DISSERTATION

THE ROLE OF CA^{2+}-DEPENDENT BINDING RESIDUES OF SYNAPTOTAGMIN IN NEUROTRANSMISSION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY BRIE PADDOCK ENTITLED THE ROLE OF CA^{2+}-DEPENDENT BINDING RESIDUES OF SYNAPTOTAGMIN IN NEUROTRANSMISSION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

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IN NEUROTRANSMISSION

Synaptic vesicle fusion and the concomitant release of neurotransmitter has long been a field of intense study. Synaptotagmin I is the Ca^{2+} sensor for this synaptic vesicle fusion, but the mechanism by which it triggers this fusion is still unclear. Synaptotagmin is composed of an intravesicular domain, a transmembrane domain through the synaptic vesicle and two tandem, cytosolic C_2 domains that both bind Ca^{2+}. Multiple Ca^{2+}-dependent interactions have been mapped to these C_2 domains, including those with SNAREs, the proposed fusion machinery, and the phospholipids present in both the vesicular and synaptic membranes. I have tested the importance for synaptic transmission of several residues that mediate phospholipid binding of both C_2 domains using site directed mutagenesis in *Drosophila* third instar larvae. These larvae all showed deficits of various severity in their evoked release consistent with the hypothesis that Ca^{2+}-dependent phospholipid binding by these residues is required for efficient synaptic transmission. I also demonstrate that Ca^{2+}-dependent phospholipid binding by C_2B is of greater physiological significance than that of C_2A. Taken together, these results suggest that Ca^{2+}-dependent phospholipid binding by the C_2 domains mediate the majority synaptotagmin's role as the Ca^{2+} sensor for evoked release.

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INTRODUCTION:

Chemical transmission relies on release of neurotransmitter, which is packaged in small, clear vesicles at the synapse. Fusion of these vesicles occurs after an action potential travels down the axon and opens voltage gated Ca^{2+} channels to allow Ca^{2+} influx into the presynaptic terminal. After this Ca^{2+} influx, multiple vesicles that are filled with neurotransmitter fuse within the submillisecond time scale, indicating that fusion is mediated by a preassembled machinery. SNAREs (soluble N-ethylmaleimide sensitive factor attachment receptor) are thought to mediate many membrane fusion events throughout the cell, including that of synaptic vesicles, but these fusion events are not Ca^{2+} dependent. Synaptotagmin I, a synaptic vesicle protein that interacts with both SNAREs and phospholipid membranes, is thought to be the Ca^{2+} sensor for synchronous, evoked neurotransmitter release. The mechanism by which synaptotagmin regulates fusion *in vivo* is still a matter of intense research.

To further understand the context in which I attempted to elucidate this mechanism, I will briefly discuss the pertinent history of and several current trends in the field of Ca^{2+}-dependent synaptic vesicle fusion. Historically, the timing and Ca^{2+} dependency of release through their suggestion of fusion machinery and a Ca^{2+} sensor, have led the field to our current state of understanding. The role of synaptotagmin, the SNAREs and the presynaptic membrane lipids in the preassembled fusion machinery will be examined. Lastly, several emerging topics in synaptic transmission and their contributions to synaptic transmission will be discussed.

A. Characteristics of Neurotransmitter Release

Chemical transmission between synapses occurs when neurotransmitter-filled vesicles fuse with the plasma membrane (1954; Heuser et al., 1979; Broadie et al., 1995). This membrane fusion event releases the neurotransmitter into the space between neurons, the synaptic cleft, where it is then free to act through receptors on the downstream cell. The release process is highly regulated and much research, including that presented here, has focused on understanding its mechanisms. Several characteristics of synaptic transmission have driven the search for the fusion machinery and its method of action. The foremost of these are 1) the timing of and 2) the Ca^{2+} dependence of synaptic transmission.

1. Timing of Synaptic Transmission

Electrophysiological techniques have enabled examination of the time course of synaptic vesicle release. Initial measurements of the synaptic delay, which is the time between action potential arrival and release of neurotransmitter, were on the millisecond to submillisecond time scale (Katz and Miledi, 1967; Llinas et al., 1981; Thomas et al., 1993). Calculations of diffusion constants predicted the time between Ca^{2+} influx and vesicle fusion to less than 200 μsec and further developments of electrophysiological techniques have supported that estimate (Reichardt and Kelly, 1983; Adler et al., 1991; Davis et al., 1999). This fast speed indicates that a precisely regulated fusion machinery is present and assembled, ready to drive neurotransmitter release in response to a signal.

The nature of both that machinery and the release signal are essential to understanding the mechanism of synaptic vesicle fusion and will be discussed below.

2. Ca^{2+} Dependence of Neurotransmitter Release

Release of neurotransmitter has long been known to be a Ca^{2+}-dependent process (del Castillo and Stark, 1952; Katz and Miledi, 1967). The advent of photolysable Ca^{2+} chelators that are concentration dependent has enabled determination of precise levels of intracellular Ca^{2+} that are required for neurotransmission (Kaplan and Ellis-Davies, 1988). The amount of Ca^{2+} required to stimulate release varies amongst systems; 1 μM intracellular Ca^{2+} is sufficient in chromaffin cells (Thomas et al., 1993; Heinemann et al., 1994), while 10-20 μM is sufficient at goldfish retinal bipolar synapses (Heidelberger et al., 1994). Many model synapses require approximately 10 μM intracellular Ca^{2+}, including rat Calyx of Held, squid giant synapse and crayfish neuromuscular junction (Hsu et al., 1996; Ravin et al., 1999; Bollman et al., 2000; Schneggenburger and Neher, 2000). These estimations may represent a lower range for the Ca^{2+} requirements as Ca^{2+} channels coincide with synaptic vesicle release sites (Roberts et al., 1990), and can create microdomains of transient, high Ca^{2+} concentration. These Ca^{2+} requirements helped to guide the search for the Ca^{2+} sensor and its mechanism of action. The current understanding of the two concepts of 1) a Ca^{2+}-sensing molecule and 2) a preassembled fusion machinery will be further examined in the following pages.

B. Molecular Determinants of Synaptic Vesicle Fusion

In the following section, I will discuss many components of the preassembled fusion machinery including the nature of the three major groups of molecular players in the process of Ca^{2+}-dependent vesicle fusion. These are synaptotagmin, the SNARE proteins, and the lipids of the fusing membranes. Synaptotagmin confers Ca^{2+}-sensitivity on membrane fusion while the SNARE proteins act as the fusion machinery. The characteristics of the membrane lipids themselves also play a role in the fusion process, and that will be further discussed.

1. Synaptotagmin: Ca^{2+} Sensor for Neurotransmitter Release

Synaptotagmin I has emerged as the primary candidate for a neural Ca^{2+} sensor, the molecule that confers Ca^{2+} sensitivity to the fusion machinery. Synaptotagmin is expressed on synaptic vesicles throughout the nervous system (Matthew et al., 1981; Perin, 1991) binds many components of the fusion machinery and has several structural characteristics that seem to support a role as neuronal Ca^{2+} sensor. Knocking out synaptotagmin abolishes synchronous, evoked neurotransmitter release in a variety of model systems from *C. elegans* to mouse (DiAntonio et al., 1993; Nonet et al., 1993; Geppert et al., 1994). This phenotype is conserved in cultured chromaffin cells (Voets et al., 2001). Not all evoked release is abolished, however, indicating that a second, asynchronous Ca^{2+} sensor may be present at the synapse (Geppert et al., 1994; Goda and Stevens, 1994; Zucker, 1999). The identity of a second sensor has remained elusive while much of the research has focused on synaptotagmin I, as it mediates the vast

majority of Ca^{2+}-dependent synaptic vesicle release. There are many isoforms of synaptotagmin, but only synaptotagmin I is discussed in this text, unless otherwise noted.

a. Synaptotagmin Structure

Synaptotagmin I is expressed on synaptic vesicles, binds many components of the fusion machinery and has several structural characteristics that seem to support a role as neuronal Ca^{2+} sensor. Synaptotagmin is comprised of an intravesicular domain, a transmembrane domain and two, large, cytosolic tandem C_2 domains [Fig 1, page 32, (Perin et al., 1991)]. The transmembrane domain passes through the vesicle membrane, anchoring the protein in place. The intravesicular domain plays a role in proper targeting of the protein to synaptic vesicles (Han et al., 2004). The C_2 domains that dominate the cytosolic portion of the protein also dominate its activity as a Ca^{2+} sensor for neurotransmission.

C_2 domains were originally identified in protein kinase C (PKC) as Ca^{2+} binding modules (Parker et al., 1986). In other proteins, they are often involved in the localization of cytosolic proteins to membranes via Ca^{2+}-dependent phospholipid binding, though not all C_2 domains bind Ca^{2+} or phospholipids (Rizo and Sudhof, 1998; Dai et al., 2004). C_2 domains are comprised of eight-stranded antiparallel β sandwich connected by flexible loops (Nalefski and Falke, 1996; Cho and Stahelin, 2006). The beta sheets are highly conserved, but the sequence of the loops varies widely amongst C_2 domains. Unlike most conserved lipid binding domains, C_2 domains do not bind a specific type of lipid, and the variable sequence of the connecting loops is thought to underlie this property (Cho and

Stahelin, 2006). This lack of specificity gives a flexibility in membrane preference that is not present in other lipid binding domains.

Synaptotagmin has two C_2 domains; C_2A which is membrane proximal and C_2B which is membrane distal. These two domains share many of the structural characteristics of other C_2 domains, though C_2B has two additional alpha helices (Sutton et al., 1995; Fernandez et al., 2001). The only change in structure of either C_2 domain in the presence of Ca^{2+} is an increased ordering of the Ca^{2+} binding loops, indicating that Ca^{2+}-synaptotagmin does not require a major conformational change to act as the Ca^{2+} sensor for neurotransmission. In both domains, Ca^{2+} binding is coordinated by aspartates in loops 1 and 3, though C_2A binds three Ca^{2+} ions while C_2B binds only two. These subtle structural differences may underlie the functional differences between synaptotagmin's two C_2 domains that will be outlined below.

b. Genetic Studies

Identification of the Ca^{2+}-coordinating of synaptotagmin's C_2 domains quickly led to site-directed mutagenesis engineered to disrupt Ca^{2+} binding. The five aspartates that coordinate Ca^{2+} binding to the C_2A domain were mutated to asparagines so that Ca^{2+} could no longer bind to the C_2A domain (Fernandez-Chacon et al., 2002; Robinson et al., 2002). Surprisingly, this mutant synaptotagmin was able to rescue the knockout phenotype. Regardless of whether all five were mutated individually or in tandem, the synapses maintained Ca^{2+} dependent release indicating that Ca^{2+} binding by the C_2A domain was not essential for synaptotagmin's role as the Ca2+ sensor (Fernandez-

Chacon et al., 2002; Robinson et al., 2002; Stevens and Sullivan, 2003). Alternatively, mutation of the homologous residues of C_2B led to severe deficits in viability and evoked release (Mackler et al., 2002; Nishiki and Augustine, 2004). *Drosophila* carrying mutations in the C_2B Ca^{2+} binding residues have decreased viability and evoked release as compared to those not expressing any synaptotagmin, indicating that the mutant protein has a dominant negative role that effects the residual Ca^{2+} sensor (Mackler et al., 2002). The differences observed between disruption of Ca^{2+} binding to the C_2A and C_2B domains indicates that the two C_2 domains do not share an equal burden of Ca^{2+} sensing in synaptic vesicle fusion.

2. SNARE Proteins

2a. Structure and Characteristics

The SNARE complex is the minimal machinery for membrane fusion (Weber et al., 1998). Three membrane associated proteins, VAMP (also called synaptobrevin), syntaxin and SNAP-25 (synaptosome associated protein of 25 kD) are assembled into the SNARE complex (Hanson et al., 1997; Lin and Scheller, 1997; Poirier et al., 1998; Sutton et al., 1998). The heterotrimeric SNARE complex consists of 4 parallel alpha helices; VAMP and syntaxin each contribute a single helix while SNAP-25 contributes two (Sollner et al., 1993; Sutton et al., 1998; Xiao et al., 2001). VAMP is classified as a vesicle SNARE (or v-SNARE) due to its anchoring in the vesicle membrane via its transmembrane domain (Sollner et al., 1993; Martin, 1997; Jahn and Sudhof, 1999; Lin and Scheller, 2000). Syntaxin and SNAP-25 are target SNAREs (t-SNAREs), syntaxin by rights of its transmembrane domain through the target membrane. SNAP-25,

however, associates with the target membrane via palmitoylation of a series of cysteines located in a largely unstructured region that connects the two alpha helices (Salaun et al., 2005). When uncomplexed, the individual members of the SNARE complex are susceptible to cleavage by clostridial neurotoxins, causing deficits in synaptic function that illustrate their importance to neurotransmitter release (Schiavo et al., 1992; Blasi et al., 1993; Schiavo et al., 1993). Syntaxin and SNAP-25 associate with each other to form a t-SNARE heterodimer (Rickman and Davletov, 2003). The entire complex assembles spontaneously (Sollner et al., 1993; Fasshauer et al., 2002). Once assembled, the complex is very stable and resistant to cleavage by clostridial neurotoxins and dissociation via SDS (Hayashi et al., 1994). These coils are thought to tighten and 'zipper up', bringing the membranes into fusable proximity. None of the three SNARE proteins, however, have ion binding sites with sufficient affinity to correspond to the levels of Ca^{2+} reached at stimulation, as discussed on page 4. This suggests further regulatory proteins are needed to confer the Ca^{2+} specificity of release (Sutton et al., 1998). While various isoforms of these proteins are expressed throughout the body and cell to perform a variety of fusion events, only the specific neuronal forms will be discussed in the following text.

2b. SNARE assembly

Examination of SNARE complex assembly through toxin studies has also increased our understanding of their function. Clostridial neurotoxins cleave only uncomplexed SNAREs (Hayashi et al., 1994). SNAREs appear to begin to assemble at their N-terminal ends with a stable intermediate before assembly of their C-terminal ends (Hua

and Charlton, 1999; Melia et al., 2002) This assembly is not enhanced by Ca^{2+} (Hu et al., 2002).

All the individual components of the SNARE complex are associated with membranes even before they assemble into the coiled coil of the complex. SNAP-25, which is largely unstructured when alone (Nicholson et al., 1998), is associated with lipid rafts through palmitoylation of several cysteines (Salaun et al., 2005). SNAP-25 and syntaxin associate to form a stable t-SNARE heterodimer on the target cell membrane (Rickman and Davletov, 2003).

2c. Concerns for Vesicle Fusion

While much of the field accepts SNAREs as the minimal machinery of fusion, there are a few lines of evidence that suggest otherwise. These include 1) a study showing Sr^{2+}-stimulated release does not require SNAREs, 2) restriction of neuronal synaptobrevin by the vesicular membrane and, mostly widely, 3) general criticisms of the membrane fusion assay used to demonstrate SNAREs as the minimal machinery for fusion.

