DISSERTATION

THE C_2B DOMAIN OF SYNAPTOTAGMIN IS CRITICAL FOR SYNAPTIC EFFICACY IN DROSOPHILA

Submitted by

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In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

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ABSTRACT OF DISSERTATION

THE C_2B DOMAIN OF SYNAPTOTAGMIN IS CRITICAL FOR SYNAPTIC EFFICACY IN DROSOPHILA

Synaptotagmin is a synaptic vesicle protein that is postulated to be the Ca^{2+} sensor for fast, evoked neurotransmitter release and also may function in vesicle docking or recycling. Genetic deletion of synaptotagmin strongly suppresses synaptic transmission in every species examined, demonstrating that synaptotagmin plays a central role in the synaptic vesicle cycle. The cytoplasmic region of synaptotagmin consists of two homologous C_2 domains, C_2A and C_2B. Early studies ascribed all of synaptotagmin's binding activities to the C_2A domain, neglecting any potential function by C_2B. Here, I have tested the importance of two highly conserved motifs in C_2B domain of synaptotagmin: a polylysine motif on β-strand four of C_2B and a cluster of acidic residues that constitute a binding pocket for Ca^{2+} ions. Biochemical studies demonstrated that synaptotagmin bound the clathrin adapter protein AP-2, neuronal Ca^{2+} channels, inositol high polyphosphates, and other synaptotagmin molecules all via the polylysine motif. Electrophysiological analyses of Drosophila site directed mutants ($K_{379,380,384}Q$) revealed that evoked transmitter release is decreased by ~36% and spontaneous release is increased two fold relative to syt^{null} flies expressing a wild type *syt* transgene, suggesting that this motif is important for full protein function. Alternatively, Ca^{2+}-binding aspartate residues may mediate synaptotagmin binding to anionic phospholipids, SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) proteins, or self-

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oligomerization. Each of these processes may constitute a necessary step in vesicle fusion, however, there has been no direct evidence that any of the Ca^{2+}-binding residues are required *in vivo*. Here, I show that mutating two of the Ca^{2+}-binding aspartate residues in the C_2B domain ($D_{416,418}N$) decreased evoked transmitter release by > 95% and decreased the apparent Ca^{2+} affinity of evoked transmitter release. Taken together, these studies demonstrate that synaptotagmin's C_2B Ca^{2+}-binding motif is essential for synaptic transmission. Furthermore, these data support the idea that synaptotagmin functions as part of the Ca^{2+} sensing mechanism in the nerve terminal.

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Chapter 1: Introduction

A putative Ca^{2+} sensor for neurotransmitter release

Synaptic transmission is one of the fastest cellular events within an organism, occurring within 60-350 microseconds (Llinas et al., 1981; Heidelberger et al., 1994). In order to maintain the fidelity of neural communication, neurotransmitter release from a nerve terminal must be prevented until Ca^{2+} influx triggers transmitter filled vesicles to fuse with the presynaptic membrane. In 1954, Del Castillo and Katz (Del Castillo and Katz, 1954) postulated the existence of a receptor "X" in nerve terminals that triggers neurotransmitter release when bound to Ca^{2+}. They predicted that the amplitude of the synaptic response would be related to the number of CaX complexes formed.

Experimentally, Dodge and Rahamimoff (Dodge and Rahamimoff, 1967) showed that the amount of neurotransmitter released was nonlinearly dependent on the extracellular Ca^{2+} concentration. On a log-log plot, the initial slope of the Ca^{2+} dose response curve was approximately four, which was interpreted to mean that, neurotransmitter release was a cooperative process involving about four Ca^{2+} ligands. They proposed two models – one stoichiometric and one stochastic – wherein four Ca^{2+} ions could cooperatively stimulate transmitter release. First, their stoichiometric model required that four Ca^{2+} ions bind to one molecule, the Ca^{2+} sensor "X" that has four independent binding sites. All four

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Ca^{2+} ions would need to bind in order for transmitter release to occur. Second, the stochastic model predicted that Ca^{2+} ions bound to Ca^{2+} sensor X in a 1:1 ratio and that four or more CaX complexes were needed "in a certain small area of the membrane" for release to occur.

Advances in molecular biology have helped to identify dozens of molecules specific to nerve terminals, many of which are capable of binding Ca^{2+}. Candidate molecules for the Ca^{2+} sensor must bind Ca^{2+} with low affinity such that they respond only to evoked Ca^{2+} influx rather than small releases from internal stores, and be located close to the release apparatus. The Ca^{2+} binding molecule, synaptotagmin, has stood out among the others due to its location as an integral membrane protein on synaptic vesicles, its low intrinsic Ca^{2+} affinity, and its ability to bind other secretory molecules in the nerve terminal. Synaptotagmin binds three Ca^{2+} ions via its C_2A domain (Ubach et al., 1998) and two ions via its C_2B domain (Fernandez et al., 2001) with affinities similar to that of neurotransmission (Heidelberger et al., 1994; Bollmann et al., 2000; Schneggenburger and Neher, 2000). In addition, synaptotagmin binds the presynaptic membrane proteins syntaxin and SNAP-25 and negatively charged phospholipids in a Ca^{2+} dependent manner (reviewed in (Tucker et al., 2002)). Based largely on evidence inferred from biochemical and genetic knock out studies, synaptotagmin has been dubbed "the Ca^{2+} sensor" in the literature (Brose et al., 1992; Geppert et al., 1994; Littleton et al., 1994). In particular synaptotagmin I is thought to be a "low affinity" sensor, while other isoforms of synaptotagmin may constitute "high affinity" sensors (Goda and Stevens, 1994; Li et al., 1995b). Although alternative molecules for Ca^{2+} sensing have been

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proposed, the very existence of a single Ca^{2+} sensor "X" continues to be a matter of debate and the role of synaptotagmin in the synaptic vesicle cycle remains at the center of scrutiny.

The synaptic vesicle cycle

Excitation-secretion coupling at nerve terminals is likely to be a well orchestrated cycle of protein-protein and protein-lipid interactions that we have yet to understand. The molecular mechanisms mediating synaptic vesicle docking, fusion, and recycling are currently a topic of intense study. Nerve terminals maintain a steady supply of vesicles that are mature and ready to fuse, known as the readily releasable pool (Rosenmund and Stevens, 1996). Among those vesicles in the readily releasable pool, some will dock at the presynaptic membrane at specialized regions called active zones. Vesicles that appear in close apposition to the presynaptic membrane are "morphologically docked". Protein-protein interactions between vesicle proteins and proteins of the plasma membrane are thought to mediate docking, yet the identity of those proteins remains in question (reviewed in (Bajjalieh, 1999)). Interactions between vesicle proteins and N- and P/Q-type Ca^{2+} channels may allow vesicles to dock close to sites of Ca^{2+} influx which is required for vesicle fusion to occur (Leveque et al., 1994; Martin et al., 1996; Sheng et al., 1997).

Ca^{2+}-triggered synaptic vesicle fusion can occur as fast as 1000-3000/second with the time lag between Ca^{2+} influx and fusion as small as approximately 200 μsec (Llinas et al., 1981; Heidelberger et al., 1994). Thus, in neurons, protein and lipid rearrangements that promote fusion must occur rapidly and with high precision. Physiology experiments predicted that at a

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synapse, the probability of synaptic vesicle fusion depends on number of quanta and individual probability of release (Del Castillo and Katz, 1954). While these synaptic properties can be estimated from synaptic recordings, individual vesicle fusions with the presynaptic membrane are elusive events that are difficult to measure directly. Recently, high resolution, amperometry experiments have demonstrated the formation of fusion pores which open when single synaptic vesicles fuse with the plasma membrane and allow the release of neurotransmitter into the synaptic cleft. Single fusion pore opening events can be recorded, much like single channel recordings from ion channels (Breckenridge and Almers, 1987; Bruns and Jahn, 1995). While fusion pores have been likened to ion channels, the molecular components of these pores remain unknown. Fusion pores are likely to consist of both lipids and members of the SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor) complex (Sollner et al., 1993a), components that have been shown to be both necessary and sufficient to facilitate vesicle fusion (Weber et al., 1998).

The SNARE complex forms a tight association between vesicle and presynaptic membrane proteins that must be dissociated after the fusion event in order for the proteins to be recycled. NSF (*N*-ethylmaleimide-sensitive factor) is an ATPase that is required for dissociation of SNARE complex (Sollner et al., 1993a; Hayashi et al., 1995), an event which occurs at some point post-fusion (Kawasaki et al., 1998; Tolar and Pallanck, 1998). Additionally, clathrin-mediated endocytosis must also occur in order to recycle membrane into synaptic vesicles (Heuser, 1989). Again, there are many other protein-protein interactions that occur here and the process is only partly understood. Endocrine cells contain many of the same nerve terminal proteins or their related isoforms (Lowe et al.,

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1988). Secretion of dense core vesicles most likely uses a similar process of docking, fusion, and recycling, albeit with much slower kinetics (reviewed in (Rettig and Neher, 2002)).

SNARE proteins – key mediators of vesicle exocytosis

It is now widely accepted that the three SNARE proteins, VAMP/synaptobrevin, SNAP-25 (synaptosome associated protein of 25 kD), and syntaxin assemble as the heterotrimeric "SNARE complex" consisting of four α-helices with their N- and C-termini arranged in parallel (Fig. 1.1) (Hanson et al., 1997a; Lin and Scheller, 1997; Poirier et al., 1998a; Sutton et al., 1998). The SNARE complex self-assembles in response to a Ca^{2+} signal and promotes synaptic vesicle fusion (Sollner et al., 1993a; Sollner et al., 1993b; Littleton et al., 1998; Chen et al., 1999). Each of the three SNARE proteins is a substrate for clostridial neurotoxins that block neurotransmitter release (Blasi et al., 1994), indicating their functional importance *in vivo*. At the synapse, VAMP (a vesicle-SNARE, or v-SNARE) is an ~11 kDa integral membrane protein of synaptic vesicles and contributes one α-helix to the SNARE complex (Sutton et al., 1998). SNAP-25 (a target-SNARE, or t-SNARE) is associated the plasma membrane via a palmitoylated loop (Veit et al., 1996) and contributes two α-helices to the SNARE complex (Sutton et al., 1998). Syntaxin (Bennett et al., 1992), also a t-SNARE, contains a transmembrane domain, a regulatory Habc domain, and an H3 domain (Zhong et al., 1997; Misura et al., 2000) that contains one α-helix of the SNARE complex (Sutton et al., 1998). The two α-helices from SNAP-25 combined with one α-helix each from VAMP and syntaxin of the SNARE

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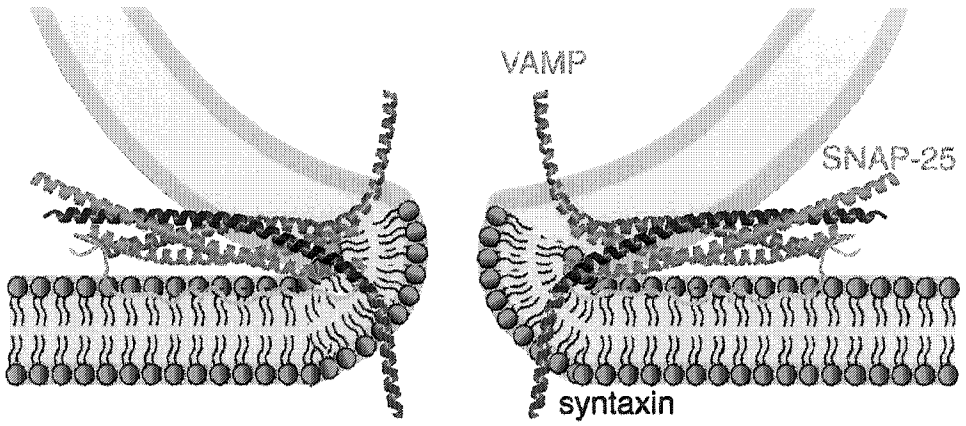

Figure 1.1 The SNARE complex (VAMP/SNAP-2/syntaxin) promotes fusion of synaptic vesicles with the presynaptic membrane. Adapted from Sutton et al., 1998.

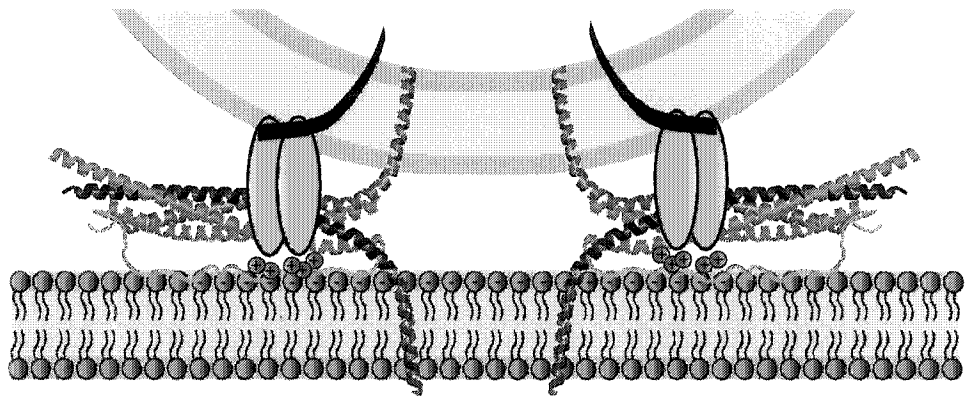

Figure 1.2 Possible model where synaptotagmin (yellow ovals) could interact with both phospholipids and the C-terminus of the SNARE complex.

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complex may loosely assemble with one another (Chen et al., 2001) prior to receiving a Ca^{2+} signal that will trigger vesicle fusion.

Upon receipt of a Ca^{2+} signal through an as yet unidentified mechanism, the SNARE complex "zips" into a coiled-coil causing a close juxtaposition of the vesicle and plasma membranes. Once fully "zippered", the SNARE complex is highly stable and resistant to SDS treatment (Hayashi et al., 1994; Fasshauer et al., 1997), clostridial neurotoxins (Hayashi et al., 1994), and trypsin digestion (Poirier et al., 1998b) *in vitro*. The C-terminal transmembrane domains of both syntaxin and VAMP are protected from trypsin digestion after the complex is formed, suggesting that the coiled coil extends into the lipid bilayers as they are fused (Poirier et al., 1998b). In fact, including the transmembrane domains in this assay increased the stability of the SNARE complex *in vitro* (Poirier et al., 1998b). Tight zippering of the SNARE complex is thought to exert sufficient force to cause mixing of the vesicle and presynaptic membrane bilayers (McNew et al., 1999; McNew et al., 2000a). The SNARE complex is the receptor for the ATPase, NSF (N-ethylmaleimide-sensitive fusion protein), and its cofactor α-SNAP. α-SNAP binds to the SNARE complex allowing the attachment of NSF, which in turn drives the disassembly of the complex *in vivo* which frees the proteins for the next round of fusion (Sollner et al., 1993a; Hayashi et al., 1995).

The assembly of the coiled-coil structure between SNARE proteins is widely believed to underlie both constitutive and regulated vesicle fusion (Hanson et al., 1997a; Weber et al., 1998; Bock and Scheller, 1999; Brunger, 2000), yet it is still unknown what confers either specificity or regulation of fusion within different cellular compartments. The proteins of the SNARE complex

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(VAMP-syntaxin-SNAP-25 (Gerst, 1999)) are highly conserved from yeast to mammals (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994; Pelham, 1999). Multiple isoforms of the SNARE proteins exist and are ubiquitous among cell types. Opinions differ on what constitutes the specificity of SNARE interactions (Calakos et al., 1994; Pevsner et al., 1994; Bock and Scheller, 1999; Yang et al., 1999; McNew et al., 2000b; Scales et al., 2000). SNARE proteins reconstituted in proteoliposomes support membrane fusion, although in a Ca^{2+} independent manner with very slow kinetics (Weber et al., 1998; Mahal et al., 2002). Since SNARE proteins assemble spontaneously (Fasshauer et al., 1997), additional proteins must be required to confer Ca^{2+} dependent regulation of fusion at synapses. Synaptotagmin is the foremost candidate for the fusion regulator of synaptic transmission.

Synaptotagmin

Synaptotagmin was originally identified using a monoclonal antibody that recognized a 65 kD protein on vertebrate synaptic vesicles, simply called p65 (Matthew et al., 1981). In 1991, p65 was cloned (Perin et al., 1990) and renamed synaptotagmin (Perin et al., 1991a). Synaptotagmin contains tandem C_2 domains that are homologous to one another and to the C_2 domain in protein kinase C (PKC) (Perin et al., 1990; Perin et al., 1991b; Gerloff et al., 1995). Ca^{2+}-dependent phospholipid binding by synaptotagmin has been widely conserved in evolution among synaptotagmin isoforms (Fukuda et al., 1996). While the interaction between synaptotagmin's C_2 domains and phospholipids appears to be a conserved interaction *in vitro*, the physiological significance of phospholipid binding has yet to be unequivocally determined.

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Most organisms contain multiple synaptotagmin isoforms with specific cellular and subcellular localizations (Ullrich and Sudhof, 1995) and with specific Ca^{2+} and phospholipid binding affinities (Li et al., 1995b; Gerst, 1999). Thirteen mammalian isoforms of synaptotagmin have now been identified (reviewed in (Sudhof et al., 2002)). In mammals, synaptotagmin I and II are the most abundant neuronal isoforms; synaptotagmin I is localized to forebrain and synaptotagmin II is in brainstem (Geppert et al., 1991) and spinal cord (Ullrich et al., 1994; Marqueze et al., 1995). Additionally, synaptotagmin III and IV are expressed at low levels in most neurons (Hilbush and Morgan, 1994; Ullrich et al., 1994; Marqueze et al., 1995). Synaptotagmin VII is another neuronal isoform that may be localized to the plasma membrane instead of to synaptic vesicles (Sugita et al., 2001). Many of the remaining isoforms are non-neuronal and their functions are unknown (Hudson and Birnbaum, 1995; Li et al., 1995b). PC –12 cells, derived from chromaffin cells, for example, appear to mainly express synaptotagmins I and IX (Zhang et al., 2002). Drosophila, on the other hand, contain as few as three (Littleton et al., 1999) and perhaps as many as six isoforms of synaptotagmin(Craxton, 2001). In Drosophila, synaptotagmin I is most abundant in neurons, while IV is expressed at lower levels (Littleton et al., 1999). While the reason for so many isoforms is still a topic of speculation, it is likely that multiple synaptotagmins provide slightly different regulation of vesicle cycling during different secretory events (reviewed in (Schiavo et al., 1998; Sudhof et al., 2002)).

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Protein structure of synaptotagmin

Synaptotagmin is comprised of an N-terminal intravesicular domain, a transmembrane domain, and two cytoplasmic C_2 domains (Perin et al., 1991a). The C_2 domains of synaptotagmin are homologous to Ca^{2+}-binding C_2 domains found in protein kinase C (Coussens et al., 1986; Knopf et al., 1986), phospholipase A2 (Clark et al., 1991), rabphilin-3 (Shirataki et al., 1993), Doc2 (Orita et al., 1995), and Munc13 (Brose et al., 1995).

The three dimensional structures of rat synaptotagmin I C_2A and C_2B as well as both linked cytoplasmic C_2 domains from mouse synaptotagmin III have been determined (Fig. 1.2) (Sutton et al., 1995; Sutton et al., 1999; Fernandez et al., 2001). Each C_2 domain consists of an eight-stranded sandwich of β-sheets with two loops constituting a Ca^{2+}-binding pocket at one end. Each binding pocket coordinates Ca^{2+} by way of five, highly-conserved, acidic residues (Shao et al., 1996; Sutton et al., 1999; Augustine, 2001). Ca^{2+} binding to synaptotagmin elicits no major conformational change (Sutton et al., 1995; Shao et al., 1996; Shao et al., 1998). Rather, Ca^{2+} binding is believed to function as an "electrostatic switch" which changes the charge in the binding pocket region thereby facilitating electrostatic interactions with effector molecules (Shao et al., 1997; Davletov et al., 1998; Zhang et al., 1998). In addition, a highly conserved cluster of lysine residues is arranged on the surface of each C_2 domain (hereafter referred to as the "polylysine motif") creating positively charged regions with which other molecules could interact (Fukuda et al., 1995a; Chapman et al., 1998; Thomas and Elferink, 1998; Sutton et al., 1999).

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Based on its *in vitro* binding attributes, much attention has been focused on synaptotagmin's C_2A domain as the low affinity Ca^{2+} sensor for synaptic transmission (Brose et al., 1992; Davletov and Sudhof, 1993; Shao et al., 1997; von Poser et al., 1997; Shao et al., 1998; Thomas and Elferink, 1998; Ubach et al., 1998; Zhang et al., 1998; Bai et al., 2000; Gerber et al., 2001; Millet et al., 2002). It was thought that Ca^{2+}-stimulated phospholipid and syntaxin binding by C_2A might provide a direct, physiological link between a Ca^{2+} signal and vesicle fusion. Neutralization of a positively charged residue near the Ca^{2+} -binding region of C_2A which interacts with phospholipids reduced evoked transmitter release and increased the Ca^{2+} requirement of release in mouse hippocampal neurons (Fernandez-Chacon et al., 2001). Neutralization of one Ca^{2+} ligand in C_2A of Drosophila (Fig. 3.1, aspartate #2 in C_2A; a key residue for all Ca^{2+}-dependent interactions *in vitro*), however, did not have significant physiological consequences (Robinson et al., 2002). Similar results were seen when the C_2A Ca^{2+}-binding aspartate residues (Fig. 3.1, aspartate #4 and #5) were individually neutralized in mice (Fernandez-Chacon et al., 2002).

In contrast, the C_2B domain participates in different interactions including Ca^{2+}-independent binding to the clathrin adapter protein AP-2 (Zhang et al., 1994), N- and P/Q-type Ca^{2+} channels (Charvin et al., 1997; Sheng et al., 1997), syntaxin (Li et al., 1995b; Kee and Scheller, 1996), SNAP-25 (Schiavo et al., 1997), β-SNAP (Schiavo et al., 1995), and members of the inositol high polyphosphate series (IHPS - IP_4, IP_5, IP_6) (Fukuda et al., 1994; Niinobe et al., 1994). Three C_2B lysine residues of mouse synaptotagmin II are critical for binding members of the

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IHPS (Fukuda et al., 1995a). Two of these same lysine residues are also critical for binding AP-2 and Ca^{2+} channels and for Ca^{2+} -dependent oligomerization of synaptotagmin molecules (Chapman et al., 1998) (see also (Ubach et al., 2001)). The C_2B domain binds negatively charged phospholipids in a Ca^{2+}-dependent manner *in vitro* as either an isolated domain (Fernandez et al., 2001; Mackler et al., 2002) or linked to C_2A as a complete cytoplasmic domain (Earles et al., 2001; Bai et al., 2002). C_2B also reportedly has Ca^{2+}-inhibited binding to SV2 (Schivell et al., 1996) *in vitro*. Due to an unfortunate mutation that prevented Ca^{2+}-dependent interactions in the most widely studied clone of rat *syt I* (Osborne et al., 1999), C_2B was largely overlooked as a non-important domain for a number of years.

Biochemical interactions with nerve terminal molecules

Reported biochemical interactions have been used to weave a complicated tapestry of potential synaptotagmin functions. For example, some models show synaptotagmin directly interacting with the lipid bilayer to promote fusion pore opening, while others show synaptotagmin altering the relationship between SNARE proteins or the conformation of the SNARE complex itself. Some of synaptotagmin's putative ligands have been shown to be artifactual (Sugita and Sudhof, 2000; Ubach et al., 2001); others have been largely ignored (Fukuda et al., 1994; Schiavo et al., 1995; Schivell et al., 1996); details of the remaining interactions continue to be hotly debated. In addition to binding Ca^{2+}, three types of Ca^{2+}-dependent interactions are generally agreed to constitute the most important functions of synaptotagmin: 1) phospholipid binding, 2) SNARE protein binding, and 3) self-oligomerization. I will briefly review these major

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interactions below, since they are most often invoked to explain the physiological results from *in vivo* synaptotagmin mutants.

Ca^{2+} and Ca^{2+} channels

Ca^{2+} binding is a key function of most C_2 domains (Nalefski and Falke, 1996; Shao et al., 1996). Since the other three important functions listed above – phospholipid binding, SNARE binding, and oligomerization – are all Ca^{2+} dependent to some degree, much work has focused on describing the mechanism of Ca^{2+} binding to synaptotagmin. At least three Ca^{2+} ions are proposed to bind to the C_2A domain via five aspartate residues, one serine, and three carbonyl groups (Ubach et al., 1998). NMR studies have determined the dissociation constants for each of the three Ca^{2+} binding sites in C_2A: site 1 = 50-75 μM, site 2 = 400-550 μM, and site 3 >> 20 mM (Ubach et al., 1998; Fernandez-Chacon et al., 2002). Ca^{2+} binding to C_2A is not cooperative, with a Hill slope of approximately 1 (Davis et al., 1999). C_2B, on the other hand, appears to bind two Ca^{2+} ions via five conserved acidic residues (either aspartate or glutamate depending on the isoform) with dissociation constants of ~400 μM and ~600 μM for each site (Fernandez et al., 2001). (Note: the five acidic residues that coordinate Ca^{2+} in each C_2 domain will hereafter be referred to as D1, D2, D3, etc. within each domain for simplicity, regardless of the species or isoform.) Such low intrinsic Ca^{2+} affinities prompted the hypothesis that the Ca^{2+} ions had incomplete coordination spheres that could be completed via interaction with other molecules (Zhang et al., 1998). Indeed, the presence of either syntaxin or phospholipids will increase the apparent Ca^{2+} affinity for the synaptotagmin

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complex (Brose et al., 1992; Chapman et al., 1995; Ubach et al., 1998; Zhang et al., 1998). Other divalent cations may substitute for Ca^{2+} in binding assays to syntaxin or phospholipids with the order of: $Ca^{2+} > Ba^{2+}$, $Sr^{2+} >> Mg^{2+}$ (Davletov and Sudhof, 1993; Chapman et al., 1995) which mirrors the ion selectivity of synaptic transmission (Augustine et al., 1987).

The acidic residues that coordinate Ca^{2+} ions in C_2A and C_2B are dispersed in the primary protein sequence, yet coalesce into two loops which form a Ca^{2+} binding pocket in the three dimensional structure (Sutton et al., 1995; Shao et al., 1996; Sutton et al., 1999). Using NMR spectroscopy, no major conformational changes were observed upon Ca^{2+} binding to the C_2A domain; instead, Ca^{2+} appears to stabilize the structure of the C_2 domain (Shao et al., 1998). Thus, synaptotagmin is thought to function as an "electrostatic switch" wherein interactions with other molecules are determined by the presence or absence of a net positive charge over the Ca^{2+} binding pocket (Shao et al., 1997; Shao et al., 1998; Ubach et al., 1998; Zhang et al., 1998). Other studies, however, employing proteolysis of synaptotagmin in its Ca^{2+}-bound versus Ca^{2+}-unbound states have declared that synaptotagmin does undergo a conformational change (Davletov and Sudhof, 1994; Desai et al., 2000). A study that utilized Tryptophan reporter fluorescence also reported small conformational changes throughout the C_2A domain in response to Ca^{2+} binding (Chapman and Davis, 1998). There is currently no consensus on whether synaptotagmin undergoes a conformational change in the presence of Ca^{2+} or not.

If synaptotagmin is the major low affinity Ca^{2+} sensor for synaptic transmission, it follows that releasable synaptic vesicles should be localized to

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nerve terminal regions that are exposed to high concentrations of Ca^{2+} during nerve terminal depolarization. Mammalian synaptotagmin has been shown to bind to N- (Yoshida et al., 1992)and P/Q-type Ca^{2+} channels (Martin et al., 1996) via a "synprint" motif on the I-II intracellular loop of the α subunit (Charvin et al., 1997; Sheng et al., 1997). This linkage may help localize vesicles to sites of Ca^{2+} influx (Klingauf and Neher, 1997). Injection of synprint peptides from N-type Ca^{2+} channels inhibited synaptic transmission (Mochida et al., 1996) and shifted the apparent Ca^{2+} affinity of release to higher values (Rettig et al., 1997). Interpretation of these experiments is extremely complicated since the synprint binding site is one of many ligands that bind to synaptotagmin via its polylysine motif on C_2B. In addition, SNAP-25 and syntaxin also bind to the synprint site of N- and P/Q-type Ca^{2+} channels reviewed in (Catterall and Department of Pharmacology, 1999). Since no synprint motif has been identified in Drosophila Ca^{2+} channels (Littleton and Ganetzky, 2000), synaptotagmin's interaction with Ca^{2+} channels via the synprint site is probably not a highly conserved function.

Phospholipids

The high degree of homology between PKC and phospholipase A2 (PLA2) prompted the hypothesis that synaptotagmin could perform Ca^{2+}-dependent membrane binding. In contrast to either PKC or PLA2, synaptotagmin does not have any enzymatic activity associated with phospholipids, therefore membrane binding must have another function (Nalefski et al., 2001). Early biochemical studies reported that synaptotagmin bound anionic phospholipids in the presence of Ca^{2+} (Brose et al., 1992) and that the isolated C_2A domain was sufficient for this reaction to occur (Davletov and Sudhof, 1993; Chapman and

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Jahn, 1994). Contrary to some original experiments, the isolated C_2B domain of rat synaptotagmin I and Drosophila synaptotagmin I bound phospholipids in a Ca^{2+}-dependent manner under some conditions *in vitro* (Fernandez et al., 2001; Mackler et al., 2002), but not others (Bai et al., 2002). The entire cytoplasmic domain of rat *syt I*, however, will bind phospholipids even when Ca^{2+}-binding aspartate residues in C_2A are mutated to asparagines, suggesting that C_2B will bind phospholipids when tethered to a non-functional C_2A domain (Earles et al., 2001). Thus, C_2A and C_2B exhibit Ca^{2+}-dependent phospholipid binding ability in isolation which probably indicates that each domain is at least partially redundant *in vivo* (Earles et al., 2001; Bai et al., 2002).

The biochemical mechanism of phospholipid binding is the topic of much investigation. From the protein structure of the C_2A domain, it is clear that Ca^{2+} ions are coordinated by several carbonyl oxygens from five aspartate residues and one serine (Sutton et al., 1995; Shao et al., 1998). Other residues around the Ca^{2+} binding pocket exhibit chemical shifts in NMR spectroscopy when examined in the presence of phospholipids: Arg_{233}, Phe_{234}, His_{198}, Val_{205}, and Phe_{206} of rat synaptotagmin I (Chae et al., 1998). Mutation of positively charged residues around the Ca^{2+} binding pocket of C_2A (Arg_{199}/Arg_{233} double mutant) decreased interactions with phospholipids, while mutation of D2N or D3N completely abolished phospholipid binding (Zhang et al., 1998). Mutation of the serine residue $S_{235}A$ which should only affect binding of the third Ca^{2+} ion also severely decreased phospholipid binding, thus the third Ca^{2+} ion is required for phospholipid binding (Zhang et al., 1998).

