Molecular Genetic Characterization of the *Drosophila* Synaptotagmin Family

by

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Molecular genetic characterization of the *Drosophila* synaptotagmin family

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Proper functioning of the nervous system requires fast, spatially-restricted neuron-neuron communication at synapses. Classic physiology studies have demonstrated the importance of calcium in regulating synaptic communication; however the molecular events underlying basic synaptic transmission and plasticity have only recently become the subject of intense investigation in neuroscience.

The synaptotagmin family of vesicle proteins has been implicated in calcium-dependent neurotransmitter release, although Synaptotagmin 1 (Syt 1) is the only isoform demonstrated to control synaptic vesicle fusion. We have characterized the six remaining synaptotagmin isoforms encoded in the *Drosophila* genome, including homologs of mammalian Synaptotagmins 4, 7, 12 and 14. Using immunolocalization and *in situ* hybridization experiments (Chapter 2), we demonstrate that each isoform has a unique subcellular localization and expression pattern, suggesting only Synaptotagmin 1 functions in synaptic vesicle exocytosis. Consistent with their distinct localizations, overexpression of Synaptotagmin 4 (Syt 4) or Synaptotagmin 7 (Syt 7) cannot functionally substitute for the loss of Syt 1 in synaptic transmission and loss-of-function mutations in Syts 4 and 7 do not have defects in neurotransmitter release (Chapter 4). Rather, Syt 4 and Syt 7 likely function in novel regulated-exocytosis pathways within neurons, distinct from synaptic vesicle cycling.

The unique ability of Syt 1, but not other Syt isoforms, to localize to synaptic vesicles prompted us to determine the domains within Syt 1 responsible for its trafficking to synaptic vesicles (Chapter 3). We find the trafficking of Syt 1 is complex, likely requiring several sorting signals present at the N-terminus and in the C2 domains. The N-terminus was required for proper targeting to presynaptic terminals, while the C2 domains were essential for internalization at synapses. Furthermore, we demonstrate the C2 domains of Syts 4,7, α, and β can not promote localization to synaptic vesicles, even if mislocalized to presynaptic terminals, further arguing only Syt 1 is present on synaptic vesicles *in vivo* (Chapter 3).

Like Synaptotagmin 1, Syt 4 is ubiquitously present at most, if not all synapses, but localizes to the postsynaptic compartment (Chapter 2). Syt 4 homologs have been identified in all metazoan genomes sequenced to date, suggesting this isoform may mediate an evolutionarily conserved role in postsynaptic vesicle trafficking. To elucidate the function of Syt 4-dependent postsynaptic vesicle trafficking we have generated and analyzed null mutations in *syt 4*. Although Syt 4 is not required for viability, embryonic neuromuscular junctions in mutant animals show a developmental delay in the formation of varicosities, a reduction in neurotransmitter release, and loss of a particular form of synaptic plasticity following high frequency stimulation, we have termed HFMR (High

Frequency-induced Miniature Release). Postsynaptic expression of a *syt4* transgene can rescue the presynaptic defects (Chapter 4), indicating Syt 4 mediates a retrograde signaling pathway at synapses. In addition, we demonstrate Syt 4 cycles through the postsynaptic plasma membrane (Chapter 4), suggesting it may regulate secretion of retrograde signals in a manner analogous to Syt 1 regulation of neurotransmitter release, presynaptically. There is mounting evidence in several experimental systems for a regulated form of postsynaptic vesicular trafficking. Dendritic release of a number of neuromodulators such as dopamine, ATP, GABA, and neuropeptides has been documented, while postsynaptic vesicles within dendritic spines and shafts have been directly visualized using electron microscopy. Studies in hippocampal culture neurons indicate that long-term labeling with FM1-43 loads dendritic organelles that undergo rapid calcium-triggered exocytosis. The localization and evolutionary conservation of Syt 4 makes it an attractive candidate for mediating a postsynaptic trafficking pathway common to all metazoan synapses. Indeed, localization studies of Syt 4 in mammals have noted the presence of the protein within dendrites and soma, similar to our studies in *Drosophila*. Utilizing the exceptional genetic tools available to *Drosophila*, we expect the characterization of Syt 4 and this novel retrograde signaling pathway will lead to new and exciting insights into the role of this protein family in fundamental synapse biology.

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Chapter 1

Molecular nature of neurotransmitter release at synapses

Bill Adolfsen and J. Troy Littleton.

Overview of Membrane Fusion

Secretory pathways can be broadly divided into constitutive and regulated modes of membrane trafficking. Constitutive secretion is found in all cell types and is the prominent mode of transport between the endoplasmic reticulum, Golgi and plasma membrane. Regulated secretion is found primarily in neurons and endocrine cells, where membrane trafficking is finely tuned to intracellular signals such as calcium (Katz, 1969). The exocytotic machinery in neurons can be viewed as a calcium-regulated system that has been superimposed on a core set of proteins that are utilized in constitutive secretion (Jahn and Sudhof, 1999; Littleton, 2000). Synaptic transmission requires that synaptic vesicles move from a reserve pool to an active cycling pool of vesicles. Synaptic vesicles that are destined to fuse with the presynaptic membrane then undergo docking reactions that position the vesicles in close proximity to the sites of fusion on the plasma membrane. Following docking, the vesicles are further prepared for fusion competence through a series of priming reactions (Sudhof, 2004). The molecular events that underlie docking and priming are unknown. However, the protein interactions mediating the last stage in exocytosis, membrane fusion, are beginning to be deciphered.

The core of the membrane fusion machine at synapses (and likely at most, if not all, sites of intracellular fusion) is built around the SNARE complex, composed of the synaptic vesicle protein, synaptobrevin/VAMP, and the plasma membrane proteins syntaxin 1 and SNAP-25 (Sollner et al., 1993). Large families of these SNAREs are present in all eukaryotes sequenced to date (Littleton, 2000) and are distributed in specific membrane compartments along the secretory pathway where they are predicted to function in compartment-specific vesicle fusion within the cell (Scales et al., 2000).

Structural analysis has shown that SNAREs assemble into a parallel coiled-coil four stranded helix with one helix each contributed by syntaxin and synaptobrevin, and two helices contributed by SNAP-25 (Figure 1-1) (Sutton et al., 1998). The parallel alignment of the helices brings the transmembrane domains of syntaxin and synaptobrevin into close contact, thereby driving the vesicle membrane into close apposition to the presynaptic membrane. Both genetic and biochemical evidence indicate SNAREs are essential for membrane fusion. Removal of the v-SNARE synaptobrevin in *Drosophila* results in the elimination of action potential evoked vesicle fusion at the neuromuscular junction and a large reduction in the frequency of spontaneous fusion (Deitcher et al., 1998; Yoshihara et al., 1999). Similarly, removal of syntaxin 1 from *Drosophila* eliminates both spontaneous and evoked fusion, without altering the docking of synaptic vesicles at presynaptic active zones (Schulze et al., 1995; Broadie et al., 1995). Temperature-sensitive paralytic mutations in *Drosophila* syntaxin 1 have also been identified that decrease synaptobrevin binding and reduce SNARE complex formation at the paralytic temperature, confirming that v-/t-SNARE interactions are required for neurotransmitter release *in vivo* (Littleton et al., 1998).

Biochemical evidence indicating the SNARE complex can mediate bilayer lipid fusion was provided by the work of Rothman and colleagues in *in vitro* liposome fusion assays. SNAREs are required and sufficient for membrane fusion in this *in vitro* fusion system (Weber et al., 1998; Nickel et al., 1999). SNARE specificity in mediating fusion has been demonstrated with several convincing controls. Using biochemically modified SNARE proteins in these assays, it appears that formation of the SNARE complex promotes bilayer fusion by exerting force on the membrane anchors of individual

Fig. 1-1

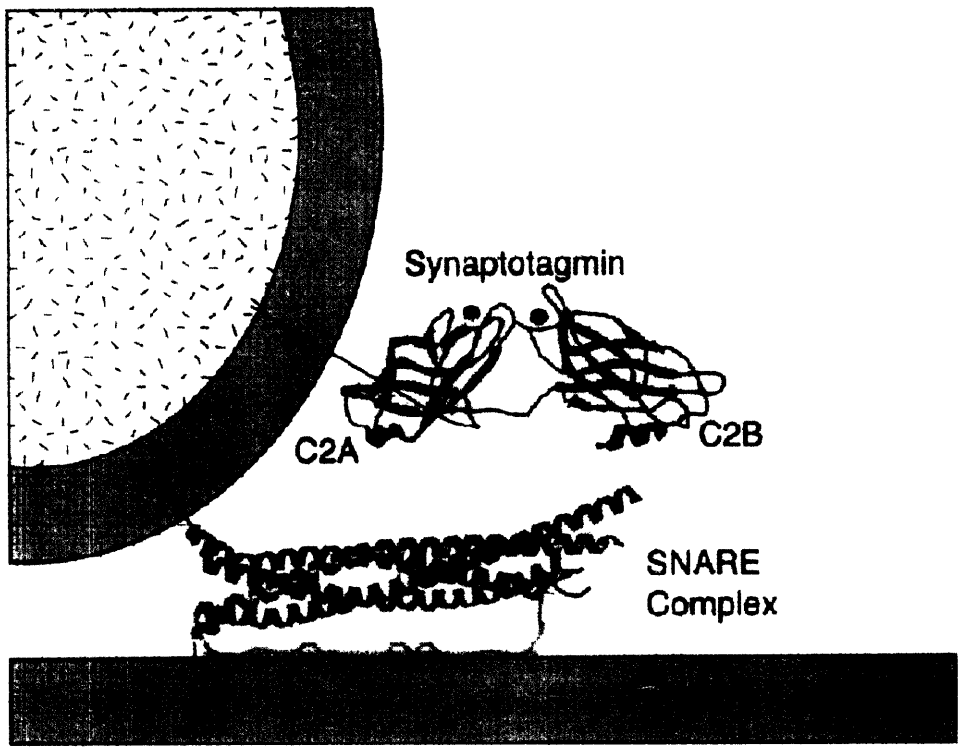

Figure 1-1. The crystal structure of the SNARE complex and synaptotagmin 3 shown schematically superimposed on a synaptic vesicle docked at the presynaptic plasma membrane. Transmembrane domains have been added to synaptotbrevin and synaptotagmin. The sites of calcium ion binding to the C2 domains of synaptotagmin are indicated by green circles. The C2 domains are shown in red (the α helix of each C2 domain is shown in blue). The intravesicular domain of synaptotagmin has been omitted. Synaptobrevin is indicated in blue, SNAP-25 in green, and syntaxin in red.

SNAREs during SNARE complex formation. This conformational arrangement is likely to lead to inward movement of lipids from the two membranes and eventual lipid mixing (McNew et al., 2000). Thus the formation of the SNARE complex has emerged as the central protein machinery that underlies membrane fusion. In addition, specific v-/t-SNARE pairing is likely to underlie compartment specificity in membrane fusion (McNew et al., 2000). Experimental determination of proteins controlling SNARE complex assembly and disassembly is likely to provide major insights into the spatial and temporal regulation of membrane fusion *in vivo* well into the next decade.

Current studies on synaptic transmission have suggested a complex picture of presynaptic nerve terminal architecture. One of the most defining features of this architecture is the extensive subcellular localization of the fusion machinery. Using electron microscopy, synaptic vesicles can be seen clustered at specific regions on the terminal plasma membrane termed active zones (Akert et al., 1969; Heuser et al., 1979). Active zones, when occupied with synaptic vesicles, are thought to host all the proteins necessary for regulated exocytosis, including presynaptic calcium channels. Electrophysiological studies have begun to elucidate some of the functional consequences of this organization. The maximal rate of calcium-triggered synaptic vesicle fusion can be as rapid as 1000–3000 vesicles/s, while the lag time between calcium influx and fusion can be as short as 100 μs (Llinas et al., 1981; Heidelberger et al., 1994; Sabatini and Regehr, 1996). Such rapid calcium sensing and membrane fusion suggest that synaptic vesicles are docked and primed prior to calcium influx and that local calcium-induced conformational changes in the exocytotic machinery result in bilayer fusion. This short time lag eliminates enzymatic steps in the final fusion event. Neurotransmitter release

has been shown to have a fourth order cooperativity with respect to calcium concentration (Dodge and Rahaminoff, 1967), suggesting multiple calcium ions bind to one or more proteins and/or lipids to trigger vesicle fusion. Current studies suggest synaptic vesicle exocytosis is triggered when the intracellular calcium concentration rises to greater than 1 μM calcium, with half-maximal fusion rates at 20 μM calcium (Bollmann et al., 2000). Such high calcium concentrations are thought to exist only near the pore of presynaptic calcium channels (Llinas et al., 1992), suggesting the release machinery and calcium channels are co-localized at sites of membrane fusion. The speed of exocytosis and the dynamics of calcium concentrations within the terminal place restrictions on how the fusion machinery can be assembled and function and point to a small number of protein rearrangements between calcium entry and bilayer membrane fusion.

A mechanistic understanding of how synaptic calcium signals are transduced into membrane fusion in still lacking. In evaluating the potential of a protein to be the calcium sensor responsible for synaptic vesicle fusion, two requirements are usually assumed. First, the protein must be able to bind calcium ions and do so with an affinity resembling that measured for neurotransmitter release at synapses. Second, since the measured time courses of the calcium transients at synapses is on the order of microseconds, the protein must be localized near the opening of presynaptic calcium channels at the active zone. Several candidate calcium binding proteins meet these requirements and have been proposed to function at synapses including calmodulin (Wang et al., 2003; Xia and Storm, 2005), neuronal calcium sensor 1 (NCS-1) (Burgoyne et al., 2004), calcium-dependent activator protein for secretion (CAPS) (Speidel et al.,

2003), and synaptotagmins (Adolfsen and Littleton, 2001; Marqueze et al., 2000; Sudhof and Rizo, 1996; Yoshihara et al., 2003).

Calmodulin

Calmodulin is an abundant highly conserved calcium binding protein found mainly in the cytosol of all eukaryotic cells. The protein is approximately 18 kD and binds four calcium ions through its four EF hand motifs, common helix-loop-helix domains known to bind divalent cations. In addition to having a putative role in neurotransmitter release, calmodulin regulates several cellular processes including muscle contraction, calcium homeostasis, and signal transduction (Burgoyne et al., 2003). Although calmodulin freely diffuses in the cytosol, the protein also associates with a number of active zone proteins which may localize it to release sites *in vivo*. In mammals calmodulin binds to the C-terminus of the synaptic vesicle protein, synaptotagmin 1 (Perin, 1996). In addition to synaptotagmin 1, calmodulin also binds to the C-terminal pore forming domain of several members of the voltage-gated calcium channel family (Black et al., 2005) and to the v-SNARE, synaptobrevin (Quetglas et al., 2002). Interactions with any of these proteins could localize calmodulin near calcium entry sites, thus meeting the fast temporal and spatial requirements of neurotransmitter release.

A second intriguing biochemical property of calmodulin is its calcium binding properties. The presence of four calcium binding sites is consistent with the calcium cooperativity (hill coefficient ~ 4) measured at most synapses. Indeed, calcium binding to each globular domain of calmodulin exhibits positive cooperativity (Linse et al., 1991).

However, how these *in vitro* biochemical properties are altered *in vivo* is still a matter of speculation.

Genetic characterization of calmodulin is hindered by the ubiquitous nature of the protein. Mutation of calmodulin in any species most likely results in several pleiotropic defects causing early lethality. Calmodulin null mutants in *Drosophila* are not embryonic lethal but die as early first instars due to maternal contribution of calmodulin transcripts (Wang et al., 2003). Both hypomorphic mutations and surviving nulls display abnormal locomotory behaviors, suggesting neurological and possibly transmission defects (Wang et al., 2003). Mosaic studies using the MARCM system in *Drosophila* should determine if any synaptic physiological defects are found in calmodulin null neurons. Functional studies in PC12 cells also indicate that calmodulin may regulate vesicle fusion *in vivo.* Calmodulin antagonists block hormone secretion in a permeabilized cell assay, arguing that calmodulin is required for the acute secretion of PC12 dense-core vesicles (Quetglas et al., 2002). Lack of physiological data from calmodulin knock-out animals, still makes this protein an attractive calcium sensor for synaptic vesicle exocytosis

Neuronal calcium sensor 1 (NCS-1)

The neuronal calcium sensor proteins are a large family of conserved proteins containing four EF hand motifs as calcium binding domains. Three of the EF hands can bind calcium with high affinity (micromolar range) (Hilfiker, 2003). Members of this superfamily include NCS-1, potassium channel-interacting proteins, visinin-like proteins, recoverins, and guanylate cyclase-activating proteins (Hilfiker, 2003). The first NCS mutant was identified and characterized in *Drosophila* and termed *frequenin* (Mallart et

al., 1991). Several lines of genetic evidence suggest that NCS proteins are not major calcium sensors involved in synaptic vesicle exocytosis. Single stimulation of the giant cervical axon pathway in *Drosophila* frequenin loss-of-function mutants results in the generation of multiple action potentials (Tanouye et al., 1981). Similarly, physiological recordings conducted at mutant larval neuromuscular junctions showed increased neurotransmitter release under high frequency stimulation (Mallart et al., 1991). The activation of neurotransmitter release in *frequenin* loss-of-function animals argues this protein is more likely to play a modulatory role rather than an essential one in neurotransmitter release. Consistent with findings in *Drosophila*, NCS null mutants in *C. elegans* do not show any obvious abnormalities in locomotion, but are defective in modulated behaviors such as isotherm tracking (Gomez et al., 2001). Interestingly, overexpression of NCS-1 in rat hippocampal cultures is able to switch short term plasticity from depression to facilitation without causing any changes in basal transmission (Sippy et al., 2003). These results suggest that NCS-1 is acting through an independent pathway from basal transmission to regulate short term synaptic plasticity. Although NCS-1 is probably not the major calcium sensor for fast synchronous transmitter release, it remains a good candidate for the asynchronous calcium sensor thought to mediate facilitation.

Calcium-dependent activator protein for secretion 1 (CAPS1)

Originally isolated as a cytosolic protein required for reconstitution of exocytosis in a semi-intact PC12 preparation, calcium-dependent activator protein for secretion (CAPS) is a 145 kD conserved protein required during the calcium-dependent step of

dense-core vesicle exocytosis (Walent et al., 1992). Subcellular localization studies have found, in addition to being distributed within the cytosol, a fraction of CAPS protein is localized to the membranes of large dense-core vesicles but absent from small clear synaptic vesicles (Berwin et al., 1998). Consistent with biochemical studies, genetic studies in *Drosophila* and *C. elegans* also indicate CAPs plays a functional role in dense-core vesicle exocytosis *in vivo*. Loss-of-function mutants in *Drosophila* CAPs display a 50 % reduction in neurotransmitter release and large accumulations of dense-core vesicles in synaptic terminals can be seen by EM microscopy (Renden et al., 2001). CAPs mutants in C. elegans are viable, however display defects in several modulated behaviors including feeding and egg-laying (Ann et al., 1997). Recently, recordings from chromaffin cells acquired from CAPs knock-out mice, suggest CAPs may function in the uptake of catecholamines in LDCVs, as mutant cells have a large reduction in the amount of catecholamine that is released, even though similar numbers of vesicles fuse upon stimulation (Speidel et al., 2003) in wild-type and mutant littermates. The synaptic phenotypes observed in CAPs null mutants from several species, indicate it is unlikely that CAPs is serving as the main calcium sensor for fast secretion of neurotransmitter at synapses.

Synapatotagmins

Several biochemical and genetic studies on synaptotagmin 1 are consistent with the idea that synaptotagmin 1 is a major calcium sensor at synapses, although it likely plays multiple roles in vesicle cycling (Schiavo et al., 1998; Marqueze et al., 2000). This conclusion is still debated, however, and the precise mechanism by which synaptotagmin

1 functions is far from elucidated. Currently, 15 synaptotagmin isoforms have been isolated in mammals, and seven and eight homologues have been found in *Drosophila* and *C. elegans,* respectively. Besides the data obtained for synaptotagmin 1, the function of the remaining synaptotagmins is relatively unclear. I will briefly review the literature on the synaptotagmin family and discuss the implications for synaptotagmin function in vesicle cycling.

Biochemistry of Synaptotagmin 1

Synaptotagmin 1 is by far the most studied isoform of the synaptotagmin family and was cloned from rat brain as a 65 kD synaptic vesicle protein with calcium-dependent phospholipid binding properties (Perin et al., 1990). Synaptotagmin 1 contains a small N-terminal intravesicular domain, a single transmembrane domain and a large cytoplasmic region (Figure 1-2). Two domains homologous to the C2 regulatory domain of protein kinase C occupy the majority of the cytoplasmic region of synaptotagmin. C2 domains are ~ 130 amino acid motifs that are found in a large array of intracellular and extracellular proteins, many of which bind calcium. The crystal structure of several C2-domain containing proteins, including the cytoplasmic domain of synaptotagmin 3 and the C2A domain of synaptotagmin 1, have been solved, demonstrating that C2 domains fold into an eight stranded β-sandwich (Sutton et al., 1995; Sutton et al., 1999). Three cytosolic loops surround the calcium-binding ligands and emerge from the core β-sandwich to form a calcium-binding pocket. The crystal structures indicate that no significant conformational changes occur upon calcium binding. Such observations favor a model of C2 domain function in which changes in surface electrostatic potential

Fig. 1-2

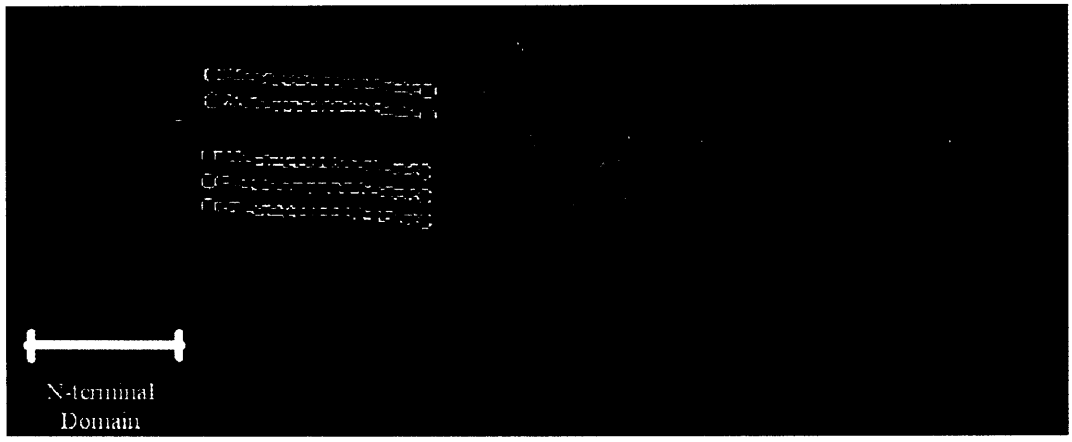

Figure 1-2. The domain structure of synaptotagmin 1. The transmembrane domain anchors synaptotagmin 1 to synaptic vesicles (indicated by phospholipids) and splits the protein into an intravesicular N-terminal domain and a cytoplasmic domain consisting of two tandem C2 domains (C2A and C2B).

mediate many of the calcium-dependent C2 domain interactions. The three loops forming the calcium binding pocket in the C2A domain of synaptotagmin 1 insert directly into phospholipid bilayers upon calcium binding (Brose et al., 1992; Davletov et al., 1993; Chapman et al., 1998). The C2A domain of synaptotagmin 1 has evolved for speed, as it interacts with membranes upon calcium binding with diffusion limited kinetics, much faster that other C2 domain-lipid interactions that have been characterized (Davis et al., 1999). In addition, the EC_{50} for calcium-dependent phospholipid binding to the C2A domain has been shown to be ~74 μM calcium, approximating the levels of intracellular calcium required to trigger synaptic vesicle fusion (Davis et al., 1999). These properties have prompted the hypothesis that synaptotagmin 1's calcium-dependent lipid binding properties may be involved in lipid rearrangements required during synaptic vesicle fusion.

The C2B domain of synaptotagmin 1 has also been shown to bind calcium and undergo local conformational changes (Desai et al., 2000). One consequence of calcium binding by the C2B domain is oligomerization of the protein via clustering by C2B-C2B interactions (Chapman et al., 1996; Sugita et al., 1996; Chapman et al., 1998; Littleton et al., 1999; Osborne et al., 1999). Calcium-dependent oligomerization of synaptotagmin is extremely rapid, requires calcium concentrations near those required for triggering fusion and is an evolutionarily conserved interaction (Davis et al., 1999; Littleton et al., 1999), suggesting that C2B domain-calcium interactions may also function in fusion. Intriguingly, the crystal structure of synaptotagmin 3 reveals that the linker between the C2A and C2B domains is unstructured and flexible. FRET studies have demonstrated that upon calcium binding, both C2 domains undergo an intramolecular interaction that

modulates the distance between the two domains (Garcia et al., 2000), suggesting that both domains may also cooperate in calcium-dependent interactions. One interaction known to require both C2 domains together is the ability of synaptotagmin to form calcium-dependent complexes with isolated t-SNAREs and the assembled SNARE complex (Davis et al., 1999; Gerona et al., 2000). Given the central role of SNARE complex formation in fusion, this interaction has also been cited as a potential coupling step in triggering rapid exocytosis. The ability of synaptotagmin to form calcium-dependent complexes with the isolated t-SNAREs syntaxin and SNAP-25 suggests that synaptotagmin may order monomeric SNAREs into helical proteins that could facilitate their incorporation into the SNARE complex. However, synaptotagmin also binds SNAREs and the assembled SNARE complex in the absence of calcium, though with a substantially reduced affinity (Davis et al., 1999). Such calcium-independent SNARE interactions could contribute to vesicle docking or perhaps function in a fusion clamp role to prevent SNARE complex formation and vesicle fusion in the absence of calcium and thus could explain why mini frequency increases in synaptotagmin 1 nulls. Botulinum A, which cleaves the extreme C-terminal of SNAP-25, disrupts calcium-dependent exocytosis. Martin and colleagues demonstrated that botulinum A also specifically blocks the binding of synaptotagmin to SNAP-25 in a calcium-dependent manner, without affecting SNARE complex formation, supporting a role for a calcium-dependent SNAP-25-synaptotagmin interaction in calcium-triggered exocytosis *in vivo* (Gerona et al., 2000). The remaining calcium-dependent interactions of synaptotagmin 1 that have been reported include a reduction in binding to the synaptic vesicle protein SV2 (Schivell

et al., 1996) and altered phosphoinositide binding (Schiavo et al., 1996). The significance of these calcium-dependent interactions is unknown.

In addition to calcium-dependent interactions, synaptotagmins have been shown to interact with a host of neuronal proteins in the absence of calcium. These interactions include binding to calcium channels (Sheng et al., 1997; Leveque et al., 1992; Yoshida et al., 1992), sodium channels (Sampo et al., 2000), the clathrin adapter AP-2 (Zhang et al., 1994), the stoned endocytotic proteins (Wu et al., 1999), β-SNAP (Schiavo et al., 1995), neurexins (Hata et al., 1993), calmodulin (Perin, 1996), inositol polyphosphates (Fukuda etal., 1994), SYNCRIP (Mizutani et al., 2000) and polyphosphoinositides (Schiavo et al., 1996)(Figure 1-3). These diverse interactions suggest synaptotagmin 1 may have important roles in a variety of presynaptic functions, potentially complicating a simplistic view of synaptotagmin solely as a calcium sensor for exocytosis. The interaction of synaptotagmin with two classes of neuronal ion channels, sodium and calcium channels, suggests synaptotagmin may be involved in the presynaptic regulation of ion channel function, though genetic evidence supporting such a role is lacking. The interaction of synaptotagmin with the clathrin adapter AP-2 is evolutionarily conserved in *Drosophila* (Littleton et al., 1999), suggesting a potential role in facilitating endocytosis of synaptic vesicles at nerve terminals. This interaction can be disrupted by binding of the C2B domain of synaptotagmin to the inositol high-polyphosphate series (Mizutani et al., 1997), suggesting the potential for modulation of synaptotagmin - AP2 binding. The interaction of synaptotagmin with the putative endocytotic proteins stnA and stnB encoded by the *stoned* locus in *Drosophila* also points towards a role in the modulation of vesicle endocytosis. Synaptotagmin's interaction with β-SNAP has been postulated to

Fig. 1-3

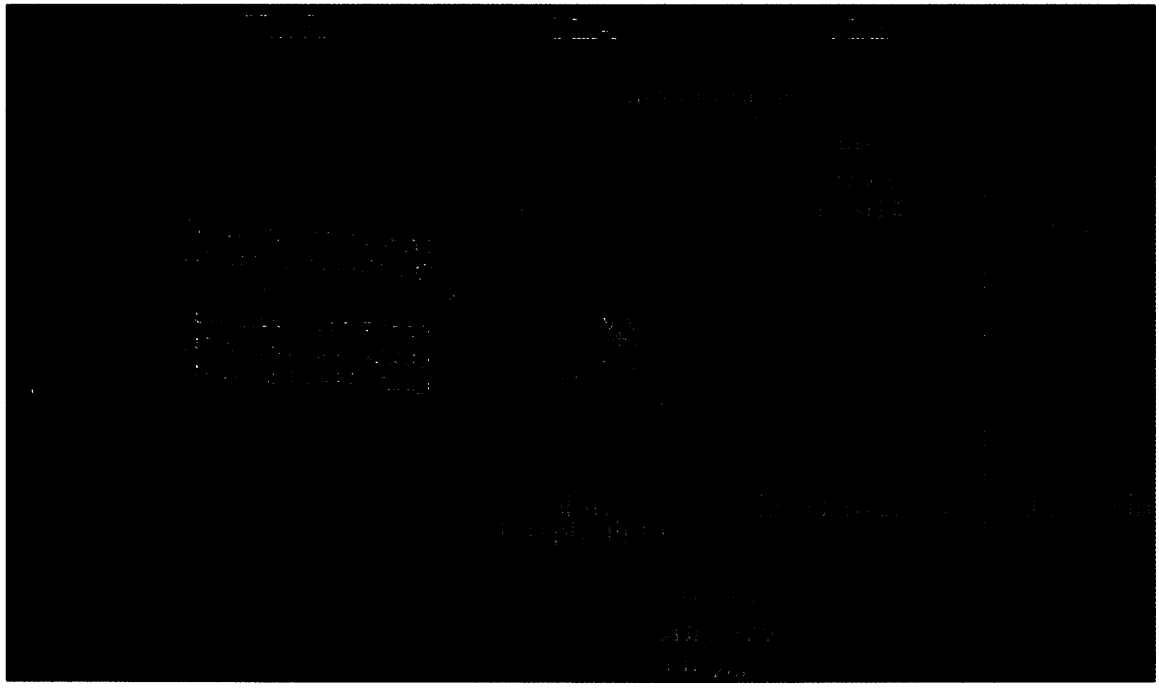

Figure 1-3. Synaptotagmin 1 biochemical interactions. Reported calcium-independent interactions are listed above the domains which mediate them. Interactions requiring both C2 domains are listed between them. Calcium-dependent interactions are listed below the synaptotagmin 1 diagram.

represent a fusion particle, but this interaction is not observed in *Drosophila*, suggesting it is not essential for synaptic vesicle trafficking (Littleton et al., 1999). Neurexins are polymorphic cell adhesion molecules localized to synapses *in vivo* (Ushkaryov et al., 1992). Their interaction with synaptotagmin could play a role in vesicle docking or in localizing synaptic vesicles to subdomains within the nerve terminal. Given the plethora of interactions now reported for synaptotagmin 1, genetic dissection of the protein's function *in vivo* via the generation of a large collection of specific loss-of-function mutants is clearly required to define which of these interactions actually occur *in vivo* and what step in vesicle cycling they mediate.

Functional analysis of synaptotagmin 1

Genetic studies have unambiguously demonstrated that disruption of synaptotagmin 1 leads to profound defects in synaptic transmission, confirming an important role in regulated secretion of synaptic vesicles. In *C. elegans*, *synaptotagmin 1* mutants are severely defective in muscle movements and show a large reduction in the number of synaptic vesicles at synapses, suggesting defects in vesicle formation or endocytosis (Nonet et al., 1993; Jorgensen et al., 1995). Removal of synaptotagmin 1 in mice disrupts the fast synchronous fusion of synaptic vesicles, without altering asynchronous or latrotoxin-induced fusion, suggesting a role for synaptotagmin in triggering synchronous vesicle release (Geppert et al., 1994). In *Drosophila*, *synaptotagmin 1* mutants are lethal and show a severe decrease in neurotransmitter release. (Littleton et al., 1993; DiAntonio et al., 1994; Littleton et al., 1994). Null mutations do not show a large depletion of synaptic vesicles as seen in *C. elegans*, but

rather have a reduced number of docked vesicles and an increase in a population of large diameter vesicles (Reist et al., 1998). Partial loss-of-function mutations that delete the C2B domain show a reduction in the number of synaptic vesicles at photoreceptor synapses under stimulation, suggesting the C2B domain is required for efficient endocytosis under conditions that drive synaptic recycling (Yoshihara and Littleton, 2002). Thus, the loss of synaptotagmin at synapses seems to disrupt, though not eliminate, synaptic vesicle endocytosis, with consequent alterations in vesicle size. Loss of synaptotagmin also alters the ability of synaptic vesicles to be maintained in a fusion-competent state at active zones. One possibility to account for the increase in spontaneous fusion and the decrease number of docked vesicles in *synaptotagmin* mutants is that synaptotagmin functions as a fusion clamp to prevent SNARE-dependent fusion until the arrival of a calcium signal. Loss of this activity in *synaptotagmin* null mutants could account for the increased rate of spontaneous fusion and decrease in the number of docked vesicles.

The defect in endocytosis in *synaptotagmin* mutants also indicates another important point in the interpretation of genetic studies. When studying *synaptotagmin* mutants that alter endocytosis, such defects have to be taken into account and can confound any interpretation of a role for synaptotagmin on the exocytotic side of the vesicle trafficking pathway. Therefore, partial loss-of-function mutations that do not alter endocytosis are necessary to address the function of synaptotagmin specifically in exocytosis. The most straight forward approach to investigate whether the calcium binding properties of the aspartic acid residues in C2A and C2B are required for exocytosis is to mutate them and evaluate the corresponding effects on synaptic

physiology. If these residues are important in coordinating calcium and triggering fusion, subtle point mutations to alter them should phenocopy the defects found in synaptotagmin 1 null animals. To date, no group has mutated all the calcium-coordinating residues in both the C2A and C2B domains. However, several recent studies have reported the alteration of some of the calcium-coordinating residues in the C2A or C2B individually.

Mutations altering two of the calcium coordinating residues (D232N and D238N) in the C2A domain were engineered into mice (Fernandez-Chacon et al., 2001). Interestingly, native protein isolated from these animals was still able to interact with phospholipids and syntaxin in a calcium-dependent manner. Consistent with the lack of disruption of known C2A interactions, physiological recordings performed on mutant animals were normal, suggesting that these C2A residues are not required for *in vivo* function (Fernandez-Chacon et al., 2001) or that calcium binding to the C2A domain may be partially redundant to C2B calcium binding (Earles et al., 2001). In order to decrease the apparent calcium affinity of the C2A domain, Fernandez-Chacon et al. mutated an arginine residue (R233Q) residing just outside the calcium binding loops. The mutation results in a two-fold decrease in the calcium affinity of synaptotagmin 1 and does not result in detectable structural or conformational changes (Fernandez-Chacon et al., 2001). R233Q knockin mouse synapses have an approximately two-fold reduction in the calcium sensitivity of neurotransmitter release, providing the best evidence to date that subtle changes in the calcium binding of synaptotagmin 1 result in corresponding changes in calcium-dependent release properties. Work in *Drosophila* has further demonstrated the importance of the C2A domain in triggering vesicle fusion (Yoshihara and Littleton,

2002). The syt^{AD1} allele encodes a premature stop codon that results in deletion of the entire C2B domain, keeping the N-terminus and C2A domain intact (DiAntonio et al., 1994). In syt^{AD1} mutants, synchronous release can still be detected although the probability of release is greatly diminished (Yoshihara and Littleton, 2002). These results are consistent with the mammalian data and confirm that the C2A domain alone is sufficient to drive synchronous fusion.