Several divalent cations, including Sr^{2+}, can stimulate membrane fusion in the place of Ca^{2+} (Goda and Stevens, 1994; Shin, 2003; Bhalla et al., 2005). This fusion is thought to occur through the same membrane fusion machinery as Ca^{2+}-dependent fusion because Sr^{2+} binds to the Ca^{2+}-binding loops of synaptotagmin (Cheng et al., 2004). Sr^{2+} binding can stimulate exocytosis, but is unable to stimulate SNARE binding to the tandem C_2 domains of synaptotagmin (Shin, 2003). Thus, the Sr^{2+} binding to the Ca^{2+}-binding site

of synaptotagmin may be driving fusion while bypassing SNARE binding (Shin, 2003). Other studies have found Sr^{2+}-stimulated binding of synaptotagmin to t-SNAREs, and these interactions were detected in both recombinant (Chapman et al., 1995) and native (Bhalla et al., 2005) proteins. Thus, the role of SNAREs in Sr^{2+}-stimulated release remains controversial, though studying Sr^{2+} release is only useful insofar as it recapitulates Ca^{2+} binding, which is the native process.

Synaptobrevin has been found to be restricted by the membrane (Quetglas et al., 2000; Hu et al., 2002; Kweon et al., 2003). Synaptobrevin, like many transmembrane proteins, is often solubilized in detergent before it is assembled into liposomes (Weber et al., 1998). This solubilization is proposed to artificially free the SNARE motif of neuronal synaptobrevin from the vesicle membrane and allow interactions with the rest of the SNARE complex (Hu et al., 2002). Thus, the physiological mechanism of release is bypassed in most of the studies defining the SNARE complex as the machinery for fusion, though one study suggests that membrane apposition can free the restricted domain (Hu et al., 2002). Further work is needed to characterize the mechanism of synaptobrevin's release by the membrane to understand the role of SNAREs in Ca^{2+}-dependent synaptic vesicle fusion.

There are many criticisms of the liposome fusion assay that first demonstrated SNAREs as the minimal machinery for fusion, including 1) protein concentrations on the liposomes in excess of physiological levels 2) inability to replicate several *in vivo* characteristics of fusion, including Ca^{2+}-dependence of fusion with synaptotagmin.

An attempt to characterize all the components of a synaptic vesicle has yielded estimates of physiological levels of protein concentrations (Takamori et al., 2006). Each vesicle is estimated to have ~70 copies of synaptobrevin and only ~15 copies of synaptotagmin (Takamori et al., 2006). While the liposomes in the early liposome fusion assays were approximately the same size, they had approximately 750 copies of synaptobrevin per liposome (Weber et al., 1998). Liposomes, however, do not share all of the organizational characteristics of synaptic vesicles, so it is possible that specialized release sites exist on vesicles. Furthermore, synaptobrevin has been localized to membrane rafts within the vesicle (Chamberlain and Gould, 2002), suggesting a mechanism for increasing local protein concentrations, possibly to levels as high as those in the liposome fusion assays. Further characterization of sites of protein localization on the synaptic vesicle are needed to determine if the *in vitro* liposome fusion assays actually mimic the physiological protein concentration requirements for fusion.

Another major concern with the liposome fusion assay is its inability to replicate some of the key characteristics of neurotransmitter release (Rizo et al., 2006). The fusion documented in these studies is slow, taking on the order of hours to complete, while synaptic vesicle fusion occurs much more quickly (see page 4 for discussion). Additionally, synaptotagmin has been shown to stimulate fusion in a Ca^{2+}-independent manner in this assay, which does not recapitulate its role as a Ca^{2+} sensor (Mahal et al., 2002). This result is not in contradiction with other roles for synaptotagmin at the synapse, however, such as a docking and priming protein(Dodge and Rahamimoff, 1967).

While Mg^{2+} inhibits neurotransmitter release at the intact synapse, another study showed that both Ca^{2+} and Mg^{2+} stimulated liposome fusion (Dodge and Rahamimoff, 1967; Liu et al., 2005). Thus, characterization of liposome fusion must be strictly correlated to *in vivo* experiments before the results can be interpreted as highly relevant for synaptic vesicle fusion and the role of SNAREs as the minimal fusion machinery is still a controversial subject.

2d. Synaptotagmin and SNAREs

Synaptotagmin's association with the fusion machinery likely plays a major role in synaptic vesicle fusion. Association with members of the SNARE complex is one of the first characteristics that led to synaptotagmin's examination as a possible Ca^{2+} sensor. There are many aspects to synaptotagmin's binding to SNAREs and understanding of these interactions can lead to a clearer understanding of how synaptotagmin drives Ca^{2+}-dependent fusion.

Synaptotagmin has many Ca^{2+}-dependent interactions with both the SNARE complex and its components. Synaptotagmin binds to the coiled coil complex of all four helices, also called the ternary SNARE complex. This binding is promoted by Ca^{2+}, though does occur in the absence of Ca^{2+} (Gerona et al., 2000; Zhang et al., 2002; Rickman and Davletov, 2003; Rickman et al., 2006; Dai et al., 2007). Synaptotagmin only associates with neuronal synaptobrevin as part of the ternary SNARE complex (Gerona et al., 2000). Synaptotagmin, however does bind to both syntaxin and SNAP-25 individually in a Ca^{2+}-dependent manner (Bennett et al., 1992; Chapman et al., 1995; Li et al., 1995; Kee and

Scheller, 1996; Shao et al., 1997; Gerona et al., 2000; Zhang et al., 2002). Two different areas of SNAP-25 have been implicated in synaptotagmin binding. Sequence alignments of SNAP-25 isoforms that participate in Ca^{2+}-regulated exocytosis identified a series of aspartates present in a stripe along one coil of SNAP-25 [D172, D179, D186, and D193, (Zhang et al., 2002)]. Mutation of these residues led to decreased synaptotagmin binding as well as decreasing Ca^{2+}-dependent release from PC12 cells (Zhang et al., 2002). NMR also showed that these residues interact with synaptotagmin in a Ca^{2+}-dependent manner (Dai et al., 2007). A second set of residues on SNAP-25's other helix has also been proposed to bind to synaptotagmin [D51, E52, and E55, (Rickman et al., 2006)]. Disruption of these residues also decreased Ca^{2+}-dependent release from PC12 cells (Rickman et al., 2006). Interestingly, the aspartates initially identified by the Martin group and aspartate/glutamic acids by Rickman and Davletov are both postulated to interact with the polylysine motif of synaptotagmin's C_2B (Fig 2B, page 34). The Ca^{2+}-independent interaction implies a pre-fusion role for the synaptotagmin/SNARE interaction, possibly in docking or priming of synaptic vesicles for release (Loewen et al., 2006; Rickman et al., 2006; Dai et al., 2007).

Many *in vitro* studies have attempted to characterize the binding partners and characteristics of synaptotagmin. Anionic phospholipid and the SNARE complex have emerged as two of the main binding partners of synaptotagmin. Many of these studies have been in disagreement, mapping mutually exclusive interactions to the same residues (Shao et al., 1997; Fernandez-Chacon et al., 2001; Wang et al., 2003). This may be explained by considering the structure of the SNARE complex. The four alpha helices

that coil to create the ternary SNARE complex form a bundle of protein that has a polar exterior with an hydrophobic core (Sutton et al., 1998; Rickman et al., 2006). In studies where phospholipids were excluded from the study, the electrical composition of the SNAREs may mimic the polar headgroups and hydrophobic core of the lipid bilayer. Unlike most lipid-binding protein domains, C_2 domains bind to targets based on electrostatics rather than shape or conformation (Cho and Stahelin, 2006), thus may artificially bind the SNARE complex if lipids are excluded. As both SNAREs and lipid bilayers are present at the fusion site, both should be present in *in vitro* studies aimed at replicating the *in vivo* environment.

3. Lipids And Synaptic Transmission

3a. Lipid Shape Theory

Membrane composition is also known to play a key role in vesicle fusion. This is not surprising, as vesicle fusion involves the rearrangement of lipid structure, at both leaflets, of both the vesicle and the target membrane. In addition to their protein components, biological membranes are composed of many different types of lipids. Each of these behaves slightly differently when packed into a membrane, largely based on their shape (Chernomordik and Kozlov, 2003). Lipids can be classified based on the sizes of their head groups and hydrophobic tails. Those with relatively larger head groups are classified as inverted cone shaped, while those with relatively smaller head groups are cone shaped (Melia et al., 2006). These shapes have consequences for the shape and stability of the bilayers formed; inverted cone lipids are most stable forming a convex

layer, while cone shaped lipids are most stable forming a concave layer (Israelachvili et al., 1977; Gruner, 1989). Indeed, when isolated in monolayers, many of these different lipids acquire spontaneous curvature (Gruner, 1989).

Because of their small diameter (approximately 40 nm), the membranes of synaptic vesicles have more curvature than cell membranes. It is easy to imagine that, to minimize the energy required to maintain the vesicle shape, the inner leaflet would be largely cone shaped lipids and the outer would be inverted cone shape (Fig 3, page 36). Addition of inverted cone lipids to the outer leaflet, however, has been shown to inhibit vesicle fusion (Melia et al., 2006). This indicates that lipid shape is relevant to vesicle fusion but, rather than the vesicle and target membrane being stabilized separately, vesicle fusion is favored by stabilizing the fusion intermediate.

3b. Lipid Rafts

Cholesterol is a major component of animal cell membranes and is a cone shaped lipid. As such, it is a source of negative curvature in membranes (Coorssen and Rand, 1989; Chen and Rand, 1997). Cholesterol is required for efficient, Ca^{2+}-dependent exocytosis (Churchward et al., 2005).

Other than shape considerations, cholesterol also has the ability to aggregate with sphingolipids in the membrane to form rafts (Simons and Ikonen, 1997; Brown and London, 2000; Ramstedt and Slotte, 2002; Simons and Ehehalt, 2002; Wang, 2003). These lipid rafts have been shown to be involved in many different cellular processes

(Allen et al., 2007; Hanzal-Bayer and Hancock, 2007). Cholesterol has been shown to be enriched on secretory vesicles as compared to their target membranes, indicating that lipid rafts may play a role in exocytosis (Zinder et al., 1978; Decker and Kinsey, 1983). Indeed, members of the SNARE complex have been shown to localize to cholesterol-based lipid rafts (Lafont et al., 1999; Chamberlain et al., 2001; Salaun et al., 2005).

3c. Phospholipids and Synaptic Transmission

Phospholipids are also important to synaptic vesicle fusion. Phospholipids are estimated to compose 50% of the synaptic vesicle membrane (Takamori et al., 2006). While phosphatidylethanolamine (PE) and phosphatidylcholine (PC) are uncharged and comprise most of these phospholipids, the remaining species carry a negative charge (Nagy et al., 1976; Takamori et al., 2006). Phosphatidylserine (PS) carries a single negative charge while the phosphatidylinositides (PI) carry multiple negative charges, creating an overall negativity on the synaptic vesicle membrane. Phosphatidylinositol-4,5-bisphosphate (PIP2) has also been shown to be undetectable on synaptic vesicle membranes but enriched on the apposed presynaptic membrane (Micheva et al., 2001). This highly structured organization of lipid components in synaptic membranes suggests a role in membrane fusion.

Despite initial excitement surrounding synaptotagmin as the Ca^{2+} sensor for evoked neurotransmitter release, determination of synaptotagmin's intrinsic Ca^{2+} affinity by NMR resulted in affinity incompatible with the levels of Ca^{2+} required for synaptic vesicle fusion [(Sutton et al., 1995), page 4]. Early work showed that synaptotagmin

binds to membranes that contain anionic phospholipids (Brose et al., 1992; Davletov and Sudhof, 1993; Chapman and Jahn, 1994; Fernandez et al., 2001; Bai et al., 2004). Recombinant cytosolic portions of synaptotagmin have been shown to bind, in a Ca^{2+}-dependent manner, to liposomes containing approximately physiological levels of phosphotidylserine. The affinity of this binding varies, however; if both recombinant C_2 domains are included, the EC_{50} is between 11 and 40 μM, if they are separate the affinity is between 49-54 μM (Fernandez-Chacon et al., 2001; Fernandez et al., 2001). Additionally, when native synaptotagmin is used, rather than recombinant, the affinity is only 3-4 μM (Fernandez-Chacon et al., 2001). Regardless of these differences, the presence of anionic phospholipids increases the affinity of synaptotagmin for Ca^{2+} (Fernandez-Chacon et al., 2001). The aspartates within the Ca^{2+} binding pocket create a largely negative field for Ca^{2+} binding (Shao et al., 1997), and the presence of adjacent anionic phospholipid head groups increases that negativity, increasing the negative field for Ca^{2+} binding. This is consistent with the fact that loops 2 and 3 of the Ca^{2+} binding pocket do not become fully ordered solely in the presence of Ca^{2+} , rather they require the binding of phospholipids (Chae et al., 1998).

Several basic residues are also found in this field of acidic charge created by anionic phospholipid headgroups and aspartate residues (Shao et al., 1997). These residues are thought to participate in synaptotagmin's interaction with anionic phospholipid headgroups (Shao et al., 1997; Fernandez-Chacon et al., 2001; Stevens and Sullivan, 2003; Wang et al., 2003; Han et al., 2004). The functional effects of disrupting this interaction are examined in chapter 1 of this dissertation.

In addition to this electrostatic interaction, the hydrophobic residues of the Ca^{2+} binding loops of synaptotagmin have also been shown to interact with phospholipids (Chapman and Davis, 1998; Davis et al., 1999; Bai et al., 2002; Bai et al., 2004; Arac et al., 2006; Herrick et al., 2006). There are hydrophobic residues present on the Ca^{2+}-binding loops of both C_2A and C_2B (gray, Fig 2, page 34). Several types of fluorescence-based assays have shown that these hydrophobic residues penetrate liposomes containing anionic phospholipids in response to Ca^{2+} (Chapman and Davis, 1998; Davis et al., 1999; Bai et al., 2002; Bai et al., 2004; Arac et al., 2006; Hui et al., 2006). Though C_2A will penetrate liposomes without being tethered to C_2B, the two act cooperatively to penetrate more deeply when they are attached (Herrick et al., 2006; Hui et al., 2006). Together, these hydrophobic residues penetrate approximately 1/3 of the way into the lipid monolayer (Herrick et al., 2006). This penetration has been mapped to a number of hydrophobic residues in the flexible Ca^{2+} binding loops that are conserved throughout evolution (Chae et al., 1998; Chapman and Davis, 1998; Davis et al., 1999; Bai et al., 2002; Gerber et al., 2002; Bai et al., 2004; Arac et al., 2006; Herrick et al., 2006). Increasing the hydrophobicity of these residues by mutation to tryptophan increases phospholipid binding and, when expressed *in vivo*, increases neurotransmitter release (Rhee et al., 2005). Thus, penetration of the hydrophobic residues may have an important physiological role in vesicle fusion. This role could be: 1) targeting of fusion, 2) destabilizing the target membrane and/or 3) bringing the two membranes into close proximity.

C_2A prefers membranes enriched with PS and C_2B prefers membranes containing PIP_2 (Bai et al., 2004). PIP_2 is enriched on the synaptic target membrane and not detectable on the membrane of the synaptic vesicle (Micheva et al., 2001). This, in combination for C_2B's preference for PIP_2, suggests a possible mechanism for steering synaptotagmin and the fusion process towards the correct membrane.

In addition to a role in membrane steering, the penetration of C_2 domains may be involved on other aspects of synaptic vesicle fusion. Significant lipid rearrangement is required to fuse two separate lipid bilayers. First the proximal leaflets fuse to form a 'hemi-fused' state [Fig. 3, page 36, (Chernomordik and Kozlov, 2005)]. The remaining leaflets then fuse through this membrane stalk and the structure transitions to a fully fused state where the contents of the synaptic vesicle are exposed to the outside of the neuron. This massive disruption of the lipid bilayers requires a great deal of energy and the insertion of hydrophobic residues of synaptotagmin may aid in that disruption (Chapman and Davis, 1998; Jahn et al., 2003; Martens et al., 2007). Indeed, a recent study has shown a strong role for the conserved hydrophobic residues of synaptotagmin's Ca^{2+}-binding loops in this membrane destabilization that leads to membrane fusion (Martens et al., 2007).