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Ca^{2+} ions bind to C_2A with higher affinity in the presence of phospholipids (Brose et al., 1992; Shao et al., 1996; Zhang et al., 1998), similar to PKC, which suggests that the anionic phospholipid head group may complete the coordination of Ca^{2+} (Bazzi and Nelsestuen, 1991). In addition, the Ca^{2+} and lipid binding sites appear to overlap (Chapman and Davis, 1998), suggesting a model wherein synaptotagmin-Ca^{2+}-phospholipids form a ternary complex or "Ca^{2+} bridge" (Bazzi and Nelsestuen, 1991). Part of the mechanism of phospholipid binding can be explained by the ability of the Ca^{2+}-binding loops of each C_2 domain to penetrate membranes (Chae et al., 1998; Chapman and Davis, 1998; Davis et al., 1999; Bai et al., 2000; Bai et al., 2002). Trp residues at positions 231 and 234 in the Ca^{2+} binding loop of rat synaptotagmin I C_2A penetrated lipid bilayers (Chapman and Davis, 1998). These experiments used synthetic liposomes that were 25% phosphatidylserine and 75% phosphatidylcholine to mimic both synaptic vesicles and plasma membranes. Trp reporter studies have also shown that C_2A can penetrate *cis* or *trans* membranes, therefore, it is not clear which membranes are penetrated by synaptotagmin's C_2 domains *in vivo* (Bai et al., 2000). Another study demonstrated membrane penetration by the C_2B domain from cytosolic phospholipase A2, yet failed to show membrane penetration by syt C_2A (Davletov et al., 1998). This study concluded that syt C_2A bound phospholipids primarily via electrostatic interactions since the binding was abolished by high salt concentrations (Davletov et al., 1998). Finally, another study has shown that syt C_2A promiscuously binds negatively charged phospholipids, regardless of

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the phospholipid head group (Zhang et al., 1998). Thus, the most parsimonious explanation is that multiple contacts involving both electrostatic and hydrophobic interactions are involved in phospholipid binding by synaptotagmin.

It is still debated, however, whether phospholipid binding by synaptotagmin is a physiologically relevant interaction. This interaction has a steep dose-response curve indicating marked cooperativity with an EC_{50} of approximately 4-6 μM free Ca^{2+} (Davletov and Sudhof, 1993). This EC_{50} appears to be well below the estimated threshold for synaptic vesicle exocytosis (> 20μM (Heidelberger et al., 1994)). Under different assay conditions, the Ca^{2+} EC_{50} for phospholipid binding to C_2A was estimated to be between 21-74 μM (Davis et al., 1999), equal to or above the minimum threshold concentration for exocytosis. A decrease in Ca^{2+}-dependent phospholipid binding has been correlated with decreased exocytosis in two *in vivo* mutagenesis studies (Fernandez-Chacon et al., 2001; Mackler and Reist, 2001). In addition, Ca^{2+}-dependent phospholipid binding is not conserved in all isoforms such as synaptotagmin IV, VI, VIII or XI (Li et al., 1995b; von Poser et al., 1997), thus, phospholipid binding likely represents only one of synaptotagmin's interactions. Interactions between synaptotagmin and members of the SNARE complex may play an additional role in regulation of membrane fusion events.

SNARE protein binding

Synaptotagmin interacts individually with the t-SNARE proteins syntaxin and SNAP-25. Synaptotagmin binds the t-SNARE syntaxin (Yoshida et al., 1992) in a Ca^{2+}-dependent manner (Chapman et al., 1995). Synaptotagmin-syntaxin

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interactions are unusually sensitive to the specific reaction conditions (for example, which protein is on the bead), making biochemical experiments difficult to interpret (Kee and Scheller, 1996). One such discrepancy in the literature involves whether synaptotagmin binds the H3 domain of syntaxin (Chapman et al., 1995; Kee and Scheller, 1996) or the Habc domain (Shao et al., 1997). A final, careful study of synaptotagmin-syntaxin binding concluded that the membrane proximal H3 domain and part of the transmembrane domain of syntaxin are required for synaptotagmin binding (Davis et al., 1999). From an NMR study, it is clear that no major conformational change occurs in synaptotagmin upon Ca^{2+}-promoted syntaxin binding (Shao et al., 1997). All three Ca^{2+} ions are reported to be required for syntaxin binding to the isolated C_2A domain (Ubach et al., 1998). A second controversy involved whether syntaxin binding requires only the C_2A domain (Li et al., 1995b) or both domains (Chapman et al., 1996). Davis et al. (Davis et al., 1999) found that high affinity syntaxin binding required all of C_2A and up to residue 337 of C_2B in rat synaptotagmin. Thus the interaction between syntaxin and synaptotagmin may be complex and very sensitive to assay conditions.

Synaptotagmin has exhibited both Ca^{2+}-independent (Schiavo et al., 1997) and Ca^{2+}-dependent (Davis et al., 1999; Gerona et al., 2000; Zhang et al., 2002) binding to the t-SNARE SNAP-25 *in vitro*. Ca^{2+}-independent binding that was originally described may have been caused by reduced Ca^{2+}-dependent interactions of t-SNARES several days after purification (Davis et al., 1999). Ca^{2+}-dependent binding of SNAP-25 to synaptotagmin appears to be mediated by a series of aspartate residues on the surface of SNAP-25 when it is in a helical

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conformation. Neutralization of these residues abolished Ca^{2+}-dependent binding to synaptotagmin and increased the Ca^{2+} requirement of synaptic transmission (Zhang et al., 2002). The region of synaptotagmin that is required for SNAP-25 binding has not been isolated, however, neutralization of Ca^{2+}-binding aspartate residues in C_2B decreased SNAP-25 binding by approximately 50% in one assay (Earles et al., 2001).

In addition, synaptotagmin can bind to the fully assembled SNARE complex *in vitro* with a Ca^{2+} EC_{50} of approximately 100 μM and may be able to interact with the SNARE complex and phospholipids simultaneously (Davis et al., 1999; Zhang et al., 2002). If the lipid binding loops surrounding the Ca^{2+}-binding pocket of both C_2 domains penetrate the presynaptic membrane in the presence of Ca^{2+} (Davis et al., 1999), the Ca^{2+} binding pocket of synaptotagmin would be unavailable to interact with the SNARE complex. This implies that the region of synaptotagmin that interacts with the SNARE complex may be on the face of the β-sandwich, not the Ca^{2+} binding loops. Synaptotagmin appears to interact with the C-terminal regions of both syntaxin and SNAP-25 nearest to their membrane anchors, perhaps making dual interaction with both SNAREs and phospholipids possible.

Oligomerization

Early studies of synaptotagmin revealed that *in vitro*, synaptotagmin appears to self-associate via two methods. First, synaptotagmin can oligomerize via a cysteine motif in its transmembrane domain independent of Ca^{2+} (Fukuda et al., 1999; Bai et al., 2000). Most attention, however, has focused on Ca^{2+}-dependent oligomerization mediated by the C_2B domain (Chapman et al., 1996;

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Damer and Creutz, 1996; Sugita et al., 1996). Neutralization of Ca^{2+}-binding aspartates in C_2B resulted in Ca^{2+}-independent oligomerization demonstrating that Ca^{2+} sensing by C_2B is required for this interaction (Desai et al., 2000). In addition to requiring Ca^{2+} binding by the aspartate residues in C_2B, a series of lysine residues on the surface of the C_2B β-sheet (the same lysine residues that bind AP-2 and the syprint peptide) are also required for Ca^{2+}-dependent oligomerization (Chapman et al., 1998). In addition, a point mutation near the second Ca^{2+} binding aspartate residue in C_2B resulted in decreased oligomerization as well (Littleton et al., 2001). The measured Ca^{2+} dependence of oligomerization, however, has varied wildly depending on the method used (Chapman et al., 1996; Sugita et al., 1996; Osborne et al., 1999).

Oligomerization of synaptotagmin was proposed to form a putative "fusion pore" between the synaptic vesicle and the presynaptic membrane. Such a structure has never been observed at synaptic terminals and although the crystal structure of synaptotagmin was known, no one knew how multiple synaptotagmin molecules would assemble. Discrepancies in biochemical findings and the overall vagueness of this interaction led some to speculate that this may be an artifact seen only *in vitro*. In 2001, Ubach et al. (Ubach et al., 2001) reported that bacterial contaminants, most likely RNA, bound tightly to the C_2B domain of recombinant synaptotagmin proteins when they were affinity purified using standard techniques. When these contaminants were removed using high salt concentrations and FPLC, Ca^{2+}-dependent oligomerization was abolished in the standard "GST pull down" assay (Ubach et al., 2001). New data using "clean" synaptotagmin, confirms that the old assays do not work using clean

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synaptotagmin, but interestingly, the phenomenon of oligomerization can still be demonstrated using a different approach (Wu et al., 2003). Chapman and colleagues applied soluble cytoplasmic domains of synaptotagmin to planar membranes and, using high resolution EM, have shown that these domains assemble into regular barrel-shaped structures (Wu et al., 2003). These barrel structures are consistent with the alignment of 7 synaptotagmin molecules along their long axis to form a putative channel – perhaps a fusion pore. Thus, although the original biochemical results may be in error, the oligomerization reaction may occur in the presence of membranes. The next step is demonstrating that these structures form *in vivo*.

Cooperation and redundancy between domains

Results of biochemical experiments, especially those involving isolated domains, and results of *in vivo* mutagenesis do not always coincide. While many of the interactions described above have been attributed solely to either the C_2A or C_2B domain, increasing evidence suggests that both domains may be necessary for many of these interactions. For example, some interactions require regions from both C_2 domains. A recent fluorescence resonance energy transfer (FRET) study revealed that Ca^{2+} binding to the cytoplasmic domain of synaptotagmin causes the C_2A and C_2B domains to move toward one another (Garcia et al., 2000). This response has an EC_{50} of 3 mM Ca^{2+}. Finally, mutation of the third and fourth Ca^{2+}-binding aspartates residues in C_2B reduced Ca^{2+}-dependent phospholipid, syntaxin and SNAP-25 binding (Earles et al., 2001); these binding interactions had been previously ascribed to only to C_2A. In addition, the presence of C_2A (with or without functional Ca^{2+} binding

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aspartates) is required for penetration of the C_2B Ca^{2+} binding loop into membranes (Earles et al., 2001; Bai et al., 2002). Therefore, Ca^{2+} binding by C_2B may be important for both the intra and inter-molecular associations of synaptotagmin. In conclusion, it is critical to bear in mind that biochemical data are often inconsistent, therefore when determining the function of synaptotagmin, studies performed *in vivo* must be considered as well.

Synaptotagmin genetic studies

Genetic knock out of synaptotagmin strongly reduces synaptic transmission in every species examined (Lin and Scheller, 2000), demonstrating that synaptotagmin plays a central role in the synaptic vesicle cycle. A variety of deficits were observed in synaptotagmin mutants in each species. Different, at times conflicting, conclusions can be drawn from each study, indicating many potential functions for synaptotagmin in the vesicle cycle.

C. elegans

A single isoform of synaptotagmin, *snt-1*, has been identified in the nematode, *C. elegans* (Nonet et al., 1993). Deficiency of this gene results in nematodes that are small, slow-growing and uncoordinated. They are also deficient in pharyngeal pumping and defecation. Yet, the animals do exhibit wavelike body movements and retract from a tactile stimulus to the head. These mutants are resistant to cholinesterase inhibitors, yet remain sensitive to acetylcholine receptor agonists, suggesting that acetylcholine release is impaired (Nonet et al., 1993). Since some synaptic activity clearly persists in these mutants, synaptotagmin is unlikely to be the sole Ca^{2+} sensor for synaptic transmission in this organism. Synaptotagmin-independent release may occur

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via an alternative fusion pathway or, more likely, through the presence of another synaptotagmin isoform(Craxton, 2001). Additionally, mutant nerve terminals were relatively depleted of synaptic vesicles, indicating that synaptotagmin may be involved in the endocytic pathway (Jorgensen et al., 1995).

Mice

Recall that *syt* I is the most abundant isoform in mammalian forebrain (Geppert et al., 1991). Mice bearing a mutation at amino acid 270 of synaptotagmin I produce only small amounts of truncated synaptotagmin. Homozygote mutant mice could breathe and respond to tactile stimulation, yet died within 48 hours of birth due to inability to suckle (Geppert et al., 1994). Electrophysiological recordings of evoked synaptic responses in cultured hippocampal neuron pairs indicated a dramatic reduction in fast transmitter release from mutant terminals. In contrast to Drosophila syt^{null} mutants described below, mouse *syt* mutant terminals did not have an increased frequency of mEJCs. In mice, action potentials were generated in mutant cells, NMDA receptor distribution was not altered, and mEPSC size was normal, all of which indicate that the defect is not post-synaptic, but rather pre-synaptic. In addition, Ca^{2+}-independent release stimulated by either 0.5 M sucrose or α-latrotoxin was similar between mutants and wild-type. This study concluded that synaptotagmin was a major Ca^{2+} sensor for fast synaptic transmission in mammals and postulated that a second, higher affinity, sensor was responsible for the remaining release.

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Drosophila

Drosophila has been an important model system for investigating neuronal function of syt^{null} mutants for several reasons. Drosophila have at most two neuronal isoforms of synaptotagmin (Littleton et al., 1999) which makes interpretation of genetic studies somewhat less complicated than in mammals. The most abundant isoform, *syt I*, is present at the well-characterized larval neuromuscular junction, where both electrophysiological and ultrastructural studies may be done. In addition, genetic manipulations in Drosophila are relatively easy when compared to mammals. Drosophila syt^{null} larvae are slow, uncoordinated and exhibit dramatically reduced Ca^{2+}-evoked synaptic transmission (DiAntonio et al., 1993; Littleton et al., 1993b). A small percentage of the Ca^{2+}-evoked release persists in these mutants, suggesting that synaptotagmin I is not the only synaptic protein competent to support Ca^{2+}-triggered vesicle fusion (Broadie et al., 1994). Other isoforms of synaptotagmin may be responsible for this residual amount of vesicle release (Littleton et al., 1999), however the mechanism for this residual Ca^{2+}-dependent release remains unknown. Drosophila syt^{null} mutants also display an increased frequency of spontaneous vesicle release consistent with a role for synaptotagmin in preventing constitutive fusion. Ultrastructural analysis of central synapses in Drosophila syt^{null} mutants revealed a decreased number of docked vesicles (Reist et al., 1998), consistent with the hypothesis that synaptotagmin stabilizes the docked state of vesicles until they are released by Ca^{2+} influx (Bennett et al., 1992; Popov and Poo, 1993). Finally, syt^{null} mutants have a decreased number of small synaptic vesicles and an elevated number of large, irregular vesicles (Reist

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et al., 1998) again indicating that synaptotagmin may also be required during vesicle recycling. Thus, syt^{null} mutants have been critical for identifying multiple potential functions of synaptotagmin, however these data do not indicate which molecular motifs within synaptotagmin may be responsible for each function. The major shortcoming of all of the syt^{null} studies was that no single synaptic function of synaptotagmin could explain all of the deficits seen in syt^{null} organisms.

Specific Aims

Genetic knockout studies have demonstrated that synaptotagmin is required for fast synaptic transmission and biochemical studies have begun to identify key residues that mediate synaptotagmin binding to a myriad of molecules. Both of these approaches have been useful for generating hypotheses about synaptotagmin function – in fact, perhaps too many functions have been assigned to synaptotagmin in an attempt to fully rationalize the results of these experiments. For example, results from both genetic knockout experiments and biochemical interactions could be used to support a role for synaptotagmin in any stage of the vesicle cycle: docking, fusion, or endocytosis. This raises the question of whether selective pressure during evolution would permit one molecule to have so many critical roles at the synapse. Disruption of any part of synaptotagmin would then be catastrophic unless there is some other mechanism of functional redundancy built in.

In this dissertation, I have taken the next step to further dissect synaptotagmin's function by making site directed mutations in specific domains. Two major conserved motifs of the synaptotagmin C_2B domain have been

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identified in biochemical binding assays and merit further investigation: a polylysine motif on the surface of the β-sandwich and a set of Ca^{2+}-binding aspartate residues within loops one and three. The functional significance of these motifs in C_2B, however, has not been tested at synapses *in vivo*. I hypothesized that since these domains were so highly conserved between species that these residues would contribute to synaptotagmin function. Using transgenic Drosophila, I have tested two main specific aims: 1) the importance of three lysine residues in the polylysine motif (Chapter 2) and 2) the contribution of four C_2B Ca^{2+}-binding aspartate residues to synaptic transmission (Chapter 3). Here I show that both motifs are important for synaptic transmission *in vivo*, thus, the C_2B domain does perform critical functions. Finally, I will propose a model wherein Ca^{2+} binding by synaptotagmin promotes vesicle fusion through binding phospholipids and/or SNAREs using Ca^{2+} as a "bridge". This model includes a role of synaptotagmin in setting the apparent Ca^{2+} affinity, but not necessarily the cooperativity, of synaptic transmission (Chapter 4).

Experimental Design and Credits

The general approach for this project is a structure/function study of the C_2B domain of Drosophila synaptotagmin I. I have taken advantage of biochemically identified residues in synaptotagmin I that are critical for select protein interactions and made homologous mutations in Drosophila cDNA using PCR-based, site-directed mutagenesis. These mutations should reduce or abolish synaptotagmin interactions in specific parts of the protein, leaving the rest of the protein functional. This mutant cDNA was inserted into a transposable DNA element (P-element) and used to transform Drosophila.

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Transformants were crossed to flies that simultaneously contained the appropriate genetic construct for neuronal expression (*P[elavGal4]*) of the mutant synaptotagmin transgene and were homozygous null for native synaptotagmin. In this way, the only synaptotagmin I produced in these flies should be the mutant synaptotagmin. Control flies included an analogous fly that expressed wild type synaptotagmin from a transgene in an otherwise syt^{null} background as a positive control and syt^{null} flies as a negative control (characterized in (Loewen et al., 2001)). For this project, I performed the site directed mutagenesis and transforming the mutant fly lines. I verified that polylysine mutant flies produced synaptotagmin protein using anti-synaptotagmin immunohistochemistry in Fig. 2.3, while Noreen Reist contributed to Fig. 3.4a. Noreen Reist, Carin Loewen, and Kim Mace used semi-quantitative Western analysis (Fig. 2.8 and 3.4) to further compare expression levels between lines to insure that the mutant transgenic lines expressed approximately the same amount of protein as the positive control. I performed all electrophysiological experiments to assay synaptotagmin function by measuring spontaneous and evoked excitatory junction potentials (EJPs) at the neuromuscular junction of third instar larvae. Since protein expression has been controlled for, any differences in synaptic transmission in the mutant lines must be due to the mutant transgene. Suzanne Royer generated the electron micrographs to look at the presence of synaptic vesicles at mutant terminals (Fig. 3.5 a,b,c). Finally, James Drummond and Iain Robinson expressed the Ca^{2+}-binding mutant cDNA constructs *in vitro* and performed biochemical assays to test oligomerization and phospholipid binding (Fig. 3.5d,e). Chapter 2 contains published data on the

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polylysine mutants (Mackler and Reist, 2001), along with an additional discussion of our current understanding of synaptotagmin oligomerization. Chapter 3 also consists primarily of our publication on the Ca^{2+} binding site mutants (Mackler et al., 2002) with some additional unpublished data and discussion. Finally, in Chapter 4, I discuss my current view of the molecular mechanism of synaptotagmin function *in vivo*.

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Chapter 2: Mutations in the C_2B polylysine motif disrupt synaptic transmission

Abstract

The synaptic vesicle protein, synaptotagmin, has been hypothesized to mediate several functions in neurotransmitter release, including: Ca^{2+} sensing, vesicle recycling, and synaptic vesicle docking. These hypotheses are based on evidence from *in vitro* binding assays, peptide and antibody injection experiments, and genetic knockout studies. Synaptotagmin contains two domains that are homologous to the Ca^{2+}-binding C_2 domain of protein kinase C. The two C_2 domains of synaptotagmin have broadly differing ligand-binding properties. We have focused on the second C_2 domain (C_2B) of synaptotagmin I, in particular on a series of conserved lysine residues on β-strand four of C_2B. This polylysine motif binds clathrin adapter protein AP-2, neuronal Ca^{2+} channels, and inositol high polyphosphates. It also mediates Ca_2-dependent oligomerization. In order to investigate the importance of these lysine residues in synaptic transmission, we have introduced *synaptotagmin I (syt)* transgenes harboring specific polylysine motif mutations into flies otherwise lacking the synaptotagmin I protein (syt^{null}). Electrophysiological analyses of these mutants revealed that evoked transmitter release is decreased by ~36% and spontaneous release is increased ~2 fold relative to syt^{null} flies expressing a wild type *syt* transgene. Synaptotagmin expression in both the mutant and wild type

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transgenic lines was equivalent as measured by semi-quantitative Western blot analysis. Thus, the alteration in synaptic transmission was due to the mutation and not synaptotagmin expression levels. We conclude that synaptotagmin interactions mediated by the C_2B polylysine motif are required to attain full synaptotagmin function *in vivo*.

Introduction

The molecular mechanisms mediating synaptic vesicle docking, fusion, and recycling are currently topics of intense study. The assembly of a coiled-coil structure between SNARE proteins on the vesicle membrane (VAMP/synaptobrevin) and the presynaptic membrane (syntaxin and SNAP-25) is widely believed to mediate vesicle fusion in both constitutive and regulated pathways (Hanson et al., 1997b; Weber et al., 1998; Bock and Scheller, 1999; Brunger, 2000). This mechanism of membrane fusion is highly conserved from yeast to mammals (Bennett and Scheller, 1993; Ferro-Novick and Jahn, 1994; Pelham, 1999). However, a number of other proteins are thought to regulate the formation of this core fusion complex (VAMP/syntaxin/SNAP-25) (Rothman and Sollner, 1997; Gerst, 1999). In nerve terminals, constitutive fusion must be prevented until an increase in Ca^{2+} triggers synaptic vesicles to fuse with the presynaptic membrane. Because the fusion process then proceeds very rapidly (Llinas et al., 1981; Augustine et al., 1991), synaptic vesicles are thought to be docked at release sites in a fusion ready state. The synaptic vesicle protein, synaptotagmin, has been hypothesized to both stabilize the docked state of vesicles (Bennett et al., 1992; Broadie et al., 1994; DiAntonio and Schwarz, 1994; Schiavo et al., 1997; Reist et al., 1998), and sense the Ca^{2+} that triggers regulated

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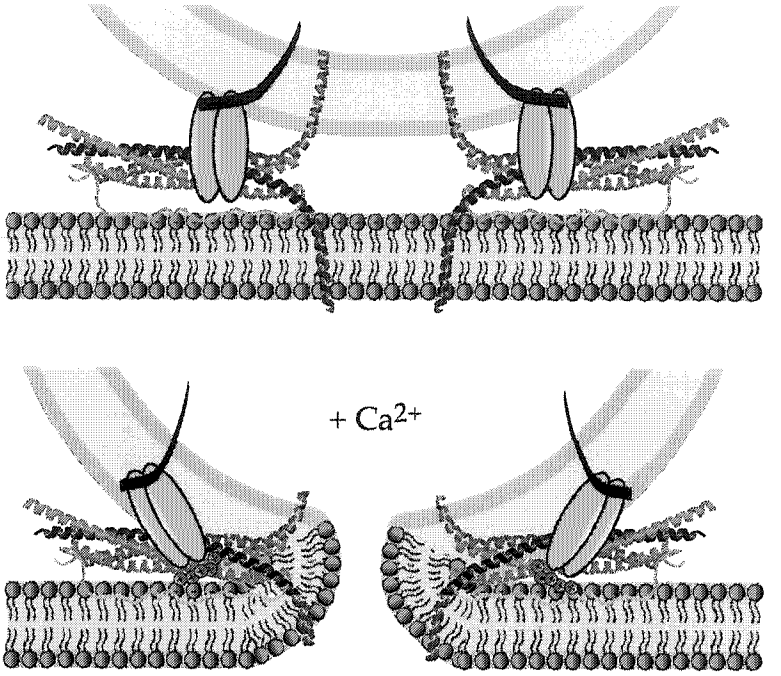

Figure 2.1 One model of possible synaptotagmin/SNARE complex interactions. Ca^{2+}-independent (upper) and Ca^{2+}-dependent (lower) interactions between synaptotagmin and the SNARE complex. Yellow ovals represent the two C_2 domains of synaptotagmin. Depicted are the Ca^{2+}-dependent interactions of synaptotagmin with both syntaxin and negatively-charged phospholipids. However, this model is also consistent with Ca^{2+}-dependent interactions between synaptotagmin and other nerve terminal molecules.

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vesicle fusion (Brose et al., 1992; Popov and Poo, 1993; Geppert et al., 1994; Bajjalieh and Scheller, 1995; Burgoyne and Morgan, 1998; Geppert and Sudhof, 1998; Schiavo et al., 1998). Synaptotagmin also may play a role during recycling of synaptic vesicles (Zhang et al., 1994; Jorgensen et al., 1995; Reist et al., 1998).

There are several models suggesting possible mechanisms for the regulation of vesicle fusion (Geppert et al., 1994; O'Connor et al., 1994; Rizo and Südhof, 1998; Weis and Scheller, 1998; Bajjalieh, 1999; Gerst, 1999; Hilfiker et al., 1999; Brunger, 2000). For purposes of illustration, Figure 2.1 shows one such model, although our data are also consistent with other models. Ca^{2+}-independent interactions between synaptotagmin and proteins of the core fusion complex (Li et al., 1995b; Kee and Scheller, 1996; Schiavo et al., 1997; Sutton et al., 1999; Leveque et al., 2000) could stimulate formation of the docked state of vesicles while preventing completion of the coiled-coil structure. *In vitro,* increased Ca^{2+} levels activate interactions between synaptotagmin's C_2 domains and a number of presynaptic components (Bennett et al., 1992; Brose et al., 1992; Davletov and Sudhof, 1993; Chapman et al., 1996; Gerona et al., 2000), while inhibiting other interactions (Kee and Scheller, 1996; Mehta et al., 1996; Schivell et al., 1996; Leveque et al., 2000). Such Ca^{2+}-dependent alterations of synaptotagmin's binding partners could facilitate completion of the core complex's coiled-coil structure, resulting in fusion (Fig. 2.1).

Functional experiments have demonstrated that the synaptic vesicle protein, synaptotagmin I, is required for efficient transmitter release. Injection of synaptotagmin peptide fragments or anti-synaptotagmin antibodies into the presynaptic terminal of squid giant synapses (Bommert et al., 1993; Mikoshiba et

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al., 1995) and PC12 cells (Elferink et al., 1993) inhibit Ca^{2+}-stimulated transmitter release. Genetic knock-out experiments in Drosophila (DiAntonio et al., 1993; Littleton et al., 1993b), *C. elegans* (Nonet et al., 1993) and mice (Geppert et al., 1994) emphasize the importance of synaptotagmin in synaptic transmission.

In Drosophila, syt^{null} mutants are slow, uncoordinated and die as first instar larvae shortly after hatching (DiAntonio et al., 1993; Littleton et al., 1993b). These larvae exhibit dramatically reduced Ca^{2+}-evoked synaptic transmission, yet a small percentage of the Ca^{2+}-evoked release persists, suggesting that synaptotagmin I is not required for Ca^{2+}-triggered vesicle fusion (Broadie et al., 1994). The Ca^{2+}-dependence of this residual release is unchanged. Since other isoforms of synaptotagmin (Littleton et al., 1999) may be responsible for this residual amount of vesicle release, it remains unclear whether synaptotagmin I is the major Ca^{2+} sensor for synaptic vesicle exocytosis. syt^{null} mutants also display an increased frequency of spontaneous vesicle release. Interestingly, over-expression of synaptotagmin I or II in *Xenopus* spinal neurons has almost the opposite effect: high frequency evoked release is increased and the spontaneous release frequency is reduced (Morimoto et al., 1998) (see also (Littleton et al., 1999)). Ultrastructural analysis of Drosophila syt^{null} mutants has revealed a decreased number of docked vesicles (Reist et al., 1998). Taken together, these results are consistent with the hypothesis that synaptotagmin stabilizes the docked state of vesicles until they are released by Ca^{2+} influx (Bennett et al., 1992; Popov and Poo, 1993; Reist et al., 1998). Finally, syt^{null} mutants have a decreased number of small synaptic vesicles (Jorgensen et al., 1995; Reist et al., 1998) and an elevated number of large, irregular vesicle (Reist et al., 1998)

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consistent with the hypothesis that synaptotagmin is also required during vesicle recycling.

Synaptotagmin comprises an N-terminal intravesicular domain, a transmembrane domain, two cytoplasmic C_2 domains, and a short C-terminal domain (Perin et al., 1991a). The C_2 domains of synaptotagmin are homologous to other Ca^{2+}-binding C_2 domains found in protein kinase C (Knopf et al., 1986), phospholipase A2 (Nalefski and Falke, 1996), rabphilin-3 (Shirataki et al., 1993), Doc2 (Orita et al., 1995), and Munc13 (Brose et al., 1995). Recently the three dimensional, crystallographic structure of both linked cytoplasmic C_2 domains from mouse synaptotagmin III has been determined (Sutton et al., 1999). Each C_2 domain consists of an eight-stranded sandwich of b-sheets with a Ca^{2+} binding pocket at one end. A highly conserved cluster of lysine residues is arranged on the surface of each C_2 domain creating positively charged regions with which other molecules could interact (Fukuda et al., 1995b; Chapman et al., 1998; Sutton et al., 1999).

Biochemical experiments have shown that each of synaptotagmin's C_2 domains has a particular complement of ligands. The C_2B domain mediates Ca^{2+}-dependent oligomerization (Chapman et al., 1996; Damer and Creutz, 1996; Sugita et al., 1996) and Ca^{2+}-inhibited binding to SV2 (Schivell et al., 1996). The C_2B domain also participates in Ca^{2+}-independent binding to the clathrin adapter protein AP-2 (Zhang et al., 1994), N- and P/Q-type Ca^{2+} channels (Sheng et al., 1996; Charvin et al., 1997), syntaxin (Li et al., 1995b; Kee and Scheller, 1996), SNAP-25 (Schiavo et al., 1997), β-SNAP (Schiavo et al., 1995), and members of the inositol high polyphosphate series (IHPS - IP_4, IP_5, IP_6) (Fukuda et al.,

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1994; Niinobe et al., 1994). Mutational analysis *in vitro* has revealed that a series of lysine residues in C_2B are required for many of these interactions. Three C_2B lysine residues (Fig. 2.2) of mouse synaptotagmin II are critical for binding IHPS (Fukuda et al., 1995b). Two of these same lysine residues (Fig. 2.2, K1 and K2) are also critical for binding AP-2 and Ca^{2+} channels, and for oligomerization of synaptotagmin molecules (Chapman et al., 1998).