In contrast to disruptions of C2A calcium binding, mutations in C2B result in more pronounced defects in vesicle fusion. Mutation of several of the aspartic acid residues in the C2B domain (D416N, D418N and a D356N, D362N double mutant) fail to rescue synaptotagmin null mutations in *Drosophila*, arguing that calcium binding to the C2B domain is essential for normal transmitter release (Mackler et al., 2002). Furthermore, neuronal overexpression of the D356N, D362N transgene resulted in a nearly 90% reduction in neurotransmitter release in wild-type backgrounds. Electron microscopy performed on these C2B mutants indicate an abundance of docked vesicles, arguing the dominant-negative C2B transgenes block release following vesicle docking (Mackler et al., 2002). Further work will have to be conducted to determine if these mutations have any effects on the ability of synaptotagmin 1 to undergo endocytosis.

In addition to the mutated C2B transgenes, the *Drosophila* synaptotagmin allele syt^{AD3} encodes a Y364N point mutation that disrupts a highly conserved residue in the C2B domain of synaptotagmin 1 (DiAntonio et al., 1994), but does not interfere with AP-2 binding or disrupt endocytosis *in vivo*. Instead, this mutation disrupts calcium-dependent oligomerization, resulting in a post-docking defect in vesicle fusion and a failure to assemble SNARE complexes (Yoshihara and Littleton, 2002). Thus, the C2B

domain is required *in vivo* for both endocytosis and calcium-dependent fusion following vesicle docking. Functional studies in cracked PC12 cells supports this model, as recombinant C2B domains introduced as dominant negatives can block exocytosis at the final calcium-triggered step in vesicle fusion (Desai et al., 2000). Injection of recombinant C2B domains that have mutations which disrupt oligomerizaton of synaptotagmin do not inhibit secretion, presumably by their inability to interact with native synaptotagmin 1 C2B domains (Desai et al., 2000).

Taken together, genetic studies demonstrate that bilayer mixing and the fusion reaction itself does not require synaptotagmin 1. Rather synaptotagmin 1 functions by triggering rapid fusion upon calcium influx and mediating efficient and rapid endocytotic retrieval of the synaptic vesicle after fusion.

In addition to genetic studies, a large number of other manipulation studies on synaptotagmin 1 have been performed. Injection of peptides from the C2 domains of synaptotagmin 1 into squid giant synapses rapidly and reversibly inhibits neurotransmitter release (Bommert et al., 1993). Injection of anti-synaptotagmin 1 C2A domain antibodies into the squid giant synapse blocks evoked release and increases the number of docked synaptic vesicles (Mikoshiba et al., 1995). This same antibody also blocks calcium-dependent phospholipid binding by the C2A domain. In contrast, injection of an anti-synaptotagmin C2B domain antibodies decreases evoked release during repetitive stimulation and also results in a 90% decrease in synaptic vesicle number (Fukuda et al., 1995). Similar findings were observed by antibody injection studies in adrenal chromaffin cells (Ohara-Imaizumi et al., 1997). Together with the results obtained from genetic studies, a simplistic and likely incomplete molecular model

underlying synaptotagmin 1's role in trafficking can be summarized as follows: 1. Synaptotagmin's ability to bind SNAREs and other effectors such as neurexins and calcium channels in a calcium-independent fashion results in synaptic vesicles that are positioned and maintained as fusion-competent vesicles at active zones. 2. The calcium-independent interaction with t-SNAREs maintains the fusion machinery in a fusion-ready state by bringing the t-SNARE complex into close approximation to the v-SNARE synaptobrevin, while preventing premature SNARE complex formation and fusion in the absence of a calcium signal. 3. Calcium entry triggers synaptotagmin oligomerization via the C2B domain, facilitates SNARE complex assembly, oligomerizes the SNARE complex into a fusion pore and promotes additional local lipid rearrangements via C2A domain-lipid interactions that culminate in rapid and efficient calcium-triggered synchronous vesicle fusion. 4. Following fusion, synaptotagmin is recognized by the AP-2 adapter proteins and together with the stoned proteins cooperate to ensure rapid endocytotic retrieval of synaptic vesicles back into the recycling pool.

Diversity of the Synaptotagmin Gene Family

Apart from synaptotagmin 1, eleven other synaptotagmins have been identified in mammals (Marqueze et al., 2000). We have identified seven synaptotagmins in *Drosophila* (Littleton, 2000), making the synaptotagmin family one of the largest protein families implicated in membrane trafficking in both invertebrates and vertebrates. Outside of mutations in mouse synaptotagmin 4 and overexpression studies on fly synaptotagmin 4, few other genetic manipulations of synaptotagmins have been reported. Thus, the precise role of these additional isoforms is unknown. One striking observation

is that most of the synaptotagmin isoforms show calcium-dependent hetero-oligomerization with each other (Desai et al., 2000; Osborne et al., 1999; Littleton et al., 1999), suggesting that hybrid synaptotagmin oligomers with novel calcium sensitivities may represent a unique mechanism for presynaptic plasticity in the nervous system. Indeed, the finding that many of the synaptotagmin isoforms express unique calcium–dependent phospholipid binding properties and different synaptotagmin-syntaxin calcium affinities (Li et al., 1995), suggests that the hetero-oligomers may possess unique trafficking properties (Hilbush et al., 1994). A description of the expression patterns and subcellular localization of each of the isoforms is required, however, to further test this model. Below we briefly summarize some of the known synaptotagmin family members and possible functions in membrane trafficking.

Expression pattern analysis, subcellular localization studies, and *in vitro* biochemical assays (Table 1-1) have been performed on many of the mammalian family members, while relatively little is known about the remaining isoforms. Two broad subfamilies have been proposed based on expression pattern. The first subgroup, which includes synaptotagmins 1-5, 10-12, is primarily expressed in the nervous system and in other endocrine organs (Mizuta et al., 1994; Babity et al., 1997; Thompson, 1996; Berton et al., 1997; Ullrich and Sudhof, 1995; Marqueze et al., 1995). The remaining synaptotagmins (6-9) show a more ubiquitous expression and are not enriched in the nervous system (Li et al., 1995). In *Drosophila*, synaptotagmins 1 and 4 are abundantly expressed in the nervous system, while synaptotagmin 7 is expressed more ubiquitously and is not enriched in the nervous system (Littleton et al., 1999). The expression pattern of the remaining synaptotagmins is unknown. Suprisingly, not all of the synaptotagmins

are predicted to be solely found on vesicles. Both synaptotagmin 3 and 6 have been found in other cellular compartments, including the plasma membrane (Butz et al., 1999). Synaptotagmin 7 has been found on lysosomes (Martinez et al., 2000), while synaptotagmin 4 has been found not only on synaptic vesicles (Littleton et al., 1999; Osborne et al., 1999), but also in the Golgi (Ibata et al., 2000; Berton et al., 2000). Synaptotagmin 2 shows very high homology to its sister isoform, synaptotagmin 1, and is likely to be functionally redundant with synaptotagmin 1. Synaptotagmin 2 and synaptotagmin 1 colocalize on the same synaptic vesicles in certain areas in rat brain. Furthermore, synaptotagmin 1 and 2 can form calcium-dependent heterodimers (Osborne et al., 1999). *In situ* hybridization studies in the rat CNS demonstrate that synaptotagmin 1 and synaptotagmin 2 show a different distribution in the developing nervous system. Northern blot analysis suggests synaptotagmin 2 is found mainly in the spinal cord while synaptotagmin 1 transcripts are abundant in brain (Geppert et al., 1991). Localization studies on synaptotagmin 3 have been performed both in pancreatic beta-cells and rat neural cells. Butz et al 1999 report that synaptotagmin 3-specific antibodies stain the synaptic plasma membrane fraction and not synaptic vesicles (Butz et al., 1999). Localization in the synaptic plasma membrane does not rule out possible functions in vesicle fusion, but may indicate that synaptotagmin 3 has an important role in other calcium-mediated presynaptic processes. In pancreatic beta-cells, synaptotagmin 3 colocalizes with the secretory granule markers, insulin and secretogranin I, suggesting it is also found in some vesicle populations (Brown et al., 2000). Surprisingly, invertebrates lack a synaptotagmin 3 homolog, suggesting this isoform is likely to serve specific functions required only in vertebrates. The subcellular localization and function

of the remaining synaptotagmins is relatively unknown. One exception to this is synaptotagmin 4, which is discussed below.

Synaptotagmin 4 has been shown to be upregulated in PC12 cells and hippocampal neurons upon depolarization (Vician et al., 1995; Ferguson et al., 1999) . It is also upregulated by kaniate-induced seizure activity in rat brain. A similar upregulation has been observed for synaptotagmin 10 (Babity et al., 1997) and syt 12 (Thompson, 1996), suggesting that modification of the expression levels of different synaptotagmin isoforms may have a functional role in neuronal plasticity. One of synaptotagmin 4's most unusual properties is an evolutionarily conserved amino acid substitution (D to S) in a key calcium binding ligand in the C2A domain that renders it incapable of calcium-dependent phospholipid binding in *Drosophila* (Littleton et al., 1999). The calcium-dependent phospholipid binding of rat synaptotagmin 4 is controversial and may depend on the phospholipid composition of vesicles (Fukuda et al., 1996; Poser et al., 1997). In *Drosophila*, upregulation of synaptotagmin 4 can lead to a decrease in both evoked and spontaneous synaptic transmission (Littleton et al., 1999). In addition, synaptotagmin 1/4 hetero-oligomers have reduced calcium-induced phospholipid-binding properties compared to synaptotagmin 1/1 homo-oligomers (Littleton et al., 1999). Thus, seizure-induced up-regulation of synaptotagmin 4 may be a conserved mechanism to down regulate neuronal activity, possibly serving a neuroprotective function. Such an upregulation may also be a mechanism for controlling long-term synaptic plasticity in the brain. Indeed, mutations in mouse synaptotagmin 4 lead to defects in both motor performance and learned behaviors (Ferguson et al., 2000). However, there are differences reported in the literature over the subcellular localization

of mammalian synaptotagmin 4. Two reports suggest synaptotagmin 4 is found on synaptic vesicles (Osborne et al., 1999; Ferguson et al., 1999), while others find the protein in the Golgi and in synaptic regions distinct from synaptotagmin 1 (Ibata et al., 2000; Berton et al., 2000). Further studies of antibody specificity will be required to explain the discrepancies in these localization studies. Mammals contain an additional homolog of synaptotagmin 4, synaptotagmin 11 (Poser et al., 1997). Further studies of these additional isoforms will be required before their function can be determined.

The recent completion of the *Drosophila* genome allows us a unique opportunity to examine the entire C2 domain-containing family in a single organism. We have characterized the C2 domain family in flies and identified a large family of C2 domain containing proteins that may function in membrane trafficking. Figure 1 shows a domain diagram of the C2 family in flies. In yeast, the C2 domain family is small and includes three synaptotagmin-related molecules termed tricalbins, each containing three C2 domains. In addition, one ubiquition ligase/Nedd4-like molecule (Rsp5p), one phosphatidyl serine decarboxylase (Psd2p) and one C2-containing protein of unknown function (296w) can be found. *Drosophila* contains an expanded repertoire of C2-domain containing proteins, suggesting novel roles for these proteins in higher metazoans. Among the C2 family are seven proteins most homologous to synaptotagmins. These include homologs of mammalian isoforms 1, 4, 7, 12, 14 and two additional synaptotagmin-like proteins, we have designated synaptotagmin α and synaptotagmin β. In addition, *Drosophila* contains a number of synaptotagmin-related proteins including one tricalbin homolog, one granulophilin homolog and one otoferlin homolog. Other C2-domain containing proteins include one Rabphilin homolog, one

Rim homolog, three proteins with some homology to Munc 13, and four additional novel C2-containing proteins. *Drosophila* also contains homologs of PIP kinase, ubiquiton ligases, and protein kinase C. Many of these proteins are conserved in both *C. elegans* and mammals, suggesting specific functions in membrane trafficking. A major goal of this thesis is to decipher the subcellular localization of the synaptotagmins, and elucidate their role in vesicle trafficking within the cell.

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Chapter 2

Synaptotagmins are trafficked to distinct subcellular domains including the postsynaptic compartment.

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and J. Troy Littleton.

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Bill Adolfsen, in majority, performed the experiments and wrote the manuscript for the published work found in both chapters 2 and 4. Sudpita Saraswati carried out the larval locomotion and physiological analysis in the overexpression studies, while Moto Yoshihara helped with imaging.

Summary

The synaptotagmin family has been implicated in calcium-dependent neurotransmitter release, although synaptotagmin 1 is the only isoform demonstrated to control synaptic vesicle fusion. Here, we report the characterization of the six remaining synaptotagmin isoforms encoded in the *Drosophila* genome, including homologs of mammalian Synaptotagmins 4, 7, 12 and 14. Like Synaptotagmin 1, Synaptotagmin 4 is ubiquitously present at synapses but localizes postsynaptically rather than presynaptically. Consistent with their distinct localizations, overexpression of Synaptotagmin 4 cannot functionally substitute for the loss of Synaptotagmin 1 in synaptic transmission. The remaining isoforms are not found at synapses (Synaptotagmin 7) or are expressed within subpopulations of neurons (Synaptotagmins α and β). Our results indicate that synaptotagmins are differentially distributed to unique subcellular compartments and that oligomerization of different isoforms on synaptic vesicles does not occur *in vivo*. In addition, the identification of a postsynaptic synaptotagmin suggests calcium-dependent membrane trafficking pathways function on both sides of the synapse.

Introduction

Regulated exocytosis is an essential cellular process that controls the trafficking of certain membrane proteins and the release of signaling molecules in response to stimulation. In neurons, synaptic release of neurotransmitters is regulated by intracellular calcium levels (Katz and Miledi, 1967) and requires SNARE complex assembly and disassembly (Chen et al., 1999; Hu et al., 2002; Littleton et al., 1998; Sollner et al., 1993). The search for synaptic calcium receptors that regulate SNARE-dependent fusion has focused on the synaptotagmins, a family of transmembrane proteins containing tandem calcium-binding C2 domains (C2A and C2B) (for review, see (Chapman, 2002; Koh and Bellen, 2003; Sudhof, 2002; Tokuoka and Goda, 2003;

Yoshihara et al., 2003). Synaptotagmin 1 (Syt 1), the first isoform extensively studied, was identified as an abundant synaptic vesicle integral membrane protein with calcium-dependent phospholipid binding properties (Perin et al., 1990). Genetic studies in *Drosophila* and mice have demonstrated that loss of Syt 1 specifically eliminates the kinetically fast synchronous component of release, without removing the slow asynchronous component (Geppert et al., 1994; Littleton et al., 1993b; Yoshihara and Littleton, 2002). Mutations in *syt 1* also disrupt the fourth order calcium dependence of fusion, suggesting Syt 1 functions as the presynaptic calcium sensor for fast synchronous release (Littleton et al., 1994; Stevens and Sullivan, 2003; Yoshihara and Littleton, 2002). The finding that the two calcium-dependent phases of release have similar calcium cooperativities (Goda and Stevens, 1994) has led to the hypothesis that another synaptotagmin isoform with distinct biochemical properties may mediate the asynchronous component of release.

Apart from Syt 1, more than a dozen additional synaptotagmins have been identified in mammals (Craxton, 2001; Sudhof, 2002) while the *C. elegans* and *Drosophila* genomes encode eight and seven synaptotagmin genes, respectively (Adolfsen and Littleton, 2001). The subcellular localizations of synaptotagmin isoforms is controversial (Marqueze et al., 2000). Nevertheless, several observations suggest that different synaptotagmin isoforms might cooperate to regulate the same exocytotic process. Localization studies in PC12 cells transfected with synaptotagmin-GFP transgenes indicate that Syts 1, 2, 4, 5 and 9 can traffic to dense core vesicles when overexpressed in cell culture (Fukuda et al., 2002; Saegusa et al., 2002; Tucker et al., 2003; Wang et al., 2001). Overexpression of C2 domains from several synaptotagmin isoforms can inhibit dense-core vesicle fusion (Shin et al., 2002; Sugita et al., 2002; Tucker et al., 2003). In addition, recombinant C2 domains of several synaptotagmin isoforms can form

calcium-dependent hetero-oligomers in GST pull-down experiments (Desai et al., 2000; Fukuda and Mikoshiba, 2000; Littleton et al., 1999; Osborne et al., 1999). Hetero-oligomerization of distinct synaptotagmins with unique calcium binding properties has been hypothesized to regulate the calcium sensitivity of neurotransmitter release (Desai et al., 2000; Littleton et al., 1999; Wang et al., 2001). Consistent with this model, overexpression of Syt 4 in *Drosophila* or mammalian PC12 cells can modulate exocytosis (Littleton et al., 1999; Wang et al., 2001).

Alternatively, each synaptotagmin isoform may participate in distinct membrane trafficking pathways. Several synaptotagmin isoforms have been reported not to co-localize with Syt 1 (Butz et al., 1999; Fukuda et al., 2001; Haberman et al., 2003; Saegusa et al., 2002). Syt 4 has been detected in both axonal and dendritic vesicular compartments using immuno-electron microscopy (Ibata et al., 2002), while Syt 7 has been localized to secretory lysosomes (Reddy et al., 2001). To extend these observations and investigate the possibility that other synaptotagmins are involved in regulating neurotransmitter release, we characterized the synaptotagmins encoded in the fully sequenced *Drosophila* genome. Of the seven *Drosophila* synaptotagmins, five are homologs of mammalian isoforms 1, 4, 7, 12, and 14. We find that synaptotagmin isoforms localize to non-overlapping subcellular compartments, suggesting they participate in the regulation of distinct membrane trafficking steps *in vivo*.

Materials and Methods

Drosophila genetics. Drosophila melanogaster were cultured on standard medium at 22°C.

Cluster Anaylsis and Dendrogram. Synaptotagmin protein sequences were collected from the *Drosophila melanogaster, Caenorhabditis elegans, Anopholes gambiae, Fugu rubripes, Mus*

musculus, Homo sapien genomes. Sequences were identified by BLAST analysis of *Drosophila* synaptotagmin protein sequences against the corresponding genomes deposited in GenBank. Collected sequences were then clustered based on homology using the ClustalW program (http://www.ch.embnet.org/software/ClustalW.html). Results were displayed as a tree diagram using the Phylodendron program (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html).

In situ hybridization. Embryos aged 0-22 hrs were collected and processed according to standard procedures. Probes of 500-700 bp long were designed to the C2 domain region of each synaptotagmin gene.

Microarray analysis. Microarrays were performed with Affymetrix *Drosophila* Genechips using biotinylated cRNA using the laboratory methods described in the Affymetrix genechip expression manual. RNA was isolated from heads or heads and bodies of Canton S males aged 3-4 days post eclosion at room temperature. All flies were sacrificed between 12 and 2 PM to reduce any circadian-dependent transcriptional changes. Affymetrix high-density oligonucleotide arrays were probed, hybridized, stained and washed in MIT's Biopolymers Facility according to manufacturer's instructions. Microarray analysis was performed using Affymetrix's Microarray Suite Vs.5 and Data Mining Tool Vs.3 statistics-based analysis software.

Western analysis. Western blots were done using standard laboratory procedures. All synaptotagmin antibodies were used at a 1:1000 dilution and detected using a goat anti-rabbit antibody conjugated to HRP (Jackson Immunolabs). Vizualization of HRP was accomplished using a SuperSignal ECL kit (Pierce).

Gradient centrifugation. Isolation of Canton S head homogenates was as previously described (Littleton et al., 1999). For rate-zonal sedimentation experiments, post-nuclear extracts were layered onto a 10-30% sucrose gradient and centrifuged at 50,000 RPM for 1 hour in a NVT65 rotor (Beckman Coulter). One milliliter fractions were collected beginning from the bottom of the gradient and proceeding to the top. After collection, fractions were mixed with an equal volume of 2X SDS-PAGE loading buffer and probed by western analysis. For equilibrium sedimentation experiments, post-nuclear extract was combined with a 26% Optiprep (Axis Shield) solution. The mixed sample was centrifuged at 60,000 RPM for 3.5 hours in a NVT65 rotor and fractions collected as for velocity experiments.

Protein Expression and Antibody purification. For the Syt 4, 7, α and β isoforms, we generated polyclonal antisera to recombinant proteins encompassing the C2 domains of each protein For Syt 14, we prepared antisera to a recombinant protein that encompassed the linker between the TM domain and C2A. For Syt 12, we generated antisera against a peptide derived from the linker domain between C2A and C2B. Each respective sequence was cloned into pGEX vectors. Recombinant GST fusion proteins were expressed and processed in *E. coli* (BL21) according to standard laboratory protocols. The fusion proteins were purified in batch using glutathione-sepharose (Amersham Pharmacia). In order to remove the GST affinity tag, protein samples were incubated with thrombin for 1 hr at room temperature. Polyclonal antibodies were generated in rabbits (Invitrogen) against the indicated domains of each synaptotagmin. Antisera was purified using affinity chromatography. The domain of each synaptotagmin was coupled to a 1 mL NHS-activated sepharose column (Amersham Pharmacia). Antisera (2 mL) injection, subsequent washes, and elution from the columns were all carried out on an AKTA FPLC

(Amersham Pharmacia). Columns were washed in 20mM sodium phosphate and eluted with 0.1 M glycine pH 2.7. To minimize denaturation of the antibody at low pH, the eluted fractions were immediately mixed with 1 M Tris pH 9. Fractions containing the desired peak were concentrated using Amicon ultra centrifugal filter devices (Millipore), aliquoted, and stored at –80°C.

Immunostaining. Embyros and larvae were immunostained as previously described (Rieckhof et al., 2003; Yoshihara and Littleton, 2002). The dilution of primary antibodies was: Syt 1 (1:1000) Syt 4 (1:500), Syt 7 (1:1000), Syt α (1:2000) and Syt β (1:500). In order to decrease background, antibodies were pre-absorbed to 0-11 hr embryos. Samples were washed, mounted in 70% glycerol, and visualized using a confocal microscope (Zeiss)

Results

Identification of *Drosophila* synaptotagmins and their evolutionary conservation.

Taking advantage of the recently completed *Drosophila* genome, we identified putative synaptotagmin genes using BLAST analysis with known mammalian synaptotagmin isoforms. We also analyzed predicted proteins that contained the characteristic tandem C2 domains found in Syt 1. Seven synaptotagmin isoforms were identified in the fly genome that showed a conserved domain structure consisting of an N-terminal transmembrane domain followed by tandem C2 domains. Six additional tandem C2 domain-encoded proteins were identified in the genome, including homologs of mammalian Granuphilin, Otoferlin, Rim, Rabphilin and Munc-

13. These proteins were not included in our analysis as they represent distinct families with higher homology to non-synaptotagmin C2 domain-containing proteins.

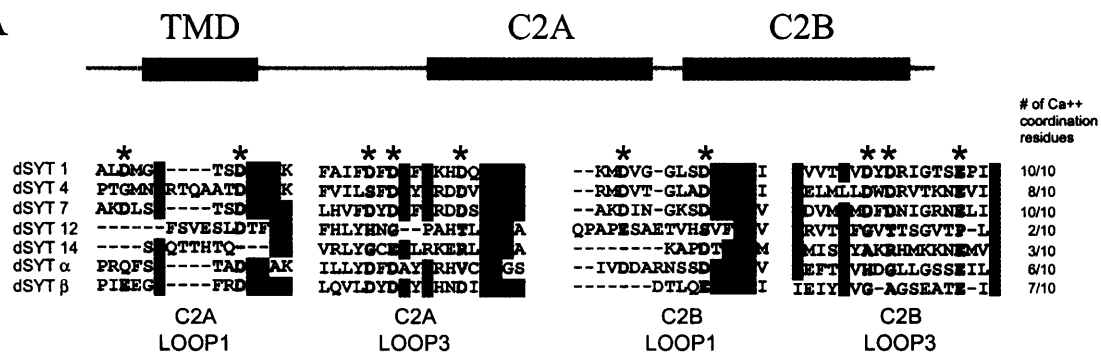
A comparison of the amino acid sequence encompassing the negatively-charged residues important for calcium coordination within each C2 domain is shown in Fig. 2-1A. Only the Syt 1 and 7 isoforms encode all the coordination residues for both C2 domains. Three of the remaining isoforms (Syt 4, α, and β) showed at least 60% conservation of these charged residues, while two isoforms (Syt 12 and 14) showed significant divergence (Fig. 2-1A), suggesting the function of some synaptotagmins may not require calcium binding.

To determine the relationship between *Drosophila* and other metazoan synaptotagmin isoforms, we performed a cluster analysis of the predicted synaptotagmin proteins encoded in currently sequenced genomes. Synaptotagmin sequences were collected from the *Caenorhabditis elegans*, *Anopholes gambiae*, *Fugu rubripes*, *Mus musculus*, and *Homo sapien* genomes as described above for *Drosophila*, and aligned using ClustalW analysis software. This analysis indicates the synaptotagmin superfamily can be divided into eight subfamilies based on sequence relationships across species (Fig. 2-1B). If the subfamily contained a previously identified mammalian isoform, we named the homologous *Drosophila* synaptotagmin according to the first mammalian subfamily member identified. If no previously characterized mammalian homolog was present in the subfamily, the *Drosophila* synaptotagmin was designated with a Greek letter.

The eight synaptotagmin subfamilies can be further categorized as conserved or divergent based on whether they contain homologs from both invertebrates and vertebrates. The Syt 1, Syt 4, Syt 7, Syt 12, and Syt 14 subfamilies are conserved, with at least one *Drosophila* member and one or more mammalian homologs. Isoforms of the Syt 1 and Syt 4 families were identified in

Fig. 2-1

A


TMD
C2A
C2B
# of Ca++ coordination residues
dSYT 1 ALDMG----TSD K FAIFDFD KHDQ --KMDVG-GLSD I VVT VDYDRIGTSEPI 10/10
dSYT 4 PTGMN RTQAATD K FVILSFD RDDV --RMDVT-GLAD I ELMLLDWDRVTKNEVI 8/10
dSYT 7 AKDLS----TSD LHVFDYD RDDS --AKDIN-GKSD V DVM MDFDNIGRNELI 10/10
dSYT 12 ------FSVESLDTF FHLYHNG--PAHTL A QPAPESAETVHSVF V RVT FGVTTSGVTP-L 2/10
dSYT 14 ----S QTTHTQ--- VRLYGCE LRKERL A --------KAPDT M MIS YAKRHMKKNEMV 3/10
dSYT α PRQFS----TAD AK ILLYDFDAY RHVC GS --IVDDARNSSD V EFT VHDGLLGSSEIL 6/10
dSYT β PIEEG----FRD LQVLDYD HNDI --------DTLQE I IEIY VG-AGSEATE-I 7/10
C2A LOOP1
C2A LOOP3
C2B LOOP1
C2B LOOP3

B

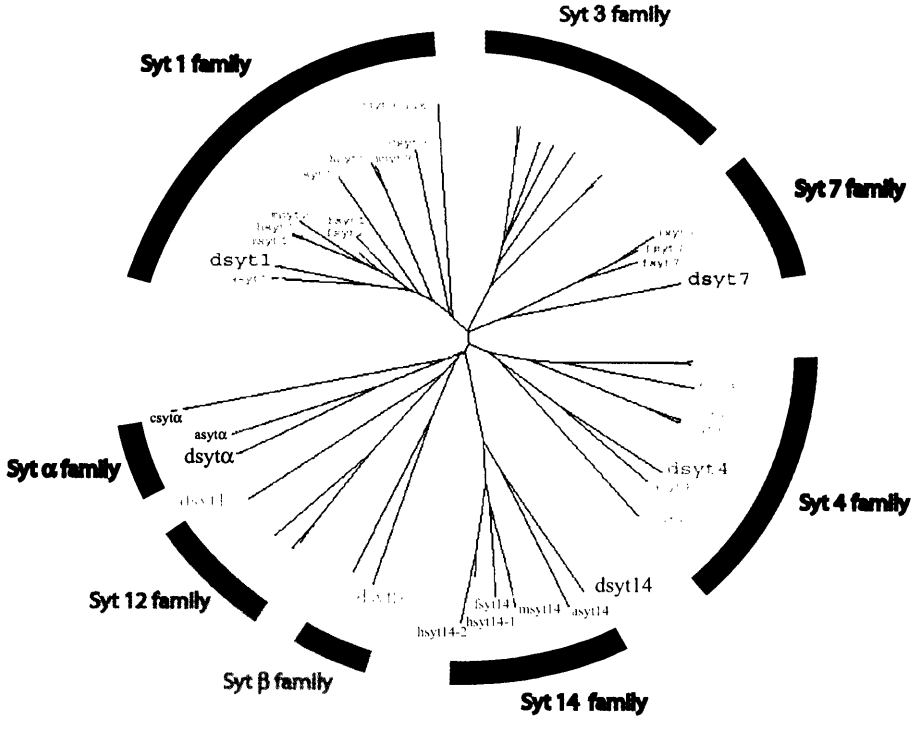

Syt 3 family
Syt 1 family
Syt 7 family
dsyt1
dsyt7
csytα
asytα
dsytα
Syt α family
dsyt4
Syt 4 family
dsyt14
Syt 12 family
Syt β family
Syt 14 family

Figure 2-1. **Conservation of *Drosophila* synaptotagmins.** (A) The general domain structure of *Drosophila* synaptotagmins is shown (top). Protein sequence alignment of loops 1 and 3 reveals the conservation of the calcium-coordinating aspartic or glutamic acid residues (*) among family members (bottom). TMD = transmembrane domain. (B) Dendrogram of synaptotagmins collected from *Drosophila melanogaster*, *Caenorhabditis elegans*, *Anopholes gambiae*, *Fugu rubripes*, *Mus musculus*, *Homo sapien*. Subfamilies are indicated by separate colors and named according to the mammalian nomenclature. Subfamilies not containing both vertebrate and invertebrate representatives were designated with Greek letters. Subfamilies were defined by major branches in the diagram and consist of members that are more highly conserved across different species than to other members within a particular species.

all vertebrate and invertebrate genomes, suggesting these two synaptotagmin families mediate an evolutionary conserved function required in all animals. The remaining conserved subfamilies (Syt 7, Syt 12, and Syt 14) contain *Drosophila* and vertebrate members, but homologs in other invertebrate genomes were not identified. The three remaining synaptotagmin subfamilies are not conserved across evolution. The Syt 3 family consists of only vertebrate members, including the mammalian 3, 5, 6 and 10 isoforms. No isoform of this subfamily was identified in any invertebrate genome. In contrast to the Syt 3 family, the invertebrate-specific Syt α and β subfamiles do not contain any vertebrate homologs.

Expression analysis of *Drosophila* synaptotagmin mRNAs

To characterize the expression profile of the *Drosophila* synaptotagmin family, we assayed their mRNA abundance and localization. The relative abundance of the mRNA transcripts and their temporal pattern of expression in embryos were determined from developmental microarray expression experiments carried out by the Berkeley Drosophila Genome Project (BDGP). Embryonic mRNA was isolated at one-hour windows throughout the first 12 hours of development and used to probe Affymetrix *Drosophila* genome arrays that include all seven *Drosophila* synaptotagmin isoforms. *syt 1* has been previously characterized (Littleton et al., 1993a) and is first expressed in the CNS at stage 13 (approximately 10 hours after egg laying (AEL)), shortly after the nervous system has differentiated (Fig. 2-2A). During axonal pathfinding and initial synapse formation at stages 14 and 15 (10-13 AEL), intense expression of *syt 1* is observed in the CNS and PNS (Fig. 2-2A). A similar developmental expression pattern is observed with the synaptic vesicle proteins, n-Synaptobrevin and Synapsin, which show no expression prior to 10 hours AEL, followed by rapid increases in mRNA

Fig. 2-2

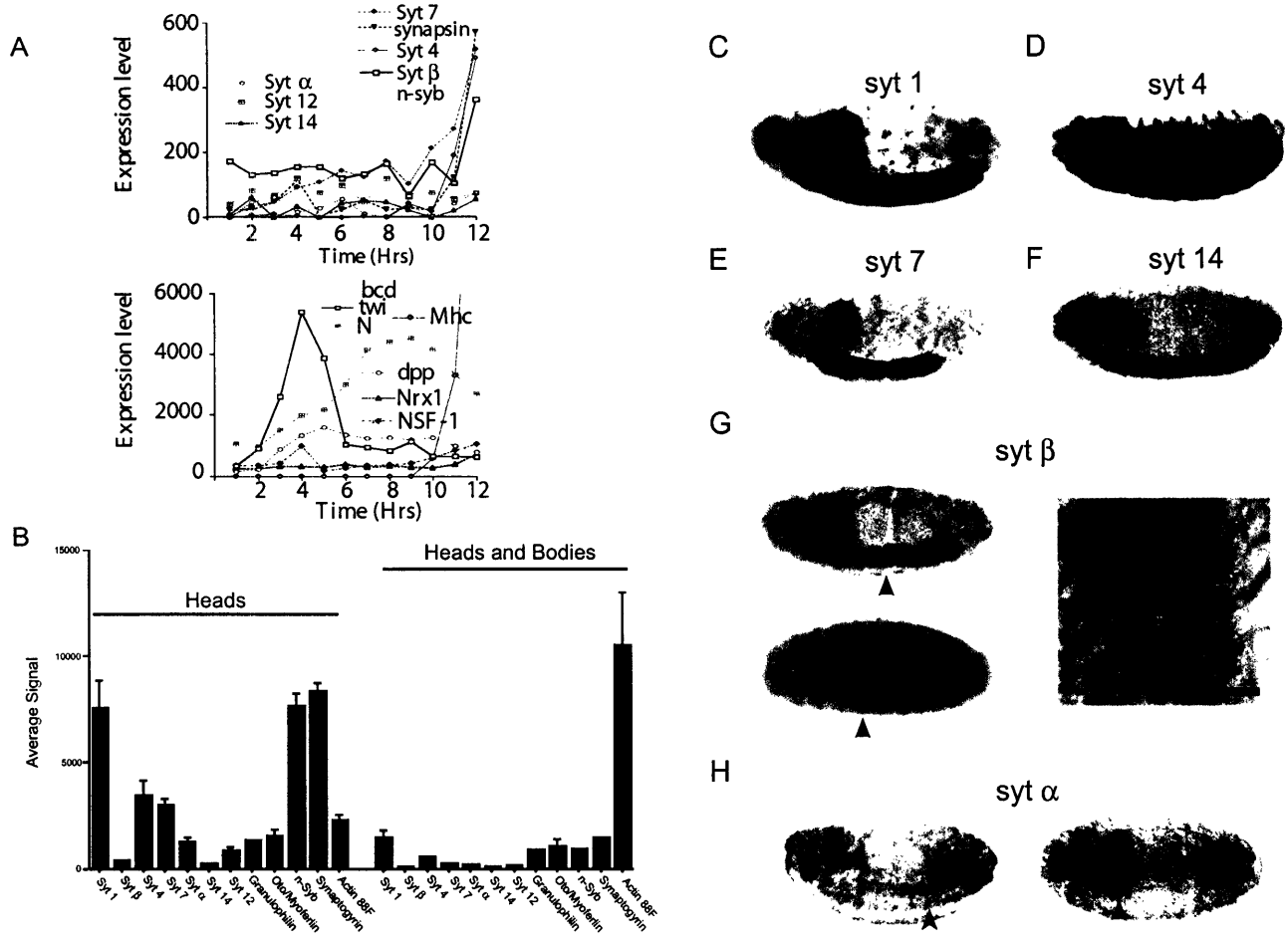

A
Expression level
Time (Hrs)
Syt α
Syt 12
Syt 14
Syt 7
synapsin
Syt 4
Syt β
n-syb
bcd
twi
N
Mhc
dpp
Nrx1
NSF-1
B
Heads
Heads and Bodies
Average Signal
C
syt 1
D
syt 4
E
syt 7
F
syt 14
G
syt β
H
syt α

Figure 2-2. **Expression analysis of the *Drosophila* synaptotagmin family.** (A) Developmental microarrays conducted by the Berkeley Drosophila Genome Project (BDGP) are shown . (Left) An outline of the major stages of development, including some of the cellular processes occurring during each stage, together with the expression of *syt 1* at each stage is shown. (Top) RNA expression levels for the *Drosophila* synaptotagmins from 0-12 hrs after egg-laying as detected by Affymetrix microarray quantification. (Bottom) Positive controls for developmentally expressed genes are shown. (B) Relative expression levels of the *Drosophila* synaptotagmins were determined by quantitative microarray analysis of either adult Canton S heads and bodies or heads only. All synaptotagmins were enriched in heads, with *syts 1, 4* and *7* being the most abundant isoforms. Error bars represent standard deviation. Embryonic expression patterns for the synaptotagmins were determined using RNA *in situ* hybridization on 0-22 hour embryos. (C) *syt 1* was abundantly expressed throughout the central and peripheral nervous systems. Similar to *syt 1*, *syt 4* (D) and *syt 7* (E) were expressed throughout the central and peripheral nervous systems. In addition to the central nervous system, *syt 7* was observed in non-neuronal tissues. (F) *syt 14* was expressed at a relatively low level in the central nervous system. (G) Abundant *syt* β signal was detected in a bilaterally symmetric population of large cells in the ventral nerve cord (arrowhead, top left) and a subset of cells in the embryonic brain (bottom left). (Right) High magnification view of the ventral nerve cord cells is shown (scale bar: 25 μm). Apart from cells in the nervous system, the *syt* β probe also detected a number of peritrachael cells (arrowhead, bottom left) present in each segment. (H) *syt* α expression was detected in a population of relatively small cells (arrow, left) in the ventral nerve cord and a subset of cells in the brain (arrowhead, right).

abundance at 11 and 12 hours AEL (Fig. 2-2A). Likewise, none of the synaptotagmins showed a peak of expression prior to 11 hours AEL, making it unlikely they function at earlier stages of development. *syt 4, 7* and β mRNA showed a similar developmental regulation, with increased expression from 10 to 12 hours of embryogenesis. The mRNAs for *syt 12, 14* and α were expressed at very low levels throughout embryonic development.

