consistent with the general finding that defects in synaptic transmission often do not alter SNARE complex levels.

Interestingly, the accumulation of SNARE complexes in *comatose* mutants is associated with decreased synaptic transmission and an accumulation of docked vesicles (Kawasaki et al., 1998). As *comatose* encodes the protein NSF (Pallanck et al., 1995), which is responsible for the ATP-dependent disassembly of SNARE complexes, it is not surprising that these mutants show a build-up of SNARE complexes *in vivo*. However, these data suggest that disassembly of SNARE complexes after docking serves a synaptic vesicle priming function and helps maintain the readily releasable pool of synaptic vesicles (Tolar and Pallanck, 1998).

Chapter 3: *Drosophila* synaptotagmin null mutants show severe alterations in vesicle populations but Ca^{2+} binding motif mutants do not.

Abstract

Synaptotagmin I is a synaptic vesicle protein postulated to mediate vesicle docking, vesicle recycling, and the Ca^{2+}-sensing required to trigger vesicle fusion. Analysis of synaptotagmin knockouts (syt^{null} mutants) in both *Drosophila* and mice support these hypotheses. While much research on the mechanisms of synaptic transmission in *Drosophila* is performed at the third instar neuromuscular junction, the ultrastructure of this synapse has never been analyzed in syt^{null} mutants. Here we report severe synaptic vesicle depletion, an accumulation of large vesicles, and decreased vesicle docking at syt^{null} third instar neuromuscular junctions.

Mutations in synaptotagmin's C_2B Ca^{2+}-binding motif nearly abolish synaptic transmission and decrease the apparent Ca^{2+} affinity of neurotransmitter release. Although this result is consistent with disruption of the Ca^{2+} sensor, redistribution of synaptic vesicles away from the site of Ca^{2+} influx could also produce a similar phenotype. To address this question, we examined vesicle distributions at neuromuscular junctions from third instar C_2B Ca^{2+}-binding motif mutants and transgenic wild-type controls. The number of docked vesicles and the overall number of synaptic vesicles in the vicinity of active zones was unchanged in the mutants. We conclude that the near elimination of synaptic transmission and the decrease in the Ca^{2+} affinity of release

observed in C_2B Ca^{2+}-binding motif mutants is not due to altered synaptic vesicle distribution, but rather is a direct result of disrupting synaptotagmin's ability to bind Ca^{2+}.

Introduction

Neurotransmitter release is triggered by Ca^{2+}-influx into the nerve terminal (Katz, 1969). Since synaptic vesicles fuse with the plasma membrane within 0.05 - 1.0 ms of Ca^{2+} entry (Llinás et al., 1981; Augustine et al., 1985; Sabatini and Regehr, 1996), the interval between Ca^{2+} entry into the terminal and synaptic vesicle exocytosis is too rapid to allow for many molecular rearrangements. Thus, synaptic vesicles undergo interactions prior to the arrival of Ca^{2+} that ready them for fast fusion (Martin, 2002). Although these interactions are not yet fully understood, they include the docking of synaptic vesicles at active zones, followed by a maturation process or priming, which allows vesicles to fuse quickly upon Ca^{2+} influx (Martin, 2002). After fusion, vesicles are re-internalized, refilled with neurotransmitter and readied for subsequent release to complete the synaptic vesicle cycle. While many of the molecular interactions underlying this cycle remain unclear, the synaptic vesicle protein synaptotagmin I is known to play an important role because the absence of synaptotagmin (syt^{null}) nearly abolishes Ca^{2+}-dependent, synchronous, neurotransmitter release (Littleton et al., 1993b; Broadie et al., 1994; Geppert et al., 1994; Loewen et al., 2001; Yoshihara and Littleton, 2002; Nishiki and Augustine, 2004b).

Synaptotagmin is composed of a short intravesicular domain, a transmembrane domain and a cytosolic domain comprised of two homologous C_2 domains, C_2A and C_2B (Perin et al., 1991b; Brose et al., 1992). Both C_2 domains contain five, highly conserved

acidic residues that coordinate Ca^{2+}-binding (Ubach et al., 1998; Fernandez et al., 2001). Ca^{2+} has been shown to promote interactions between synaptotagmin and numerous presynaptic molecules: syntaxin, snap-25, t-SNARE heterodimers, complete SNARE complexes, other molecules of synaptotagmin, anionic phospholipids, and PIP_2 containing membranes (Brose et al., 1992; Chapman et al., 1995; Li et al., 1995a; Li et al., 1995b; Schiavo et al., 1996; Schiavo et al., 1997; Shao et al., 1997; Davis et al., 1999; Gerona et al., 2000; Earles et al., 2001; Mackler et al., 2002; Zhang et al., 2002; Wu et al., 2003; Bai et al., 2004b). In addition, synaptotagmin stimulates membrane fusion mediated by neuronal SNAREs reconstituted into vesicles (Mahal et al., 2002), and under certain conditions, this stimulation is also Ca^{2+}-dependent (Tucker et al., 2004).

Whether any or all of these Ca^{2+}-dependent interactions are required for synaptic transmission *in vivo* remains unclear. Nonetheless, the plethora of Ca^{2+}-dependent interactions coupled with synaptotagmin's ability to bind Ca^{2+} directly, and the observation that syt^{null} mutations abolish fast, Ca^{2+}-triggered exocytosis led to the hypothesis that synaptotagmin is a Ca^{2+}-sensor for neurotransmitter release [reviewed by (Südhof, 2004)]. Although exocytosis must also involve another Ca^{2+}-sensor (or sensors) since the residual neurotransmitter release seen in syt^{null} mutants is still Ca^{2+}-dependent, strong evidence supports the hypothesis that synaptotagmin I is the main Ca^{2+} sensor. In *Drosophila*, when two of the Ca^{2+}-binding acidic residues in synaptotagmin's C_2B domain are mutated to asparagines (syt^{DN}) and the mutant protein is expressed as a transgene in the syt^{null} background ($P[syt^{DN}]$), the apparent Ca^{2+}-affinity of synaptic transmission is reduced and Ca^{2+}-triggered, synchronous neurotransmitter release is almost entirely eliminated (>95% decrease) (Mackler et al., 2002). These experiments

demonstrate that synaptotagmin's ability to bind Ca^{2+} by the C_2B domain is critical for synaptic transmission *in vivo*, and they support the hypothesis that synaptotagmin is the main Ca^{2+}-sensor for neurotransmitter release.

In addition to Ca^{2+}-sensing, synaptotagmin likely mediates other steps in the synaptic vesicle cycle. Previous work has shown that synaptotagmin is critical for synaptic vesicle docking and maintenance of vesicle populations at some synapses. syt^{null} mutations in *C. elegans* and *Drosophila* first instars cause severe synaptic vesicle depletion at central synapses (Jorgensen et al., 1995; Reist et al., 1998). In *Drosophila*, the overall number of synaptic vesicles surrounding active zones is decreased by ~50%, while the number of morphologically docked vesicles is decreased by ~75%. This severe decrease in synaptic vesicles immediately adjacent to the presynaptic membrane at central synapses in *Drosophila* syt^{null} mutants implicates synaptotagmin in vesicle docking (Reist et al., 1998). In addition, *Drosophila* first instar syt^{null} mutants exhibit a dramatic increase (>3 fold) in the number of large, irregularly shaped vesicles near central nervous system synapses (Reist et al., 1998). The overall synaptic vesicle depletion and the accumulation of large, irregular vesicles observed in syt^{null} mutants implicate synaptotagmin in synaptic vesicle endocytosis. Indeed, functional experiments demonstrate that synaptotagmin plays an important role in endocytosis (von Poser et al., 2000; Jarousse and Kelly, 2001; Jarousse et al., 2003; Poskanzer et al., 2003; Llinás et al., 2004; Nicholson-Tomishima and Ryan, 2004).

While the ultrastructure of *Drosophila*, first instar, central synapses support a role for synaptotagmin in maintaining synaptic vesicle populations and docking synaptic vesicles at active zones, the ultrastructure of another synapse does not. Cultured mouse

hippocampal synapses exhibit neither synaptic vesicle depletion nor decreased synaptic vesicle docking (Geppert et al., 1994). Therefore, before physiological deficits can be ascribed to alterations in synaptic vesicle populations, it is critically important to analyze the ultrastructure of the specific synapse under investigation. Since much of the research on synaptotagmin function in *Drosophila* is performed at third instar neuromuscular junctions (Littleton et al., 1993b; DiAntonio and Schwarz, 1994; Littleton et al., 1994; Littleton et al., 1999; Littleton et al., 2001b; Loewen et al., 2001; Mackler and Reist, 2001; Mackler et al., 2002; Robinson et al., 2002; Poskanzer et al., 2003), we analyzed the synaptic ultrastructure of this synapse in syt^{null} mutants.

We also examined the synaptic ultrastructure at $P[syt^{DN}]$ third instar neuromuscular junctions. Since $P[syt^{DN}]$ mutants contain mutations in key Ca^{2+}-binding residues and exhibit a decrease in the apparent Ca^{2+} affinity of neurotransmitter release, the simplest explanation for the electrophysiological defects seen in $P[syt^{DN}]$ mutants is that one or more Ca^{2+}-dependent interactions critical for triggering fusion have been disrupted. Indeed, the disruption in Ca^{2+}-evoked release correlates with a disruption in Ca^{2+}-dependent phospholipid binding (Mackler et al., 2002), an interaction postulated to be important for fusion (Fernández-Chacón et al., 2001). However, defects in synaptic vesicle docking, or endocytosis could also contribute to the decrease in evoked release observed in these mutants. To address whether the $P[syt^{DN}]$ mutants have docking or endocytic deficits, we completed an ultrastructural analysis of synaptic vesicle distributions at third instar neuromuscular junctions, the same synapse used to document the disruption of evoked transmitter release (Mackler et al., 2002). The number of vesicles in the vicinity of active zones was determined for both small, clear, synaptic

vesicles as well as for two populations of larger diameter vesicles: those with a diameter of 50-90 nm postulated to be recycling intermediates (Kosaka and Ikeda, 1983b; Fergestad et al., 1999; Wucherpfennig et al., 2003), and a second population of larger diameter, irregularly shaped structures that may be part of a degradative pathway (Jia et al., 1993a; Koenig et al., 1993; Wucherpfennig et al., 2003). Consistent with results from central synapses of syt^{null} first instars (Reist et al., 1998), the neuromuscular junctions of syt^{null} third instars exhibited synaptic vesicle depletion as well as increased numbers of large vesicles. Although the synaptic vesicles present in syt^{null} terminals were targeted to active zones (vesicle depletion was most severe away from active zones), these vesicles were not efficiently docked. Surprisingly, the syt^{null} ultrastructural phenotype was completely rescued by expression of the syt^{DN} mutant transgene. $P[syt^{DN}]$ mutants showed no synaptic vesicle depletion, no increase in large vesicles and no disruptions in synaptic vesicle docking. Thus, although the syt^{DN} mutation results in a more severe decrease in synaptic transmission than the syt^{null} mutation (Mackler et al., 2002), the syt^{DN} mutant transgene effectively rescues the severe ultrastructural abnormalities observed at syt^{null} neuromuscular junctions. We conclude that the absence of synaptotagmin I results in both synaptic vesicle biogenesis as well as docking deficits at third instar neuromuscular junctions. In addition, the decrease in neurotransmitter release recorded in $P[syt^{DN}]$ mutants is not due to altered synaptic ultrastructure, indicating that synaptotagmin's ability to bind Ca^{2+} by the C_2B domain is not required for synaptotagmin's vesicle biogenesis and synaptic vesicle docking activities.

Methods

Fly Strains: syt^{AD4} is a null mutation in the synaptotagmin I gene with a stop codon at amino acid 32 (DiAntonio and Schwarz, 1994). The *synaptotagmin* null mutants (syt^{null}) had the genotype *yw;* syt^{AD4}/syt^{AD4} *P[elavGal4* w^+*].* The wild-type strain (syt^{wt}) used was Oregon R. The Ca^{2+}-binding mutants (*P[*syt^{DN}*]*) were syt^{null} larvae that expressed synaptotagmin from a mutant transgene. In this mutation, the third and fourth Ca^{2+}-binding aspartates in C_2B (D416 and D418) were mutated to asparagines (Mackler et al., 2002). These flies had the genotype: *yw;* syt^{AD4}/syt^{AD4} *P[elavGal4* w^+*];P[UAS* $syt^{C2B\text{-}D3,4N}$ w^+*]/+*. Transgenic controls (*P[*syt^{wt}*]*) expressed synaptotagmin from a wild-type transgene in a syt^{null} background (Mackler and Reist, 2001; Mackler et al., 2002). These flies had the genotype: *yw;* syt^{AD4}/syt^{AD4} *P[elavGal4* w^+*];P[UAS* syt^{wt} w^+*]/+*.

Electron Microscopy: Third instars were processed according to standard procedures (Reist et al., 1998). They were dissected in ice cold, Ca^{2+} free HL3 saline, fixed for 1 h in ice cold 1% acrolein, 2.5% glutaraldehyde in 0.1M cacodylate (Cac) buffer, pH 7.2, post-fixed in 0.5% OsO4, 0.8%KFeCn in O.1 M Cac for 1 h, incubated in 5% uranyl acetate for 1 h to overnight, dehydrated and embedded in EmBed 812 araldite. 70 nm sections were post-stained with uranyl acetate and Reynold's lead citrate. Boutons were imaged at 12K and 30K magnification using a JEOL JEM 2000 EX-II TEM operated at 100 kV. Negatives were scanned at 1500 dpi into a Macintosh G4 computer using an AGFA Duoscan T2500.

Analysis: The average diameter of small, clear, synaptic vesicles (35 ± 5 nm, STD) was determined by measuring the diameter of 216 small, clear, synaptic vesicles in a wild-type terminal. Vesicles with diameters >50 nm were defined as large vesicles. Images from control and experimental larvae were coded and randomized for blind analysis. Only active zones with visible t-bars and clear presynaptic membranes were selected for analysis. Using the public domain, Object Image program (http://simon.bio.uva.nl/object-image.html), 150 nm of presynaptic membrane was marked on both sides of the t-bar. A straight line was drawn between the center of each vesicle and the presynaptic membrane closest to it (see Fig. 3.2E). This distance was measured. Only vesicles whose centers were both closest to marked, presynaptic membrane and within 200 nm of the marked presynaptic membrane were included in the analysis. To examine the distribution of vesicles with respect to the presynaptic membrane, the data were pooled into eight, 23 nm bins. The ninth (last) bin contained the vesicles in the remaining analyzed area (16 nm). Docked vesicles were defined as those vesicles whose centers were within 23 nm of the presynaptic membrane. 84 active zones from three syt^{wt} and 81 active zones from three syt^{null} larvae were analyzed. 99 active zones per genotype from four $P[syt^{DN}]$ and four $P[syt^{wt}]$ larvae were analyzed. Fisher's protected Least Squares Difference (LSD) was used to generate p-values for pairwise comparisons. All other multiple comparisons were tested using Tukey's W procedure. Statistical calculations were performed using SAS software 6.12 (SAS Institute, Cary, NC).

Results

To determine whether synaptic vesicle depletion or abnormal vesicle distributions contribute to the nearly complete inhibition of evoked neurotransmitter release recorded at third instar neuromuscular junctions in syt^{null} mutants (Loewen et al., 2001) and C_2B Ca^{2+}-binding motif mutants ($P[syt^{DN}]$, (Mackler et al., 2002), we analyzed the ultrastructure of these synapses. Figure 3.1 and 3.2 show representative micrographs of neuromuscular junctions from syt^{null}, syt^{wt}, $P[syt^{wt}]$ and $P[syt^{DN}]$ third instars. At the larval neuromuscular junction, a typical active zone contains a thickened presynaptic membrane, marked by an electron dense body capped with a meshwork of filaments. In cross section, the dense body with overlying filaments often appears t-shaped and, as such, is referred to as a t-bar [Fig. 3.1, small arrowheads, (Atwood et al., 1993; Koenig and Ikeda, 1999)]. All four genotypes examined exhibited typical active zone organization, having t-bars associated with a thickened, presynaptic membrane. In syt^{wt}, $P[syt^{wt}]$ and $P[syt^{DN}]$ larvae, numerous small, clear, synaptic vesicles (Fig. 3.1, small arrows) were located throughout the presynaptic boutons of the neuromuscular junction, especially in the vicinity of t-bars. In contrast, syt^{null} terminals exhibited severe synaptic vesicle depletion (Fig. 3.1, syt^{null}). In areas distant to active zones, syt^{null} terminals were often devoid of synaptic vesicles. The few small, clear vesicles present in these terminals tended to be closely associated with the active zone (Fig. 3.1, syt^{null}, top). In addition to the obvious decrease in small, clear, synaptic vesicles, syt^{null} terminals also exhibited an increase in larger membranous structures. These included a commonly encountered 50-90 nm vesicle population (Fig. 3.1, large arrows), as well as larger structures (Fig. 3.1, large arrowheads). Two examples from syt^{null} mutants demonstrate the range of

Figure 3.1 *syt*^{null} mutants show severe alterations in vesicle populations but C_2B Ca^{2+}-binding motif mutants do not. Electron micrographs show the synaptic ultrastructure of neuromuscular junctions in *syt*^{null} (left column), *syt*^{wt} (right, top), *P[syt*^{wt}*]* (right, middle), and *P[syt*^{DN}*]* (right, bottom) third instars. *syt*^{null} mutants show severe synaptic vesicle (small arrows) depletion, as well as an accumulation of both 50-90 nm vesicles (large arrows) and larger membranous structures (large arrowheads). Two examples from *syt*^{null} mutants demonstrate the range of abnormal vesicle populations encountered. Both *P[syt*^{wt}*]* and *P[syt*^{DN}*]* terminals contain numerous synaptic vesicles throughout the nerve terminal, including areas around active zones, which are marked by t-bars (small arrowheads). Bar = 100 nm.