The insertion of the hydrophobic residues may also serve to simply bring the vesicle and target membrane into closer proximity. Various docking and priming reactions serve to hold the vesicles within 3-4 nm of the target membrane previous to Ca^{2+} influx (Martens et al., 2007). Synaptotagmin is thought to be involved in this preassembled fusion

machinery, either through its interactions with the SNARE complex or the target membrane (Zhang et al., 2002; Rickman and Davletov, 2003; Bai et al., 2004; Arac et al., 2006; Dai et al., 2007). The C_2B domain, in particular, has areas of charge away from the Ca^{2+} binding loops that have been implicated in membrane binding, including a polylysine motif along the side of the beta sheets and an alpha helix on the opposite side of the Ca^{2+} binding pockets (Fernandez et al., 2001; Arac et al., 2006). Several residues on the end of C_2B opposite of the Ca^{2+}-binding pockets have been shown to interact with liposomes in response to Ca^{2+} binding, though they are at the opposite end of the C_2 domain from the Ca^{2+} binding site [N396, R398, V419 in Fig 2C, page 34 (Arac et al., 2006)].

While this study did not examine any residues on the back of C_2A, earlier studies found residues there that penetrate liposomes [F153 (Chapman and Davis, 1998), His 254 (Chae et al., 1998)]. Figure 2C shows the position of these residues relative to the rest of the molecule. This interaction was not as strong as the one observed for F231 and F234 of the Ca^{2+} binding pocket, and was explained as the result of a possible alternate Ca^{2+} binding site within C_2A (Chae et al., 1998; Chapman and Davis, 1998). No such Ca^{2+} binding site has been observed (Sutton et al., 1995; Fernandez-Chacon et al., 2001) and this interaction, like those of N396, R398 and V419, may be the result of the back end of the C_2 domains interacting with the vesicle membrane while the Ca^{2+} binding pockets interact with the target membrane (Arac et al., 2006). This interaction may play a role in either the proximity model or the lipid disruption model outlined above. Indeed, these

models are not mutually exclusive and membrane penetration by the C_2 domains may act in all of the modes described above.

C. Additional Considerations in Synaptic Vesicle Fusion

1. Complexin

Complexin had recently emerged as another player in the Ca^{2+}-dependent vesicle fusion process. Complexins are small, soluble proteins present in nerve terminals (Ishizuka et al., 1995; McMahon et al., 1995; Takahashi et al., 1995). Mammals have four isoforms, two of which are recently discovered (Reim et al., 2005). A complexin I/II double knock out mouse has impaired synchronous, Ca^{2+}-triggered release, suggesting that it plays a role in this process (Reim et al., 2001). Complexins bind to the assembled SNARE complex, in the groove between syntaxin and synaptobrevin, but not to the individual components (McMahon et al., 1995; Pabst et al., 2000; Tokumaru et al., 2001; Chen et al., 2002; Hu et al., 2002). These two facts together indicate that complexin may promote fusion by stabilizing the ternary SNARE complex. The similarity to the synaptotagmin knockout phenotype suggests that they play a role in the same process, though complexin has been ruled out as the Ca^{2+} sensor because it doesn't bind Ca^{2+}.

Complexin overexpression studies, however, find an inhibition of evoked release indicating that its role is more complicated than simply a promoter of release (Ishizuka et al., 1995; Ono et al., 1998; Giraudo et al., 2006; Schaub et al., 2006). It has been suggested that complexin's binding to the SNARE complex may arrest it in the coiled coil and prevent any zippering that then drives fusion (Bracher et al., 2002).

Interestingly, neurons from complexin I/II double knockout mice display no change in spontaneous release rates, suggesting that they do not act as a fusion clamp (Reim et al., 2001). These two theories, however, are mutually exclusive, suggesting a more complicated role for complexin. As mice lacking complexins I and II have deficits in synchronous, Ca^{2+}-dependent transmitter release though complexin lacks any metal binding sites, complexin may interact with synaptotagmin to coordinate Ca^{2+}-dependent release.

Complexin has not been shown to directly bind to synaptotagmin though the two appear to cooperate to confer Ca^{2+} sensitivity to synaptic vesicle exocytosis. For example, complexin inhibits SNARE-mediated fusion of liposomes, but that inhibition is relieved and fusion enhanced by addition of synaptotagmin and Ca^{2+} (Schaub et al., 2006). Similar results were obtained from a 'flipped cell' system, in which cytoplasmic mixing of cells expressing the membrane associated proteins on the outside of their membranes is used as a measure of membrane fusion (Giraudo et al., 2006). Though synaptotagmin does not bind to complexin, they compete for binding to the SNARE complex (Tang et al., 2006; Dai et al., 2007) see however (Schaub et al., 2006). The differences observed in these three studies may come from the Ca^{2+}-dependence of synaptotagmin's ability to compete complexin off of the SNARE complex (Dai et al., 2007). Complexin binds to the SNARE complex, stabilizing and arresting it in a partially assembled state (Giraudo et al., 2006). During an action potential, Ca^{2+} increases drastically in the presynaptic terminal, binding to synaptotagmin and allowing it to displace complexin. This theory explains how overexpression of complexin can inhibit Ca^{2+}-dependent fusion by decreasing

synaptotagmin's ability to compete it from the SNARE complex, but decreasing complexin levels is also deleterious because it stabilizes the SNAREs prior to fusion.

2. Ca^{2+} Cooperativity of Neurotransmitter Release

While Ca^{2+} promotes release of neurotransmitter, increasing Mg^{2+} inhibits that release and this antagonist relationship led to the idea of a molecule that bound cations to influence release probability (del Castillo and Katz, 1954). The non-linear relationship between external [Ca^{2+}] and release, however, confounded further examination of this principle until Jenkinson proposed a cooperative interaction (Jenkinson, 1957). Because he only considered a cooperativity coefficient of 2, his proposition failed to effectively fit the data.

The finding that release of neurotransmitter relies on the fourth power of the activated complex 'CaX' has long driven research in Ca^{2+}-dependent neurotransmitter release. Dodge and Rahamimoff propose two possible models to explain this fourth power (1967). The stoichiometric model involves four divalent cations binding to and activating a single sensor, CaX, to promote release, and this is the one for which they are most often cited (Reid et al., 1998; Stevens and Sullivan, 2003). The stoichiometric model seemed further supported when synaptotagmin was proposed as a Ca^{2+} sensor and bound 4-5 Ca^{2+} ions (Sutton et al., 1995; Ubach et al., 1998; Fernandez et al., 2001).

The Dodge and Rahamimoff equation involves many different parameters, including a dissociation constant for Mg^{2+}, based on the Ca^{2+}/Mg^{2+} antagonistic relationship. Rather

than measure all of these different parameters, many researchers use the Hill Equation (Hill, 1910) to approximate the power relationship initially described by Dodge and Rahamimoff (Fernandez-Chacon et al., 2001; Mackler and Reist, 2001; Wang et al., 2003). While many of the point mutations in synaptotagmin change the Ca^{2+} affinity of neurotransmitter release, there is less support for a change in the power relationship (cooperativity) of release (Littleton et al., 1994; Stewart et al., 2000; Yoshihara and Littleton, 2002; Okamoto et al., 2005; Loewen et al., 2006; Tamura et al., 2007). It has been suggested that substituting asparagine (N) for aspartic acid (D) used in the site-directed synaptotagmin mutagenesis of synaptotagmin's Ca^{2+}-binding residues may mimic Ca^{2+} binding. Thus, the Ca^{2+} dependence studies do not reveal a change in the power relationship because the mimicked Ca^{2+} binding is sufficient for the measured output, which is evoked release (Stevens and Sullivan, 2003). This is a viable explanation within the parameters of Dodge and Rahamimoff's stoichiometric paradigm, but another possibility also exists.

Dodge and Rahamimoff also proposed another model, the stochastic model, in which the fourth power is the result of four activated complexes promoting fusion, rather than four cations binding to a single sensor (Dodge and Rahamimoff, 1967). A study has detected a change in the power relationship of release involved hypomorphs of members of the SNARE complex, the minimal machinery for fusion, rather than Ca^{2+} binding to synaptotagmin (Stewart et al., 2000). Thus, the cooperativity may be described by the stochastic model, if the activated complex is considered to be Ca^{2+}+synaptotagmin+SNAREs. The Ca^{2+}-dependence of evoked release from complexin

knock out mice could provide further insight into this model, but has not yet been examined. Discerning which of these models accurately described the reality of the release machinery requires further study.

3. Spontaneous Release

All of the previous discussion has focused on the machinery necessary to regulate and drive the unfavorable fusion of two apposing membranes. Occasionally, fusion of a single neurotransmitter occurs in the absence of the Ca^{2+} signal that triggers evoked release of a pool of vesicles. This spontaneous release, also called miniature excitatory junction potentials (mEJPs) was first seen by Katz and colleagues and led to the theory of quantal release (del Castillo and Katz, 1954). Now, more than fifty years later, the precise mechanisms underlying spontaneous release are still not known.

Spontaneous release persists in synaptotagmin^{null} animals, indicating that synaptotagmin plays a regulatory, rather than direct, role in fusion. For example, spontaneous release is abolished in syntaxin nulls (Broadie et al., 1995; Schulze et al., 1995). It does persist, however, in both autaptic hippocampal cultures from synaptobrevin KO mice and at the neuromuscular junction of *Drosophila* embryos lacking synaptobrevin, though at reduced frequency (Deitcher et al., 1998; Schoch et al., 2001). A temperature sensitive mutation in SNAP-25 in *Drosophila* that decreases evoked release actually enhances spontaneous release (Rao et al., 2001). As this is not a true knock out system, the relevance of these results for the mechanism and regulation of spontaneous synaptic vesicle fusion is difficult to interpret.

In contrast with their drastic decrease in evoked release, *Drosophila* synaptotagmin^{null} and hypomorphs exhibit an increased rate of spontaneous release when examined at the neuromuscular junctions (NMJs) of embryos (Broadie et al., 1994; DiAntonio and Schwarz, 1994), L1s (Littleton et al., 1993) and L3s (Loewen et al., 2001). This finding is not universal in *Drosophila* studies, however. No increase in spontaneous release was seen in synaptotagmin^{null} embryos by another group (Yoshihara and Littleton, 2002). Finally, when synaptotagmin is transiently and acutely inactivated at *Drosophila* L3 NMJ, no persistent increase in spontaneous release was observed (Marek and Davis, 2002). While no differences were found in the rate of spontaneous release, this release was examined early, either as embryos or due to acute inactivation. This suggests a possible developmental defect in *Drosophila* synaptotagmin^{null} larvae that results in an increased spontaneous release rate, but no work has further examined this hypothesis.

Hippocampal autapses from mouse synaptotagmin I knockouts do not exhibit an increase in spontaneous release (Geppert et al., 1994). This difference suggested that, despite the high sequence homology, synaptotagmin I may play different roles in vertebrates and *Drosophila.* Spontaneous release in synaptotagmin I knockouts in vertebrates wasn't examined again for some time. Recently, another study found an increase in spontaneous release frequency in cultured cortical neurons (Maximov and Sudhof, 2005; Pang, 2006) from synaptotagmin I KO mice, suggesting that the lack of an increased rate is an artefact of the hippocampal autaptic system.

Synaptotagmin II is highly homologous to synaptotagmin I, though the two have different expression patterns. Synaptotagmin I is expressed at low levels throughout the nervous system, but enhanced in rostral areas, such as the frontal cortex (Geppert et al., 1991). In mammals, synaptotagmin II is enhanced in caudal areas, such as the cerebellum, brainstem and spinal cord (Geppert et al., 1991). In synaptotagmin II knock out mice, the rate of spontaneous release is increased at the NMJ (Pang, 2006). This is consistent with the *Drosophila* studies, because synaptotagmin I rather than synaptotagmin II that is expressed at the *Drosophila* NMJ.

If synaptotagmin does act to repress the rate of spontaneous fusion, what is the mechanism of that repression? Are conserved synaptotagmin motifs, such as those responsible for interactions with the SNARE complex or phospholipids, responsible for such a role? Mutation of several highly conserved, functional motifs within synaptotagmin may elucidate part of its role of 'fusion clamp', but much work remains to further our understanding of this role.

D. Rationale and Specific Aims

1. Rationale:

Synaptic vesicle fusion is a highly regulated process and many aspects of this regulation are not yet understood. While *in vitro* studies are vital to dissecting the mechanism of synaptic vesicle fusion, any failure to recapitulate the native environment of the fusion machinery decreases the usefulness of these studies. For example, synaptotagmin's *in vitro* activity changes with changing levels of Ca^{2+}, other salts, anionic phospholipids

(PS, PIP2, PIP3), SNARE fusion machinery and other protein components, adding many caveats to the understanding of its role in fusion (Schiavo et al., 1996; Bai et al., 2002; Bai et al., 2004; Schaub et al., 2006; Tang et al., 2006; Dai et al., 2007). Thus, I proposed to study the role of synaptotagmin in synaptic vesicle fusion *in vivo*, where all other players remain intact, even those not yet identified.

Synaptotagmin is the Ca^{2+} sensor for synchronous neurotransmitter release, but the mechanism of its role in fusion remains unclear. Deletion of synaptotagmin abolishes Ca^{2+}-dependent, synchronous release in many systems (Nonet et al., 1993; DiAntonio and Schwarz, 1994; Geppert et al., 1994; Voets et al., 2001), but little is gleaned about its interaction with other components of the fusion machinery through these studies. Several functional motifs essential for synaptotagmin function have been identified, and previous work in this lab has identified the Ca^{2+}-binding motif by the second C_2 domain to be essential for synaptotagmin function (Mackler et al., 2002).

As the Ca^{2+} sensor, synaptotagmin must couple Ca^{2+} binding to the fusion machinery. It is this interaction with the fusion machinery components that I examined. Several *in vitro* studies have identified a conserved phospholipid binding site at the tip of each of synaptotagmin's C_2 domains (Chae et al., 1998; Zhang et al., 1998; Davis et al., 1999; Fernandez-Chacon et al., 2001; Bai et al., 2002; Gerber et al., 2002; Stevens and Sullivan, 2003; Wang et al., 2003; Bai et al., 2004; Han et al., 2004). While mutation of these residues disrupts Ca^{2+}-dependent phospholipid binding by synaptotagmin, the functional consequence of this disruption remains unclear. I proposed to disrupt this

interaction at an intact synapse in *Drosophila* to conclusively determine its significance to synaptotagmin's role as the Ca^{2+} sensor.

2. Specific Aim 1:

Are the conserved basic residues within the C_2A or C_2B domains that interact with negatively charged phospholipid bilayers required for function?