To compare the expression levels between adults and embryos, we performed quantitative microarray analysis using Affymetrix genome arrays and mRNAs isolated from whole animals or from heads only (greatly enriching for neuronal transcripts). *syt 1* was the most abundant transcript, enriched in heads over whole animals as expected, and was comparable in expression to other synaptic vesicle protein mRNAs, including n-Synaptobrevin and Synaptogyrin (Fig. 2-2B). *syt 4* and *syt 7* mRNAs were also relatively abundant, while the remaining synaptotagmin mRNAs were expressed at low levels. None of the synaptotagmins showed increased expression in whole animal versus head extracts, suggesting preferential enrichment of the isoforms in non-neuronal tissue is unlikely. We conclude that the mRNAs for *syts 1, 4*, and *7* are abundantly expressed in adults and embryos, with expression coinciding with maturation of the nervous system. The remaining synaptotagmin mRNAs were expressed at much lower levels, suggesting they are low abundance transcripts or have restricted patterns of expression.

To identify the expression patterns for the synaptotagmin family, we performed RNA *in situ* hybridization experiments on 0-22 hr embryos using RNA probes specific to each isoform (Fig. 2-2C-H). Similar to *syt 1*, *syt 4* and *syt 7* mRNA were abundantly expressed throughout the CNS (Fig. 2-2D,E). In addition to the CNS staining, *syt 7* mRNA was expressed in a number of tissues outside the nervous system, indicating a more ubiquitous expression. *syt 14* was

expressed at low levels in the CNS, and was not detected in other tissues (Fig. 2-2F). The divergent synaptotagmins, *syt* α and *syt* β, displayed a highly restricted expression pattern in subsets of CNS cells. *syt* β was expressed in a few bilaterally symmetrical large cells found in each segment of the ventral nerve cord (VNC) (Fig. 2-2G). Expression was also detected in peritracheal cells and within a small population of neurons in each brain lobe. The *syt* α isoform was found in a distinct population of smaller cells within each VNC segment, and in a subset of neurons within each brain lobe (Fig. 2-2H). *syt 12* mRNA was not detected by microarray analysis in embryos or adults, or by *in situ* analysis, suggesting it is expressed at very low levels, or expressed in a specific developmental window between the first instar larval and pupal stage. None of the synaptotagmins were expressed during developmental stages prior to the formation of the nervous system. Together with the microarray analysis, our data indicate that Syts 1 and 4 are expressed in most, if not all, neurons. Syt 7 is also abundantly expressed, but in a ubiquitous pattern both within and outside of the nervous system. The remaining synaptotagmins display restricted expression in the nervous system, labeling only specific subpopulations of cells.

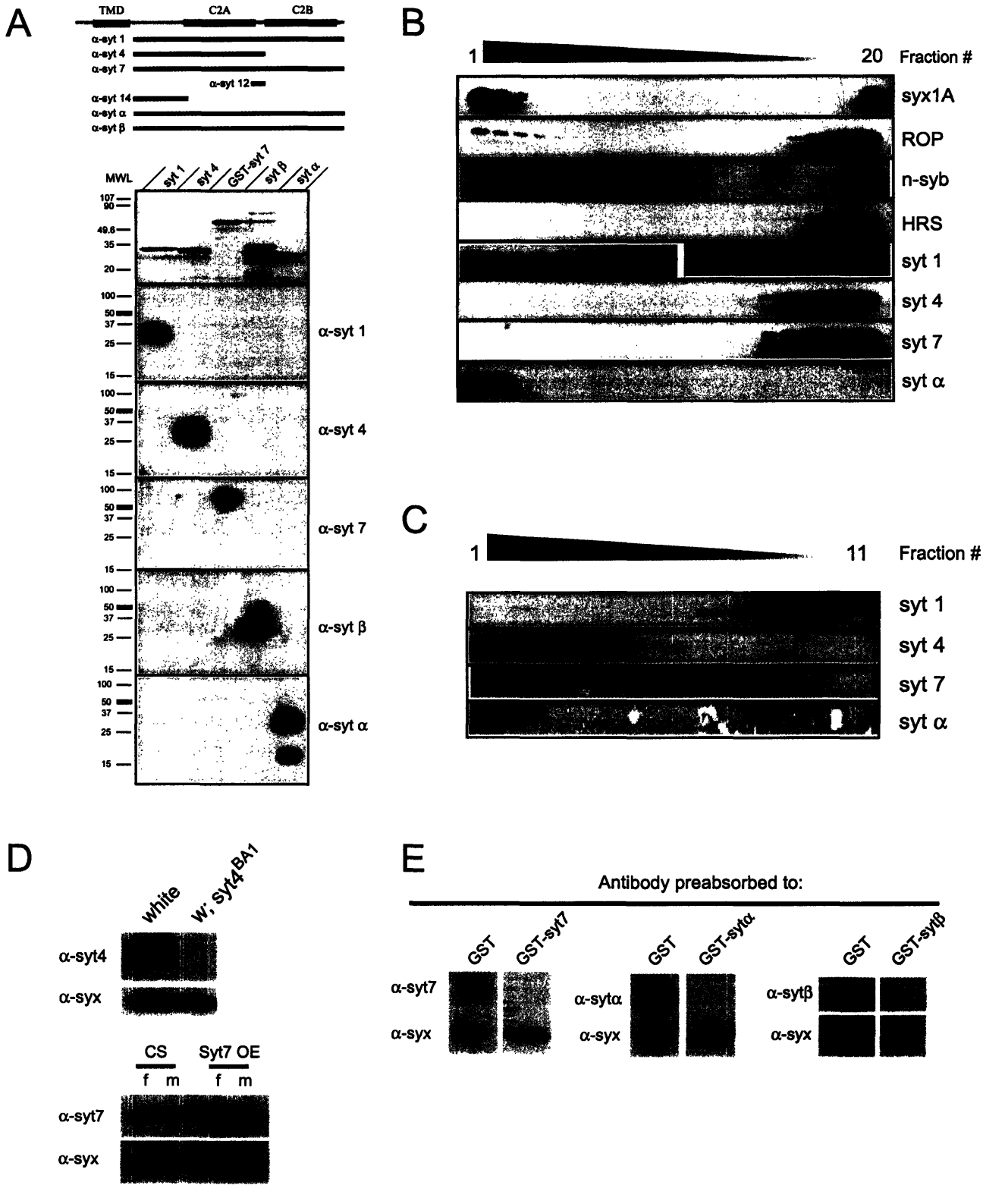
Generation of anti-synaptotagmin antisera and characterization of compartmental localization

The subcellular localization of mammalian synaptotagmins has been controversial (Marqueze et al., 2000). Much of the debate likely reflects the lack of isoform-specific antibodies that work well on tissue. Isoform specificity has also been problematic in *Drosophila*. Previously described Syt 4 antisera display cross-reactivity with Syt 1, and the antigen detected by the antisera is not removed in animals lacking the *syt 4* locus. Therefore, to define the subcellular localization of the *Drosophila* synaptotagmins, we generated isoform-specific antisera to each synaptotagmin using multiple host animals, and carried out control experiments

to confirm their specificity. Antisera to *Drosophila* Syt 1 have been previously generated and tested for specificity (Littleton et al., 1993a). The reactivity of the purified antisera to purified *Drosophila* synaptotagmins is shown in Figure 2-3A. The synaptotagmin antisera correctly recognized their respective recombinant proteins and did not cross-react with other isoforms. In addition, pre-incubation of Syt 7, Syt α, and Syt β antisera with excess recombinant antigen abolished immunodetection by western blot (Fig. 2-3E). The most definitive confirmation of isoform specificity is to demonstrate that immunoreactivity is lost in mutant animals. This has been determined for our antisera to Syt 1 and 4 (Fig. 2-3D), proving these antisera display isoform specificity. We have not yet generated mutations in the remaining synaptotagmins, so their specificity is still tentative using these rigorous requirements. However, as described below, the localization patterns for each isoform are unique and correspond with *in situ* results, indicating these antisera are likely to be isoform specific as well.

We were particularly interested in investigating if synaptotagmins reside on synaptic vesicles or instead mark unique membrane compartments. To characterize the compartmental localization of synaptotagmins, we determined their subcellular distribution on gradients prepared from *Drosophila* brain homogenates. We performed velocity gradient subcellular fractionation experiments using 10-30% sucrose gradients to separate Canton S head extracts. Fractions containing plasma membrane, synaptic vesicle and cytosol compartments were identified using known markers (Syntaxin 1A, n-Synaptobrevin and ROP respectively). As shown in Fig. 2-3B, Syt 1 co-migrates with other synaptic vesicle proteins such as n-Synaptobrevin, and shows a peak in plasma membrane fractions 1 and 2, as well as the synaptic vesicle peak in fractions 7-10. In contrast, Syt 4 and Syt 7 did not co-migrate with Syt 1, suggesting they reside in compartments that are biochemically separate from Syt 1-containing

Fig. 2-3


A
TMD
C2A
C2B
α-syt 1
α-syt 4
α-syt 7
α-syt 12
α-syt 14
α-syt α
α-syt β
MWL
syt 1
syt 4
GST-syt 7
syt β
syt α
α-syt 1
α-syt 4
α-syt 7
α-syt β
α-syt α
B
1
20
Fraction #
syx1A
ROP
n-syb
HRS
syt 1
syt 4
syt 7
syt α
C
1
11
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syt 4
syt 7
syt α
D
white
w; syt4BA1
α-syt4
α-syx
CS
Syt7 OE
f m
f m
α-syt7
α-syx
E
Antibody preabsorbed to:
GST
GST-syt7
α-syt7
α-syx
GST
GST-sytα
α-sytα
α-syx
GST
GST-sytβ
α-sytβ
α-syx

Figure 2-3. **Specificity of synaptotagmin antibodies and biochemical analysis of the synaptotagmin subcellular compartments** (A) (Top) Diagram indicating portion of protein used to generate each synaptotagmin antisera. Recombinant synaptotagmin proteins were purified as GST fusion proteins as described. With the exception of Syt 7, each GST fusion protein was cleaved with thrombin to remove the GST moiety. Removing GST from the Syt 7 C2 domains resulted in increased degradation, so this moiety was left attached. (Bottom, Top panel) Recombinant C2 domains of the indicated synaptotagmins were equally loaded onto a 10% polyacrlymide gel, subjected to SDS-PAGE electrophoresis, and stained with coomassie blue (Bottom, top panel). Protein preparations were then diluted 1/20 and subjected to western anaylsis with the indicated polyclonal antibodies. Each antibody is specific for the synaptotagmin isoform that served as its specific antigen. (B) Post-nuclear fractions of Canton S head extracts were separated on 10-30% sucrose gradients. Isolated fractions were probed for a number of subcellular markers by Western analysis. Syx-1a and a pool of the ROP protein are known to localize to the plasma membrane (left most fractions). Synaptic vesicle fractions were identified using the Syt 1 and n-Synaptotbrevin antibodies, while cytosolic fractions were indicated by immunostaining for Hrs and ROP. Syt 4 and Syt 7 were not detected in synaptic vesicle or plasma membrane fractions, but rather found near the top of the gradient. Syt α co-migrated with plasma membrane markers. The last collected fraction (right most lane) often contained contaminants from the residual membrane debris extracted from the tube sides in the final step. (C) Equilibrium density gradient fractions were probed for synaptotagmins to detect the localization of their respective compartments. Under these conditions, synaptic vesicles (Syt 1) migrate at the top of the gradient. The remaining synaptotagmins migrated to the bottom of the gradients, indicating their localization to compartments more dense than synaptic vesicles.

(D) (Top) Western blots of adult head extracts collected from wild-type and a syt 4 null mutant ($syt4^{BA1}$) were probed with the syt 4 antibody. Syt 4 immunoreactivity could not be detected in syt 4 null animals confirming the specificity of the antibody. (Bottom) Adult head extract isolated from either wild-type (Canton-S) animals or animals overexpessing the syt 7 transgene were analyzed by western analysis using the syt 7 antibody. Extracts were collected from females (f) (C155/+; +/+; UAS-syt7/+) and males (m) (C155; +/+; UAS-syt7/+) separately. (E) Specificity of the syt 7, syt α, and syt β antibodies was determined using western analysis on Canton-S adult head extract. Antibodies were incubated overnight at 4^0C either with sepharose beads containing the respective GST fusion proteins or GST alone. Except for the syt β blots, which were developed at the same time, equivalent exposure times were determined by the intensity of the syntaxin signal.

synaptic vesicles. Syt 4 and Syt 7 are also not found in plasma membranes, but enrich in distinct compartments migrating in fractions 17-19. Syt α was primarily enriched in fractions containing plasma membrane proteins, and was absent from the synaptic vesicle fraction and the compartments containing Syt 4 and Syt 7. Antibodies against the Syt β, Syt 14 and Syt 12 homologs did not give a signal from brain extracts, indicating a low expression level as suggested by the microarray and *in situ* results.

To confirm that Syt 1-containing synaptic vesicles can be separated from other synaptotagmin compartments, we performed equilibrium density gradient centrifugation experiments using a 26% self-forming Optiprep gradient (Fig. 2-3C). As with velocity gradients, the Syt 1 compartment was clearly separable from the remaining synaptotagmins. In addition, Syt 4 and Syt 7 immunoreactivity showed distinct peaks of enrichment on Optiprep gradients, suggesting they are likely to reside on non-overlapping compartments as well. The Syt 4 and Syt 7 compartments migrated farther down the gradient than Syt 1, indicating the vesicular compartments containing these synaptotagmins are denser than synaptic vesicles. We conclude from these experiments that the remaining synaptotagmins are not present on synaptic vesicles *in vivo*, indicating Syt 1 is the only synaptic vesicle isoform in *Drosophila*.

Subcellular localization of *Drosophila* synaptotagmins

To characterize the subcellular distribution of the synaptotagmins, we examined their localization in *Drosophila* embryos and larvae using immunocytochemistry. The subcellular localization of Syt 1 has been extensively characterized in *Drosophila* (Littleton et al., 1993a; Littleton et al., 1995) and the protein localizes to synaptic vesicles at presynaptic terminals. Similar to Syt 1 and consistent with our *in situ* localization data, the Syt 4 protein was found

concentrated in the synaptic neuropil of the larval CNS (Fig. 2-4A), suggesting localization to mature synapses. This immunostaining is abolished in animals containing overlapping deficiencies (Df(3R)Antp1/ Df(3R)dsx29) that remove the *syt 4* locus. However, during embryonic development the subcellular localization of Syt 4 is clearly distinct from Syt 1. As shown in Fig. 4B, Syt 4 is abundant in neuronal cell bodies in the developing CNS at a time in which Syt 1 and other axonal markers such as Fas II have already trafficked to axons, indicating Syt 4 is differentially sorted during the establishment of neuronal polarity. The consequences of this differential sorting are apparent at mature third instar larval neuromuscular junctions (NMJs), where Syt 4, in striking contrast to Syt 1, localizes postsynaptically (Fig. 2-4 C, D). Co-staining with Syt 1 antisera demonstrates no overlap in the distribution of the two proteins, confirming Syt 4 is not a synaptic vesicle protein. Rather, Syt 4 antisera specifically labels the postsynaptic side of NMJs (Fig. 2-4 C), surrounding the outside of presynaptic terminals (labeled by anti-HRP) in a punctate pattern, suggesting Syt 4 is the first identified molecular component of a postsynaptic vesicular compartment. Co-staining experiments carried out in animals overexpressing a myc-tagged postsynaptic glutamate receptor subunit, mycGluRIIA, reveals that Syt 4 localizes to regions adjacent to postsynaptic receptor clusters, and does not directly co-localize with postsynaptic density proteins (Fig. 2-4E, F). Although there is no overlap between Syt 4 and Syt 1 staining (Fig. 2-4D), and the majority of Syt 4 labeling is clearly postsynaptic (Fig. 2-4C), we cannot rule out the possibility that a small component of Syt 4 is present presynaptically. However, as described below, overexpression of Syt 4 in neurons does not shift its localization to synaptic vesicles, confirming the differential sorting of the two synaptotagmins. Based on the function of Syt 1 as a presynaptic calcium sensor, our

Fig. 2-4

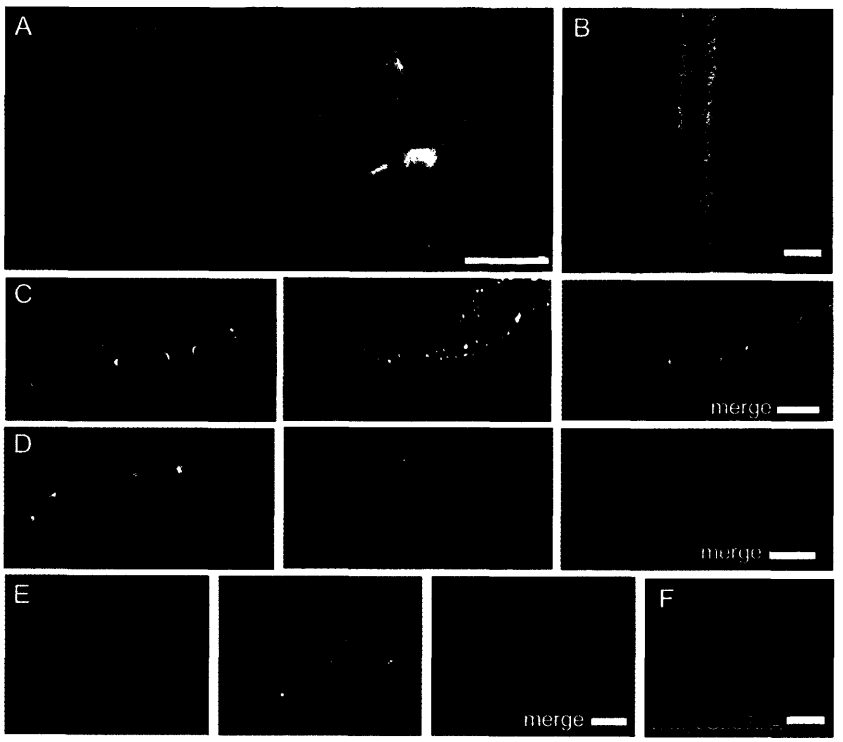

Figure 2-4. **Localization of *Drosophila* synaptotagmin 4** (A) First instar central nervous system immunostained with anti-Syt 4 (magenta) and a neuronal marker, anti-HRP (green) (scale bar: 50 μm). Syt 4-specific signal was concentrated in the neuropil of the ventral ganglion where synapses occur. (B) Early stage 17 embryo co-stained with anti-Syt 4 and anti-Fas II antibodies (scale bar: 20 μm). Fas II is found in axonal tracts in the central nervous system (CNS), while Syt 4 was localized to CNS cell bodies during axonal pathfinding. (C) Third instar neuromuscular synapses were imaged on muscle fibers 6 and 7. Syt 4 co-labeling was performed with a number of synaptic antibodies, including the presynaptic membrane marker anti-HRP, (scale bar: 5 μm), the synaptic vesicle marker, anti-Syt 1 (D) (scale bar: 5 μm), and the postsynaptic marker, anti-myc antibody to detect myc-tagged GluRIIA (E) (scale bar: 5 μm). (F) Higher magnification of a synaptic varicosity co-stained with anti-Syt 4 (magenta) and anti-myc antibodies to detect tagged GluRIIA (green), indicating that Syt 4 concentrates at regions surrounding glutamate receptor clusters (scale bar: 2 μm).

localization studies make Syt 4 a strong candidate for a putative calcium sensor mediating postsynaptic vesicle fusion.

Similar to Syt 4, the Syt 7 isoform was not found in axonal tracts during embryonic neuronal development, but instead localized to cell bodies of developing neurons (Fig. 2-5A). This segregation of the synaptotagmins was maintained in mature third instar larvae. Unlike Syt 1 or Syt 4, Syt 7 was not detected at NMJ synapses, but rather in a distinct post-Golgi vesicular compartment that was present not only in muscles (Fig. 2-5B,C), but in many other tissues, including imaginal discs (Fig. 2-5D). In muscle, anti-Syt 7 staining was observed in small clusters found throughout the sarcoplasm.

The four remaining *Drosophila* synaptotagmin isoforms are expressed at very low abundance compared to Syt 1, Syt 4, and Syt 7. Consistent with our *in situ* and microarray analysis, antisera to Syt 12 and Syt 14 revealed no staining for these two isoforms in either embryos or at mature synapses in third instar larva. In contrast, the two divergent synaptotagmins, Syt α and Syt β, were detected in subsets of neurons in both the CNS and periphery that corresponded with their *in situ* expression patterns. In mature embryos, the Syt α protein was found in a small population of bilaterally symmetrical VNC neurons (Fig. 2-6B). In third instar larvae, Syt α immunoreactivity was observed in the mushroom body and in several large CNS cell bodies (Fig. 2-6A). Subsets of synaptic tracts that innervate the ventral ganglion and a number of ventral ganglion cell bodies were also labeled. Syt α was not detected at any peripheral motor synapses, but rather localized to the neurosecretory lateral bipolar dendritic neuron (LBD) within each abdominal segment of the larva (Fig. 2-6C). Specific localization in the LBD neuron and a CNS localization pattern similar to that observed for known neuropeptides

Fig. 2-5

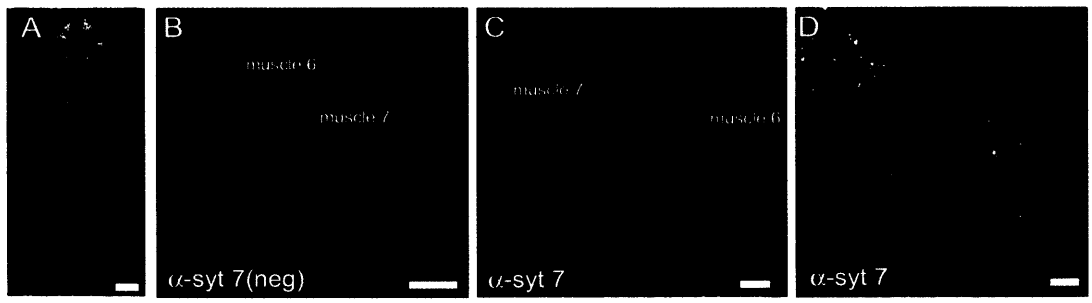
A
B
muscle 6
muscle 7
α-syt 7(neg)
C
muscle 7
muscle 6
α-syt 7
D
α-syt 7

Figure 2-5. **Localization of *Drosophila* synaptotagmin 7** (A) Early stage 17 embryo labeled with anti-Syt 1 (magenta) and anti-Syt 7 (green) antibodies (scale bar: 20 μm). (B) Third instar neuromuscular junction stained with anti-Syt 7 antibody preabsorbed to the recombinant GST-Syt 7 fusion protein (scale bar: 50 μm). (C) Third instar neuromuscular junction stained with anti-Syt 7 antibody preabsorbed to recombinant GST protein reveals vesicular staining throughout the muscle at sites beneath the plasma membrane (scale bar: 20 μm). (D) Third instar imaginal disc stained with anti-Syt 7 antibody (scale bar: 20 μm) reveals widespread immunolocalization of Syt 7 to cell bodies.

Fig. 2-6

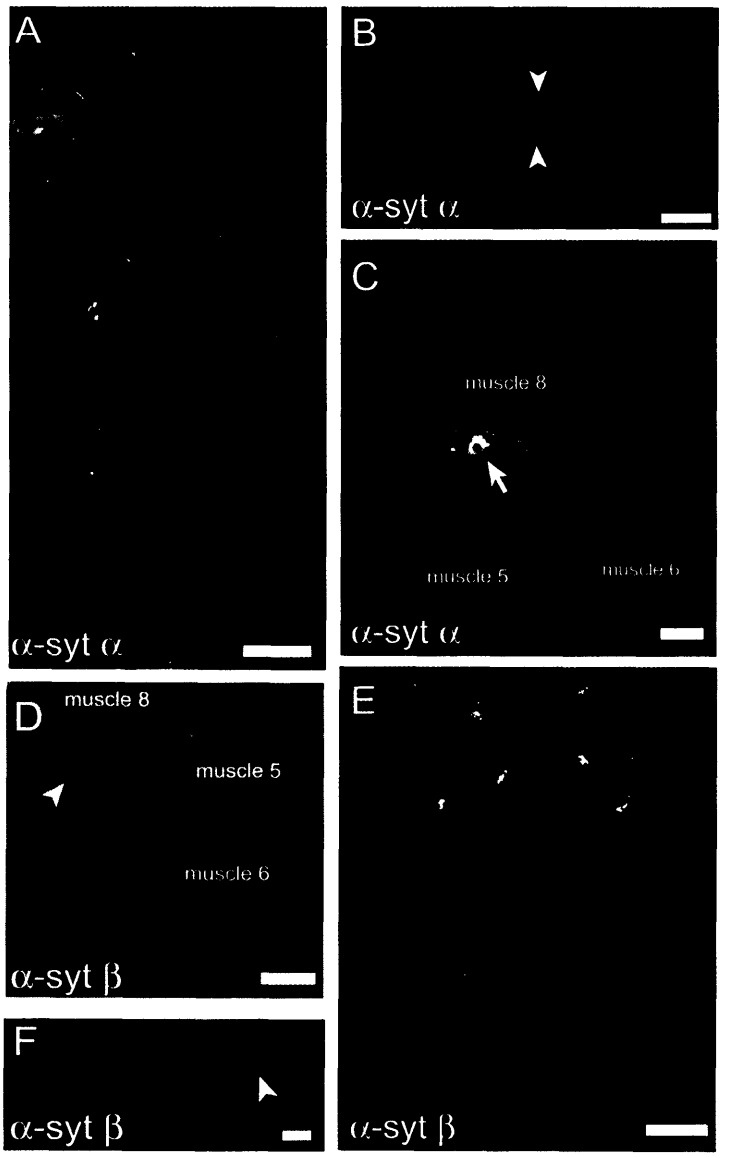
A
α-syt α
B
α-syt α
C
muscle 8
muscle 5
muscle 6
α-syt α
D
muscle 8
muscle 5
muscle 6
α-syt β
E
α-syt β
F
α-syt β

Figure 2-6. **Localization of *Drosophila* synaptotagmins α and β** (A) Third instar CNS stained with the anti-Sytα antibody (scale bar: 50 μm). Staining was observed in the mushroom bodies in the brain hemispheres (arrows) and several cell bodies in the CNS. (B) Early stage 17 embryo stained with the anti-Sytα antibody (scale bar: 50 μm). Specific signal was detected in two populations of cells in the CNS, one bilaterally symmetric pair (arrowheads), and another present in the midline. (C) Third instar neuromuscular preparation stained with the anti-Sytα antibody. Signal was detected in the lateral bipolar dentritic neuron (arrow) (scale bar: 20 μm). (D) Syt β antibody staining of third instar neuromuscular junctions (scale bar: 50 μm). Fluorescence image was overlayed onto the DIC image to indicate muscle positions. Syt β staining was observed only at motor terminals innervating muscle fiber 8. (E) Third instar CNS stained with the anti-Syt β antibody (scale bar: 50 μm). The antibody labels a number of cell bodies throughout the CNS. The immunolocalization was distinct from anti-Syt α staining, suggesting distinct subpopulation of neurons expressing each isoform. (F) Staining of peritracheal cells located at tracheal branchpoints in late stage embryos is also observed with the anti-Syt β antibody, consistent with *in situ* labeling of the same cells (scale bar: 20 μm).

suggests Syt α may function in trafficking of specific subclasses of neuropeptides and/or neuromodulators.

Similar to Syt α, Syt β was detected in a restricted population of cells. As observed with *in situ* hybridization experiments, Syt β was found in peritracheal cells that surround tracheal branchpoints in embryos (Fig. 2-6F) and larvae. Syt β was also detected in several ventral ganglion synaptic tracts and large cell bodies. In the larval brain, Syt β immunoreactivity was absent from the mushroom bodies and instead concentrated in a pair of bilaterally symmetric cell bodies in the brain lobes that innervated the ventral ganglion. At peripheral NMJs, Syt β is present at synapses of single motorneuron that innervates muscle fiber 8 and that release the neuropeptide, leukokinin (Cantera and Nassel, 1992). Presynaptic localization of Syt 1 and Syt β was observed at muscle fiber 8 synapses, accounting for the only case of co-localization of any two synaptotagmin isoforms at the same synapse detected in our analysis. Whether Syt 1 and Syt β are found on the same population of vesicles in this single cell type is unknown. In summary, we have identified and localized the members of the synaptotagmin family in *Drosophila*. Our findings indicate that only the Syt 1 and Syt 4 isoforms are ubiquitously present at synapses, while the other isoforms are non-synaptic (Syt 7), expressed at very low levels (Syt 12 and Syt 14) or in subsets of putative neurosecretory cells (Syt α and Syt β).

Discussion

Genetic analysis has demonstrated that Syt 1 is essential for calcium-dependent synchronous release, underlying the fourth order cooperativity of synaptic vesicle fusion (Geppert et al., 1994; Stevens and Sullivan, 2003; Yoshihara and Littleton, 2002). However, removal of Syt 1 does not abolish the asynchronous calcium-dependent form of release (Goda

and Stevens, 1994; Yoshihara and Littleton, 2002). These observations are consistent with the current two calcium-sensor model for synaptic transmission (Yamada and Zucker, 1992), with Syt 1 functioning as the calcium sensor regulating the fast synchronous component of release and an unidentified calcium sensor mediating the slow asynchronous component. Other synaptotagmin isoforms are obvious candidates for the asynchronous calcium sensor. In addition, synaptotagmins have unique calcium binding properties (Sugita et al., 1996) and undergo hetero-oligomerization *in vitro* (Desai et al., 2000; Littleton et al., 1999). Together with the observation that several isoforms undergo activity-regulated transcription (Vician et al., 1995), several plasticity models have been proposed suggesting differential expression of synaptotagmin isoforms on synaptic vesicles might regulate presynaptic release probability (Littleton et al., 1999; Wang et al., 2001) or transitions from full fusion to kiss-and-run (Wang et al., 2003). These hypotheses require that synaptotagmins have a similar expression pattern as Syt 1, and localize presynaptically at synaptic terminals. We have addressed these hypotheses *in vivo* by performing an extensive expression and localization study of the entire synaptotagmin family in *Drosophila melanogaster*. Our localization data argue against the possibility that other synaptotagmin isoforms function with Syt 1 to regulate neurotransmitter release. Instead, the remaining synaptotagmin isoforms likely regulate distinct membrane trafficking steps *in vivo*.

Syt 4 localizes to the postsynaptic side of the synapse, suggesting it regulates a postsynaptic membrane trafficking pathway. Indeed, there is mounting evidence in several experimental systems for a regulated form of postsynaptic vesicular trafficking. Dendritic release of a number of neuromodulators such as dopamine, ATP, GABA, and neuropeptides has been documented (Araneda and Bustos, 1989; Cheramy et al., 1981; Heeringa and Abercrombie, 1995; Isaacson, 2001; Israel and Meunier, 1978; Ludwig, 1998; Ludwig et al., 2002; Pow and

Morris, 1989). Electron microscopy has also directly visualized postsynaptic vesicles within dendritic spines and shafts (Cooney et al., 2002). Studies in hippocampal culture neurons indicate that long-term labeling with FM1-43 loads dendritic organelles which undergo rapid calcium-triggered exocytosis that is blocked by tetanus toxin (Maletic-Savatic and Malinow, 1998). In addition, pharmacological blockage of postsynaptic membrane fusion reduces LTP (Lledo et al., 1998), suggesting postsynaptic vesicle trafficking contributes to synaptic plasticity. Our localization studies indicate Syt 4 is the first identified molecular marker for a postsynaptic trafficking pathway. Mammalian Syt 4 has been localized within dendrites and soma (Ibata et al, 2002), suggesting Syt 4 and possibly the related Syt 11 homolog, may function postsynaptically. Although the exact role for regulated postsynaptic fusion remains unclear, possibilities include the release of retrograde signals, trafficking of postsynaptic receptors, and/or trafficking of synaptic cell adhesion proteins.

Syt 7 is the only other abundant isoform of the *Drosophila* synaptotagmin family. Unlike Syt 1 or Syt 4, Syt 7 does not localize to synapses and is expressed broadly outside of the nervous system. Syt 7 is found in both neurons and muscle but does not traffic to synapses. Rather, Syt 7 resides on a population of post-Golgi vesicles that are often clustered throughout the sarcoplasma of muscle or found in neuronal cell bodies. The localization of mammalian Syt 7 is controversial. It has been reported that Syt 7 is found in the presynaptic plasma membrane (Sugita et al., 2001) and regulates the switching between slow and fast forms of endocytosis (Virmani et al., 2003). Syt 7 has also been reported to localize to secretory lysosomes and mediate calcium-dependent membrane repair (Chakrabarti et al., 2003; Martinez et al., 2000). Our localization studies in *Drosophila* suggest an evolutionary conserved role for Syt 7 in this regard, consistent with the hypothesis that Syt 7 functions as a ubiquitous calcium sensor for a

vesicular trafficking event that is non-synaptic and present in many cell types. We found no evidence suggesting Syt 7 functions as a plasma membrane calcium sensor in neurons. However, future genetic characterization of Syt 7 will be needed to convincingly rule out this possibility, though mice lacking Syt 7 show no obvious neurological phenotypes (Chakrabarti et al., 2003).