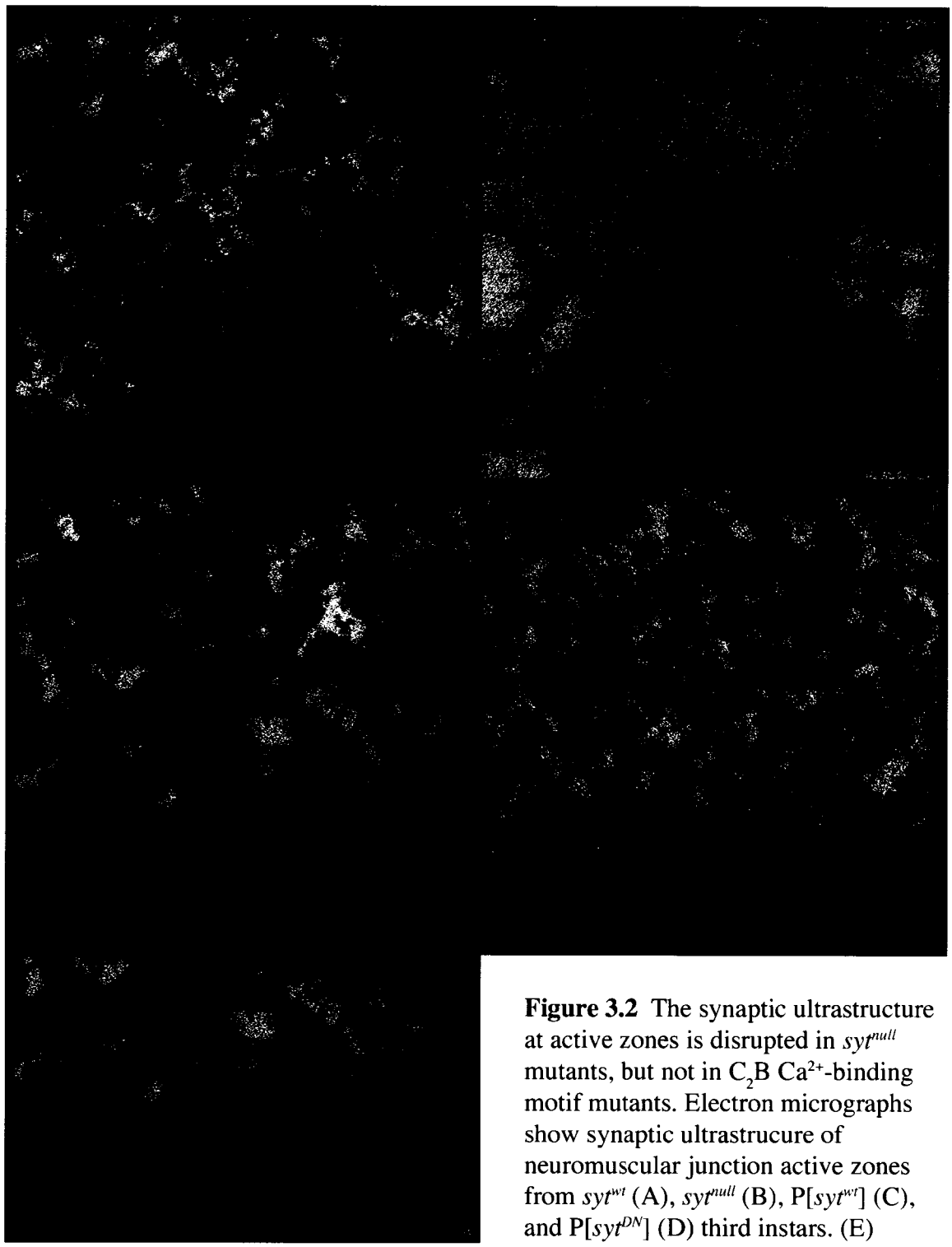

Figure 3.2 The synaptic ultrastructure at active zones is disrupted in syt^{null} mutants, but not in C_2B Ca^{2+}-binding motif mutants. Electron micrographs show synaptic ultrastrucure of neuromuscular junction active zones from syt^{wt} (A), syt^{null} (B), P[syt^{wt}] (C), and P[syt^{DN}] (D) third instars. (E) illustrates the marking protocol used to determine synaptic vesicle distributions on a syt^{wt} terminal.

abnormal vesicle populations encountered, from terminals that were largely depleted of vesicles (Fig. 3.1, syt^{null}, top) to terminals containing numerous large vesicles (Fig. 3.1, syt^{null}, bottom).To quantify alterations in vesicle populations near release sites, we counted the number of vesicles near active zones (Fig. 3.2). We limited our analysis to vesicles in the area immediately surrounding t-bars, where synaptic vesicle release preferentially occurs (Heuser et al., 1979; Govind et al., 1980; Propst and Ko, 1987; Jia et al., 1993a; Broadie et al., 1995; Fergestad et al., 1999). 150 nm of presynaptic membrane on either side of t-bars was marked. Only vesicles whose centers were closest to the marked presynaptic membrane (as opposed to unmarked membrane in the section) and whose centers were within 200 nm of the nearest marked presynaptic membrane were included in the analysis. Figure 3.2E illustrates our marking protocol on a $P[syt^{wt}]$ terminal.

At third instar neuromuscular junctions in syt^{null} mutants, the overall number of small, clear, synaptic vesicles in the vicinity of active zones was decreased by 48% compared to syt^{wt} larvae (Fig. 3.3, $p<0.001$). Expression of either the syt^{wt} or syt^{DN} transgene rescued the synaptic depletion observed in syt^{null} terminals; the number of small, clear, synaptic vesicles surrounding t-bars in both $P[syt^{wt}]$ and $P[syt^{DN}]$ larvae was not significantly different from that in syt^{wt} larvae (Fig. 3.3, Tukey's 95% confidence level). Thus endocytic defects resulting in synaptic vesicle depletion could contribute to the decrease in neurotransmitter release observed in syt^{null} mutants (Loewen et al., 2001). However, since no vesicle depletion was observed in $P[syt^{DN}]$ mutants, the decrease in neurotransmitter release recorded in $P[syt^{DN}]$ mutants (Mackler et al., 2002) does not result from an insufficient supply of synaptic vesicles.

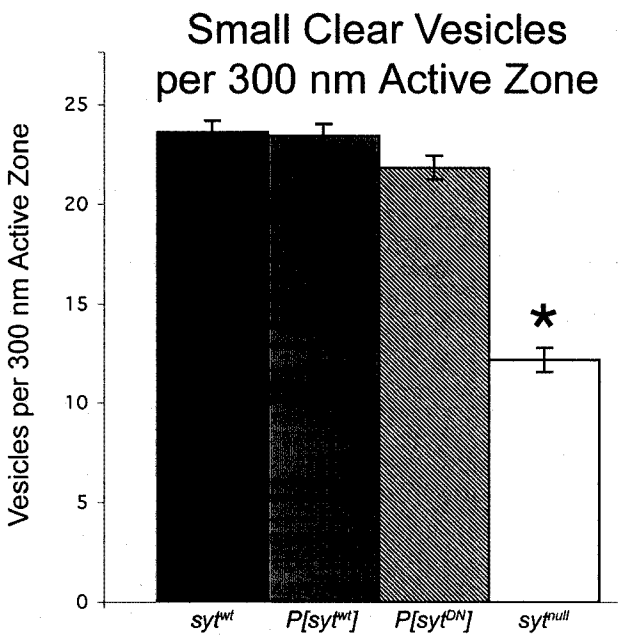

Figure 3.3 Small, clear vesicles are depleted at active zones in syt^{null} mutants, but not in C_2B Ca^{2+}-binding motif mutants. The number of small, clear synaptic vesicles per 300 nm of active zone membrane was determined for syt^{wt}, $P[syt^{wt}]$, $P[syt^{DN}]$ and syt^{null} third instars. At syt^{null} active zones, synaptic vesicles are decreased by 48% compared to syt^{wt} larvae ($p<0.001$). Neither $P[syt^{wt}]$ nor $P[syt^{DN}]$ terminals were significantly different from syt^{wt} terminals (Tukey's 95% confidence level), demonstrating that both the mutant and wild-type synaptotagmin transgenes rescue the syt^{null} ultra-structural phenotype. Total synapses analyzed: syt^{wt}, 84; syt^{null}, 81; $P[syt^{wt}]$, 99; $P[syt^{DN}]$, 99. Error bars, SEM.

Since displacement of synaptic vesicles further from the Ca^{2+} channels located in the presynaptic membrane could contribute to the increased EC_{50} for Ca^{2+} observed in $P[syt^{DN}]$ mutants (Mackler et al., 2002), we measured the distance between the center of each synaptic vesicle and the nearest presynaptic membrane, as indicated in figure 3.2. The data were pooled into 23 nm bins. Figure 3.4A shows histograms of the synaptic vesicle distribution for each genotype. The first bin shows the number of synaptic vesicles whose centers were within 23 nm of the presynaptic membrane; we define these vesicles as morphologically docked. To facilitate the comparison of vesicle populations at each distance in mutant and control terminals, we regraphed the distribution data as a percentage of syt^{wt} (Fig. 3.4B). As already shown in figure 3.1 (syt^{null}, upper panel), small, clear, synaptic vesicles that remain in the syt^{null} mutants are preferentially localized near t-bars. While syt^{null} mutants show vesicle depletion at almost every distance measured, the distribution of synaptic vesicles in the vicinity of active zones is

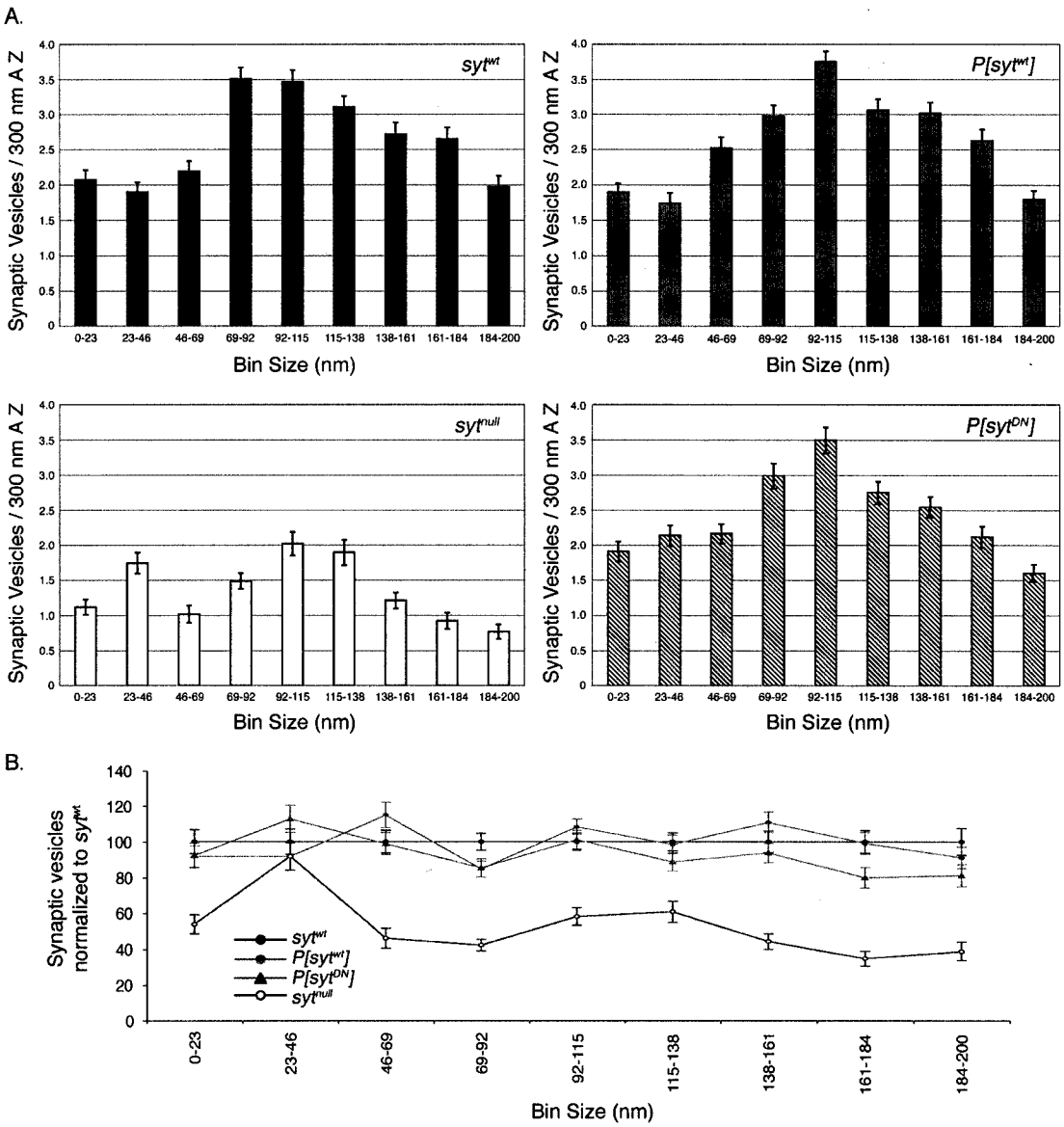

Figure 3.4 Distribution of small, clear synaptic vesicles in the vicinity of active zones. **(A)** The distance of vesicles from the presynaptic membrane is determined for each genotype (see Fig. 2E and Materials and Methods), and a histogram of their distribution is shown (means ± SEM). All bins are 23 nm except the last bin, which is 16 nm. **(B)** To compare vesicle populations at each distance, the data from each mutant genotype are plotted as a percentage of syt^{wt}. $P[syt^{wt}]$ and $P[syt^{DN}]$ mutants are not significantly different from wild-type at any distance, whereas syt^{null} larvae show significant vesicle depletion at every distance except 23-46 nm from the presynaptic membrane (p=0.49).

not uniform (Fig. 3.4B). The number of morphologically docked vesicles in syt^{null} larvae was decreased by 46% compared to controls ($p<0.001$), yet the number of vesicles near, but not touching the presynaptic membrane (i.e., in the next bin out), was not significantly different from controls ($p=0.49$). This finding suggests that the few small, clear, synaptic vesicles seen in syt^{null} terminals are efficiently targeted toward release sites. However, once these vesicles reach the active zone, they fail to stably dock at the presynaptic membrane. Such vesicles may spontaneously fuse with the presynaptic membrane, resulting in the increased frequency of spontaneous, miniature excitatory junctional potentials seen in syt^{null} third instar neuromuscular junctions (Loewen et al., 2001). Alternatively, these vesicles may disengage from docking sites, which would result in the relative build-up of synaptic vesicles just short of the presynaptic membrane. Importantly, expression of either the syt^{wt} or syt^{DN} transgene rescued the disruption of synaptic vesicle distribution seen in the syt^{null} mutants. In both $P[syt^{wt}]$ and $P[syt^{DN}]$ larvae the number of docked vesicles is not different from syt^{wt} (Tukey's 95% confidence level). Vesicle distribution at sites further from the presynaptic membrane is also not significantly different from that in syt^{wt} larvae (Tukey's 95% confidence level). Thus, neither an inability of synaptic vesicles to dock efficiently at active zones nor altered synaptic vesicle distribution can explain either the decrease in evoked neurotransmitter release or the shift in apparent Ca^{2+} affinity of release recorded in $P[syt^{DN}]$ mutants (Mackler et al., 2002).

In addition to severe depletion of small, clear, synaptic vesicles, syt^{null} terminals frequently contained numerous large membranous structures (Fig. 3.1, large arrows and large arrowheads). The number of large, membranous structures near active zones at

third instar neuromuscular junctions was analyzed for all genotypes and was increased 3.1 fold in syt^{null} mutants compared to syt^{wt} (Fig. 3.5A, $p<0.001$). This is remarkably similar to the increase (3.4 fold) reported in syt^{null} first instars at central synapses (Reist et al., 1998). Again, expression of either the syt^{wt} or syt^{DN} transgene rescued the syt^{null} ultrastructural phenotype; neither $P[syt^{wt}]$ nor $P[syt^{DN}]$ mutants showed a significant increase in large membranous structures compared to syt^{wt} terminals (Fig. 3.5A, Tukey's 95% confidence level).

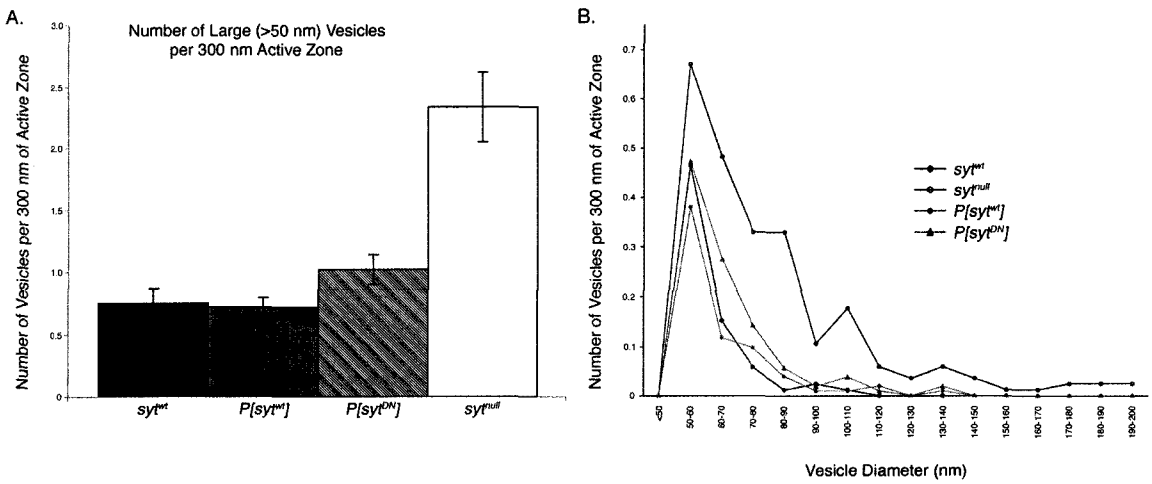

Figure 3.5 Large diameter membranous structures are increased in syt^{null} mutants, but not in C_2B Ca^{2+}-binding motif mutants. **(A)** The number of large (>50 nm in diameter) membranous structures per 300 nm of active zone membrane analyzed was determine for syt^{wt}, $P[syt^{wt}]$, $P[syt^{DN}]$ and syt^{null} third instars. At syt^{null} active zone, large structures were increased 3.1 fold ($p<0.001$). Neither $P[syt^{wt}]$ nor $P[syt^{DN}]$ terminals were significantly different from syt^{wt} terminals (Tukey's 95% confidence level). Error bars denote SEM. **(B)** The majority of large vesicles in all four genotypes have diameters of 50-90 nm. syt^{null} mutants have more 50-90 nm vesicles than syt^{wt}, $P[syt^{wt}]$, $P[syt^{DN}]$ mutants. Membranous structures larger than 90 nm in diameter are rare in syt^{wt}, $P[syt^{wt}]$ and $P[syt^{DN}]$ mutants, whereas they are found more frequently in syt^{null} larvae. The total number of synapses analyzed was: syt^{wt}, 84; syt^{null}, 81; $P[syt^{wt}]$, 99; and $P[syt^{DN}]$, 99.