While much attention has been focused on the role of synaptotagmin's C_2A domain during synaptic transmission (Davletov and Sudhof, 1993; Mikoshiba et al., 1995; Shao et al., 1997; von Poser et al., 1997; Geppert and Sudhof, 1998; Shao et al., 1998; Ubach et al., 1998; Zhang et al., 1998), especially in terms of Ca^{2+} sensing (Brose et al., 1992; Geppert et al., 1994), increasing biochemical evidence suggests that C_2B may play a pivotal role in synaptotagmin function. The functional significance of these biochemical interactions, however, has not been tested *in vivo*. Here, we demonstrate a system for testing the functional effects of synaptotagmin mutations at synapses using transgenic Drosophila. Previous wild type *syt* transgenes have produced only weak rescue of the synaptotagmin null phenotype due to very low expression of the transgenic protein (DiAntonio and Schwarz, 1994). Indeed most provide such weak levels of expression that little to no synaptotagmin complex (Li et al., 1995b; Kee and Scheller, 1996; Schiavo et al., 1997; Sutton et al., 1999; Leveque et al., 2000) could stimulate formation of the docked state of protein could be detected at larval neuromuscular junctions. Limited expression of the transgene represents a severe limitation for structure/function studies; if wild type synaptotagmin from a transgene provides poor rescue of the null phenotype,

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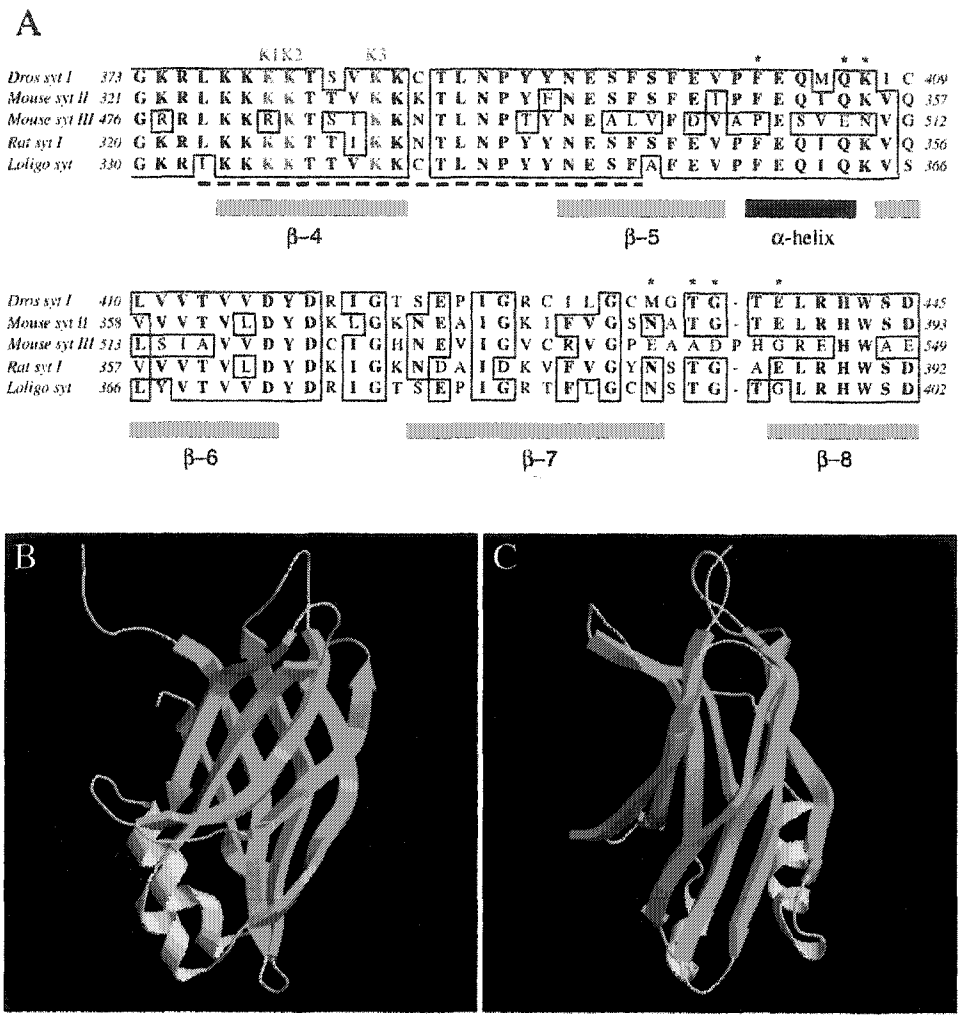

Figure 2.2 Three lysine residues in C_2B that mediate multiple synaptotagmin interactions are highly conserved among different synaptotagmin isoforms. **A:** ClustalW sequence alignment of the C_2B region containing these lysines from Drosophila synaptotagmin and other synaptotagmin isoforms used in previous investigations. The three lysine residues (K1, K2, and K3) are conserved in Drosophila synaptotagmin. Residues permissive for IHPS binding (asterisks) are also highly conserved in Drosophila. Dashed line represents the 20 amino acid peptide from *Loligo* used in injection studies by Bommert et al. (1993). Secondary structures are indicated below the sequence (colored bars) and were placed according to Sutton et al. (1999). **B:** Overall structure of the C_2B domain of synaptotagmin III (PDB file 1 DQV) adapted from Sutton et al. (1999), using MolScript (Kraulis, 1991) and Raster 3D (Merritt and Bacon, 1997) software (adapted by copyright permission of the Rockefeller University Press). Yellow arrows indicate β-sheets, white coils indicate α-helices, and the red sphere indicates Mg^{2+}. The location of the three lysine residues of the C_2B polylysine motif of Drosophila synaptotagmin I are highlighted in blue. **C:** Same as B, rotated to display the cup-shaped region bordered by these lysine residues.

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it becomes impractical to try and assess any deficits due to specific mutations. Therefore, we used a Gal4/UAS amplification system (Brand and Dormand, 1995) to enhance expression of *syt* transgenes in neurons, which resulted in significant rescue of the syt^{null} phenotype. We then used this system to examine the functional importance of the C_2B polylysine motif (Fig. 2.2) for synaptic transmission in Drosophila.

Experimental procedures

Site-Directed Mutagenesis

Lysine residues 379, 380, and 384 (Fig. 2.2) of the Drosophila synaptotagmin I C_2B domain were mutated to glutamines by PCR. A specifically mutated oligonucleotide was paired with a wild-type oligonucleotide that flanked a unique *Eag* I restriction site. The PCR product was then gel purified and used as a macroprimer in a second round of PCR with a wild-type oligonucleotide that flanked a unique *Eco* RV restriction site. This second round PCR product was restriction cut with *Eco* RV and *Eag* I and ligated into a Drosophila *syt* cDNA construct in pBluescript II (which had been modified to lack most of the multi-cloning site). The entire region generated by PCR was sequenced to confirm that the only changes in the *syt* cDNA coded for the three specific K to Q mutations ($K_{379,380,384}Q$). The mutant *syt* gene was then subcloned into pUAST (Brand and Perrimon, 1993) for P-element mediated transformation of Drosophila.

Fly Strains

The pUAST vector containing either wild type *syt* cDNA or the $syt^{K_{379,380,384}Q}$ mutant cDNA was used to transform *yw* flies via standard P-

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element-mediated transformation. Lines carrying the transgenes on the third chromosome were maintained as stocks in combination with the syt^{null} mutation, *AD4* (*w;*syt^{AD4}*/Gla Bc;P[*syt^{wt}*]* and *w;*syt^{AD4}*/Gla Bc;P[*$syt^{K379,380,384Q}$*]*). To generate Drosophila where the only source of synaptotagmin is from the transgene, these lines were crossed to a syt^{null} mutant line carrying a neuronal source of Gal4 (*w;*syt^{AD4} *P[elav-Gal4]/Gla Bc*). From this cross, third instar larvae lacking black cell (*Bc*) or adults without glazed eyes (*Gla*) were selected. We will refer to these lines as syt^{null} + *P[*syt^{wt}*]* (*w;*syt^{AD4}*/*syt^{AD4} *P[elav-Gal4];P[*syt^{wt}*]/+*) and syt^{null} + *P[*syt^{KQ}*]* (*w;*syt^{AD4}*/*syt^{AD4} *P[elav-Gal4];P[*$syt^{K379,380,384Q}$*]/+*). Two independently transformed lines carrying the same $syt^{K379,380,384Q}$ mutations are designated syt^{null} + *P[*syt^{KQ12}*]* and syt^{null} + *P[*syt^{KQ59}*]*. A line homozygous for the wild type *syt* gene (*w;Sco/Gla Bc*) and a line heterozygous for wild type *syt* gene (*w;*syt^{AD4}*/Gla Bc*) were used as controls.

Immunohistochemistry

Third instar larvae were dissected in HL3 saline (70 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES)(Stewart et al., 1994), fixed 30 minutes in 1% formaldehyde in phosphate-buffered saline (PBS) pH=7.4, rinsed in PBS containing 0.1% Triton X-100 (PBST), incubated overnight in anti-synaptotagmin antibody, Dsyt-2 (Littleton et al., 1993a), diluted 1:400 in PBST containing 10% normal goat serum (PBST-NGS) at 4°C. The preparations were washed 1-3 hours in PBST, incubated 1 hour in fluorescein-conjugated goat-anti-rabbit IgG (Jackson Immunoresearch Laboratories) diluted 1:400 in PBST-NGS, washed 1 hour in PBST, and mounted in Citifluor AF-1 (Ted Pella, Inc.). Whole mounts were imaged on an Olympus

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1X70 laser-scanning confocal microscope. Optical sections were projected using Olympus Fluoview software. Images were resized and combined into a single figure prior to contrast adjustment using Adobe Photoshop software. The gamma setting of a Codonics NP-1600 Photographic Network Printer was adjusted to match image appearance on the screen.

Electrophysiology

Electrophysiology experiments were performed at room temperature in HL3 saline on third instar larval fillet preparations pinned in a Sylgard (Dow Corning) coated dish. Muscle fiber 6 from abdominal segments 3 and 4 were used exclusively. Fibers were impaled with 10-30 MΩ electrodes filled with 3M KCl. Intracellular recordings of spontaneous miniature excitatory junctional potentials (mEJPs) were collected for 3 minutes using an AxoClamp 2B and digitized using a MacLab4s A/D converter (Chart software, CB Sciences). Spontaneous events were manually identified and their mean mEJP frequency was analyzed using Mini Analysis Software (Synaptosoft, Inc., www.synaptosoft.com). Evoked excitatory junction potentials (EJPs) were generated by stimulating segmental nerves with 5-10 μm diameter glass suction electrodes filled with HL3. Nerves were stimulated with 1 millisecond pulses of 30-50 nA at 0.5 Hz. Stimulation protocols and analyses were both accomplished using the MacLab4s and its associated Scope software. The resting membrane potential of each muscle fiber was normally between -45 and -65 mV. However, the membrane potential was maintained at -55mV by passing a DC bias current so that mEJP and EJP amplitude comparisons could be made between preparations. Statistical comparisons of means between groups were performed

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via a one-way ANOVA and Bonferroni's Multiple Comparison Test using Prism software (GraphPad Software).

Immunoblotting

For blots of Drosophila heads, ten heads per line were solubilized in 20-50 μl of buffer (5% SDS, 10% glycerol in 0.1 M Tris buffer, pH=8.5), boiled five minutes, and briefly centrifuged to pellet non-solubilized debris. Total protein concentration was measured using a BCA protein assay kit (Pierce) and dithiothreitol (DTT) was subsequently added to a final concentration of 50 mg/ml. All three lines were processed in parallel and run on the same gel. Samples were diluted to 0.75 mg/ml, boiled 5 minutes, electrophoresed by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore) according to standard techniques (BioRad). Blots were incubated for 30 minutes in blocking solution (5% milk, 4% NGS, 1% BSA, 0.05% Tween-20 in PBS), rinsed briefly in PBS containing 0.05% Tween 20 (PBS-Tw), and incubated overnight in primary antibody diluted in PBS-Tw with 10% normal goat serum (PBS-Tw-NGS) at 4° C. After a 1 hour wash in PBS-Tw, bands were visualized on hyperfilm (Amersham) using the ECL or ECL Plus Western analysis systems (Amersham). Blots were then rinsed for 5 minutes in water, stripped for 5 minutes in 0.2 N NaOH, rinsed for 5 minutes in water, reblocked, and reprobed with another primary antibody. Primary antibodies used were: an anti-synaptotagmin rabbit antiserum (Dsyt-2, diluted 1:2,000) and an anti-actin mouse monoclonal antibody (MAB 1501 [Chemicon], diluted 1:20,000). Equal protein loading was confirmed by Coomassie brilliant blue staining of the polyacrylamide gel and anti-actin staining of the blot. By probing a dilution series (from 7.5 - 0.5 μg total protein

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per lane) with the Dsyt-2 antibody, an optimal range of 2.0 - 2.5 μg total protein per lane was determined (data not shown).

For blots of third instar larval central nervous systems (CNS), the CNS from each of four larvae per line were dissected in HL3, solubilized in 20 μl of protein loading buffer (5% SDS, 10% glycerol, 50 mg/ml DTT in 0.1 M Tris buffer), boiled ten minutes, and briefly centrifuged to pellet non-solubilized debris. All lines were processed in parallel and run on the same gel. The entire sample from each line was electrophoresed by SDS-PAGE and processed as above. Approximately equal protein loading was confirmed by Coomassie brilliant blue staining of the polyacrylamide gel and anti-actin staining of the blot.

Results

Gal4/UAS Amplification Greatly Enhances Transgenic Rescue of the syt^{null} Phenotype

In order to enhance neuronal expression of a syt^{wt} transgene, we made wild type *syt* rescue constructs that take advantage of *Gal4/UAS* amplification system (Brand and Perrimon, 1993). To achieve pan neuronal expression, *Gal4* was placed under the control of the *elav* promoter (Robinow and White, 1988), and our *syt* constructs were placed under the control of the upstream activating sequence (*UAS*). Thus, Gal4 is expressed in neurons which then induces expression of our transgenic synaptotagmin constructs. This amplification procedure dramatically improved the expression of the synaptotagmin transgene in the nervous system compared to previously used wild type rescue constructs (DiAntonio and Schwarz, 1994). However, wild type levels of synaptotagmin expression were not attained (data not shown). Use of this system provided

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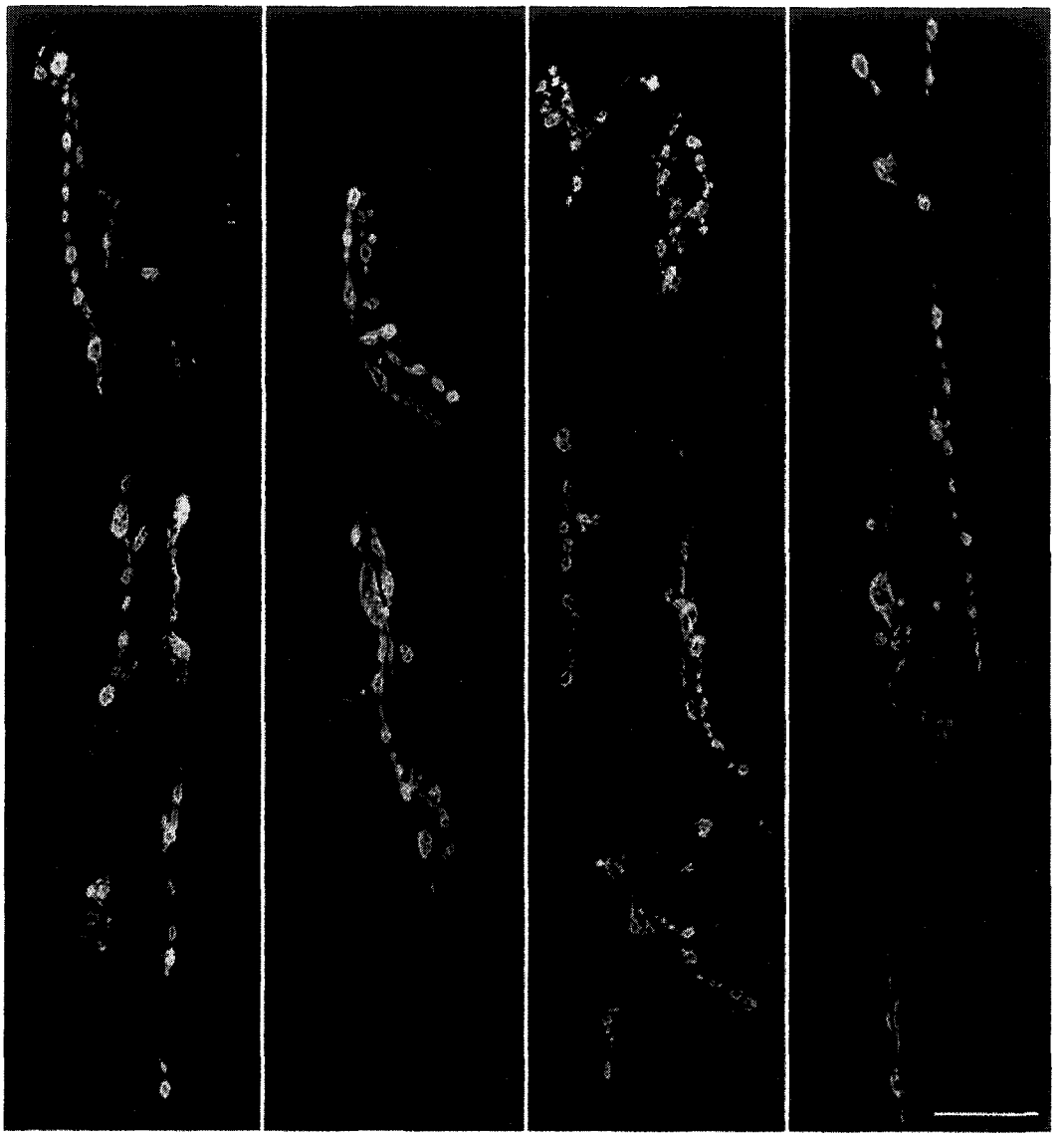

Figure 2.3 Synaptotagmin is expressed at larval neuromuscular junctions in all of the *syt* transgenic lines used in this study. Anti-synaptotagmin antibodies label synaptic boutons of larvae that are homozygous for native *syt* (+/+) and null mutants carrying a *syt* transgene. Scale: 20 μM.

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close to Mendelian rescue of syt^{null} mutant lethality; 85 ± 6% (mean ± SEM) of expected progeny survive. However, the syt^{null} adults that were rescued with the syt^{wt} transgene were distinguishable from wild type adults due to slightly uncoordinated movements.

To determine whether the synapse to be used for electrophysiological analyses expressed the *syt* transgene, third instar larvae were stained with anti-synaptotagmin antibodies. While syt^{null} larvae exhibit no detectable synaptotagmin immunoreactivity either in whole mount or on Western blots (Broadie et al., 1994; DiAntonio and Schwarz, 1994), bright labeling of neuromuscular junctions on muscle fiber number six was observed in syt^{null} mutant larvae rescued with the syt^{wt} transgene (-/-; $P[syt^{wt}]$, Fig. 2.3). Thus, the wild type *syt* transgene is expressed at the synapse under investigation.

We examined the level of synaptotagmin function attained using this *elav-Gal4/UAS-*syt^{wt} transgenic system by measuring both evoked (Fig. 2.4) and spontaneous (Fig. 2.5) neurotransmitter release at the neuromuscular junction. Control larvae that were either homozygous (+/+) or heterozygous (+/-) for native synaptotagmin were compared to syt^{null} mutant larvae rescued with the syt^{wt} transgene (-/-; $P[syt^{wt}]$). Mean EJP amplitudes averaged 39.7 ± 0.7 mV (mean ± SEM, n =11 fibers) for the native *syt* homozygous line and 37.6 ± 0.96 mV (mean ± SEM, n = 14) for the heterozygous line (Fig. 2.4). The slight difference in EJP amplitude between these native *syt* lines was not significant ($p > 0.05$, one-way ANOVA). The mean EJP amplitude for the syt^{null} + $P[syt^{wt}]$ line was 67% of the homozygote value (26.5 ± 1.0 mV, mean ± SEM, n = 9; Fig. 2.4). This value, although clearly an improvement over previous rescue lines

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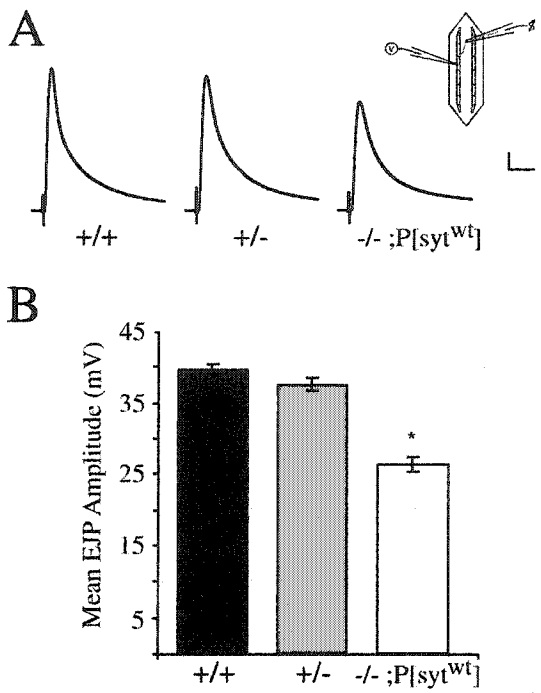

Figure 2.4 The *elav-GAl4/UAS-syt*^{wt} transgenic system provides substantial rescue of the EJP amplitude of *syt*^{null} flies. **A:** Representative EJP traces recorded from larval muscle fiber six in three lines expressing wild type synaptotagmin: *w;Sco/Gla Bc (+/+)*, homozygous for native *syt*; *w;syt*^{AD4}*/Gla Bc (+/-)*, heterozygous for native *syt*; and *syt*^{null} + *P[syt*^{wt}*] (-/-;P[syt*^{wt}*])*, *syt*^{null} mutants expressing wild type *syt* from a transgene. Each trace represents an average of 30 sweeps of EJPs recorded from a single muscle fiber. The inset schematic displays the orientation of recording and stimulating electrodes on a third instar larval fillet preparation. **B:** The mean EJP amplitude of the *syt*^{null} + *P[syt*^{wt}*]* transgenic line was 67% of that of the *syt* homozygous line ($p < 0.001$, one-way ANOVA, Bonferroni's). Scale: 5 mV, 10 msec.

(DiAntonio and Schwarz, 1994), still represents a significant reduction in EJP amplitude compared to either native *syt* line ($p < 0.001$, one-way ANOVA, Bonferroni's). The decrease in evoked transmitter release in the *syt*^{null} + *P[syt*^{wt}*]* line is consistent with the decrease in synaptotagmin expression, the decrease in viability, and the slight decrease in coordination seen in this line.

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Spontaneous transmitter release was very similar among all three lines (Fig. 2.5). Mean mEJP frequencies of *syt* homozygous and heterozygous control lines were almost identical (1.9 ± 0.3 Hz, mean ± SEM, n = 8 and 1.8 ± 0.3 Hz, mean ± SEM, n = 10; p > 0.05, one-way ANOVA; Fig. 2.5a,b). The *syt^{null} +P[syt^{wt}]*

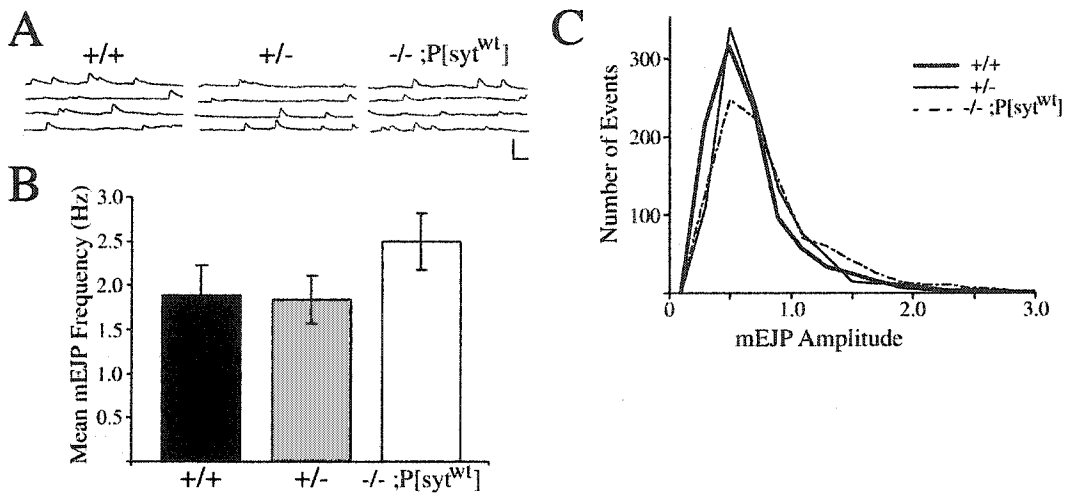

Figure 2.5 The *elav-GAl4/UAS-syt^{wt}* transgenic system rescues the mEJP frequency of *syt^{null}* flies. **A:** Representative recordings of mEJPs in third instar larvae of the three Drosophila lines shown in Fig. 2.4. mEJPs are recognizable by their slope. **B:** Calculated mean mEJP frequencies for all three lines were not significantly different from one another (p > 0.05 one-way ANOVA, Bonferroni's). **C:** Frequency distribution curves of mEJP amplitudes from each line calculated from 1000 events compiled into 0.2 mV bins. The distribution of mEJP amplitudes (quantal sizes) was unchanged in the *syt^{wt}* transgenic line. Accordingly, the mean mEJP amplitude of the *syt^{wt}* transgenic line was not significantly different from other controls (+/+, 0.70 ± 0.04 mV, mean ± SEM; +/-, 0.72 ± 0.02 mV, mean ± SEM; -/-; P[syt^{wt}], 0.87 ± 0.07 mV, mean ± SEM; p > 0.05 one-way ANOVA, Bonferroni's). Scale: 4 mV, 100 msec.

line exhibited a slightly elevated mEJP frequency relative to these lines (2.5 ± 0.3 Hz, mean ± SEM, n = 8) that was not statistically significant (p > 0.05, one-way ANOVA; Fig. 2.5a,b). The *syt^{null} + P[syt^{wt}]* line exhibited a slightly elevated mEJP frequency relative to these lines (2.5 ± 0.3 Hz, mean ± SEM, n = 8) that was not statistically significant (p > 0.05, one-way ANOVA, Bonferroni's; Fig. 2.5a,b). Both the distribution of quantal sizes (mEJP amplitude) and mean quantal size

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did not differ among these three lines (Fig. 2.5c). The nearly complete rescue of mEJP frequency by the *elav-Gal4/UAS-syt^{wt}* construct suggests that spontaneous transmitter release is less affected by synaptotagmin expression level than is evoked transmitter release.

Together with the bright synaptotagmin immunolabeling seen at larval neuromuscular junctions (Fig. 2.3), these results constitute a significant improvement in the level of rescue of *syt^{null}* mutants achieved by the Gal4/UAS expression system. The significant reduction in EJP amplitude in the *syt^{null}* + *P[syt^{wt}]* transgenic line, however, demonstrates the necessity of using this line as a control for transformed lines containing mutated *syt* P-elements. Therefore, our *elav-Gal4/UAS-syt^{KQ}* transgenic lines were only compared to *elav-Gal4/UAS-syt^{wt}* transgenic lines to avoid overestimating the deficits caused by the mutations.

Disruption of the C_2B Polylysine Motif Impairs Synaptotagmin Function

A series of lysine residues within the C_2B domain of synaptotagmin (Fig. 2.2, heavy box) have been shown to be involved in numerous binding interactions (see above). Mutation of all three lysines to glutamines ($K_{327,328,332}Q$ - mouse *syt II*) blocks IHPS binding *in vitro* (Fukuda et al., 1995a); mutation of residues homologous to the first two of these lysines to alanines ($K_{326,327}A$ - rat *syt I*) blocks the binding of AP-2 and Ca^{2+} channels as well as oligomerization (Chapman et al., 1998). In an effort to determine whether the biochemical interactions mediated by these lysine residues *in vitro* are important for synaptic transmission *in vivo*, we generated mutations in Drosophila *syt* cDNA

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($K_{379,380,384}Q$) homologous to the $K_{327,328,332}Q$ mutation in mouse *syt II* and examined the physiological consequences.

The syt^{KQ} construct was expressed in Drosophila nervous system using the same *elav-Gal4/UAS-syt* transgenic system described above for expression of the wild type transgene. The $P[syt^{KQ}]$ transgene partially rescues the lethality of syt^{null} mutants; 35 ± 7 to 65 ± 6% ($P[syt^{KQ59}]$ and $P[syt^{KQ12}]$ respectively, mean ± SEM) of expected progeny survive to adulthood. Therefore these mutant lines do provide some synaptotagmin function. However, the $P[syt^{KQ}]$ transgenic lines were easily distinguished from $P[syt^{wt}]$ transgenic lines due to distinctly uncoordinated behavior. Mutant larvae crawled sluggishly and moved their mouth hooks at a slower frequency. Mutant adult flies exhibited slow, uncoordinated and arrhythmic limb placement while climbing the walls of the vial. Similar results were obtained for two independent polylysine mutant lines, $syt^{null} + P[syt^{KQ12}]$ and $syt^{null} + P[syt^{KQ59}]$. Thus, these $P[syt^{KQ}]$ transgenes provided less rescue of the syt^{null} uncoordinated behavioral phenotype than the $P[syt^{wt}]$ transgenic line.

Prior to an analysis of synaptic function, synaptotagmin expression by the $syt^{null} + P[syt^{KQ}]$ lines was compared to the $syt^{null} + P[syt^{wt}]$ line at the synapse under investigation, neuromuscular junctions on larval muscle fiber number six. Control and experimental third instar larvae were stained with an anti-synaptotagmin antibody, which was then visualized using a fluoresceinated secondary antibody. Bright labeling of neuromuscular junctions was seen in the $syt^{null} + P[syt^{wt}]$, $syt^{null} + P[syt^{KQ12}]$ and $syt^{null} + P[syt^{KQ59}]$ transgenic lines (Fig. 2.3). These results demonstrate robust expression of transgenic synaptotagmin at

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the synapse under investigation in the syt^{null} + $P[syt^{wt}]$ and syt^{null} + $P[syt^{KQ}]$ mutant lines.