Each C_2 domain of synaptotagmin contains a conserved basic residue in loop 3 of the Ca^{2+}-binding pocket (Fig. 1, page 32). This basic residue is required for efficient Ca^{2+}-dependent binding of phospholipid membranes by each C_2 domain (Fernandez-Chacon et al., 2001; Wang et al., 2003). This interaction has been postulated to mediate a critical step in Ca^{2+}-triggered fusion, but cell culture studies have yielded conflicting results (Fernandez-Chacon et al., 2001; Stevens and Sullivan, 2003; Wang et al., 2003; Han et al., 2004; Li et al., 2006). To conclusively test the functional significance of these residues for synaptic transmission, I mutated each of these arginines to a glutamine and expressed the mutant synaptotagmin in the absence of any wild type synaptotagmin at an intact *Drosophila* synapse. Both spontaneous and evoked release was measured at the NMJ of third instar larvae and compared to that of a transgenic control expressing wild type synaptotagmin with the same neuronal expression system. The Ca^{2+}-dependence of release was also determined by measuring evoked release at a variety of Ca^{2+} concentrations. In addition to revealing a critical role for these phospholipid binding residues within the Ca^{2+}-binding pockets of synaptotagmin, I have demonstrated that the

phospholipid binding residues of the C_2B domain is of greater consequence to synaptic transmission than that of C_2A.

3. Summary

In the following chapter, I present my analyses of two *Drosophila* synaptotagmin mutants that disrupt Ca^{2+}-dependent phospholipid binding. These results demonstrate that this interaction by the C_2 domains of synaptotagmin is essential for its role as Ca^{2+} sensor for evoked neurotransmitter release. This work continues analyses defining specific motifs within synaptotagmin that are responsible for aspects of its role in synaptic vesicle fusion.

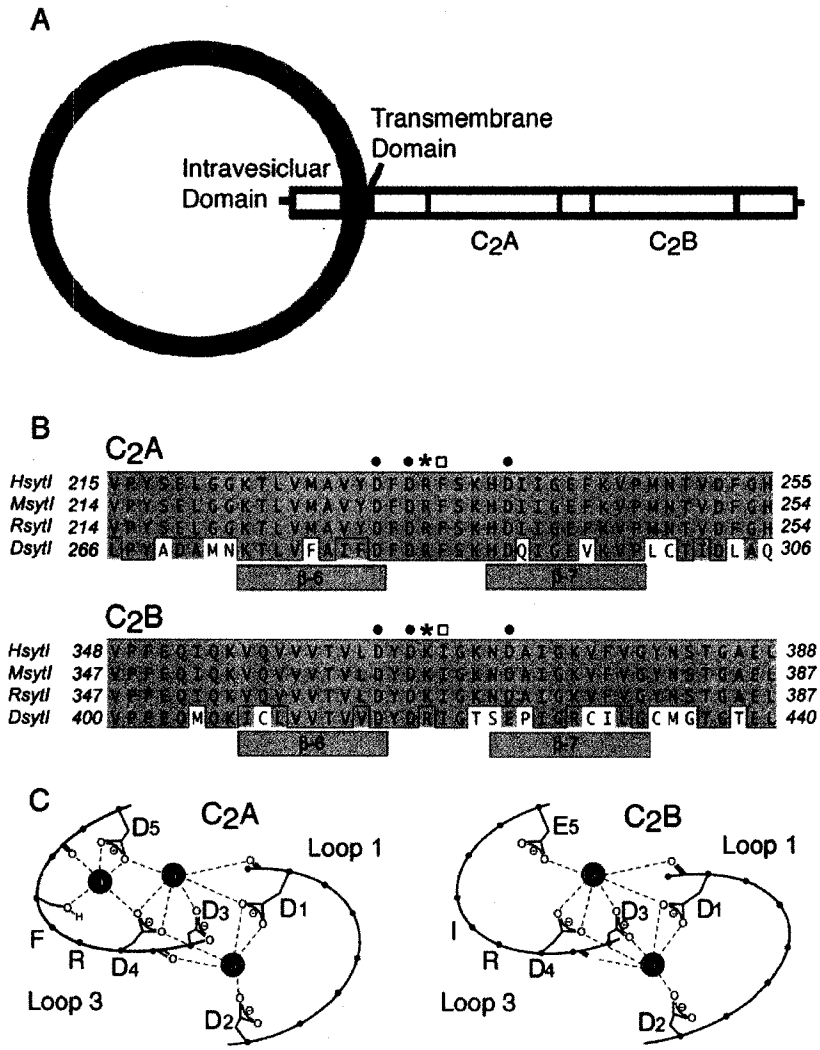

Fig 1. Synaptotagmin Structure. A: Domain structure of synaptotagmin, illustrating organization of the C2 domains, the membrane proximal C_2A and membrane distal C_2B. B: Alignment of human, rat, mouse and *Drosophila* synaptotagmin I C_2A and C_2B domains. Loop 3 is located between β-6 and β-7. Bars indicate β-sheets, asterisks indicate the conserved basic residues, dots indicate Ca^{2+} binding residues, open boxes indicate conserved hydrophobic residues. Within the alignment, conserved residues are

shown in gray, identical residues are boxed. C: Schematic projection of loops 1 and 3 that form the Ca^{2+}-binding pockets of both C_2 domains. Adapted from Fernandez et al (2001) using the *Drosophila* sequence.

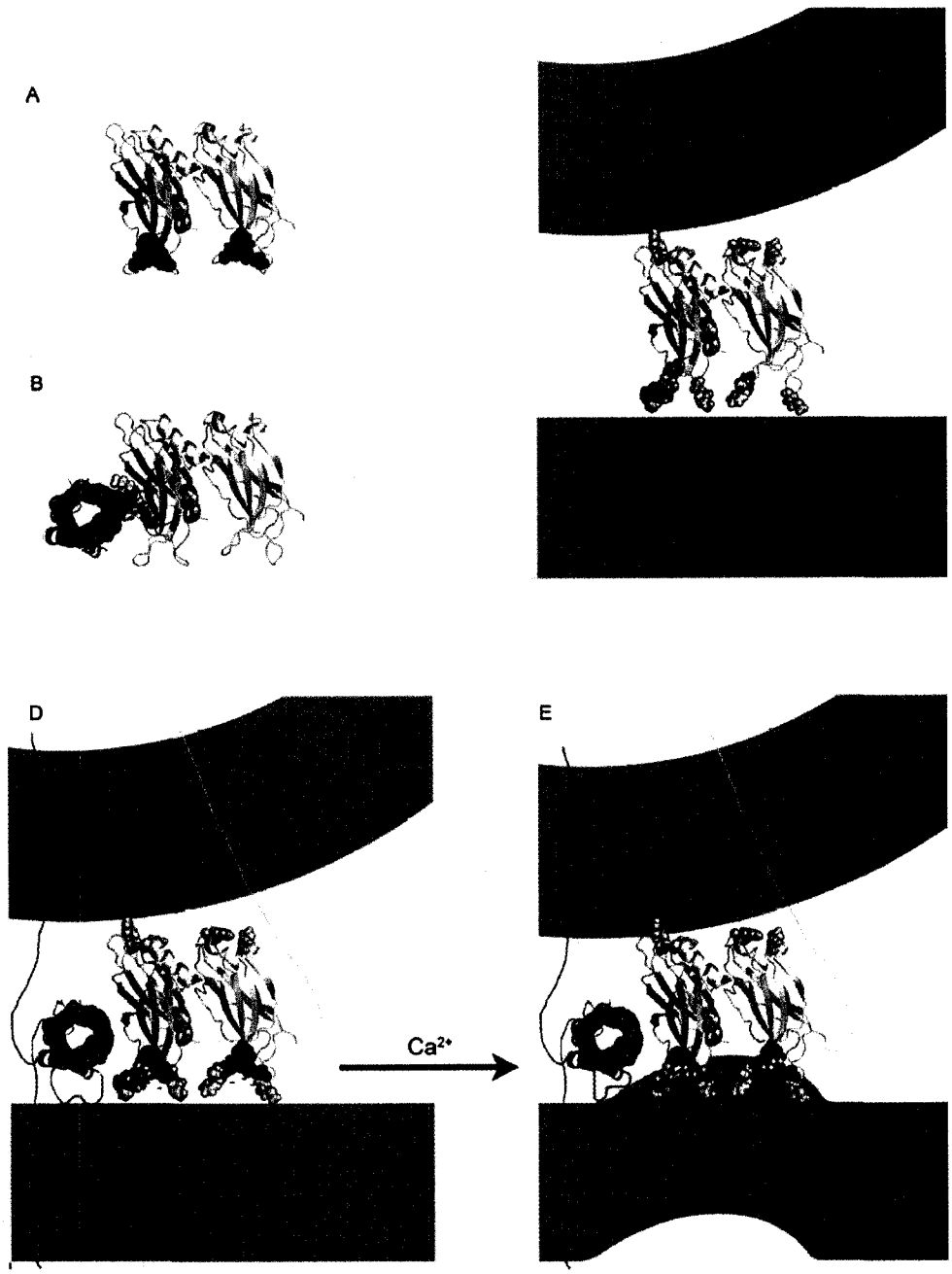

Fig 2. Structure and interactions of synaptotagmin's C_2A and C_2B domains. The nuclear magnetic resonance structures of the C_2A (PDB file 1BYN) and C_2B (PDB file 1K5W) domains of synaptotagmin are yellow in every panel, except for specifically highlighted

residues, while C_2A is on the right and C_2B on the left. A: The Ca^{2+}-coordinating residues of both domains are highlighted in red, with their coordinated Ca^{2+} ions in green. B: The interaction between the polylysine motif of synaptotagmin's C_2B and the SNARE complex, shown here as the crystal structure of the core complex (PDB file 1SFC, containing syntaxin [red], SNAP-25 [green], and VAMP/synaptobrevin [blue]). C: Synaptotagmin's residues that interact with phospholipids are highlighted, hydrophobics in gray and basic residues in blue. The gray boxes represent the phospholipid bilayers of the synaptic vesicle (top) and presynaptic membrane (bottom).

D and E: Composite of panels A, B and C to illustrate the simultaneous interactions of synaptotagmin in synaptic vesicle fusion. D shows Ca^{2+} free, while E shows the Ca^{2+} driving the membrane penetration. The membranes, the transmembrane domains, and the link between C_2A and C_2B were added in Adobe Photoshop.

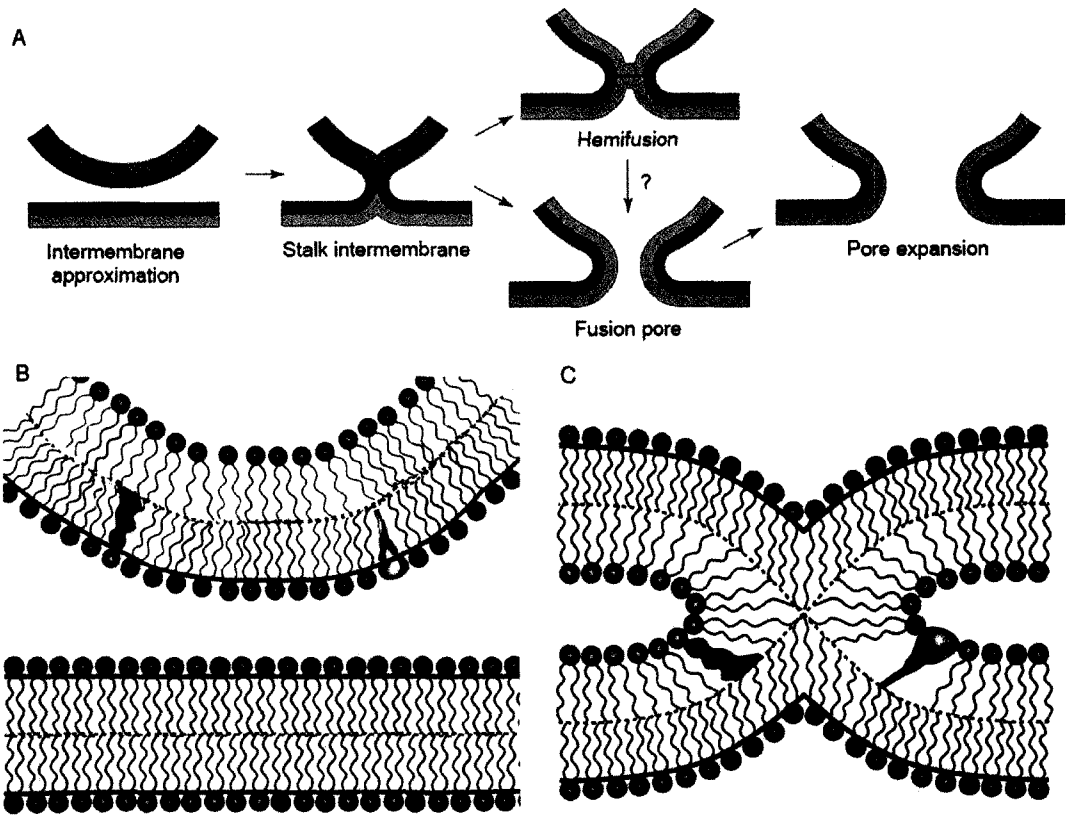

Fig 3. Lipid fusion; intermediates and lipid shape. Lipid bilayer of vesicle shown in blue, lipid bilayer of presynaptic membrane shown in red, fused leaflets shown in purple to indicate lipid mixing that occurs upon fusion.. Cone shaped lipid shown in magenta, inverted cone shaped lipid shown in green. A: Adapted from Rizo et al (2006). B: Unfused but apposed membranes, green cone shaped lipid stabilizing the curve of the vesicle membrane (right), while the magenta inverted cone lipid does not favor this arrangement (left). C: Hemifused membranes. The magenta inverted cone lipid stabilizes this method of curvature (left) while the green cone shaped lipid does not favor this formation (right). Possible protein components of fusion omitted for clarity. Adapted from Chernomordik and Kozlov (2005).

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Ca^{2+}-Dependent, Phospholipid-Binding Residues of Synaptotagmin are Critical for Excitation-Secretion Coupling

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Abbreviations: Synaptotagmin (syt), Soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE), phosphotidylinositol 4,5-bisphosphate (PIP_2), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidyl inositol phosphate (PIP), phosphatidyl inositol (PI), phosphate buffered saline (PBS), phosphate buffered saline with Triton (PBST), excitatory junctional potentials (EJP), miniature excitatory junctional potentials (mEJP), synaptosome associated protein of 25 kD (SNAP-25)

ABSTRACT:

Synaptotagmin I is the Ca^{2+} sensor for fast, synchronous release of neurotransmitter, however, the molecular interactions that couple Ca^{2+} binding to membrane fusion remain unclear. The structure of synaptotagmin is dominated by two C_2 domains which interact with negatively-charged membranes upon binding Ca^{2+}. *In vitro* work has implicated a conserved basic residue at the tip of loop 3 of the Ca^{2+}-binding pocket in both the C_2A and C_2B domain in coordinating this electrostatic interaction with anionic membranes. Yet a recent study of cultured hippocampal neurons suggests that this basic residue of the C_2A domain, but not of the C_2B domain, functions during vesicle fusion at a synapse. To examine the functional significance of each of these residues *in vivo*, we mutated either the C_2A or the C_2B basic residue and assessed synaptic transmission at an intact synapse, the neuromuscular junction in transgenic *Drosophila*. The conserved basic residue at the tip of the Ca^{2+}-binding pocket of both the C_2A and C_2B domains were required for efficient evoked transmitter release. Our results support the hypothesis that an interaction between synaptotagmin and the presynaptic membrane mediated by the basic residues at the tip of both the C_2A and C_2B Ca^{2+}-binding pockets is critical for coupling Ca^{2+} influx with vesicle fusion.