The diameters of the large membranous structures near active zones were also determined (Fig. 3.5B). The majority of these structures in all genotypes were large vesicles with a diameter of 50-90 nm (Fig 3.1, large arrows). Few structures with diameters larger than 90 nm were observed in the area around active zones in syt^{wt}, $P[syt^{wt}]$, and $P[syt^{DN}]$ larvae. However, syt^{null} active zones contained many membranous structures with diameters larger than 90 nm (Fig. 3.5 and Fig. 3.1, large arrowheads). Curiously, some of the large diameter structures observed in syt^{null} terminals were reminiscent of membranous structures involved in degradation pathways, such as multivesicular bodies (Fig. 3.1, syt^{null}, upper panel, large arrowhead). These were rarely encountered in syt^{wt}, $P[syt^{wt}]$ or $P[syt^{DN}]$ terminals.

Discussion

Synaptic ultrastructure is severely disrupted at third instar neuromuscular junctions in syt^{null} larvae with the overall number of small, clear, synaptic vesicles being severely depleted (Fig. 3.1). However, the synaptic vesicles that remain in these syt^{null} terminals are efficiently targeted to active zones, such that the number near, but not touching, the presynaptic membrane (i.e., Fig. 3.4B, bin #2) is the same as in controls. Despite this targeting to synapses, the number of morphologically docked synaptic vesicles at syt^{null} third instar neuromuscular junctions is decreased by 46%. These results are similar to findings from first instar central synapses (Reist et al., 1998). syt^{null} third instar neuromuscular junctions also show a 2.6 fold increase in large (50-90 nm) diameter vesicles (Fig. 3.5). Vesicles of this diameter at *Drosophila* neuromuscular junctions are

postulated to be recycling intermediates and are often referred to as endosomes (Kosaka and Ikeda, 1983b; Fergestad et al., 1999; Wucherpfennig et al., 2003).

Synaptotagmin has long been postulated to play a role in endocytosis (Nonet et al., 1993; Jorgensen et al., 1995; Reist et al., 1998; von Poser et al., 2000; Jarousse and Kelly, 2001; Jarousse et al., 2003; Llinás et al., 2004). Recently, functional studies have demonstrated that endocytosis is slowed twofold in syt^{null} mice (Nicholson-Tomishima and Ryan, 2004) and even more severely impaired in syt^{null} *Drosophila* third instars (Poskanzer et al., 2003). Both the synaptic vesicle depletion and the accumulation of large diameter vesicles in syt^{null} third instar neuromuscular junctions are consistent with synaptotagmin's proposed role in endocytosis.

Larger vesicles/cisternae (i.e., membranous structures >90 nm in diameter, Fig. 3.5) have been previously observed at *Drosophila* NMJs (Jia et al., 1993a; Koenig et al., 1993; Wucherpfennig et al., 2003). Interestingly, syt^{null} active zones exhibit a 16.2 fold increase in the number of these larger vesicles. Some of these structures are likely part of a degradative pathway as they include accumulations of multivesicular bodies, which are thought to play a role in protein and lipid degradation. Multivesicular bodies were rarely observed near syt^{wt}, $P[syt^{wt}]$ or $P[syt^{DN}]$ active zones. In *Drosophila*, multivesicular bodies have been shown to accumulate in nerve terminals of both potassium channel and stoned protein mutants. The reason for this accumulation is unknown. However, synaptotagmin protein is degraded in *stoned* mutants; therefore, in the case of these mutants, the accumulation of multivesicular bodies may be secondary to their loss of synaptotagmin.

While synaptic ultrastructure is severely disrupted in syt^{null} mutants, expression of the syt^{DN} mutant protein completely rescued these ultrastructural defects at third instar neuromuscular junctions. This finding is somewhat surprising since synaptic transmission is more severely disrupted in the *P[syt^{DN}]* larvae than in syt^{null} larvae (Mackler et al., 2002). In the vicinity of t-bars, we found no difference in the number of small, clear, synaptic vesicles between *P[syt^{DN}]* mutants and *P[syt^{wt}]* or syt^{wt} larvae (Fig. 3.3). Since no significant synaptic vesicle depletion occurs in *P[syt^{DN}]* mutants, we conclude that their defect in synaptic transmission is not due to impaired synaptic vesicle recycling leading to an insufficient supply of synaptic vesicles. Consistent with this finding, *P[syt^{DN}]* mutants do not show an accumulation of large diameter membranous structures in the vicinity of t-bars (Fig. 3.5A). Nor does displacement of synaptic vesicles away from sites of Ca^{2+} influx contribute to the increased EC_{50} for Ca^{2+} in *P[syt^{DN}]* mutants, since the distribution of synaptic vesicles with respect to the presynaptic membrane is unaltered (Fig. 3.4). In particular, *P[syt^{DN}]* mutants do not have decreased numbers of morphologically docked vesicles compared to either *P[syt^{wt}]* or syt^{wt} larvae. Since alterations in synaptic ultrastructure cannot explain the >95% decrease in synchronous neurotransmitter release recorded in *P[syt^{DN}]* mutants, our findings support the hypothesis that Ca^{2+}-sensing by synaptotagmin is specifically disrupted in these mutants.

How Ca^{2+}-bound synaptotagmin triggers synaptic vesicle fusion remains a fundamental question of synaptic transmission. One possibility is via synaptotagmin's Ca^{2+}-dependent interaction with t-SNARE heterodimers (Gerona et al., 2000; Bai and Chapman, 2004) or complete SNARE complexes (Davis et al., 1999; Bai and Chapman,

2004). Synaptotagmin mutants with disrupted t-SNARE heterodimer binding also show disrupted synaptic transmission (Bai et al., 2004b). Thus, the severe decrease in neurotransmitter release recorded in C_2B Ca^{2+}-binding motif mutants could be due to disrupted t-SNARE binding. However, whether or not Ca^{2+}-dependent t-SNARE binding is disrupted in these mutants is currently unknown.

A second possibility of how Ca^{2+}-bound synaptotagmin triggers synaptic vesicle fusion is via synaptotagmin's Ca^{2+}-dependent interaction with anionic phospholipids. Some mutations in both C_2A and C_2B that disrupt evoked transmitter release *in vivo* show a good correlation to disruptions in Ca^{2+}-dependent phospholipid binding *in vitro* (Fernández-Chacón et al., 2001; Mackler et al., 2002). In the C_2B Ca^{2+}-binding motif mutants, the severity of the decrease in phospholipid binding measured *in vitro* mirrors the severity of the disruption in synaptic transmission seen *in vivo* (Mackler et al., 2002). In C_2A, a mutation ($R_{233}Q$ in rat) that decreases synaptotagmin's Ca^{2+}-dependent affinity for negatively charged phospholipids by ~two-fold *in vitro*, also decreases the apparent Ca^{2+} affinity of neurotransmitter release by ~two-fold *in vivo* (Fernández-Chacón et al., 2001). These findings suggest that Ca^{2+}-dependent phospholipid binding by both C_2 domains are critical interactions for triggering fusion. However, other C_2A mutations are difficult to reconcile with this hypothesis. Several different mutations in C_2A's Ca^{2+}-binding motif that disrupt Ca^{2+}-dependent phospholipid binding by C_2A *in vitro* do not disrupt fast synchronous synaptic transmission *in vivo* (Fernández-Chacón et al., 2002; Robinson et al., 2002).

Closer scrutiny of the mutations in the context of synaptotagmin's Ca^{2+} and phospholipid binding activity may help explain this anomaly. In C_2A, five aspartates

partially coordinate the binding of three Ca^{2+} ions (Sutton et al., 1995; Shao et al., 1996; Ubach et al., 1998). In the absence of Ca^{2+}, these negatively charged aspartates are thought to repel the negatively charged plasmamembrane. However, when Ca^{2+} binds to these aspartates, the negatively charged plasma membrane completes the coordination of the Ca^{2+} ions, thus creating a triad between synaptotagmin, Ca^{2+} and the plasma membrane (Zhang et al., 1998b). Important concomitant interactions are thought to occur between a conserved arginine ($R_{233}Q$ in rat) and the negatively charged plasma membrane (Shao et al., 1997; Zhang et al., 1998b; Fernández-Chacón et al., 2001) as well as insertion of hydrophobic residues of synaptotagmin into the plasma membrane (Chae et al., 1998; Chapman and Davis, 1998) . Membrane insertion may act to destabilize the membrane, thereby facilitating the opening of the fusion pore (Jahn et al., 2003). In C_2A Ca^{2+}-binding motif mutants where either the forth or fifth negatively charged aspartates were neutralized, binding of the second and third Ca^{2+} ions was disrupted, but binding of the first Ca^{2+} ion remained intact. One Ca^{2+} ion bound, in combination with partial neutralization of the repulsive force of the negative charges, may still permit interactions between the conserved arginine and hydrophobic residues of C_2A with the plasmamembrane to facilitate fusion when an intact C_2B domain is also present. Indeed, when the C_2A Ca^{2+}-binding motif mutant is fused to an intact C_2B domain, *in vitro* phospholipid binding is not different from controls (Fernández-Chacón et al., 2002). Such coordinated action of the C_2A and C_2B domains during phospholipid binding could explain why C_2A Ca^{2+}-binding motif mutants show little synaptic transmission defects even though the isolated C_2A domain does not bind phospholipids well.

In conclusion, *P[syt^{DN}]* mutants do not show altered synaptic vesicle distribution or synaptic vesicle depletion. Thus, the cause of the severe decrease in evoked release recorded in these mutants (Mackler et al., 2002) is inconsistent with a synaptic vesicle recycling defect. Instead, it is likely due to the inability of mutant synaptotagmin to bind Ca^{2+}.

Unlike neuromuscular junctions of *P[syt^{DN}]* mutants, neuromuscular junctions of *syt^{null}* mutants have dramatically altered synaptic ultrastructure, including severe synaptic vesicle depletion, and an increase in large diameter vesicles that are postulated to be endosomal intermediates. Numerous functional studies implicate synaptotagmin in endocytosis (von Poser et al., 2000; Jarousse and Kelly, 2001; Jarousse et al., 2003; Poskanzer et al., 2003; Llinás et al., 2004; Nicholson-Tomishima and Ryan, 2004). The ultrastructural phenotype of *syt^{null}* mutants also supports a role for synaptotagmin in synaptic vesicle endocytosis at neuromuscular junctions. The ultrastructural phenotype of *syt^{null}* mutants also supports a role for synaptotagmin in synaptic vesicle docking at neuromuscular junctions, as docked vesicles are decreased in *syt^{null}* mutants at this synapse. Other studies have implicated synaptotagmin in docking vesicles at *Drosophila* central synapses (Reist et al., 1998), the squid giant synapse (Fukuda et al., 2000b), and in neuroendocrine cells (Chieregatti et al., 2002; Chieregatti et al., 2004). Finally, the accumulation of large membranous structures in *syt^{null}* mutants, including multivesicular bodies, suggests a novel function for synaptotagmin in a degradative pathway.

Acknowledgments

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Chapter 4: Synaptotagmin's C_2B Polylysine Motif is Required for Synaptic Vesicle Recycling, but not for Endocytosis *In vivo*.

Abstract

Synaptotagmin I is a multifunctional, synaptic vesicle protein required for efficient synaptic transmission. Synaptotagmin's C_2B domain contains a highly conserved polylysine motif necessary for synaptotagmin function. However, the polylysine motif's role in the synaptic vesicle cycle is unclear. *In vitro* experiments implicate this motif in three steps within the synaptic vesicle cycle: 1) synaptic vesicle endocytosis via a Ca^{2+}-independent interaction with AP-2 and/or PIP_2, 2) regulation of Ca^{2+}-triggered fusion via Ca^{2+}-dependent oligomerization, and 3) synaptic vesicle docking/priming via a Ca^{2+}-independent interaction with PIP_2 and/or t-SNARE heterodimers (syntaxin/SNAP-25). First, since we found no change in the rate of FM 1-43 dye uptake in *Drosophila* C_2B polylysine motif mutants, this polylysine motif isn't required for synaptic vesicle endocytosis. Second, the time constant of recovery from synaptic depression evoked by high frequency stimulation is >twofold slower in the mutants. Since the rate of recovery from depression is independent from the rate of fusion, the polylysine motif must be required for a step in synaptic vesicle recycling other than fusion itself. Third, the mutants exhibit greater synaptic facilitation and augmentation than controls, demonstrating a decreased release probability. Slowed

recovery from depression and synaptic/facilitation/augmentation are consistent with a disruption in synaptic vesicle docking/priming. Thus, while we do not exclude some role for the polylysine motif in fusion, a disruption of synaptic vesicle docking/priming could account for all of the deficits observed in the C_2B polylysine motif mutants.

Introduction

Synaptotagmin I (syt) is a synaptic vesicle protein whose cytoplasmic region contains two C_2 domains, C_2A and C_2B (Perin et al., 1991a; Brose et al., 1992). Although synaptotagmin is a Ca^{2+}-sensor for synaptic vesicle exocytosis (Brose et al., 1992; Geppert et al., 1994) via its C_2B Ca^{2+}-binding motif (Fig. 4.1, circles) (Mackler et al., 2002), it likely mediates other molecular interactions via additional functional motifs.

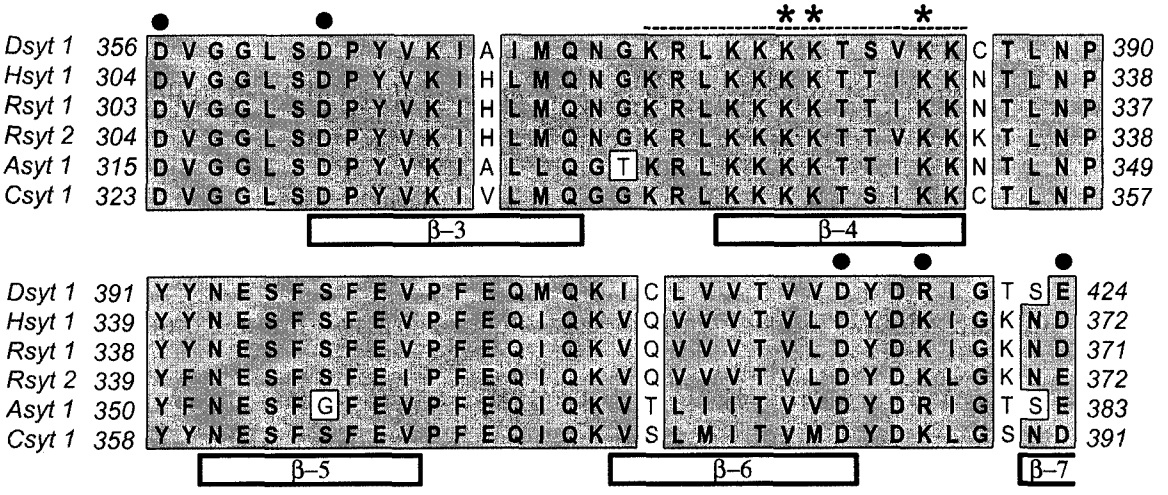

Figure 4.1 Synaptotagmin's C_2B polylysine region is highly conserved throughout evolution. ClustalW sequence alignment of 56 highly conserved amino acids from synaptotagmin's C_2B domain. Dotted line denotes the polybasic region. Asterisks mark the polylysine motif. Circles mark the Ca^{2+}-binding motif. Bars indicate ß-sheets within the C_2B domain.

Synaptotagmin's C_2B domain contains a highly conserved polybasic region (Fig. 4.1, dashes). Three lysines within this region (the polylysine motif, Fig. 4.1, asterisks) are required for full synaptotagmin function. In *Drosophila*, when these lysines are mutated to glutamines (syt^{KQ}) and the syt^{KQ} transgene is expressed in a syt^{null} background ($-/-; P[syt^{KQ}]$), evoked transmitter release is decreased compared to transgenic wild-type controls ($-/-;P[syt^{wt}]$) [(Mackler and Reist, 2001) and see Fig. 2A].

The function of the polylysine motif within the synaptic vesicle cycle remains unknown. Results from *in vitro* experiments suggest this motif may mediate three steps in the synaptic vesicle cycle. First, functional studies indicate that synaptotagmin I is required for synaptic vesicle endocytosis (von Poser et al., 2000; Jarousse and Kelly, 2001; Jarousse et al., 2003; Poskanzer et al., 2003; Llinás et al., 2004; Nicholson-Tomishima and Ryan, 2004). Synaptotagmin's C_2B domain may mediate this role (von Poser et al., 2000; Jarousse and Kelly, 2001; Littleton et al., 2001b; Jarousse et al., 2003; Llinás et al., 2004). Specifically, synaptic vesicle endocytosis may be mediated by the polylysine motif in synaptotagmin's C_2B domain (Takei and Haucke, 2001) via Ca^{2+}-independent interactions with the clathrin adaptor protein, AP-2 (Zhang et al., 1994; Chapman et al., 1998; Haucke and De Camilli, 1999; Haucke et al., 2000; Littleton et al., 2001b; Grass et al., 2004) and/or with PIP_2 (Bai et al., 2004a). Both AP-2 and PIP_2 are molecules that play a role in clathrin mediated endocytosis (Cremona and De Camilli, 2001; Hurley and Wendland, 2002).