Synaptic function of each mutant line was first assessed by comparing evoked transmitter release at larval neuromuscular junctions to that of the syt^{null} + $P[syt^{wt}]$ line (Fig. 2.6). As reported above, the mean EJP amplitude of

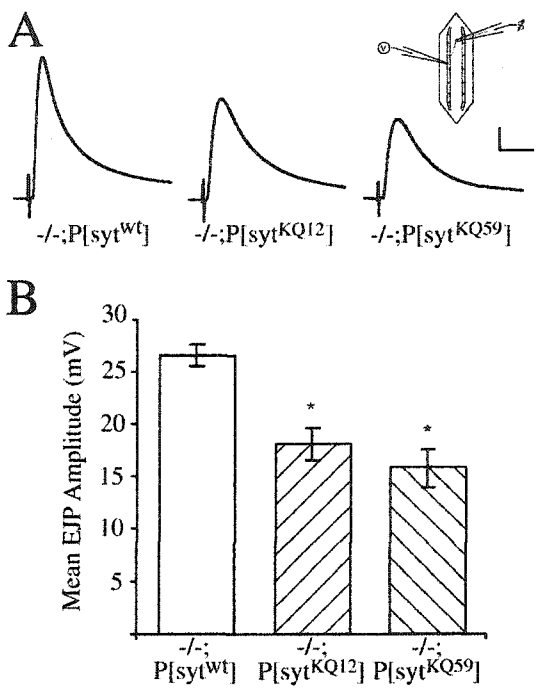

Figure 2.6 EJP amplitude is reduced in C_2B polylysine mutants. Data for the transgenic control, syt^{null} + $P[syt^{wt}]$, is repeated from figure four for comparison. **A:** Representative EJP traces recorded from larval muscle fiber number six of syt^{null} + $P[syt^{wt}]$, syt^{null} + $P[syt^{KQ12}]$, and syt^{null} + $P[syt^{KQ59}]$. Each trace represents the average of 30 consecutive sweeps from the same muscle fiber. The inset schematic displays the orientation of recording and stimulating electrodes on a third instar larval fillet preparation. **B:** Compared to syt^{null} + $P[syt^{wt}]$, mean EJP amplitude was significantly smaller in the syt^{null} + $P[syt^{KQ}]$ mutants ($p < 0.001$, one-way ANOVA, Bonferroni's). Scale: 5 mV, 10 msec.

transgenic syt^{wt} larvae averaged 26.5 ± 1.0 mV (mean ± SEM, n = 9 fibers, Figs. 2.4b and 2.6b). The mean EJP amplitude of syt^{null} + $P[syt^{KQ12}]$ was 18.1 ± 1.6 mV (mean ± SEM, n = 9), which did not differ significantly from that of the other mutant, syt^{null} + $P[syt^{KQ59}]$, which averaged 15.8 ± 1.8 mV (mean ± SEM, n = 9, p

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> 0.05, one-way ANOVA; Fig. 2.6b). In contrast, the mutant lines exhibited a significant reduction (~32-40%) in evoked transmitter release when compared to syt^{null} + $P[syt^{wt}]$ ($p < 0.001$, one-way ANOVA, Bonferroni's; Fig. 2.6). These results suggest that mutation of synaptotagmin's C_2B polylysine motif causes a decrease in evoked transmitter release.

To determine whether the lysine residues are involved in the regulation of spontaneous transmitter release by synaptotagmin, we measured the amplitude and frequency of mEJP events on larval muscle fiber number six in $P[syt^{KQ}]$ and $P[syt^{wt}]$ transgenic lines. Mean mEJP amplitudes for both mutant lines were not statistically different from the transgenic wild type control (Fig. 2.7, legend). In addition, the frequency distribution curves for quantal sizes from all three transgenic lines were qualitatively the same (Fig. 2.7c). However, mean mEJP frequency of both syt^{null} + $P[syt^{KQ}]$ mutant lines (Fig.2. 7a,b; syt^{null} + $P[syt^{KQ12}]$, 5.1 ± 0.6 Hz, mean ± SEM, n = 8; syt^{null} + $P[syt^{KQ59}]$, 4.6 ± 0.4 Hz, mean ± SEM, n = 6) was approximately twice that of the syt^{null} + $P[syt^{wt}]$ control line (Figs. 2.5a,b and 2.7a,b; 2.5 ± 0.3 Hz, mean ± SEM, n = 8; $p < 0.001$ and $p < 0.05$ respectively, one-way ANOVA, Bonferroni's). Mean mEJP frequencies of the mutant lines were not significantly different from one another ($p > 0.05$, one-way ANOVA). Thus, mutation of the C_2B polylysine motif results in an increased rate of spontaneous transmitter release.

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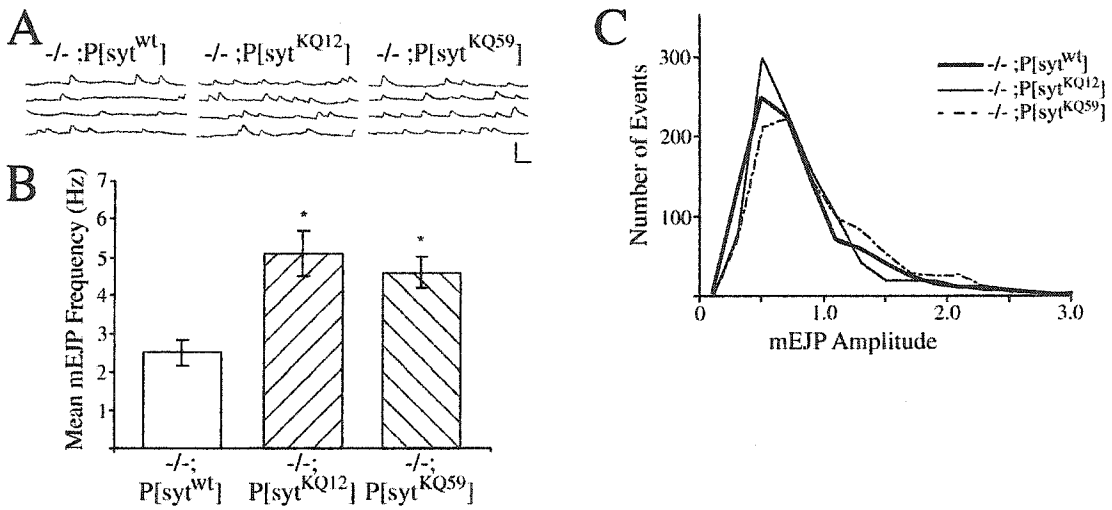

Figure 2.7 Miniature EJP frequency is elevated in C_2B polylysine mutants. Data for the transgenic control, syt^{null} + $P[syt^{wt}]$, is repeated from figure five for comparison. **A:** Representative recordings of spontaneous mEJPs from larval muscle fiber six of syt^{null} + $P[syt^{wt}]$, syt^{null} + $P[syt^{KQ12}]$, and syt^{null} + $P[syt^{KQ59}]$. Each set of traces represents 4 seconds of continuous recording. Miniature EJPs are readily recognizable by their slope. **B:** Calculated mean mEJP frequencies of each mutant differed from the control ($p < 0.001$, one-way ANOVA, Bonferroni's), but lines 12 and 59 were not different from one another ($p > 0.05$, one-way ANOVA). **C:** Frequency distribution curves of mEJP amplitudes calculated as in figure five. The distribution of mEJP amplitudes (quantal sizes) was similar among all three transgenic lines. Accordingly, mean mEJP amplitude of the syt^{null} + $P[syt^{KQ}]$ transgenic lines were not significantly different from control or one another (-/-;P[syt^{wt}], 0.87 ± 0.07 mV, mean ± SEM; -/-;P[syt^{KQ12}], 0.81 ± 0.04 mV, mean ± SEM; -/-;P[syt^{KQ59}], 1.00 ± 0.06 mV, mean ± SEM; $p > 0.05$ one-way ANOVA, Bonferroni's). Scale: 4 mV, 100 msec.

Impaired Synaptic Transmission in the $syt^{K379,380,384Q}$ Transgenic Lines is Due to the Mutation, not Gene Dosage

Similar, robust staining at larval neuromuscular junctions (Fig. 2.3) suggests that these *syt* transgenes are expressed at similar levels, however, this labeling procedure is not quantitative. Since the physiological phenotype of the C_2B polylysine motif mutants is similar to, albeit less severe than, that of the syt^{null} phenotype, one possibility is that the *apparent* phenotype of the mutant

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may be simply due to a gene dosage effect; the mutant *syt* transgene may produce less total synaptotagmin protein. To obtain a more quantitative estimate of relative synaptotagmin expression levels in the control and mutant transgenic lines, Western blots of Drosophila heads (Fig. 2.8a) and larval CNS (Fig. 2.8b) were probed with anti-synaptotagmin antibodies. Approximately equal loading of total protein for the syt^{null} + $P[syt^{wt}]$, the syt^{null} + $P[syt^{KQ59}]$, and the syt^{null} + $P[syt^{KQ12}]$ transgenic lines (Fig. 2.8, lanes 1-3 respectively) was verified by actin

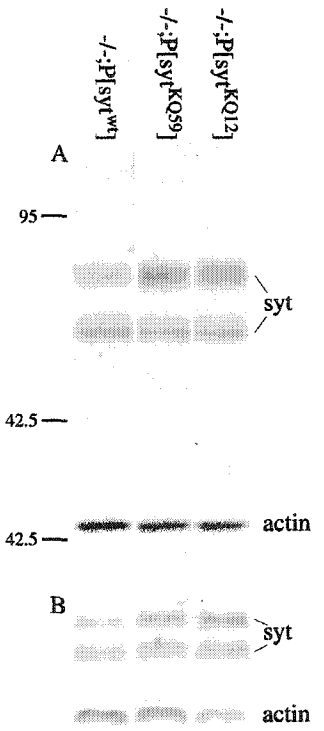

Figure 2.8 Wild type and polylysine mutant *syt* transgenes express similar amounts of synaptotagmin. **A:** Western blots of homogenized fly heads from the indicated lines were probed with an anti-synaptotagmin antibody, Dsyt-2. To confirm equal protein loading, the same blot was stripped and reprobed with an anti-actin antibody, MAB 1501. **B:** Western blots of homogenized third instar larval CNS were probed with the same antibodies.

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staining. Comparison of the mutant and wild type transgenic lines consistently revealed slightly higher synaptotagmin expression in the mutant lines compared to the wild type transgenic line at both developmental stages examined. Thus the decrease in evoked transmitter release and the increase in spontaneous release frequency seen in the *syt* C_2B polylysine motif mutants is specific for this mutation.

Discussion

We have demonstrated that the *UAS/Gal4* system amplifies the expression from a wild type *syt* transgene (in otherwise syt^{null} larvae) such that >60% of synaptotagmin function is rescued. This dramatic increase in the level of synaptotagmin expression from a transgene is now sufficient for structure/function analyses to test whether specific, site-directed mutations within synaptotagmin are important for function. Accordingly, we have used this system to demonstrate *in vivo* that an individual amino acid motif within synaptotagmin, the C_2B polylysine motif, is indeed required to achieve complete synaptotagmin function during synaptic transmission.

Several amino acid motifs within synaptotagmin are hypothesized to be critical for function based on their interactions with various nerve terminal molecules *in vitro*. However, the physiological relevance of these biochemical interactions is unclear (Fukuda and Mikoshiba, 1997; Burgoyne and Morgan, 1998), since none of these motifs have previously been tested *in vivo*. Analysis of syt^{KQ} mutants revealed that evoked neurotransmitter release is reduced by 32-40% while spontaneous release is increased 85-105% over wild type transgenic controls. Since the Gal4/UAS promoter system utilized in these studies provides

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approximately equivalent expression levels from both wild type and mutant synaptotagmin transgenes, the phenotypic differences between these lines must be due to the mutation, not gene dosage. The phenotype of the syt^{KQ} mutants is similar to, albeit much less severe than, the syt^{null} phenotype (Broadie et al., 1994), demonstrating that the polylysine mutation results in a partial loss of synaptotagmin function.

Where in the vesicle cycle might this loss of function occur? Synaptotagmin has been postulated to mediate several stages of the synaptic vesicle cycle, including: 1) the Ca^{2+} sensing required to trigger vesicle fusion (Brose et al., 1992; Geppert et al., 1994; Sudhof, 1995), 2) synaptic vesicle recycling (Zhang et al., 1994; Fukuda et al., 1995b; Jorgensen et al., 1995; Reist et al., 1998), and finally, 3) synaptic vesicle docking at release sites (Bennett et al., 1992; Leveque et al., 1992; Popov and Poo, 1993; Sollner et al., 1993b; Schiavo et al., 1995; Reist et al., 1998). Much support for each of these hypotheses comes from various biochemical experiments, some of which involve the lysine residues noted in Figure 2.2. While the present study demonstrates that the C_2B polylysine motif is important for full synaptotagmin function, it is difficult to discern whether any, some, or all of the *in vitro* interactions mediated by this motif contribute to the physiological deficits we see in the mutants. Each of the hypothesized functions and each of the *in vitro* interactions involving the C_2B polylysine motif are discussed below.

Synaptotagmin was first hypothesized to mediate a Ca^{2+}-sensing function based on the Ca^{2+}-dependent interaction between the C_2A domain of synaptotagmin with phospholipids and syntaxin (Perin et al., 1990; Bennett et al.,

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1992; Brose et al., 1992; Chapman and Jahn, 1994). Specifically, the Ca^{2+}-binding pocket of C_2A has been proposed to fulfill this function (Shao et al., 1996; Ubach et al., 1998). Since the C_2B polylysine motif is not located near either of synaptotagmin's Ca^{2+}-binding domains, a role for the polylysine mutants in Ca^{2+}-sensing was not pursued in this study.

Synaptotagmin was postulated to be important for synaptic vesicle recycling based on *in vitro* interactions between synaptotagmin's C_2B domain and the clathrin adapter protein, AP-2 (Zhang et al., 1994). The finding that tyrosine-based endocytic motifs, commonly present in endocytic cargo proteins (e.g., SV2), stimulate the interaction between synaptotagmin and AP-2 (Haucke and De Camilli, 1999) further supports an endocytic role for synaptotagmin. Consistent with this hypothesis, nerve terminals in syt^{null} mutants of Drosophila (Reist et al., 1998) and *C. elegans* (Jorgensen et al., 1995) exhibit depletion of synaptic vesicles. In addition, Drosophila null mutant terminals also exhibit accumulations of larger membranous structures that could represent recycling intermediates or failed attempts at vesicle biogenesis (Reist et al., 1998). *In vitro* binding of synaptotagmin to AP-2 requires two of the three lysines mutated in this study; mutation of K1 and K2 of rat *syt I* (Fig. 2.2) to alanines prevents AP-2 binding to synaptotagmin (Chapman et al., 1998). Thus, the syt^{KQ} mutation could cause a recycling deficit by disrupting synaptotagmin's interaction with AP-2. If this interaction were important during endocytosis, a resulting vesicle depletion could contribute to the observed decrease in evoked transmitter release. Experiments examining the ability to maintain evoked responses during high frequency stimulation may indicate whether such vesicle depletion is

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occurring in these mutants. An increased rate of fatigue would prompt detailed morphometric analysis of synaptic ultrastructure in the syt^{KQ} mutants to answer this question. However, such a deficit cannot explain the increase in spontaneous transmitter release observed in the syt^{KQ} mutants.

Biochemical interactions between synaptotagmin, syntaxin, and Ca^{2+}-channels led to the hypothesis that these proteins are involved in docking synaptic vesicles near the site of Ca^{2+} influx at active zones (Bennett et al., 1992; Leveque et al., 1992; Sollner et al., 1993b). Bommert et al. (Bommert et al., 1993) provided physiological support for synaptotagmin's involvement in synaptic transmission by demonstrating that injection of a 20 amino acid synaptotagmin peptide, which included the polylysine region of C_2B (Fig. 2.2, dashed line), into squid nerve terminals reduced synaptic transmission. Peptide injection experiments, however, cannot rule out non-specific interactions, such as interference with the function of one or more of the other presynaptic proteins that contain C_2-domains. Strong support for the docking hypothesis was provided by morphometric analyses of active zone ultrastructure in *syt* mutants of Drosophila (Reist et al., 1998). These studies established that the number of morphologically docked vesicles at both central synapses and neuromuscular junctions were severely depleted in Drosophila *syt* mutants. Genetic manipulations further demonstrated that the decreases in synaptic vesicle docking and evoked transmitter release persisted even in the absence of the overall vesicle depletion and elevated spontaneous release usually seen in *syt* mutants. Thus synaptotagmin likely acts to stabilize the docked state of vesicles in Drosophila [see, however,[Geppert, 1994 #138]].

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The polylysine motif of C_2B could contribute to this docking function. Peptides that include this region not only blocked synaptic transmission, as mentioned above, but also increased the number of morphologically docked vesicles (Bommert et al., 1993). This result might suggest that the peptide interfered directly with vesicle fusion resulting in an accumulation of docked vesicles mediated by other molecular interactions. Alternatively, since synaptic vesicles in these squid terminals contain native synaptotagmin, the injected peptide may block synaptic transmission by preventing synaptotagmin interactions that are required to *release* vesicle docking - in other words, vesicles become "hyperdocked."

One possible mechanism whereby synaptotagmin may stabilize vesicle docking is through its interactions with the core fusion complex (Fig. 2.1) (Sollner et al., 1993b; Sutton et al., 1999; Gerona et al., 2000; Leveque et al., 2000). Sutton et al. (Sutton et al., 1999) have suggested that the cup-shaped polybasic regions of C_2A and C_2B, which include the polylysine motifs of each C_2 domain, may mediate an electrostatic interaction with the negatively-charged portion of the cylindrical, core fusion complex. Thus, in resting terminals, synaptotagmin may stabilize docking and prevent fusion by holding the core fusion complex in an intermediate state prior to fusion (Fig. 2.1, left). Ca^{2+}-influx could then alter the interaction between synaptotagmin and the core fusion complex (Gerona et al., 2000; Leveque et al., 2000), stimulating complete formation of the coiled-coil structure (Fig. 2.1, right) postulated to mediate fusion of the vesicle with the presynaptic membrane. According to this scenario, the syt^{KQ} mutation could cause a partial disruption of the interaction between synaptotagmin and the core

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fusion complex due to the removal of 3 basic residues in this cup-shaped, polybasic region of C_2B (see Fig. 2.2c). By disrupting this interaction, docking may be destabilized and more spontaneous fusion events could occur thereby contributing to a reduction of docked vesicles. Fewer docked vesicles available for fusion could then result in decreased evoked transmitter release. We favor this proposed mechanism of action because it can account for both the increased frequency of spontaneous release and the decreased amplitude of evoked release seen in the C_2B polylysine mutants. Morphometric analysis of synaptic ultrastructure in these mutants will be required to assess whether this motif is involved in docking.

An alternate mechanism that could contribute to vesicle docking is the interaction between synaptotagmin, in the vesicle membrane, and neuronal Ca^{2+} channels, in the presynaptic membrane. K1 and K2 (Fig. 2.2) of the C_2B polylysine motif of rat synaptotagmin interact with the second I-II intracellular loop (the synprint site) of neuronal Ca^{2+} channels (Charvin et al., 1997; Sheng et al., 1997; Chapman et al., 1998). While this interaction may play a role in vesicle docking in mammals, disruption of such an interaction is unlikely to contribute to the deficits in Drosophila syt^{KQ} mutants, since Drosophila Ca^{2+} channels do not contain the synprint motif (Littleton and Ganetzky, 2000).

Additional biochemical interactions involving these three lysine residues include the synaptotagmin-IHPS interaction and synaptotagmin oligomerization. Each of these interactions, which could be involved in any part of the vesicle cycle, will be considered below. When IHPS molecules are injected into the squid giant axon (Llinas et al., 1994) or adrenal chromaffin cells (Ohara-Imaizumi

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et al., 1997), they block spontaneous and evoked transmitter release. IHPS has been shown to bind to mouse synaptotagmin II via the three lysine residues highlighted in Figure 2.2 (Fukuda et al., 1995b). Antibodies directed against the C_2B domain of synaptotagmin block both IHPS binding to synaptotagmin and the inhibitory action of IHPS in nerve terminals (Fukuda et al., 1995b). Thus the inhibitory action of IHPS is likely to be mediated by IHPS binding to these three lysine residues. By a mutational analysis of different isoforms of mouse synaptotagmin that bind the IHPS to different degrees, Ibata et al. (Ibata et al., 1998) have identified a permissive region in the C-terminal portion of C_2B that is important for IHPS binding. All three lysine residues required for IHPS binding are conserved in Drosophila synaptotagmin I (Fig. 2.2) and key residues of the permissive region (Fig. 2.2a, *s above sequence) are also conserved. Thus, IHPS is likely to bind to this site of Drosophila synaptotagmin I.

IHPS binding to the polylysine motif of C_2B may inhibit synaptic transmission by preventing presynaptic proteins (such as AP-2 or other synaptotagmin molecules) from binding to these same lysine residues. Indeed, IHPS has been shown to interfere with AP-2 binding by synaptotagmin (Mizutani et al., 1997). Thus, the absence of this multi-functional binding site would result in decreased synaptotagmin function. Our finding that the syt^{KQ} mutation results in a partial loss of synaptotagmin function supports this hypothesis.

The C_2B domain mediates Ca^{2+}-dependent oligomerization of synaptotagmin (Chapman et al., 1996; Damer and Creutz, 1996; Sugita et al., 1996) via the C_2B polylysine motif; mutation of 2 of these lysine residues (K1 and

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K2, Fig. 2.2) to alanines abolished synaptotagmin I self-association *in vitro* (Chapman et al., 1998). Recent evidence indicates that multiple isoforms of synaptotagmin co-localize to the same population of synaptic vesicles and are capable of forming hetero-oligomers at Ca^{2+} levels approximating that which triggers exocytosis (Osborne et al., 1999). Taken together, these data suggest that oligomerization may occur *in vivo* as well (Littleton et al., 1999; Osborne et al., 1999). Multiple isoforms of synaptotagmin have recently been identified in Drosophila and Drosophila synaptotagmin I and synaptotagmin IV have been shown to form hetero- and homo-oligomers *in vitro* (Littleton et al., 1999). Since overexpression of synaptotagmin IV in otherwise wild type flies resulted in decreased evoked transmitter release, Littleton et al. (Littleton et al., 1999) proposed that adjusting the ratio of different isoforms of synaptotagmin may provide a key mechanism for regulating the efficacy of transmitter release. Indeed, since our syt^{null} + $P[syt^{wt}]$ transgenic control line expresses less synaptotagmin I than wild type flies (and thus also decreases the *syt* I/*syt* IV ratio), the decrease in evoked transmitter release we observed would be consistent with this hypothesis. However, synaptotagmin IV overexpression also resulted in decreased mEJP frequency while in our syt^{null} + $P[syt^{wt}]$ transgenic line mEJP frequency remained unaltered or was slightly increased.

In summary, the polylysine motif of C_2B has been implicated in specific synaptotagmin functions based on numerous biochemical interactions with other presynaptic molecules. While the current study does not differentiate between several possible roles within the vesicle cycle for this motif, we have demonstrated conclusively that the C_2B polylysine motif is required to achieve

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full synaptotagmin function. In the absence of these lysine residues, evoked transmitter release is reduced by ~36% while spontaneous release frequency is increased by approximately twofold. Future morphometric analyses of presynaptic ultrastructure detailing the overall number and distribution of synaptic vesicles may be able to distinguish whether synaptotagmin's C_2B polylysine motif is specifically involved in vesicle recycling, vesicle docking or both.

Additional unpublished discussion

Since the work above was published in 2001, there have been many twists and turns in the story of synaptotagmin function that have since influenced my interpretation of this paper. I will briefly discuss two major updated findings below. First, accumulating evidence suggests that mini frequency or rate of spontaneous vesicle fusions is probably not diagnostic of synaptotagmin function *in vivo*. Second, bacterial contaminants, reportedly nucleic acids, that adhere to the recombinant C_2B domain have obfuscated many previously reported biochemical interactions of synaptotagmin, notably the Ca^{2+} dependent oligomerization interaction mediated by the polylysine motif.

The most puzzling result from this chapter is the increased mini frequency in K-Q mutants. Every synaptotagmin mutant in Drosophila that has been examined at *the third instar stage* to date (including the Ca^{2+} binding site mutants discussed in Chapter 3) has displayed an increased mini frequency (Fig. 2.7). Indeed, simply decreasing expression of synaptotagmin also increases mini frequency (Fig. 2.5). syt^{null} and C_2B Ca^{2+} binding site mutant larvae have an incredibly high mini frequency (see Chapter 3 and (Loewen et al., 2001)). In

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contrast, syt^{null} mutants examined as early embryos (Yoshihara and Littleton, 2002) exhibited no change in the frequency of spontaneous release. Likewise, spontaneous release was not altered when two mutations were made in mouse synaptotagmin, both a knockout (Geppert et al., 1994) and a site directed "knock-in" (Fernandez-Chacon et al., 2001). In both mouse mutants, cultured hippocampal neurons were used to assay synaptic transmission since *syt I* is expressed in forebrain, not at the neuromuscular junction.

Until recently, our lab and others have tried to rationalize the decrease in evoked release and increase in mini frequency with some function of synaptotagmin whereby it acts as both a positive and negative regulator of release probability. Increasingly, however, it appears that the elevation in mini frequency in some synaptotagmin mutants may be due to the preparation itself. In developing synapses from either embryonic Drosophila or perinatal mice, spontaneous release is not elevated, perhaps as a function of either a low number of fusible vesicles or fewer active zones. A low evoked release probability in the absence of synaptotagmin does not appear to affect the ability of the neuron to make or maintain contact with the post-synaptic cell. In contrast, it is possible that the increased mini frequency in the third instar larval preparation is a compensatory reaction by the neuron to maintain contact with the muscle when evoked release is compromised. Alternatively, a postsynaptic cue from inactive muscles could provoke a compensatory reaction from the neuron to increase spontaneous release. In fact, spontaneous release in Drosophila has been shown to correlate with clustering of post-synaptic glutamate receptors (Saitoe et al., 2001). Thus, increased mini frequency may simply be a compensatory

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mechanism to keep the synapse intact. Increased production of cAMP is sufficient to elevate mini frequency at the Drosophila NMJ (Yoshihara et al., 1999; Suzuki et al., 2002) and is a mechanism worthy of further investigation.

In 2001, Ubach, et al. (Ubach et al., 2001) published a paper describing bacterial contaminants adhered to recombinant synaptotagmin C_2B domains when they were purified using standard protocols. This group found that when they washed the glutathione beads bound to fusion proteins with high salt concentrations, cleaved the cytoplasmic domains of synaptotagmin from the beads and further purified the protein with FPLC, the protein was no longer capable of Ca^{2+} dependent oligomerization in the standard GST pull-down assay. This radical finding strongly suggested that the interaction that many groups have reported and that we have tried to incorporate into a model for synaptotagmin function, may be nothing more than an artifact.

The oligomerization story doesn't end there, however. A very recent publication confirmed that "cleaning up" the recombinant synaptotagmin abolished Ca^{2+} dependent oligomerization in the standard assay (Wu et al., 2003). Remarkably, they were able to demonstrate oligomerization of recombinant synaptotagmin cytoplasmic domains in the presence of Ca^{2+} and a lipid (25 % phosphatidylserine/75 % phosphatidylcholine) monolayer. They used high resolution EM to perform single particle analysis and described 11 nm X 11 nm heptameric barrel structures that correlated with a reconstruction of seven synaptotagmin particles bound together in a barrel-shaped structure. Mutation of $K_{326,327}A$ in rat *syt I* (Fig 2.2) prevented formation of these structures. Thus, under some circumstances, oligomerization of synaptotagmin

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still occurs after all. It remains to be demonstrated, however, that these structures form *in vivo* or if there is even sufficient space between vesicle and presynaptic membrane at the active zone. It is tempting to speculate that a regular arrangement of synaptotagmin molecules that occurs following contact with phospholipids may strategically place the t- and v-SNAREs in a position wherein fusion is imminent. Perhaps mutation of lysine residues that facilitate the regular arrangement of synaptotagmin reduce the efficiency of synaptic vesicle exocytosis.

Indeed, it has been difficult to imagine a mechanism where Ca^{2+} dependent oligomerization of synaptotagmin molecules may function in the vesicle cycle. Previously, it was suggested that oligomerization of synaptotagmin could form a fusion pore (Desai et al., 2000; Wang et al., 2001; Tucker and Chapman, 2002), similar to that described for viral fusion (reviewed in (Jahn and Sudhof, 1999; Bentz and Mittal, 2000)). If the polylysine motif does regulated oligomerization *in vivo*, perhaps the best test of the hypothesis that oligomerization directly promotes membrane fusion is the data presented in this Chapter. Here, I mutated the homologous amino acids in Drosophila that are required for oligomerization by rat *syt I* and found only a ~40 % decrease in evoked release. Certainly, if the major mechanism of vesicle fusion were through synaptotagmin pore formation, there would have been a more significant defect in synaptic transmission.

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Chapter 3: Ca^{2+}-binding aspartate residues in C_2B are required for synaptic transmission

Abstract

Synaptotagmin is a synaptic vesicle protein that is postulated to be the Ca^{2+} sensor for fast, evoked neurotransmitter release (Augustine, 2001). Genetic knock out of synaptotagmin strongly suppresses synaptic transmission in every species examined (Lin and Scheller, 2000), demonstrating that synaptotagmin plays a central role in the synaptic vesicle cycle. The cytoplasmic region of synaptotagmin contains two C_2 domains, C_2A and C_2B. Five, highly-conserved, acidic residues in both the C_2A and C_2B domains of synaptotagmin coordinate the binding of Ca^{2+} ions (Shao et al., 1996; Sutton et al., 1999; Fernandez et al., 2001) and biochemical studies have characterized several *in vitro* Ca^{2+}-dependent interactions between synaptotagmin and other nerve terminal molecules (Rizo and Südhof, 1998). There has been no direct evidence, however, that any of the Ca^{2+}-binding sites within synaptotagmin are required *in vivo*. Here, we show that mutating two of the Ca^{2+}-binding aspartate residues in the C_2B domain ($D_{416,418}N$ in Drosophila) decreased evoked transmitter release by > 95% and decreased the apparent Ca^{2+} affinity of evoked transmitter release. These studies demonstrate that synaptotagmin's C_2B Ca^{2+}-binding motif is essential for synaptic transmission.

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Introduction

The C_2B domain has been shown to be critical for synaptotagmin function *in vivo* (Bommert et al., 1993; Fukuda et al., 1995b; Desai et al., 2000; Littleton et al., 2001; Mackler and Reist, 2001), yet most of the Ca^{2+}-dependent, biochemical interactions of synaptotagmin were originally mapped to the C_2A domain (Li et al., 1995b). Thus, the importance of Ca^{2+} binding by the C_2B domain has been largely neglected. The recent finding that the C_2B domain mediates Ca^{2+} dependent interactions with phospholipids (Fernandez et al., 2001; Bai et al., 2002) has heightened interest in Ca^{2+} binding by C_2B. However, the effects of mutations of the acidic residues that coordinate Ca^{2+} binding in the C_2B domain have never been examined at intact synapses. We mutated the C_2B aspartate residues to assess the importance of the C_2B Ca^{2+}-binding motif. Specifically, we mutated the third and fourth aspartate residues that coordinate Ca^{2+}-binding in the C_2B domain of Drosophila synaptotagmin to asparagines ($D_{416,418}N$, referred to henceforth as B-D3,4N, Fig. 3.1) and expressed the mutant protein in a syt^{null} line of Drosophila. Thus, the only source of synaptotagmin I in these lines (*P[sytB-D3,4N]*) was from the mutant transgene. As a negative control, we used the syt^{null} line alone, and as a positive control, we used the same syt^{null} line expressing synaptotagmin from a transgene bearing no mutations (Mackler and Reist, 2001) (*P[*syt^{wt}*]*).