The two divergent synaptotagmins, Syt α and Syt β, are found only in invertebrate genomes and localize to distinct subsets of cells in the CNS and periphery. Interestingly, the staining patterns for these synaptotagmins are similar to those observed for a number of neuropeptides. Peripheral Syt β staining is restricted to muscle fiber 8 synapses that are known to release the neuropeptide, leukokinin (Cantera and Nassel, 1992). In the CNS, Syt β was observed in a pair of bilateral neurons that may be the DPM neurosecretory neurons known to secrete the amnesiac neuropeptide (Waddell et al., 2000). Indeed a similar expression pattern is observed in α-amnesiac immunostaining experiments (data not shown). The only staining outside the nervous system is detected at tracheal branch points, where a group of myomodulin-releasing neurosecretory cells are located (O'Brien and Taghert, 1998). These localization studies suggest Syt β is a candidate calcium sensor for mediating dense core vesicle fusion and release of neuropeptides. Similar to Syt β, Syt α showed specific expression in another set of putative CNS neuropeptide-releasing neurons, as well as within the mushroom bodies. In the periphery, staining was restricted to the LBD neurosecretory neuron, consistent with a role in neuropeptide release. It is interesting to note that Syt α co-migrated with the plasma membrane compartment during subcellular fractionation experiments, suggesting it may regulate fusion via a similar mechanism proposed for certain mammalian plasma membrane synaptotagmins (Butz et al., 1999). Whether the plasma membrane localization reflects a post-fusion event is currently unknown. Syt α and Syt β both have relatively strong conservation of the calcium-binding

aspartate residues in C2A and C2B, including conservation of the fourth calcium-binding aspartate residue, the most important residue for calcium binding in the Syt 1 C2A domain (Stevens and Sullivan, 2003). The relatively high conservation of calcium binding residues, coupled with the localization to putative neurosecretory cells, indicate the invertebrate specific Syt α and Syt β may function in a similar capacity to the vertebrate specific Syt 3/5/6/10 family, which has also been postulated to function in neuropeptide release (Saegusa et al., 2002). In addition, the localization of Syt α in mushroom bodies and the possible localization of Syt β in DPM neurons makes these isoforms attractive candidates for potential roles in vesicular trafficking pathways contributing to neuronal plasticity attributed to these two brain regions in olfactory learning and memory.

Although we collected data for all of the abundant synaptotagmin isoforms, we were unable to localize the two remaining synaptotagmins, Syt 12 and Syt 14. It is possible that the proteins are below the detection level of our antisera, consistent with the quantitative microarray experiments indicating these isoforms are expressed at low levels in both embryos and adults. Alternatively, these isoforms may be expressed in developmental windows or tissues that were not investigated. Recent work on mammalian Syt 14 suggests it is not found in the nervous system, but rather localizes to the kidneys and testes (Fukuda, 2003). The subcellular localization of mammalian Syt 12 is unknown, but is only expressed in the nervous system between post-natal days 7 and 21 in mice (Potter et al., 2001). Unlike the other synaptotagmins, these two isoforms lack most of the calcium coordination residues in C2A and C2B in both vertebrates and flies, indicating they may function in a trafficking pathway not regulated by calcium.

Fig. 2-7

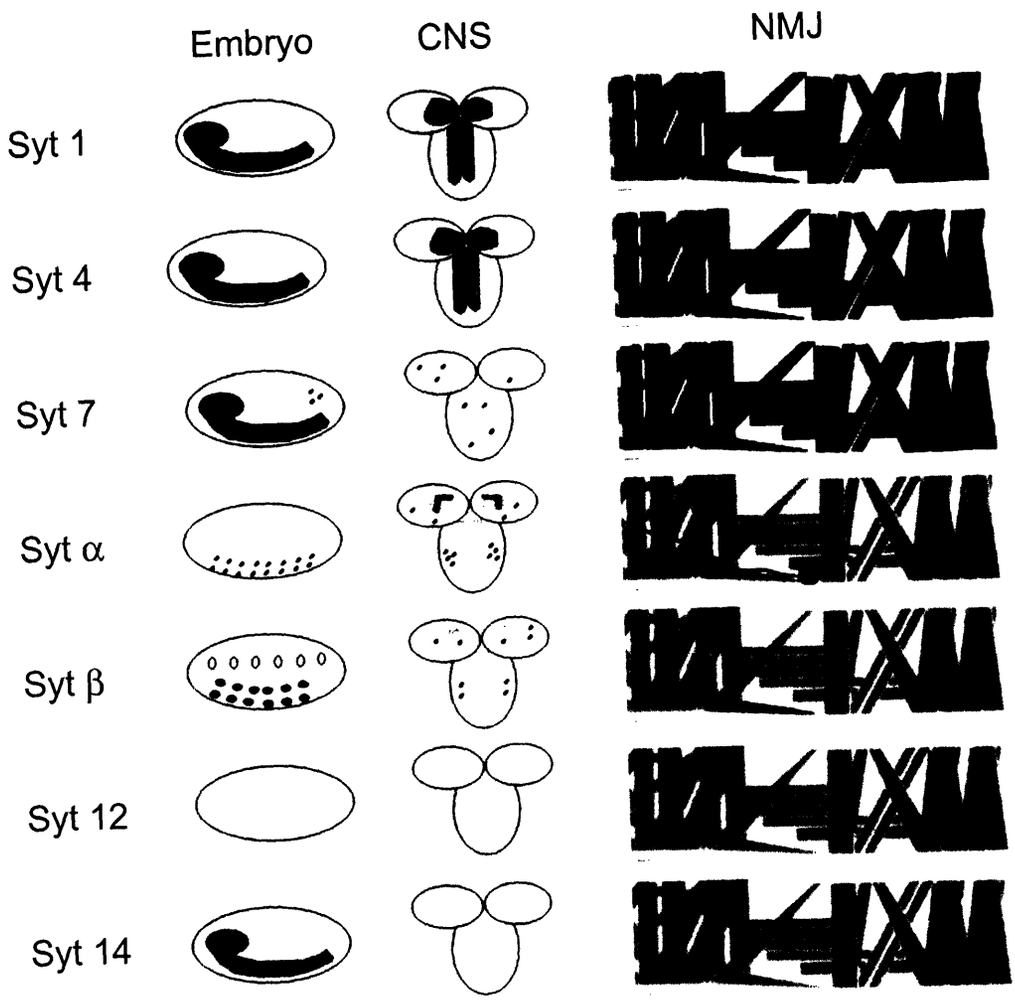

Figure 2-7. **Summary of the expression pattern of the *Drosophila* synaptotagmin family.** The results from embryonic *in situ* experiments are shown in the left panel, while the two right panels highlight protein expression in the third instar larval CNS and periphery.

In summary, *Drosophila* synaptotagmin isoforms identify unique membrane trafficking compartments. A summary of the expression of both the mRNA and protein for each synaptotagmin family member is shown in Fig. 2-7. Our data indicate that only the Syt 1 isoform is found on synaptic vesicles and so argue against hetero-oligomerization models. Based on localization arguments, our findings also argue against the possibility that another synaptotagmin isoform mediates the residual calcium-dependent asynchronous release observed in *syt 1* null mutants.

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Chapter 3

Localization of Synaptotagmin 1 to Synaptic Vesicles requires specific C2 domain trafficking signals

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and J. Troy Littleton.

Bill Adolfsen performed the majority of the work presented in this chapter. Sudpita Saraswati helped perform the fly crosses and immunostaining. Enrico Montana cloned and analyzed the syt1(N-terminal domain)-GFP construct, while Melissa Mihelidakis cloned the Syt 1 chimeras.

SUMMARY

To mediate synaptic vesicle function, a repertoire of specialized synaptic vesicle proteins is specifically trafficked to presynaptic terminals and must be selectively maintained during several rounds of vesicle/plasma membrane mixing. The mechanisms responsible for this specialized form of neuronal trafficking have remained unclear. To understand how proteins are trafficked and maintained on synaptic vesicles, we have initiated an analysis of synaptotagmin (Syt) trafficking in *Drosophila* neurons. We find the trafficking of Syt 1 requires sequences within the N-terminal domain (intravesicular+transmembrane domain+linker region) and C2 domains. Furthermore, the C2 domains of Syts 4, 7, α, and β can not substitute for the Syt 1 C2 domains to confer synaptic vesicle localization at synapses, indicating these isoforms can not localize to synaptic vesicles even when targeted to presynaptic terminals. Our results suggest the trafficking Syt 1 to mature synaptic vesicles is complex, most likely, requiring several signals throughout the protein. In addition, a putative model of Syt 1 trafficking is discussed.

INTRODUCTION

Neurons have evolved to become highly compartmentalized to allow rapid information transfer in the brain. Ion channels responsible for propagating the action potential are localized mainly to the axon and axon hillock. Proteins required for the release of neurotransmitter at synapses are trafficked to presynaptic nerve terminals, while postsynaptic receptors responsible for neurotransmitter binding are localized to dendrites. This elaborate spatial organization ensures that proteins required for a common cellular process reside in close physical proximity, while occluding

unproductive protein interactions or activities. While much is known about the general mechanisms used by all eukaryotic cells to traffic secreted and membrane proteins, relatively little is known about the trafficking mechanisms in various specialized cells, such as neurons, where trafficking can occur on a sub-millisecond time scale. The localization of proteins to synaptic vesicles presents a particularly challenging problem, if one considers that presynaptic terminals may be several cell body lengths away from the site of protein production. Furthermore, once proteins have reached terminals, the rapid turn-over of synaptic vesicles implies efficient retrieval mechanisms must exist to maintain synaptic vesicle identity.

Proper localization of many membrane proteins requires clathrin-mediated endocytosis. Internalization signals, including dileucine (LL) and tyrosine (YXXØ; Y = tyrosine, X = any amino acid, Ø = hydrophobic amino acid) motifs located on the cytosolic domains of several membrane proteins, initiate the assembly of clathrin coats by binding clathrin adaptor proteins (AP1-3)(Schekman and Orci, 1996; Sorkin, 2004; Traub, 2003). Clathrin-mediated endocytosis is postulated to also function at synapses where it directs the retrieval of synaptic vesicle proteins after calcium-evoked exocytosis (Jarousse and Kelly, 2001b; Royle and Lagnado, 2003). To understand how proteins are localized to synaptic vesicles, we have begun to investigate the intracellular trafficking of synaptotagmins.

Synaptotagmins constitute a large family of single-pass transmembrane proteins implicated in vesicular trafficking. Nineteen synaptotagmin isoforms have been identified in humans, while the genomes of mice, *Drosophila*, and *C.elegans* have 14, 7, and 8 synaptotagmin isoforms, respectively (Craxton, 2004). Each synaptotagmin

isoform is composed of similiar sequence features including a short intravesicular N-terminal domain, a transmembrane domain followed by an unstructured linker sequence, and two tandem C2 domains (C2A and C2B) residing at the C-teminus. Over the past decade, genetic evidence from several laboratories has convincingly demonstrated that Syt 1 is required for both synaptic vesicle exocytosis (DiAntonio and Schwarz, 1994; Geppert et al., 1994; Littleton et al., 1994) and endocytosis (Jorgensen et al., 1995; Littleton et al., 2001; Poskanzer et al., 2003; Zhang et al., 1994) at synapses. However, the function of the majority of synaptotagmin isoforms remains unclear. To begin elucidating the function of the remaining synaptotagmin isoforms, we have characterized the expression and localization patterns of the remaining six synaptotagmin isoforms in *Drosophila* (Adolfsen et al., 2004). Based on immunohistochemical and subcellular fractionation studies, we found only Syt 1 was localized to synaptic vesicles and each of the remaining isoforms localized to distinct subcellular compartments even when expressed in the same cell.

To determine the sequence motifs within Syt 1 responsible for its localization to synaptic vesicles, we generated a number of synaptotagmin chimeras and determined their subcellular localization *in vivo*. We demonstrate that the N-terminal portion of the protein, including the intravesicular domain, transmembrane domain, and linker region, is sufficient to target the protein to presynaptic terminals, while the C2 domains are required to localize the protein to synaptic vesicles once at terminals. Furthermore, we show the C2 domains of Syt 1 are unique in their ability to localize the Syt 1 protein to synaptic vesicles, as swapping the C2 domains of synaptotagmins 4, 7, α, and β for the Syt 1 C2 domains results in mislocalization of the Syt 1 N-terminal domain at synapses.

These results suggest the trafficking of Syt 1 to synaptic vesicles is complex, requiring specific sequences within the N-terminal and C2 domains, and provide further evidence that only Syt 1 contains the appropriate trafficking signals for localizing to synaptic vesicles.

MATERIAL AND METHODS

Construction of Synaptotagmin 1 chimeras. The sequences of the Syt 1 chimeras are list below. Chimeras were generated using fusion PCR. Briefly, the N-terminal domain of Syt 1 was amplified using a 5' primer encoding an appropriate restriction site (in lower case below) used for final cloning and a 3' chimeric primer encoding the 3' end of the Syt 1 N-terminal domain and the 5' portion of the other Syt C2 domains. The Syt C2 domains of Syt 4, 7, a, and b were amplified using a 5' chimeric primer (reverse complement of chimeric primer used in first PCR) and a 3' primer encoded a restriction site and complementary to the 3' end of the appropriate C2 domains. To generate the final chimeric DNA, a fusion PCR was set-up using a small aliquot of the initial two PCRs as templates and the two outside primers with restriction tags as primers. The resulting fused PCR product was gel purified, digested with the appropriate restriction enzymes, ligated into pUAST, and transformed into XL-blue competent cells. Correct clones were identified by conventional DNA sequencing.

syt 1-4
gaagatctATGTACCCTTACGATGTTCCTGATTACGCCAGCCTGCCGCCAAATGCA
AAATCGGAAACGGACGCCAAACCGGAAGCGGAACCAGCGCCAGCGTCTGAG
CCGGCAGCGGATCTGGAGTCCGTGGACCAGAAGCTGGAAGAAACACATCAC
TCCAAATTCCGTGAAGTGGACAGGCAGGAGCAGGAGGTGCTCGCCGAGAAG
GCGGCGGAAGCGGCCAGTCAAAGAATCGCACAGGTGGAATCAACAACACGG

AGTGCTACCACAGAGGCTCAGGAATCCACCACCACCGCAGTGCCGGTGATCA
AAAAGATCGAGCATGTCGGCGAAGTGGTCACCGAGGTGATCGCGGAGCGCA
CGGGGCTGCCCACATGGGGCGTGGTGGCCATCATCATACTCGTGTTCCTCGTC
GTCTTTGGTATAATCTTCTTCTGTGTGCGGAGATTCCTGAAGAAGCGAAGAAC
CAAAGATGGAAAGGGTAAGAAGGGTGTCGACATGAAGTCGGTACAGTTGTT
GGGATCGGCGTACAAGGAGAAAGTTCAGCCTGATATGGAGGAACTCACCGA
AAATGCCGAGGAGGGTGACGAGGAGGACAAGCAGAGCGAGCAGAAGCTGG
GTACCATCTATTTTAAGCTGCGCTACTTGGCCGAAAGGAACGCCCTTATGGTG
TCCATCATCCGTTGTCGCGGACTCCCCTGCAAGGGAGGATCGAGCGGAACCG
GAGATATTCCCACTGGCATGAATGGACGCACTCAGGCGGCAACGGATCCGTA
TGTCAAGCTGCAGCTGCTGCCGGACAAGCAGCACAAGGTCAAGACCCGGGTG
GTGCGAAATACCCGGAACCCGGTTTACGACGAGGACTTCACCTTCTACGGCC
TGAACATGAACGACCTGCAGAACATGTCGCTGCACTTTGTCATCCTCAGCTTC
GATCGATACTCACGTGACGACGTCATTGGCGAAGTGGTGTGCCCATTGACCT
CCATCGAGATCGGGGACATCTCCAAAGAGGCTTTGTCCATCAGCAAAGAGAT
CCAGCCACGGAGCTTGAAGATTCGAGCCCAGGGTCGCGGGGAGCTGCTCATC
TCCCTCTGCTGGCAACCAGCTGCCGGTCGCCTCACCGTCGTCTTGCTGAAGGC
CCGAAACTTACCGCGCATGGATGTCACCGGACTGGCCGATCCGTATGTTAAG
ATATATCTCCTCTACAATGGCCAACGCATCGCCAAGAAGAAGACGCACGTGA
AGAAACGAACTCTGAGCCCCGTTTTCAACGAGAGCTTCGCATTCGATATTCCC
GCCGCCGAAGGCGCTGGCGCCAGTCTTGAGGGTGTGTCTTTGGAACTGATGC
TGCTCGACTGGGATCGCGTGACCAAGAATGAGGTCATCGGTCGGCTGGAGCT
GGGCGGCCCGAACTCGAGCAGCACCGCCTTGAACCACTGGAACGAGGTTTGC
AACTCGCCGCGCCGCCAGATCGCCGAGTGGCACAAGCTGAACGAGTAGtctaga
gc

syt1-7

gaagatctATGTACCCTTACGATGTTCCTGATTACGCCAGCCTGCCGCCAAATGCA
AAATCGGAAACGGACGCCAAACCGGAAGCGGAACCAGCGCCAGCGTCTGAG
CCGGCAGCGGATCTGGAGTCCGTGGACCAGAAGCTGGAAGAAACACATCAC
TCCAAATTCCGTGAAGTGGACAGGCAGGAGCAGGAGGTGCTCGCCGAGAAG
GCGGCGGAAGCGGCCAGTCAAAGAATCGCACAGGTGGAATCAACAACACGG
AGTGCTACCACAGAGGCTCAGGAATCCACCACCACCGCAGTGCCGGTGATCA
AAAAGATCGAGCATGTCGGCGAAGTGGTCACCGAGGTGATCGCGGAGCGCA
CGGGGCTGCCCACATGGGGCGTGGTGGCCATCATCATACTCGTGTTCCTCGTC
GTCTTTGGTATAATCTTCTTCTGTGTGCGGAGATTCCTGAAGAAGCGAAGAAC
CAAAGATGGAAAGGGTAAGAAGGGTGTCGACATGAAGTCGGTACAGTTGTT
GGGATCGGCGTACAAGGAGAAAGTTCAGCCTGATATGGAGGAACTCACCGA
AAATGCCGAGGAGGGTGACGAGGAGGACAAGCAGAGCGAGCAGAAGGTTGG
GCAAATACATTTCTCCTTGGAGTATGATTTCCAAAACACAACATTAATTTTGA
AAGTTCTTCAAGGAAAAGAGCTTCCAGCAAAAGATTTGAGTGGTACTTCCGA
TCCATATGTTCGTGTAACGCTGTTACCAGATAAAAAGCATAGATTGGAAACC
AAAATAAAACGGCGAACCCTTAATCCACGATGGAATGAAACATTTTATTTTG
AAGGTTTTCCAATTCAAAAACTTCAATCACGTGTTTTACACTTACATGTTTTTG
ATTACGATCGGTTTTCAAGAGATGACTCCATAGGGGAAGTATTTCTTCCTTTA
TGTCAGGTTGACTTTGCTGGAAAACAATCTTTTTGGAAGGCTTTAAAGCCCCC

TGCAAAGGATAAATGCGGAGAACTTCTCTCCTCCCTTTGCTATCACCCGTCGA
ACTCAATTTTGACTTTAACACTAATTAAAGCGAGAAATTTGAAAGCCAAAGA
TATCAACGGAAAATCTGATCCATACGTAAAGGTGTGGCTACAGTTTGGCGAT
AAAAGGGTAGAAAAAAGAAAAACGCCTATATTTACGTGTACATTAAATCCAG
TATTTAATGAATCCTTTAGTTTTAACGTACCATGGGAAAAAATAAGAGAATGT
TCTTTGGATGTTATGGTGATGGATTTTGATAACATTGGAAGGAACGAGTTAAT
AGGACGAATACTATTAGCTGGAAAAAACGGTTCTGGAGCATCGGAAACTAAA
CATTGGCAGGACATGATCTCAAAGCCCAGACAAACTGTAGTACAATGGCATC
GCTTAAAACCTGAGTAAtctagagc

syt 1-α

gaagatctATGTACCCTTACGATGTTCCTGATTACGCCAGCCTGCCGCCAAATGCA
AAATCGGAAACGGACGCCAAACCGGAAGCGGAACCAGCGCCAGCGTCTGAG
CCGGCAGCGGATCTGGAGTCCGTGGACCAGAAGCTGGAAGAAACACATCAC
TCCAAATTCCGTGAAGTGGACAGGCAGGAGCAGGAGGTGCTCGCCGAGAAG
GCGGCGGAAGCGGCCAGTCAAAGAATCGCACAGGTGGAATCAACAACACGG
AGTGCTACCACAGAGGCTCAGGAATCCACCACCACCGCAGTGCCGGTGATCA
AAAAGATCGAGCATGTCGGCGAAGTGGTCACCGAGGTGATCGCGGAGCGCA
CGGGGCTGCCCACATGGGGCGTGGTGGCCATCATCATACTCGTGTTCCTCGTC
GTCTTTGGTATAATCTTCTTCTGTGTGCGGAGATTCCTGAAGAAGCGAAGAAC
CAAAGATGGAAAGGGTAAGAAGGGTGTCGACATGAAGTCGGTACAGTTGTT
GGGATCGGCGTACAAGGAGAAAGTTCAGCCTGATATGGAGGAACTCACCGA
AAATGCCGAGGAGGGTGACGAGGAGGACAAGCAGAGCGAGCAGAAGCGGG
GTAATTTGCATGTGAGTCTTGGCTACGATCCAGTTGGAGGATTGCTAAATGTT
CGACTCCTGGAGGCCCAGAATCTGCAACCGAGGCAATTTAGTGGAACTGCCG
ACCCATATGCCAAAGTCCGCTTGCTGCCGGATAAAAAGAACTTTTGGCAGAC
GCGTATACACAAGAGGACCCTGAATCCAGTTTTCGACGAGCAGTTTGTTTTTG
AGGTAACAGCCGGAGTAATTGACAAGCGTACTGTGGAAATTTTACTATACGA
CTTTGATGCTTATTCCCGGCACGTTTGCATCGGAGGAAGTAAGCTACATTTGG
CCAACTTGGATCTCAGCGAGCAATTGAAGTTGTGGACTCCTCTTAGTTCTGCC
TCGGCCCAAGATATGAAGGTGGATTTGGGCGATATAATGGTGTCCCTAGCCT
ATTTGCCATCAGCGGAGCGTTTGATGGTCGTCCTTATCAAGGCACGAAACCTG
CGGATTGTCGACGATGCCCGGAACTCCTCGGACCCTTATGTAAAGGTAACCC
TCCTCGGTCCCGGTGGCAAAAAGATAAAGAAACGCAAGACGGGCGTTCAAA
GGGGCACCCTGAACCCAGTCTATAATGAGGCTCTTGCTTTCGATGTGGCCAA
GGAGACTTTAAAAAACTGTGTACTAGAATTCACCGTAGTCCACGATGGCCTA
TTGGGATCAAGTGAAATATTAGGACGAACTCTCATTGGAAACTCTCCGGAAG
TACGCACCGAGGAAAAAATATTTTTTGAAGAAGTTTTTCGTGCCAAAAATGC
TACGGCTCAATGGGTTCCACTGCAAGAACCGGCAAACAATTTGGCCACATCG
GCCAAAAGTTCAAAGAACTAGtctagagc

syt 1-β

gcggtaccATGTACCCTTACGATGTTCCTGATTACGCCAGCCTGCCGCCAAATGCA
AAATCGGAAACGGACGCCAAACCGGAAGCGGAACCAGCGCCAGCGTCTGAG
CCGGCAGCGGATCTGGAGTCCGTGGACCAGAAGCTGGAAGAAACACATCAC
TCCAAATTCCGTGAAGTGGACAGGCAGGAGCAGGAGGTGCTCGCCGAGAAG

GCGGCGGAAGCGGCCAGTCAAAGAATCGCACAGGTGGAATCAACAACACGG
AGTGCTACCACAGAGGCTCAGGAATCCACCACCACCGCAGTGCCGGTGATCA
AAAAGATCGAGCATGTCGGCGAAGTGGTCACCGAGGTGATCGCGGAGCGCA
CGGGGCTGCCCACATGGGGCGTGGTGGCCATCATCATACTCGTGTTCCTCGTC
GTCTTTGGTATAATCTTCTTCTGTGTGCGGAGATTCCTGAAGAAGCGAAGAAC
CAAAGATGGAAAGGGTAAGAAGGGTGTCGACATGAAGTCGGTACAGTTGTT
GGGATCGGCGTACAAGGAGAAAGTTCAGCCTGATATGGAGGAACTCACCGA
AAATGCCGAGGAGGGTGACGAGGAGGACAAGCAGAGCGAGCAGAAGGTGG
GTCGTTTGCATTTGCGCGTTAAGTACGACTACCACCTGTTCGATCTGACGGTT
CATCTGATTGAAGCTCACAATCTCAGCCCCATCGAAGAGGGTGGATTCCGGG
ATCCCTATGTCCGCCTGATGTTGCAACCAGAAGTGGACAGCCGGAAGCGACA
GACCCACATTCACAGGGGCGAATCCAATCCCTACTTTGACCAGCATTTCAAG
TTCCCGGTTTCTCGGGATCAGCTCCAGGGCAAGGAACTGATCCTCCAAGTGCT
CGACTACGATCGCTATTCGCACAACGACATTATCGGCGAGGTTCGCATCTCTG
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TACCAAGAAACCCAAGGAGGATCGACCGGAACTGCTGTGCTCCTTGAACTAT
TTGCCGCAGGCCGAGCGATTGACGGTTGTCATTATGAAGGCTAGAAATTTGG
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ATCAAGAAGAAGAAGACGAGCATTACAAAGTCGGACGATCCCACCAATCCC
ATTTGGAACGAGGCGTTCACGTTTAATTTGCAATCAAATTATCTTCACAATGC
AGCCATCGAGATCTATGTGGTGGGTGCCGGGAGCGAGGCCACGGAAATCGG
ATGCTGCGGACTGGGCCCCCAGGAAAGTGGAACTGGATGCCAGCACTGGCAC
GATATGATTAACAATGCCCGAAAACCTACGGCTATGTGGCACTATATCCGCT
AGtctagagc

Construction of non-chimeric synaptotagmin transgenes. In order to clone the syt α-HA transgene, the *syt α* ORF was PCR amplified from a *Drosophila* head cDNA library using a 5' primer encoding an Xho I restriction site (5'-GCCCTCGAGATGGACATTGTCATACGTGAGG-3') and a 3' primer complimentary to the 3' end of the *syt α* ORF excluding the stop codon (5'-GCTCTAGACTAGGCATAGTCCGGGACGTCATACGGATAGCCGCCGCCGTTCT TTGAACTTTTGGCCGATGTGGCC-3'). In addition, the 3' primer encoded a short linker composed of three glycines followed by the Haemagglutin (HA) tag, a stop codon, and an Xba I restriction site. The PCR product was gel-purified using standard agarose gel electrophoresis, digested with Xho I and Xba I, ligated into pUAST, and transformed

in XL-blue competent cells. Correct clones were identified by DNA sequencing. For the syt 7-CFP transgene, cyan fluorescent protein (Clontech) was fused to the C-terminal of the *syt 7* ORF using fusion PCR. The *syt 7* ORF was PCR amplified using a 5' primer containing an EcoRI restriction tag (5'-GCGAATTCATGGCAAGCATAGTACTTATAG-3') and a 3' chimeric primer (5'-GAACAGCTCCTCGCCCTTGCTCACCATTCCTCCTCCCTCAGGTTTTAAGCGAT GCCATTGTAC-3') containing sequence corresponding to the last 25 nucleotides of the *syt 7* ORF and the first 25 nucleotides of the CFP ORF. The CFP ORF was PCR amplified with a 5' chimeric primer (reverse complement of chimeric primer used in *syt 7* PCR) and 3' primer containing a Xba I restriction site (5'-GCTCTAGATTACTCAGGTTTTAAGCGATGC-3'). Both PCR products were gel-purified (Qiagen gel extraction kit) on agarose gels and used as templates for a fusion PCR using the 5' and 3' restriction-tagged end primers. The resulting fused PCR product was digested with EcoRI and Xba I, ligated into pUAST, and transformed into XL-blue competent cells. Correct clones were identified by conventional DNA sequencing.

Drosophila genetics. Drosophila melanogaster were cultured on standard medium at 22°C. All transgenic constructs were sent to Model System Genomics at Duke university for injection.

Immunostaining and Imaging. Third instar larvae were dissected in HL3 saline (NaCl, 70 mM; KCl, 5; $MgCl_2$, 4; $NaHCO_3$, 10; Trehalose, 5; Sucrose, 115; HEPES-NaOH, 5; pH 7.2) and fixed with 4% formaldehyde solution (HL3 + 4% formaldehyde). Fixed larvae were blocked in BSA solution (PBS + 0.5% triton + 2% bovine serum albumin) and

incubated overnight with primary antibody (α-Syt 1 diluted 1/1000, α-HA rat monoclonal diluted 1/1000). After four 15 minute washes in PBS, the larvae were incubated overnight with the appropriate secondary antibodies (goat anti-rabbit Cy2 and donkey anti-rat rhodamine diluted 1/500 in BSA solution. Stained larvae were then washed four times (15 minutes each) in PBS, mounted on glass slides in 70% glycerol, and imaged using a confocal microscope (Zeiss).

Western analysis. Western blots were done using standard laboratory procedures. All antibodies were used at a 1:1000 dilution and detected using either goat anti-rabbit or donkey anti-rat antibodies conjugated to HRP (Jackson Immunolabs). Vizualization of HRP was performed using a SuperSignal ECL kit (Pierce).

RESULTS

The synaptotagmin 1 C2 domains are required for synaptic vesicle localization

Our previous studies revealed that five out of the seven synaptotagmins encoded within the *Drosophila* genome localize to distinct subcellular compartments, with synaptotagmin 1 (Syt 1) being the only isoform found on synaptic vesicles (Adolfsen et al., 2004; chapter 2). This differential targeting indicates each isoform must have unique trafficking signals which direct it to unique subcellular compartments. To identify trafficking signals Syt 1 that target it to synaptic vesicles, we generated transgenic *Drosophila* expressing Syt 1 chimeras and followed their subcellular localization *in vivo*. The chimeric transgenes generated in our study are shown in Figure 3-1A. For

Fig. 3-1

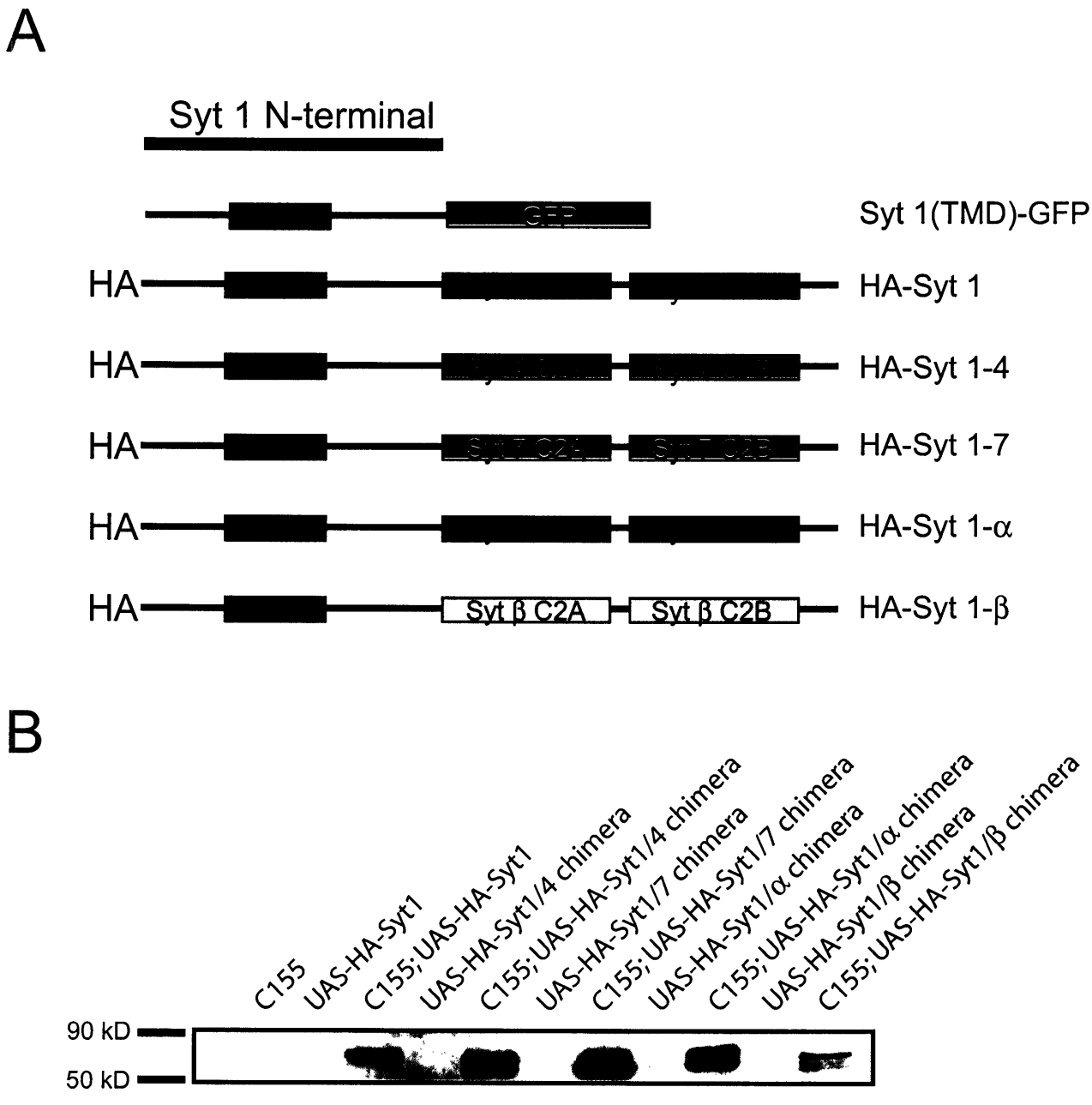
A
Syt 1 N-terminal
GFP
Syt 1(TMD)-GFP
HA
HA-Syt 1
HA
HA-Syt 1-4
HA
HA-Syt 1-7
HA
HA-Syt 1-α
HA
Syt β C2A
Syt β C2B
HA-Syt 1-β
B
C155
UAS-HA-Syt1
C155;UAS-HA-Syt1
UAS-HA-Syt1/4 chimera
C155;UAS-HA-Syt1/4 chimera
UAS-HA-Syt1/7 chimera
C155;UAS-HA-Syt1/7 chimera
UAS-HA-Syt1/α chimera
C155;UAS-HA-Syt1/α chimera
UAS-HA-Syt1/β chimera
C155;UAS-HA-Syt1/β chimera
90 kD
50 kD

Fig. 3-1. Synaptotagmin 1 chimeric transgenes are expressed and produce full-length protein products. (A) Schematic diagram of the chimeric transgenes used in this study. HA signifies a haemagglutin epitope tag, with the initial red box in each transgene representing the Syt 1 transmembrane domain. (B) Western blot of total adult head extracts (1 fly head equivalent was loaded per lane) collected from transgenic animals expressing the Syt 1 chimeric transgenes using the *elav-Gal4*^{C155} Gal4 driver. The blot was probed with an anti-HA rat monoclonal primary antibody (1/1000) and visualized with a HRP conjugated anti-rat secondary antibody.

immunolocalization of each transgene *in vivo* we placed haemagglutinin (HA) epitope tags on the N-terminus of each chimera, except in the case of the syt1(TMD)-GFP chimera. To confirm the N-terminal HA-tag did not disrupt the localization of Syt 1, we generated a full-length Syt 1 transgene with an N-terminal HA tag to serve as a positive control. To ensure each transgene was expressed and produced the predicted full-length product, we performed western analysis on total head extracts collected from adults expressing each transgene using the pan-neuronal Gal4 driver, *elav-Gal4*^{C155}. As shown in Fig 3-1B, western analysis using anti-HA antisera indicated that each transgene was expressed in a Gal4-dependent manner and produced the full-length protein product predicted by molecular weight.