A second proposed function for synaptotagmin's polylysine motif is regulation of Ca^{2+}-dependent fusion (Chapman et al., 1998; Wu et al., 2003). This motif mediates Ca^{2+}-dependent oligomerization of synaptotagmin *in vitro* (Chapman et al., 1998; Wu et

al., 2003), which has been proposed to regulate the opening, dilation, or stability of the fusion pore (Wang et al., 2001; Bai and Chapman, 2004).

Finally, the polylysine motif may participate in Ca^{2+}-independent docking and/or priming of synaptic vesicles. In the absence of Ca^{2+}, synaptotagmin preferentially binds to PIP_2 containing membranes, potentially mediating a *trans* interaction between the synaptic vesicle and the plasma membrane (Bai et al., 2004a) where PIP_2 is predominantly located (Holz et al., 2000; Micheva et al., 2001). *In vitro*, this interaction increases the rate of synaptotagmin penetration into lipid membranes upon Ca^{2+} influx, which is postulated to be important for fusion (Bai et al., 2004a). Also, synaptotagmin's Ca^{2+}-independent interaction with t-SNARE heterodimers may prime vesicles by bringing the synaptic vesicle v-SNARE (synaptobrevin/VAMP) into close proximity with the t-SNARE heterodimer, preparing the vesicle for Ca^{2+}-triggered fusion via formation of the SNARE complex (Rickman et al., 2004c).

Using electrophysiology, we demonstrate that the polylysine motif participates in a step of synaptic vesicle recycling other than fusion itself. As synaptic vesicle recycling is the recreation of a fusion competent vesicle following exocytosis, it consists of many steps, which include but are not limited to: endocytosis, clathrin uncoating, refilling with neurotransmitter, docking, and priming. Ultrastructural analysis and an FM 1-43 assay demonstrate that the polylysine motif is not required for synaptic vesicle endocytosis. The recycling defect seen in the polylysine motif mutants is more severe after stimulation ceases (when intracellular Ca^{2+} levels are low) than during stimulation (when intracellular Ca^{2+} levels are elevated). Thus, it is likely that the polylysine motif functions during a Ca^{2+}-independent step in the synaptic vesicle cycle. Finally, we

demonstrate that the polylysine motif mutants have a decreased probability of release. All of these results are consistent with the hypothesis that the polylysine motif plays an important role in Ca^{2+}-independent docking/priming of synaptic vesicles.

Materials and Methods

Fly stocks: Synaptotagmin's polylysine motif (Fig. 4.1) was mutated ($K_{379,380,384}Q$) and the mutant synaptotagmin was expressed from a transgene in a *synaptotagmin* null (syt^{AD4}) line. The experimental flies (*-/-;P[syt^{KQ}]*) had the genotype *yw; syt^{AD4} P[elav-Gal4]/syt^{AD4};P[UAS syt^{K379,380,384Q}]/+* and were generated as previously described (Mackler and Reist, 2001). The control flies (*-/-;P[syt^{wt}]*) expressed wild-type *synaptotagmin I* from a transgene (Mackler and Reist, 2001) and had the genotype *yw; syt^{AD4} P[elav-Gal4]/syt^{AD4};P[UAS syt^{wt}]/+*. Two independent transgenic wild-type synaptotagmin lines and two independent transgenic C_2B polylysine motif lines were generated. Since both lines of a given genotype had similar levels of evoked release [data not shown and (Mackler et al., 2002)], one control line and one mutant line were used in subsequent experiments.

Solutions: Standard saline for these experiments is defined as: 5mM KCl, 2mM $CaCl_2$, 130 mM NaCl, 2 mM $MgCl_2$, 36mM sucrose, 5 mM HEPES, pH 7.3 (Jan and Jan, 1976). In Ca^{2+}-free saline, the $CaCl_2$ was replaced by additional $MgCl_2$. Stimulating saline contained 90 mM KCl, 5 mM $CaCl_2$, 45 mM NaCl, 2mM $MgCl_2$, 36mM sucrose, 5mM HEPES, pH 7.3. HL3 saline contained: 5mM KCl, 70mM NaCl, 20mM $MgCl_2$, 10mM

$NaHCO_3$, 5mM HEPES, 115mM sucrose, 5mM trehalose and 1.5, 2.0 or 5.0 mM $CaCl_2$ (Stewart et al., 1994).

Dye Uptake Assay: Synaptic boutons in *Drosophila* third instars were labeled with the activity-dependent, fluorescent dye, FM 1-43 (Molecular Probes, Eugene, OR). Larvae were dissected in Ca^{2+}-free saline, stimulated for 6 minutes with stimulating saline, and the stimulating saline was replaced with standard saline containing 3μM TTX for a variable length of time ($\Delta t = 0$, 30, 60 or 180 seconds). TTX was added to block spontaneous activity coming from the attached CNS. After Δt, the preparation was incubated in standard saline + 3 μM TTX + 4μm FM 1-43 dye for 6 minutes and then washed for 15 minutes with three changes of Ca^{2+}-free saline.

Fluorescence Microscopy and Image Processing: FM 1-43 labeled preparations were viewed using a 40X/0.80 NA water immersion objective lens (Leica, Bannockburn, IL) on a Leica DMRA light microscope (Nussloch, Germany) equipped with epifluorescence optics (#51019 EGFP/DsRed filter, Chroma, Brattleboro, VT) and fitted with a microstepping servomotor in the z-axis. Images were captured (usually 1.0 s/frame) with a Hamamatsu CCD camera (C4742-95). A through focal series using 0.4μm steps was taken of each labeled neuromuscular junction (NMJ). All imaging was accomplished within 2 minutes of first exposure to light. Images were acquired, stored and processed using Open Lab 2.2.0 software (Improvision, Inc., Boston, MA) on a Mac G-4 platform.

The average fluorescence intensity of 12-15 brightly stained boutons with diameters $\geq$ 2 μm were measured at each neuromuscular junction. Only boutons on

muscle fibers 6 and 7 from segment 3 or 4 were used. Only one neuromuscular junction per preparation was imaged. Nine neuromuscular junctions (from nine larvae) were analyzed for each genotype. Each bouton was imaged in its optimal focal plane. Images were imported into the public domain Object Image program (http://simon.bio.uva.nl/object-image.html). Background fluorescence was measured over nearby muscle nuclei and was subtracted from the fluorescence intensity of each bouton. Statistical comparisons between genotypes were tested with a mixed model ANOVA using SAS software 6.12 (SAS Institute, Cary, NC).

Electrophysiology: Electrophysiology experiments were performed at room temperature in HL3 saline containing variable levels of Ca^{2+} (1.5mM, 2mM or 5mM). Recordings were from muscle fiber six from abdominal segments three and four of third instar larval fillet preparations. Fibers were impaled with 15-45 MΩ electrodes filled with a solution of three parts 2M potassium citrate to one part 3M potassium chloride. Segmental nerves were stimulated with a 5-10 μm diameter glass micropipette filled with HL3 to evoke EJPs (excitatory junctional potentials). Nerves were stimulated with 1ms pulses at the indicated frequency using an AxoClamp 2B (Molecular Devices, Union City, CA) and digitized using a MacLab4s A/D converter (Chart or Scope software, ADInstruments, Colorado Springs, CO). The resting membrane potential of each muscle fiber was normally between (-65 and -45 mV) but was maintained at about -55mV by passing a bias current. EJP amplitudes were determined using Chart or Scope software. Using SigmaPlot 2001 software (SPSS Inc., Chicago, IL), the time constant of recovery was

determined by fitting the data with a single exponential (3 parameters). The last four points from figure 4.5 were used to calculate the time constant of recovery.

EM: Third instars were dissected in cold, Ca^{2+}-free HL3 saline, and then embedded, sectioned and stained as previously described (Reist et al., 1998). Electron micrographs of neuromuscular junctions on muscle fiber number 6 or 7 from abdominal segments 3 and 4 were taken at 15,000X magnification on a JEOL JEM 2000 EX-II transmission electron microscope operated at 100 kV. Images were scanned using an Agfa DuoScan T2500 and adjusted for contrast using Adobe Photoshop software.

Results

Synaptotagmin polylysine motif mutants and transgenic wild-type controls show impaired synaptic vesicle recycling.

Evoked transmitter release in 1.5 mM Ca^{2+} is decreased ~40% in polylysine motif mutants compared to transgenic wild-type controls (Mackler and Reist, 2001). However, in the presence of 5 mM Ca^{2+}, evoked release is only decreased ~20%. The average excitatory junctional potential (EJP) amplitude evoked by 30 pulses at 0.05 Hz is 36.4 ± 0.9 mV for *-/-;P[syt^{KQ}]* (n=8 fibers) and 45.9 ± 1.1 mV for *-/-;P[syt^{wt}]* (n=8 fibers) (Fig. 4.2A). Thus, Ca^{2+} may provide partial rescue of the polylysine motif mutation. However, as non-linear summation is more of an issue in this range of EJP amplitudes, the degree of rescue may be minimal. While the polylysine motif is thus required for efficient evoked transmitter release, the specific role of the motif in the synaptic vesicle cycle is unknown.

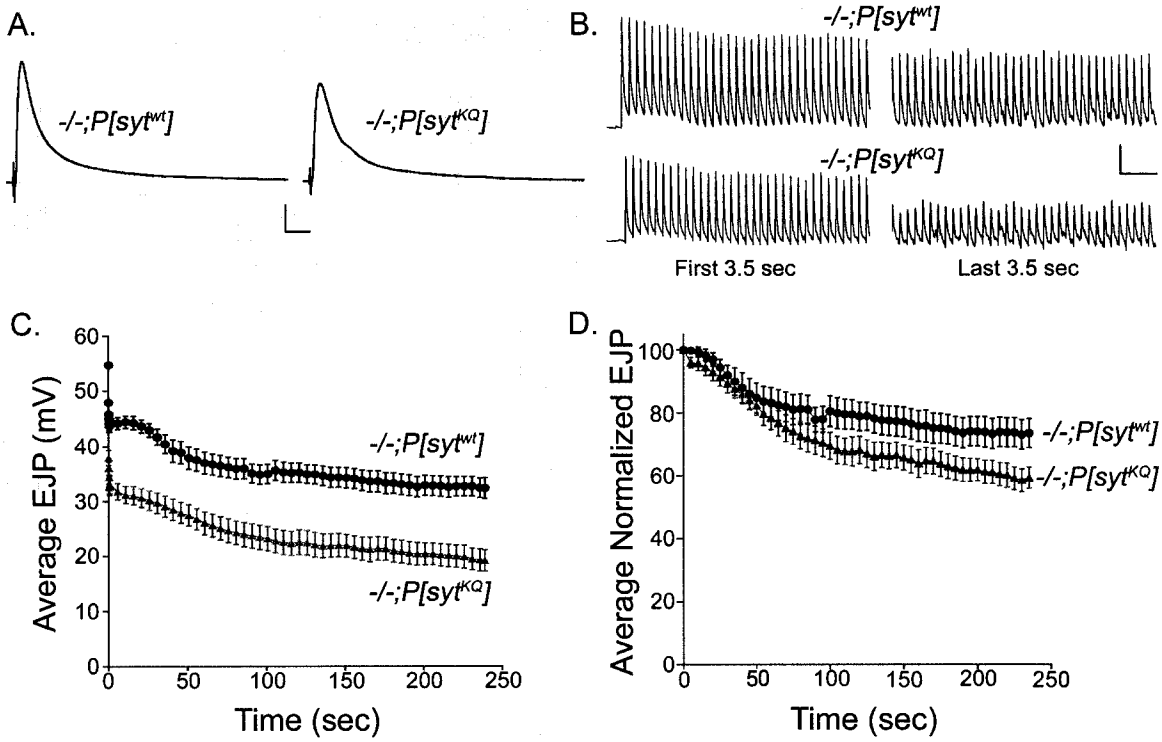

Figure 4.2 Synaptotagmin polylysine motif mutants show impaired synaptic transmission. **(A)** Representative traces showing evoked release during 0.05 Hz stimulation in 5 mM Ca^{2+} saline. Each trace is an average of 30 individual traces from a single muscle fiber. The mean EJP amplitude in *-/-;P[syt^{wt}]* was 45.9 ± 1.1 mV (mean ± SEM, n=8 fibers) and in *-/-;P[syt^{KQ}]* mutants was 36.4 ± 0.9 mV (mean ± SEM, n=8 fibers). Scale bar = 10 mV, 20 ms. **(B)** *-/-;P[syt^{wt}]* and *-/-;P[syt^{KQ}]* larvae were stimulated at 10 Hz in 5 mM Ca^{2+} saline for four min. Representative traces showing the first and last 3.5 sec of evoked release for both *-/-;P[syt^{wt}]* and *-/-;P[syt^{KQ}]* larvae. Scale bar = 15 mV, 0.5 sec. **(C)** Average EJP amplitudes evoked during four min of 10 Hz stimulation in 5 mM Ca^{2+} saline. Except for the first nine points and the last point, the EJP amplitudes were binned at 5 sec intervals, and the average of the bin is plotted. The first nine points are not binned. The last point is the average EJP amplitude of a 3 sec bin. *-/-;P[syt^{wt}]*, black, circles, n=8 NMJs. *-/-;P[syt^{KQ}]*, grey, circles, n=10 NMJs. **(D)** The same EJP amplitudes evoked during four min of 10 Hz stimulation in 5 mM Ca^{2+} saline and plotted in C were binned at 5 sec intervals and normalized to the first 5 sec bin.

To examine whether the polylysine motif functions in maintaining the supply of synaptic vesicles for release, we examined the ability of larvae to sustain neurotransmitter release at the neuromuscular junction during high frequency stimulation. During high

frequency stimulation, neurotransmitter release at *Drosophila* neuromuscular junctions is maintained both by mobilizing vesicles from a reserve pool, as well as recycling vesicles that have undergone release (Delgado et al., 2000). The relative contribution of each of these processes in maintaining the supply of vesicles for release likely depends on the stimulus frequency (Richards et al., 2003). When the rate of re-supply is slower than the rate of synaptic vesicle exocytosis, the result is a net loss of synaptic vesicles available for release, which is reflected in decreasing EJP amplitudes. Thus, the degree of synaptic depression reflects the balance between synaptic vesicle exocytosis and synaptic vesicle re-supply, which would include both mobilization and recycling.

During 10 Hz stimulation in 5 mM Ca^{2+}, the larval neuromuscular junction exhibited synaptic depression (Fig. 4.2, B, C and D). To address whether the polylysine motif (Fig. 4.1, asterisks) in the C_2B domain plays a role in maintaining the supply of vesicles for release, we examined the ability of the polylysine motif mutants to maintain neurotransmitter release during high frequency stimulation compared to a transgenic control line *(-/-;P[syt^{wt}])*. Similar levels of synaptotagmin expression are provided by the polylysine motif transgene and the control transgene (Mackler and Reist, 2001). Thus, the only difference between the transgenic polylysine motif mutants and the transgenic wild-type controls is the mutation in synaptotagmin's C_2B polylysine motif (Mackler and Reist, 2001). If the polylysine motif were critical for maintaining synaptic vesicle supply, the *-/-;P[syt^{KQ}]* larvae should exhibit substantially more depression than the *-/-;P[syt^{wt}]* larvae.

EJP amplitudes were binned at 5 sec intervals and normalized to the first EJP amplitude bin (Fig. 4.2D). EJPs from transgenic wild-type larvae (Fig. 4.2D, *-/-;P[syt^{wt}]*)

decreased to 83% of the first 5 sec. bin after 60 sec. of 10 Hz stimulation, and to 73% after 4 minutes of stimulation. EJP amplitudes from *-/-;P[syt^{KQ}]* larvae did decrease to a greater extent than the *-/-;P[syt^{wt}]* controls, 78% vs. 83% after 60 sec. of 10 Hz stimulation, and 60% vs. 73% after 4 minutes of stimulation. This result indicates that rate of synaptic vesicle re-supply relative to the rate of exocytosis is slower in the polylysine motif mutants than it is in controls. However, the increased synaptic depression in the polylysine motif mutants is not severe, and *-/-;P[syt^{KQ}]* larvae are able to maintain release relatively well for at least four minutes of 10 Hz stimulation. The relatively mild impairment in synaptic vesicle re-supply in the polylysine motif mutants may be because the demand on the vesicle re-supply system is less in these mutants than it is in controls; *-/-;P[syt^{KQ}]* mutants release fewer vesicles with each stimulus (Fig. 4.2A).

Synaptic ultrastructure is not altered in polylysine motif mutants.

While the decreased ability to maintain neurotransmitter release during high frequency stimulation indicates that *-/-;P[syt^{KQ}]* mutants have some defect in maintaining synaptic vesicle release, it does not tell us where in the process the defect is occurring. It could be in either synaptic vesicle mobilization or synaptic vesicle recycling.