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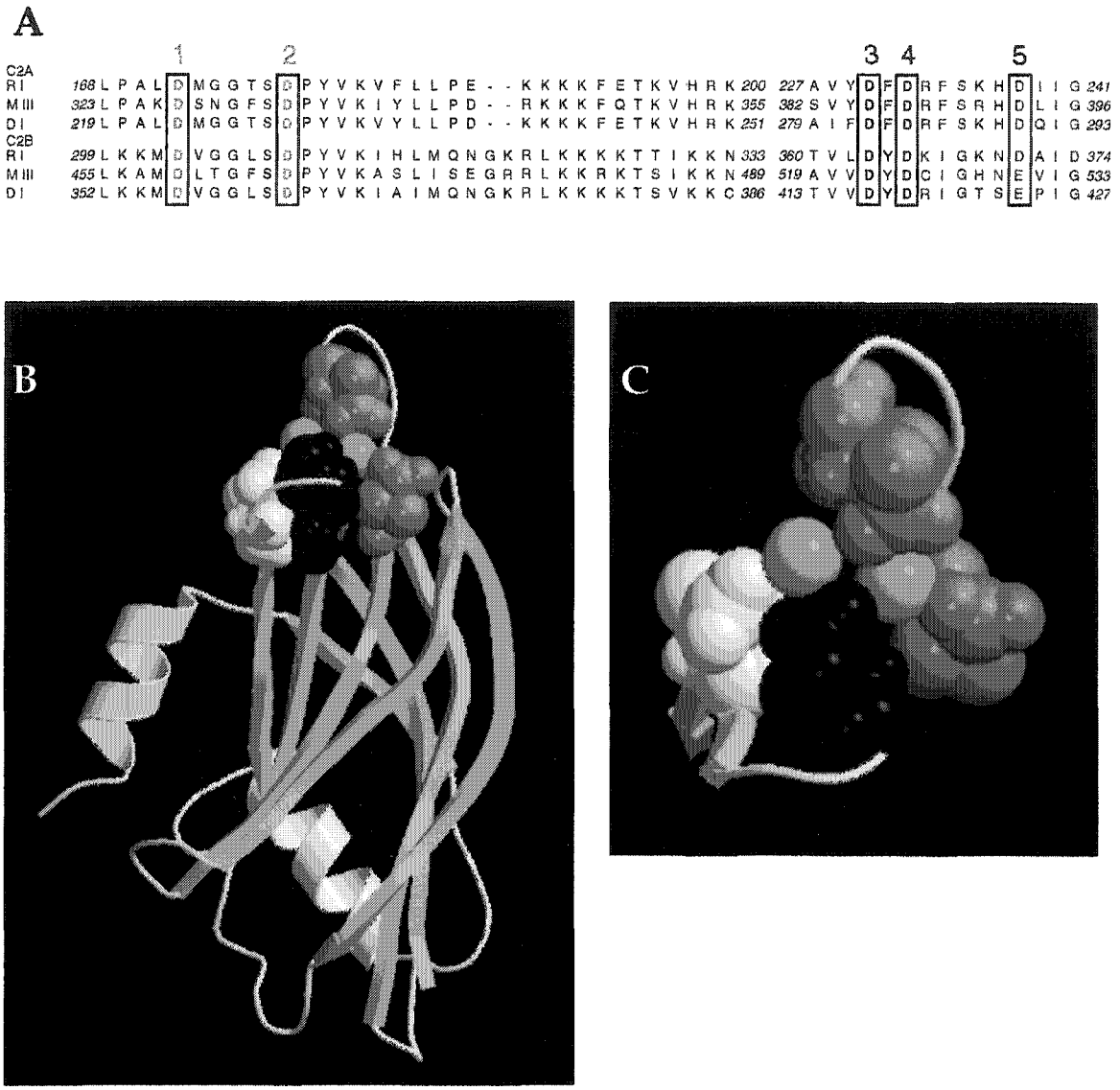

Figure 3.1 Ca^{2+}-binding aspartate residues are conserved among synaptotagmin isoforms. **A:** ClustalW alignment of the partial amino acid sequence of C_2A and C_2B domains of rat *syt I* (R I), mouse *syt III* (M III), and Drosophila *syt I* (D I). The five conserved acidic residues that coordinate the binding of multiple Ca^{2+} ions are boxed and color coded. **B:** Three-dimensional structure of rat *syt I* adapted from Fernandez et al. (2001) illustrating the Ca^{2+} binding aspartate residues (spacefilled); Ca^{2+} ions are green. D1 and D2 are orange, D3 and D4 are red, D/E5 is white. Molecular model generated from PDB file 1K5W using Molscript (Kraulis, 1991) and Raster3D (Merritt and Bacon, 1997) software. **C:** Close up view of **B** looking down on the Ca^{2+} binding pocket.

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Methods

Site directed mutagenesis and fly strains

Mutant Drosophila *syt* I cDNA was generated by PCR and used to transform Drosophila. Either C_2B aspartate residues 416 and 418 (Fig. 3.1, residues labeled 3 and 4) or 356 and 362 (Fig. 3.1, residues labeled 1 and 2) were mutated to asparagines. Mutant, antisense oligonucleotides were used with Drosophila *syt* I cDNA to generate the $D_{416,418}N$ (B-D3,4N) and $D_{356,362}N$ (B-D1,2N) mutations by PCR (tgcagcggccgatgggttcggaggtgccaatacgatTgtagtTcac gacggtcacaacg and gattgcaactttcacatatggatTagacagtccgcccacgtTcatcttcttcaag ttcttggcc, respectively). These mutant cDNAs were subsequently used to make the polypeptides for the binding experiments.

Drosophila were transformed as previously described (Mackler and Reist, 2001). syt^{AD4} is a null mutation in the Drosophila *syt* I gene (DiAntonio et al., 1993). The syt^{null}, negative control line used was *yw;*syt^{AD4}*/*syt^{AD4} *P[elavGal4].* To maximize neuronal expression of *syt* transgenes in the syt^{null} mutant line, we used the Gal4/UAS amplification system in combination with the pan neuronal *elav* promoter (Robinow and White, 1988; Brand and Perrimon, 1993; Mackler and Reist, 2001). The genotype of the transgenic lines used were: P[syt^{wt}] = *yw;* syt^{AD4}*/*syt^{AD4} *P[elavGal4]; P[UAS*syt^{wt}*]/+*, P[sytB-D3,4N] = *yw;* syt^{AD4}*/*syt^{AD4} *P[elavGal4]; P[UASsytB-D3,4N]/+*, wt with P[B-D3,4N] = *yw;*syt^{AD4} *P[elavGal4]/CyO, y+; P[sytB-D3,4N]/+*, and wt with P[B-D1,2N] = *yw;*syt^{AD4} *P[elavGal4]/CyO, y+; P[sytB-D1,2N]/+*. The *syt* heterozygous control lines that contained the mutant transgene but did not express it lacked the Gal4 required to drive transgene expression (wt = *yw;*syt^{AD4}*/CyO, y+; P[sytB-D3,4N]/+* or

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yw;syt^{AD4}*/CyO, y+; P[sytB-D1,2N]/+* as indicated). Expression levels in the control heterozygotes are similar to that of *syt* heterozygotes that do not contain any transgenes, while expression levels in the transgene expressing lines are similar to *syt* homozygotes (data not shown). Evoked transmitter release is not significantly different in *syt* heterozygotes and homozygotes (Mackler and Reist, 2001).

Electrophysiology

Spontaneous and evoked potentials were recorded from third instar muscle fibers as previously described (Loewen et al., 2001) in standard HL3 saline (Stewart et al., 1994) containing 1.5 mM Ca^{2+} unless otherwise indicated. For Ca^{2+} dose response experiments, preparations were bathed in HL3 containing from 0.4 mM to 5 mM Ca^{2+} (without adjusting Mg^{2+}). EJP values from control fibers recorded in ≥ 0.8 mM Ca^{2+} were corrected for non-linear summation(Stevens, 1976) prior to calculation of mean quantal content (mean EJP amplitude/mean mEJP amplitude). To prevent overestimating nerve-evoked transmitter release in *syt*^{null} and *P[sytB-D3,4N]* larvae, we restricted the measurement interval for EJP amplitudes to a 30 msec interval immediately following the stimulus artifact.

In addition, when fibers exhibited a mean evoked response of ≤ 5 mV, we subtracted an estimate of the spontaneous release after Petersen et al. (Petersen, 1997). We estimated the contribution of spontaneous release that may contaminate the 30 msec measurement interval by calculating the amplitude during an equivalent 30 msec interval, 200 msec after the stimulus artifact; for each fiber, this estimate was then subtracted from the mean EJP amplitude for

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that fiber. We verified that these estimates were not different from similar measurements from unstimulated fibers. Ca^{2+} dose response curves were plotted and fit to the logistic equation ($y = m^1/[1+(m^2/m^0)m^3]$, where m^1 = maximal response, $m^2 = EC_{50}$, $m^0 = [Ca^{2+}]$ and m^3 = cooperativity coefficient) using Kaleidagraph software (Synergy Software, Reading, PA). Statistical comparisons of means were tested with a Satterthwaite 2-sided t-test using SAS software 6.12 (SAS Institute, Cary, NC). Failure rate was calculated as previously described(Del Castillo and Katz, 1954). Differences between failure rates were tested with a z-test calculated manually. Transmitter release was also evoked by focal application of a hypertonic, 2.0 M sucrose solution to fibers in standard HL3 saline.

Immunohistochemistry

Larval whole mounts were dissected, fixed, and stained as previously described (Mackler and Reist, 2001). The anti-synaptotagmin antibody, Dsyt-CL1, was diluted 1:4,000. Images were acquired on an Olympus 1X70 laser-scanning confocal microscope, projected using NIH Image J software, and adjusted for contrast using Adobe Photoshop software.

Western analysis

Central nervous systems from third instar larvae were dissected, solubilized, measured for protein concentration, electrophoresed by SDS-PAGE, and transferred to membranes as previously described (Loewen et al., 2001; Mackler and Reist, 2001). Membranes were probed(Mackler and Reist, 2001) with the following antibodies: an antibody generated against the intravesicular domain of Drosophila synaptotagmin (Dsyt-CL1, diluted 1:10,000), and an anti-

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actin monoclonal antibody (MAB 1501, Chemicon, diluted 1:20,000). Approximately equal protein loading was confirmed by Coomassie brilliant blue staining of the polyacrylamide gel and anti-actin staining of the blot.

Oligomerization

B-D1,2N, B-D3,4N, and wild type polypeptides of synaptotagmin incorporating either amino acids 317-474 which encode the C_2B domain or 191-474 which encode both C_2 domains (C_2A-C_2B) were made by amplifying Drosophila *syt* I cDNA using oligonucleotides GCGACCGAATTCTAGTT GAAGGAGAGGGCG (C_2B) or AGCAGAGAATTCAGAAGCTGGGGCGCC (C_2A-C_2B) with CCGCCGAAGCTTTTACTTCATGTTCTT (both C_2B and C_2A-C_2B), followed by subcloning into pGEX-KG using EcoR I and Hind III. Polypeptides were expressed as GST fusion proteins. Soluble proteins were prepared by thrombin cleavage.

Soluble C_2A-C_2B domains were assayed for binding to immobilized GST-C_2A-C_2B domains according to the procedures of Desai et al. (Desai et al., 2000). Binding was wild type to wild type or mutant to mutant in buffer containing 2.5 mM $CaCl_2$ with or without 5 mM EGTA. Protein levels were quantified on Coomassie blue stained gels by comparison with BSA standards and were normalized to 12% of the total soluble protein used in the assay. There was no binding to GST alone. Binding assays were carried out three times.

Lipid binding

Lipid binding was assayed by the binding of liposomes to GST-C2B fusion proteins (Davletov and Sudhof, 1993). Tritiated liposomes were prepared from 12.5 mg of phosphatidylcholine and 5 mg of phosphatidylserine (Sigma, Poole,

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UK) with 20 μCi of 1,2-dipalmitoyl,L-3-phosphatidyl[N-methyl-3H]choline (Amersham, UK) and suspended in 5 ml buffer A (0.1M NaCl, 50 mM HEPES, pH 7.2) by vigorous shaking followed by sonication. Prior to use liposomes were centrifuged at 5000 rpm for 20 min to remove aggregates. 50 μg of recombinant protein bound to glutathione agarose beads were mixed with 175 μg 3H phospholipids in a final volume of 0.1 ml of the test solutions (Buffer A with additions of EGTA and $CaCl_2$, calculated with MaxChelator, http://www.stanford.edu/~cpatton/maxc.html, to give the desired free Ca^{2+}). The mixture was incubated at room temperature with vigorous shaking for 15 min, then washed 3 times with 1 ml of test solution.

Liposome binding was quantified by scintillation counting. Data points were obtained in triplicate. Data shown is representative of three separate experiments.

Electron Microscopy

Third instar larvae 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 from abdominal segments 3 and 4 were taken at 12,000X magnification on a JEOL 2000 EX-II 200 keV transmission electron microscope. Images were scanned using an Agfa DuoScan T2500 and adjusted for contrast using Adobe Photoshop software.

Results and Discussion

Ca^{2+}-evoked neurotransmitter release in *P[sytB-D3,4N]* mutant larvae was reduced to a level even less than that seen in the syt^{null} negative control (Fig. 3.2a,b). Spontaneous and evoked potentials were recorded from third instar

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muscle fibers as previously described (Loewen et al., 2001), except that evoked values were corrected for contamination by spontaneous release when appropriate (Petersen, 1997). When motor nerves of third instar larvae were stimulated in standard HL3 saline (Stewart et al., 1994) containing 1.5 mM Ca^{2+}, the mean amplitude of evoked excitatory junction potentials (EJPs) in muscles from *P[syt^{wt}]* larvae was 25.0 ± 1.88 mV with no failures (n = 13), while mean EJP amplitude in *P[sytB-D3,4N]* larvae was only 0.21 ± 0.06 mV (n = 11) with a 78% failure rate. By comparison, mean EJP amplitude from *syt^{null}* larvae was 1.70 ± 0.28 mV (n = 10) with a 15% failure rate.

In contrast to the decrease in evoked transmitter release, *P[sytB-D3,4N]* and *syt^{null}* mutant synapses exhibited an elevated rate of spontaneous vesicle fusion events (*P[syt^{wt}]*, 2.9 ± 0.5 Hz; *P[sytB-D3,4N]*, 6.6 ± 1.4 Hz; *syt^{null}*, 6.6 ± 0.8 Hz). The high frequency of miniature excitatory junction potentials (mEJPs) in mutant larvae shows that synaptic vesicles can fuse with the presynaptic membrane. The mean mEJP amplitude for *P[sytB-D3,4N]* and *syt^{null}* larvae were almost identical (0.99 ± 0.11 mV, n = 11 and 1.04 ± 0.08 mV, n = 10, respectively; p = 0.68), and slightly larger than that of *P[syt^{wt}]* larvae (0.72 ± 0.09 mV, n = 11; $p < 0.05$). However, the frequency distribution curves of spontaneous mEJP amplitudes were remarkably similar among all genotypes (Fig. 3.2c) indicating that the majority of vesicle fusions in all genotypes result in the same quantal sizes. The small number of larger events may represent either fusion of larger vesicles or an increased number of double and triple events due to the increased mini frequency in the *syt^{null}* and *P[sytB-D3,4N]* mutants. To further examine the fusibility of vesicles in *P[sytB-D3,4N]* mutant terminals, we focally applied a

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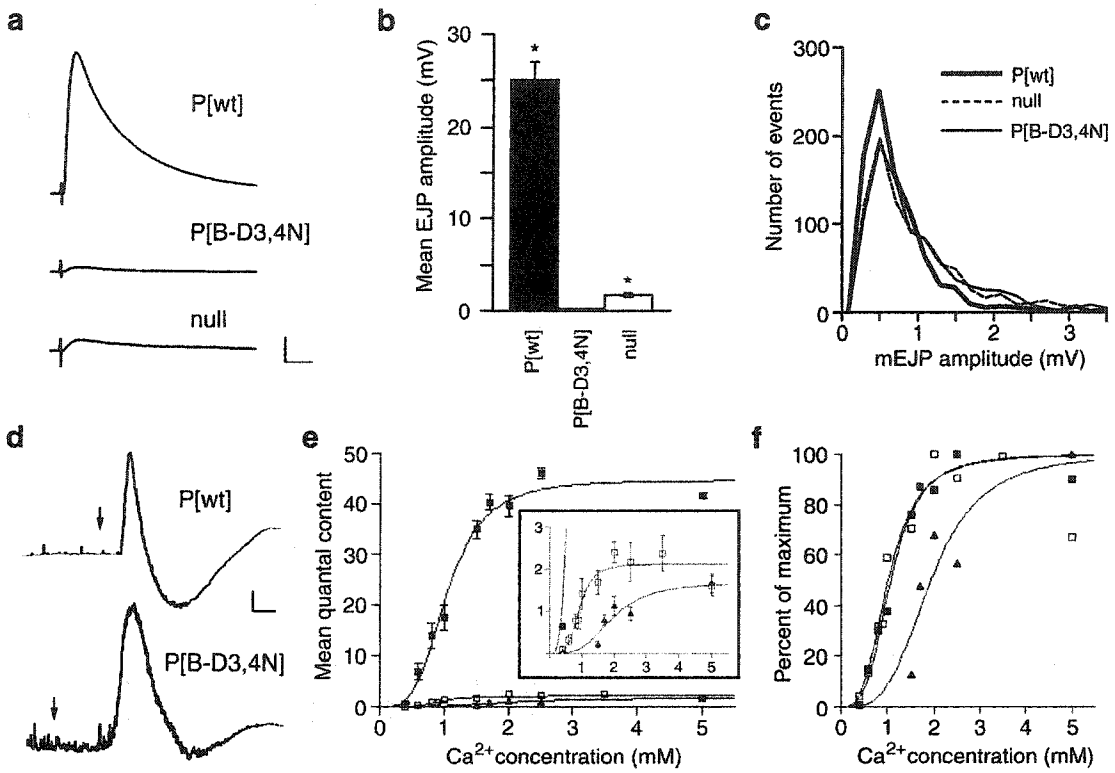

Figure 3.2 Fast, Ca^{2+}-evoked synaptic transmission is nearly abolished and the apparent Ca^{2+} affinity of evoked transmitter release is decreased in a C_2B Ca^{2+}-binding motif mutant. **A:** Averages of 30 evoked EJPs recorded in saline containing 1.5 mM Ca^{2+}. P[wt], syt^{null} expressing the *P[syt^{wt}]* transgene; P[B-D3,4N], syt^{null} expressing the *P[sytB-D3,4N]* transgene; null, syt^{null}. Scale bars: 5 mV, 20 msec. **B:** Mean EJP amplitude ± SEM. Asterisks indicate results that are significantly different from P[B-D3,4N] ($p < 0.001$, Satterthwaite t-test). **C:** Frequency distribution curves of mEJP amplitudes calculated from 850 individual events from each line compiled into 0.2 mV bins. **D:** Representative responses from P[wt] and P[B-D3,4N] fibers to focal application (arrows) of 2.0 M sucrose. Scale bars: 5 mV, 1.25 sec. **E:** Linear plot of $[Ca^{2+}]$ versus mean quantal content. Each point represents the mean quantal content of 6-10 muscle fibers from ≥ 3 larvae. Closed squares, *P[syt^{wt}]*; open squares, syt^{null}; triangles, *P[sytB-D3,4N]*. Error bars where visible indicate SEM. **Inset**: enlarged to show *P[sytB-D3,4N]* and syt^{null} curves. **F:** Ca^{2+} dose response data normalized to illustrate the rightward shift of the *P[sytB-D3,4N]* curve. Symbols as in (e).

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hyperosmotic solution (2.0 M sucrose) to elicit neurotransmitter release independent of nerve stimulation. Though variable, sucrose-stimulated release was comparable in *P[syt^{wt}]* and *P[sytB-D3,4N]* larvae (Fig 3.2d). Taken together, these data demonstrate that synaptic vesicles are capable of fusing with the presynaptic membrane. However, in *P[sytB-D3,4N]* mutants, nerve stimulation triggers the fusion of even fewer vesicles than in *syt^{null}* mutants.

To determine the Ca^{2+} dependence of neurotransmitter release, we measured EJP amplitude at extracellular Ca^{2+} concentrations ranging from 0.4 mM to 5 mM Ca^{2+} for *P[syt^{wt}]*, *P[sytB-D3,4N]*, and *syt^{null}* mutants (Fig. 3.2e). Although they differed greatly in maximal response, *P[syt^{wt}]* and *syt^{null}* fibers showed a similar Ca^{2+} dependence (Fig. 3.2e,f) with EC_{50} values of 1.05 and 0.93 mM, respectively. The maximal response in *P[sytB-D3,4N]* fibers was reduced even more than in *syt^{null}* fibers. Moreover, *P[sytB-D3,4N]* fibers showed a significantly shifted Ca^{2+} dependence compared to *P[syt^{wt}]* and *syt^{null}*, with an EC_{50} of 1.95 mM (Fig. 3.2e,f). To facilitate comparison of the *P[syt^{wt}]* and *P[sytB-D3,4N]* mutants, the data were plotted as a percentage of the maximal response in each line (Fig. 3.2f). The rightward shift of the dose response curve in the *P[sytB-D3,4N]* mutants demonstrates that the apparent Ca^{2+} affinity of synaptic transmission is reduced by the B-D3,4N mutation.

Since evoked release was even lower in *P[sytB-D3,4N]* mutants than in *syt^{null}* mutants, we examined whether the mutant protein dominantly inhibits synaptic transmission. We analyzed evoked release in larvae that coexpressed native synaptotagmin and the transgenic B-D3,4N mutant synaptotagmin (wt with P[B-D3,4N]). The control line only expressed synaptotagmin from the

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native gene, and not from the mutant transgene. Under standard recording conditions, the control line (wt, Fig. 3.3) displayed a mean EJP amplitude of 42.8 mV ± 1.1 (n = 9). In contrast, evoked transmitter release in larvae expressing both the native and the B-D3,4N mutant synaptotagmin (wt with B-D3,4N, Fig. 3.3) was reduced by 53% to 20.3 ± 3.2 mV (n = 11, $p < 0.001$). Thus, the B-D3,4N mutant protein inhibited synaptic transmission even in the presence of wild type synaptotagmin.

We also mutated the first and second of the aspartate residues that coordinate Ca^{2+}-binding in the C_2B domain to asparagines ($D_{356,362}N$, referred to henceforth as B-D1,2N, Fig. 3.1). However, when crossed into the syt^{null} background, none of the embryos bearing the B-D1,2N mutant transgene were able to hatch. The late embryonic lethality suggests that the B-D1,2N mutation may be even more detrimental to the organism than the B-D3,4N mutation.

Physiological analysis of larvae that coexpressed native synaptotagmin and the transgenic B-D1,2N mutant synaptotagmin (wt with P[B-D1,2N], Fig. 3.3) support this hypothesis. The B-D1,2N mutant protein inhibited evoked transmitter release by at least 90.5% (4.09 ± 1.33 mV, n = 9, $p < 0.0001$) in the presence of wild type synaptotagmin. The only common feature between these mutations is that each is lacking two of the aspartate residues that coordinate Ca^{2+}-binding in the C_2B domain of synaptotagmin. Thus, two independent mutations that disrupt the Ca^{2+}-binding motif of C_2B each inhibit evoked transmitter release at Drosophila synapses.

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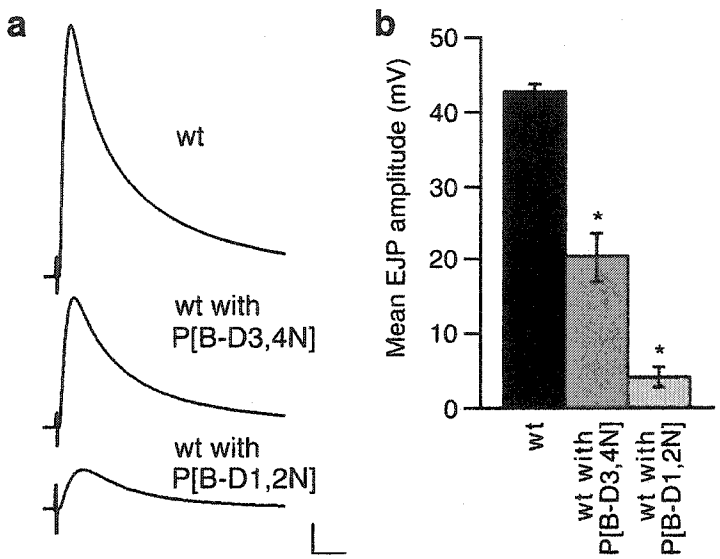

Figure 3.3 Expression of B-D3,4N or B-D1,2N mutant synaptotagmin inhibits synaptic transmission in otherwise wild-type synapses. **A:** Average of 30 evoked EJPs from three genotypes: wt, a line heterozygous for the native *syt* gene which contains the *P[sytB-D3,4N]* transgene but lacks transgene expression; wt with P[B-D3,4N], a *syt* heterozygote that expresses the B-D3,4N mutant protein; wt with P[B-D1,2N], a *syt* heterozygote that expresses the B-D1,2N mutant protein. Scale bars: 5 mV, 20 msec. **B:** Mean EJP amplitude ± SEM for all three lines. Results that are significantly different from wt are indicated by asterisks (*, $p < 0.001$, Satterthwaite t-test).

To verify transgene expression in the syt^{null} background, we stained *P[*syt^{wt}*]* and *P[sytB-D3,4N]* larval whole mounts (Fig. 3.4) and Western blots of larval central nervous systems (CNS, Fig. 3.4b) with an anti-synaptotagmin antibody (Loewen et al.). Both demonstrated abundant transgene expression. Thus in the syt^{null} background, the B-D3,4N mutant protein is stably expressed in the larval nervous system and is appropriately targeted to neuromuscular junctions. To demonstrate transgene expression in the *syt* heterozygous background, similar Western blots were analyzed from the following lines: wt with *P[sytB-D3,4N]*, wt with P[*syt*B-D1,2N], and their paired wt controls, each of

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which carry a transgene but do not express it. Fig. 3.4c demonstrates that both of the transgene expressing lines (lanes 1 and 3) expressed similar levels of

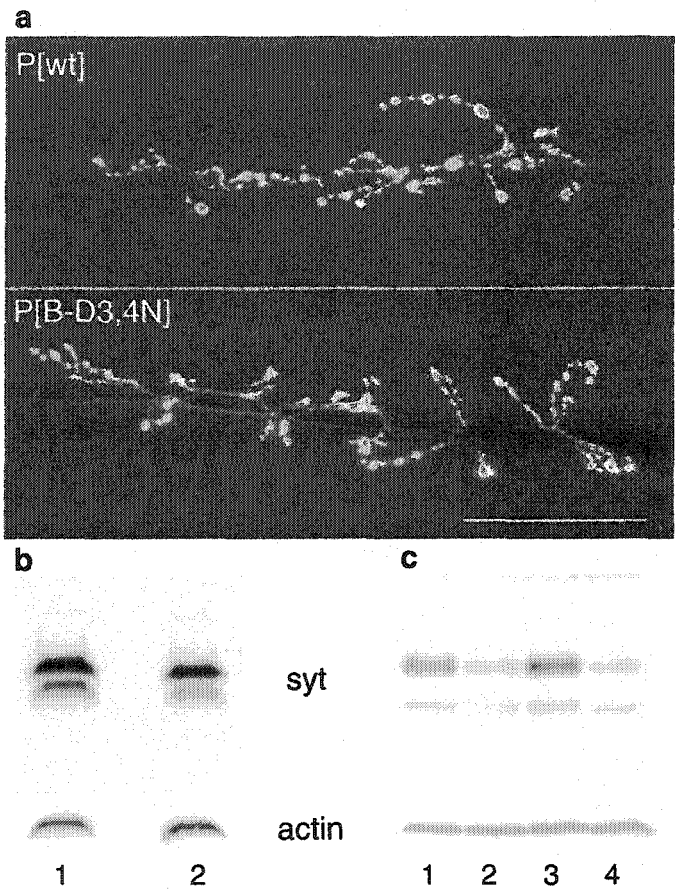

Figure 3.4 Synaptotagmin expression in the nervous system of *P[syt^{wt}]*, *P[sytB-D3,4N]* and P[*syt*B-D1,2N] transgenic lines. **A:** Anti-synaptotagmin immunolabeling of *P[syt^{wt}]* and *P[sytB-D3,4N]* larvae shows that the wild type and mutant transgenes are both abundantly expressed at neuromuscular junctions, the synapse used for all analyses. Scale bar: 50 μm. **B:** Western blot showing synaptotagmin expression (seen as a doublet in Drosophila(Littleton et al., 1993a)) in the CNS of *syt^{null}* larvae bearing synaptotagmin transgenes. Lane 1, *P[syt^{wt}]*; lane 2, *P[sytB-D3,4N]*. **C:** Western blot showing synaptotagmin transgene expression in *syt* heterozygotes. Lane 1, wt with P[B-D3,4N]; lane 2, wt without P[B-D3,4N] expression; lane 3, wt with P[B-D1,2N]; lane 4, wt without P[B-D1,2N] expression.

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synaptotagmin, while the control lines (lanes 2 and 4) expressed significantly less. Thus the mutant transgenes are also expressed in the *syt* heterozygous background. Coupled with the finding that evoked release was even lower in *P[sytB-D3,4N]* mutants than in syt^{null} mutants (which exhibit no detectable synaptotagmin expression (Loewen et al.)), the expression data suggest that the electrophysiological deficits observed in these C_2B Ca^{2+}-binding motif mutants are unlikely to be a result of insufficient expression or mislocalization of the mutant transgenic protein.