INTRODUCTION:

Synaptotagmin is an integral membrane protein located on synaptic vesicles that functions as the Ca^{2+} sensor for the synchronous release of neurotransmitter upon Ca^{2+} influx (Brose et al., 1992; Geppert et al., 1994; Augustine, 2001; Mackler et al., 2002; Yoshihara and Littleton, 2002; Nishiki and Augustine, 2004). Consistent with this role, synaptotagmin's cytosolic domain contains two C_2 domains, C_2A and C_2B, that coordinate the binding of Ca^{2+} ions. While Ca^{2+} binding by the C_2B domain is essential for synchronous, evoked transmitter release, Ca^{2+} binding by the C_2A domain plays only a modest role in neurotransmission (Fernandez-Chacon et al., 2002; Mackler et al., 2002; Robinson et al., 2002; Stevens and Sullivan, 2003; Nishiki and Augustine, 2004; Pang et al., 2006). The C_2 domains of synaptotagmin interact with members of the SNARE (soluble N-ethylmaleimide sensitive factor attachment receptor) protein family as well as with anionic phospholipids in a Ca^{2+}-dependent manner *in vitro* and these interactions are postulated to link Ca^{2+} binding to vesicle fusion (Perin et al., 1990; Brose et al., 1992; Chapman et al., 1994; Earles et al., 2001; Fernandez-Chacon et al., 2001; Fernandez et al., 2001; Bai et al., 2002; Mackler et al., 2002).

Both C_2A and C_2B bind membranes containing anionic phospholipids in a Ca^{2+}-dependent manner with each C_2 domain exhibiting a differential preference for specific anionic phospholipids (Davletov and Sudhof, 1993; Chapman and Jahn, 1994; Fernandez et al., 2001; Bai et al., 2002; Tucker et al., 2003; Bai et al., 2004). C_2A associates preferentially with phosphotidylserine (PS)-containing membranes in a strictly Ca^{2+}-dependent manner, while C_2B associates preferentially with phosphotidylinositol 4,5-bisphosphate (PIP_2)-containing membranes in both a Ca^{2+}-independent and a Ca^{2+}-

dependent mode (Schiavo et al., 1997; Tucker et al., 2003; Wang et al., 2003; Bai et al., 2004). Since PIP_2 is exclusively partitioned to the presynaptic membrane and is undetectable in synaptic vesicle membranes (Micheva et al., 2001), the C_2B interaction may assist in targeting synaptic vesicles to the presynaptic membrane (Bai et al., 2004). The magnitude of deficits in Ca^{2+}-triggered fusion in several synaptotagmin mutants parallels the severity of the decrease in Ca^{2+}-dependent phospholipid binding (Fernandez-Chacon et al., 2001; Mackler et al., 2002; Sorensen et al., 2003; Wang et al., 2003; Nishiki and Augustine, 2004; Li et al., 2006; Pang et al., 2006) see however (Fernandez-Chacon et al., 2002; Robinson et al., 2002). Thus, a Ca^{2+}-dependant interaction between synaptotagmin and the presynaptic membrane is postulated to be critical in mediating Ca^{2+}-triggered vesicle fusion.

Biochemical studies have identified specific residues in both the C_2A and C_2B domains of synaptotagmin that are required for Ca^{2+}-dependent phospholipid binding *in vitro*. At the tip of the Ca^{2+}-binding loops of each C_2 domain of synaptotagmin there is a conserved basic residue (Fig. 1, ⊕, arginine or lysine) which is thought to mediate an electrostatic interaction at the interface between synaptotagmin and the head groups of anionic phospholipids in the presynaptic plasma membrane (Chae et al., 1998; Davletov et al., 1998; Fernandez-Chacon et al., 2001; Fernandez et al., 2001; Wang et al., 2003). This interfacial interaction may contribute to the electrostatic switch that drives vesicle fusion upon Ca^{2+} influx (Shao et al., 1997; Davletov et al., 1998; Ubach et al., 1998; Murray and Hoenig, 2002). Indeed, in C_2A, this basic residue is also necessary for efficient synaptic transmission in cultures of hippocampal neurons, PC12 cells and chromaffin cells (Fernandez-Chacon et al., 2001; Sorensen et al., 2003; Wang et al.,

2003; Han et al., 2004). Curiously, a recent report contradicted the finding that the C_2B basic residue is important for Ca^{2+}-dependent phospholipid interactions and also indicated that this basic residue in C_2B is not involved in Ca^{2+}-evoked neurotransmitter release in cultured neurons (Li et al., 2006).

Given that 1) several reports indicate that residues located at the tip of the Ca^{2+}-binding pockets, including the basic interfacial residues, in both the C_2A and C_2B domains are critical for Ca^{2+}-dependent phospholipid interactions (Chae et al., 1998; Chapman and Davis, 1998; Davis et al., 1999; Fernandez-Chacon et al., 2001; Bai et al., 2002; Gerber et al., 2002; Frazier et al., 2003; Shin, 2003; Wang et al., 2003; Han et al., 2004; Arac et al., 2006) and 2) various fusion assays using PC12 cells, chromaffin cells, hippocampal neuron cultures, or liposome fusion also implicate these residues as functionally important for Ca^{2+}-triggered fusion (Fernandez-Chacon et al., 2001; Sorensen et al., 2003; Wang et al., 2003; Rhee et al., 2005; Martens et al., 2007), we decided to assess the functional significance of the basic interfacial residues in the C_2A and C_2B domains *in vivo* using a transgenic *Drosophila* system to express mutant synaptotagmin in the nervous system (Brand and Perrimon, 1993; Yao and White, 1994; Mackler and Reist, 2001). We individually mutated the conserved basic residue in each C_2 domain to a glutamine, which disrupts the interfacial interaction between synaptotagmin and anionic phospholipids (Fernandez-Chacon et al., 2001; Wang et al., 2003). Here we demonstrate that the basic residues at the tip of the C_2A and C_2B Ca^{2+}-binding pockets that mediate interactions with anionic phospholipids are each critical for synaptotagmin function. Indeed the C_2B interfacial mutation results in a more severe disruption of synaptic transmission than the C_2A mutation.

MATERIALS AND METHODS:

Site-directed mutagenesis

In order to neutralize the positive charge without disrupting the structure of the C_2 domain (Fernandez-Chacon et al., 2001), arginine residues 285 and 419 (Fig. 1, ⊕) of *Drosophila* synaptotagmin I (*syt*) were mutated to glutamines using the polymerase chain reaction (PCR). To mutate arginine 285, a specifically mutated oligonucleotide (CGAGAACTGATCGAAGTCGAAAATGGC) was paired with a wild type oligonucleotide that flanked a unique *StyI* site. The PCR product was gel purified and used as a macroprimer in a second round of PCR with a wild type oligonucleotide that flanked a unique *EcoRV* site. This second-round, mutant PCR product was then subcloned into an otherwise wild type *Drosophila syt* cDNA construct in pBluescript II (Mackler and Reist, 2001). To mutate arginine 419, a specifically mutated oligonucleotide (TGCAGCGGCCGATGGGTTCGGAGGTGCCAATCTGATCGTAGTCCACGACGGT CACAACG) containing a unique *EagI* site was paired with a wild type oligonucleotide that flanked a unique *EcoRV* site. That mutant PCR product was then gel purified and and subcloned into the *Drosophila syt* cDNA construct in pBluescript mentioned above. DNA sequencing confirmed that either R285Q or R419Q was the only mutation harbored in the entire region generated by PCR. Each mutant *syt* cDNA was subcloned into a pUAST vector to place the mutant *syt* gene under the control of the UAS promoter (Brand and Perrimon, 1993).

Generation of mutant transgenic lines

Drosophila embryos were transfected with the mutant pUAST plasmids as previously described (Mackler and Reist, 2001). At least two lines carrying separate insertions of the mutant *syt* transgenes were isolated for each genotype. Expression of each transgene was localized to the nervous system using the elav promoter to drive Gal4 and the Gal4/UAS system was used to amplify expression levels (Brand and Perrimon, 1993; Yao and White, 1994). Standard genetic techniques were used to cross the transgenes into the syt^{null} background in order to express the transgene in the absence of endogenous synaptotagmin I for all experiments. The genotypes of the mutant lines were *yw;* syt^{AD4} *elav GAL4/*syt^{AD4}*; P[UAS* $syt^{A\text{-}R285Q}$*]/+*, and *yw;* syt^{AD4} *elav GAL4/*syt^{AD4}*; P[UAS* $syt^{B\text{-}R419Q}$*]/+* which are written as $P[syt^{A\text{-}RQ}]$ and $P[syt^{B\text{-}RQ}]$, respectively, in the text. The genotype of the control was *yw;* syt^{AD4} *elav GAL4/*syt^{AD4}*; P[UAS* $syt^{wild\ type}$*]/+* which is written as $P[syt^{WT}]$ in the text.

Immunolabeling

Standard saline was HL3 [5 mM KCl, 1.5 mM $CaCl_2$, 70 mM NaCl, 20 mM $MgCl_2$, 10 mM $NaCHO_3$, 5 mM HEPES, 115 mM sucrose and 5 mM trehalose (Stewart et al., 1994)]. For immunolabelling of the neuromuscular junction, third instars of the indicated genotypes were dissected in Ca^{2+}-free HL3 saline to expose their body wall muscles and fixed in 4% paraformaldehyde in phosphate buffered saline (PBS). This whole mount preparation was incubated overnight in anti-synaptotagmin antibody [Dsyt-CL1, (Mackler et al., 2002)], diluted 1:1000 in PBST-NGS [PBS with 0.1% Triton, 1% BSA and 1% Normal Goat Serum (NGS from Jackson ImmunoResearch, West Grove, PA)],

washed in PBST for 30-60 minutes, incubated in an Alexa fluor conjugated anti-rabbit antibody (Molecular Probes, Carlsbad, CA) diluted 1:5000 in PBST-NGS for one hour, washed in PBST 1-2 hours, mounted in Citifluor (Ted Pella Inc, Redding, CA) and then visualized on a Zeiss LSM 510Meta confocal microscope equipped with an Argon laser (Carl Zeiss Microimaging Thornwood, NY). Emissions were collected using a band pass 505-530 emission filter at 40X with a pinhole set for 1 Airy Unit.

Immunoblotting

Similar levels of transgene expression were verified by Western analysis. The nervous system of a single third instar of the indicated genotype was homogenized in protein loading buffer (Biorad; Hercules, CA). Proteins were separated via SDS-PAGE and transferred to a PVDF membrane as previously described (Mackler and Reist, 2001). All antibodies were diluted in PBS with 0.05% Tween with 10% NGS. Blots were probed with Dsyt-CL1 diluted between 1:1250 and 1:5000, and an anti-actin antibody (MAB 1501, Chemicon, Temecula, CA), diluted between 1:20,000 and 1:80,000. Actin levels were used to normalize for equal protein loading. These antibodies were visualized with HRP-tagged donkey anti-rabbit IgG, diluted 1:10,000 to 1:20,000, and HRP-tagged donkey anti-mouse IgG, diluted 1:2500 to 1:40,000 (Jackson ImmunoResearch, West Grove, PA). The HRP-tagged antibodies were detected using a Supersignal West Dura Extended Duration Substrate kit (Pierce, Rockford, IL) in an Epichemi3 Darkroom (UVP, Upland, CA).

The synaptotagmin:actin signal ratio was determined for each CNS, then normalized to the mean synaptotagmin:actin ratio of the *P[syt^{WT}]* lanes on each blot to allow comparison of signal between multiple blots.

Electrophysiology

Evoked and spontaneous excitatory junctional potentials (EJPs) were recorded from muscle 6 of segments 3 and 4 of third instars as previously described (Loewen et al., 2001). Briefly, third instar larvae were dissected in Ca^{2+}-free HL3 to expose the body wall musculature. After changing to HL3 saline containing 1.5 mM Ca^{2+}, muscle 6 was impaled with a recording electrode having a resistance between 10-40 MOhms. Evoked EJPs were generated by stimulating segmental nerves with a suction electrode filled with HL3. The Ca^{2+} dependence curve was generated by evoking EJPs in external Ca^{2+} concentrations ranging from 0.6 to 5.0 mM. Muscles were impaled in 1.5 mM Ca^{2+} HL3 and recordings in several different Ca^{2+} concentrations were obtained from each muscle fiber. The trehalose was varied between 0.5 to 5.0 mM, though this had no effect on evoked release (data not shown). The predicted maximal response was calculated by fitting the Hill equation to the mean response at each extracellular Ca^{2+} concentration (Kalediagraph, Reading, PA). The Ca^{2+} cooperativity coefficient was estimated from the slope of a double log plot of EJP amplitude vs Ca^{2+} concentration (Kalediagraph, Reading, PA). All events were collected using an AxoClamp 2B (Axon Instruments, Union City, CA) and digitized using a MacLab4s A/D converter (ADInstruments, Colorado Springs, CO). Spontaneous events were recorded in Chart Software, evoked

events were recorded in Scope software (ADInstruments, Colorado Springs, CO). Spontaneous fusion events were identified manually, blind to genotype.

RESULTS:

The conserved basic residue at the tip of synaptotagmin's C_2A and C_2B Ca^{2+}-binding pockets are both required for efficient synaptic transmission

A highly conserved basic residue is present in loop 3 of the Ca^{2+} binding pocket in both the C_2A and C_2B domains of *Drosophila* synaptotagmin I (Fig. 1, ⊕). To examine the role of these residues during synaptic transmission, we separately mutated the conserved basic residue in each C_2 domain to a glutamine. We will denote the mutation of these residues as $syt^{A\text{-}RQ}$ and $syt^{B\text{-}RQ}$. All experiments were performed on third instars expressing the indicated form of synaptotagmin from a transgene in the absence of endogenous synaptotagmin I. To indicate their transgenic origin, we will refer to the C_2A mutants as *P[syt$^{A\text{-}RQ}$]*, the C_2B mutants as *P[syt$^{B\text{-}RQ}$]*, and the transgenic controls as *P[syt^{WT}]*. Finally, since the random insertion of a transgene could potentially disrupt another functionally important gene, two independent insertions of each transgene were examined to ensure that any deficits found resulted from the mutation rather than the insertion site.

Evoked excitatory junctional potentials (EJPs) and spontaneous miniature excitatory junctional potentials (mEJPs) were recorded from larval neuromuscular junctions in HL3 saline containing 1.5 mM Ca^{2+}. Mutation of either of the conserved basic residues that mediate synaptotagmin's interfacial interaction with anionic phospholipids decreased the evoked response (Fig. 2). The mutation within the C_2A

domain reduced evoked release by approximately 50% as compared to the transgenic wild type control. Evoked release in *P[syt$^{A\text{-}RQ}$]* was 13.6 ± 2.1 mV (line 7) or 11.5 ± 0.6 mV (line 8) compared to 27.4 ± 1.5 mV in *P[syt^{WT}]* (Fig. 2B, asterisks $p<0.0001$). This decrease is similar to the result observed in a homologous mutation in mice and rats (Fernandez-Chacon et al., 2001; Sorensen et al., 2003; Wang et al., 2003; Han et al., 2004). The mutation within the C_2B domain reduced evoked release by approximately 80% compared to the transgenic control. Evoked release in the *P[syt$^{B\text{-}RQ}$]* mutants was 5.4 ± 0.7 mV (line 3) or 4.4 ± 0.7 mV (line 4), as compared to 27.4 ± 1.5 mV in *P[syt^{WT}]* (Fig. 2B, double asterisks $p<0.0001$). The level of evoked release remaining in the *P[syt$^{B\text{-}RQ}$]* mutants is significantly less than that in the *P[syt$^{A\text{-}RQ}$]* mutants (Fig. 2B, asterisks vs. double asterisks $p<0.001$). No difference in mean EJP amplitude was found between the insertions of a given genotype for either *P[syt$^{A\text{-}RQ}$]* or *P[syt$^{B\text{-}RQ}$]* ($p>0.2$). Thus, the reduction in evoked release results from the specific synaptotagmin mutations and not from insertion of the transgene disrupting an unspecified gene.