One possibility is that endocytosis is disrupted in the polylysine motif mutants. Functional studies implicate synaptotagmin I in endocytosis (von Poser et al., 2000; Jarousse and Kelly, 2001; Jarousse et al., 2003; Poskanzer et al., 2003; Llinás et al., 2004; Nicholson-Tomishima and Ryan, 2004) and the polylysine motif of synaptotagmin's C_2B

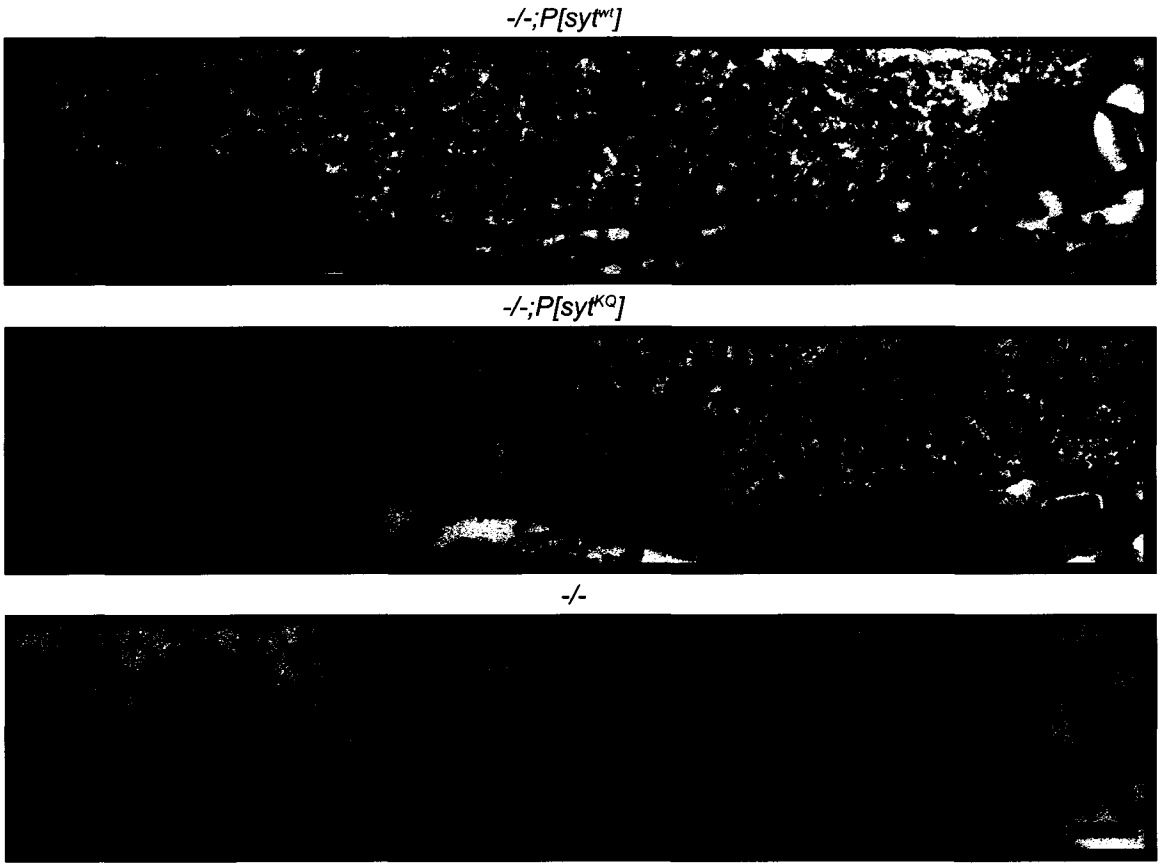

Figure 4.3 Synaptic vesicles are abundant in nerve terminals of polylysine motif mutants. Electron micrographs showing the synaptic ultrastructure of neuromuscular junctions in transgenic controls (top panel), polylysine motif mutants (middle panel), and syt^{null} mutants (bottom panels). syt^{null} munants show severe synaptic vesicle depletion as well as an accululation of larger membranous structures, consistant with an endocytosis defect. In contrast, numerous synaptic vesicles are found throughout the nerve terminal, including areas around active zones, in both transgenic lines, demonstrating that both mutant and wild-type synaptotagmin transgenes rescue the syt^{null} ultrastrucural phenotype. Bar = 200.

domain is postulated to mediate this role (Takei and Haucke, 2001). A common ultrastructural phenotype seen at neuromuscular junctions in endocytic mutants is severe synaptic vesicle depletion (Koenig et al., 1989; Zhang et al., 1998a; Verstreken et al., 2002; Verstreken et al., 2003). Indeed, syt^{null} mutations in *Drosophila* exhibit such depletion, along with an accumulation of larger membranous structures [Fig. 4.3, bottom panels, see also (Reist et al., 1998) and Chapter 3]. We wondered if the polylysine motif mutants would also exhibit such depletion, indicative of an endocytic defect. Thus, we examined the synaptic ultrastructure of *-/-;P[syt^{KQ}]* mutants (Fig. 4.3).

The synaptic ultrastructure observed in syt^{null} mutants (Fig. 4.3, bottom panel) is consistent with synaptotagmin's demonstrated role in synaptic vesicle endocytosis (Poskanzer et al., 2003; Nicholson-Tomishima and Ryan, 2004). However, the boutons of *-/-;P[syt^{wt}]* larvae (Fig. 4.3, top panel) and *-/-;P[syt^{KQ}]* mutants (Fig. 4.3, middle panel) contain numerous synaptic vesicles located at active zones and throughout the nerve terminal, and no accumulation of larger membranous structures. Thus, the polylysine motif mutant transgene and the wild-type transgene are able to rescue the ultrastructural deficits seen in syt^{null} mutant terminals. This finding suggests that synaptotagmin's polylysine motif is not critical for synaptic vesicle endocytosis.

The rate of synaptic vesicle endocytosis is not impaired in synaptotagmin polylysine motif mutants.

To examine endocytic function in *-/-;P[syt^{KQ}]* mutants directly, we used an FM 1-43 dye uptake assay. FM 1-43 fluoresces when it inserts into lipid membranes. If it is present when synaptic vesicle membrane is internalized, these synaptic vesicles will be

fluorescently labeled. Subsequently, the dye can be washed from the outside of cells, leaving only the internalized synaptic vesicles labeled. Thus, FM 1-43 makes it possible to examine the process of endocytosis directly (Betz et al., 1992).

Figure 4.4A illustrates the endocytic assay we used to test the polylysine motif's involvement in endocytosis. *Drosophila* third instars were stimulated for six minutes with a high potassium stimulating saline. Stimulation was terminated when the stimulating saline was replaced by standard saline plus 3 μM TTX. TTX was included to block any spontaneous action potentials coming from the attached CNS. The standard saline + TTX was left on the preparation for a variable amount of time ($\Delta t = 0$, 30, 60 or 180 sec). After Δt, the preparation was bathed in standard saline containing TTX and FM 1-43 for six minutes to label endocytosing vesicles. Finally the preparation was washed for 15 minutes with three changes of Ca^{2+}-free saline to remove extracellular FM 1-43 from the plasma membrane. Ca^{2+}-free saline was used to minimize vesicle fusion during the washing stage. After washing, preparations were imaged with a fluorescence microscope and the fluorescence intensity of the FM 1-43 labeled synaptic boutons was measured.

Because FM 1-43 is not included during the stimulation period, but is instead added after stimulation ceases, the above protocol allowed us to determine the rate of internalization independent of the rate of exocytosis. However, only those synaptic vesicles that remain on the surface at the end of the stimulation can be labeled. Thus, a strong stimulus was needed to ensure maximal exocytosis so that the amount of synaptic vesicle membrane awaiting endocytosis from the plasma membrane after the stimulation stopped would provide sufficient fluorescent signals for analysis. In 1.5 mM Ca^{2+} saline,

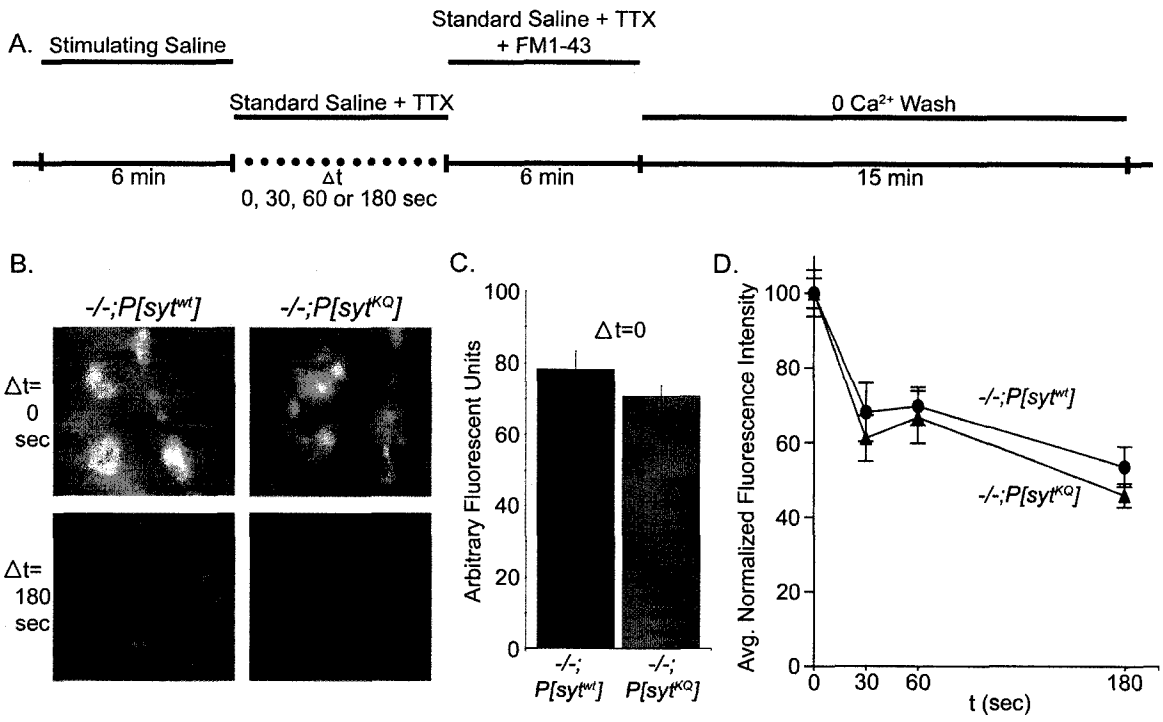

Figure 4.4 The rate of endocytosis is not significantly different in synaptotagmin polylysine motif mutants. **(A)** Larvae were stimulated for six minutes in stimulating saline. This saline was subsequently replaced with standard saline containing 3 μM TTX for a variable length of time (Δt = 0, 30, 60 or 180 sec). After Δt, the preparation was incubated in standard saline + 3 μM TTX + 4 μM FM 1-43 dye for six minutes. To remove non-internalized dye, the preparation was washed for 15 minutes with three changes of Ca^{2+}-free saline. **(B)** Synaptic boutons on muscle 6 and 7 in *-/-;P[syt^{wt}]* controls and *-/-:P[syt^{KQ}]* mutants were labeled with FM 1-43 as described in A. Labeling is brightest in both genotypes at Δt = 0 sec (top panels), and is dimmer after Δt = 180 sec (bottom panels). **(C)** At Δt = 0, the average fluorescence intensity of boutons from polylysine motif mutants was ~10% less than controls. **(D)** Fluorescence intensity of synaptic boutons is graphed as a function of the time between stimulus termination and application of the dye. *-/-;P[syt^{wt}]*, circles, n=135 boutons from 9 preps for each Δt. *-/-:P[syt^{KQ}]*, triangles, n=132-135 boutons from 9 preps for each Δt. To account for difference between genotypes in the absolute number of synaptic vesicles released during stimulation, fluorescence values for each genotype were normalized to the fluorescence intensity of that genotype at Δt = 0 sec. 95% C.I. = -11.6, 2.75.

evoked release is decreased by ~40% in the polylysine mutants (Mackler and Reist, 2001). This decreased release results in fewer vesicles cycling. Thus, FM 1-43 labeling achieved by the above protocol was quite dim in the polylysine motif mutants when the stimulating saline contained standard levels of Ca^{2+}. Because our goal in these experiments was to determine whether endocytosis is the major defect in the polylysine motif mutants, we increased the Ca^{2+} in the stimulating saline to 5 mM to increase FM 1-43 labeling. In 5 mM Ca^{2+}, the average EJP amplitude in polylysine motif mutants (~36 mV, Fig. 4.2A) is ~90% of the average EJP amplitude recorded from true wild-type larvae in 1.5 mM Ca^{2+} [~40 mV, (Mackler and Reist, 2001)]. Thus, increasing Ca^{2+} to 5 mM increased neurotransmitter release in *-/-;P[syt^{KQ}]* larvae to a level that is similar to the level of release recorded in true wild-type larvae in saline containing standard Ca^{2+} concentrations. Therefore, the strong stimulus used to evoke maximal release in these experiments was a high K^+, high Ca^{2+} saline.

At all time points, the mean fluorescence of *-/-;P[syt^{KQ}]* boutons was lower than the mean fluorescence for controls (Fig. 4.4C and data not shown). This is most likely because the mutants exocytose fewer vesicles than the controls during the stimulation. The absolute amount of fluorescence at delta t=0 is correlated with the amount of membrane awaiting endocytosis after the stimulation stops. Figure 4.4C shows the amount of FM 1-43 labeling at delta t=0 sec for both mutants and controls. Under these stimulation conditions (high K^+, high Ca^{2+} for 6 min.), the amount of membrane awaiting endocytosis after stimulation is less in the polylysine motif mutants, but only by ~10%. To determine the kinetics of synaptic vesicle internalization independent of this ~10% difference, we normalized our data according to previous methods used to compare

endocytic rates between preparations with different amounts of exocytosis (Ryan et al., 1996b; Ryan et al., 1996a; Stimson et al., 2001). The fluorescence value of each bouton was normalized to the mean fluorescence value of boutons of the corresponding genotype at Δt=0 sec.

During the delay period between the end of stimulation and addition of FM 1-43 dye, Δt, some fraction of the synaptic vesicle membrane remaining on the plasma membrane undergoes endocytosis. As Δt increases, more and more vesicle membrane is internalized, leaving less vesicle membrane on the surface to be labeled when FM 1-43 is subsequently applied. Therefore, as Δt increases, less FM 1-43 is internalized and nerve terminal labeling becomes progressively dimmer. This is illustrated in Figure 4.4B which shows FM 1-43 labeled synaptic boutons on muscle 6 and 7 in both *-/-;P[syt^{wt}]* and *-/-;P[syt^{KQ}]* larvae after Δt=0 sec and Δt=180 sec. In both *-/-;P[syt^{KQ}]* and *-/-;P[syt^{wt}]* larvae, labeling was brightest at Δt=0 sec and dimmest at Δt=180 sec. Loading was dependent on both stimulation and Ca^{2+} (data not shown).

If endocytosis were impaired in *-/-;P[syt^{KQ}]* larvae, then during any given Δt they should not be able to endocytose as large a fraction of the remaining synaptic vesicle membrane as controls. As a result, a larger proportion of synaptic vesicle membrane would remain in the plasma membrane when the FM 1-43 was applied, leading to proportionally more FM 1-43 internalization. Thus, if *-/-;P[syt^{KQ}]* mutants had endocytic deficits, then after any given Δt, *-/-;P[syt^{KQ}]* terminals should exhibit a normalized fluorescence that was brighter than controls. This was not observed. The normalized results are plotted in figure 4.4D. At each time point, the normalized fluorescence of the mutant terminals was slightly lower than that of controls. Lower

fluorescence values would suggest a faster rate of recycling in the mutants, not an impaired rate. However, the differences between *-/-;P[syt^{KQ}]* and *-/-;P[syt^{wt}]* were not statistically significant. A mixed model ANOVA was used to generate a 95% confidence interval for the true difference between *-/-;P[syt^{KQ}]* and *-/-;P[syt^{wt}]* which included 0 (–11.6, 2.75). We conclude that the polylysine motif in synaptotagmin's C_2B domain is not required for synaptic vesicle endocytosis.

Synaptotagmin polylysine motif mutants show marked defects in recovery from synaptic depression.

To further characterize the synaptic vesicle cycle defect caused by the polylysine motif mutation, we examined the rate of recovery from synaptic depression in both transgenic mutants and controls. As recovery from synaptic depression occurs by synaptic vesicle recycling rather than vesicle mobilization (Pyle et al., 2000; Richards et al., 2003), this experiment allowed us to examine the role of the polylysine motif specifically in the recycling process. It also allowed us to determine whether Ca^{2+}-dependent fusion is the sole defect in the mutants, as would be expected if the only role of the polylysine motif were to regulate fusion via Ca^{2+}-dependent oligomerization. If a disruption in fusion were the primary defect in these mutants, they should recover from synaptic depression as quickly as controls.

Larvae were stimulated at 10 Hz for 30 sec to induce synaptic depression and recovery was examined 5 sec or 15 sec after the stimulation ceased (Fig. 4.5).

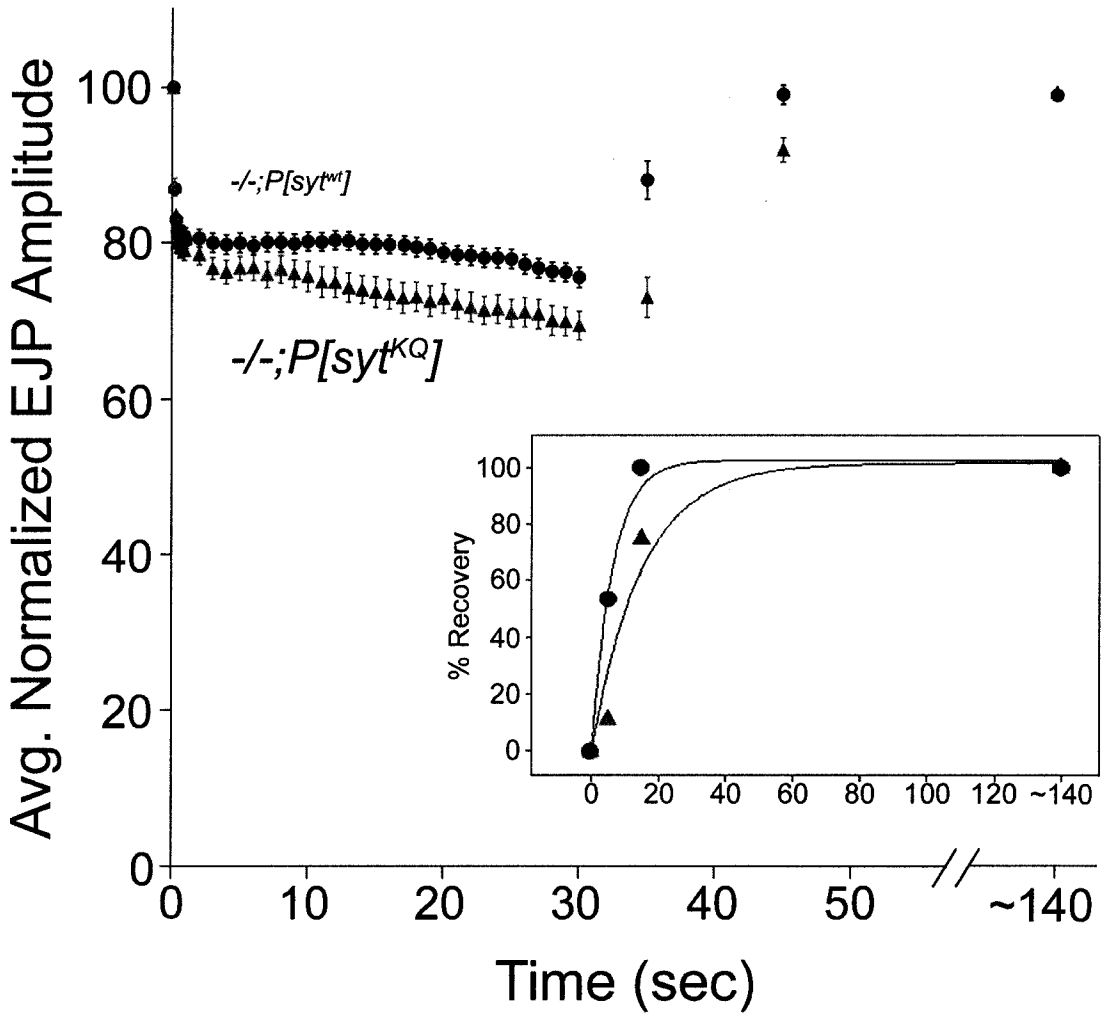

Figure 4.5 *-/-;P[syt^{KQ}]* mutants recover more slowly from synaptic depression than *-/-;P[syt^{wt}]* controls. Synaptic depression was elicited by 30 sec of stimulation in 5 mM extracellular Ca^{2+}. Recovery was monitored by recording the EJP amplitude of a single shock either 5 sec or 15 sec after the 10 Hz stimulation stopped. Only larvae with >95% recovery by ~140 sec were included. EJP amplitudes were normalized as in figure 2. After the first 9 points, the points plotted during the 10 Hz stimulation are averages of 10 EJPs. *-/-;P[syt^{wt}]*, black, circles, n=10 NMJs from 7 preps for 5 sec recovery and n=14 NMJs from 7 preps for 15 sec recovery. *-/-;P[syt^{KQ}]*, grey, triangles, n=10 NMJs from 5 preps for 5 sec recovery and n=14 NMJs from 11 preps for 15 sec recovery. Error bars denote SEM. To determine the time constant of recovery, the last four points were re-plotted in the inset. Inset: The last four points were normalized, so that the first point of each genotype showed 0% recovery and the last point showed 100% recovery, using the following equation: y = ylast*(y-y0)/(ylast-y0), where y0 = the normalized EJP of the last point in the 10 Hz train and ylast is the normalized EJP of the last recovery shock at ~140 sec for each genotype. The points were replotted so that the first point is at 0 sec (rather than at 30 sec). Recovery curves for each genotype were fitted with a single exponential.