The C_2B domain of synaptotagmin has been proposed to play a role in vesicle recycling (Zhang et al., 1994). This hypothesis is supported by ultrastructural observations of syt^{null} mutants (Jorgensen et al., 1995; Reist et al., 1998); first instar syt^{null} mutants exhibit substantial depletion of synaptic vesicles and increased numbers of large vesicles, consistent with impaired recycling. If the electrophysiological deficits in our C_2B Ca^{2+}-binding motif mutants were the result of a recycling defect, one would expect *P[sytB-D3,4N]* mutant terminals (Fig. 3.5b) to exhibit at least as much vesicle depletion as syt^{null} mutant terminals (Fig. 3.5c). Qualitative observation of synaptic ultrastructure revealed that the *P[sytB-D3,4N]* mutants did exhibit some increase in large vesicles compared to controls (Fig. 3.5a,b); however, the abundance of synaptic vesicles in *P[sytB-D3,4N]* mutant terminals suggests that recycling is not the major defect in these mutants. Since the >95% decrease in evoked transmitter release in the *P[sytB-D3,4N]* mutant is not due to an absence of synaptic vesicles, the decreased release is likely a result of decreased release probability per vesicle.

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In order to address the molecular mechanisms that may underlie the dramatic decrease in synaptic transmission caused by C_2B Ca^{2+}-binding motif mutations, we tested wild type and mutant Drosophila synaptotagmin I for Ca^{2+}-dependent interactions *in vitro*. Syntaxin binding by the C_2B domain was not Ca^{2+} dependent (Kee and Scheller, 1996) and the C_2B Ca^{2+}-binding motif mutations did not alter this interaction (data not shown). As previously seen for rat synaptotagmin (Desai et al., 2000), we found that the B-D3,4N mutation caused a 25% reduction in Ca^{2+}-dependent oligomerization (Fig. 3.5d, $p = 0.052$). Unlike the studies of the rat protein, however, the Ca^{2+}-independent oligomerization was unchanged by the mutation. Ca^{2+}-dependent phospholipid binding by the C_2B domain (Fernandez et al., 2001; Bai et al., 2002), on the other hand, was dramatically altered by the C_2B Ca^{2+}-binding motif mutations (Fig. 3.5e). Compared to wild type, the B-D3,4N mutation reduced Ca^{2+}-dependent phospholipid binding by 65% at 200 μM Ca^{2+} and by 95% at 2 mM Ca^{2+} ($p < 0.001$), while increasing Ca^{2+}-independent binding. The B-D1,2N mutation reduced Ca^{2+}-dependent phospholipid binding even more severely, down to levels similar to background binding to GST alone (Fig. 3.5e). These mutations have also been shown to disrupt Ca^{2+}-dependent interactions between rat *syt* I and phospholipids (Earles et al., 2001). Both the oligomerization assay and the phospholipid-binding assay demonstrate that the C_2B Ca^{2+}-binding motif mutations disrupt Ca^{2+}-dependent interactions of synaptotagmin.

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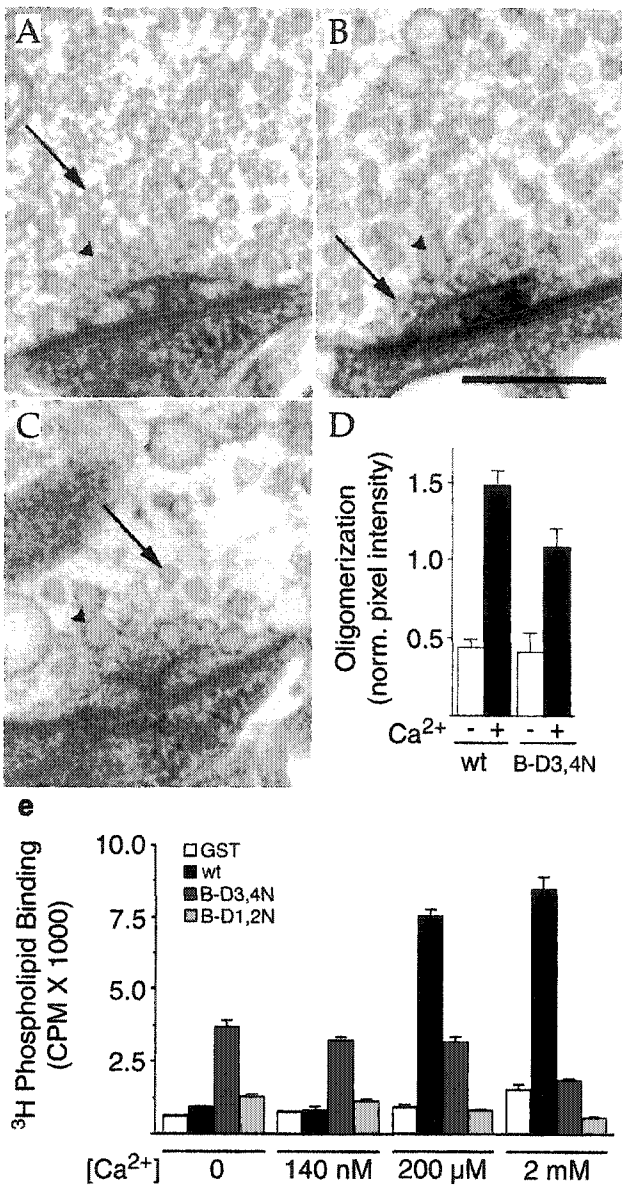

Figure 3.5 While defects in recycling and Ca^{2+}-dependent oligomeriztion cannot account for the severe decrease in synaptic transmission, decreased Ca^{2+}-dependent phospholipid binding may. Representative nerve terminals from neuromuscular junctions of *P[sytwt]* (**A**), *P[sytB-D3,4N]* (**B**), and *sytnull* (**C**) third instar larvae. Although there is an increase in the number of large vesicles (arrowheads), synaptic vesicles (arrows) are abundant in *P[sytB-D3,4N]* mutant nerve terminals. Scale bar: 250 nm. **D:** Ca^{2+}-dependent oligomeriztion was decreased by ~25% in C_2A-C_2B domains bearing the B-D3,4N mutation (mean ± SEM). **E:** Ca^{2+}-dependent phospholipid binding to GST-C_2B is severely reduced or abolished by the B-D3,4N or B-D1,2N mutation, respectively. Background liposome binding is shown in the GST alone columns.

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The decreases in Ca^{2+}-dependent phospholipid binding by the mutant C_2B domains closely reflect the severity of the electrophysiological deficits seen *in vivo*. The B-D3,4N mutation severely decreased Ca^{2+}-dependent phospholipid binding, while the B-D1,2N mutation completely blocked it. This correlation suggests that Ca^{2+}-dependent phospholipid binding by the C_2B domain may be a critical step in evoked transmitter release. Ca^{2+}-dependent phospholipid binding by the C_2A domain is also thought to be important for evoked transmitter release. A mutation in the C_2A domain that decreased synaptotagmin's phospholipid binding *in vitro* also decreased evoked transmitter release (Fernandez-Chacon et al., 2001). However, alterations of the C_2A Ca^{2+}-binding motif do not appear to inhibit evoked release *in vivo* (Robinson et al., 2002). Since the Ca^{2+}-binding pockets of each C_2 domain are located in close proximity to one another (Sutton et al., 1999; Garcia et al., 2000), Ca^{2+} binding by the C_2B domain may facilitate membrane binding by the C_2A domain.

Regardless of whether the C_2A and C_2B domains function cooperatively, our data showing that mutation of two aspartates in C_2B inhibited evoked transmitter release by >95% demonstrate that the C_2B Ca^{2+}-binding motif plays a central role in synaptotagmin function. Since synaptic vesicles in the *P[sytB-D3,4N]* mutant terminals were plentiful and fusion competent, the dramatic decrease in evoked release is unlikely to result from a vesicle recycling or biogenesis defect. These results are the first to show a disruption of synaptic transmission caused by mutation of a Ca^{2+}-binding motif and demonstrate that

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the C_2B Ca^{2+}-binding motif of synaptotagmin is essential for synaptic transmission *in vivo*.

Additional unpublished results and discussion

This publication was the first to demonstrate that any Ca^{2+} binding residue in a nerve terminal molecule was important for synaptic transmission *in vivo*. Standing alone, these data do not "prove" that synaptotagmin is "the Ca^{2+} sensor", nor do they explain the exact mechanism of synaptotagmin function. These data do, however, strongly support the hypothesis that synaptotagmin is involved in Ca^{2+} sensing since both the probability of release and the apparent Ca^{2+} affinity of synaptic transmission were reduced. Unfortunately, the *P[sytB-D3,4N]* mutants have a probability of release that is almost lower than our detection limits. Perhaps the most significant shortcoming of this publication was that I was unable to accurately measure the cooperativity coefficient for synaptic transmission in the *P[sytB-D3,4N]* mutants. This measurement would put to rest much controversy over the role of synaptotagmin in Ca^{2+} cooperativity of evoked release. (The significance of a change in Ca^{2+} affinity vs. cooperativity will be discussed at length in Chapter 4). Other students in our lab are currently making single point mutations in the Ca^{2+} binding aspartates in the hope that those mutants will have a less severe phenotype that permits more accurate measurements of evoked release.

Since making new mutants can take a year or more, I decided to look at the Ca^{2+} dependence of synaptic transmission in wild type lines where I co-expressed either the B-D3,4N or B-D1,2N mutant synaptotagmin (Fig. 3.6). Evoked release was suppressed at all Ca^{2+} concentrations in both mutant lines.

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The EC_{50} was 0.8 ± 0.03 for wt and 1.0 ± 0.07 for wt with P[B-D3,4N] while the EC_{50} for wt with P[B-D1,2N] was shifted to 1.9 ± 0.68. The cooperativity coefficients were calculated accurately for all lines: wt, 3.67; wt with P[B-D3,4N], 2.74; and wt with P[B-D1,2N], 1.84. Since over-expression of a mutant protein is similar to a peptide injection experiment, the only conclusion that can be drawn from these data is that the P[B-D1,2N] protein is more deleterious to function than the P[B-D3,4N] protein. I cannot determine where in the vesicle cycle the mutant proteins are acting, therefore, it is not possible to determine a mechanism of synaptotagmin function from these electrophysiology data alone. The best explanation for the data is derived from a stochastic model of synaptic vesicle

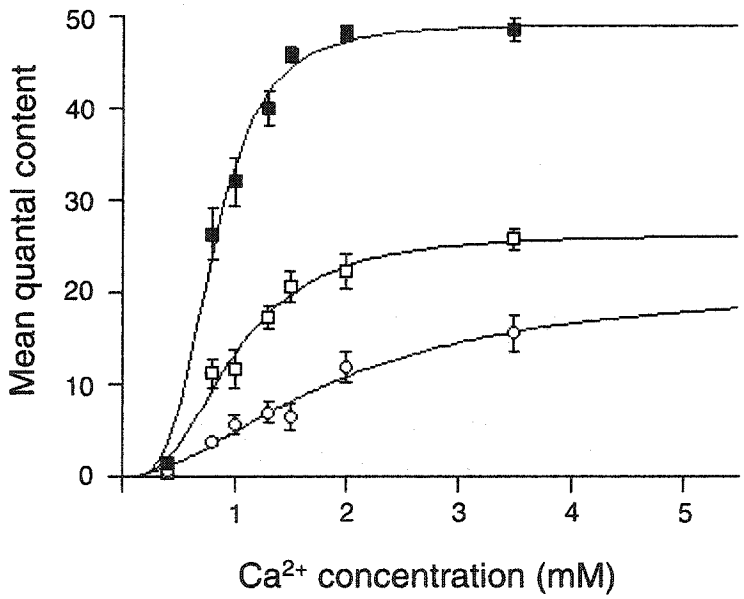

Figure 3.6 P[B-D1,2N] or P[B-D3,4N] mutant proteins suppress evoked release even in the presence of wild type synaptotagmin. Each data point represents the average quantal content of 10-20 muscle fibers. Error bars where visible are SEM. Filled squares, wt; open squares, wt with P[B-D3,4N]; open circles wt with P[B-D1,2N]. Abbreviations as in Fig. 3.3.

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release. A certain number of functional synaptotagmin molecules may be needed for a vesicle to target properly and fuse. In the case of over-expression of a mutant protein, the mutant protein may displace the wild type some percentage of the time, thereby "poisoning" the vesicle and preventing fusion. The fusion events that do occur would be the result of vesicles at the active zone that happened to contain the correct number of wild type synaptotagmin molecules and were able to fuse with normal kinetics. The change in cooperativity in the D1,2N mutant may indicate that the mutant protein is interfering with the efficiency of the normally precise cooperative mechanism. Thus, over-expression of synaptotagmin with a mutated Ca^{2+} binding motif still cannot directly elucidate the mechanism of Ca^{2+} cooperativity.

In Chapter 4, I will argue that a change in apparent Ca^{2+} affinity is the most significant evidence that Ca^{2+} sensing has been altered. There are factors, other than direct alteration of the Ca^{2+} sensor, however, that may increase the Ca^{2+} requirement of synaptic transmission. First, we have not shown that Ca^{2+} binding to synaptotagmin itself has been altered. Second, perhaps the neutralization of negatively charged aspartates has permitted novel Ca^{2+}-independent interactions to occur that disrupt the efficacy of synaptic transmission or perhaps we simply have a mis-folded protein causing aberrant molecular interactions. Finally, if synaptotagmin were necessary for docking, the mutation may have caused vesicles to be farther away from Ca^{2+} microdomains that has increased the effective Ca^{2+} concentration needed for synaptic transmission to occur.

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Ca^{2+} binding to synaptotagmin is rarely measured directly in this field since very few labs have access to NMR facilities and the experiments are not trivial. Rather, Ca^{2+} binding is measured indirectly, in terms of the capacity of the synaptotagmin construct to participate in recognized Ca^{2+}-dependent interactions. Thus, while we have biochemical evidence for decreases in Ca^{2+}-dependent interactions with the B-D3,4N construct, we do not have evidence that the intrinsic affinity of the C_2B binding site for Ca^{2+} has changed. From the Ca^{2+} dependence curves in Fig. 3.2e,f you might expect that if the only change the mutation caused was a decrease in Ca^{2+} affinity for synaptotagmin's binding site, the B-D3,4N curve would be shifted to the right, but have the same maximum response as *P[syt^{wt}]*. Most likely, some other critical interaction downstream of Ca^{2+} binding is impaired which prevents extracellular Ca^{2+} from rescuing the amplitude of evoked release.

Induction of non-specific interactions is always a caveat of mutagenesis studies. Neutralizing multiple charges in a highly charged part of the molecule could destabilize the entire tip of the C_2B domain. Ideally, circular dicroism or NMR should be performed on the mutated C_2B domains to demonstrate that their tertiary structure is intact. It is encouraging that the D2N mutation in rat *syt I* C_2B domain resulted in a well-structured domain that failed to bind Ca^{2+} (Fernandez et al., 2001). It may be useful to mutate the same residues to alanine or another small amino acid, in case the results from *P[sytB-D3,4N]* mutants were due to the large asparagine side chains not only neutralizing charge, but disrupting the structure. Single mutations of aspartates to asparagines in C_2B may be less disruptive to the Ca^{2+} binding pocket and perhaps provide a less

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severe phenotype. Hopefully the results of this study will generate interest in the field so that others will pursue NMR studies of this mutation in C_2B.

Finally, a disruption in Ca^{2+} influx or a change in Ca^{2+} buffering within the nerve terminal may be indistinguishable from a change in Ca^{2+} sensing. Ca^{2+} imaging of nerve terminals would rule out the possibility that Ca^{2+} influx through presynaptic Ca^{2+} channels has somehow been altered by mutation of synaptotagmin. Note that all three dose-response curves in Figure 3.2e,f are saturated at about 3 mM extracellular [Ca^{2+}]. One interpretation of this effect is that presynaptic Ca^{2+} channels are saturated at this point (Dr. Iain Robinson, personal communication); there is no gain in Ca^{2+} influx by using much higher concentrations of extracellular Ca^{2+}. If Ca^{2+} influx in the nerve terminals could be enhanced, I could examine the response of the *P[sytB-D3,4N]* mutants in higher Ca^{2+} concentrations. The use of a Ca^{2+} ionophore or caged Ca^{2+} may help to determine if the vesicles in B-D3,4N mutant terminals are releasable under extremely high concentrations of Ca^{2+}.

If the mutated aspartate residues in C_2B are critical for localization of vesicles to active zones or participate in docking, vesicles in these mutants may be located farther away from active zones and/or sites of Ca^{2+} influx. According to Ca^{2+} microdomain models, this may result in a change in Ca^{2+} dependence (Klingauf and Neher, 1997). The polylysine residues tested in Chapter 2 are reported to bind the "synprint" site of Ca^{2+} channels in other organisms; Drosophila apparently do not contain a homologous synprint site (Littleton and Ganetzky, 2000), so unfortunately, our polylysine mutants do not address the disjunction of synaptotagmin from sites of Ca^{2+} influx. If vesicles

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were relatively farther away from site of Ca^{2+} influx, then use of a Ca^{2+} ionophore to massively and non-specifically raise intracellular Ca^{2+} may be useful to determine the maximal amount and rate of vesicle release in the *P[sytB-D3,4N]* mutants. Carin Loewen in our lab is currently conducting detailed morphological analyses of nerve terminals from EM images to count docked vesicles and measure distances of vesicles from the plasma membrane. These studies should determine if there is a major docking deficit, however, a very small change in vesicle distance might not be detected.

In conclusion, there is still much work to be done to further dissect the mechanism of synaptotagmin function. These data have significantly contributed to our understanding of synaptotagmin by focusing attention on the C_2B domain as an important region of the molecule. Further physiological experiments on both double and single point mutants may provide substantial biophysical insight into the mechanisms of apparent Ca^{2+} affinity vs. cooperativity. Careful biochemical studies may further define potential molecular interactions to be interpreted in tandem with results from physiological studies. There are likely to be a few more surprises to come.

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Chapter 4: Further discussion and conclusions: Is synaptotagmin the Ca^{2+} sensor?

"Even though a Hill coefficient provides no insight into the reason for the sigmoid behavior, it is still useful to tabulate values of the Hill coefficient derived from such plots as semiquantative measures of the degree of sigmoidicity"

- Jack Kyte in Mechanism in Protein Chemistry

History and development of the Ca^{2+} sensor hypothesis

In Chapter 1, I introduced the historical concept of a Ca^{2+} sensor for synaptic transmission, a hypothesis that was formed before any of the molecular components of the synaptic terminal had been cloned or crystallized. The early model for the Ca^{2+} sensor assumed that the Ca^{2+} sensor for quantal release could bind both Ca^{2+} and Mg^{2+}, except that Ca^{2+} promoted transmitter release whereas Mg^{2+} did not (Dodge and Rahamimoff, 1967). Since Mg^{2+} and Ca^{2+} are both present in the extracellular and intracellular neuronal compartments and may compete for the same binding site(s), it was assumed that the fraction of "Ca^{2+} sensors" that are bound to Ca^{2+} could be calculated by the equation:

$$[Ca^{2+}X] = \frac{W[Ca^{2+}]}{1 + \frac{[Ca^{2+}]}{K_1} + \frac{[Mg^{2+}]}{K_2}} \qquad (1)$$

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Where X = Ca^{2+} sensor, W = constant, K_1 = dissociation rate for Ca^{2+}, K_2 = dissociation constant for Mg^{2+}. Dodge and Rahamimoff (Dodge and Rahamimoff, 1967) showed that, in low levels of Ca^{2+}, the amount of neurotransmitter released was nonlinearly dependent on the extracellular Ca^{2+} concentration, indicating that more that one Ca^{2+} ion may work cooperatively to facilitate transmitter release. In order to incorporate this finding into the mathematical model above, they added an exponent (*n*) to both sides of equation 1 to describe the Ca^{2+} dependence of evoked transmitter release:

$$EJP = [Ca^{2+}X]^{n} = \left(\frac{W[Ca^{2+}]}{1 + \frac{[Ca^{2+}]}{K_1} + \frac{[Mg^{2+}]}{K_2}} \right)^{n} \quad (2)$$

Experimentally, their data fit equation (2) using a cooperativity coefficient of n = 4. They proposed two models – one stoichiometric and one stochastic – that could explain how 4 Ca^{2+} ions could trigger neurotransmitter release. Their stoichiometric model required that 4 Ca^{2+} ions bind to one Ca^{2+} sensor molecule with four independent binding sites; all 4 Ca^{2+} ions would need to bind in order for transmitter release to occur. Their stochastic model, on the other hand, predicted that Ca^{2+} ions bound to the Ca^{2+} sensor in a 1:1 ratio; thus, 4 or more Ca^{2+}-bound complexes were needed for release to occur. To understand how they extrapolated from a cooperativity coefficient of 4 to 4 binding sites for Ca^{2+}, I need to digress into a brief discussion of allosteric enzyme interactions.

Dodge and Rahamimoff's idea of using the exponent of the power relationship to determine the degree of cooperativity was not novel, but rather an application of a current theory in enzyme kinetics. In the early 1960s, Jaque

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Monod, Jeffreys Wyman and Jean-Pierre Changeaux proposed a mathematical model for allosteric enzymes to explain real world enzyme reactions that did not obey simple Michaelis-Menton kinetics (Monod et al., 1965). Briefly, for an enzyme and its substrate, if you plot reaction rate (V/Vmax) vs. substrate concentration ([S]) on linear axes, you will either get a hyperbolic curve if the reaction follows Michaelis-Menton kinetics or a sigmoid curve if the interaction is allosteric. An allosteric interaction is where binding of one substrate molecule to an enzyme (usually a multi-subunit complex) favors the binding of the next substrate molecule(s) (usually in an adjacent subunit); substrate binding in an allosteric interaction is said to be "cooperative".

If you plot reaction rate vs. substrate concentration on a log-log plot, also known as a Hill plot, over some portions of the curve, the previously sigmoid function is now linear. The initial slope of this relation (before saturation) indicates the degree of cooperativity, *n*. In years past, before computers that easily perform non-linear regression analyses, the slope of the relation on a Hill plot was used to estimate the exponent, *n*, in kinetic equations such as equation 2. The exponent, *n*, is also called a "cooperativity coefficient". The cooperativity coefficient derived in this way is also called the "Hill slope". A Hill slope of 1 indicates no cooperativity, and therefore a single binding site, whereas a Hill slope > 1 indicates positive cooperativity with more that one binding site; the steeper the slope, the more cooperative the binding. For a sigmoid dose-response curve with a very steep slope, you can image that most binding will occur over a narrow range of substrate concentrations. In other words, an enzyme with strong positive cooperativity is "tuned" to bind substrate over a narrow range of substrate concentrations and is very sensitive to small changes

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in substrate concentration. The Hill slope is often used as a measure of the number of binding sites, which happens to work for highly cooperativity molecules such as hemoglobin. However, in Monod et al. 1965 the authors clearly state that the coefficient *n* used in their kinetic equations is distinct from the number of binding sites.

Returning now to neurotransmitter release, Dodge and Rahamimoff applied this theory of allosteric enzyme interactions to the relationship between end plate potential (e.p.p.), as a measure of "reaction rate", vs. "substrate", as Ca^{2+}concentration. They observed a steep, sigmoid curve when the dose-response curve was plotted on linear axes and a Hill slope of approximately 4 when the data were plotted on double log axes. They postulated that 4 Ca^{2+} ions with 4 individual binding sites were required for transmitter release. For several years now, other labs studying the mechanisms of transmitter release have used the cooperativity coefficient as a direct measure of the number of Ca^{2+} ions needed for vesicle release (Goda and Stevens, 1994; Heidelberger et al., 1994; Beutner et al., 2001). I doubt that Dodge and Rahamimoff intended their model, which was stretching the boundaries of biophysics, to be held as synaptic dogma for so many years.

Regardless, there are several rational reasons to question the use of *n* to calculate the number of binding sites. First, it is critical to understand that using the Hill Slope to determine the number of binding sites is simply an estimate derived from the experimental measurement of neurotransmitter release. The Hill Slope as an estimate of the number of binding sites should only apply in ideal cases where there is extreme positive cooperativity and where the binding

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of ligand is virtually all or none (Weiss, 1997). The process of neurotransmitter release is undoubtedly more complicated than binding of enzyme and substrate and the method used to determine "reaction rate" much less precise. The cooperativity coefficient of neurotransmission is measured by EJP or EJC amplitude, both of which are an output of an unknown number of molecular interactions. The amplitude of evoked release depends on many steps including Ca^{2+} entry, diffusion, and presumably, on the molecular steps involved in vesicle docking, priming, and fusion. While synaptic transmission is fast (100-200 μsec), we have no reason to assume that Ca^{2+} binding to the release apparatus is all-or-none. Furthermore, it is troubling that estimates of n for neurotransmission are rarely an integer, which is what you would expect for a number of binding sites. For all of these reasons, the application of the Hill slope to determine binding sites should be avoided. The current imaging capabilities of NMR and X-ray crystallography provide a better glimpse at the number of binding sites that a molecule possesses. Other experimental methods are needed to determine which Ca^{2+} binding sites are important for synaptic transmission. At best, the Hill slope should only be used to determine a relative degree of cooperativity in an intermolecular interaction, as yet another tool to probe the properties of synaptic transmission.

Despite these criticisms and despite all of our current biochemical knowledge of the synaptic terminal, physiologists continue to use the cooperativity coefficient to discuss Ca^{2+} binding sites required for vesicle release. The search for the Ca^{2+} sensor has often turned on whether or not mutation of synaptotagmin alters cooperativity, and therefore the number of Ca^{2+} binding

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sites. Recent evidence suggests that the mechanisms for Ca^{2+} cooperativity and Ca^{2+} affinity of neurotransmitter release may be experimentally separable. Here I will discuss the hypothesis that changes in Ca^{2+} sensing may be reflected physiologically by a change in apparent Ca^{2+} affinity, and not necessarily Ca^{2+} cooperativity. Finally, I will propose two models for synaptotagmin function that each accounts for both of these mechanisms.

Mechanism of Ca^{2+} cooperativity

The measurement of the Hill slope, hereafter referred to simply as "cooperativity", of transmitter release has become a common tool for analyzing synaptic function, even though the molecular interaction that underlies the cooperative action of Ca^{2+} is still unknown. As the prime candidate for the neuronal Ca^{2+} sensor, synaptotagmin has been thought to be the molecule that must confer Ca^{2+} cooperativity to synaptic transmission. Several different experiments, however, have failed to clearly demonstrate that synaptotagmin, acting as a solitary Ca^{2+} sensor, underlies the phenomenon of Ca^{2+} cooperativity: 1) syt does not bind Ca^{2+} cooperatively by itself *in vitro*, 2) genetic deletion of synaptotagmin does not alter cooperativity, 3) disruption of SNARE components, however, do change cooperativity. Instead, I will argue that synaptotagmin may contribute to the cooperative mechanism, but that SNARE proteins are essential for it.

First, the dissociation constants for Ca^{2+} binding sites within synaptotagmin are not consistent with synaptotagmin as a sole Ca^{2+} sensor that cooperatively binds 4 Ca^{2+} ions. Results of NMR experiments show that the dissociation constants for Ca^{2+} binding sites in C_2A and C_2B are such that the

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Ca^{2+} ions deepest in the binding pocket have lower dissociation constants than those of the more exposed Ca^{2+} binding sites (Ubach et al., 1998; Fernandez et al., 2001; Fernandez-Chacon et al., 2002). These data show that the "later" Ca^{2+} ions that bind to either C_2 domain have lower intrinsic binding affinities, not higher as you would expect if Ca^{2+} binding caused allosteric interactions within the Ca^{2+} binding pocket. Indeed, Ca^{2+} binding to C_2A alone does not itself appear to be a cooperative process (Davis et al., 1999). Thus, a stoichiometric model where the cooperativity of synaptic transmission is governed by 3-6 Ca^{2+} ions binding to one molecule of synaptotagmin seems extremely unlikely. Given what we now know about the protein structure and binding attributes of synaptotagmin and the other molecules at the synapse, it is not logical to try and force synaptotagmin to fit this model.

It is important to remember, however, that the NMR studies above were done in the absence of synaptotagmin-binding molecules and may not reflect the true intrinsic Ca^{2+} affinity of synaptotagmin in its native environment. The results of X-ray crystallography and NMR studies both determined that the Ca^{2+} binding pocket in each of synaptotagmin's C_2 domains does not completely coordinate Ca^{2+}, but rather creates a region of positive charge on the end of the C_2 domain when Ca^{2+} is bound (Sutton et al., 1995; Shao et al., 1996; Shao et al., 1998). Synaptotagmin's binding partners, including phospholipids, syntaxin, SNAP-25 and perhaps the SNARE complex itself, may help complete the coordination sphere of Ca^{2+}, forming a "Ca^{2+} bridge"(Bazzi and Nelsestuen, 1991). Both phospholipids and syntaxin have been shown to increase the apparent Ca^{2+} affinity of the synaptotagmin complex (Brose et al., 1992;

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Chapman et al., 1995; Ubach et al., 1998; Zhang et al., 1998). From these data, it is tempting to postulate that synaptotagmin's Ca^{2+} binding pocket is only one half of the actual binding site for Ca^{2+} in synaptic transmission. The complete Ca^{2+} binding site may only form when synaptotagmin is in close proximity to one of its effectors molecules. A potential mechanism for Ca^{2+} cooperativity in exocytosis would be if formation of one Ca^{2+} binding site between synaptotagmin and its effector increased the probability of forming other equivalent Ca^{2+} binding sites. A certain minimum number of Ca^{2+} ions would be required to "bridge" synaptotagmin and its effector in order for fusion to occur. If synaptotagmin, specifically one or both of its Ca^{2+} binding pockets, were intimately involved in the cooperative mechanism, you would expect that deletion or mutation of synaptotagmin would dramatically affect cooperativity.

Genetic experiments that deleted *syt I*, the predominant isoform in mouse brain and Drosophila NMJ, however, failed to show a clear role of synaptotagmin in Ca^{2+} cooperativity. First, cultured mouse autaptic hippocampal neurons of syt^{null} mice were used to measure the cooperativity of evoked release. Geppert, et al. (Geppert et al., 1994) found that fast synaptic transmission was nearly abolished, however, there was no difference in n between syt^{null} and control mice for the remaining small fast component or the slow component of exocytosis. If synaptotagmin is the Ca^{2+} sensor, why was any Ca^{2+} evoked release, either fast or slow, remaining at this synapse? The presence of other isoforms of synaptotagmin at most synapses have complicated all *syt* deletion studies to date. In mouse, syt^{null} experiments are confounded by the presence of synaptotagmin III at hippocampal synapses (Ullrich et al., 1994),

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therefore the cooperativity of the release that they measured was likely due to a redundant molecule. Regardless of not having altered cooperativity, the authors were convinced that synaptotagmin was the Ca^{2+} sensor since "fast" synaptic transmission had been abolished in the *syt* knockouts.