Mutation of either of the conserved basic residues increases the rate of spontaneous release at third instar neuromuscular junctions (Fig. 3A). The mutation within the C_2A domain at least doubled the rate of mEJPs, with a frequency of 3.5 ± 0.4 Hz (line 7) or 2.8 ± 0.3 Hz (line 8) in *P[syt$^{A\text{-}RQ}$]* compared to 1.4 ± 0.1 Hz in *P[syt^{WT}]* (Fig. 3A, asterisks $p<0.0001$). The mutation within the C_2B domain increased the rate of mEJPs by approximately 40%, to 1.9 ± 0.2 Hz (line 3) or 2.0 ± 0.3 Hz (line 4) as compared to 1.4 ± 0.1 Hz for *P[syt^{WT}]* (Fig. 3A, double asterisks $p<0.05$). The frequency of mEJPs in the *P[syt$^{A\text{-}RQ}$]* and *P[syt$^{B\text{-}RQ}$]* mutants was not significantly different ($p>0.05$, single asterisks vs. double asterisks, one way ANOVA). No difference in mEJP

frequency was found between independent insertions of the synaptotagmin gene for *P[syt$^{A\text{-}RQ}$]* or *P[syt$^{B\text{-}RQ}$]* (Fig. 3A, $p>0.2$). The amplitude of mEJPs was unchanged in the mutants (*P[syt^{WT}]* 1.10 ± 0.03 mV; *P[syt$^{A\text{-}RQ}$]* line 7, 1.00 ± 0.08 mV; *P[syt$^{A\text{-}RQ}$]* line 8, 1.24 ± 0.06 mV; *P[syt$^{B\text{-}RQ}$]* line 3, 1.09 ± 0.06 mV; *P[syt$^{B\text{-}RQ}$]* line 4, 1.10 ± 0.07 mV, $p>0.1$, one way ANOVA). In addition, we compared the frequency of quantal amplitudes for each mutant line (Fig. 3B, *P[syt^{WT}]*, *P[syt$^{A\text{-}RQ}$]* line 8 and *P[syt$^{B\text{-}RQ}$]* line 3 shown). The constant mEJP amplitude indicates that neither the C_2A nor the C_2B interfacial mutation perturbs synaptic vesicle filling or the postsynaptic machinery and is consistent with previous studies of spontaneous release characteristics in syt$^{A\text{-}RQ}$ (Sorensen et al., 2003).

The decreased evoked release observed in *P[syt$^{A\text{-}RQ}$]* and *P[syt$^{B\text{-}RQ}$]* mutants is not the result of protein misexpression

It is conceivable that the decreased evoked release demonstrated in both the *P[syt$^{A\text{-}RQ}$]* and *P[syt$^{B\text{-}RQ}$]* mutants results from protein misexpression. To assess the expression level of each transgenic line, we probed Western blots of larval central nervous systems with an anti-synaptotagmin antibody (Fig. 4A, *P[syt^{WT}]*, *P[syt$^{A\text{-}RQ}$]* line 8 and *P[syt$^{B\text{-}RQ}$]* line 3 shown). The two independent lines of both *P[syt$^{A\text{-}RQ}$]* and *P[syt$^{B\text{-}RQ}$]* used for the electrophysiological experiments expressed approximately the same amount of transgenic synaptotagmin as the transgenic control line (Fig. 4B). Thus, the deficits in evoked release seen in both the *P[syt$^{A\text{-}RQ}$]* and *P[syt$^{B\text{-}RQ}$]* mutants are not the result of insufficient expression of the transgene. To determine whether the mutant proteins were appropriately localized to synaptic sites, the neuromuscular junctions of

mutant and control transgenic larvae were immunolabelled with an anti-synaptotagmin antibody. In all lines, transgenic synaptotagmin was properly localized to the neuromuscular junction (Fig. 4C, *P[syt^{WT}]*, *P[syt$^{A\text{-}RQ}$]* line 8 and *P[syt$^{B\text{-}RQ}$]* line 4 shown). Thus, the decrease in evoked release did not result from either a deficiency in gene dosage or improper localization.

The syt$^{A\text{-}RQ}$ and syt$^{B\text{-}RQ}$ interfacial mutations change the Ca^{2+} affinity but not the cooperativity of release

Release of neurotransmitter has long been known to be a Ca^{2+} dependent, cooperative process (Dodge and Rahamimoff, 1967). The Ca^{2+}-cooperativity ("n") of release may represent the mean number of Ca^{2+} ions utilized to trigger a vesicle fusion event (Dodge and Rahamimoff, 1967; Stevens and Sullivan, 2003; Tamura et al., 2007). To assess the Ca^{2+} dependence of the release properties in the interfacial mutants, evoked release was measured at a variety of extracellular Ca^{2+} concentrations, ranging from 0.6-5.0 mM. At all Ca^{2+} concentrations, the interfacial mutants exhibit a decrease in evoked transmitter release compared to control (Fig. 5A). To determine whether mutation of either of these basic residues changes the Ca^{2+} cooperativity of release, we plotted the mean EJP amplitude vs extracellular Ca^{2+} concentration on a double log plot in non-saturating Ca^{2+} ranges (Fig. 5B). As estimated from the slope of these double log plots, n = 3.2 for *P[syt^{WT}]*, n = 3.0 for *P[syt$^{A\text{-}RQ}$]*, and n = 2.9 for *P[syt$^{B\text{-}RQ}$]* similar to previously recorded values (n = 3.0-3.6) at wild type neuromuscular junctions in *Drosophila* (Littleton et al., 1994; Stewart et al., 2000; Yoshihara and Littleton, 2002; Okamoto et al., 2005). Thus neither of the interfacial mutations changes the Ca^{2+} cooperativity of

release. This finding is consistent with the hypothesis that synaptotagmin's interaction with phospholipids functions downstream of Ca^{2+} binding and does not effect the number of Ca^{2+} ions needed to trigger vesicle fusion.

To assess the apparent Ca^{2+} affinity of release, we fit the Hill equation to the data and normalized to the predicted maximal response within each line. Fig. 5C shows that the apparent Ca^{2+} affinity of release *in vivo* was decreased in both the *P[syt$^{A\text{-}RQ}$]* (EC_{50} = 2.0 ± 0.1 mM) and *P[syt$^{B\text{-}RQ}$]* (EC_{50} = 2.3 ± 0.2 mM) mutants as compared to the transgenic wild type control (EC_{50} = 1.4 ± 0.1 mM). A rightward shift of the EC_{50} for Ca^{2+}-evoked transmitter release was previously seen for syt$^{A\text{-}RQ}$ mutants in several cell culture systems including: hippocampal autapses, chromaffin cells, and PC12 cells (Fernandez-Chacon et al., 2001; Sorensen et al., 2003; Wang et al., 2003). While confirming the effect of the C_2A mutation, our experiments further demonstrate that at an intact synapse, the syt$^{B\text{-}RQ}$ mutation also results in a severe disruption of Ca^{2+}-evoked transmitter release suggesting that the function of the basic residue at the tip of loop three is conserved.

DISCUSSION:

The C_2A and C_2B domains are structurally highly homologous and exhibit many similar biochemical interactions *in vitro* (Geppert et al., 1991; Sutton et al., 1995; Chae et al., 1998; Shao et al., 1998; Ubach et al., 1998; Fernandez et al., 2001; Cheng et al., 2004). Analysis of synaptotagmin's biochemical interactions have provided critical insights into the molecular mechanisms mediating synaptic vesicle fusion. The precise experimental conditions *in vitro*, however, can have dramatic effects on the observed

interactions. Biochemical studies examining the interfacial interaction between the highly-conserved, positively-charged residue at the tip of the C_2A Ca^{2+}-binding pocket and negatively-charged phospholipid membranes show contradictory results when the isolated C_2A domain is used: Fernandez-Chacon et al (2001) document an ~two-fold decrease in the Ca^{2+} affinity of the interaction when this residue is neutralized by replacement with a glutamine ($syt^{A\text{-}RQ}$) while Zhang et al.(1998) found no decrease in Ca^{2+}-dependent phospholipid interactions resulting from the $syt^{A\text{-}RQ}$ mutation, despite examination of interactions with PS/PC, PI/PC, PIP/PC and PIP_2/PC liposomes. Studies using the tandem C_2AB domains, on the other hand, support the ~two-fold decrease in Ca^{2+}-dependent phospholipid binding (Fernandez-Chacon et al., 2001; Wang et al., 2003; Li et al., 2006). Studies examining the interaction between the homologous residue in C_2B and negatively charged phospholipid membranes show variable results using tandem C_2AB domains: Wang et al. (2003) find that the C_2B mutation results in an analogous ~two-fold decrease in the Ca^{2+}-affinity for negatively charged phospholipid membranes composed of PS/PC, whereas Li et al. (2006) find no change when PS/PC membranes are used. Thus, Ca^{2+}-dependent, interfacial interactions between these positively charged residues and negatively-charged phospholipid membranes appears to be quite sensitive to the exact experimental conditions.

Consistent with the majority of studies on the basic residues, biochemical studies of other residues also support the hypothesis that the Ca^{2+}-dependent interaction between each C_2 domain and anionic phospholipids is conserved and mediated by residues at the tip of the Ca^{2+}-binding pockets. At the tip of each Ca^{2+}-binding pocket, immediately adjacent to the conserved basic residue (Fig. 1, ⊕), there is a hydrophobic residue (Fig. 1,

open box). In both C_2A and C_2B these hydrophobic residues have been demonstrated to interact with anionic phospholipids in a Ca^{2+}-dependent manner (Chae et al., 1998; Chapman and Davis, 1998; Bai et al., 2002; Gerber et al., 2002; Frazier et al., 2003), with the hydrophobic residues inserting into the hydrophobic core of the membrane. Increasing the hydrophobicity of three hydrophobic residues located around the rim of each Ca^{2+}-binding pocket, by substitution with tryptophans, substantially increased the Ca^{2+} affinity of the C_2 domain interactions with negatively charged liposomes (Rhee et al., 2005). Taken together, these results provide strong support for the hypothesis that a Ca^{2+}-dependent interaction between phospholipids and both the C_2A and C_2B domains are mediated by residues located at the tip of the Ca^{2+}-binding pockets.

But are these biochemically identified, Ca^{2+}-dependent interactions between synaptotagmin and anionic membranes relevant for synaptic transmission? Results from cultured cells provide strong support for the hypothesis that residues at the tip of the C_2A Ca^{2+}-binding pocket mediate a functionally significant interaction. In cultured hippocampal neurons, PC12 cells, and chromaffin cells, the $syt^{A\text{-}RQ}$ mutation decreased evoked transmitter release by shifting the apparent Ca^{2+} affinity for neurotransmitter release by ~two-fold (Fernandez-Chacon et al., 2001; Sorensen et al., 2003; Wang et al., 2003; Han et al., 2004) providing a good correlation between decreases in evoked release and the studies that show decreased phospholipid binding. However, even in the C_2A domain, this result is not unanimous. One study could not detect a significant decrease in the amplitude of evoked release in cultured hippocampal neurons expressing either the syt^{WT} or the $syt^{A\text{-}RQ}$ mutation although they did see a decrease in the Ca^{2+} affinity of release in the mutants (Stevens and Sullivan, 2003). The homologous mutation in C_2B

has been examined twice in cultured cells and these experiments produced contradictory results. When used to rescue cultured hippocampal neurons from *syt*^{null} mutant mice, this mutation showed no decrease in Ca^{2+}-evoked release (Li et al., 2006), while in cultured rat PC12 cells, the C_2B mutation resulted in an ~two-fold decrease in the Ca^{2+} affinity of evoked release (Wang et al., 2003). Thus, the relevance of the C_2B interaction remained inconclusive.

We, therefore, tested the function of the conserved, basic residues at the tip of the C_2A and C_2B Ca^{2+}-binding pockets during evoked transmitter release *in vivo* using an intact synapse, the *Drosophila* neuromuscular junction. We examined Ca^{2+}-evoked transmitter release in transgenic larvae expressing either the syt$^{A\text{-}RQ}$ or syt$^{B\text{-}RQ}$ mutant protein. Consistent with the findings from the majority of the cultured cell experiments, we found that the syt$^{A\text{-}RQ}$ mutation decreased evoked transmitter release by ~50% in 1.5 mM Ca^{2+} due to a decrease in the apparent Ca^{2+} affinity of release. Thus, the positively charged residue at the tip of the Ca^{2+}-binding pocket of the C_2A domain is required for efficient evoked transmitter release at intact synapses.

Importantly, we found that the syt$^{B\text{-}RQ}$ mutation actually decreased Ca^{2+}-evoked transmitter release more than the syt$^{A\text{-}RQ}$ mutation. In 1.5 mM Ca^{2+}, evoked release was decreased by ~80% in the *P[syt$^{B\text{-}RQ}$]* mutant lines. Since this decrease was seen in two independent transgenic lines, it was not caused by the random insertion sites of the transgene. Western analysis and immunohistochemical localization studies demonstrate approximately equal levels of transgene expression and appropriate synaptic localization in the mutant and control lines. Therefore the decrease in evoked release at the neuromuscular junction is a direct result of the syt$^{B\text{-}RQ}$ mutation.

Our results are consistent with the decrease in evoked release seen in rat PC12 cells expressing the mutant C_2B protein (Wang et al., 2003). When using high K^+ to trigger release from PC12 cells, these authors found that the *rate* of evoked release was decreased by ~50% in both the C_2A and C_2B basic residue mutations. Interestingly, the cumulative amount of release was lower in the C_2B basic residue mutant than in the C_2A mutant, although this effect was not quantified. Both our results at intact synapses and the results from rat PC12 cells are in direct contrast with the lack of effect seen at hippocampal autapses (Li et al., 2006). Why cultured hippocampal syt^{null} neurons transfected with this C_2B mutant did not exhibit deficits in evoked release is difficult to assess. Yet the reported amplitude of evoked release for cultured hippocampal neurons is highly variable; the amplitude of release at cultured hippocampal autapses for the $syt^{A\text{-}RQ}$ mutation has been shown as 90% of control (n=7, (Stevens and Sullivan, 2003), as 51% of control (n=35, Li 06), and as 25% of control (n=25, (Han et al., 2004).

Importantly, the replacement of three hydrophobic residues around the rim of either the C_2A *or* the C_2B Ca^{2+}-binding pocket with residues of increased hydrophobicity (Trp) results in an increase in the Ca^{2+} affinity of evoked release at cultured hippocampal autapses. This increase correlated with an increase in Ca^{2+} affinity of phospholipid binding *in vitro* (Rhee et al., 2005). Thus, even at hippocampal autapses, the tip of the C_2B Ca^{2+}-binding pocket is available and able to interact with phospholipid membranes during synaptic transmission.

Our current data supports the model (Fig. 6) that a conserved, Ca^{2+}-dependent interaction between the anionic presynaptic membrane and the positively-charged residue at the tip of the Ca^{2+}-binding pocket in both the C_2A and C_2B domains pulls the synaptic

vesicle toward the presynaptic membrane in preparation for fusion (Shao et al., 1996; Arac et al., 2006). The insertion of hydrophobic residues on the tips of the C_2 domains may, in addition, destabilize the presynaptic membrane aiding the fusion reaction (Chapman and Davis, 1998; Jahn et al., 2003; Martens et al., 2007).