Since the localization of each Syt isoform is unique, we reasoned the trafficking signals most likely reside in non-conserved domains that differ between each isoform. The N-terminal domains of synaptotagmins, consisting of the intravesicular domain, transmembrane domain (TMD), and the linker connecting the TMD to the C2A domain, are highly variable among all the synaptotagmin isoforms (Craxton, 2004). Furthermore, the trafficking of most membrane proteins depend on signal sequences residing at the N-terminus, which are first to emerge from the ribosome during protein translation and translocation into the endoplasmic reticulum (Corsi and Schekman, 1996; Rapoport et al., 1996). Thus, we first analyzed whether the Syt 1 N-terminal domain alone could localize to synaptic vesicles.

To determine the localization of the Syt 1 N-terminal domain *in vivo*, we generated transgenics expressing the N-terminal domain fused to eGFP (Syt 1(TMD)-GFP) under control of the Gal4/UAS system (Fig 3-2). When expressed throughout the

Fig. 3-2

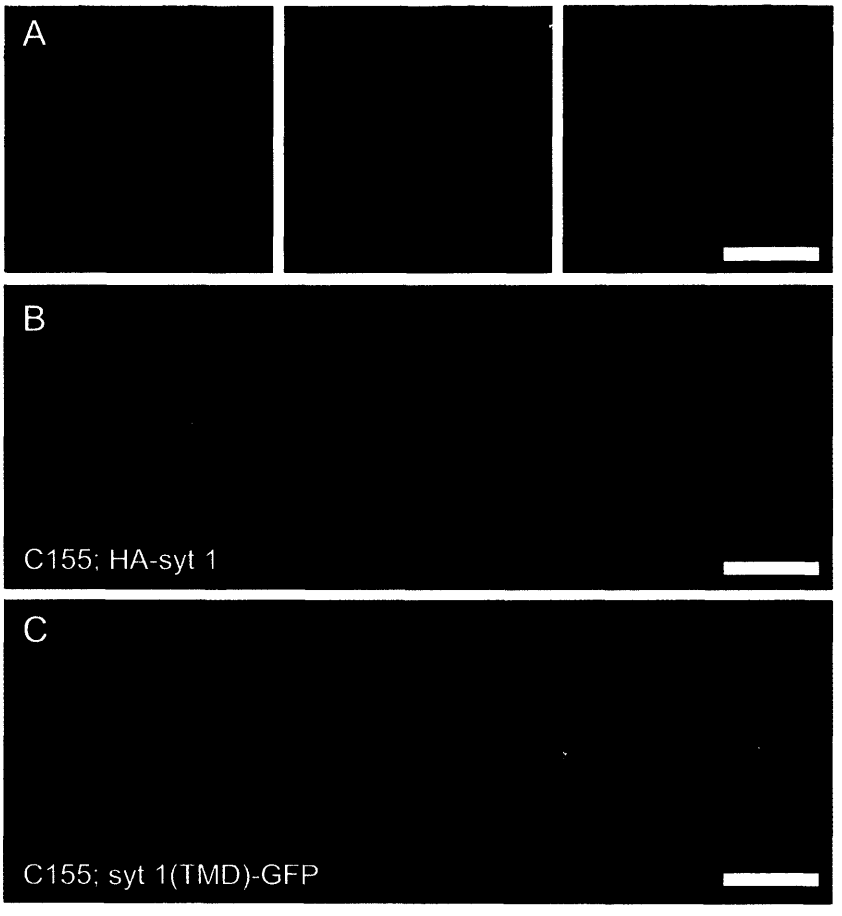

Fig. 3-2. The N-terminal domain (alone) of synaptotagmin 1 does not localize to synaptic vesicles. (A) Ventral ganglion (VG) from a third instar larvae expressing the Syt 1(TMD)-GFP using the neuronal *elav-Gal4*^{C155} Gal4 driver. Most GFP signal was found concentrated within the dense synaptic neuropil region of the VG similar to the native localization pattern observed for Syt 1 and other synaptic proteins (scale bar: 40 μm). Images were acquired with a 40X water immersion lens on a confocal microscope (Zeiss) (B) Neuromuscular junction stained with the anti-HA monoclonal antibody and expressing a syt 1 transgene harboring an HA epitope tag. When overexpressed, the transgenic Syt 1 protein localizes to the characteristic bouton halo pattern observed with endogenous Syt 1 (scale bar: 20 μm). (C) The N-terminal Syt 1(TMD)-GFP transgene does not localize in a Syt 1-like pattern, rather it is found surrounding boutons in a punctuate pattern (scale bar: 20 μm).

nervous system of third instar larvae using the pan-neuronal driver, *elav-Gal4*^{C155}, strong GFP localization to the synaptic neurophil of the ventral ganglion was observed, indicating the transgenic protein was being targeted to synapses (Fig 3-2A). However, when the motor terminals at the neuromuscular junctions were imaged, the Syt 1(TMD)-GFP transgene (Fig 3-2C) failed to localize in a pattern characteristic of the endogenous Syt 1 protein (Fig 3-2B). Whereas immunostaining of native Syt 1 forms a characteristic halo within synaptic boutons, the Syt 1(TMD)-GFP was mainly localized in a punctate pattern at the bouton periphery. These experiments suggest that the N-terminal domain alone of Syt 1 is sufficient for targeting to synapses, but lacks either synaptic vesicle targeting or retention signals, which presumably reside within the C2 domains.

The C2 domains alone are not sufficient to localize synaptotagmins to their native compartments

The finding that the Syt 1 N-terminal domain was not sufficient to confer synaptic vesicle localization, prompted us to test whether the C2 domains of synaptotagmins were sufficient to localize the protein to its native compartment. The *Drosophila* Syt 7 isoform localizes to a prominent 'lysosomal-like' compartment found throughout the sarcoplasm of third instar muscles (Fig. 3-3A). When a Syt 1 transgene is expressed in muscles, using a myosin heavy chain Gal4 (MHC-Gal4), the protein localizes almost exclusively within the subsynaptic reticulum (SSR) at neuromuscular synapses (Fig 3-3B). We took advantage of the clear differential localization of these two synaptotagmin isoforms within muscles, to investigate whether expression of a Syt 1-7 chimeric (N-terminal domain of Syt 1 fused to the C2 domains of Syt 7) protein would localize to the Syt 1 SSR compartment or the Syt 7 'lysosomal-like' compartment. If C2 domains are

Fig. 3-3

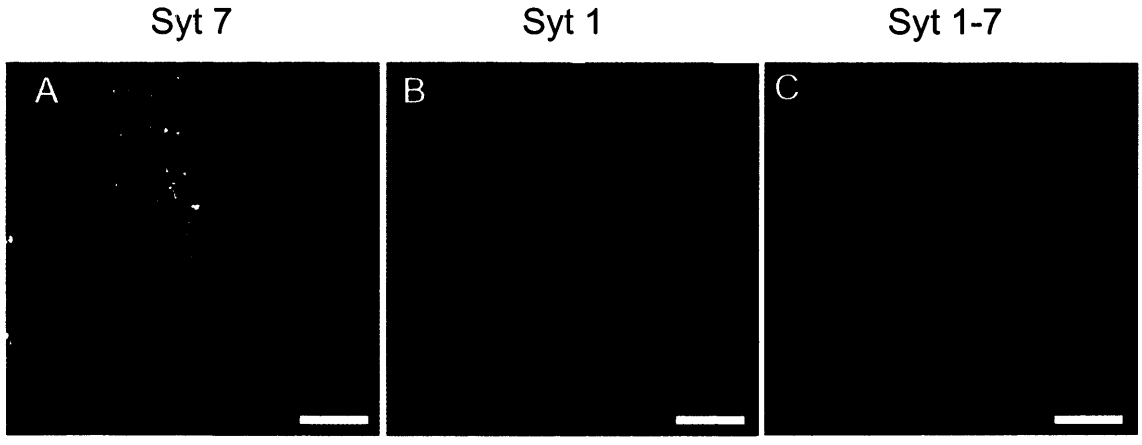

Fig. 3-3. The synaptotagmin C2 domains alone are not sufficient to confer native localization. (A) Expression of a C-terminal CFP-tagged *syt 7* transgene within the muscle using the MHC-Gal4 driver. The transgenic fusion protein localizes to the native Syt 7 'lysosomal-like' compartment found throughout the sarcoplasm (scale bar: 40 μm). (B) An HA-Syt 1 fusion protein localizes to the subsynaptic reticulum (SSR) when expressed in the muscle using MHC-Gal4 (scale bar: 25 μm). (C) The HA-Syt 1-7 fusion protein localizes to the SSR, similar to the HA-Syt 1 protein, suggesting that the Syt 7 C2 domains alone are not sufficient to localize the Syt 1-7 chimeric protein to the endogenous Syt 7 subcelluar compartment within muscle (scale bar: 30 μm).

sufficient to localize synaptotagmins to their native compartments, we expected the Syt 1-7 chimera should localize to the Syt 7 native compartment. When muscles expressing the chimeric protein were immunostained using the anti-HA epitope antibody, the chimeric protein was found almost exclusively in the SSR, not the native Syt 7 compartment (Fig 3-3C), arguing C2 domains alone are not sufficient to localize synaptotagmins to their native compartments. These experiments, along with the Syt 1(TMD)-GFP studies, favor a model of synaptotagmin trafficking, in which proper localization depends on trafficking signals present in both the N-terminus and C2 domains.

The N-terminal domain of synaptotagmins specify synaptic targeting

To account for our previous results, we hypothesized that the N-terminal domain of synaptotagmin 1 might be responsible for the initial targeting to synapses, while the C2 domains are required to maintain Syt 1 on cycling synaptic vesicles once at synapses. Indeed, several studies have demonstrated the requirement of the C2B domain for proper endocytosis of Syt 1 and synaptic vesicles during fast synaptic transmission (Jorgensen et al., 1995; Littleton et al., 2001; Poskanzer et al., 2003). If this hypothesis is correct, lack of C2 domains in our Syt1(TMD)-GFP studies would be predicted to disrupt the endocytosis of the transgenic protein and result in localization to the plasma membrane, although initial synaptic targeting is normal.

To more rigorously test whether the N-terminal domain of Syt 1 was actively targeted to presynaptic motor terminals, we generated a Syt 1-α chimeric transgene (Syt 1 N-terminal fused to the C2 domains of Syt α). Interestingly, when full length Syt α is expressed in all motor neurons using the pan-neuronal driver, *elav-Gal4*^{C155}, the protein

localizes to terminals known to have large quantities of dense core vesicles (type II and type III), but is absent from the majority of boutons containing abundant clear synaptic vesicles (type I)(Fig 3-4). We took advantage of this observation and asked whether fusing the Syt 1 N-terminal domain to the Syt α C2 domains would now localize these domains to type I boutons. As shown in figure 3-4B, pan-neuronal expression of the Syt 1-α fusion protein, with the *elav-Gal4*^{C155}, driver in motor neurons resulted in clear localization to type I boutons at muscle fiber 12, although the fusion protein still failed to colocalize with native Syt 1 at terminals. These findings demonstrate the importance of the N-terminal domain of Syt 1 in synaptic targeting and suggest that different synaptotagmin isoforms may only be targeted to certain types of synapses even when abundantly expressed.

The C2 domains of other synaptotagmins can not localize the N-terminal domain of synaptotagmin 1 to synaptic vesicles

Interestingly, the failure of the Syt 1-α chimera to colocalize with native Syt 1, even though it was properly targeted, suggests that not every pair of C2 domains can confer synaptic vesicle localization. We next investigated whether the C2 domains of several other abundant synaptotagmin isoforms could rescue the localization defect observed in the Syt 1(TMD)-GFP experiments. We generated several chimeric transgenes encoding the N-terminal of Syt 1 fused to the C2 domains of synaptotagmins 4 (Syt 4), 7 (Syt 7), and β (Syt β). If any of these C2 domains contained the appropriate synaptic vesicle trafficking signals, we predicted the transgene should colocalize with native Syt 1 at motor terminals. However, when we expressed each of these transgenes

Fig. 3-4

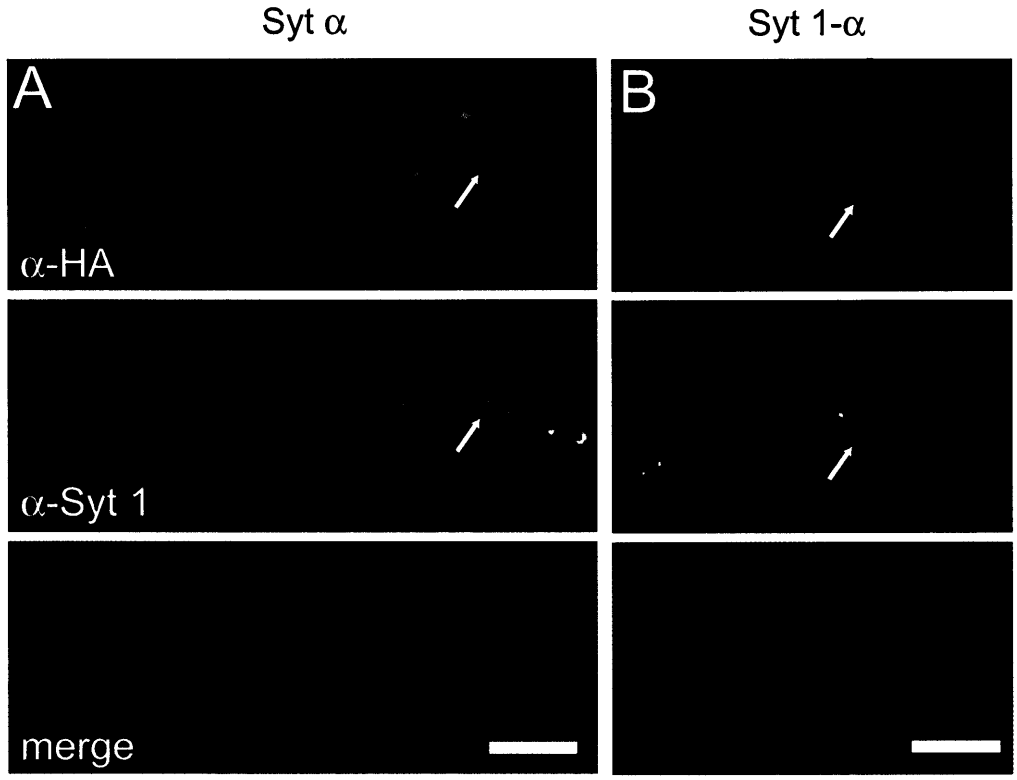

Fig. 3-4. The N-terminal domain of Syt 1 is sufficient for targeting to presynaptic nerve endings. (A) The Syt α-HA fusion protein localizes to type II and III boutons when expressed in all motor neurons using the *elav-Gal4*^{C155} driver. Abundant Syt α staining can be seen in type III boutons innervating muscle 12, however near background levels can only be detected in the type I synapses innervating the same muscle (arrows)(scale bar: 20 μm) (B) Exchange of the Syt α N-terminal domain for the Syt 1 N-terminal domain results in targeting to type I boutons at muscle fiber 12 (scale bar: 20 μm), suggesting the Syt 1 N-terminal domain is sufficient to target Syt α C2 domains to type I presynaptic nerve terminals, although it fails to confer localization to synaptic vesicles.

in third instar motor neurons, none of the chimeric transgenes colocalized with native Syt 1, even though they were properly targeted to type I boutons (Fig 3-5). Representative high magnification images of Syt 1-7 chimeras clearly show the transgenic protein fails to colocalize with endogenous Syt 1 and accumulates at the periphery of each bouton (Fig 3-6). These results suggest the C2 domains of Syt 1 uniquely specify localization to synaptic vesicles. In addition, these results indicate the Syt 4, Syt 7, and Syt β isoforms can not localize to synaptic vesicles, even when targeted to presynaptic nerve terminals.

DISCUSSION

Our initial experiments indicate the trafficking of Syt 1 to synaptic vesicles is complex, most likely requiring several sorting signals present within the N-terminal and C2 domains. We show the N-terminal domain of Syt 1 can properly target to presynaptic terminals, suggesting this domain may control trafficking events occurring between the cell body and presynaptic nerve endings. The Syt 1 N-terminal domain may traffick to presynaptic nerve terminals by mediating a number of targeting decisions, including packaging into synaptic vesicle precursors and/or association with the microtubule anterograde transport apparatus. Further experiments are needed to more rigorously address these possibilities.

Consistent with mammalian studies (Blagoveshchenskaya et al., 1999; Jarousse et al., 2003), we find trafficking of *Drosophila* Syt 1 to synaptic vesicles also requires localization or retention signals in the C2 domains. Although the N-terminal domain is sufficient for targeting to presynaptic nerve terminals, it is not sufficient for synaptic vesicle localization once at terminals. The localization of the Syt 1 chimeric proteins to the periphery of synaptic boutons suggests these proteins are unable to be internalized

Fig. 3-5

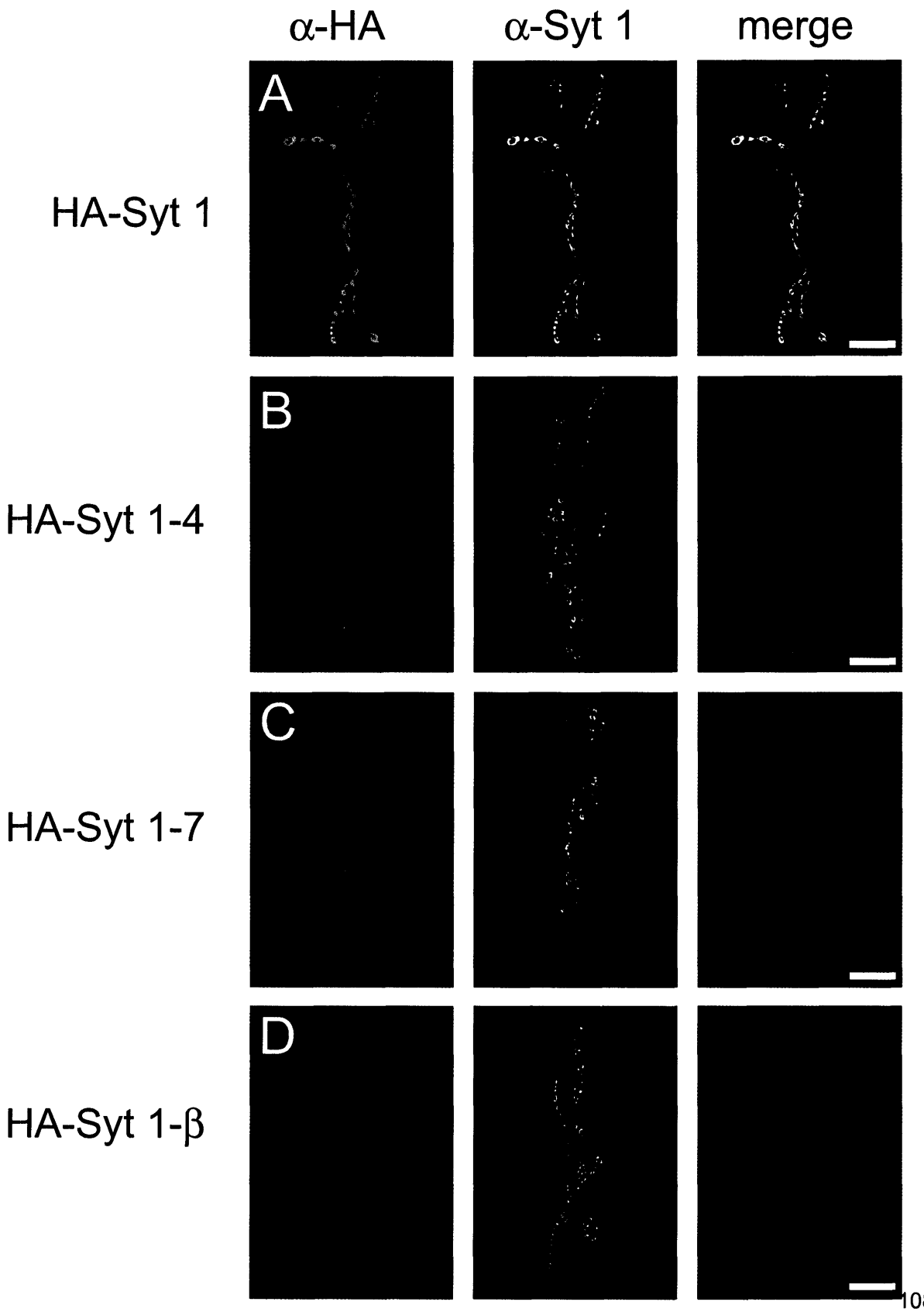

Fig. 3-5. The C2 domains of Syt 4, 7, and β fail to rescue the localization defect seen in Syt 1(TMD)-GFP transgenic animals. All transgenes were expressed in motor neurons using the *elav-Gal4*^{C155} Gal4 driver. (A) The HA-Syt 1 trangenic protein localizes to boutons in the characteristic halo pattern known for native Syt 1 (scale bar: 30 μm). Chimeric proteins, consisting of the C2 domains of Syt 4 (B)(scale bar: 30 μm), Syt 7 (C)(scale bar: 30 μm), and Syt β (D)(scale bar: 30 μm) fused to the N-terminal domain of Syt 1, fail to colocalize with endogenous Syt 1 at motor boutons, even though they are properly targeted to these terminals.

Fig 3-6

A

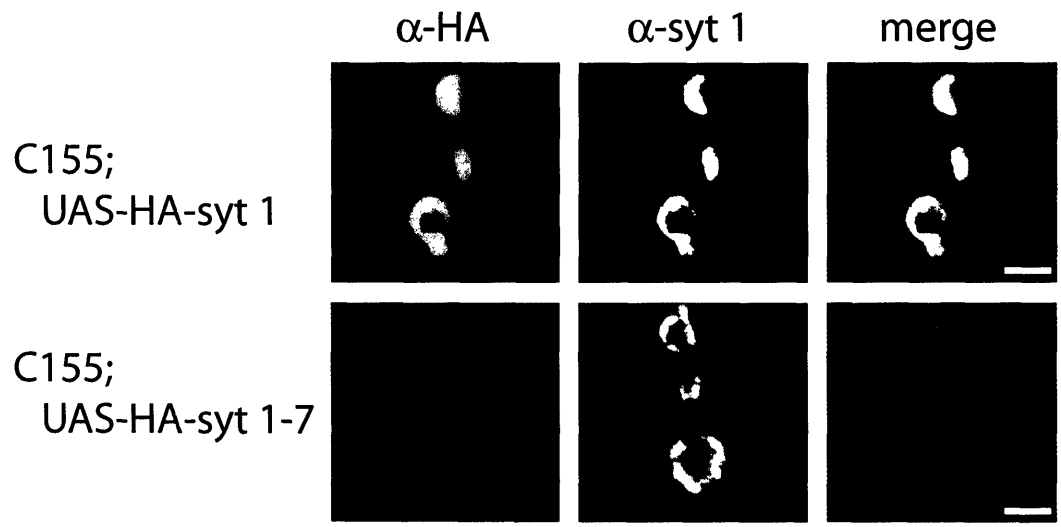

Fig 3-6 The Syt 1 chimeras accumulate at the bouton periphery. (Top panels) The HA-Syt 1 transgenic protein colocalizes with endogenous Syt 1 at the center of synaptic boutons (scale bar: 5 μm). The HA-Syt 1 transgene was expressed in motor neurons using the C155 Gal4 driver and endogenous Syt 1 was labeled using the Syt 1 polyclonal antibody. (Bottom panels) The HA-Syt 1-7 chimeric protein fails to colocalize with endogenous Syt 1 within synaptic boutons and accumulates at the periphery of the bouton (scale bar: 5 μm).

following exocytosis and become trapped at the plasma membrane. This is consistent with several reports indicating the C2B domain of Syt 1 is required for proper endocytosis at synapses (Jorgensen et al., 1995; Littleton et al., 2001; Poskanzer et al., 2003; Zhang et al., 1994) and internalization in PC12 cells (Jarousse and Kelly, 2001a; Jarousse et al., 2003). However, our finding that the N-terminal domain of *Drosophila* Syt 1 is not sufficient to localize to synaptic vesicles *in vivo*, contrasts with a number of studies in mammals, where posttranslational modification of the N-terminus of Syt 1 was sufficient to confer vesicle localization (Han et al., 2004; Kang et al., 2004). In PC12 cells, N-glycosylation of the luminal domain of Syt 1 is required for internalization and is sufficient to switch the localization of the Syt 7 isoform from the plasma membrane to intracellular vesicles (Han et al., 2004). The authors of this study concluded the luminal domain, in addition to any set of C2 domains, was sufficient to confer vesicle localization in PC12 cells. Our results argue the C2 domains of Syt 1 are unique in their ability to confer synaptic vesicle localization in *Drosophila*, as the C2 domains of Syt 4, 7, α, and β fail to localize to synaptic vesicles even when fused to the N-terminal domain of Syt 1. Our data indicate the N-terminal domain of *Drosophila* Syt 1is not sufficient to confer synaptic vesicle localization, or that the C2 domains of the other *Drosophila* Syt isoforms may inhibit putative localization signals in the Syt 1 N-terminal domain. The second of these possibilities is unlikely, since the majority of reported modifications which regulate the localization of mammalian Syt 1 reside within the luminal domain and are separated from the C2 domains by a lipid bilayer. One exception is the reported palmitoylation of cysteine residues following the transmembrane domain (Heindel et al., 2003; Kang et al., 2004), which also is required for internalization in PC12 cells. However, inhibition of

this putative N-terminal signal seems unlikely in flies, since no cysteine residues are found in the variable linker of *Drosophila* Syt 1. Thus, our Syt 1 chimera experiments indicate that localization signals most likely reside within the C2 domains.

The novel finding that the C2 domains of Syts 4, 7, α, and β fail to localize to synaptic vesicles even when fused to the N-terminal domain of Syt 1, implies there are unique sequences within the Syt 1 C2 domains that direct trafficking to synaptic vesicles. Two possible mechanisms may account for these observations. One possibility is that the C2 domains of Syt 1 contain a unique targeting signal that is not present within the C2 domains of the other Syt isoforms (Fig 3-7A). In support of this hypothesis, mammalian Syt 1 encodes a dileucine-like motif (ML; M=methionine, L=leucine) at its C terminus, which mediates targeting of Syt 1 to synaptic-like vesicles in PC12 cells (Blagoveshchenskaya et al., 1999). This dileucine-like motif has been conserved in *Drosophila* Syt 1 (IL motif), but is absent in Syts 4, 7, α, and β.

A second possible mechanism that could account for our results was uncovered from studies of mammalian Syt 1 internalization in PC12 cells (Jarousse and Kelly, 2001a; Jarousse et al., 2003). In these studies, the authors show Syt 1 contains a tryptophan-based internalization signal at its C terminus, which is sufficient to drive internalization of a CD4 transmembrane domain in PC12 and CHO cells (Jarousse and Kelly, 2001a). When this internalization signal is placed back into the context of the C2B domain the resulting CD4 chimeric protein can be efficiently internalized in PC12 cells (neuroendocrine cell lineage), but fails to internalize in CHO cells (ovary cell lineage), indicating the C2B domain regulates this latent internalization signal in a cell-dependent manner. One attractive hypothesis is that all synaptotagmin isoforms have a

Fig. 3-7

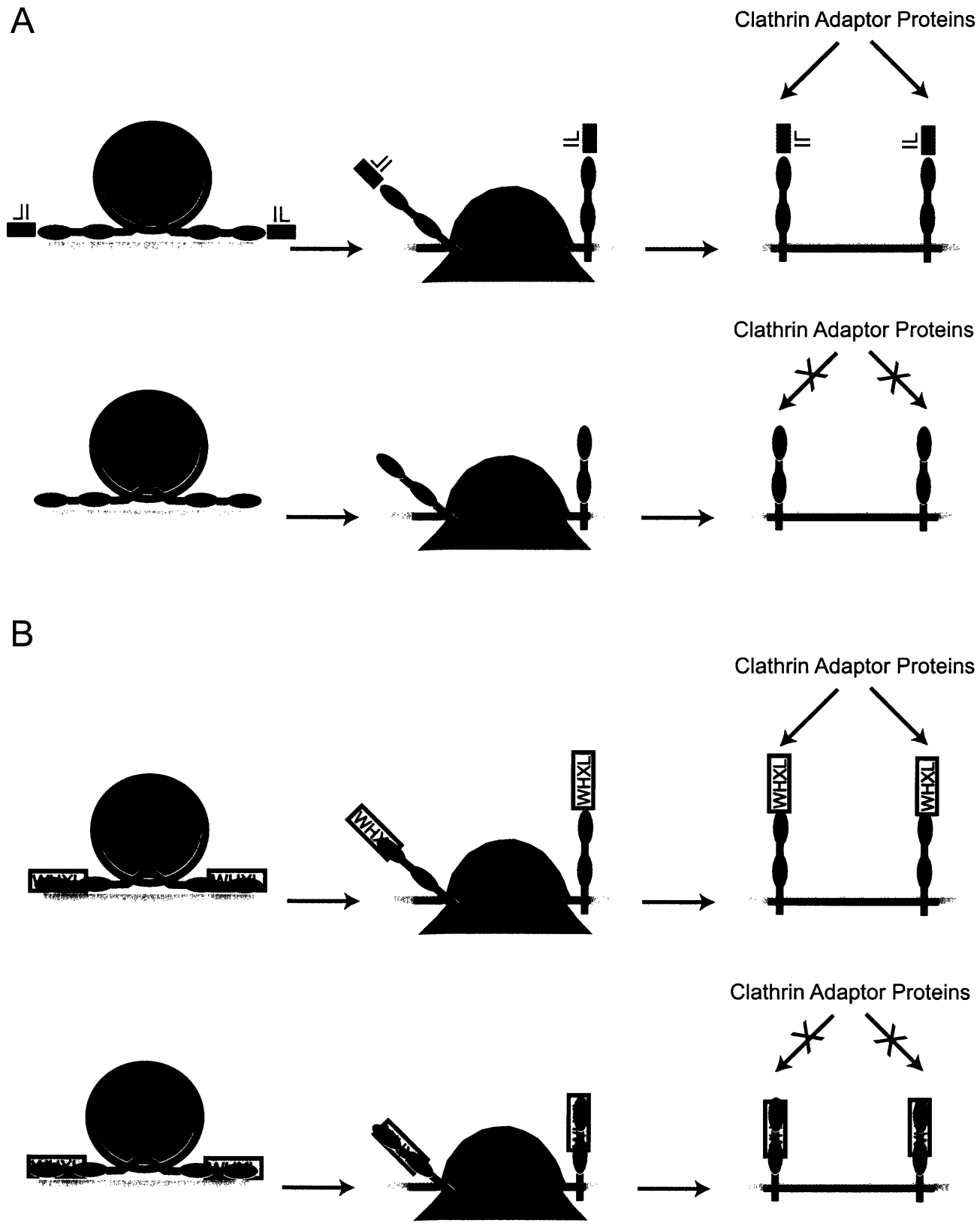
A
Clathrin Adaptor Proteins
Clathrin Adaptor Proteins
B
Clathrin Adaptor Proteins
WHX
WHXL
WHXL
Clathrin Adaptor Proteins

Fig 3-7. Two models of Syt 1 internalization at synapses. C2 domains of Syt 1 are shown as red ovals, while C2 domains of Syts 4,7, α, and β are indicated are green ovals. (A) The C2B domains of mammalian and *Drosophila* Syt 1 encode a dileucine-like motif (dark blue box) near their C-termini. Since the C2 domains of Syts 4, 7, α, and β lack this motif, they can not be internalized at presynaptic nerve terminals and become trapped at the plasma membrane. (B) The C2 domains of all synaptotagmins contain a tryptophan-based motif (WHXL) which is normally sequestered or inhibited by the C2 domains (Shown behind the C2 domains). Activation of this signal may depend on a regulatory within the C2 domains, which during the exocytic process exposes the WHXL motif. Regulating the internalization signal in this manner ensures the signal is only exposed following fusion when Syt 1 needs to be internalized from the plasma membrane. Although the C2 domains of Syts 4, 7, α, and β contain this motif, it can not be properly exposed during synaptic vesicle exocytosis because these isoforms do not normally function in this process.

common internalization signal, but this signal is only available in certain subcellular contexts (Jarousse and Kelly, 2001a; Jarousse et al., 2003). Interestingly, the tryptophan-based motif (WHXL; W=Tryptophan, H=Histidine, X = any amino acid, L = leucine) previously implicated in the internalization of both mammalian Syt 1 is found in all synaptotagmins identified to date, including the seven isoforms in *Drosophila*, suggesting this pathway may be utilized to internalize all Syt isoforms and has been highly conserved through evolution. Internalization of Syt 7 in PC12 cells also utilizes the WHXL motif, but the regulation of this signal differs from Syt 1, supporting the idea that separate pairs of C2 domains regulate the internalization signal differently (Dasgupta and Kelly, 2003). According to this hypothesis, even though the C2 domains of Syts 4, 7, α and β encode the C-terminal internalization signal, the activation of this signal depends on proper regulation of the C2 domains (presumably the C2B domain), which in the case of Syts 4, 7, α and β does not occur in presynaptic terminals with small clear synaptic vesicles. Initial inhibition of the internalization signal by the C2 domains may ensure this signal is not exposed prematurely, when the protein is localized on synaptic vesicles. Rather, the molecular rearrangments that occur during or after fusion may activate the internalization signal to properly recruit the endocytotic machinery, when the protein is present on the plasma membrane (Figure 3-7B) and needs to be retrieved.

In summary, we find the N-terminal domain of Syt 1 is not sufficient to localize to synaptic vesicles *in vivo,* rather signals residing in the C2 domains are also essential. The C2 domains of Syts 4, 7, α and β can not functionally substitute for the Syt 1 C2 domains to confer synaptic vesicle localization, further providing additional evidence these isoforms are not present on synaptic vesicles *in vivo*. Our results support a model

of Syt 1 trafficking that requires unique sequences present in the C2 domains of Syt 1 to confer synaptic vesicle targeting. Future experiments, discussed below, should be informative in determining the exact mechanism of Syt 1 internalization at synaptic terminals in *Drosophila.*

Future Directions

Several additional questions need to be addressed to resolve the mechanism that mediates specific Syt 1 targeting. First, is the WHXL motif alone sufficient to localize to synaptic vesicles at presynaptic terminals in *Drosophila*? To address this question, we need to construct a transgene that encodes the N-terminal domain followed by the final 29 amino acids of the C-terminus (which includes WHXL + IL motif) of Syt 1 and determine its localization at NMJs. The N-terminal domain will ensure the transgenic protein is targeted to presynaptic terminals, while the C terminus alone will ensure the WHXL motif is constitutively active, since it can not be regulated by the C2 domains, and the IL motif is also present. If our hypotheses are correct, the transgenic protein will colocalize with endogenous Syt 1 in boutons, suggesting it is properly targeted to synaptic vesicles. To further demonstrate the C-terminal motifs are sufficient to localize to synaptic vesicles, an additional transgene encoding the transmembrane region of CD8 followed by the Syt 1 C-terminus should be generated. The CD8 domain targets to the plasma membrane when expressed in *Drosophila* neurons, while addition of the C-terminal domain should internalize the transgenic protein to synaptic vesicles (shown by colocalization with endogenous syt 1 at synaptic boutons). If this first set of experiments support our hypotheses, it would be interesting to extend the analysis to the C-termini

found in all the *Drosophila* synaptotagmin isoforms. Localization of all CD8-C-terminus chimeras to synaptic vesicles, would argue the WHXL motif is sufficient to internalize synaptotagmins and imply the internalization mechanism is not cell-specific, rather the C2 domain regulation of the WHXL motif is responsible for the apparent cell-specific internalization observed. If only the CD8-Syt 1-C-terminal fusion localized to synaptic vesicles, we would conclude unique sequences, possibly the IL motif, within the Syt 1 C-terminus promote synaptic vesicle localization. Further mutation analysis would be required to identify the sorting motif.