EJP amplitudes were normalized to the EJP amplitude elicited by the first pulse in the train. Only larvae that exhibited ≥95% recovery at an extended time point were used in this assay. While -/-;*P[syt^{KQ}]* larvae exhibited only ~6% more synaptic depression than -/-;*P[syt^{wt}]* larvae after 30 sec of 10 Hz stimulation, the recovery from this depression in -/-;*P[syt^{KQ}]* larvae was >twofold slower. Single exponential fits to the recovery curves gave time constants of 6.2 sec for -/-;*P[syt^{wt}]* and 15.0 sec for -/-;*P[syt^{KQ}]* (Fig. 4.5, inset).

The slower rate of recovery in -/-;*P[syt^{KQ}]* mutants demonstrates that a major defect caused by the polylysine motif mutation occurs prior to the Ca^{2+}-dependent fusionstep. Although a slowed recovery from synaptic depression could be explained by slowed endocytosis, we have already ruled out endocytosis as the major defect in -/-;*P[syt^{KQ}]* larvae (see above). Thus, the slowed recovery must be due to a defect in a step of synaptic vesicle recycling that occurs after endocytosis and before fusion. Furthermore, as intracellular Ca^{2+} levels are likely quite low during the slow recovery period in -/-;*P[syt^{KQ}]* mutants (see discussion), this defect is likely in a Ca^{2+}-independent process.

Synaptotagmin polylysine motif mutants have a decreased release probability.

Results from *in vitro* experiments have implicated the polylysine motif in endocytosis, Ca^{2+}-dependent fusion, and Ca^{2+}-independent docking/priming of synaptic vesicles. Our *in vivo* data show that the polylysine motif does not play a significant role in synaptic vesicle endocytosis, but that it does play a role prior to Ca^{2+}-dependent fusion. To test whether this motif is involved in synaptic vesicle docking/priming, we

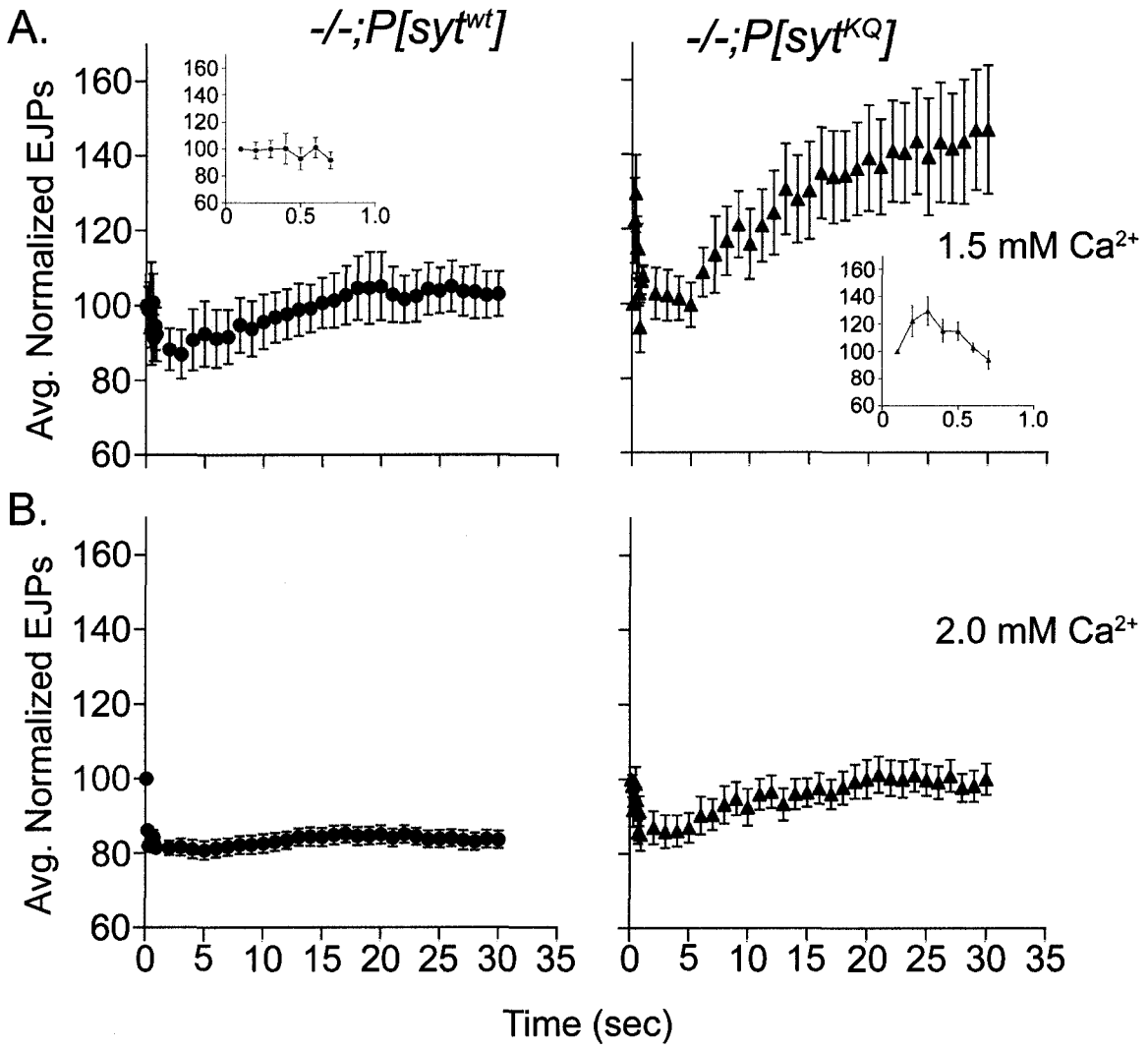

Figure 4.6 -/-; *P[syt^{KQ}]* mutants have a decreased release probability compared to -/-; *P[syt^{wt}]* controls. Amplitudes of EJPs evoked by 30 sec of 10 Hz stimulation in 1.5 mM (A) or 2.0 mM (B) extracellular Ca^{2+}. EJP amplitudes for each genotype were normalized as in figure 2. After the first 9 points, the points plotted are averages of 10 EJPs. At 1.5 Mm Ca^{2+}, in addition to a large degree of synaptic augmentation, -/-; *P[syt^{KQ}]* mutants also exhibit facilitation (panel A right, see inset for expanded time scale), whereas -/-; *P[syt^{wt}]* controls do not (panel A left, see inset for expanded time scale). At 2.0 mM Ca^{2+}, the degree of augmentation in -/-; *P[syt^{KQ}]* mutants is more similar to that of -/-; *P[syt^{wt}]* controls in 1.5 mM Ca^{2+} than -/-; *P[syt^{wt}]*controls in 2.0 mM Ca^{2+}. -/-; *P[syt^{wt}]*, circles, n=6 NMJs from 5 preps for 1.5 mm Ca^{2+} and 10 NMJs from 7 preps for 2.0 mM Ca^{2+}. -/-; *P[syt^{KQ}]*, triangles, n=6 NMJs for 1.5 mM Ca^{2+} and 9 NMJs from 9 preps for 2.0 mM Ca^{2+}.

investigated the effect of extracellular Ca^{2+} on synaptic augmentation and facilitation. A deficit in docking/priming should result in a decreased release probability that can be detected by an increase in synaptic augmentation and facilitation (Zucker and Regehr, 2002). EJPs were evoked by 10 Hz stimulation for 30 seconds. *-/-;P[syt^{KQ}]* mutants exhibited larger synaptic augmentation than *-/-;P[syt^{wt}]* larvae in 1.5 mM extracellular Ca^{2+} (Fig. 4.6A) and still exhibited synaptic augmentation at 2.0 mM extracellular Ca^{2+}, a concentration where *-/-;P[syt^{wt}]* larvae no longer augment (Fig. 4.6B). In addition to greater synaptic augmentation, *-/-;P[syt^{KQ}]* mutants also exhibited facilitation during the first few pulses of 10 Hz stimulation in 1.5 mM Ca^{2+}; but *-/-;P[syt^{wt}]* larvae did not (Fig.4.6A, see insets). These data indicate that *-/-;P[syt^{KQ}]* mutants have a decreased release probability compared to *-/-;P[syt^{wt}]* larvae, consistent with a defect in synaptic vesicle docking/priming.

Discussion

The polylysine motif in synaptotagmin I's C_2B domain is highly conserved throughout evolution and between synaptotagmin isoforms [Fig. 4.1, and (Rickman et al., 2004c)]. Since the polylysine motif is critical for efficient synaptic transmission *in vivo* (Mackler and Reist, 2001), it must play a functional role in the synaptic vesicle cycle. Three molecular mechanisms have been proposed to mediate this role: 1) synaptic vesicle endocytosis via Ca^{2+}-independent interactions with AP-2 and/or PIP_2 containing membranes; 2) regulation of Ca^{2+}-triggered fusion via Ca^{2+}-dependent self-oligomerization; 3) docking and/or priming of synaptic vesicles via Ca^{2+}-independent interactions with t-SNARE heterodimers and/or PIP_2 containing membranes.

We have employed a variety of techniques at intact neuromuscular junctions that allowed us to examine the role of the polylysine motif in the synaptic vesicle cycle. Although our *in vivo* studies demonstrate that the polylysine motif plays a role in overall synaptic vesicle recycling, they rule out a major role for the polylysine motif in synaptic vesicle endocytosis. We further demonstrate that the polylysine motif must participate in a step other than fusion itself, and that the polylysine motif mutants have a decreased probability of release. These results support the hypothesis that the major role of synaptotagmin's polylysine motif is in Ca^{2+}-independent docking and/or priming of synaptic vesicles.

During high frequency stimulation, EJP amplitudes decrease in *-/-;P[syt^{KQ}]* mutants to a slightly greater extent than transgenic controls. This indicates that *-/-;P[syt^{KQ}]* mutants do have some defect in maintaining a supply of synaptic vesicles for release. However, the electrophysiological phenotype of these mutants is not consistent with the hypothesis that their major defect is strictly endocytic. In synaptojanin and endophilin mutants, evoked release at low frequency stimulation is not different than controls. However, during four minutes of 10 Hz stimulation, the mutants exhibit significantly more synaptic depression than controls (Verstreken et al., 2002; Verstreken et al., 2003). In contrast, *-/-;P[syt^{KQ}]* mutants do have a defect in evoked release during low frequency stimulation [Fig. 4.2A, and (Mackler and Reist, 2001)]. However, unlike endophilin and synaptojanin mutants, polylysine motif mutants are able to maintain release almost as well as controls during four minutes of 10 Hz stimulation (Fig 4.2C and D). Indeed, if endocytosis is completely blocked, as it is in *shibire* mutants at the nonpermissive temperature, evoked release is decreased by >90% after four minutes of

10 Hz stimulation (Delgado et al., 2000). Dap160/Intersection is another protein that has been implicated in synaptic vesicle endocytosis and in synaptic growth (Marie et al., 2004). Dap160/Intersection mutations also cause more severe synaptic depression during high frequency stimulation than polylysine motif mutations. Furthermore, unlike polylysine motif mutations, Dap160/Intersection mutations do not change synaptic vesicle release probability (Marie et al., 2004).

The ultrastructural phenotype of -/-;*P[syt^{KQ}]* mutants is also not consistent with the hypothesis that the polylysine motif functions primarily during synaptic vesicle endocytosis. Ultrastructural analysis of nerve terminals in strictly endocytic mutants show severe synaptic vesicle depletion (Koenig et al., 1989; Zhang et al., 1998a; Guichet et al., 2002; Verstreken et al., 2002; Verstreken et al., 2003). Indeed, *syt^{null}* mutants in *Drosophila* and *C. elegans* also show severe synaptic vesicle depletion [(Jorgensen et al., 1995; Reist et al., 1998), see also Fig. 4.3 and Chapter 3], consistent with synaptotagmin's role in endocytosis (von Poser et al., 2000; Jarousse and Kelly, 2001; Jarousse et al., 2003; Poskanzer et al., 2003; Llinás et al., 2004; Nicholson-Tomishima and Ryan, 2004). However, nerve terminals in -/-;*P[syt^{KQ}]* mutants do not exhibit synaptic vesicle depletion (Fig. 4.3). Instead, the polylysine motif mutant synaptotagmin transgene rescues the *syt^{null}* ultrastructural phenotype, and suggests that the defect in polylysine motif mutants is not a strictly endocytic one.

We directly measured the rate of synaptic vesicle internalization in -/-;*P[syt^{KQ}]* mutants and controls using an FM 1-43 assay. In this assay, synaptic vesicle release was evoked by 5 mM Ca^{2+}/90 mM K^{+} saline for 6 min. This assay showed that the rate of endocytosis in -/-;*P[syt^{KQ}]* mutants is not significantly different from -/-;*P[syt^{wt}]* larvae

(Fig. 4.4C). Previous studies indicate that the rate of endocytosis may slow as more and more membrane is internalized (Wu and Betz, 1996; Sun et al., 2002), [see however, Sankaranarayanan and Ryan, (2000) and Yamashita et al., (2005)]. Thus, the trend towards faster internalization in the polylysine motif mutants may be due to the fact that they have ~10% less membrane to endocytose after stimulation than do their controls; the boutons of polylysine motif mutants are ~90% as bright as control boutons at $\Delta t=0$ (Fig. 4.4C).

Multiple studies have indicated a functional role for synaptotagmin I in synaptic vesicle endocytosis *in vivo*. Fluorescein-assisted light inactivation (FALI) of synaptotagmin I after exocytosis but before endocytosis completely blocked internalization of n-synaptobrevin and FM 4-64 (Poskanzer et al., 2003). Neurotransmitter release was completely blocked in squid giant axons that were injected with an antibody against synaptotagmin's C_2B domain (Llinás et al., 2004). This complete block of neurotransmitter release was accompanied by synaptic vesicle depletion and a >fourfold reduction in the number of clathrin-coated vesicles, consistent with a severe endocytic defect. In hippocampal synapses from syt^{null} mice, endocytosis was slowed by >twofold (Nicholson-Tomishima and Ryan, 2004). And finally, nerve terminals in synaptotagmin I null mutants are severely depleted of synaptic vesicles [(Jorgensen et al., 1995; Reist et al., 1998), see also Fig. 4.3 and Chapter 3]. While the C_2B domain has been implicated in synaptotagmin's endocytic function (Jarousse and Kelly, 2001; Littleton et al., 2001b; Jarousse et al., 2003; Llinás et al., 2004), our studies clearly demonstrate that the polylysine motif within the C_2B domain is not involved. Our results do not, however, rule out a role for other motifs within the C_2B domain of

synaptotagmin in synaptic vesicle endocytosis. Indeed, the conserved WHXL motif in synaptotagmin's C_2B domain has been shown to be critical for internalization of synaptotagmin in PC12 cells (Jarousse et al., 2003).

At neuromuscular junctions in both *Drosophila* and frog, intracellular Ca^{2+} levels rise quickly to a plateau during stimulation (Wu and Betz, 1996; Karunanithi et al., 1997; Suzuki et al., 2000; Macleod et al., 2002). The level of the plateau depends on both the stimulation frequency (Karunanithi et al., 1997; Suzuki et al., 2000; Macleod et al., 2002) and the extracellular Ca^{2+} concentration (Karunanithi et al., 1997; Suzuki et al., 2000). However, the duration of the plateau only appears to last as long as the stimulation (Wu and Betz, 1996; Karunanithi et al., 1997; Suzuki et al., 2000; Macleod et al., 2002). The plateau has been demonstrated to last for at least 10 sec of 10 Hz stimulation in *Drosophila* (Karunanithi et al., 1997), and for 300 sec of 30 Hz stimulation in frog (Wu and Betz, 1996). After stimulation ceases, the majority of the increased intracellular Ca^{2+} is removed within a few seconds; a slower process then restores Ca^{2+} back down to resting levels (Wu and Betz, 1996; Karunanithi et al., 1997; Suzuki et al., 2000; Macleod et al., 2002).