Similar results were obtained in Drosophila syt^{null} mutants were *syt I* was originally thought to be the only isoform of *syt* in Drosophila (DiAntonio et al., 1993). Broadie et al. (Broadie et al., 1994) recorded EJCs from syt^{null} first instar larvae (the larvae did not survive longer under their conditions, see also (Loewen et al., 2001; Mackler et al., 2002)) and found a dramatic reduction in transmitter release, but no difference in n between syt^{null} and control. The authors argued that synaptotagmin could not be the Ca^{2+} sensor because residual vesicle exocytosis remained and that cooperativity was not altered. We now know that *syt IV* also exists at Drosophila NMJs (Littleton et al., 1999), so that like in mice, some other molecule, perhaps *syt IV* which is normally expressed at low levels, was facilitating transmitter release with similar kinetics. When I examined the same syt^{null} mutants as third instar larvae, I found similar results; EJP amplitude was greatly reduced in syt^{null} mutants but the kinetics of the Ca^{2+} dose-response curves were very similar to control (Mackler et al., 2002). Taken together, genetic knockout studies from both mice and Drosophila suggest that the Ca^{2+} sensing mechanism in neurons has built in redundancy, either through another isoform of *syt* or a similar molecule. In addition, this implies that the Ca^{2+} evoked release that remains in the absence of the major isoform of syt uses the same, or similar, cooperative mechanism to induce vesicle exocytosis.

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In order to specifically test the importance of the synaptotagmin's Ca^{2+} binding pocket, our lab and others designed transgenic Drosophila or mice bearing site specific mutations in the Ca^{2+} binding residues of the C_2A (Fernandez-Chacon et al., 2002; Robinson et al., 2002) and C_2B domains (Mackler et al., 2002)of *syt I*. Surprisingly, mutation of critical Ca^{2+}-binding aspartate residues to asparagine in C_2A did not significantly affect any aspect of synaptic transmission, including cooperativity (Fernandez-Chacon et al., 2002; Robinson et al., 2002). The highly conserved aspartate residue, D2, that appeared to be important for Ca^{2+} dependent interactions with phospholipids *in vitro* (Li et al., 1995b; Bai et al., 2000), apparently is not part of the cooperative Ca^{2+} sensing mechanism *in vivo*. Mutation of dual Ca^{2+} binding aspartate residues in the C_2B domain, however, reduced both the amplitude of evoked release and the sensitivity to external Ca^{2+} concentration (apparent Ca^{2+} affinity) so severely that it was impossible to accurately measure *n* in this study (Chapter 3). Members of the Reist lab are now making single point mutants in Drosophila Ca^{2+} binding aspartates that will hopefully not be as severely impaired and will help determine if Ca^{2+} binding residues in C_2B contribute to the mechanism of cooperativity. Currently, this hypothesis cannot be ruled out.

There is some evidence to suggest that the C_2B domain does in some way contribute to cooperativity, though not necessarily via the Ca^{2+} binding domain. Littleton, et al. (Littleton et al., 1994) published results of Ca^{2+} dependence measurements on several Drosophila *syt* mutants, including one that lacked the entire C_2B domain of *syt I* (AD1 allele (DiAntonio et al., 1993)). This study found

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that AD1 combined with a *syt I* hypomorphic allele, T41, reduced the apparent Ca^{2+} affinity of synaptic transmission and reduced Ca^{2+} cooperativity from 3.5 in wild type to 1.6 in the mutant. At the time, this study was criticized for using an uncharacterized hypomorphic allele, rather than a syt^{null} or deficiency allele to complement AD1. Littleton and co-workers recently duplicated this experiment using AD1 combined with N13 (a syt^{null} allele) (Yoshihara and Littleton, 2002). Recording from Drosophila embryos, they found that AD1 mutants in this genetic background had an *n* of 0.77 compared to 3.5 in wild type. In both studies, a different synaptotagmin C_2B point mutant, AD3 (Y334N) (DiAntonio et al., 1993), displayed a shift in apparent Ca^{2+} affinity, yet no change in cooperativity (Littleton et al., 1994; Yoshihara and Littleton, 2002). The data from AD1 and AD3 mutants were the first to suggest that both the apparent Ca^{2+} affinity and cooperativity could be differentially affected by mutations in the C_2B domain. Thus, synaptotagmin mutagenesis studies to date have shown that if synaptotagmin contributes to the mechanism of cooperativity, it does so over a restricted part of the molecule, perhaps confined to the C_2B domain, and may or may not specifically involve the C_2B Ca^{2+} binding residues. With this hypothesis in mind, it would be worthwhile to examine the Ca^{2+} dependence of the polylysine mutants discussed in Chapter 2 since this is a highly charged region of the C_2B domain and its function *in vivo* remains unknown.

Interestingly, Ca^{2+} cooperativity has been altered in other Drosophila synaptic protein mutants, without changing the apparent Ca^{2+} affinity. For example, the *dunce* mutant, deficient in a phosphodiesterase involved in neuronal cAMP signaling, exhibited a decreased Ca^{2+} cooperativity (Zhong and

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Wu, 1991). Intriguingly, specific disruption of SNARE proteins can also reduce the Ca^{2+} cooperativity of synaptic transmission. A careful examination by Stewart et al. (Stewart et al., 2000) reported that *n* was reduced from 3.4 in wild type to 2.6 and 2.4, respectively, in Drosophila syntaxin and VAMP hypomorphs (nulls were lethal). Interestingly, the apparent Ca^{2+} affinity of synaptic transmission was the same as control for both of these mutants. Again, these data support the hypothesis that Ca^{2+} affinity and cooperativity have slightly different underlying mechanisms. It has been suggested that the SNARE complex may be a Ca^{2+} sensor itself (Chen et al., 1999; Gerona et al., 2000; Zhang et al., 2002).

Vesicle fusion is thought to occur when SNARE proteins form a trans complex which zippers from the N-terminal toward the C-terminal (Lin and Scheller, 1997; Hua et al., 1999). Recent biophysical data suggest that stoichiometrically, three SNARE complexes are required for a single vesicle fusion (Hua and Scheller, 2001). (Interestingly, the authors point out that this roughly corresponds to the power relationship of ~ 3 for synaptic transmission.) SNARE proteins are thought to be largely unstructured until they form α-helices when complexed with one another in a coiled-coil (Hayashi et al., 1994; Sutton et al., 1998). Prior to fusion, the complex is thought to exist in a loosely coiled state, thereby making a vesicle "fusion competent". Although *in vitro*, SNARE complex formation occurs in the absence of either synaptotagmin or Ca^{2+} (Hayashi et al., 1995), *in vivo*, Ca^{2+} may be the trigger for final zippering of the SNARE complex at the C-terminus that initiates vesicle fusion (Chen et al., 1999; Melia et al., 2002; Zhang et al., 2002). Theoretically, this interaction with Ca^{2+}

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may either occur directly by Ca^{2+} binding to the SNARE complex C-terminus, or through the indirect "bridging" of Ca^{2+} between the complex and synaptotagmin.

Much is still unknown about synaptotagmin-SNARE interactions, most importantly, which residues of synaptotagmin interact with the SNARE complex. Do those residues reside in the C_2B domain and do they include the Ca^{2+} binding residues or some other face of the molecule? Currently, I am attempting to perform *in vitro* binding assays between synaptotagmin C_2B Ca^{2+}-binding-site mutants and syntaxin-SNAP-25 heterodimers using "properly" purified C_2AB cytoplasmic domains (Wu et al., 2003). Perhaps the results of this effort will address the question of whether or not the C_2B Ca^{2+} binding residues are important for this interaction. Furthermore, is the interaction between synaptotagmin and SNAREs important physiologically? If we knew which of synaptotagmin's residues were critical for SNARE binding *in vitro*, we could test their function *in vivo* through site-directed mutagenesis. Further experiments are needed to tease away the cooperative mechanism from the rest of the molecular machinery at the synapse.

Currently, SNARE proteins are hypothesized to function at or immediately following the Ca^{2+}-dependent step in exocytosis. The formation of the SNARE complex may be responsible for the apparent cooperativity seen *in vivo*. Mutation of synaptotagmin *in vivo*, however, is most often correlated with a change in apparent Ca^{2+} affinity without altering cooperativity. While synaptotagmin's role remains unresolved, one possible model (hereafter referred to as Model I, Fig. 4.1) of vesicle fusion involves at least three SNARE complexes,

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each coupled with a Ca^{2+}-bound synaptotagmin molecule, in order for a single vesicle to fuse. The Ca^{2+} binding site on the SNARE complex, which has been suggested based on the crystal structure (Sutton et al., 1998) may not form until the complex is at least partway coiled. The cooperativity of this macromolecular interaction could be derived from the complete "zippering" of one SNARE complex in response to Ca^{2+}-dependent synaptotagmin binding, perhaps allowing the other SNARE complexes to fully zipper through formation of additional Ca^{2+} binding sites. Ca^{2+} bridging between synaptotagmin and the SNARE complex would direct the final formation of the SNARE complex. In this model, either the Ca^{2+} binding pocket of the synaptotagmin C_2B domain may contribute to this cooperative interaction.

Alternatively, another model (Model II, Fig. 4.2) would require that Ca^{2+}-dependent phospholipid binding by synaptotagmin initiate the process of SNARE coiling by bringing the vesicle close to the presynaptic membrane, allowing v- and t-SNARES to engage one another in a stochastic manner (Hu et al., 2003). Here, coiling of one SNARE complex increases the probability of coiling of the subsequent complexes; Ca^{2+} was only needed to start the process. In this model synaptotagmin may not directly interact with the SNARE complex or it may interact independent of Ca^{2+} bridging. There is very recent data using purified native proteins (to avoid the bacterial contaminant issue with recombinant synaptotagmin) that show a high affinity Ca^{2+}-independent interaction between synaptotagmin and syntaxin-SNAP-25 heterodimers (Rickman et al., 2003). Thus, a new model of Ca^{2+}-dependent exocytosis is beginning to resolve with SNARE proteins conferring cooperativity to the

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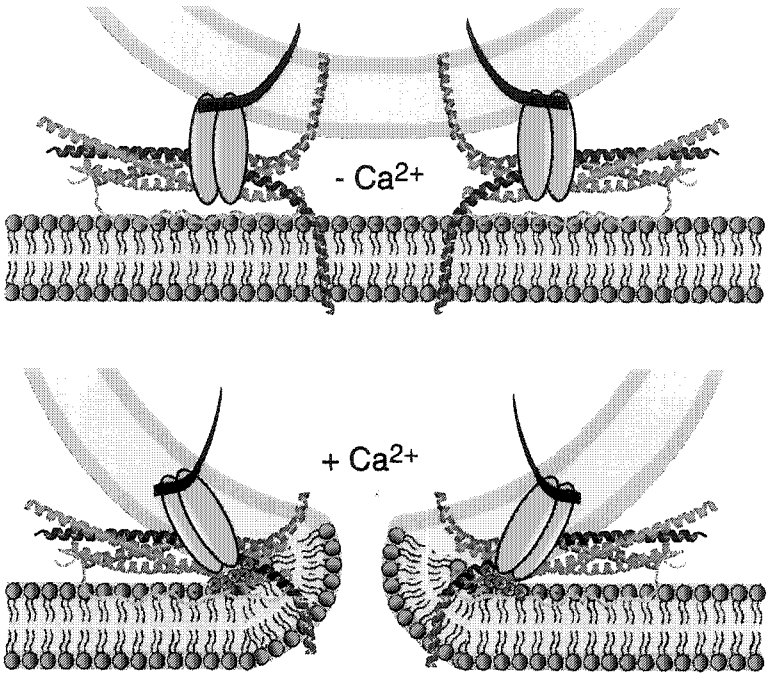

Figure 4.1 Model I. Ca^{2+}-dependent synaptotagmin (yellow ovals) binding to the SNARE complex triggers fusion. Ca^{2+} -independent interaction between synaptotagmin and SNAREs may mediate docking. Cooperativity is derived from formation of Ca^{2+} binding sites on coiled SNARE complexes. At least three SNARE complexes may be required for fusion.

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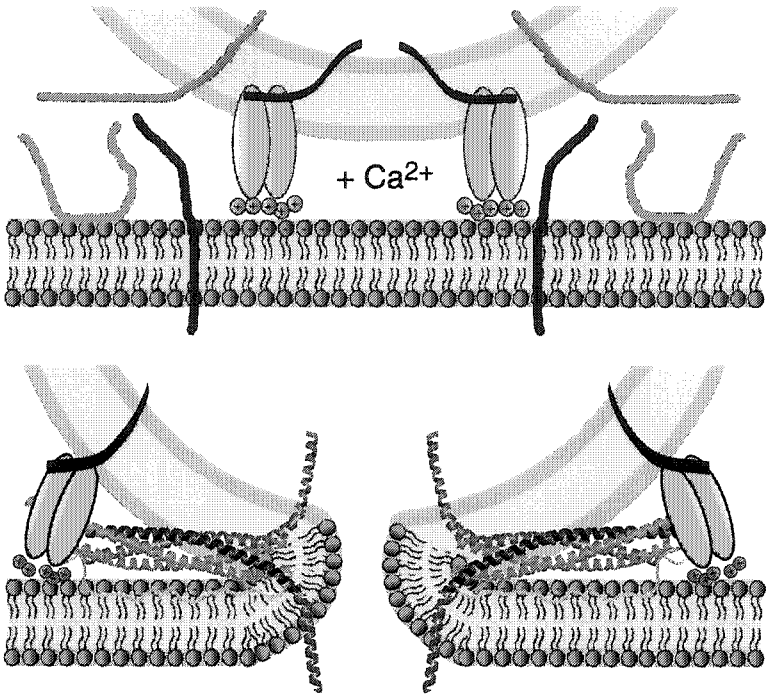

Figure 4.2 Model II. Ca^{2+}-dependent synaptotagmin (yellow ovals) binding to negatively charged phospholipids allows the v-SNARE, VAMP, to associate with t-SNAREs, SNAP-25 (green), and syntaxin (red). Cooperativity is derived from the stochastic interaction between v- and t-SNAREs which permits coiling of each SNARE complex. Complete zippering of three or more complexes would be sufficent for membrane fusion. Synaptotagmin may not directly interact with SNAREs.

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process and synaptotagmin, perhaps through its Ca^{2+}-dependent interaction with phospholipids, setting the apparent Ca^{2+} affinity of synaptic transmission.

Mechanism of apparent Ca^{2+} affinity

We know from Dodge and Rahamimoff and others that increasing $[Mg^{2+}]$ without altering $[Ca^{2+}]$ shifts the cation dose-response curve of synaptic transmission to the right of the Ca^{2+} dose-response curve without changing the cooperativity coefficient (Dodge and Rahamimoff, 1967; Augustine et al., 1987). It is also well known that Sr^{2+} can substitute for Ca^{2+} in evoked release, yet is much less effective at triggering fast synaptic transmission at the same concentration (Dodge et al., 1969; Augustine et al., 1987; Goda and Stevens, 1994). Sr^{2+} substitution most likely does not change cooperativity (Meiri and Rahamimoff, 1971; Augustine and Eckert, 1984; Goda and Stevens, 1994; Shin et al., 2003) (see also (Xu-Friedman et al., 2000)). Sr^{2+} has been presumed to target the Ca^{2+} sensor, probably synaptotagmin, but until recently, this was unsubstantiated. A recent study by Shin, et al.(Shin et al., 2003) has demonstrated that Sr^{2+} failed to evoke any fast, synchronous component of transmitter release in syt^{null} hippocampal cells, suggesting that *syt I* is a major target for Sr^{2+}, as well as Ca^{2+}, at the synapse.

This finding correlates well with what is known about the Sr^{2+} binding properties of synaptotagmin. In biochemical experiments, other divalent cations may substitute for Ca^{2+} in triggering synaptotagmin I C_2A domain binding to syntaxin or phospholipids with the order of: $Ca^{2+} > Ba^{2+}, Sr^{2+} >> Mg^{2+}$ (Davletov and Sudhof, 1993; Chapman et al., 1995; Li et al., 1995a). Indeed, the C_2A domains of synaptotagmin I and II have a dramatically lower apparent

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affinity for Sr^{2+} than Ca^{2+} when assayed by cation-dependent phospholipid binding (Li et al., 1995a). Shin, et al.(Shin et al., 2003) determined that Sr^{2+} is most effective at substituting for Ca^{2+} in phospholipid binding mediated exclusively by the C_2B domain of *syt I*. Both Sr^{2+} and Ba^{2+} were able to mediate phospholipid binding by the C_2B and C_2AB cytoplasmic fragment of synaptotagmin (Shin et al., 2003). Thus, it is likely that substitution of cations other than Ca^{2+} does not change the nature of the cooperative intermolecular interaction required for synaptic transmission, but rather, other cations function as weakly effective agonists with a lower affinity for synaptotagmin's Ca^{2+}-binding site. If this were true (as proposed in Model II), you would expect that by altering the Ca^{2+} binding sites of synaptotagmin, thereby changing the intrinsic affinity of the binding pocket for Ca^{2+}, you should also decrease the apparent Ca^{2+} affinity of synaptic transmission without altering cooperativity.

Indeed, mutation of several different synaptotagmin residues from both the C_2A and C_2B domains does reduce the apparent Ca^{2+} affinity of synaptic transmission as discussed above. In C_2B, mutation of the Ca^{2+} binding aspartates (Mackler et al., 2002), the point mutant AD3 (DiAntonio et al., 1993; Littleton et al., 1994; Littleton et al., 2001; Yoshihara and Littleton, 2002) (where the mutation is adjacent to D2), and the AD1 mutant that lacks the entire C_2B domain (DiAntonio et al., 1993; Littleton et al., 1994; Littleton et al., 2001; Yoshihara and Littleton, 2002) all exhibited a reduced amplitude of exocytosis accompanied by a reduced apparent Ca^{2+} affinity. High concentrations of Ca^{2+} were able to partially rescue evoked release in the AD3 line, but not the other

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C_2B mutants (Littleton et al., 2001; Yoshihara and Littleton, 2002). Mutation of mouse C_2A residue R233Q, a residue adjacent to the Ca^{2+} binding pocket that contributes to Ca^{2+}-dependent phospholipid binding, also decreased both the amplitude of exocytosis and the Ca^{2+} affinity (Fernandez-Chacon et al., 2001). Unexpectedly, mutation of Ca^{2+} binding aspartate residues in C_2A did not alter Ca^{2+} affinity in either Drosophila or mouse (Fernandez-Chacon et al., 2002; Robinson et al., 2002). Of all of the synaptotagmin mutations that decrease apparent Ca^{2+} affinity, only the AD1 mutant changed the cooperativity of synaptic release as well. Together these data suggest that synaptotagmin, as a "Ca^{2+} sensor", is more intimately involved in determining the apparent Ca^{2+} affinity of evoked release than cooperativity.

Is the mechanism controlling apparent Ca^{2+} affinity linked to phospholipid binding by synaptotagmin? Unfortunately, the correlation between the decreased Ca^{2+} affinity caused by these mutations *in vivo* and phospholipid binding by mutant recombinant synaptotagmin *in vitro* has not been precise. For example, both AD1 and AD3 mutants bind phospholipids as well as control (Littleton et al., 2001). This is not surprising since the AD1 mutant is essentially an isolated C_2A domain which readily binds phospholipids *in vitro* (Davletov and Sudhof, 1993; Chapman and Jahn, 1994). Curiously, the D-N mutation in the C_2A Ca^{2+} binding site in Drosophila that did not alter synaptic transmission disrupted Ca^{2+} dependent phospholipid binding (Li et al., 1995b; Fernandez-Chacon et al., 2002; Robinson et al., 2002). On the other hand, both Drosophila C_2B D-N and mouse C_2A R233Q mutants both had severe deficits in

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Ca^{2+} dependent phospholipid binding of *isolated* C_2 domains that *did* correlate with the severity of their physiological phenotypes (Fernandez-Chacon et al., 2001; Mackler et al., 2002). When the C_2AB fragments bearing the D3,4N or D1,2N mutations in Drosophila were assayed for phospholipid binding in the same assay, however, they were not different than control (data not shown). Thus, the physiology of these mutants does not reliably predict the outcome of *in vitro* phospholipid binding experiments, most likely because of peculiar biochemical properties of synaptotagmin. Many experiments have shown that C_2 domains can function as independent phospholipid binding "modules" (Davletov and Sudhof, 1993; Ubach et al., 1998; Desai et al., 2000; Nalefski et al., 2001; Ubach et al., 2001; Bai et al., 2002). In addition, there also appears to be functional redundancy between the two C_2 domains when the whole cytoplasmic domain is present (Earles et al., 2001; Bai et al., 2002). If phospholipid binding is a major function of synaptotagmin that determines apparent Ca^{2+} affinity, it will need to be demonstrated by an experimental design other than liposome binding assays.

One lab has taken a slightly different approach to this problem by using Sr^{2+} to distinguish between cation-dependent phospholipid vs. SNARE binding in synaptotagmin function. As stated before, Sr^{2+} was able to mediate cation-dependent phospholipid binding *in vitro* via the C_2B domain alone or in combination with C_2A, even if C_2A was mutated to prevent cation binding (Shin et al., 2003). In contrast, in the same study, Sr^{2+} was unable to stimulate binding of synaptotagmin I (isolated domains or the cytoplasmic fragment) to SNAREs using three different experimental approaches to address the same question.

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Since Sr^{2+} nearly abolishes the fast component of synaptic transmission, and requires synaptotagmin, the authors proposed that, at least in neurons, fast synaptic transmission is mediated by Ca^{2+}-dependent interaction between the C_2B domain of *syt I* and phospholipids and not SNAREs. This argument is bolstered by the recent evidence for a high affinity Ca^{2+}-independent interaction between synaptotagmin and SNAREs as mentioned above (Rickman et al., 2003). Synaptotagmin has been reported to interact simultaneously with phospholipids and SNARE complexes in the presence of Ca^{2+} (Davis et al., 1999) although the molecular mechanism is still unknown. These data support Model II discussed above, where the Ca^{2+} binding domain mediates Ca^{2+}/Sr^{2+} binding to phospholipids, while synaptotagmin binding to the SNARE complex is either non-existent or Ca^{2+}-independent. This remains a novel hypothesis that needs to be rigorously tested.

We still cannot completely rule out a role for Ca^{2+}-dependent synaptotagmin-SNARE interactions in determining the apparent Ca^{2+} affinity of synaptic transmission (Model I). Different serotypes of Botulinum neurotoxins (BoNt) cleave SNAP-25 at specific sites with variable effects on vesicle secretion that may provide insight into synaptic function. BoNT A cleaves the 9 C-terminal amino acids of SNAP-25 and partially inhibits exocytosis while decreasing the apparent Ca^{2+} affinity in both PC-12 cells and neurons while BoNT E cleaves 26 amino acids from the C-terminus of SNAP-25 and nearly blocks exocytosis (Lundh et al., 1977; Capogna et al., 1997; Gerona et al., 2000). The cooperativity of evoked exocytosis, however, was unaltered by either BoNT A or E in the majority of studies (Lundh et al., 1977; Capogna et al., 1997; Gerona

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et al., 2000) (see also(Simpson, 1978)). Interestingly, BoNT A, but not BoNT E reduction in synaptic transmission can be partially rescued by stimulation in high extracellular Ca^{2+} concentration or by using a Ca^{2+} ionophore (Lundh et al., 1977; Capogna et al., 1997; Xu et al., 1998; Gerona et al., 2000). One study also found that BoNT A toxicity can also be partially rescued with Sr^{2+} (Capogna et al., 1997). This finding again raises the question of where Sr^{2+} is acting at the synapse. If Sr^{2+} does not mediate Ca^{2+}-dependent SNARE binding (Shin et al., 2003), that effectively rules out Model I. Model II provides a simple explanation where increasing the Ca^{2+} concentration increases the "on-time" of Ca^{2+} bridging of the synaptotagmin-phospholipid complex keeping the vesicle closely apposed to the presynaptic membrane. This would allow more time for the stochastic action of t-SNAREs "finding" their cognate v-SNAREs. Data demonstrating that the BoNT A cleavage of SNAP-25 does not alter formation or disassembly of the SNARE complex, but rather reduces its stability (Pellegrini et al., 1995), lends strong support for this model.

Despite the cohesiveness of Model II in explaining both Ca^{2+} cooperativity and apparent Ca^{2+} affinity, Model I that includes a Ca^{2+}-dependent interaction between synaptotagmin and SNAREs cannot be completely dismissed yet. From the crystal structure, the SNARE complex does appear to contain a putative cation-binding site at its C-terminus (Fasshauer et al., 1998; Sutton et al., 1998), made in part by aspartate residues from SNAP-25 (Zhang et al., 2002). Ca^{2+} reportedly promotes synaptotagmin binding to SNAP-25/syntaxin heterodimers as well as to heterotrimeric SNARE complexes (Davis et al., 1999; Gerona et al., 2000; Littleton et al., 2001). Mutagenesis of SNAP-25 indicates that several

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aspartate residues on the surface of the C-terminal α-helix are critical for Ca^{2+}-dependent binding of synaptotagmin (Gerona et al., 2000; Zhang et al., 2002). The BoNT A cleavage site occurs in between two of these aspartate residues (BoNT cleavage reviewed in (Pellizzari et al., 1999)). BoNT-E cleavage blocks Ca^{2+} dependent binding of synaptotagmin to SNAP-25 and BoNT-A modifies this interaction (Gerona et al., 2000). If Ca^{2+}-dependent binding of synaptotagmin to the SNARE complex were important for complete zippering (Model I), the ability of additional Ca^{2+} to rescue synaptic transmission following BoNT A cleavage would function through Ca^{2+} bridging between synaptotagmin and the SNAP-25 C-terminus. *In vitro* assays targeting the ability of synaptotagmin Ca^{2+} binding site mutants (D1,2N and D3,4N) to engage SNARE heterodimers may begin to address the feasibility of this model.

Conclusion

In conclusion, through the synthesis of both biochemical and genetic studies, two main models of synaptotagmin function have emerged (which I have presented as Model I and Model II). While early genetic studies suggested that synaptotagmin functions in many steps in the vesicle cycle, these two relatively simple models can explain results from most studies. These models focus on synaptotagmin's role as part of a Ca^{2+} sensing mechanism that acts prior to fusion, but does not rule out an additional function in endocytosis. Inconsistencies in biochemical procedures have plagued this field and have added unnecessary confusion to models of synaptotagmin function. The majority of these studies need to be repeated using current recombinant protein purification procedures. Novel ways to examine synaptotagmin-phospholipid

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interactions need to be developed. Most importantly future experiments need to be designed to *specifically* address how synaptotagmin-SNARE and synaptotagmin-phospholipid interactions differentially contribute to synaptic transmission *in vivo*.

The experiments needed to address this problem, however, are not trivial to perform, especially in neurons. Very few studies to date have used an *"in vivo"* mutagenesis approach to study specific residues in the SNAP-25 C-terminus (Zhang et al., 2002). Only one study so far using transfected hippocampal neurons in a slice preparation has addressed the effect of specific mutations of SNAP-25 *in vivo* (Finley et al., 2002). This group transfected neurons with a BoNT E resistant (D179K) mutant SNAP-25 and then treated the preparation with BoNT E to abolish release via native SNAP-25. The mutation conferring BoNT E resistance rescued synaptic transmission as well as the native protein, while site-directed mutants containing additional mutations provided much less rescue of EPSC amplitude and resulted in significantly more failures. Using this technique, they were able to test the function of several different motifs in SNAP-25. Unfortunately, this study did not address the Ca^{2+} dependence of these mutations, perhaps because of the challenging nature of this experiment.

To do an analogous test in Drosophila and assay for synaptic transmission deficits would be equally challenging. First, Drosophila neurons are resistant to Botulinum toxins because Drosophila SNAP-25 lacks the toxin recognition site (Washbourne et al., 1997), however, with some work, one could be engineered. Second, a genetic strategy other than the UAS/Gal4 expression system will have

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to be used for analysis of SNAP-25 mutants, because in *SNAP-25^{null}* mutants, SNAP-24 is upregulated and functionally rescues this mutant (Vilinsky et al., 2002). If targeted gene replacement of SNAP-25 with a mutant form were possible in Drosophila, one could mutate homologous residues in the C-terminus that would correspond to BoNT A treatment in mammals, which should reduce, but not abolish synaptic transmission. You could then assay the ability of Ca^{2+} to rescue synaptic transmission in the presence of various site–directed synaptotagmin mutants. In particular, it would be important to test if Ca^{2+} binding aspartate residues or the polylysine motif in synaptotagmin is required to rescue synaptic transmission. The ability of Sr^{2+} to partially mimic the effect of Ca^{2+} may be further used to separate the two mechanisms of phospholipid vs. SNARE binding as suggested previously (Shin et al., 2003). If Ca^{2+} but not Sr^{2+} rescued evoked release and the Ca^{2+} binding aspartate residues were required for this response, this would provide strong evidence in favor of Model II. We must find a way to determine which interactions described *in vitro* are occurring as a part of synaptotagmin's normal function *in vivo*.

Finally, with the prospect of challenging and creative approaches to studying synaptic transmission on the horizon, recall the "simple" model of the Ca^{2+} sensor "X" postulated almost 40 years ago by Dodge and Rahamimoff that requires the action of 4 Ca^{2+} ions in a "certain small area". Since then it has become clear that the mechanism of Ca^{2+} sensing at the synapse is anything but simple. This mechanism most likely functions in a stochastic manner through the interaction of Ca^{2+} with a macromolecular complex that involves synaptotagmin in addition to either phospholipids and/or the SNARE proteins.

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Although most attention has focused on synaptotagmin as "the Ca^{2+} sensor" I predict that a paradigm shift is underway, wherein both phospholipids and/or SNAREs will soon be considered an integral part of the Ca^{2+} sensor "X" as well.

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Appendix A: Creating high-resolution protein structure images

1) Download PDB file from the Brookhaven protein database on a Mac in the lab. Example 1DQV.pdb. (lys.pdb is an edited version of this .pdb file which only has the C2B domain in it. To alter an existing .pdb file, open into a text reader and make changes.) If you do not have to make major changes to the .pdb text and just want to go forward with it, open RasMac and open the .pdb file. You should see wireframe model of the molecule. From the *molecule* window (not the text command window) choose "Save as". Give the file a new name (ie. c2b.pdb). Use this RasMol version of the .pdb file to go forward. If you open this into a text reader you will see that a lot of the header info and duplications have been removed. Burn the new .pdb file onto a CD. (In theory the zip drive on the SGI should also work, however as of 3/02 it was not working).

2) Hit any key to wake up the SGI Octane computer. Double click to the Reist user icon and login as "reist" with the password "chara00".

3) Insert CD and drag your .pdb file from the CD icon to the Reist user directory (folder on the desktop, which you can also double click to get a big window that shows what's in your directory - use as if on a Mac). To make a new folder, from the Reist directory window, choose Actions fi Add new folder. Highlight the name and retype with what you want to call it. Then, HIT RETURN to save that new folder name, otherwise it will revert back.

   Note: Your .pdb file and all your molscript files will need to be in the same folder (= directory)!!

4) From the Toolchest (box of menus in upper left corner of screen), select Desktop fi Open Unix Shell. You will get a dark blue window with an "octane%" prompt. In order to type in this window it must be up front and the mouse pointer MUST be positioned within the window. To select this window to come to the front, you must click on the top bar. The rest of this protocol assumes that you know how to do this.