Second, is the internalization signal present in Syt 1 inactive when placed in a different subcellular context? To address this question, experiments using a transgene encoding the N-terminal domain of Syt 4 fused to the Syt 1 C2 domains will be informative. If this transgenic protein can not colocalize with endogenous Syt 4 on postsynaptic vesicles, when expressed specifically in muscle, it suggests the Syt 1 C2 domains can not activate the universal internalization signal when present in the Syt 4 subcellular environment.

ACKNOWLEDGEMENTS

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Chapter 4

Genetic and functional analysis of Synaptotagmins 4, 7, and α.

Bill Adolfsen, Motojiro Yoshihara, Sudpita Saraswati, Jie Zhang,
and J. Troy Littleton.

Bill Adolfsen performed the majority of the work presented in this chapter, with exception of the embryonic physiological analysis conducted on the *syt 4* null. Moto Yoshihara was responsible for all embryonic physiology experiments and results from these studies have been submitted for publication, Yoshihara M, Adolfsen B, Galle K, & Littleton JT. (submitted). As already mentioned, Sudpita Saraswati carried out the larval locomotion and physiological analysis for the overexpression studies, while Jie Zhang was an integral part in the synaptotagmin α excision screen.

SUMMARY

Apart from synaptotagmin 1, the role of the remaining synaptotagmin isoforms in neurotransmitter release remains unclear. To rigorously investigate whether the remaining abundant neuronal isoforms, Syt 4 and Syt 7, participate in synaptic vesicle fusion, we generated loss-of-function mutants and analyzed their physiology at the *Drosophila* neuromuscular junction. We find that neither loss-of-function mutations in *syt 4* nor *syt 7* results in neurotransmitter release defects, suggesting these isoforms function in distinct regulated-exocytosis pathways in neurons. To begin to uncover the endogenous functions of the remaining Syt isoforms, we characterized the neuronal phenotypes of *syt 4* and *syt α* null mutants. Physiology analysis of *syt 4* null mutants at the embryonic neuromuscular junction indicates Syt 4 is required postsynaptically to induce large changes in presynaptic release properties following high frequency stimulation (we have termed High Frequency-Induced Miniature Release (HFMR)). We postulate Syt 4 functions in a postsynaptic regulated exocytosis pathway required to secrete retrograde signals which modulates presynaptic release properties. The identity of the retrograde signals awaits further study. Consistent with the idea that different synaptotagmin isoforms participate in separate regulated exocytosis pathways within neurons, null mutations in a putative dense-core vesicle synaptotagmin, Syt α, phenocopy amnesiac mutations suggesting Syt α may be required for secretion of neuropeptides important for middle-term memory.

INTRODUCTION

Fast and reliable communication at synapses is dependent on the tightly-regulated secretion of small neurotransmitters by calcium at presynaptic nerve terminals (Katz, 1971). The molecular identity of the receptor(s) responsible for transducing the calcium signal into fusion of synaptic vesicles and release of neurotransmitter into the synaptic cleft is still highly debated (Jahn and Sudhof, 1999). The synaptic vesicle protein, Synaptotagmin 1 (Syt 1), has emerged as the most likely candidate for encoding the presynaptic calcium receptor (Fernandez-Chacon et al., 2001; Geppert et al., 1994; Littleton et al., 1994; Perin et al., 1991). Syt 1 is tethered to synaptic vesicles by a single transmembrane domain near its N-terminus and contains two cytosolic C2 domains (C2A and C2B) that can bind calcium and phospholipids with near diffusion-limited kinetics (Davis et al., 1999). As expected for a putative calcium sensor, Syt 1 knock-outs in both mice and *Drosophila* show complete loss of fast, synchronous synaptic vesicle fusion at presynaptic terminals (Geppert et al., 1994; Littleton et al., 1993b).

Interestingly however, a slower, asynchronous form of vesicle fusion persists at Syt 1 knock-out synapses, indicating a form of calcium-sensing can still occur even in the absence of Syt 1 (Geppert et al., 1994; Yoshihara and Littleton, 2002). This observation can be interpreted in at least two ways. First, if one calcium sensor was responsible for the evoked release measured at synapses, loss of that calcium sensor should result in complete loss of all calcium-dependent vesicle fusion, synchronous or asynchronous, at synapses. If this model were correct, it would argue that Syt 1 is not the calcium sensor for neurotransmitter release, but rather an adaptor-like protein that may interact with the true calcium sensor and alter its calcium binding properties and kinetics. Alternatively, two calcium sensors responsible for vesicle fusion in presynaptic terminals may exist.

One would be Syt 1, which is required for the fast, synchronous vesicle fusion pathway, and the other unknown calcium sensor would control the slow, asynchronous pathway. Indeed, the sensitivities of the two components of release to the divalent cation Sr^{2+} differs, suggesting Sr^{+2}, like calcium, interacts at two independent sites (Li et al., 1995a). The best way to decipher between these two models is to identify the asynchronous calcium sensor and determine its null synaptic phenotype. If the null phenotype of the asynchronous calcium sensor is loss of both the fast and asynchronous vesicle fusion pathways, the single calcium sensor model would likely be correct. If the null phenotype was only loss of the slow, asynchronous fusion pathway specifically, and double mutants with *syt 1* resulted in lack of both slow and fast fusion pathways, the two calcium sensor model would be more accurate.

Other synaptotagmin isoforms are obvious candidates for encoding the asynchronous calcium sensor. To test whether other synaptotagmin isoforms may regulate the slow, asynchronous fusion pathway, we investigated the role of the two remaining *Drosophila* pan-neuronal isoforms, Syt 4 and Syt 7, in synaptic transmission. Here we demonstrate that neither overexpression of *syt 4* nor *syt 7* (data not shown) can rescue the transmission defects measured in *syt 1* null animals. Furthermore, loss-of-function mutations in *syt 4* and *syt 7* do not disrupt basal synaptic transmission or facilitation, suggesting that neither of these isoforms are required for synaptic vesicle fusion and neither are likely to encode the asynchronous calcium sensor at presynaptic terminals. Rather, different synaptotagmin isoforms function in distinct vesicular trafficking pathways. We provide evidence that Syt 4 functions in vesicular trafficking events within the postsynaptic compartment to control the secretion of retrograde signals

that acutely modulate basic synaptic properties. In addition, we show that another putative dense-core vesicle synaptotagmin isoform, Syt α, is required for short-term memory in *Drosophila*, making it a good candidate calcium sensor required for the secretion of neuropeptides important for middle-term memory.

MATERIAL AND METHODS

Cloning the syt7-CFP construct. Cyan fluorescent protein (Clontech) was fused to the C-terminal of the *syt 7* ORF using fusion PCR. The *syt 7* ORF was PCR amplified using a 5' primer containing an EcoRI restriction tag (5'-GCGAATTCATGGCAAGCATAGTACTTATAG-3') and a 3' chimeric primer (5'-GAACAGCTCCTCGCCCTTGCTCACCATTCCTCCTCCCTCAGGTTTTAAGCGAT GCCATTGTAC-3') containing sequence corresponding to the last 25 nucleotides of the *syt 7* ORF and the first 25 nucleotides of the CFP ORF. The CFP ORF was PCR amplified with a 5' chimeric primer (reverse complement of chimeric primer used in *syt 7* PCR) and 3' primer containing a Xba I restriction site (5'-GCTCTAGATTACTCAGGTTTTAAGCGATGC-3'). Both PCR products were gel-purified (Qiagen gel extraction kit) on agarose gels and used as templates for a fusion PCR using the 5' and 3' restriction-tagged end primers. The resulting fused PCR product was digested with EcoRI and Xba I, ligated into pUAST, and transformed into XL-blue competent cells. Correct clones were identified by conventional DNA sequencing.

Cloning the sytα-HA construct. The *syt α* ORF was PCR amplified from a *Drosophila* head cDNA library using a 5' primer encoding an Xho I restriction site (5'-GCCCTCGAGATGGACATTGTCATACGTGAGG-3') and a 3' primer complimentary

to the 3' end of the *syt α* ORF excluding the stop codon (5'-GCTCTAGACTAGGCATAGTCCGGGACGTCATACGGATAGCCGCCGCCGTTCT TTGAACTTTTGGCCGATGTGGCC-3'). In addition, the 3' primer encoded a short linker composed of three glycines followed by the Haemagglutin (HA) tag, a stop codon, and an Xba I restriction site. The PCR product was gel-purified using standard agarose gel electrophoresis, digested with Xho I and Xba I, ligated into pUAST, and transformed in XL-blue competent cells. Correct clones were identified by DNA sequencing.

Cloning the syt4 pHluorin construct. Ecliptic pH-sensitive GFP or pHluorin (Miesenbock et al., 1998) was fused to the N-terminal of the *syt 4* ORF using fusion PCR. The pHluorin ORF was PCR amplified using a 5' primer (included first 10 amino acids of syt 4 before the transmembrane domain) containing a Bgl II restriction site (5'-GAAGATCTATGGCCGAAGAGTATATCCCAGATAGTAAAGGAGAAGAACTTTT CACTGGAGTT-3') and a 3' chimeric primer complimentary to the final 25 nucleotides of the pHluorin ORF (excluding the stop codon) and the first 25 nucleotides of the *syt 4* ORF(5'GGCGGGGACAATCGTGTCCATGACGCTGGCTTTGTATAGTTCATCCAT GCCATGTGTAATCCC-3'). Similiarly, the *syt 4* ORF was PCR amplified using a 5' chimeric primer (reverse compliment of the 3' chimeric primer used for pHluorin PCR) and a 3' primer containing an Xba I restriction site (5'-GCTCTAGACTACTCGTTCAGCTTGTGCCAC-3'). Both PCR products were gel-purified (Qiagen) on agarose gels and used as templates for a fusion PCR using the 5' and 3' restriction-tagged end primers. The resulting fused PCR product was digested with Bgl II and Xba I, ligated into pUAST, and transformed into XL-blue competent cells. Correct clones were identified by conventional DNA sequencing.

Synaptotagmin 4 P-element excision screen. A P-element (*P{EPgy2}SytIV*^{EY12073}) inserted approximately 100 bp upstream of the first exon of *syt 4* was obtained from the *Drosophila* gene disruption project (Bellen et al., 2004). Standard genetic crosses were performed to mobilize the P-element (Fig. 4-1) and generate a number of excision chromosomes that were subsequently put in *trans* to a *syt 4* deficiency, *rn16.* Genomic DNA was isolated from over 400 viable excision/rn16 lines and was screened for deletions by PCR using a pair a primers approximately 200 bps on either side of the p-element insertion. The screening primers were: psyt45' 5'-GCTGCGCTCTCTGAAACTTGGTCTGAACCG-3' and psyt43' 5'-CAGAGATTCTCGGAAAAGCCATAGTAGCCAC-3'. Lines that failed to produce expected PCR products were subsequently screened by western analysis using the Syt 4 polyclonal antibody. Four small deletion lines removing the first two exons or ~1 KB of the *syt 4* genomic locus were isolated. One of these lines, *syt4*^{BA1}, was functionally characterized further.

Generation of synaptotagmin 7 hypomorphs by transgenic RNAi. A ~3.2 kb genomic fragment consisting of the first two exons and introns (including all splice donor & acceptor sites) of the *syt 7* locus was PCR amplified from genomic DNA using primers tagged with EcoRI and Bgl II restriction sites, syt7gen5'-eco 5'-CGGAATTCTTCGACGTGTTTTCATTGGCATAAGTAGCTC-3' and syt7gen3'-bgl 5'-GAAGATCTCTGAAAGATTGTGTTAAGTAAAATTGTTGG-3'. The PCR product was digested with EcoRI and Bgl II, ligated into the pUAST vector, and transformed into

Fig. 4-1

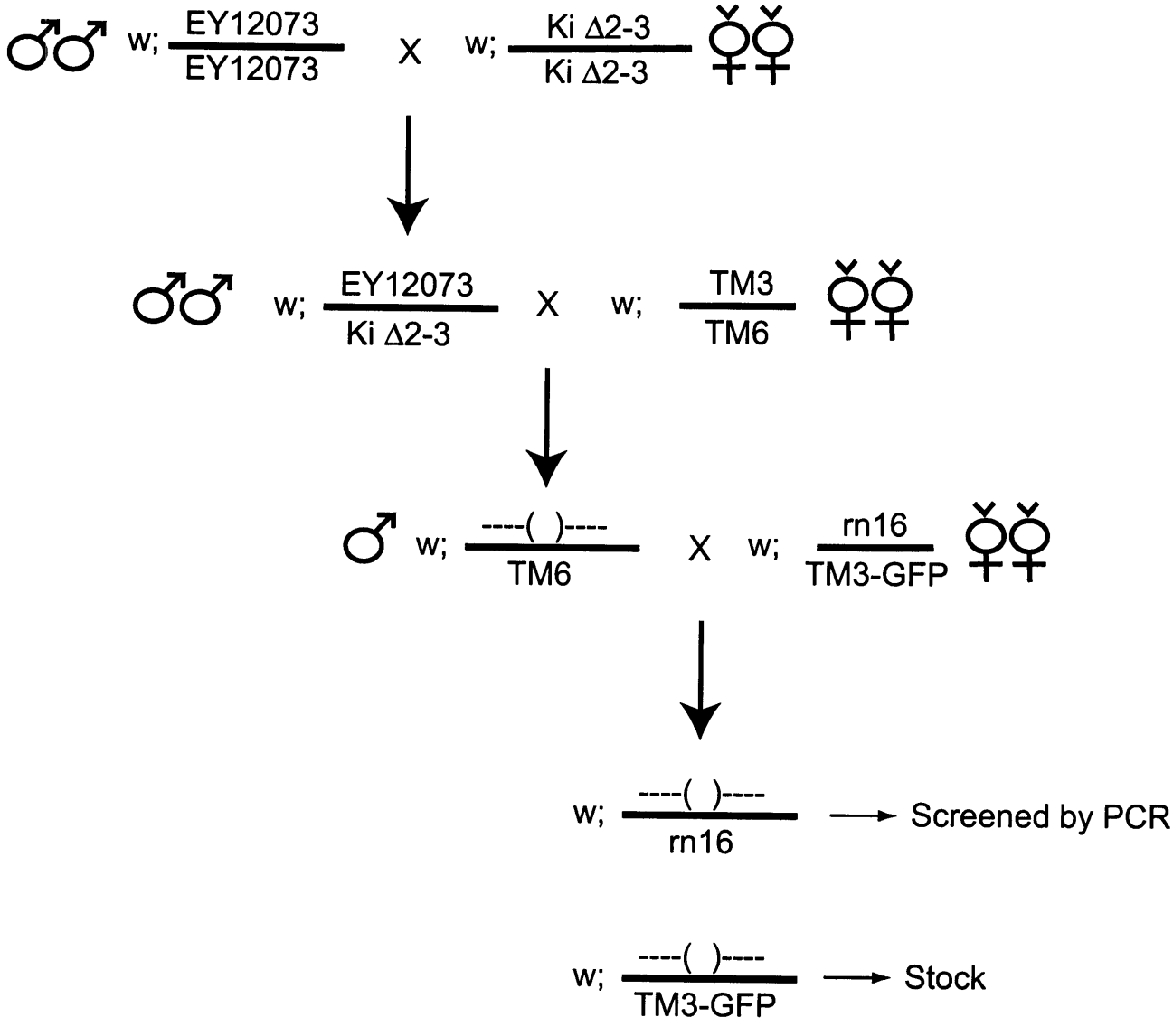

w; EY12073 / EY12073 X w; Ki Δ2-3 / Ki Δ2-3
w; EY12073 / Ki Δ2-3 X w; TM3 / TM6
w; ----( )---- / TM6 X w; rn16 / TM3-GFP
w; ----( )---- / rn16 → Screened by PCR
w; ----( )---- / TM3-GFP → Stock

Fig. 4-1. Synaptotagmin 4 excision screen. EY12073 is a viable p-element insertion (*P{EPgy2}SytIV*^{EY12073}) generated by the *Drosophila* gene disruption project (Bellen et al., 2004). Single excision chromosomes were generated by common p-element mobilization crosses and screened by PCR over the rn16 deletion which removes the *syt 4* locus along with the flanking genes *Gld* and *rotund.* Candidate lines which failed PCR anaylsis were further characterized by western blot and immunohistochemistry using the Syt 4 polyclonal antibody.

XL-blue chemically competent cells. The reverse cDNA sequence encoding the first two exons (~300 bp) of *syt 7* was then PCR amplified from a cDNA library using primers tagged with Bgl II and Kpn I, syt7revC3'-bgl 5'-GAAGATCTCCGAAGACTATAACTACTAGATTCCTCATC-3' and syt7revC5'-kpn 5'-GGGGTACCTTCGACGTGTTTTCATTGGCATAAGTAGCT-3'. The resulting PCR product was directionally cloned into a pUAST-syt7genomic clone using the Bgl II and Kpn I restriction sites and transformed into SURE2 chemically compentent cells (Strategene) (Fig. 4-2).

Synaptotagmin α P-element excision screen. A P-element (*P{SUPor-P}CG5559^{KG02182}*) inserted within the first predicted intron of the *syt α* locus was obtained from the *Drosophila* gene disruption project (Bellen et al., 2004). The p-element was mobilized by standard genetic crosses (Fig. 4-3) to generate over 100 excision lines. Viable homozygous excision lines were screened by PCR using primers flanking the insertion site, p55595' 5'-GCCTAGTGTTATCAGAGTAGTCTAG-3' and p55593' 5'-CACAGATGGGCTGATGGAAACACCACAC-3'. Lines failing to produce predicted PCR products were further screened by western analysis using the Syt α polyclonal antibody. One imprecise excision line (*sytα^{JZ1}* or JZ1) dramatically reduced Syt α protein levels compared to a precise excision line (*sytα^{S44}* or S44) and was further characterized

Larval Locomotion Analysis. To quantify larval locomotion, late third instar larvae grown at 25°C were collected and placed on a flat layer of 2.9% agar supplemented with grape

Fig. 4-2

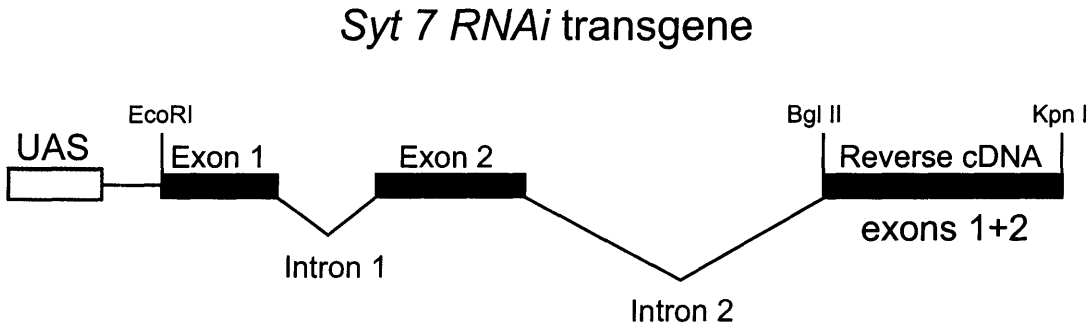

Syt 7 RNAi transgene
EcoRI
Bgl II
Kpn I
UAS
Exon 1
Exon 2
Reverse cDNA
exons 1+2
Intron 1
Intron 2

Fig. 4-2. Synaptotagmin 7 'knock-down' construct. The initial genomic region of the *syt 7* locus including exons and introns 1 & 2 was fused to the reverse cDNA coding for exons 1 & 2 maintaining the splice acceptor and donor sites in the two introns.

Fig. 4-3

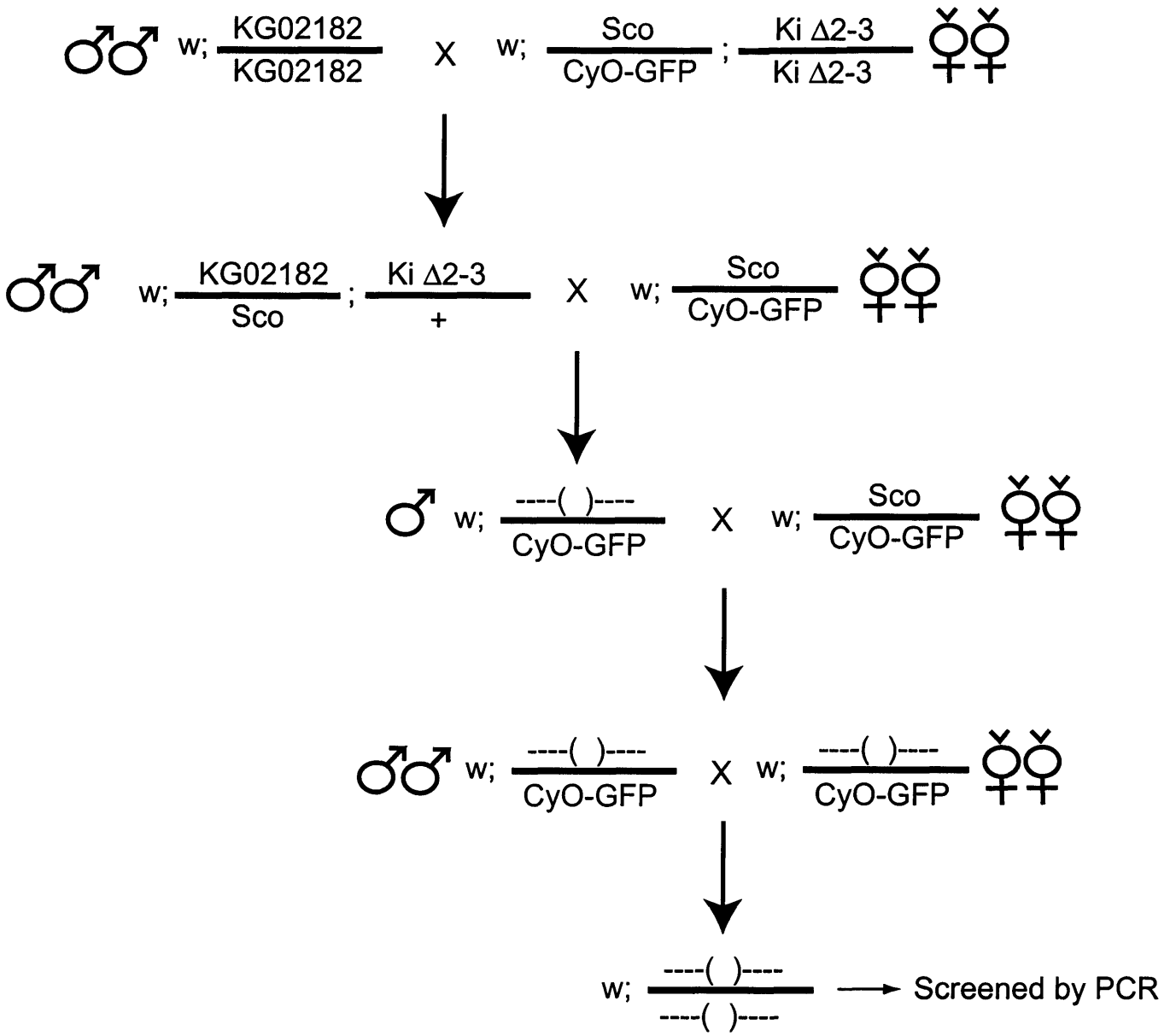

♂♂ w; KG02182 / KG02182 X w; Sco / CyO-GFP ; Ki Δ2-3 / Ki Δ2-3 ♀♀
♂♂ w; KG02182 / Sco ; Ki Δ2-3 / + X w; Sco / CyO-GFP ♀♀
♂ w; ----( )---- / CyO-GFP X w; Sco / CyO-GFP ♀♀
♂♂ w; ----( )---- / CyO-GFP X w; ----( )---- / CyO-GFP ♀♀
w; ----( )---- / ----( )---- → Screened by PCR

Fig. 4-3. Synaptotagmin α excision screen. KG02182 is a viable p-element insertion (P{SUPor-P}CG5559^{KG02182}) obtained from the *Drosophila* gene disruption project (Bellen et al., 2004). The KG02182 p-element was mobilized by common genetic techniques and viable excision events were screened as homozygotes using PCR analysis on isolated genomic DNA. Candidate lines which failed PCR analysis were further characterized by western blot and immunohistochemistry using the Syt α polyclonal antibody.

juice (Loewen et al., 2001). Quantification of larval locomotion parameters was as previously described (Saraswati et al., 2004). For quantification of cycle duration, video recording of locomotion was performed using a digital video camera (Canon XL1S) attached to a 16 X zoom lens. Cycle duration was reconstructed offline by digitizing frame-by-frame locomotor contractions.

Third Instar Electrophysiology Analysis. Electrophysiological analysis of wandering stage third instar larva was performed in *Drosophila* saline (NaCl, 70 mM; KCl, 5; $MgCl_2$, 4; $NaHCO_3$, 10; Trehalose, 5; Sucrose, 115; HEPES-NaOH, 5; pH 7.2) using an Axoclamp 2B amplifer (Axon Instrument) at 22°C as previously described (Rieckhof et al., 2003). Calcium concentrations varied with experiment and are indicated in the figure legends. Evoked EJPs were recorded from muscle fiber 6 and 7 of segments A3 and A4 for the rescue of synaptotagmin 1 nulls by overexpression of synaptotagmin 4 experiments. All EJPs from synaptotagmin 4 null animals and synaptotagmin 7 knock-down animals were recorded from muscle fiber 6 in segment A3.

Embryonic electrophysiology. Synaptic currents were recorded using the patch clamp technique in whole-cell configuration from *Drosophila* embryonic muscle fiber 6 in segments A2–A5 maintained at a holding potential of -60 mV. Embryos were aged 21–22 hr after fertilization and recorded in HL3.1 saline (in mM: NaCl, 70; KCl, 5; $MgCl_2$, 5; $CaCl_2$, 0.5; $NaHCO_3$, 10; Trehalose, 5; Sucrose, 115; HEPES-NaOH, 5; pH 7.2) as described (Yoshihara and Littleton, 2002) using an Axopatch 200B amplifier and pCLAMP 8.0.2 (Axon Instrument) at 23°C –24°C. Before recording, embryo fillets were

treated for 1 min with 0.4 mg/ml collagenase (type IV; Sigma, St. Louis, Missouri) in 0.1 mM Ca^{2+} saline. The internal solution in patch pipettes contained (in mM): CsCl, 158; ATP, 2; EGTA, 5; HEPES-NaOH, 10, pH 7.1. For BAPTA experiments and the accompanying control experiments, EGTA was not included. With this internal solution a liquid junction potential of the recording electrode is calculated to be –3.7 mV using the junction potential calculator in pCLAMP 8.0.2 (Axon Instrument). Thus, actual membrane potential during the voltage-clamping recording is estimated to be –63.7 mV. A patch pipette with a low resistance less than 1.7 Mohm was used for BAPTA experiments and the accompanying control experiments. Motor nerves were positioned in a suction electrode at their site of emergence from the CNS for stimulation, and 5 μA of positive current was passed for 1 msec through a suction electrode containing HL3.1 saline. Pulling or damaging motor neurons induced abnormal discharges, and we did not include results from such preparations into our analyses. BAPTA used in this study is from Sigma. StatView 5.0.1 (SAS Institute Inc., North Carolina) was used for statistical analysis.

Learning and memory assay. Olfactory learning and memory assays were performed as described in Quinn et al. (1974). Briefly, populations were exposed separately to each odor (octanol or 4-methylcyclohexanol) for 30 seconds with one odor being paired with shock for a given assay. Thirty second rest periods were used in between each odor presentation and each population was exposed to each odor three times. The training protocol is diagrammed in each learning assay figure. The learning score was calculated as the number of flies avoiding the conditioned odor minus the number of flies avoiding

the unconditioned odor divided by the total number of flies in the experiment. A single learning score is the average score from two different populations of flies from a given genotype tested with each odor. Microsoft Excel was used to perform the Student's paired t-tests.

Immunostaining. Third instar larvae were dissected in HL3 saline (NaCl, 70 mM; KCl, 5; $MgCl_2$, 4; $NaHCO_3$, 10; Trehalose, 5; Sucrose, 115; HEPES-NaOH, 5; pH 7.2) and fixed with 4% formaldehyde solution (HL3 + 4% formaldehyde). Fixed larvae were blocked in BSA solution (PBS + 0.5% triton + 2% bovine serum albumin) and incubated overnight with α-Syt 1 primary antibody diluted 1/1000. After four 15 minute washes in PBS, the larvae were incubated overnight with a goat anti-rabbit Cy3 secondary antibody diluted 1/500 in BSA solution. Stained larvae were then washed four times (15 minutes each) in PBS, mounted on glass slides in 70% glycerol, and imaged using a confocal microscope (Zeiss).

Western analysis. Western blots were done using standard laboratory procedures. All antibodies were used at a 1:1000 dilution and detected using either a goat anti-rabbit or a donkey anti-rat antibody conjugated to HRP (Jackson Immunolabs). Vizualization of HRP was accomplished using a SuperSignal ECL kit (Pierce).

RESULTS

$Syt4^{BA1}$ is a putative synaptotagmin 4 null mutant

To elucidate the endogenous function of Syt 4, we generated putative null mutations in *syt 4* using P-element mutagenesis. From our screen, we were able to isolate two classes of deletions in the *syt 4* locus. The first class consisted of rather large homozygous viable deletions (~ 45kb) that removed the entire *syt 4* ORF along with the neighboring *rotund* locus (rn) suggesting that *syt 4* is not an essential gene. Due to the lack of single gene specificity these deletions were not pursued further. The second class of deletions were approximately 1kb in size, as determined by PCR analysis, and removed the first two exons of *syt 4* including the ATG start codon. In order to determine whether these deletions removed the Syt 4 protein, we performed immunohistochemistry and western analysis using the Syt 4 polyclonal antibody (Adolfsen et al., 2004). Immunostaining of one such deletion, $syt4^{BA1}$, lacked Syt 4 immunoreactivity in both the central nervous system and muscles in wandering third instar larvae (Fig. 4-4A) as compared to a precise excision control line (pre1). Futhermore, western analysis of adult head extract isolated from $syt4^{BA1}$ animals indicated that the Syt 4 protein was absent (Fig. 4-4B). $Syt4^{BA1}$ animals are homozygous viable and have no obvious developmental or behavioral abnormalities.

Syt 7 transgenic RNAi lines knock down the expression of synaptotagmin 7

The *Drosophila syt 7* locus resides on the small fourth chromosome. Few convenient P-elements are available in the vicinity of the *syt 7* locus, making traditional reverse genetic mutagenesis approaches difficult. Several recent studies have shown that RNA interference is an efficient technique to 'knock-down' the expression of endogenous genes (Mello and Conte, 2004). In *Drosophila*, transgenic lines expressing

Fig. 4-4

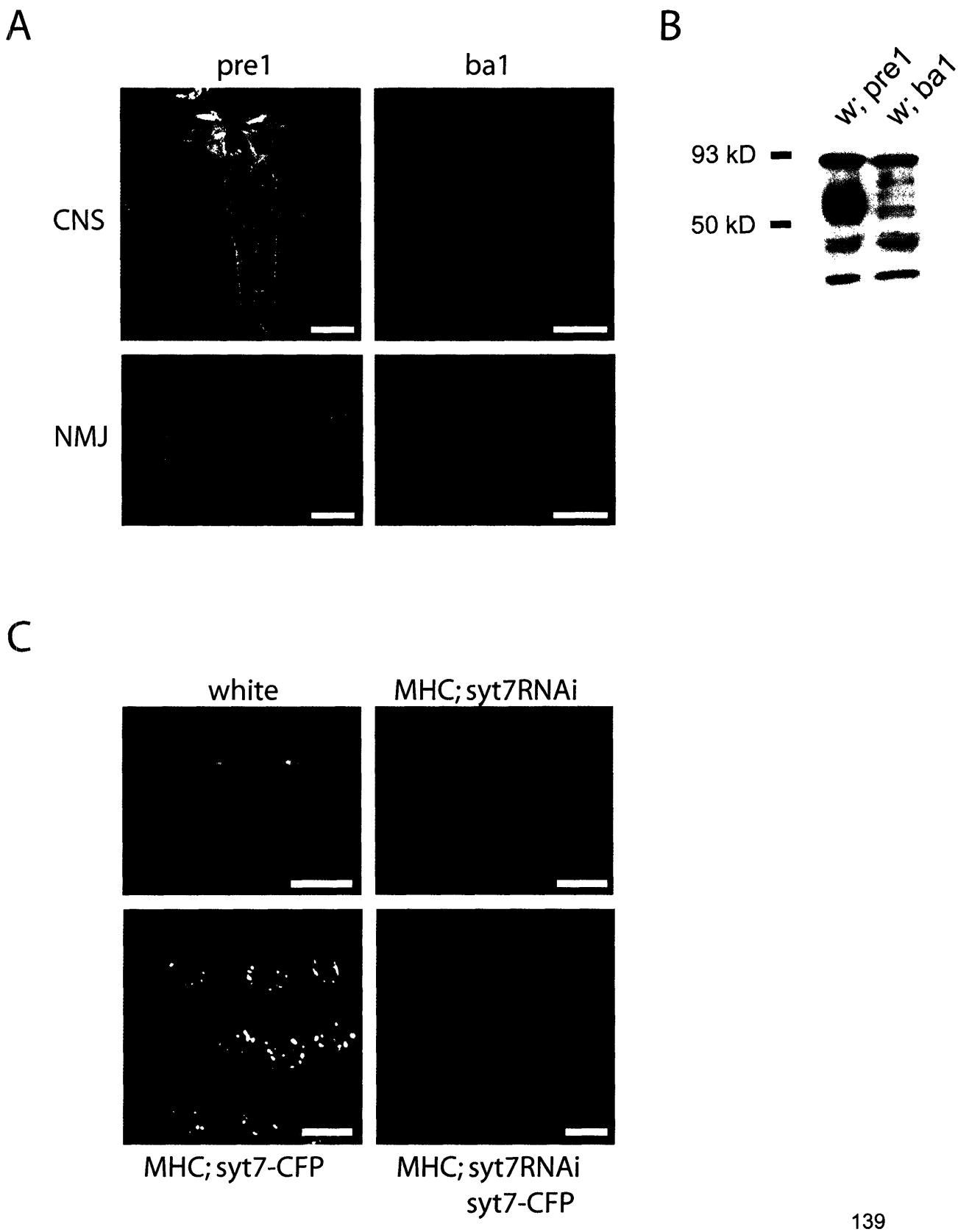

A
pre1
ba1
CNS
NMJ
B
w; pre1
w; ba1
93 kD
50 kD
C
white
MHC; syt7RNAi
MHC; syt7-CFP
MHC; syt7RNAi
syt7-CFP

Fig. 4-4. Generation of synaptotagmin 4 and synaptotagmin 7 loss-of-function mutants. (A) Putative synaptotagmin 4 nulls (ba1) and precise excision control larvae (pre1) were dissected and stained together within the same eppendorf tube using the Syt 4 polyclonal antibody. Larval brains (top, scale bar: 100μm) and neuromuscular junctions (bottom, scale bar: 30μm) from ba1 and pre1 animals were imaged using identical confocal settings to measure the relative Syt 4 protein levels between the two genotypes. (B) Western analysis of total protein extracted from adult head preparations collected from pre1 and ba1 lines. One fly head equivalent of protein was loaded into each lane and blotted using the Syt 4 polyclonal antibody. Although several non-specific bands are detected, the ba1 head extract is missing an abundant band corresponding to the predicted molecular weight of Syt 4. (C, top) Third instar muscles from *white* and *Mhc-Gal4; syt 7 RNAi* larvae stained with the Syt 7 polyclonal antibody (scale bar: 60 μm). (C, bottom) Muscle specific expression of a *syt7-CFP* transgene with or without coexpression of the *syt 7 RNAi* transgene (scale bar: 30 μm). Larvae coexpressing the two transgenes have dramatically reduced Syt 7-CFP levels in the muscle compared to control siblings.

genomic-cDNA fusions predicted to form double stranded RNA after splicing are potent silencers of neuronal and non-neuronal gene expression (Kalidas and Smith, 2002). In order to investigate *syt 7* loss-of-function phenotypes, we generated transgenic flies expressing a genomic-cDNA syt7 RNAi fusion under control of the Gal4/UAS system. To determine if expression of the construct could reduce Syt 7 protein levels *in vivo* we performed immunostaining experiments using the Syt 7 polyclonal antibody (Adolfsen et al., 2004). As shown in fig. 4-4C, muscle specific expression of the *syt 7 RNAi* construct, using the myosin heavy chain (*Mhc*) Gal4 driver, reduced the levels of native Syt 7 protein to below detection. For further validation that the *syt 7 RNAi* construct was functional, we tested for the ability of the *syt 7 RNAi* construct to reduce the expression of a *syt 7-CFP* (cyan fluorescent protein fused to the c-terminal of syt 7) transgene when coexpressed within the muscle. Flies homozygous for *Mhc-Gal4* driver and carrying one copy of the *syt7 RNAi* transgene over a GFP-labeled second chromosome balancer (*UAS-syt 7 RNAi/CyO-GFP; Mhc-Gal4*) were crossed to a homozygous *UAS-syt 7-CFP* line. Third instar larvae carrying the *UAS-syt 7 RNAi* transgene and control siblings carrying the balancer chromosome were dissected and the muscles were imaged by confocal microscopy using a CFP filter set. CFP fluorescence measured in muscles 6 and 7 in larvae carrying the *syt7 RNAi* transgene was dramatically reduced compared to balancer larvae controls (Fig 4-4C), suggesting that the RNAi transgene could efficiently target and reduce *syt 7-CFP* transgene RNAs.