The ability of *-/-;P[syt^{KQ}]* mutants to maintain neurotransmitter release during 30 sec of high frequency stimulation is less affected (>90% of *-/-;P[syt^{wt}]*) than their ability to recover from this synaptic depression (>twofold slower). This difference may be because intracellular Ca^{2+} is higher during stimulation compared to after stimulation (Wu and Betz, 1996; Karunanithi et al., 1997; Suzuki et al., 2000; Macleod et al., 2002). Elevated intracellular Ca^{2+} during a stimulus train is thought to accelerate the rate of recovery from synaptic depression during the train compared to the rate of recovery post

stimulation (Zucker and Regehr, 2002). Our observation that -/-;*P[syt^{KQ}]* mutants have greater difficulty recovering from depression [when intracellular Ca^{2+} levels have fallen] than maintaining neurotransmitter release [when intracellular Ca^{2+} levels are elevated] suggests that the major defect in -/-;*P[syt^{KQ}]* mutants is likely in a Ca^{2+}-independent process. It also suggests that the elevated intracellular Ca^{2+} level that occurs during the stimulus train partially masks the defect caused by the polylysine motif mutation. During the stimulus train, Ca^{2+}-dependent processes that aren't impaired by the mutation partially rescue the Ca^{2+}-independent defect. Increased intracellular Ca^{2+}during stimulation may explain why a recent study found little change in synaptic vesicle priming in *syt^{null}* mice (Nicholson-Tomishima and Ryan, 2004). Nicholson-Tomishima and Ryan (2004) examined synaptic vesicle priming *during* 20 Hz stimulation.

The >twofold slowing of recovery from synaptic depression in -/-;*P[syt^{KQ}]* mutants compared to controls, indicates that the polylysine motif plays a role in synaptic vesicle recycling at a step other than fusion itself. If the primary defect in -/-;*P[syt^{KQ}]* mutants were in Ca^{2+}-triggered fusion itself, -/-;*P[syt^{KQ}]* mutants should recover from synaptic depression as quickly as controls, but they do not. Thus, the primary defect in -/-;*P[syt^{KQ}]* mutants cannot be solely in the fusion step.

What is the role of synaptotagmin's C_2B polylysine motif in the synaptic vesicle cycle? We suggest that the most straightforward explanation for all the results presented here is that the polylysine motif plays a critical role in synaptic vesicle docking and /or priming at release sites. Although a quantitative analysis of synaptic vesicle localization is required to determine whether or not -/-;*P[syt^{KQ}]* mutants exhibit a docking defect, docked vesicles are easily found in micrographs of -/-;*P[syt^{KQ}]* nerve terminals (Fig 4.3,

middle panel). Thus, we favor priming, rather than docking, as the major role for synaptotagmin's C_2B polylysine motif in the synaptic vesicle cycle. Consistent with a priming defect, *-/-;P[syt^{KQ}]* mutants have a decreased probability of release compared to *-/-;P[syt^{wt}]* larvae, and are >twofold slower to recover from synaptic depression than *-/-;P[syt^{wt}]* larvae.

In vitro binding data support the hypothesis that Ca^{2+}-independent docking and/or priming are impaired in *-/-;P[syt^{KQ}]* mutants. The C_2B polylysine motif exhibits Ca^{2+}-independent interactions with t-SNARE heterodimers (syntaxin/SNAP-25) (Rickman et al., 2004c) and PIP_2 containing membranes (Bai et al., 2004a). Synaptotagmin's interaction with t-SNARE heterodimers may prime vesicles by bringing the synaptic vesicle v-SNARE (synaptobrevin/VAMP) into close proximity with the t-SNAREs (Rickman et al., 2004c). Synaptotagmin's interaction with PIP_2, located predominately in the plasmamembrane (Holz et al., 2000; Micheva et al., 2001), may facilitate a *trans* interaction between synaptotagmin in the synaptic vesicle membrane and PIP_2 in the plasma membrane. This Ca^{2+}-independent interaction has been proposed to mediate docking or priming of vesicles because it increases the rate of synaptotagmin's Ca^{2+}-dependent penetration into lipid membranes (Bai et al., 2004a), a step thought to be important for fusion. Thus, disruption of either of these interactions could impair Ca^{2+}-independent docking or priming while leaving many interactions proposed to mediate Ca^{2+}-dependent docking and priming intact (Augustine, 2001).

In summary, electron micrographs show that *-/-;P[syt^{KQ}]* larvae do not exhibit synaptic vesicle depletion. An FM 1-43 assay demonstrates that the rate of endocytosis is also not impaired in these mutants. *-/-;P[syt^{KQ}]* larvae exhibit only minor increases in

synaptic depression during high frequency stimulation when Ca^{2+} levels are elevated. However, *-/-;P[syt^{KQ}]* larvae are slow to recovery from synaptic depression, when intracellular Ca^{2+} levels are low, indicating that a Ca^{2+}-independent process is likely disrupted in these larvae. The decreased probability of release observed in *-/-;P[syt^{KQ}]* mutants, coupled with their slow rate of recovery from depression, is consistent with the hypothesis that Ca^{2+}-independent docking and/or priming of synaptic vesicles is disrupted in these mutants. Thus, we suggest that a major role of synaptotagmin's polylysine motif during the synaptic vesicle cycle *in vivo* is to provide Ca^{2+}-independent synaptic vesicle docking and/or priming.

Acknowledgments

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Chapter 5: Conclusions

Synaptotagmin I is a synaptic vesicle protein known to play a role in the synaptic vesicle cycle. Ca^{2+}-dependent synaptic transmission is severely disrupted when *synaptotagmin* is knocked-out in *C. elegans* (Jorgensen et al., 1995), *Drosophila* (DiAntonio et al., 1993; Littleton et al., 1993b; Broadie et al., 1994; Littleton et al., 1994; Loewen et al., 2001; Yoshihara and Littleton, 2002), and mice (Geppert et al., 1994; Nishiki and Augustine, 2004b). Although it is postulated to play a role in numerous steps of the synaptic vesicle cycle, the full role of synaptotagmin in synaptic transmission remains unknown. The overarching goal of my graduate research was to further dissect synaptotagmin's function in the synaptic vesicle cycle. I accomplished this goal through a series of experiments I conducted on *Drosophila* third instars harboring mutations in two different motifs of synaptotagmin, the C_2B Ca^{2+}-binding motif, and the C_2B polylysine motif, as well as on larvae that expressed no synaptotagmin protein at all. In this final chapter, I will summarize and discuss my results in the context of synaptotagmin's role in synaptic transmission.

A. Synaptotagmin Synchronizes Neurotransmitter Release

In chapter 2, I report experiments I conducted, along with Dr. Jennifer Mackler, to characterize syt^{null} third instars (Loewen et al., 2001). These experiments demonstrate that synaptotagmin does not play a role in synaptic growth, as the syt^{null} mutation did not

disrupt synaptic arborization. Thus, any defect in synaptic transmission observed in *syt*^{null} third instars is not due to gross morphological changes. Electrophysiological recordings from *syt*^{null} neuromuscular junctions (performed by Dr. Mackler) demonstrate that synaptic transmission persists in *syt*^{null} mutants. Indeed, these mutants can survive to adulthood under certain culturing conditions. However, the remaining EJP amplitudes are severely decreased and spontaneous release is dramatically increased (Loewen et al., 2001). These electrophysiological results are similar to what has been reported for *Drosophila syt*^{null} embryos (DiAntonio et al., 1993; Broadie et al., 1994; Yoshihara and Littleton, 2002).

The increased spontaneous fusion rate observed in *Drosophila syt*^{null} mutants supports the hypothesis that synaptotagmin may act as a fusion clamp, inhibiting synaptic vesicle fusion until the Ca^{2+} signal arrives (Popov and Poo, 1993). In addition, it has been recently reported that *syt*^{null} embryos exhibit a Ca^{2+}-dependent increase in asynchronous release that continues up to 1 sec post stimulus (Yoshihara and Littleton, 2002). Similar results have been demonstrated in *syt*^{null} mouse hippocampal synapses (Shin et al., 2003; Nishiki and Augustine, 2004b) and PC12 cells (Shoji-Kasai et al., 1992). Thus, in addition to inhibiting spontaneous fusion, synaptotagmin also confers synchronicity of fusion with Ca^{2+} influx. In this case, asynchronous release is less likely to occur in wild-type synapses because vesicles instead fuse synchronously upon Ca^{2+} influx, and are therefore not available for subsequent asynchronous release.

How does synaptotagmin confer synchronicity of fusion with Ca^{2+} influx? Synaptotagmin performs at least three functions that likely contribute to this

synchronizing role: 1) synaptic vesicle docking, 2) synaptic vesicle priming, and 3) Ca^{2+}-sensing to trigger fusion.

B. Synaptotagmin is Important for Synaptic Vesicle Docking

In Chapter 3, I report that syt^{null} third instars exhibit severe synaptic vesicle depletion (~50% of controls). Vesicles that remain in nerve terminals are targeted to active zones, but these vesicles do not dock efficiently with the presynaptic membrane; syt^{null} mutants have approximately half the number of docked vesicles as controls. This result is similar to what has been reported for central synapses in syt^{null} first instars (Reist et al., 1998). Thus, synaptotagmin appears to be an important synaptic vesicle docking protein.

Very few proteins have been implicated in synaptic vesicle docking. Interestingly, the formation of the SNARE complex is not what docks vesicles at active zones, as syntaxin and synaptobrevin/VAMP knockouts do not have decreased numbers of docked vesicles (Broadie et al., 1995). SNAP-25/23, however, plays a role in granule cell docking (Chieregatti et al., 2002; Chieregatti et al., 2004), and may play a role in synaptic vesicle docking (Schiavo et al., 1997). This docking in granule cells is controlled by intracellular Ca^{2+} and involves an interaction between SNAP-25/23 and synaptotagmin (Chieregatti et al., 2002; Chieregatti et al., 2004). Ca^{2+} has also been demonstrated to increase synaptic vesicle docking at *Drosophila* neuromuscular junctions (Koenig et al., 1993).

The region of synaptotagmin that mediates its interaction with SNAP-25/23 has not been precisely mapped. However, both Ca^{2+}-binding domains are likely important;

in vitro, both Ca^{2+}-binding domains must be knocked-out to fully inhibit Ca^{2+}-dependent binding of recombinant synaptotagmin to GST-SNAP-25 (Earles et al., 2001). If docking involves a Ca^{2+}-dependent interaction between SNAP-25 and the Ca^{2+}-binding motifs in both of synaptotagmin's C_2 domains, then Ca^{2+}-binding motif mutations in the C_2B domain alone should not affect docking as the Ca^{2+}-binding motif in the C_2A domain would remain intact. Indeed, in Chapter 3, I report that synaptic vesicle docking is unaltered in *Drosophila* mutants lacking the Ca^{2+}-binding motif in synaptotagmin's C_2B domain. Alternatively, antibody injection into squid giant terminals suggests that synaptotagmin's docking function may be mediated by a conserved WHXL motif found in its C_2B domain (Fukuda et al., 2000b).

How might synaptic vesicle docking confer synchronicity of fusion with Ca^{2+} influx? Synaptic vesicle docking may locate synaptic vesicles appropriately close to Ca^{2+} channels, thus close to the site of Ca^{2+} entry, such that they are able to fuse quickly upon Ca^{2+} influx. This hypothesis is strengthened by co-immunoprecipitation experiments that suggest synaptotagmin may interact with N-type (Bennett et al., 1992; Leveque et al., 1992; Yoshida et al., 1992) and P/Q-type (Martin-Moutot et al., 1996) Ca^{2+} channels. However, the importance of synaptotagmin's interaction with Ca^{2+} channels *in vivo* has recently been called into question (Zamponi, 2003). Invertebrates express only a single Ca_v2 channel, and it lacks the synprint site (Spafford et al., 2003); yet invertebrates are certainly capable of synchronous neurotransmitter release.

Alternatively, synaptic vesicle docking at active zones may facilitate synaptic vesicle priming, the next step in the synaptic vesicle cycle required for synchronous neurotransmitter release. The molecular interactions that underlie synaptic vesicle

priming are just beginning to be understood, and they appear to involve the formation of *trans*-SNARE complexes [reviewed by (Rettig and Neher, 2002)].

C. Synaptotagmin Participates in Synaptic Vesicle Priming

In Chapter 4, I report four experimental findings that led me to conclude that the major function of synaptotagmin's C_2B polylysine motif is synaptic vesicle priming.

1. The polylysine motif mutants exhibit greater synaptic depression than controls during high frequency stimulation. This indicates that they have a defect in maintaining a supply of vesicles for release. Unfortunately, the mechanism underlying synaptic depression is quite complicated (see section below on synaptic depression), which makes it impossible to discern the exact defect from these recordings.

2. Synaptic vesicle recycling consists of all the steps after synaptic vesicle fusion that recreate another fusion competent vesicle. As recovery from synaptic depression has been demonstrated to occurs via synaptic vesicle recycling (Pyle et al., 2000; Richards et al., 2003), and recovery from synaptic depression is greater than twofold slower in the polylysine motif mutants, these mutants must have a defect in synaptic vesicle recycling. Furthermore, the slowed recovery from depression also indicates that the defect cannot be solely in the fusion step.

3. Although synaptic vesicle recycling consists of multiple steps, *in vitro* experiments only implicate synaptotagmin in three of them: synaptic vesicle endocytosis, synaptic vesicle docking, and synaptic vesicle priming. The electrophysiological phenotype of polylysine motif mutants is quite different from that seen in *Drosophila* harboring mutations in strictly endocytic proteins; polylysine motif mutants show

decreased release during low frequency stimulation but are able to maintain this level of release fairly well during high frequency stimulation. Furthermore, the ultrastructural phenotype of the polylysine motif mutants is also inconsistent with that seen in *Drosophila* endocytic mutants, as the polylysine motif mutants do not exhibit synaptic vesicle depletion.

An alternative explanation is that polylysine motif mutants do have an endocytic defect, but that it does not manifest itself in synaptic vesicle depletion because the polylysine motif mutants also exhibit decreased release. However, this alternative seems unlikely for two reasons. First, syt^{null} mutants exhibit almost no evoked transmitter release and yet their terminals are severely depleted of synaptic vesicles. Second, the nerve terminals of endocytic mutants (AP180, synaptojanin and endophilin) that exhibited synaptic vesicle depletion, were examined at rest, often in 0 or very low extracellular Ca^{2+} (Zhang et al., 1998a; Verstreken et al., 2002; Verstreken et al., 2003). Thus, very little release should have occurred in these terminals, yet they still exhibited synaptic vesicle depletion.

However, as neither the electrophysiological nor the ultrastructural phenotype of the polylysine motif mutants directly addresses the endocytic function in these mutants, I examined endocytosis directly using an FM 1-43 assay. These experiments clearly demonstrate that the polylysine motif mutants do not have a severe endocytic defect. An important consideration is whether one would expect a severe endocytic defect in the polylysine motif mutants if this motif mediates synaptotagmin's role in endocytosis. In Chapter 2, I report that syt^{null} mutants can survive to adulthood under proper culturing conditions (Loewen et al., 2001). Certainly, some level of endocytosis must persist in

these flies to permit survival. Indeed, endocytosis is not completely abolished in *syt*^{null} hippocampal synapses (Nicholson-Tomishima and Ryan, 2004). However, it is >twofold slower (Nicholson-Tomishima and Ryan, 2004). An even more pronounced slowing of endocytosis was observed in *Drosophila* neuromuscular junctions when synaptotagmin function was eliminated (Poskanzer et al., 2003). Thus, if the polylysine motif does mediate synaptotagmin's endocytic role, these mutants should have severe endocytic defects. However, the FM 1-43 experiments demonstrate that they do not. An important corollary of this conclusion is that the endocytic role of synaptotagmin is mediated by a motif other than the polylysine motif.

4. Although polylysine motif mutants do not exhibit an endocytic defect, they do have a decreased probability of release. This is consistent with a priming defect. Of course, a decreased probability of release does not directly prove that the polylysine motif is involved in synaptic vesicle priming; and, I cannot rule out the possibility that the polylysine motif functions in a step of the synaptic vesicle cycle in which it has not yet been implicated. However, a priming defect is the simplest explanation for all the defects observed in the polylysine motif mutants. In this model, the priming that occurs via the polylysine motif increases both the speed and efficacy of synaptic vesicle priming. Thus, priming is both slower and less effective in the polylysine motif mutants. Slower priming explains both the increased synaptic depression, and the slowed recovery from this depression that was recorded in the polylysine motif mutants. However, if priming was only slowed in the mutants, then given a long enough priming period, they should be able to release as many vesicles per stimulus as controls, but they do not. Even during very low frequency stimulation, the polylysine motif mutants do not release as much as

controls. Electron micrographs show that this decreased release is not due to synaptic vesicle depletion. The increased synaptic facilitation and augmentation recorded in the polylysine motif mutants suggests that this decreased release is due to a lower probability of release. Less effective priming explains this decreased probability of release. In summary, I conclude that the polylysine motif plays a role in synaptic vesicle priming that serves to increase the speed of priming and the probability of synaptic vesicle fusion.

Model for the polylysine motif's role in synaptic vesicle priming

In vitro, synaptotagmin's polylysine motif undergoes a Ca^{2+}-independent interaction with PIP_2 containing membranes (Bai et al., 2004a) and/or t-SNARE complexes (Rickman et al., 2004a). *In vitro*, the speed of synaptotagmin's Ca^{2+}-dependent membrane penetration is slowed in the absence of the polylysine motif (Bai et al., 2004a). Also, the polylysine motif facilitates a *trans* interaction between synaptotagmin on the synaptic vesicle and PIP_2 in the plasma membrane, prior to Ca^{2+}-influx (Bai et al., 2004a).