Our results demonstrate that the basic interfacial residues of the C_2A and C_2B domains are both required for efficient, evoked transmitter release at an intact synapse, indicating that the function of this basic residue is conserved in the C_2A and C_2B domains. Yet other studies have discovered dramatic functional differences between the C_2A and C_2B domains during synaptic transmission. The most striking difference is the relative importance of Ca^{2+} binding by the C_2B vs the C_2A domain in triggering synchronous vesicle fusion upon Ca^{2+} influx. While Ca^{2+} binding by the C_2A domain may modulate fusion, mutations within the C_2A Ca^{2+}-binding motif result in either subtle or no disruptions in evoked transmitter release (Fernandez-Chacon et al., 2002; Robinson et al., 2002; Stevens and Sullivan, 2003; Pang et al., 2006). On the other hand, Ca^{2+} binding by the C_2B domain is essential for triggering synaptic transmission; mutations within this motif inhibit synchronous transmitter release by up to 99% (Mackler and Reist, 2001; Nishiki and Augustine, 2004; Tamura et al., 2007). Thus Ca^{2+} binding by the C_2B domain plays the pivotal role in triggering fast, synchronous vesicle fusion.

The key difference between the C_2A and C_2B domains may lay within their polylysine motifs (Li et al., 2006; Loewen et al., 2006; Dai et al., 2007). The polylysine motif of the C_2B domain mediates a unique set of interactions that are *not* shared by the C_2A domain (Zhang et al., 1994; Chapman and Davis, 1998; Thomas and Elferink, 1998; Takei and Haucke, 2001), including Ca^{2+}-independent interactions with PIP_2 and SNARE

proteins (Zhang et al., 2002; Bai et al., 2004; Rickman et al., 2004; Rickman et al., 2006; Dai et al., 2007). Since the polylysine motif is located along the side of the C_2B domain, an interaction with the SNARE proteins could serve to hold the Ca^{2+}-binding pocket of the C_2B domain in close proximity to the presynaptic membrane, priming vesicles for immediate fusion upon Ca^{2+} influx (Loewen et al., 2006). In Fig. 6, we chose to diagram the interaction between the C_2B polylysine motif and SNAP-25 within the SNARE complex (Zhang et al., 2002; Rickman et al., 2004; Rickman et al., 2006; Dai et al., 2007), because mutation of the C_2B polylysine motif disrupts synaptotagmin's ability to increase the speed of SNARE-mediated liposome fusion in the absence of any PIP_2 (Loewen et al., 2006). However, an interaction with PIP_2, which is located specifically in the presynaptic membrane, has also been proposed to serve this purpose (Bai et al., 2004; Arac et al., 2006). Thus, while the conserved basic residues of each C_2 domain are mediating similar Ca^{2+}-dependent reactions with anionic phospholipids (Fig. 6 and Wang 03), it may be the relative positioning of the C_2A vs. C_2B domains with respect to the SNARE complex that determines the relative importance of the Ca^{2+}-binding sites for triggering vesicle fusion. A remarkably similar model, postulating a direct interaction between the polylysine motif of the C_2B domain and SNAP-25 within the SNARE complex resulting in a more distally positioned C_2A domain has recently been proposed based on the Ca^{2+}-dependent action of the C_2B domain in displacing complexin from membrane-anchored SNARE complexes (Dai et al., 2007).

In summary, the initiation of synchronous synaptic vesicle fusion by Ca^{2+} requires the coordinated interactions of many molecules in the presynaptic terminal. Examination of isolated interactions *in vitro* provide critical insights into the molecular

mechanisms that could mediate the fusion event; however, only the analysis of synaptic transmission at intact synapses can determine which interactions are likely to function *in vivo*. Our findings demonstrate that the positively-charged residue located at the tip of each Ca^{2+}-binding pocket is required for efficient synaptic transmission, indicating that the function of this region of the C_2 domains is likely conserved. These results support the hypothesis that these residues mediate the Ca^{2+}-dependent interaction of both the C_2A and C_2B domains with anionic phospholipids requisite for efficient excitation-secretion coupling during synaptic transmission.

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FIGURES:

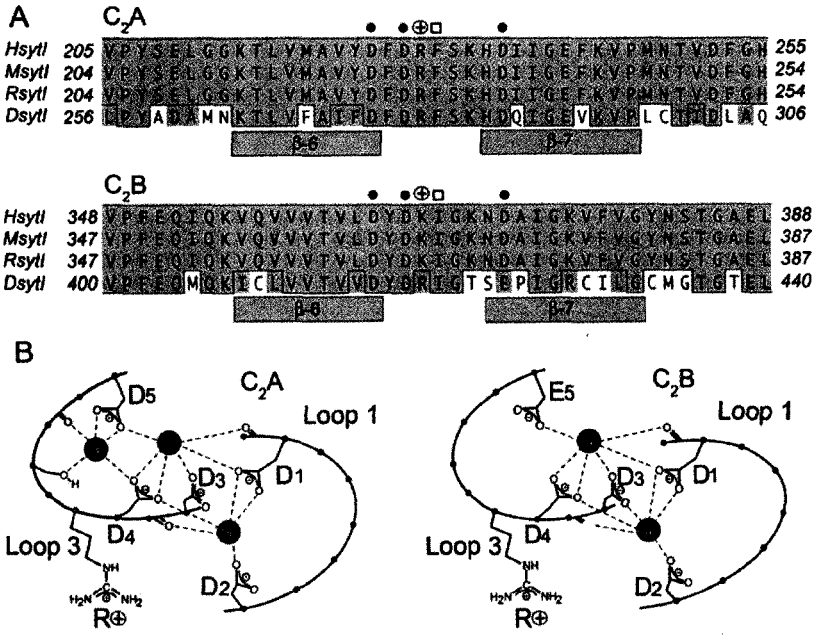

Fig 1. Both the C_2A and C_2B domains of synaptotagmin I have a conserved basic residue at the tip of the Ca^{2+}-binding pocket. A: Alignment of synaptotagmin I from human, rat, mouse and *Drosophila*. Bars indicate beta-sheets, ⊕ indicate the conserved basic residues, dots indicate Ca^{2+} binding residues, open boxes indicate conserved hydrophobic residues. Within the alignment, conserved residues are shown in gray, identical residues are boxed. B: Schematic representation of loops 1 and 3 that form the Ca^{2+}-binding pockets of both C_2 domains. Adapted from Fernandez et al (2001) using the *Drosophila* sequence to show the conserved basic residues (⊕) examined in this study.

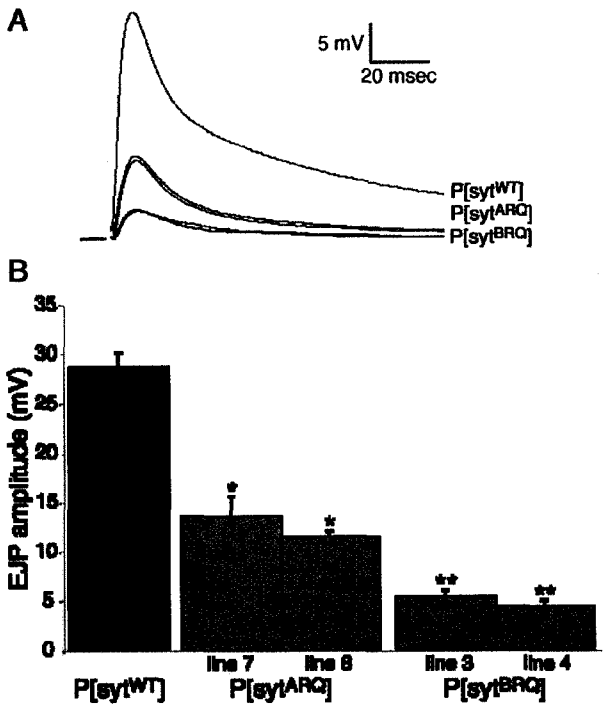

Fig 2. Evoked release is reduced in phospholipid-binding mutants of both C_2 domains. A: Representative traces recorded from larval muscle fiber 6. Each trace represents the mean of 30 consecutive sweeps from the same muscle fiber. B: Compared with *P[syt^{WT}]*, the mean EJP amplitude of all *P[syt^{RQ}]* lines was significantly decreased; $p<0.0001$, single and double asterisks, one way ANOVA, *P[syt^{WT}]*, n=16; *P[syt$^{A\text{-}RQ}$]* line 7, n=12; *P[syt$^{A\text{-}RQ}$]* line 8, n=14; *P[syt$^{B\text{-}RQ}$]* line 3, n=16; *P[syt$^{B\text{-}RQ}$]* line 4, n=13. Additionally, the evoked responses of the *P[syt$^{B\text{-}RQ}$]* lines were significantly lower than that of the *P[syt$^{A\text{-}RQ}$]* lines ($p < 0.01$, single vs. double asterisks), but no differences were found between *P[syt$^{A\text{-}RQ}$]* lines 7 and 8, or between *P[syt$^{B\text{-}RQ}$]* lines 3 and 4 ($p > 0.2$).

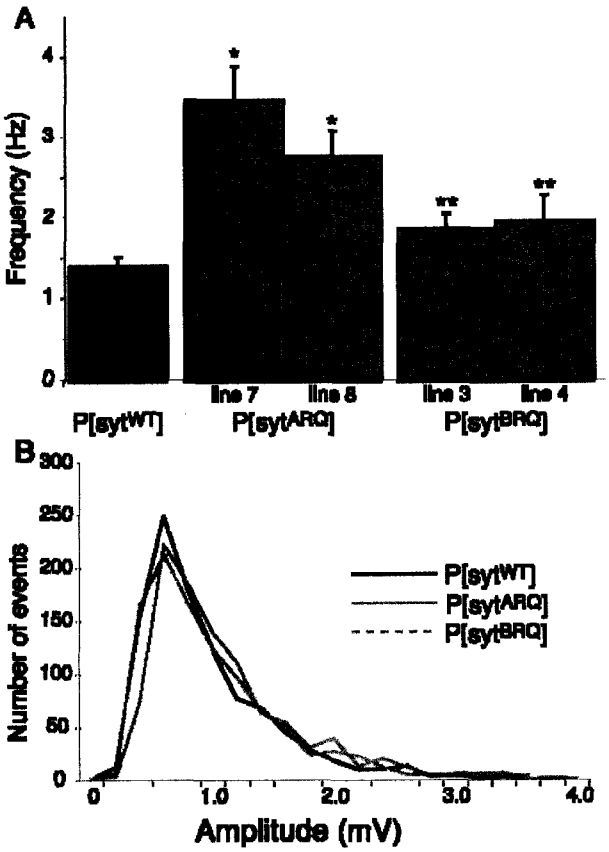

Fig 3. mEJP frequency is increased in phospholipid-binding mutants of both C_2 domains. A: Mean mEJP frequencies of each mutant and the transgenic control. *P[syt^{WT}]* n=55 fibers; *P[syt$^{A\text{-}RQ}$]* line 7, n=7 fibers; *P[syt$^{A\text{-}RQ}$]* line 8, n=17 fibers; *P[syt$^{B\text{-}RQ}$]* line 3, n=27 fibers; *P[syt$^{B\text{-}RQ}$]* line 4, n=12 fibers. Compared with *P[syt^{WT}]*, all genotypes had an increased mEJP frequency ($p<0.05$), though no difference was detected between *P[syt$^{A\text{-}RQ}$]* lines 7 and 8 ($p>0.1$) or between *P[syt$^{B\text{-}RQ}$]* lines 3 and 4 ($p>0.1$). B: Frequency distribution curves of mEJP amplitudes calculated from 1000 individual events per transgenic line in 0.2 mV bins. *P[syt^{WT}]*, *P[syt$^{A\text{-}RQ}$]* line 8, *P[syt$^{B\text{-}RQ}$]* line 3 shown.

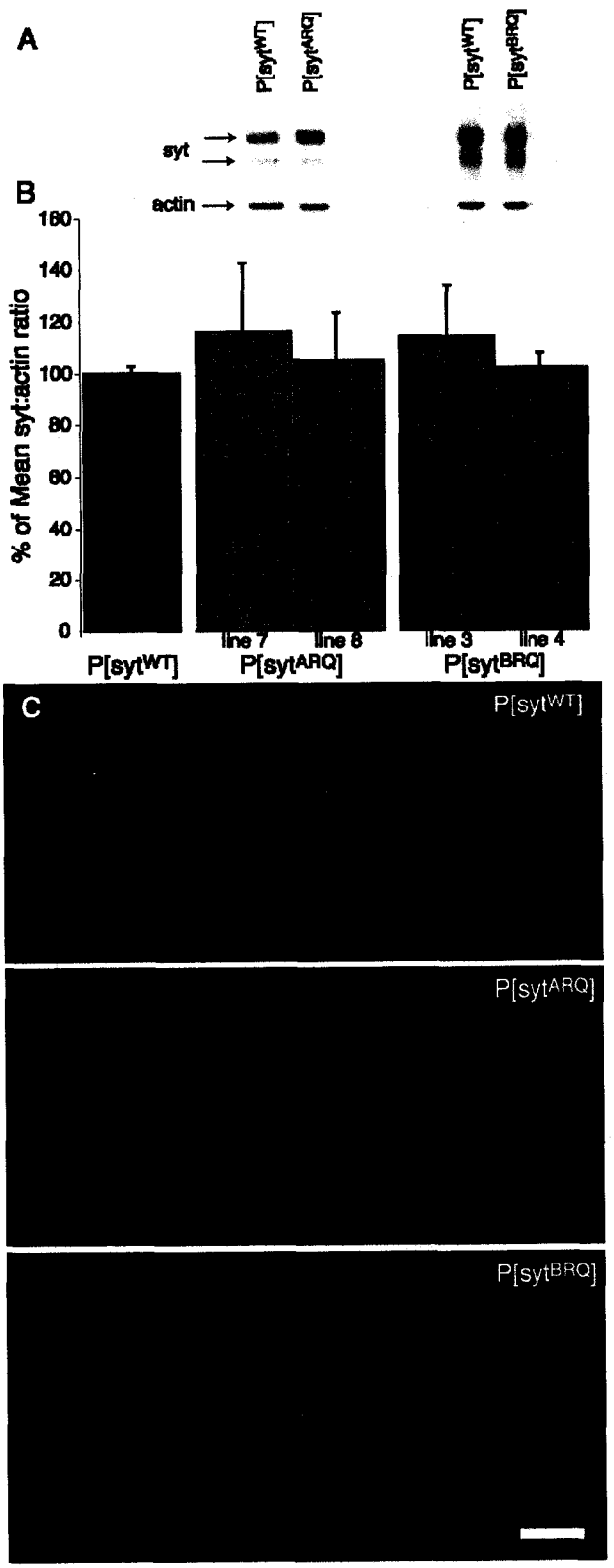

Fig 4. Synaptotagmin expression is unaltered in phospholipid-binding mutants. A: Synaptotagmin is expressed at similar levels in each of the transgenic synaptotagmin lines. Representative Western blots of homogenized central nervous systems of third instars from the indicated lines were probed with an anti-synaptotagmin antibody. To confirm equal loading, they were also probed with an anti-actin antibody. B: Comparison of synaptotagmin/actin ratio normalized to the mean ratio of the transgenic control for *P[syt^{WT}]*, n=45; *P[syt$^{A\text{-}RQ}$]* line 7, n=6; *P[syt$^{A\text{-}RQ}$]* line 8, n=14; *P[syt$^{B\text{-}RQ}$]* line 3, n=18; *P[syt$^{B\text{-}RQ}$]* line 4, n=10. An ANOVA showed no significant difference between any of the genotypes ($p > 0.1$). C: Synaptotagmin is localized to the larval neuromuscular junction in each of the transgenic synaptotagmin lines. Representative confocal Z-stack projections for *P[syt^{WT}]*, *P[syt$^{B\text{-}RQ}$]* line 8, and *P[syt$^{B\text{-}RQ}$]* line 4. Scale bar is 10 uM.