Sytα^{JZ1} is a putative synaptotagmin α null mutant

In order to functionally characterize the *syt α* gene, we generated a deletion within the *syt α* genomic locus using p-element mutagenesis. Approximately 100 excision lines were screened by PCR to isolate potential imprecise excisions. Promising candidates were then screened by western blot analysis using the Syt α polyclonal antibody. One of the lines from our screen, $syt\alpha^{JZ1}$ (JZ1), failed to produce detectable amounts of the Syt α protein as measured by western analysis, suggesting this line was a severe *syt α* hypomorph or a null mutant. As shown in Fig. 4-5B, synaptotagmin α protein levels in adult heads from JZ1 animals were far below that seen in precise excision control animals (S44). We also confirmed the specificity of the Syt α immunostaining observed in third instar larvae previously reported (Adolfsen et al., 2004) by performing immunohistochemistry experiments on dissected JZ1 and S44 larvae (Fig. 4-5A). While S44 control animals displayed significant immunostaining (data not shown) throughout the larval central nervous system and at the lateral bipolar dendritic neuron in the periphery, no staining could be observed at either of these locations in JZ1 larvae, confirming that JZ1 is likely a severe hypomorph or null mutant and the Syt α polyclonal antibody is specific.

To facilitate gain-of-function and rescue experiments, we generated a number of transgenic lines expressing the *syt α* cDNA fused with a C-terminal HA epitope tag under control of the Gal4/UAS system. Neuronal expression of one such line using the $elav^{C155}$ (C155) driver induced abundant expression of the transgene within adult heads, whereas absence of the Gal4 driver resulted in little or no transgene expression (Fig. 4-5C).

Overexpression of Syt 4 can not rescue Syt 1 null mutants

Fig. 4-5

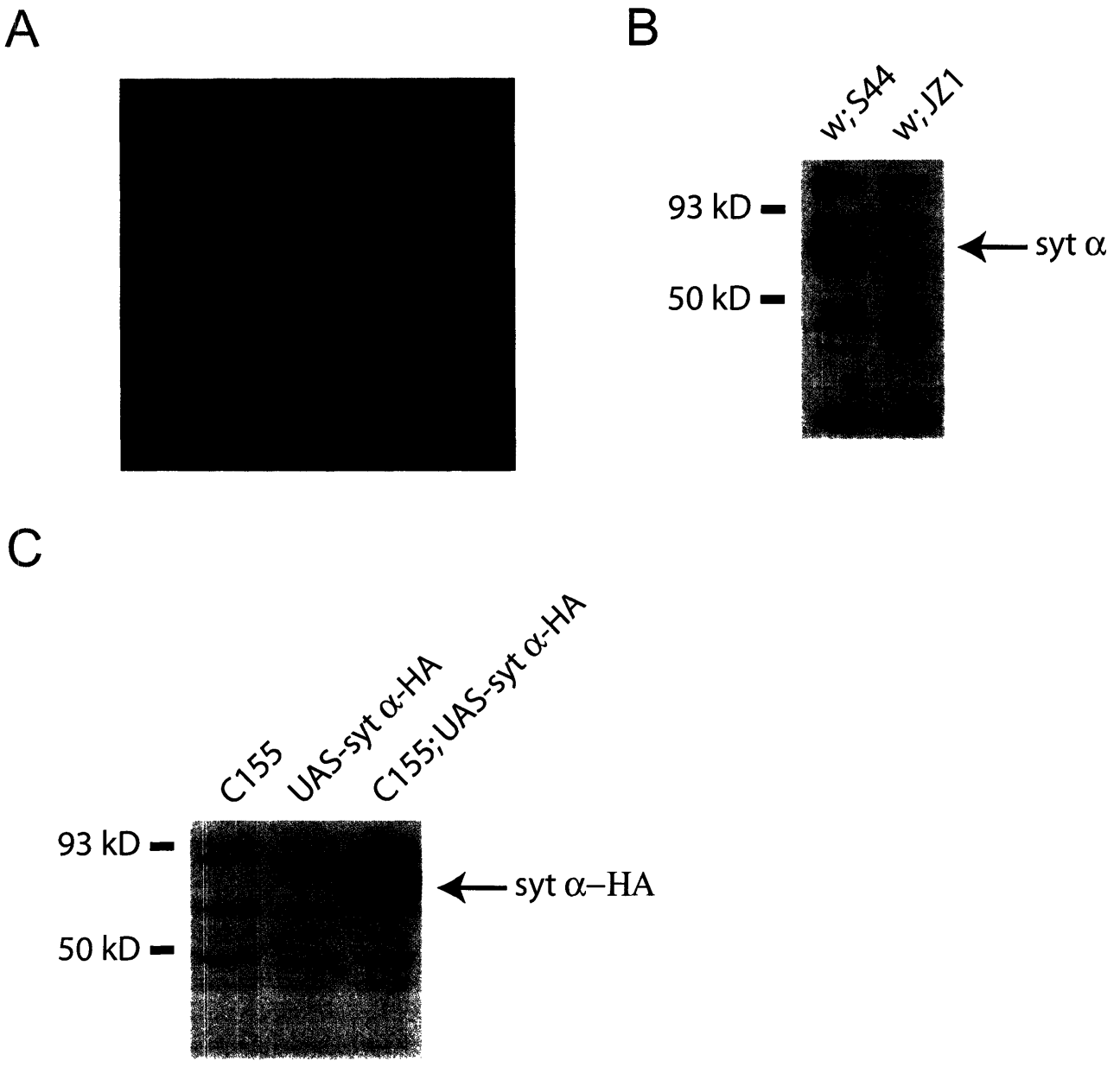

A
B
w;S44
w;JZ1
93 kD
50 kD
syt α
C
C155
UAS-syt α-HA
C155;UAS-syt α-HA
93 kD
50 kD
syt α–HA

Fig. 4-5. Generation of a synaptotagmin α mutant and transgene. (A) Immunostaining of third instar larval brains in S44 and JZ1 lines using the Syt α polyclonal antibody. No immunostaining could be detected in JZ1 larval brains as compared to S44 control brains which were incubated within the same staining chamber. (B) Western blot analysis of total protein extracted from adult heads. One fly head equivalent was loaded per lane and then blotted using the Syt α antibody. As indicated, the Syt α specific band is missing in the JZ1 line, suggesting it is a null mutant. (C) Regulated expression of a C-terminal HA epitope-tagged *syt α* cDNA transgene using the *elav*^{C155} (C155) Gal4 driver. One fly head equivalent of total adult head protein from animals of the indicated genotypes was loaded per lane, transferred to nitrocellulose, and then western blotted using a rat monoclonal anti-HA antibody. As indicated, lines carrying both the C155 driver and *UAS-syt α-HA* transgene properly expressed the transgene at the predicted molecular weight.

The localization of synaptotagmin isoforms to distinct subcellular compartments suggests they function in unique trafficking pathways. Although synaptotagmins may function similiarly to control vesicular fusion, our results indicate the proteins are non-redundant due to differential trafficking. This conclusion conflicts with a recent report indicating overexpression of Syt 4 using a UAS construct we previously generated can fully rescue synaptic transmission defects in *syt 1* mutants in *Drosophila* (Robinson et al., 2002). These results do not fit with our observation that Syt 4 is absent from synaptic vesicles and localizes postsynaptically. In addition, the results seem contradictory to the finding that the *syt 4* gene is co-expressed in all neurons with *syt 1*, yet there is a complete absence of the calcium-dependent synchronous component of release when Syt 1 is removed. We examined this apparent contradiction by quantifying the behavior and physiological rescue of *syt 1* mutants by overexpression of Syt 4 or Syt 1. Syt 4 transgenic expression can be easily followed by PCR analysis using primers that span intronic sequences, which are not present in the cDNA used for transgene construction (Littleton et al., 1999) (Fig. 4-6A). In addition, immunocytochemical analysis reveals a dramatic overexpression of UAS-Syt 4 in the CNS when driven exogenously by the pan-neuronal driver $GAL4^{elav\text{-}C155}$ (Fig. 4-6B). One potential explanation for the reported rescue of *syt 1* mutants by Syt 4 overexpression would involve a compartmental shift of Syt 4 when overexpressed, resulting in inappropriate targeting to synaptic vesicles. Therefore, we tested whether Syt 4 would target to Syt 1-containing synaptic vesicles when overexpressed. Consistent with the strong upregulation of the UAS-*syt 4* gene by the C155 driver, we detected presynaptic localization of Syt 4 following overexpression (Fig. 4-6C). However, immunostaining of Syt 4 and Syt 1 revealed a non-overlapping

Fig. 4-6

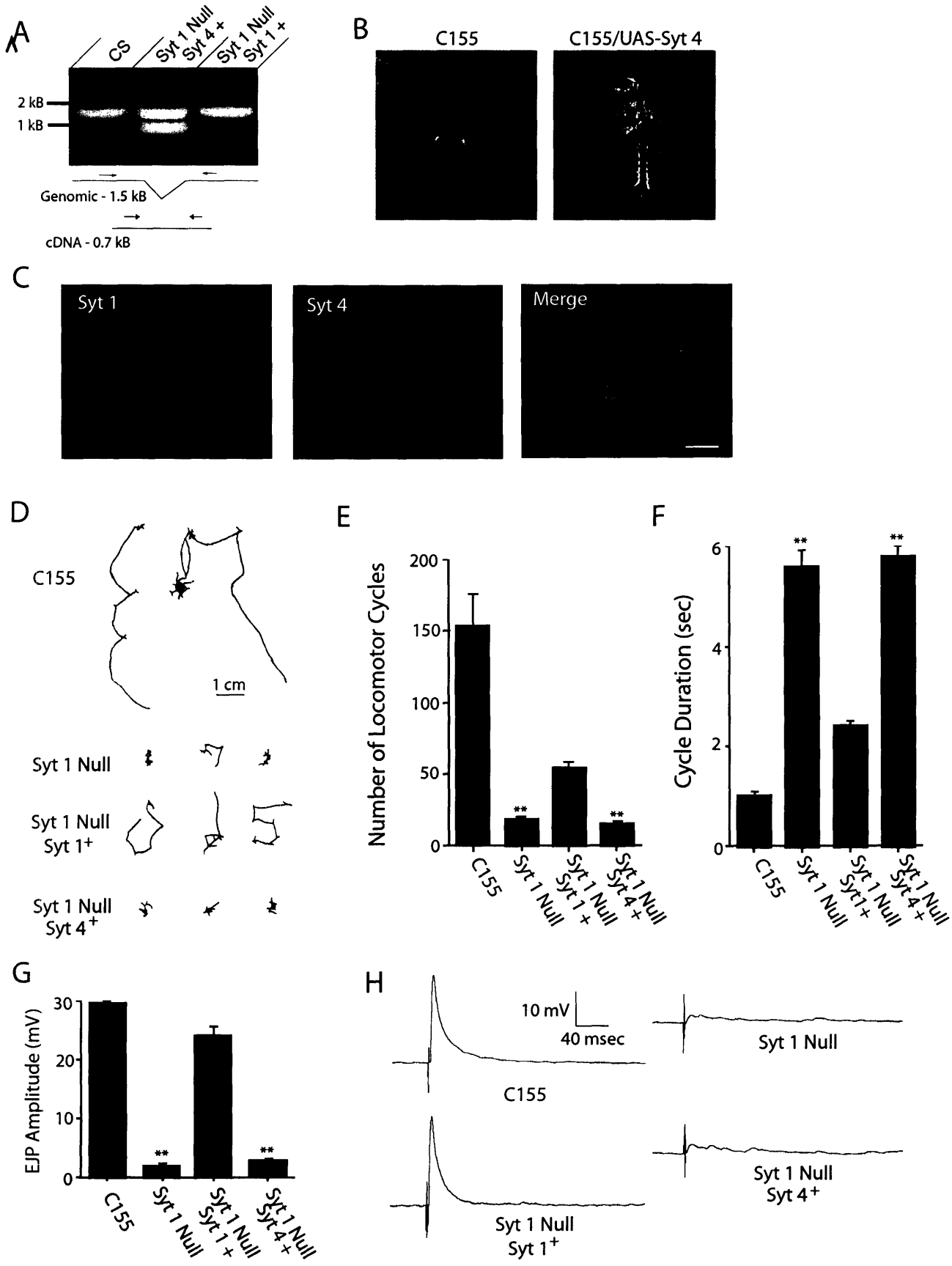

A
CS
Syt 1 Null Syt 4 +
Syt 1 Null Syt 1 +
2 kB
1 kB
Genomic - 1.5 kB
cDNA - 0.7 kB
B
C155
C155/UAS-Syt 4
C
Syt 1
Syt 4
Merge
D
C155
1 cm
Syt 1 Null
Syt 1 Null Syt 1+
Syt 1 Null Syt 4+
E
Number of Locomotor Cycles
200
150
100
50
0
**
C155
Syt 1 Null
Syt 1 Null Syt 1 +
Syt 1 Null Syt 4 +
F
Cycle Duration (sec)
6
4
2
0
**
**
C155
Syt 1 Null
Syt 1 Null Syt1+
Syt 1 Null Syt 4+
G
EJP Amplitude (mV)
30
20
10
0
**
**
C155
Syt 1 Null
Syt 1 Null Syt 1 +
Syt 1 Null Syt 4 +
H
10 mV
40 msec
C155
Syt 1 Null
Syt 1 Null Syt 1+
Syt 1 Null Syt 4+

Figure 4-6. Syt 4 cannot rescue release defects in *syt 1* mutants. (A) PCR confirmation of the *syt 4* trangene in rescued animals was obtained by priming across a small intron, revealing a larger 1.5 kB band from the native genomic locus, and a 0.7 kB band specifically from animals containing the UAS-*syt 4* cDNA lacking the intron. (B) Immunostaining with anti-Syt 4 antibodies from control and overexpressing lines. The confocal settings were identical between the two pictures, and the signal intensity was set to a low level to highlight the strong upregulation of Syt 4 in the third instar CNS of animals containing UAS-*syt 4* and the $C155^{Elav\text{-}GAL4}$ driver. (C) Overexpression of Syt 4 in transgenic animals resulted in presynaptic localization of Syt 4. However, Syt 4 staining was specifically excluded from the Syt 1-positive synaptic vesicle domains, indicating that overexpression of Syt 4 does not cause abnormal sorting of the protein to synaptic vesicles. (D) Traces of the crawling pattern of control, *syt 1* null mutants, and rescued lines containing UAS-*syt 1* or UAS-*syt 4* are shown for a 4 minute imaging period. Quantification of the number of locomotor cycles during 4 mins (E) and the cycle duration (F) are shown. Error bars are SEM. In contrast to the behavioral rescue observed by neuronal expression of Syt 1, no rescue were observed with Syt 4 overexpression. The number of animals examined were: $C155^{elav\text{-}GAL4}$, n=5; $syt^{AD4}/Df(2L)N13$, n=17; $C155^{elav\text{-}GAL4}$/UAS-*syt 1*; $syt^{AD4}/Df(2L)N13$, n=15; and $C155^{elav\text{-}GAL4}$/UAS-*syt 4*; $syt^{AD4}/Df(2L)N13$, n=8. (G) Mean evoked EJP amplitudes for responses peaking in the first 20 msec recorded in 1.5 mM extracellular calcium for the indicated genotypes. In contrast to the rescue observed with *syt 1* transgenic expression, Syt 4 had no effect on neurotransmission in the *syt 1* null mutant. Average muscle resting potentials were unchanged between the genotypes and were: $C155^{elav\text{-}GAL4}$, 59.3

+3.9 Std; *syt*^{AD4}*/Df(2L)N13*, 61.1 + 5.2 Std; C155$^{elav\text{-}GAL4}$/UAS-*syt 1*; *syt*^{AD4}*/Df(2L)N13*, 63.7 + 3.6 Std; and C155$^{elav\text{-}GAL4}$/UAS-*syt 4*; *syt*^{AD4}*/Df(2L)N13*, 61.5 + 4.4 Std. The number of muscles examined were: C155$^{elav\text{-}GAL4}$, n=26; *syt*^{AD4}*/Df(2L)N13*, n=17; C155$^{elav\text{-}GAL4}$/UAS-*syt 1*; *syt*^{AD4}*/Df(2L)N13*, n=10; and C155$^{elav\text{-}GAL4}$/UAS-*syt 4*; *syt*^{AD4}*/Df(2L)N13*, n=16. (H) Representative traces of evoked responses in the indicated genotypes. In contrast to the fast release observed in control and Syt 1 rescued animals, Syt 4 rescued animals and the *syt 1* null mutant both showed only slow EJPs, reflecting asynchronous synaptic transmission. Statistical significance (**) was determined by Student t-test, $P<0.05$.

pattern of expression, with Syt 4 excluded from Syt 1-positive synaptic vesicle microdomains (Fig. 4-6C). Thus, although overexpression of Syt 4 can lead to presynaptic accumulation of the protein, it is not targeted to synaptic vesicles as indicated by absence of co-localization with Syt 1. Our results indicate differential targeting of Syt 1 and Syt 4 to distinct post-Golgi vesicles, as reported in mammalian neurons (Ibata et al., 2002). Therefore, if Syt 4 were capable of substituting for Syt 1, it would represent a unique mechanism bypassing the requirement that the protein be present on synaptic vesicles.

To directly test if Syt 4 could rescue synaptic transmission in *syt 1* mutants, we overexpressed the protein in the *syt 1* null background (syt^{AD4} in trans to *Df(2L)N13*) using the C155 driver. To obtain quantitative information on the behavioral rescue, we performed larval locomotion assays to examine the output of the central motor pattern generator (Suster and Bate, 2002). *Drosophila* larvae perform rhythmic waves of body wall muscle contractions that result in peristaltic propagation over a surface. A stereotypic pattern of locomotion can by achieved by allowing wandering stage third instar larva to crawl over an agar surface in constant light. We video-recorded larval crawling to analyze locomotion parameters, including the number of locomotor cycles and cycle duration. Representative traces of crawling patterns from the control line *C155* and the *syt 1* null mutant (syt^{AD4}/*Df(2L)N13*) are shown in Fig. 4-6D. In contrast to the robust locomotion observed in control animals, the lack of Syt 1 dramatically slows larval locomotion. In addition to a decrease in distance traveled and locomotor cycle number (Fig. 4-6E), *syt 1* null mutants display an increase in the duration of a single locomotor cycle from one second to approximately six seconds (Fig. 4-6F). Transgenic expression

of the *syt 1* gene in the null background was able to partially restore all the behavioral defects observed (Fig. 4-6D-F). The lack of a full rescue likely reflects differences in the timing and abundance of the Syt 1 protein from the transgene compared to native regulation from the genomic locus. In contrast to Syt 1, Syt 4 overexpression did not rescue any aspect of the behavioral defects (Fig. 4-6D-F). Similar results were observed when UAS-*syt 1* and UAS-*syt 4* were driven with a third chromosome elav-GAL4 driver (data not shown).

To directly examine synaptic transmission, we performed synaptic physiology at the third instar NMJ (Fig. 4-6G-H). Consistent with previous observations, synaptic transmission was severely decreased in *syt 1* null mutants, which showed the characteristic slow rise and decay reflecting asynchronous release and a loss of synchronous fusion. Overexpression of Syt 1 was able to restore evoked excitatory junctional potential (EJP) amplitudes to near wild type levels. In addition, overexpression of Syt 1 completely restored the fast rise and decay of the EJP, reflecting synchronous release. Similar to the lack of behavioral rescue, overexpression of Syt 4 in the *syt 1* null mutant had no effect on the synaptic transmission defects. Only slow release reflecting the asynchronous component of fusion was observed in Syt 4 rescued animals, and EJP amplitude was unchanged from the null mutant alone. These behavioral and physiological data indicate that Syt 4 cannot functionally substitute for Syt 1 when overexpressed, consistent with the observation that Syt 4 defines a unique postsynaptic membrane trafficking pathway. In addition to Syt 4, we also tested the ability of Syt 7 to rescue Syt 1 defects. Similar to the Syt 4 experiments, overexpression of Syt 7 could not substitute for Syt 1 in neurotransmitter release (data not shown).

Synaptotagmin 4 and synaptotagmin 7 mutants do not have defects in classical presynaptic neurotransmitter release

Although several studies support the role of synaptotagmin 1 in presynaptic neurotransmitter role, no loss-of-function studies have investigated whether other synaptotagmin isoforms also participate in classical synaptic vesicle fusion. In *syt 1* null animals, fast synchronous vesicle fusion is completely absent, however a slower calcium-dependent asynchronous form of fusion is still present (Geppert et al., 1994; Yoshihara and Littleton, 2002), leading some to hypothesize the existence of a second independent calcium sensor. Synaptotagmins 4 and 7 are good candidates for encoding the asynchronous calcium sensor since they are well-conserved through evolution (Craxton, 2004) and are abundantly expressed throughout the nervous system in both vertebrates and invertebrates (Adolfsen et al., 2004; Li et al., 1995b). To test whether Syt 4 or Syt 7 may function as a second calcium sensor in neurotransmitter release, we generated loss-of-function mutations in each and performed electrophysiological recordings at the larval neuromuscular junction. Although we have previously shown that Syt 4 mostly localizes postsynaptically at the neuromuscular junction (NMJ) and Syt 7 is non-synaptic (Adolfsen et al., 2004), we could not be certain that some Syt 4 or Syt 7 might localize presynaptically and was thus below the detection limit of our antibody. Therefore, to rigorously test the hypothesis that Syt 4 or Syt 7 may function in synaptic vesicle fusion, we performed electrophysiological analysis on *syt 4* and *syt 7* loss-of-function mutants.

We first investigated whether *syt 4* or *syt 7* mutants displayed defects in quantal content, by measuring excitatory junctional potentials (EJPs) at the third instar NMJ at two different calcium concentrations. We performed recordings at 0.4 mM calcium, to determine if there where any drastic changes in quantal content. As shown in Fig 4-7A.,

Fig. 4-7

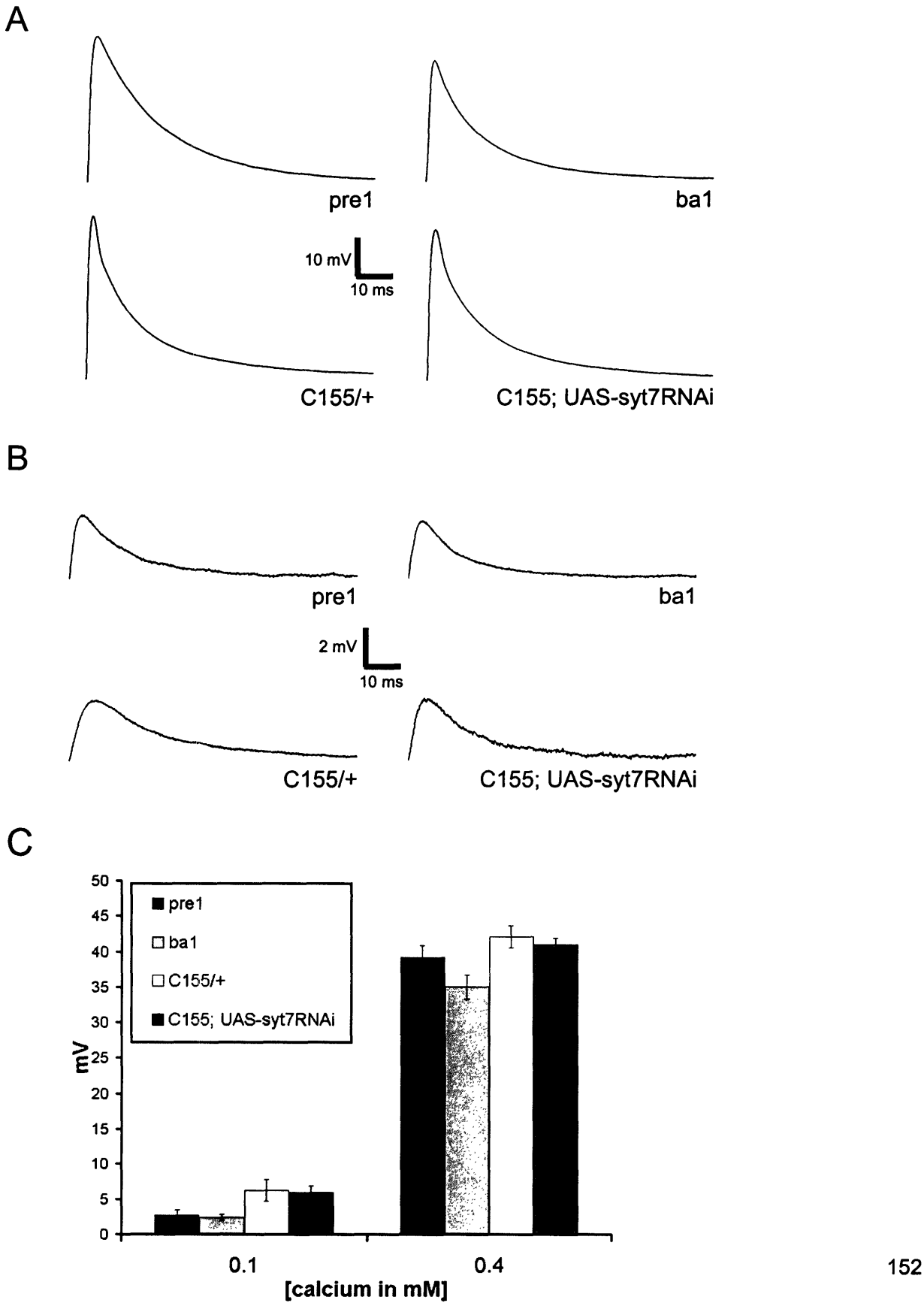

A
pre1
ba1
10 mV
10 ms
C155/+
C155; UAS-syt7RNAi
B
pre1
ba1
2 mV
10 ms
C155/+
C155; UAS-syt7RNAi
C
pre1
ba1
C155/+
C155; UAS-syt7RNAi
mV
50
45
40
35
30
25
20
15
10
5
0
0.1
0.4
[calcium in mM]

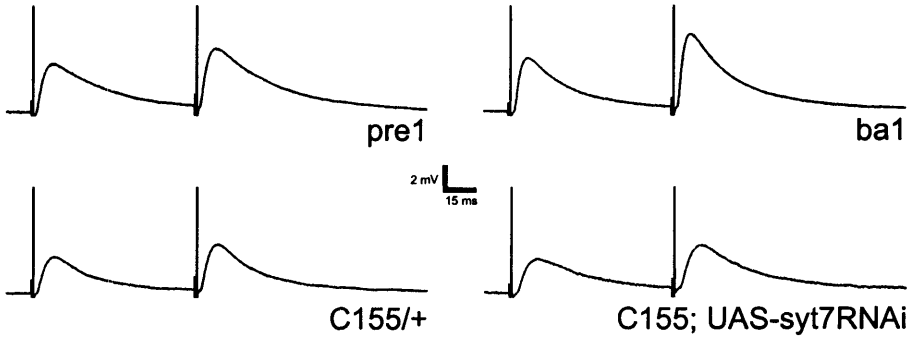
Fig. 4-7. Quantal content is unaltered in *syt 4* and *syt 7* mutants. (A) Representitive EJP traces recorded in 0.4 mM Ca^{++} HL3 saline from muscle 6 in segment A3 for each of the indicated genotypes (pre1, n = 9 muscles, avg. RP = -69 ± 6 mV; ba1, n = 9 muscles, avg. RP = -66 ± 5 mV; C155/+ n = 9, avg. RP = -75 ± 6 mV; C155; syt7RNAi n = 9, -73 ± 3 mV). (B) Sample EJP traces in 0.1 mM Ca^{++} HL3 for the indicated genotypes (pre1, n = 10 muscles, avg. RP = -64 ± 4 mV; ba1, n = 9 muscles, avg. RP = -68 ± 7 mV; C155/+ n = 8, avg. RP = -62 ± 6 mV; C155; syt7RNAi n = 8, -62 ± 4 mV). (C) Quantification of EJPs from all measured genotypes. Error bars are SEM and no statistical difference was observed between mutant and control lines as measured by a paired t-test. Avg. RP = average resting potential in mV and standard deviation.

we did not observe any statistical differences in the evoked excitatory EJP amplitudes between the *syt 4* or *syt 7* mutants ($syt4^{ba1}$ and C155; syt7RNAi) and their appropriate controls ($syt4^{pre1}$ and C155/+), suggesting that robust transmission is still intact in these mutant animals. Next, we investigated the possibility that loss of *syt 4* or *syt 7* may have more subtle effects on quantal content. We measured EJPs at a low calcium concentration (0.1 mM), where nonlinear summation effects were minimal and subtle defects in quantal content could accurately be measured. Again, we could not detect any changes in the EJP amplitudes in the *syt 4* or *syt 7* mutant animals (Fig. 4-7B). These results indicate that loss of Syt 4 or Syt 7 does not effect fast synaptic transmission at this synapse.

Although these experiments demonstrate that *syt 4* and *syt 7* are not required for fast synchronous fusion, they do not rule out the possibility that *syt 4* or *syt 7* may be required for the slow asynchronous release still present in *syt 1* null synapses, which is essentially masked when Syt 1 is present (Yoshihara and Littleton, 2002). Interestingly, robust paired-pulse facilitation is observed at *syt 1* null synapses, suggesting that the asynchronous calcium sensor may be required for paired-pulse facilitation (ppf)(unpublished data, Littleton lab). To begin to investigate the possibility that *syt 4* or *syt 7* may encode the asynchronous calcium sensor, we measured the degree of paired-pulse facilitation at the NMJ in *syt 4* and *syt 7* mutants using low calcium salines (0.1 mM). As shown in Fig 4-8, the amount of facilitation measured at 75 ms pulse intervals was statistically the same for all the genotypes tested, indicating that neither *syt 4* nor *syt 7* likely encodes the facilitation calcium receptor. Future electrophysiological studies on

Fig. 4-8

A


pre1
ba1
2 mV
15 ms
C155/+
C155; UAS-syt7RNAi

B

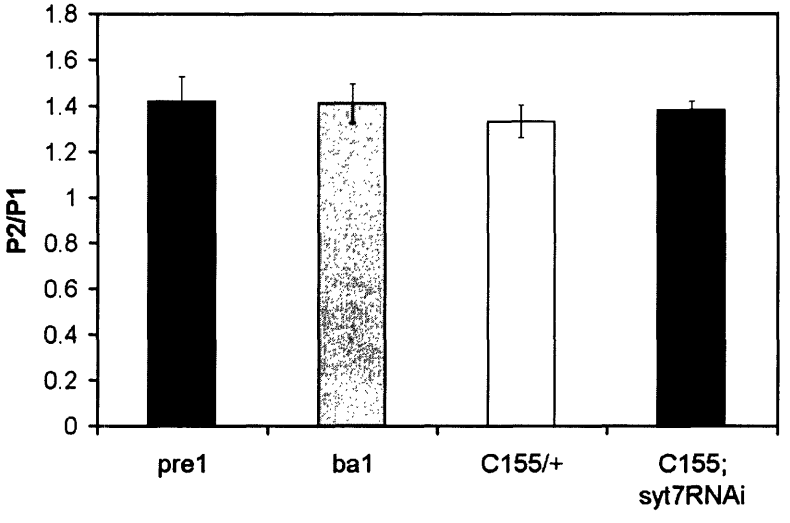

P2/P1
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
pre1
ba1
C155/+
C155; syt7RNAi

Fig. 4-8. Paired-pulse facilitation is normal in *syt 4* and *syt 7* mutants. (A) Representative paired-pulse traces from *syt 4* (ba1) and *syt 7* (C155; UAS-syt7RNAi) mutants. Recordings were performed in 0.1 mM calcium and stimulation pulses were spaced 75 ms apart to minimize baseline shift due to the first EJP. (B) Quantification of paired-pulse facilitation in all genotypes tested (pre1, n = 10 muscles, avg. RP = -63 ± 7 mV; ba1, n = 9 muscles, avg. RP = -59 ± 7 mV; C155/+ n = 9, avg. RP = -63 ± 5 mV; C155; syt7RNAi n = 7, -60 ± 4 mV). No statistical difference was observed between the mutants and their appropriate controls as measured by a Student's paired t-test. Error bars are SEM and Avg. RP = average resting potential in mV and standard deviation.

the *syt 4; syt 1* and *syt 7; syt 1* double mutants should allow an even more rigorous test of the hypothesis that *syt 4* or *syt 7* encodes the asynchronous calcium sensor.