   You need to know how to navigate directories (folders) in the UNIX environment. Type a given command after the octane% prompt and hit return.

   To ask what directory you are currently in:
   octane% pwd

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It tells you:
usr/people/reist

This is the main reist folder or root directory, but you want to be in the new folder where you put the .pdb file. Use the "cd" command to "change directories".

To change to the "root" directory at any time:

octane% cd

It will not tell you anything after this. You can use "pwd" to double check if you want.

At any time to ask what files are in your current directory:

octane% ls

It tells you a list of your files:
file1.pdb
file2.in
file3.ps

When you first open the unix shell you are in root. To change to the directory with your .pdb file type:

octane% cd foldername

To check that your file is there:

octane% ls
ratc2b.pdb

Now that you are in the correct directory you can proceed.

5) To open the .pdb file in Rasmol, type "rasmol c2b.pdb" at the octane% prompt. This basic syntax: "program filename" is how you tell the computer to use that program to open that file. The file will open as a wireframe model.

6) Select Display fi cartoons from the menu in the molecule window to change to strands/loops/helices.

7) The dark blue UNIX shell window will now show the Rasmol> prompt like you would see in the command window of RasMac. Use this to perform any commands in your Rasmol model.

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For example:

Rasmol > select asp303
Rasmol> spacefill
Rasmol> color red

This will help you decide what rotation you want to get the best view -- these changes WILL NOT be preserved in Molscript.

8) Drag the molecule in Rasmol until you get approximately what rotation, you want. You will have more color and secondary structure options in Molscript so don't bother setting that up here. Rasmol's secondary structure determinations are based on its own best guess and do not necessarily agree with published NMR or crystallography data. Consult the appropriate reference and you can reassign secondary structures in Molscript. Use this reference to determine the residue numbers of the residues that you have mutated or want to highlight.

9) You now need to construct an input file to take the molecule into the next program, Molscript. Input files are indicated by the suffix ".in". You can use the Rasmol file as a starting point, but it's best to also have a template to work from as well. If you are only working with rat syt I C2B from the ratc2b.pdb file, use the "dntemplate.in" input file as a starting point for assigning and coloring secondary structures. Otherwise you can also use any input file for simply understanding the syntax to assign colors, shading, and structures to other molecules.

10) Save your Rasmol file as a Molscript input file by using the write command after the Rasmol> prompt in the command window (UNIX shell):

****DO NOT give this file the same name as any of your .pdb files or else it will overwrite it and not warn you that this is happening. ****

Rasmol> write molscript d12n.in

To now quit Rasmol:

Rasmol> quit

Now the prompt returns to:
octane%

You have just written a basic input file into your current directory. The only info you will care about from this input file is the rotational coordinates. You need to put those coordinates into the dntemplate.in to begin making your final input file. Note that the dntemplate.in uses the ratc2b.pdb file in the same folder and has all the strands arranged according to Sutton's mouse sytIII paper. It also has two space-filled mutations in aspartate residues. You

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will need to add to or remove these mutations, but at least this preserved the nomenclature which was not easy to figure out.

11) Now you need to open both your Rasmol-made input file and the dntemplate input file in a text editor program called "Nedit" in order to merge them. To do this:

octane% nedit d12n.in
octane% nedit dntemplate.in

Alternatively, once the first nedit window is open, you can choose "open" and open the second file into its own window, like you would in any other program.

12) Now, select the rotation information from the Rasmol file, cut and paste it over the rotation information in the dntemplate.in file. Choose "Save as" and give this input file a new name.in. Always use the suffix ".in" for your input files. Change colors or residue mutations according to the nomenclature used in the template.

13) Once that is done, you are ready to take a low resolution look at the molecule:

octane% molscript <d12n.in> d12n.ps

This should open your Rasmol - made input file containing commands to construct the image in Molscript and convert it to a low resolution Post-script file. Look in the directory window, find and double click this file. Double clicking on the new d12n.ps icon in the directory will open the image in Adobe ShowPS software. If you like what you see, go on to the next step. If not, go back to Nedit and edit your input file. The Molscript manual will help with new commands and colors if you need something that is not in the template file. Remember to save the file each time before trying to view it.

14) To begin processing the Molscript file for final output resolution in yet another program, Raster3D, type:

octane% molscript -r <d12n.in> d12n.r3d

This will convert your Molscript file to a Raster3D file. A bunch of junk will scroll past on the Unix shell screen. Wait until it is done and you see a new octane% prompt.

15) When that is complete, type exactly "render -sgi <d12n.r3d> d12n.rgb". When stuff is done scrolling past, look in your user directory for a file called "render.rgb" and double click on it. It should open Imageview software and show you your final image. Choose SAVE AS and save the file as a TIFF (use the scrolling menu of filetypes in that window) and with whatever name you want with the suffix ".tif". It will appear in the selected directory.

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Tranferring the UNIX TIFF files to the Mac:

1) Make sure that you are in the directory that contains the TIFF files you wish to transfer. See step # 4 above.

2) From the dark blue UNIX shell window, type:

octane% ftp chiron.cvmbs.colostate.edu

It will ask for your login and password. Type each then hit return.

Once logged in:

octane% bin

It will say: type set to I

Then change directories on Chiron to put your files into the "OpenAccess" folder:

octane% cd /openaccess

It will say: CWD command successful

Then upload your files one at a time:

octane% put d12n.tif

You will watch it loading then it will say it was successful.

Then quit Chiron:

octane% quit

3) When you're done, type "logout" at the octane% prompt to leave the UNIX shell. Close all windows. Then go to the toolchest and choose Desktop fi logout.

To find your files on Chiron on the Mac:

1) Go to the Apple menu and select Chooser. From that window, choose AppleShare then choose Chiron from the list. Log in to Chiron and choose the "OpenAccess" folder. Your files will have a Unix icon and will be loose in the folder. Drag them onto the Mac.

2) Open files using Photoshop and edit as needed. They will come in as 72 dpi and will be upside down!! Use "rotate canvas" to flip them vertically. You will probably

3) Remember to delete your files off of Chiron after they have been transferred.

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When that is done and you want to print to the Codonics, follow these instructions exactly:

1) Open Netscape with two browser windows (File -> new browser) and go to the Departmental Codonics web page (http://codonics.cvmbs.colostate.edu/) on both windows. In one window click "Printer Status", in the other, click "Send a Print".

2) In "Printer Status", look over the settings to make sure the correct ribbon is in -- for molecular models, I use the CMY laminate for color.

3) In the "Send a Print" window, first scroll down and click the link to User Settings. Type in our username which is "fruitfly". Choose the Gamma, Contrast and TCR as desired; leave all else default and ignore MCM unless you know more about this thing than I do. IN GENERAL if you print without adjusting the Gamma, your image will come out much blacker than what you see on the monitor. So, IN GENERAL try setting Gamma = 2.5 in order to see the same brightness as on the monitor.

4) Go back to "Send a Print" by clicking the link at the bottom of the page. NOTE: these will be your settings unless you change them *immediately* before you print a subsequent image. Make sure "fruitfly" is in the username box. Click "Browse" to browse for the .tif file you wish to print. Click the "unscaled" button under the "Device" box. Click "Print".

5) Monitor the printer status on the "Printer Status" page. When the "1" has left the "jobs in the print queue", go look for your print. For problems with printing or print quality, other than those described here, see Mac.

dninput file:

```
! File: d34n.in
! Creator: RasMol Version 2.6
! Version: MolScript v1.3

plot
 window 60;

 read mol "ratc2b.pdb";
 transform atom *
  by centre position atom *
  by rotation x 180.0
  by rotation z 93.0341
  by rotation y -47.8355
  by rotation x -133.306;

set segments 200;
        set segmentsize .5;
        set shininess .5;
```


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```
        set shading .75;
        set colourparts on;
        set strandwidth 1.0;
        set strandthickness 1.0;
        set smoothsteps 1;
        set splinefactor 1.0;
 turn residue A272;
set residuecolour from A272 to A283 yellow;
 strand from A272 to A283;
set residuecolour from A283 to A287 white;
 coil from A280 to A287;
set residuecolour from A287 to A300 yellow;
 strand from A287 to A300;
set residuecolour from A300 to A308 white;
 coil from A300 to A308;
set residuecolour from A308 to A318 yellow;
 strand from A308 to A318;
set residuecolour from A318 to A323 white;
 coil from A318 to A323;
set residuecolour from A323 to A332 yellow;
 strand from A323 to A332;
set residuecolour from A332 to A336 white;
 coil from A332 to A336;
set residuecolour from A336 to A347 yellow;
 strand from A336 to A347;
set residuecolour from A347 to A356 white;
 coil from A347 to A356;
set residuecolour from A356 to A364 yellow;
 strand from A356 to A364;
set atomcolour in residue A363 rgb .95 .05 .15;
      cpk in residue A363;
set residuecolour from A364 to A369 white;
 coil from A364 to A369;
set atomcolour in residue A365 rgb .95 .05 .15;
      cpk in residue A365;
set residuecolour from A369 to A378 yellow;
 strand from A369 to A378;
set residuecolour from A378 to A385 white;
 coil from A378 to A385;
set residuecolour from A365 to A395 white;
 helix from A385 to A395;
set residuecolour from A395 to A402 white;
 coil from A395 to A402;
set residuecolour from A402 to A407 yellow;
 strand from A402 to A407;
set residuecolour from A407 to A409 white;
 coil from A407 to A409;
set residuecolour from A409 to A417 white;
 helix from A409 to A417;
```


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```
set residuecolour from A417 to A419 white;
  coil from A417 to A419;
set atomradius in residue A500 1.5;
        set atomcolour in residue A500 rgb .50 1 0;
        cpk in residue A500;
set atomradius in residue A501 1.5;
        set atomcolour in residue A501 rgb .50 1 0;
        cpk in residue A501;

end_plot
```

Sample input file to spacefill D1 and D2 in Drosophila C_2B:

```
! File: d12n.in
! Creator: RasMol Version 2.6
! Version: MolScript v1.3

plot
  window 60;

  read mol "ratc2b.pdb";
  transform atom *
    by centre position atom *
    by rotation x 180.0
     by rotation z 93.0341
    by rotation y -47.8355
    by rotation x -133.306;

  set segments 200;
        set segmentsize .5;
        set shininess .5;
        set shading .75;
        set colourparts on;
        set strandwidth 1.0;
        set strandthickness 1.0;
        set smoothsteps 1;
        set splinefactor 1.0;
  turn residue A272;
set residuecolour from A272 to A283 yellow;
  strand from A272 to A283;
set residuecolour from A283 to A287 white;
  coil from A280 to A287;
set residuecolour from A287 to A300 yellow;
  strand from A287 to A300;
set residuecolour from A300 to A308 white;
  coil from A300 to A308;
set atomcolour in residue A303 rgb 1 .65 0;
```


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```
      cpk in residue A303;
set residuecolour from A308 to A318 yellow;
 strand from A308 to A318;
set atomcolour in residue A309 rgb 1 .65 0;
      cpk in residue A309;
set residuecolour from A318 to A323 white;
 coil from A318 to A323;
set residuecolour from A323 to A332 yellow;
 strand from A323 to A332;
set residuecolour from A332 to A336 white;
 coil from A332 to A336;
set residuecolour from A336 to A347 yellow;
 strand from A336 to A347;
set residuecolour from A347 to A356 white;
 coil from A347 to A356;
set residuecolour from A356 to A364 yellow;
 strand from A356 to A364;
set residuecolour from A364 to A369 white;
 coil from A364 to A369;
set residuecolour from A369 to A378 yellow;
 strand from A369 to A378;
set residuecolour from A378 to A385 white;
 coil from A378 to A385;
set residuecolour from A365 to A395 white;
 helix from A385 to A395;
set residuecolour from A395 to A402 white;
 coil from A395 to A402;
set residuecolour from A402 to A407 yellow;
 strand from A402 to A407;
set residuecolour from A407 to A409 white;
 coil from A407 to A409;
set residuecolour from A409 to A417 white;
 helix from A409 to A417;
set residuecolour from A417 to A419 white;
 coil from A417 to A419;
set atomradius in residue A500 1.5;
      set atomcolour in residue A500 rgb .50 1 0;
      cpk in residue A500;
set atomradius in residue A501 1.5;
      set atomcolour in residue A501 rgb .50 1 0;
      cpk in residue A501;

end_plot
```


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Appendix B: Drosophila transformation

A few weeks before:

1) Culture 10-20 bottles of yw flies @25°C. Make sure the bottles you use have lots of food in them, but don't get sloppy.

2) Culture 4-5 bottles of yw; Sco/Cyo flies @25°C.

One to two days before you want to inject:

1) Make a batch of fly vials, to have on hand when the embryos start hatching.

2) Make molasses plates: One 4x batch makes around 40 plates, this is usually enough for a week. Pour the autoclaved syrup mix into the lids of 35mm petri dishes arranged in clean 150mm petris. Put the lids on right away, these plates are like food to escapees. Parafilm the 150mm plates after the small plates have cooled. You can either PARAFILM THEM and put them in the fridge or leave them on the benchtop, the important thing is that they not dry out or pull away from the sides of the dish.

3) Flip enough yw's so that you get around 8 very crowded bottles (with no food). Put a grape plate over the top, tape it in place or flies will get out. Invert the bottle, date the bottom. Change out the plates once a day till you are ready to inject. The theory is that they will be thirsty when you finally put a plate on that has yeast paste, but another theory is that they don't like to lay on the new plates until the walls have reached a certain egg density.

4) Make agar plates: Use the same agarose that you would for a DNA gel, but not the really expensive low-melt stuff. In a pinch, the agar used for making fly food will also work, but it tends to weep more, so your flies don't stick to the tape as well. One 50ml, 2% batch (1.0g agarose/50 ml dH20) poured into a 60mm petri dish and left to cool will work for a week of casual injecting, or a day or two of intensive injecting. If you parafilm the petri dish after it is cool, it will last longer, especially if put in the fridge.

5) Make up DNA solution:

3 ml Wings Clipped helper DNA (~2μg/ml)
3 ml mutant maxi-prep DNA (~4μg/ml)
12 ml DNA injection buffer
12 ml sterile water
Store in 1.5ml screw cap tube in freezer

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6) Pull electrodes:

Settings on John Walrond's puller as of 6/3/99 are:

line 1 660, 50, 60, 255; line 2 660, 200, 60, 255

Store in large petri on benchtop.

The day you inject:

1) Fill electrodes. Fill tip with DNA delivered from a pulled 1cc syringe. Backfill with light mineral oil delivered from a 3cc syringe with a spinal needle. Keep electrodes for one day only.

2) Make fresh 50% bleach. This is by far and away the most important component we have found to this whole process. Use bleach that has been in a tightly capped container --Safeway brand works fine. Use tap water to dilute it out. Make fresh bleach every day you plan to inject. 150mls of 50% will definitely get you through a day of intensive injecting. If you are unsure of the bleach, pitch it and get new.

3) Fill the humidifier, using de-ionized water. Construct a paper funnel to direct the mist onto your workspace below the microscope. It is essential that you have mist on the eggs while you are lining them up.

4) Open a new cell strainer. It's ok to use old ones in a pinch, but you get a build-up of debri in the old ones over the course of the day, and it just goes a little faster if you don't have to sweep debris out of your way while you're lining up.

5) Clean out the watch glass, and fill the rinse water squirt bottle. Otherwise, the watch glass will develop bleach crystals overnight. This has not been proven to be bad, but given the trouble we've had with bleach in the past, it's a good idea to start fresh.

6) Put fresh desiccant in the desiccation chamber. Use enough to cover about half the bottom of the chamber. The chamber is a yellow pipette tip box. The desiccant sits in the bottom of the box, and the slides with the eggs are placed on the honeycombed platform, and the top put on. This is not an airtight system, though, and the desiccant does turn from blue to purple over a few days. The desiccation time was originally set with fresh desiccant, and you do get slightly better injections with fresh desiccant than with old. Dessication time should be 5-6 minutes.

7) Other things you will need:

paper towel on which to blot the strainer, post-bleaching
razor blade to cut agar and tape
slides with a white marking area
a spatula to get the agar out of the petri dish
a very small paint brush to manipulate eggs
a slightly larger paintbrush to transfer eggs

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double-sided sticky tape, in a dispenser
kimwipes to blot strainer
fresh yeast paste with dH20, stored in 2-5 ml culture tube or fruity baby food
Halocarbon 700 oil, in a 1 ml syringe, no needle.
a spinal needle
kimwipes
at least 2 timers
a bent spatula to munch up food in vials
a tool to pick up larvae (pin on a wood stick)

Notes: Five factors that absolutely affect injections: The bleach, the desiccation time, the age of the embryos, and keeping the slides flat and hydrated while the embryos are developing. The thickness of the oil is a factor, but a little too thick won't kill them as long as you are checking the slides at least twice a day. Too thin will kill them quickly, however. Desiccating the slides right on top of the desiccant seems to be too harsh on them, they need to be separated from the desiccant, or out in the air. Keeping the oil where you want it increases survivability a fair bit. Oxygen flowing in the development chamber doesn't seem to help very much now that we have properly working bleach, and it causes the food to dry out extremely quickly. A drop of water or baby food in the oil will cause many of the L1's to congregate, which can be a good thing, if you want them all in one place.

The injection procedure:

Lining up eggs:

1) Take fresh grape plates out, and put a tiny drop of yeast on each one. Exchange the plates that are on the yw bottles for the yeasted ones, and set a timer for 40-50 minutes.

2) While this timer is going, make sure you have your lining up area set up.

3) Just before the timer goes off, get another set of plates ready with a spot of baby food (or yeast) on them. Check the plates on the bottles after 30 minutes to assess how the flies are laying. If the numbers of embryos are good, flip the plates out for fresh.

4) Start another timer for 26 minutes. This is the amount of time you have to get the embryos dechorionated, lined up on the agar, and transferred to tape.

5) Turn on the humidifier, and make sure that the mist is hitting the area under the dissecting scope. Put some sort of block in front of the air intake to the light source, watery mist might short out the power supply.

6) Using the larger of the two paintbrushes, wipe the surfaces of the plates to remove the embryos. Using a slightly damp paintbrush will make this easier. Put the embryos in the sieve, which is set in the watch glass, and filled with

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Nanopure water. When you've transferred all the eggs, dump the water into the waste, fill the sieve with bleach solution (make sure that bubbles trapped under the sieve are removed) and start a 45 second timer.

7) While the timer is going, cut a piece of agar that is about 12 mm wide, and about an inch to two inches long. The length depends on how many embryos you want to line up.
8) Use the scooper to transfer it from the petri dish to a slide. If you are a lefty, have the white end of the slide on the right, vice versa if you're a righty. The width of the agar should be a bit less than the width of your tape, which will be placed lengthwise down a second slide, later.

9) After the timer goes off, remove the sieve from the watch glass, blot it on the paper towels. Dump the bleach in the watch glass out, and rinse the sieve in the watch glass 3x with water, blotting between each time. A final rinse with the sieve above the waste container , at an angle, will help wash all the eggs to one area of the sieve, which makes it easier to remove them.

10) Transfer the eggs to the agar block. Blot the sieve really, really well with a kimwipe and transfer eggs with larger paintbrush. Sort out all the obviously unsuitable eggs: the ones that are very clear with little floaties, and the ones that are more transparent at the ends than in the middle. What you want is an egg that has no chorion and is uniformly opaque white. The more selective you are at this stage, the more smoothly injections will go, and the fewer you will have to pop with the electrode.

11) Line up the embryos: 20-25 parallel embryos per column seems to be the best spacing. Try for 5-10 columns (50-100 embryos per slide). You want the "toothed" end of the embryo (anterior), to be pointing away from the white end of the slide, whether you are right or left handed. When you go to inject the embryos, you will want to inject the pole cell end (posterior), which is at the opposite end from the "tooth". Also, the anterior end is slightly narrower than the posterior end. Sometimes the "tooth" is not visible, and the relative sizes of each end are the only way to tell them apart.

12) It is important to get the embryos parallel, and the columns straight and parallel -- it makes it easier to inject. When the 26 minutes are up, or when you run out of suitable embryos, stop and check once more that the lines and embryos are straight, and make sure there are no larvae scudding about.

13) Put a piece of double-sided sticky tape on a new glass slide, length-wise. If you haven't injected yet that day, pull tape off the roll so that fresh tape is exposed. Make sure the tape is stuck down uniformly by pressing your finger into the tape. This will leave fingerprints, but doesn't seem to affect embryos sticking to the tape, and does help with keeping the embryos at the same level in the oil. We thought that the tape might be toxic to the embryos, but it does not appear to be. Try and get the tape straight down the middle, if it is crooked, all your lines will be crooked when injecting, and that increases the time needed. Make sure that the white ends of the slides are on the same side. Press the taped

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slide down onto the agar. Press firmly enough that the embryos make dents in the agar, and so that you can see agar sticking to the tape between eggs. Lift straight up. Do not rock side to side, end to end, or anything. You want the embryos to stick to the tape exactly as you lined them up. With a razor blade, cut the tape so that only about 1/16th of an inch of tape is around the block of embryos. This means less oil, which is better for the developing embryos.

14) Place the slide, eggs up, into the desiccation chamber, and put the lid on. For summer/fall injections, 6 minutes in the chamber seems to be the best. If it is actively raining, you might think about increasing to 7 minutes, but see how the first slide of the day is, and then make adjustments. During the winter, the time may have to be decreased, but this has yet to be determined.

15) After desiccating, remove the slide from the chamber, apply an 8-10 mm dollop of Halocarbon 700 oil to the slide, on top of the eggs, and spread it so that it covers all the eggs with the spinal needle. This oil will keep the de-chorionated eggs from drying out, but still allow enough oxygen to penetrate so they don't suffocate. The most important thing here is to make it as absolutely thin as possible, without exposing the tops of the eggs. If you spread the oil so that it just goes over the edges of the tape, it has less of a tendency to ball up and expose the eggs on the edges.

16) Mark the slide with the date, the number of embryos on the slide, the desiccation time, and the DNA you're going to inject it with. Record this on a log sheet.

Injecting embryos:

17) The stage is vertically fixed, so it is the eyepieces that move vertically. Move the electrode up with the micromanipulator so that it is well above the oil on the blank slide. Remove the blank, and place the embryo slide in the holder such that the white side is towards the left. If you've removed the embryos from the agar correctly, this will mean that all the posteriors are pointed towards the electrode, which is also to the left. Looking through the microscope, with the electrode still above the plane of the slide, move the stage so that the electrode is positioned at the last row of embryos to be injected, so that all subsequent stage movements will drag the electrode backwards, not push the electrode forwards. This will help avoid snags that could break the tip of the electrode.

18) Under transillumination, it is easy to tell which embryos are too old to inject. Any embryo that has a clear ring around the edge, or clear ends, is too old. Any embryo that is obviously cellularized (with what looks like a notochord) is too old. You want embryos that have their contents uniformly distributed, with no clear anywhere. All others must be popped with the electrode, because there is no possibility that the DNA could incorporate correctly after the pole cells cellularize, and the clear spot at the posterior end is where the pole cells are.

19) If the desiccation time is correct, when you advance the electrode into the embryo, it will dimple, then it will pop through the membrane. If the time is too

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short, when you insert the electrode, a spurt of egg contents will come out. If too long, you will be able to push the membrane about half the length of the embryo before piercing it. The goal is to dimple the egg, pierce it, inject a small amount of DNA (a clear spot will be formed in the posterior 1/3 of the embryo is done correctly), and remove the electrode without any liquid coming out of the embryo. The finer the electrode, the easier it will slide in and out of cell without causing trauma.

20) After injecting or popping all embryos on the slide, return to the lab, and re-evaluate the oil. Make sure it is evenly spread, and is covering the edges of the tape. This also gives it a greater tendency of spreading out, however, so wipe the slide well, just outside of the oil. Spread a thin line of yeast paste around the oil, keeping it outside of the oil. The space between the oil and the food allows the larvae to get completely out of the oil, which they prefer. Try to keep the food from hitting the edge of the slide, because this can encourage wandering, and you don't want to lose any survivors.

21) In a clean 150 mm petri dish, place two kimwipes that have been soaked with water. It is important to use a fresh dish here, because mold and bugs will grow in the dishes, given enough time, water, and yeast. Put the slide, eggs up, in the dish, cover, and put on a flat space on the bench.

22) Check the slide under the dissecting scope 2x/day for the next two days. Have at least one vial of food available that has been churned up to make it softer, then flattened out. You will want one vial for every 25 larvae. The reason to flatten out the churned up food is so that it is easier to keep the vials hydrated later. You will get a few hatchers on the first day, but the big hatch will be on the morning of the second day. You will not get very many on the evening of the second day, and after three days, anything that has not hatched is dead. There is a log sheet to record date, DNA, slide #, desiccation time, # on slide, # hatched, etc. If you get very few survivors, there are a few things that could have happened:

a) The oil was too thick, or too thin. Shriveled, crispy-looking eggs means the oil was too thin. Developed, but for some mysterious reason, unhatched embryos can mean too that the oil was too thick.

b) The bleach was bad. If one slide of the day doesn't hatch well, it's most likely the oil. If all don't hatch well, think about the bleach. Dechorionating times longer than 30 seconds can indicate a problem with the bleach.

c) Too many old embryos on the slide that had to be popped, will give you a bad yield. This is why early sorting is important.

d) A bad electrode can also be the culprit, but again, it should not be all the slides from the day, assuming you changed electrodes at least once.

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e) Incorrect desiccation time can also be a problem, but that should be corrected within a few slides of the day.

23) Once the larvae are put into vials, and incubated at 25°C, it will take about 10-12 days for them to hatch into adults. Hydration is very important during this time. When it gets close to the hatching date, you need to check the vials twice a day, because it is good to get the females as virgins, if at all possible. Good notes as to who came from what vial, on what day, and at what time, are very important here. These data should be recorded on a log sheet.

24) When you have adults, cross each individual with three-four yw;Sco/CyO's of the opposite gender, put at 25°C, and record on the log sheet. After 10 days, remove the parents.

25) Start checking progeny at around 14 days, for red eyes. Record each date the number, gender, and eyes color of progeny. You will only be able to check the vial for a maximum of 12 days after the first progeny hatched, otherwise you could start running into F2's.

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Appendix C: Intracellular Recording: Synaptic Potentials in Muscle

Introduction:

In this lab you will record both spontaneous and evoked excitatory junction potentials (EJPs) from the abdominal muscles of third instar Drosophila larvae. You will each analyze the amplitude of evoked EJPs and the frequency and amplitude of spontaneous mEJPs. To demonstrate the effect of extracellular Ca^{2+} concentration on synaptic potentials, each group will perform their experiments in saline containing different unknown Ca^{2+} concentrations.

Objectives:

1) To understand he basic concepts of synaptic vesicle release

2) To record and interpret both spontaneous and evoked EJPs at the Drosophila NMJ

3) To understand the effect of extracellular Ca^{2+} concentration on synaptic transmission

4) To recognize the benefits and limitations of the Drosophila NMJ as a model system

Procedure:

1) A third instar larval preparation will be dissected and provided to you pinned in a Sylgard dish. The prep will be immersed in Drosophila HL3 saline of an unknown Ca^{2+} concentration. "Standard" Drosophila HL3 saline contains: 70 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 20 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM trehalose, 115 mM sucrose, 5 mM HEPES. The possible test salines may have one of the following Ca^{2+} concentrations: 0.4 mM, 0.8 mM, 1.0 mM, 1.5 mM, 5 mM.

2) Your microelectrodes will be pulled for you, but you will need to fill them with the appropriate salt solutions: **The sharp intracellular electrodes should be filled with 3M KCl; the blunt, suction electrodes should be filled with the same saline in which your prep is immersed.**

3) Place the recording dish on the stage and stick down with wax while watching through the eyepieces to make sure the prep is centered in the field of view. Place the ground wire in the bath and top off saline if necessary. Place the suction electrode in the right micromanipulator. Place the sharp intracellular electrode in the left micromanipulator. One at a time, lower the electrodes into the saline and bring them into view just above the prep. Position the suction

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electrode over a segmental nerve; apply suction using the attached syringe until the nerve is sucked into the electrode.

4) "Zero" the sharp intracellular electrode while it is in the bath using the following method:

1) "Remove" the junction potential at the electrode tip by turning the input offset dial until Vm reads 000.

2) Apply a 1.0 nA current using the step command. "Remove" the electrode resistance by turning the bridge dial until Vm reads 000.

5) Now penetrate the membrane of muscle 6 in the segment just caudal to the nerve you've sucked up by GENTLY advancing the electrode until you read a membrane potential on the Axoclamp.

6) Record the following information on your worksheet: filename, muscle fiber number, segment number, left or right, starting membrane potential.

7) To record mEJPs: Start the program "Chart" on the Mac. You will see a new window open up. If the membrane potential is not at -55 mV, pass DC bias current to "clamp" the membrane potential at -55 mV. Now, simply click on "Start' in the bottom right hand corner to start recording. Monitor the membrane potential during the recording session to make sure it stays at -55; adjust current if necessary . Record for 1 minute (monitor time in upper right hand corner of screen), then click "Stop". Save your file.

8) To record EJPs: Set stimulus isolator to 50 nA. Start the program "Scope" on the Mac. You will see a new window open up. Again,monitor the membrane potential to keep it at -55 mV. The program is set to record 10 evoked events, timed every 30 seconds apart. When ready, click on "Start" in the lower left-hand corner of the screen.

9) When the run is finished, save your file. Carefully withdraw your electrode from the muscle fiber and expel the nerve from the suction electrode. Repeat steps 4-8 for at least one other muscle fiber. Let your partner have a turn at the controls!!

10) I will transfer you files to another computer for analysis using the same Chart and Scope software. You will each analyze mEJP frequency and amplitude as well as evoked EJP amplitude. Use an Excel spreadsheet to calculate a mean mEJP frequency, and mEJP and EJP amplitudes for your prep. I will compile everyone's data into a few graphs showing the dependence of synaptic transmission on extracellular Ca^{2+} concentration.

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Questions:

1. Diagram the major steps of the synaptic vesicle cycle. Be sure to indicate where Ca^{2+} acts in the vesicle cycle.

2. How would you expect high extracellular Ca^{2+} concentrations to effect mEJP frequency?

mEJP amplitude?

evoked EJP amplitude?

3. If you created a Drosophila mutant in which the Ca^{2+} affinity of the "Ca^{2+} sensor" molecule on synaptic vesicles was increased and you performed the same electrophysiological assays we did today, what results would you expect and why? If you viewed the mutant synapses with electron microscopy, what distribution of synaptic vesicles within the nerve terminal would you expect?

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Name: _____
Date: _____

Unkown saline: _____

Known Ca^{2+} concentration: _____

Filename	Vm	Muscle/Segment	mEJP frequency	mEJP amplitude	EJP amplitude

Mean mEJP frequency: _____

Mean mEJP amplitude: _____

Mean evoked EJP amplitude: _____

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