In vivo, a Ca^{2+}-independent, *trans* interaction between synaptotagmin's polylysine motif and PIP_2 or t-SNARE complexes associated with the plasma membrane may prime synaptic vesicles by promoting the formation of "tight" *trans*-SNARE complexes (Fig. 1.1). The interaction may facilitate the formation of "tight" *trans*-SNARE complexes by providing a tether from the synaptic vesicle membrane to the plasma membrane, which may help overcome the repulsive force between the two membranes. Alternatively, the interaction may serve to optimally position synaptotagmin relative to the other SNARE proteins. As the formation of "tight" SNARE complexes appears to involve the C-

terminus of SNAP-25 and synaptotagmin, this optimal position may facilitate a subsequent Ca^{2+}-dependent interaction between synaptotagmin and SNAP-25. Or, this position might facilitate synaptotagmin's Ca^{2+}-dependent interaction/penetration of the plasma membrane upon Ca^{2+} influx (Fig.1.1). A final hypothesis involves synaptotagmin in synaptic vesicle priming by its interaction with the soluble protein, β-SNAP. This interaction has been mapped to the polylysine motif (Schiavo et al., 1995). Recall that SNAP's function is to recruit NSF to *cis*-SNARE complexes. These *cis*-SNARE complexes are subsequently disassembled by NSF's hydrolysis of ATP. NSF mutants in *Drosophila* show an accumulation of docked vesicles and an activity-dependent decrease in neurotransmitter release (Kawasaki et al., 1998). They also show an increase in SNARE complex levels (Tolar and Pallanck, 1998). These results suggest that NSF functions downstream of docking, in a pre-fusion, priming step to maintain a supply of readily releasable vesicles. NSF and α-SNAP have also been implicated in priming in PC12 cells, chromaffin cells, and squid giant axons [reviewed by (Li and Chin, 2003)]. β-SNAP has been demonstrated to prime chromaffin granules (Sudlow et al., 1996; Xu et al., 2002). If the priming function of β-SNAP were mediated by its interaction with synaptotagmin's C_2B polylysine motif, it would be disrupted in the polylysine motif mutants. Consistent with this hypothesis, I have preliminary data (not shown), which suggests that SNARE complex levels may be elevated in polylysine motif mutants. However, these results have not yet been replicated.

In summary, synaptotagmin may confer synchronicity of release by priming vesicles for fusion. Although the molecular interactions underlying this priming function have not yet been identified, they may involve an interaction between the polylysine

motif and PIP_2 or t-SNARE complexes in the plasma membrane, or between the polylysine motif and β-SNAP.

Synaptic Depression

The degree of synaptic depression that occurs during high frequency stimulation is widely used as a measure of recycling ability (Delgado et al., 2000; Verstreken et al., 2002; Verstreken et al., 2003; Marie et al., 2004). However, measuring the degree of synaptic depression may not be a reliable assay for synaptic vesicle recycling, because, as I discuss below, multiple mechanisms underlie synaptic depression during high frequency stimulation.

A simple depletion model (Liley and North, 1953) can account for many aspects of synaptic depression. In this model the amount of release evoked by an action potential (R_a) is proportional to both the size of the readily releasable pool (RRP, the pool of vesicles that are immediately available for release) and the fraction (F) of this pool that is released by the action potential.

$$R_a = RRP * F$$

This simple depletion model incorporates two assumptions. The first is that F remains constant. The second is that the RRP is slowly replenished (with a time constant of many seconds) during and after repetitive stimulation. This model predicts that during high frequency stimulation, R_a will eventually decline to nearly zero. Unfortunately, this simple depletion model does not adequately describe synaptic depression at many synapses. After an initial rapid decline, R_a often reaches a steady state that is higher than

the model would predict. Experimental evidence from calyx of Held synapses suggests that the inadequacy of the simple depletion model lies in its two assumptions.

First, F is not constant. Instead, at calyx of Held synapses, F decreases during repetitive stimulation (Wu and Borst, 1999). This decrease seems to be due to the selective release during stimulus trains of a "pool" of synaptic vesicles that intrinsically has a lower probability of release (Wu and Borst, 1999; Sakaba and Neher, 2001b). However, Wu and Borst (1999) also presented data suggesting that F increases when intracellular Ca^{2+} increases. Thus, F might be higher during the stimulation train when intracellular Ca^{2+} levels are elevated, which would help maintain release (R_a is larger when F is larger). This hypothesis is supported by experiments which demonstrate that increasing presynaptic Ca^{2+} facilitates the release of vesicles with an intrinsically low probability of release (Sakaba and Neher, 2001b). Thus, even though neurotransmitter release during high frequency stimulation is maintained by synaptic vesicles with an intrinsically low probability of release, the increased intracellular Ca^{2+} levels that occur during high frequency stimulation increase these vesicles' probability of release, such that their apparent probability of release is more similar to that of the vesicles with an intrinsically high probability of release (Sakaba and Neher, 2001a). Thus, although F is not constant, during high frequency stimulation, F can increase so that it behaves more or less like a constant.

Liley and North (1953) observed that recovery from synaptic depression is slow. This slow recovery is the second assumption of their simple depletion model. However, if F behaves similarly to a constant, yet neurotransmitter release (R_a) is maintained better than the simple depletion model predicts, then the recovery of the RRP must be faster

than originally thought. Indeed, experimental evidence has shown this to be the case. First, Wu and Borst (1999) demonstrated that after stimulation ceases, the RRP recovers with a time constant of ~0.2 sec at calyx of Held synapses. Sakaba and Neher (2001a) reported a similar time constant. However, although the RRP recovers quickly after stimulation, F does not (Wu and Borst, 1999). Thus, R_a recovers slowly after depression because F recovers slowly. In addition, F is not facilitated as much during the recovery period (after stimulation ceases) because intracellular Ca^{2+} levels fall during this period.

Further analysis of the recovery of the RRP demonstrated that the two vesicle "pools", those with a high probability of release and those with a low probability of release, recover differently. Although the low probability release vesicles recover quickly (time constant ~0.2 sec), the vesicles with a high probability of release recover more slowly (time constant ~4.5 sec) (Wu and Borst, 1999; Sakaba and Neher, 2001a). Furthermore, the speed of recovery of the high probability release vesicles, which is intrinsically quite slow, increases with elevated intracellular Ca^{2+} (Sakaba and Neher, 2001a). [Recovery of the low probability release vesicles seems to be Ca^{2+} independent (Wu and Borst, 1999; Sakaba and Neher, 2001a).]

In summary, synaptic transmission is maintained at a level higher than the simple depletion model predicts because recovery of the RRP is faster than originally thought. This is both because recovery of the low probability vesicles is fast and because recovery of high probability (intrinsically slowly recovering) synaptic vesicles increases during high frequency stimulation due to increased intracellular Ca^{2+} levels.

As the level of synaptic depression is intimately connected to the recovery of the RRP, understanding how the RRP is refilled is a critical component of understanding

synaptic depression. During high frequency stimulation, neurotransmitter release at the *Drosophila* neuromuscular junction is maintained via both mobilization of vesicles from a reserve pool as well as recycled synaptic vesicles (Delgado et al., 2000). However, to what extent each of these processes contributes to release during high frequency stimulation is unclear.

Studies in various organisms have attempted to address this issue by stimulating in the presence of a fluorescent membrane styryl dye and then correlating the amount of neurotransmitter released with the number of labeled synaptic vesicles. At neuromuscular junctions in both frog (Wu and Betz, 1996; Richards et al., 2000; Richards et al., 2003) and *Drosophila* (Kuromi and Kidokoro, 2000), terminals fluoresce more and more brightly as the duration of a 30 Hz stimulation train increases. The increase in fluorescence is presumably because a greater number of synaptic vesicles have been mobilized and taken up the dye. Similar results are seen at calyx of Held synapses where increasing the length of high K^+ stimulation from 1 minute to 15 minutes increases the number of synaptic vesicles containing photoconverted label (de Lange et al., 2003). Thus, increasing stimulus duration can increase the number of synaptic vesicles mobilized during stimulation trains.

The magnitude of the increase in vesicle mobilization due to increasing stimulus duration seems to depend on stimulation frequency. Less of an increase in fluorescence is seen when 3 Hz stimulation is used instead of 30 Hz stimulation (Kuromi and Kidokoro, 2000). Indeed, increasing stimulus frequency can also increase vesicle mobilization. In *Drosophila* (Kuromi and Kidokoro, 2000) and frog (Richards et al., 2003) neuromuscular junctions, the same number of stimuli given at a higher frequency

result in terminals that are brighter then if the stimuli are given at a lower frequency. This was the case even though at the higher stimulus frequency less quanta had been released due to synaptic depression (Richards et al., 2003). These data indicate that during low frequency stimulation, neurotransmitter release is at least partly maintained by recycling vesicles that re-release multiple times. Re-release of vesicles has also been demonstrated in calyx of Held synapses, where only a small fraction (5-10%) of the total vesicles in the terminal participate in release/recycling during continuous stimulation at either 5 or 20 Hz (de Lange et al., 2003). The re-release of recycling vesicles has also been demonstrated in hippocampal synapses where the number of labeled vesicles can be much less than the number of quanta released [reviewed by (Harata et al., 2001)]. Thus, the extent to which neurotransmitter release during a stimulation train depends on vesicle mobilization versus synaptic vesicle recycling seems to depend on the stimulation conditions.

In summary, synaptic depression and its recovery are complicated. They are determined by both the rate of replenishment of the RRP and the rate at which F returns to its pre-depression level, both of which can depend on intracellular Ca^{2+} concentrations. Moreover, during stimulation, replenishment of the RRP probably occurs by both vesicle mobilization and recycled synaptic vesicles; the relative level of contribution of each of these processes varies with the stimulation conditions. Thus, the level of synaptic depression observed during high frequency stimulation depends on many issues and likely the action of multiple proteins.

D. Synaptotagmin Senses Ca^{2+} to Trigger Fusion

I have discussed how synaptotagmin's proposed ability to dock synaptic vesicles at active zones and prime synaptic vesicles for fusion may be critical for synchronous neurotransmitter release. However, the most important function of synaptotagmin *in vivo* appears to be its ability to stimulate fusion up binding of Ca^{2+}, especially via its C_2B domain (Fernández-Chacón et al., 2001; Fernández-Chacón et al., 2002; Mackler et al., 2002; Robinson et al., 2002).

Synaptotagmin's ability to bind Ca^{2+} directly, its Ca^{2+}-dependent interactions with presynaptic molecules, and the observation that syt^{null} mutations abolish fast, Ca^{2+}-triggered exocytosis led to the hypothesis that synaptotagmin is the main Ca^{2+}-sensor for fast, synchronous release [reviewed by (Südhof, 2004)]. Strong evidence supporting this hypothesis comes from *Drosophila*, where it was demonstrated that synaptotagmin's ability to bind Ca^{2+} by the C_2B domain is critical for synaptic transmission. When two of the Ca^{2+}-binding acidic residues in synaptotagmin's C_2B domain were mutated to asparagines, the apparent Ca^{2+}-affinity of synaptic transmission was reduced and Ca^{2+}-triggered, synchronous neurotransmitter release was almost entirely eliminated (Mackler et al., 2002).

Although the above result is consistent with synaptotagmin's role as a Ca^{2+}-sensor to trigger fusion, it may also be explained by altered synaptic vesicle distribution. An apparent decrease in both synaptic transmission and Ca^{2+} affinity would also be observed if synaptic vesicles were no longer located appropriately close to the site of Ca^{2+} influx. In Chapter 3, I present data that implicates synaptotagmin in maintaining a supply of synaptic vesicles, including docked vesicles, in the nerve terminal. My ultrastructural

analysis of active zones from *syt*^{null} third instar neuromuscular junctions, shows that the lack of synaptotagmin leads to a severe decrease in synaptic vesicles, including docked vesicles. Thus, the severe decrease in neurotransmitter release recorded in *syt*^{null} mutants is likely due in part to this severe disruption in synaptic ultrastructure.

To determine if altered synaptic ultrastructure contributes to the severe decrease in evoked release recorded in C_2B Ca^{2+}-binding motif mutants, I completed an ultrastructural analysis of active zones at neuromuscular junctions from these mutants. I quantified the number of docked vesicles and the overall number of synaptic vesicles in the vicinity of active zones. No significant differences were observed between mutants and controls. Thus, I conclude that the decrease in neurotransmitter release and Ca^{2+} affinity recorded in C_2B Ca^{2+}-binding motif mutants is not due to altered synaptic vesicle distribution, but that it is more likely the result of the inability of the protein to bind Ca^{2+} directly and undergo a critical Ca^{2+}-dependent molecular interaction.

E. Summary

Synaptotagmin is an important molecule for synaptic transmission. However, its full role in the synaptic vesicle cycle has been unclear. Part of the uncertainty is likely a result of the multi-functional nature of this protein. It doesn't have one role in the cycle, but many. My ultrastructural analysis of *syt*^{null} active zones supports a role for synaptotagmin in synaptic vesicle docking. Although this analysis doesn't reveal the molecular interaction(s) that mediate this function, my ultrastructural analysis of C_2B Ca^{2+}-binding motif mutants demonstrates that it is not via the C_2B Ca^{2+}-binding motif, as these mutants do not have docking defects. The normal synaptic ultrastructure observed in C_2B Ca^{2+}-binding motif mutants, along with the severe decrease in evoked release

recorded from these mutants (Mackler et al., 2002), supports a role for synaptotagmin in the Ca^{2+}-sensing to trigger fusion. In addition, my ultrastructural analysis of *syt*^{null} active zones supports a role for synaptotagmin in synaptic vesicle endocytosis, as synaptic vesicles are severely depleted in these mutants and larger membranous structures accumulate in their terminals. The ultrastructural analysis does not reveal the molecular interaction(s) that mediate synaptotagmin's role in endocytosis. However, my experiments on the C_2B polylysine motif mutants reveal that it is not via the C_2B polylysine motif. Instead, the C_2B polylysine motif likely mediates synaptotagmin's role in synaptic vesicle priming. As many of the molecular interactions that underlie synaptotagmin's various roles in the synaptic vesicle cycle remain unclear, the future of synaptotagmin research is replete with potential discoveries.

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Appendix A: SNARE Complex Westerns

1. Pour SDS-acrylaminde separating gel, such that the bottom 1/3 of the gel is 15% acrylamide and the top 2/3 is 9%.

2. Pour SDS-acrylamide stacking gel.

3. Make up Squishing Buffer: (from Dr. David Deithcher)

100 μl 50% glycerol	= 10.0%
25 μl 20% SDS	= 1.0%
6.2 μl β-mercaptoethanol	= 175.0 mM
31.2 Tris, pH 7.5	= 62.5 mM
337.6 ddH_20	
bromophenol blue	
500 μl	

4. Make up 1X Running Buffer:

29.0 gms glycine
6.0 gms TRIS BASE
5 mls 20% SDS
ddH_20 to 1 liter

5. Put Gel, Squishing Buffer, and 1X Running Buffer in the cold room.

6. Age match experimental and control flies. Anesthetize flies with $C0_2$ and cut off heads with clean razor blade. Immediately place heads into plastic mortar (Kontes Glass Company, New Jersey) on ice.

7. Turn on Boiler.

8. In cold room, squish heads thoroughly with pestle (Kontes Glass Company, New Jersey) in Squishing Buffer on ice. Rinse pestle with additional Squishing Buffer and collect rinse in the mortar. Keep samples on ice in the cold room.

9. Dilute sample with additional Squishing Buffer such that a dilution series from ~1.5 – 0.20 heads/lane can be run. Keep samples on ice in the cold room.

10. Boil a sample as a negative control. (Boiling disassembles SNARE complexes.)

11. Spin samples at top speed in microfuge in cold room for ~5 minutes.

12. Load gel.

13. Run Gel at 60V for 2 1/2 hours in cold room.

14. Make up Blotting Buffer: 3.03 gms Tris (25 mM)
    14.4 gms glycine (192 mM)
    ddH_20 to 1 liter

15. Put Blotting Buffer in cold room.

16. Place gel in Blotting Buffer in cold room for 30 min.

17. Blot soak in cold room: 1st in methanol, 2nd in ddH_20, and 3rd in Blotting Buffer.

18. Wet Transfer: 30V, 90 mA overnight in the cold room. In the morning, turn up to 80V, 350 mA for 1 1/2 hours.

19. Probe with anti-syntaxin antibody.

LIST OF ABBREVIATIONS

1. syt	synaptotagmin
2. syt^{wt}	wild-type synaptotagmin
3. $syt^{null} = syt^{AD4}$	synaptotagmin null mutation
4. syt^{AD3}	synaptotagmin Y to N mutation in C_2B domain
5. syt^{DN}	synaptotagmin DD to NN mutation in the C_2B Ca^{2+}-binding motif
6. syt^{KQ}	synaptotagmin KKK to QQQ mutation in the C_2B polylysine motif
7. $P[syt^{wt}] = -/-;P[syt^{wt}]$	*Drosophila* mutant with a wild-type synaptotagmin transgene inserted via P-element mediated transformation into its third chromosome and expressed in a syt^{null} background.
8. $P[syt^{DN}] = -/-;P[syt^{DN}]$	*Drosophila* mutant with a syt^{DN} transgene inserted via P-element mediated transformation into its third chromosome and expressed in a syt^{null} background.
9. $P[syt^{KQ}] = -/-;P[syt^{KQ}]$	*Drosophila* mutant with a syt^{KQ} transgene inserted via P-element mediated transformation into its third chromosome and expressed in a syt^{null} background.
10. SNARE	Soluble NSF Attachment Protein Receptor
11. NSF	N-ethylmaleimide Sensitive Factor
12. VAMP	Vesicle Associated Membrane Protein
13. SNAP-25	Synapse Associated Protein of 25 KD
14. PIP_2	Phosphotidylinositol-4,5-bisphosphate
15. AZM	Active Zone Material
16. IMPs	Intramembrane Particles

17. DCG	Dense Core Granule
18. RRP	Readily Releasable Pool
19. RP	Reserve Pool
20. IRP	Immediately Releasable Pool
21. EJC	Evoked Junctional Current
22. EJP	Evoked Junctional Potential
23. mEJP	mini Excitatory Junctional Potential
24. IHPS	Inositol High Polyphosphate Series
25. CNS	Central Nervous System
26. NMJ	Neuromuscular Junction