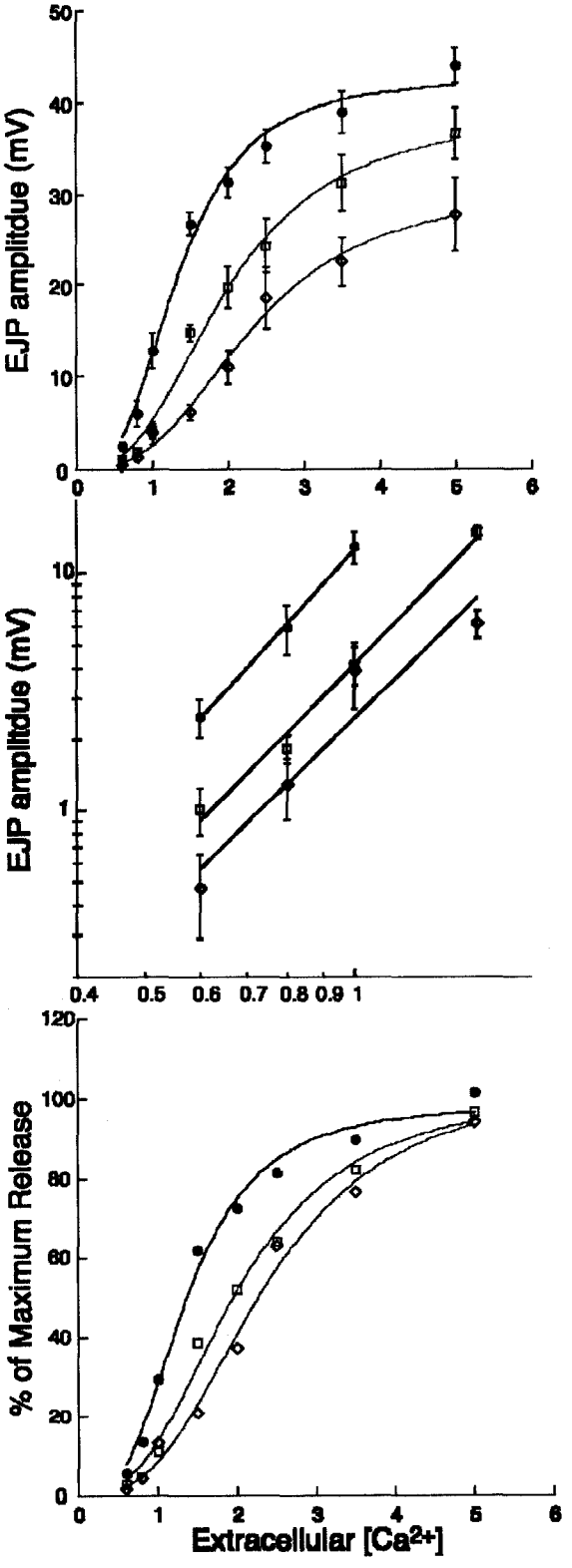

Fig 5. The phospholipid-binding mutants decrease the apparent Ca^{2+} affinity but do not effect the Ca^{2+} cooperativity of release. A: EJPs were evoked in *P[syt^{WT}]*, *P[syt$^{A\text{-}RQ}$]* line 8, and *P[syt$^{B\text{-}RQ}$]* lines 3 and 4 by 0.05 Hz stimulation and 10 sweeps were averaged for each fiber at each $[Ca^{2+}]$. The responses from lines 3 and 4 of *P[syt$^{B\text{-}RQ}$]* were not significantly different, so the results were pooled. For all genotypes at all $[Ca^{2+}]$, n=10-18 muscle fibers, except for 1.5 mM Ca^{2+}, at which n=40-49 muscle fibers. The Hill equation was fit to the data, error bars are SEM. B: The EJP amplitudes within the non-saturating range of Ca^{2+} were plotted on a double log plot and a linear regression line was used to determine the slope (n) (*P[syt^{WT}]*: n = 3.2, R=0.999; *P[syt$^{A\text{-}RQ}$]*: n = 3.0, R=0.999; *P[syt$^{B\text{-}RQ}$]*: n = 2.9, R=0.933). C: The EJP amplitudes at each Ca^{2+} concentration were normalized to the maximum predicted by the Hill equation for each genotype and replotted to illustrate the shift in EC_{50}. P[*syt^{wt}*]: EC_{50} = 1.4 ± 0.1 mM; *P[syt$^{A\text{-}RQ}$]*: EC_{50} = 2.0 ± 0.1 mM; *P[syt$^{B\text{-}RQ}$]*: EC_{50} = 2.3 ± 0.2 mM. For all panels, filled circles indicate *P[syt^{WT}]*, open squares indicate *P[syt$^{A\text{-}RQ}$]* and open diamonds indicate *P[syt$^{B\text{-}RQ}$]*.

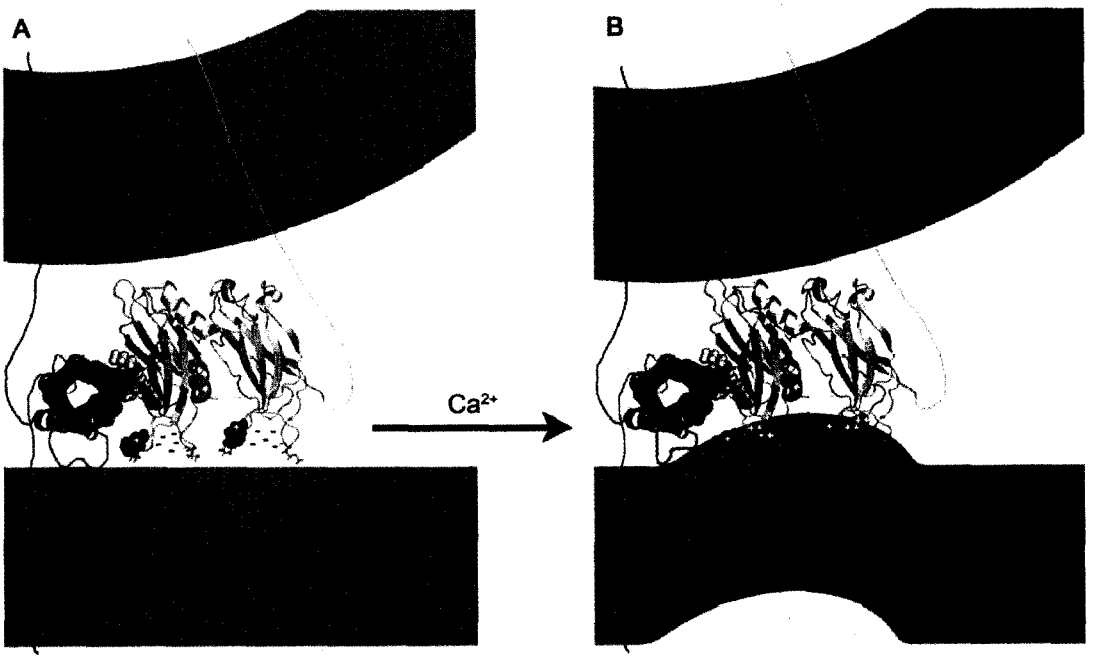

Fig 6. Model of the role played by the conserved basic residues in Ca^{2+}-dependent interfacial interactions with the anionic presynaptic membrane. The crystal structure of the core complex (PDB file 1SFC, containing syntaxin [red], SNAP-25 [green], and VAMP/synaptobrevin [blue]), the nuclear magnetic resonance structures of the C_2A (PDB file 1BYN) and C_2B (PDB file 1K5W) domains of synaptotagmin (yellow), and Ca^{2+} (pink) are shown to scale using PyMOL Molecular Graphics System (DeLano Scientific, Palo Alto, CA). The membranes, the transmembrane domains, and the link between C_2A and C_2B were added in Adobe Photoshop. A: The Ca^{2+}-

independent priming interaction between the C_2B polylysine motif (yellow, space-filled residues) and SNAP-25 [green, space-filled residues, (Zhang et al., 2002; Rickman and Davletov, 2003; Loewen et al., 2006)] holds the C_2A and C_2B Ca^{2+} binding sites in close proximity to the presynaptic membrane. In the absence of Ca^{2+}, the high concentration of negative charge in the Ca^{2+}-binding pockets repulse the negatively charged presynaptic membrane, preventing synaptotagmin's conserved basic interfacial residues (blue, space-filled residues) from interacting with the membrane. B: Upon Ca^{2+} entry, the negative charge of the Ca^{2+}-binding pockets are neutralized by the bound Ca^{2+}, which initiates the electrostatic switch: a strong attraction of the negatively-charged, phospholipid head groups by the bound Ca^{2+} and the basic residues at the tips of Ca^{2+}-binding pockets that draws the synaptic vesicle towards the presynaptic membrane. Insertion of the hydrophobic residues at the tips of the C_2 domains into the core of the presynaptic membrane may help trigger fusion by promoting a local Ca^{2+}-dependent buckling of the plasma membrane (Chapman and Davis, 1998; Martens et al., 2007). The Ca^{2+} induced increase in positive charge at the end of the C_2B domain also likely increases the strength of the electrostatic interaction between the C_2B polylysine motif and the SNARE complex resulting in simultaneous binding of the SNARE complex and the presynaptic membrane (Loewen et al., 2006; Dai et al., 2007).

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Chapter 2:

Conclusions and Further Directions

Further Directions:

Synaptotagmin is the Ca^{2+} sensor for fast synchronous neurotransmitter release, yet the molecular interactions that couple Ca^{2+} binding to vesicle fusion remain unclear. Deletion of synaptotagmin I abolishes Ca^{2+}-dependent, synchronous release in many systems (Nonet et al., 1993; DiAntonio and Schwarz, 1994; Geppert et al., 1994; Voets et al., 2001), but little is gleaned about its interaction with other components of the fusion machinery through these studies. *In vitro* studies have identified a host of important conserved sites within synaptotagmin, but deficits in *in vitro* studies do not always correlate to functional deficits in synaptic transmission (Bai et al., 2002; Fernandez-Chacon et al., 2002; Robinson et al., 2002; Zhang et al., 2002; Bai et al., 2004). Only *in vivo* analysis correlating the *in vitro* interactions of these conserved sites to deficits in synaptic transmission will give an accurate understanding of their role in synaptic transmission. *In vivo* studies on site-directed mutants have begun to identify individual motifs within synaptotagmin that are critical for Ca^{2+}-triggered synchronous release. For example, mutations of the C_2B Ca^{2+}-binding motif decrease both Ca^{2+}-dependent phospholipid binding *in vitro* (Mackler et al., 2002) and synchronous synaptic transmission *in vivo* (Mackler et al., 2002; Nishiki and Augustine, 2004). These studies demonstrate that Ca^{2+} binding by the C_2B domain is essential for synaptotagmin function. In addition, they are consistent with the hypothesis that Ca^{2+}-dependent phospholipid binding by the C_2B domain is functionally important, but mutation of the

Ca^{2+} binding motif would disrupt all Ca^{2+}-dependent interactions of the C_2B domain, not just phospholipid binding.

Ca^{2+}-dependent phospholipid binding is one of the most prevalent functions of C_2 domains, often involved in translocating them to the cell membrane (Cho and Stahelin, 2006). Both C_2 domains of synaptotagmin I have been shown to interact with anionic phospholipids. The proposed 'electrostatic switch' mechanism involves the Ca^{2+} ions, charges at the tips of the Ca^{2+} binding pockets of synaptotagmin and anionic phospholipid headgroups (Shao et al., 1997; Davletov et al., 1998; Ubach et al., 1998; Murray and Hoenig, 2002). Another mode of phospholipid interaction has also been proposed for the Ca^{2+}-binding pockets of synaptotagmin. Specifically, the Ca^{2+}-binding loops of both C_2 domains have several conserved hydrophobic residues that have been shown to penetrate phospholipid membranes *in vitro* (Chapman and Davis, 1998; Davis et al., 1999; Bai et al., 2002; Bai et al., 2004; Arac et al., 2006; Herrick et al., 2006). The extent and strength of this interaction has been shown to be largely dependent on the composition of the anionic phospholipids in liposomes used (Bai et al., 2004; Arac et al., 2006). However, these studies have relied on fluorescent reporters that are, themselves, hydrophobic which may cause a non-physiological membrane penetration.

Each C_2 domain of synaptotagmin contains a highly conserved hydrophobic residue at the tip of loop 3 in the Ca^{2+} binding pocket (Fig 1, open boxes, page 32). During synaptotagmin's Ca^{2+}-dependent interaction with anionic phospholipids *in vitro*, these hydrophobic residues insert into the bilayer while the adjacent basic residues interact with

the negatively charged phospholipid head groups (Chae et al., 1998; Chapman and Davis, 1998; Bai et al., 2002; Bai et al., 2004; Arac et al., 2006; Herrick et al., 2006). A recent study found that enhancing the hydrophobicity of multiple hydrophobic residues around the rim of each C_2 domain enhanced the Ca^{2+}-sensitivity of release, suggesting that facilitating Ca^{2+}-dependant membrane penetration of either C_2A or C_2B facilitated synaptic transmission. (Rhee et al., 2005). However, introducing increased hydrophobicity may create an interaction that would not normally occur. To ultimately determine the role of these residues, it will be necessary to examine the *in vivo* consequences of replacing these hydrophobic residues with non-hydrophobic residues.

In vivo disruption of the conserved membrane penetration residues at the tip of the C_2 domains would provide insight into the role of synaptotagmin's membrane penetration in synaptic transmission. In addition to that insight, examination of these conserved residues in both C_2A or C_2B would provide information on the relative importance of phospholipid penetration by each C_2 domain.

Conclusions:

Synaptotagmin is the Ca^{2+} sensor for fast, synchronous neurotransmitter release. Many molecular interactions have been identified, including those with the SNARE complex and phospholipids. Which of these interactions underlie its role as Ca^{2+}-sensor remain to be determined. Here we have examined the role of synaptotagmin's phospholipid binding by disrupting Ca^{2+}-dependent phospholipid binding sites of *Drosophila* synaptotagmin. Specifically, we have demonstrated that a conserved basic residue in both the C_2A and C_2B domains of synaptotagmin are essential for synchronous evoked release. This is consistent with synaptotagmin's binding to phospholipids contributing to its role as the Ca^{2+} sensor. Additionally, we have demonstrated that phospholipid binding by synaptotagmin's C_2B domain plays a more critical role than that of the C_2A domain. This has led to the development of a model in which interactions between the tips of the C_2 domains of synaptotagmin with the pre-synaptic membrane underlies its role as the Ca^{2+} sensor for fast, synchronous neurotransmitter release (Chapter 1, Fig 6, page 74). This model can be further tested by disruption of the membrane penetration residues, as described in the further directions section (page 87).

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