Synaptic development is normal in synaptotagmin 4 null mutants

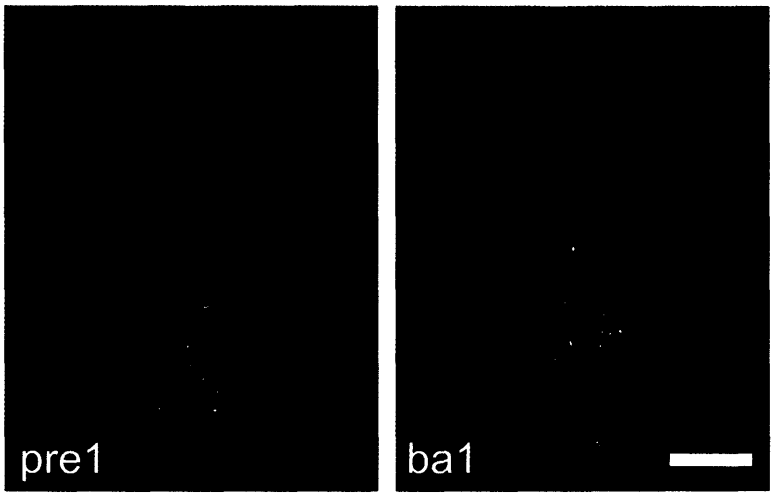
The intriguing localization of Syt 4 to the postsynaptic side of most, if not all, NMJ synapses prompted us to determine whether *syt 4* was required for normal synapse formation and/or development. $Syt4^{BA1}$ third instar larvae were dissected and motor neuron boutons were visualized using the α-Syt 1 polyclonal antibody (Littleton et al., 1993a). We quantified the number of synaptic boutons innervating muscles 6 and 7 from segment A3 in ba1 and pre1 (precise excision 1) animals and found that there is no statistical difference in the number of boutons between these two genotypes (Fig. 4-9). These results argue that *syt 4* is not essential for normal synaptic development at the NMJ in *Drosophila*.

Synaptotagmin 4 and calcium are required postsynaptically for high frequency-induced miniature release.

We have provided numerous lines of evidence that the abundant synaptotagmin isoforms 4 and 7 do not function as calcium sensors on classical Syt 1-containing synaptic vesicles. First, localization studies conducted on the whole *Drosophila* synaptotagmin family indicate that only Syt 1 is found presynaptically on synaptic vesicles, whereas Syt 4 was localized postsynaptically and Syt 7 was ubiquitously expressed and localized to a 'lysosomal-like' compartment found throughout the muscle and other non-neuronal tissues (Chapter 1). Second, neither overexpression of Syt 4 or Syt 7 could rescue neurotransmitter release defects in *syt 1* null animals, most likely due

Fig. 4-9

A



B

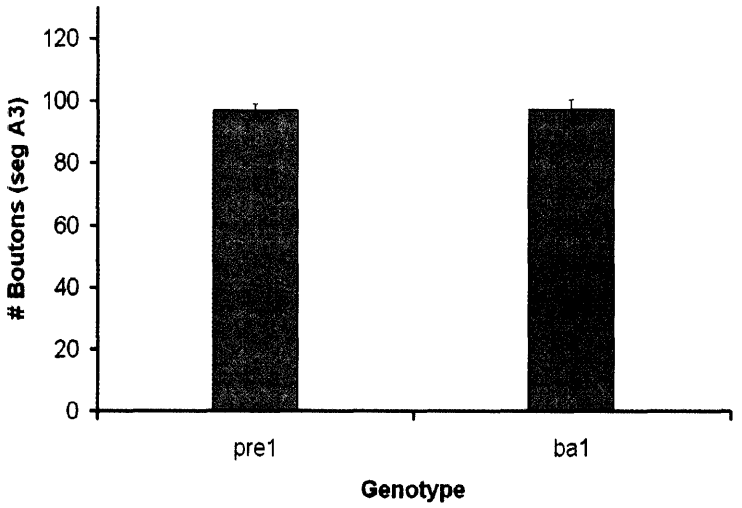


Fig. 4-9. Synaptic growth is unaltered in *syt 4* mutants. (A) Neuromuscular synapses imaged on muscles 6 and 7 in segment A3 stained with the α-Syt 1 antibody. (scale bar: 30 μM) (B) Quantification of the number of boutons in *syt 4* mutant (ba1) and control (pre1) animals (pre1, n = 15 muscles; ba1 n = 15 muscles). No statistical difference was found between *syt 4* mutants and control animals as measured by a Student's pairwise t-test. Error bars are SEM.

to the differential trafficking of these isoforms or the lack of functional redundancy between the different isoforms (Chapter 4). Third, neither the C2 domains of Syt 4 or Syt 7 contain the appropriate trafficking signals to remain localized on synaptic vesicles (Chapter 3) even when targeted to type I synapses. Finally, loss-of-function mutations in *syt 4* and *syt 7* do not have alterations in neurotransmitter release at the *Drosophila* NMJ (Chapter 4). These results argue that each synaptotagmin functions in distinct, potentially calcium-regulated, membrane trafficking pathways *in vivo*. To uncover the endogenous function of synaptotagmin 4 we have carried out a detailed electrophysiological analysis at the embryonic neuromuscular junction.

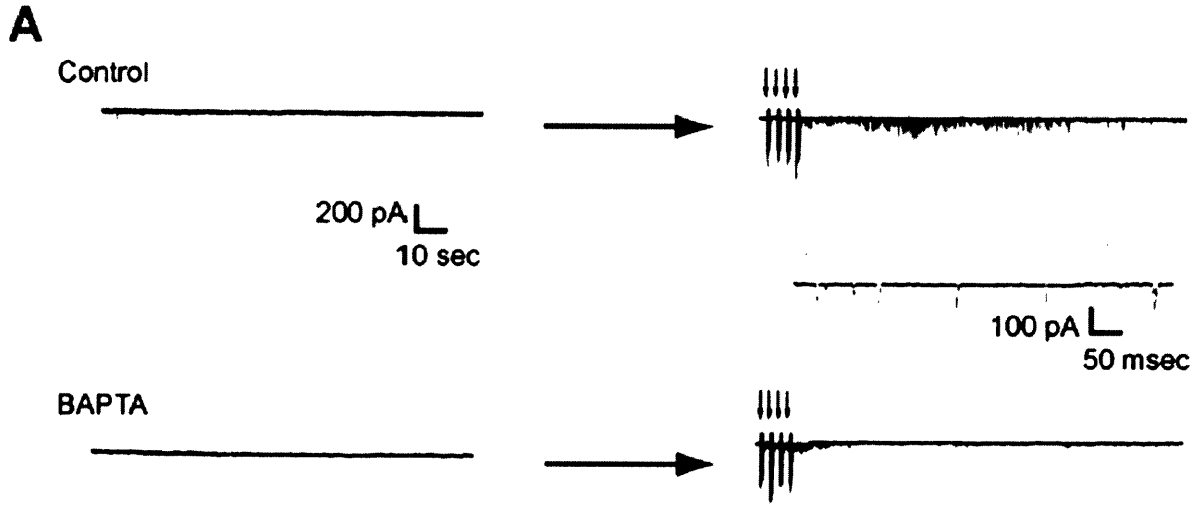
High frequency stimulation of neurons in many regions of the mammalian CNS is known to induce various forms of synaptic plasticity (Bliss and Collingridge, 1993), leading to long-lasting changes in synaptic function. To genetically dissect the mechanisms underlying activity-dependent synaptic plasticity, we tested whether newly formed *Drosophila* glutamatergic neuromuscular junctions (NMJs) show physiological changes following high frequency stimulation (four one-second 100 Hz stimuli spaced 2 seconds apart). The *Drosophila* embryonic neuromuscular junctions examined in our recordings have less than 20 active zones, with miniature frequency per active zone (~0.001 Hz) similar to that of mammalian CNS synapses. Within one minute following high frequency stimulation of embryonic NMJs, a 100-fold increase in the frequency of miniature excitatory synaptic currents (minis) was observed (Fig. 4-10), from a baseline of 0.03 Hz to frequencies more than 5 Hz. The high frequency-induced miniature release (termed HFMR) continued for a few minutes to as long as 20 minutes, before subsiding

to baseline levels. These results indicate that acute activity-dependent alterations in presynaptic function occur at *Drosophila* NMJs following high frequency stimulation

To determine the role of postsynaptic Ca^{2+} in HFMR, we perfused postsynaptic muscles with Ca^{2+} chelators using the patch recording electrode. While perfusion of EGTA for 2.5 minutes caused a modest suppression of HFMR, the fast Ca^{2+} chelator BAPTA induced strong suppression by 2.5 minutes perfusion. Longer perfusion with BAPTA for 5 minutes prior to stimulation abolished HFMR (Fig. 4-10), indicating HFMR is induced following postsynaptic Ca^{2+} influx. Given the dramatic increase in presynaptic release that occurs during HFMR, we conclude that postsynaptic Ca^{2+} influx triggers release of a retrograde signal to induce HFMR by altering presynaptic function.

Ca^{2+}-induced vesicle fusion in presynaptic terminals provides a temporally controlled and spatially restricted signal essential for synaptic communication. Analogous to presynaptic release, postsynaptic vesicles within dendrites have been visualized by TEM (Pow and Morris, 1989), and dendritic release of several neuromodulators has been reported (Ludwig and Pittman, 2003). Recent studies have also shown that postsynaptic vesicles undergo rapid Ca^{2+}-triggered exocytosis (Maletic-Savatic and Malinow, 1998) that is required for LTP in the mammalian hippocampus (Lledo et al., 1998). We postulated that postsynaptic vesicle fusion might underlie the Ca^{2+}-dependent release of retrograde signals that triggers altered presynaptic function at *Drosophila* NMJs. The synaptic vesicle protein Synaptotagmin 1 (Syt 1) functions as the major Ca^{2+} sensor for vesicle fusion at presynaptic terminals (Geppert et al., 1994; Yoshihara and Littleton, 2002), but is not localized postsynaptically. Recently, we have found that another isoform of the synaptotagmin family, Synaptotagmin 4 (Syt 4), is

Fig. 4-10


A
Control
200 pA
10 sec
100 pA
50 msec
BAPTA

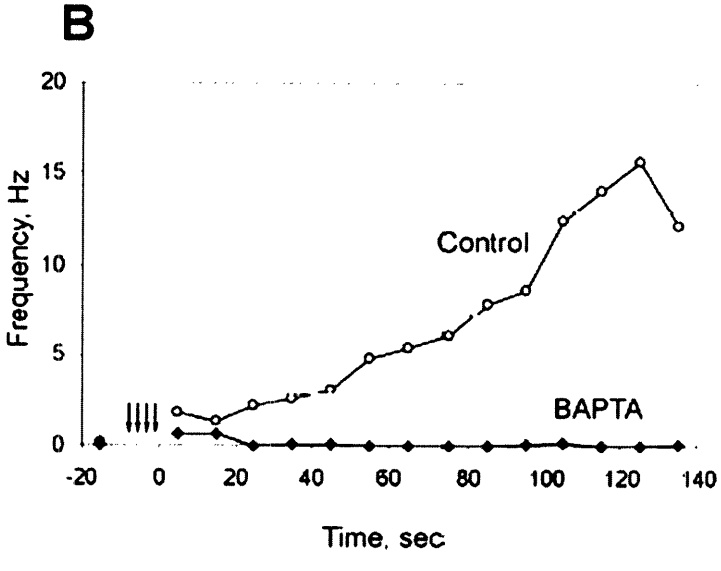

B
Frequency, Hz
20
15
10
5
0
Control
BAPTA
-20
0
20
40
60
80
100
120
140
Time, sec

Fig. 4-10. High frequency stimulation triggers enhanced presynaptic miniature release that requires postsynaptic Ca^{2+} for induction. (A) Motor nerves were stimulated four times with one-second 100 Hz stimuli spaced 2 seconds apart in 0.5 mM extracellular Ca^{2+}. Representative sample traces from whole cell voltage clamp recordings of postsynaptic muscle cells are shown. Whereas spontaneous release is rarely seen without stimulation at 0.5 mM Ca^{2+} (upper left in A), high frequency stimulation (represented by arrows) induces a robust induction of miniature synaptic current (HFMR) (upper right in A). The lower panel shows representative traces when a Ca^{2+} chelator, BAPTA, was included in the internal solution of the patch electrode at 5 mM. In both cases, stimulation was performed 5 minutes after the start of whole cell clamping to allow sufficient perfusion of BAPTA into the muscle. (B) Time courses of HFMR for a representative example from control and BAPTA-treated muscle cells. At each 10-second interval, miniature synaptic currents were counted and are displayed as mean frequency. The first time point represents averaged miniature frequency for five minutes prior to stimulation. Following the brief enhanced release just after tetanic stimulation (due to sustained Ca^{2+} elevation by tetanus during the initial stimulation), miniature release increases gradually in control animals, whereas HFMR is abolished by perfusion with BAPTA. (C) Quantification of miniature frequency at 1 minute following the end of tetanic stimulation (calculated mean between 50 - 70 seconds following stimulation), compared to miniature frequency before stimulation (mean for 5 minutes prior to stimulation). The number of samples analyzed were: control (6); BAPTA perfusion (5). Double asterisks indicates $p < 0.01$ by the paired t-test.

present in the postsynaptic compartment (Adolfsen et al., 2004), suggesting Syt 4 might function as a postsynaptic Ca^{2+} sensor. Indeed, recent biochemical studies have demonstrated that *Drosophila* Syt 4 can efficiently bind Ca^{2+} (Dai et al., 2004). To investigate whether Syt 4 was involved in HFMR, we characterized the embryonic physiological phenotype of the *syt 4* null mutant. Mutants lacking Syt 4 hatch from the egg case 21 hours after egg laying at 25°C, similar to wild type, and grow to fully mature larvae that pupate and eclose with a normal time course. In contrast to the robust enhancement of presynaptic release seen in controls (Fig. 4-11A), the increase of miniature release was eliminated in *syt 4* mutants (Fig. 4-11B). Postsynaptic expression of a UAS-*syt 4* transgene using the muscle specific driver, *Mhc-Gal4,* was able to completely restore HFMR in the null mutant (Fig. 4-11C), demonstrating that postsynaptic Syt 4 is required for triggering enhanced presynaptic function. In contrast, presynaptic expression of a UAS-*syt 4* transgene using the neuron-specific driver, *elav-Gal4,* did not restore HFMR in the null mutant (Fig. 4-11D), suggesting that Syt 4 function is not required in the presynaptic compartment to induce HFMR.

Synaptotagmin 4 pHluorin fusion localizes to synapses when expressed postsynaptically

The localization of Syt 4 and the requirement of Syt 4 postsynaptically for HFMR, suggests that calcium-regulated exocytosis functions on both sides of the synapse. We provided initial electrophysiological evidence to support the hypothesis that postsynaptic exocytosis may be responsible for the release of retrograde signals during high frequency stimulation, and thus a possible conserved mechanism to modulate synaptic transmission. Our retrograde signal hypothesis implies that Syt 4-containing

Fig. 4-11

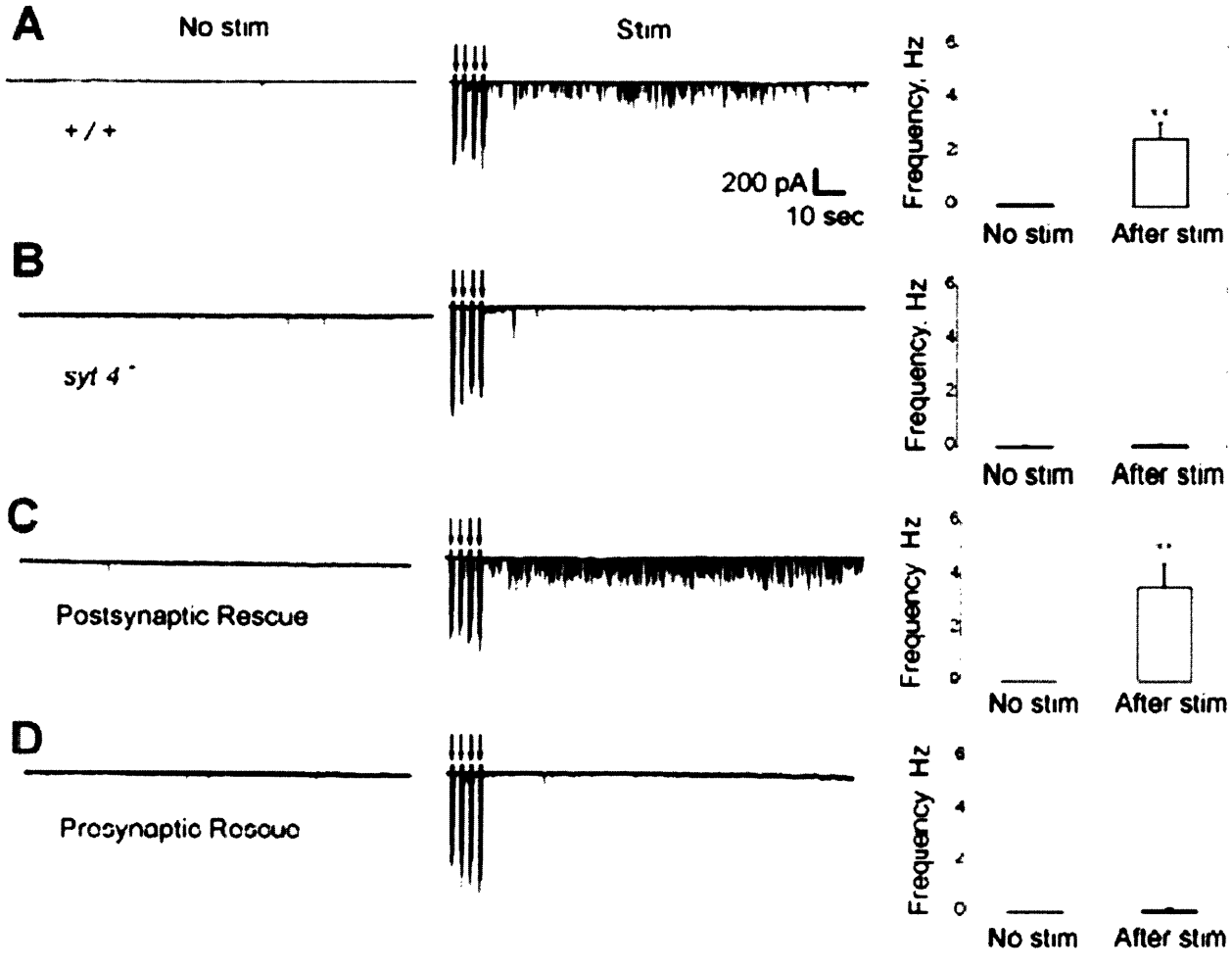

A
No stim
Stim
+ / +
200 pA
10 sec
Frequency, Hz
No stim
After stim
B
syt 4
C
Postsynaptic Rescue
D
Presynaptic Rescue

Fig. 4-11. High frequency stimulation-induced miniature release is abolished in the absence of postsynaptic Syt 4. (A-E) Stimulation protocol was the same as that in Fig. 4-9. Left panels are representative traces without high frequency stimulation. Middle panels are representative traces when stimulated. Whereas spontaneous release is rarely seen without stimulation at 0.5 mM Ca^{2+} (left in A), high frequency stimulation induces a robust HFMR response (middle in A). The induction of increased presynaptic release is abolished in the *syt 4* null mutant (*syt* 4^{BA1}, B; rn^{16} showed indistinguishable phenotypes), but restored in *Mhc-Gal4/UAS-syt 4* rescued synapses (rn^{16} background, C). Presynaptic expression by *elav-gal4 /UAS-syt 4* did not rescue the loss of HFMR (D).

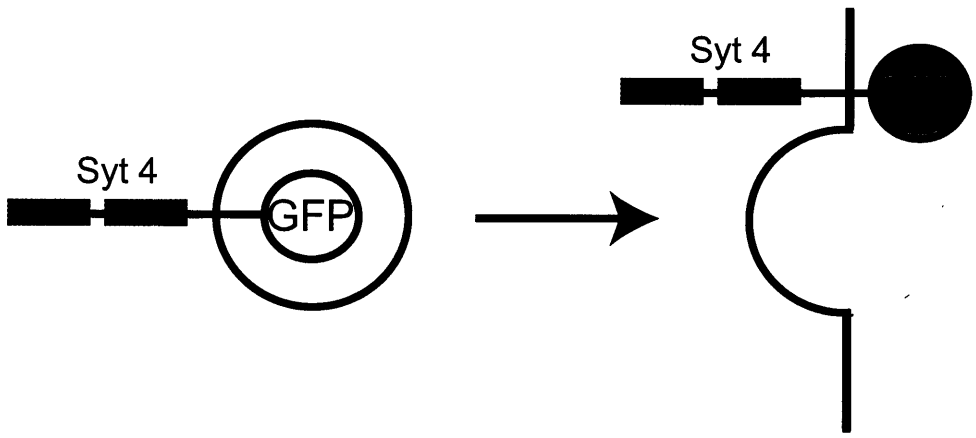
vesicles may be fusing with the postsynaptic membrane in a manner quite similar to the cycling of Syt 1 synaptic vesicles presynaptically. GFPs sensitive to pH (or pHluorins) have been used successfully to follow the fusion of synaptic vesicles in real time (Miesenbock et al., 1998) and we reasoned that a Syt 4 transgene fused with a pHluorin in the intravesicular domain might result in GFP signal juxtaposed to the synaptic boutons innervating the muscle, implying Syt 4 vesicles were fusing with the postsynaptic plasma membrane. Surprisingly, this is what we observed. Expression of the pHluroin-Syt 4 transgene, specifically in the muscle using a myosin heavy chain promoter GAL4 (MHC-Gal4), resulted in strong postsynaptic staining at the NMJ (Fig. 4-12). High magnification images showed that the pHluorin-Syt 4 fusion protein was localized to sub-bouton microdomains at the synapse, arguing that Syt 4 is efficiently trafficked to the postsynaptic apparatus and cycles to the plasma membrane. Future real-time imaging experiments using this tagged transgene should allow determination of the kinetics of this postsynaptic fusion event.

Synaptotagmin 4 mutants do not have learning or short term memory defects

The absence of high frequency-induced miniature release (HFMR) in *syt 4* null mutants, allowed us to investigate the potential importance of this synaptic property in behavioral conditioning and learning. Additionally, some studies have suggested *syt 4* may be required for certain forms of learning, as *syt 4* knock-out mice have defects in some learning paradigms (Ferguson et al., 2000). However, the interpretations from these genetic studies may be hindered by a second *syt 4*-like gene in mammals, *syt 11*. Studies utilizing the siphon-withdrawal reflex in *Aplysia* have hypothesized the existence

Fig. 4-12

A



B

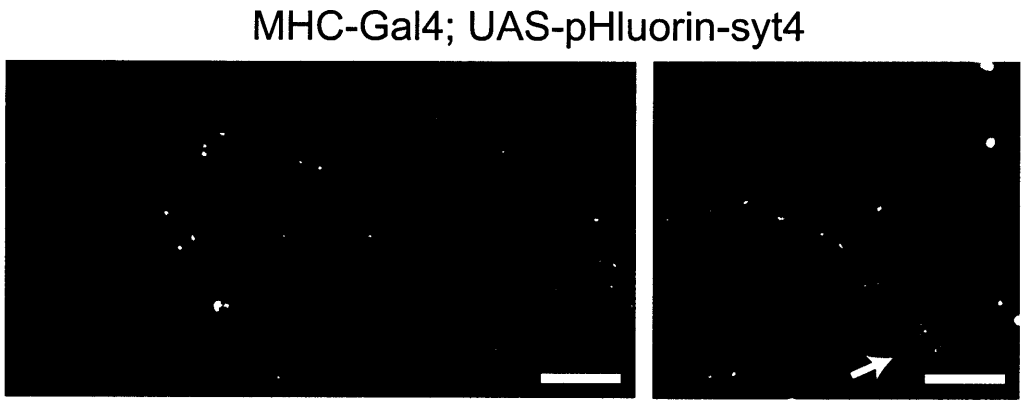
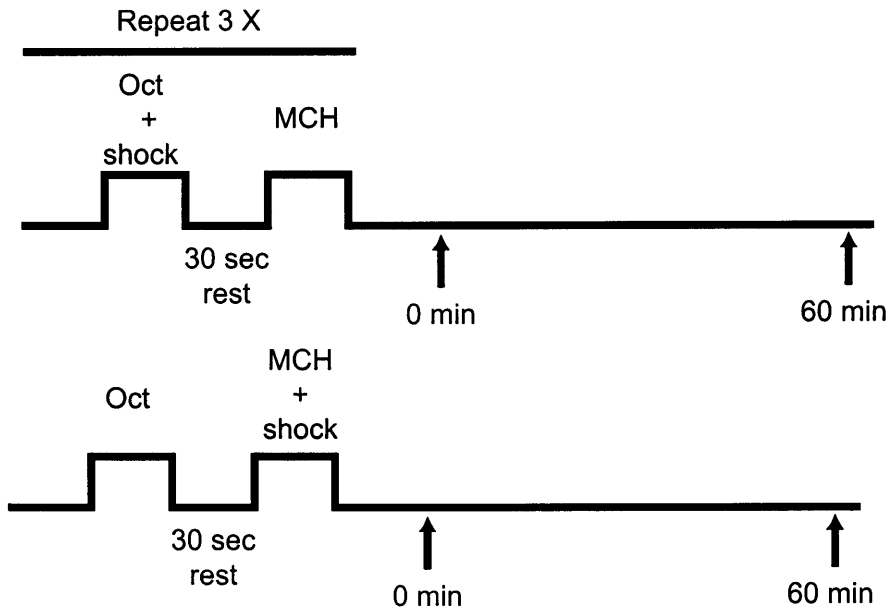


Fig. 4-12. Syt 4 trafficks to the postsynaptic plasma membrane. (A) Diagram illustrating the pHluorin-Syt 4 fusion protein. The pHluorin tag is ecliptic pHluorin previously reported (Miesenbock et al., 1998). (B, left) High magnification (100X oil immersion lens, N.A.) images of third instar neuromuscular synapses expressing the pHluorin-Syt 4 transgene using the muscle-specific, myosin heavy chain Gal4 (MHC-Gal4) driver (scale bar: 10 μm). (B, right) A single confocal section illustrating the sub-bouton localization of Syt 4 to plasma membrane microdomains (scale bar: 5 μm).

of an unknown retrograde signal that may be required for more persistent memories in this cellular model of memory (Antonov et al., 2003). One intriguing hypothesis is that Syt 4 may be involved in the release of such a retrograde signal. As a starting point to investigate the role of *syt 4* in the CNS, we conducted learning and memory assays on mutant animals to assess whether Syt 4 may be involved in associative conditioning. $Syt4^{BA1}$ and $Syt4^{PRE1}$ control animals were grown at 25°C and trained using the QHB learning assay (Quinn et al., 1974). Both populations were tested for odor learning, measured immediately after training, and odor memory, measured 60 minutes post-training. Immediately after training, PRE1 control animals obtained an average learning score of 0.34, consistent with scores previously reported for wild-type flies (Waddell et al., 2000) (Fig 4-13). The *syt 4* null animals obtained an average learning score of 0.30, slightly below that measured for PRE1 but statistically insignificant as measured by a Student's paired t-test. Similar results were obtained when we looked at 60 minute memory. Both PRE1 and BA1 scored statistically similar when odor association was tested one hour after training (Fig 4-13). These results suggest *syt 4* mutants do not have obvious defects in learning or short-term memory as measured by this assay. However, in order to more systematically investigate the role of *syt 4* in learning and memory, experiments using other learning paradigms, such as courtship learning and long-term memory assays, should be conducted. *Syt 4* knock-out mice perform well in certain conditioning tests, such as cued fear conditioning, but score poorly in contextual fear conditioning assays (Ferguson et al., 2000). These studies hint that the requirement for *syt 4* function in learning may be complex and that different neural networks responsible for different learned behaviors may depend on *syt 4* function to varying degrees.

Fig. 4-13

A


Repeat 3 X
Oct
+
shock
MCH
30 sec
rest
0 min
60 min
Oct
MCH
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shock
30 sec
rest
0 min
60 min

B

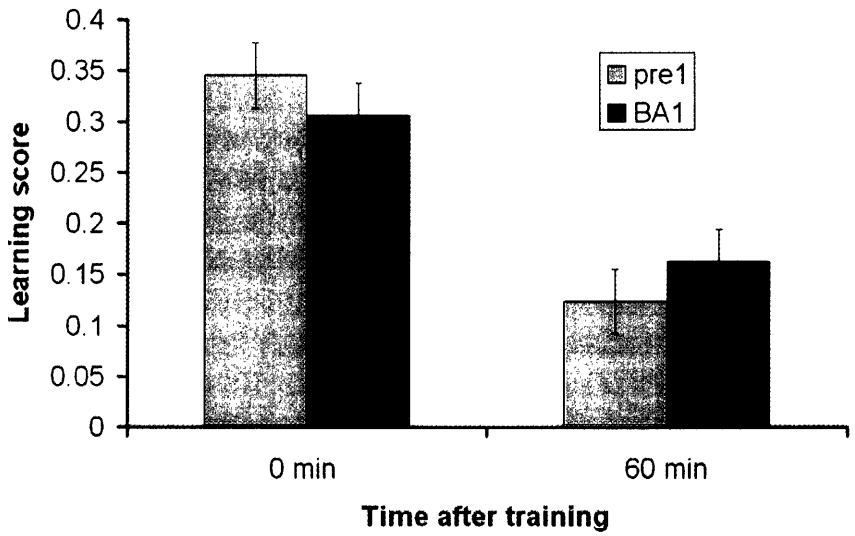

0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Learning score
pre1
BA1
0 min
60 min
Time after training

Fig 4-13. Odor conditioning is normal in *syt 4* null animals. (A) The training and testing protocol used was as previously described in (Quinn et al., 1974). (B) The average learning scores for each genotype determined immediately and 60 minutes post-training. Eight learning assays (N = 8) were averaged together for the 0 minute scores and five learning assays (N = 5) were performed for the 60 minute scores. No statistical difference was observed between the BA1 and PRE1 populations at 0 or 60 min post-training as measured by a Student's paired t-test. Error bars are SEM.

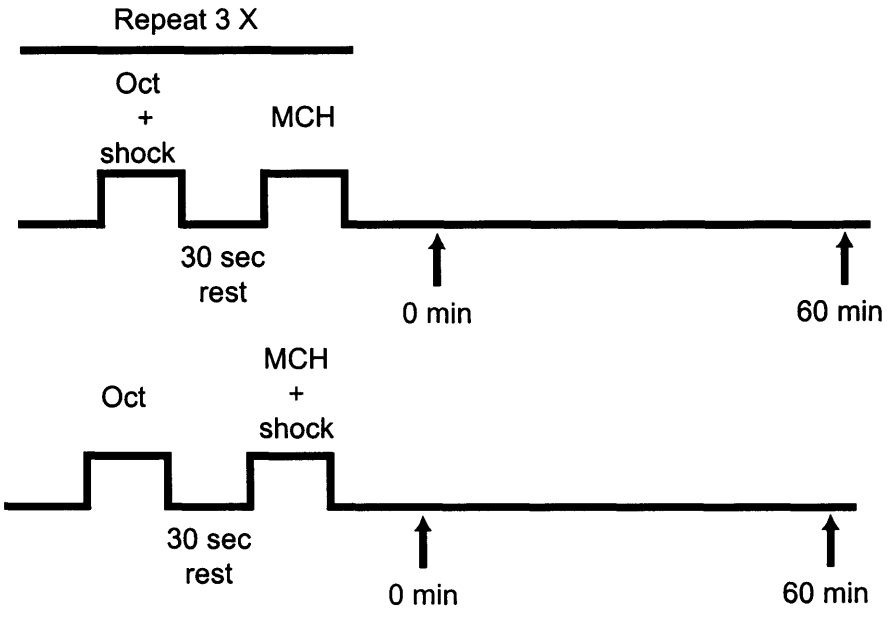
Synaptotagmin α mutants have defects in short term memory but not learning

The localization of synaptotagmin α (Syt α) to the mushroom bodies, neural structures required for olfactory conditioning in several insect species (), prompted us to investigate the role of Syt α in *Drosophila* learning and memory. Syt α null mutants (JZ1) and precise excision control animals (S44) were tested for learning and short-term memory defects using the QHB assay (Quinn et al., 1974). When tested immediately after training, both S44 and JZ1 flies learn to similar degrees (Fig 4-14), suggesting Syt α is not required for initial learning. However, when we tested the S44 and JZ1 populations one hour after training, we observed a significant decrease in the performance of the *syt α* null flies as compared to S44 controls (Fig 4-14), indicating Syt α is required for short-term memory. Interestingly, the performance observed in *syt α* null animals using the QHB assay phenocopies the defects previously reported for amnesiac mutants (Waddell et al., 2000). One intriguing hypothesis is that Syt α may control the secretion of the amnesiac neuropeptide from dense-core vesicles in dorsal paired medial (DPM) neurons. Future rescue experiments using the *syt α* transgene and a DPM-specific Gal4 driver will allow us to test if *syt α* is required in the DPMs for short-term memory. Additionally, subcellular gradient and tissue colocalization studies using the Syt α and amnesiac antibodies will allow us to determine if these two proteins reside on the same subcellular compartment, namely dense-core vesicles.

DISCUSSION

Fig. 4-14

A


Repeat 3 X
Oct
+
shock
MCH
30 sec
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0 min
60 min
Oct
MCH
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shock
30 sec
rest
0 min
60 min

B

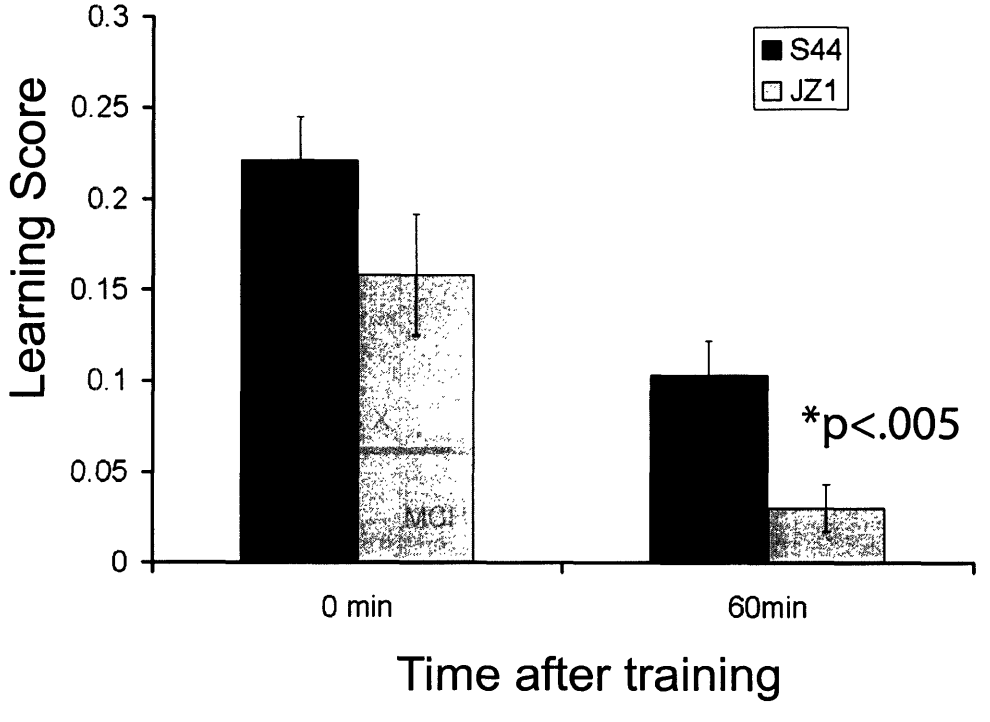
Learning Score
0.3
0.25
0.2
0.15
0.1
0.05
0
S44
JZ1
*p<.005
0 min
60min
Time after training

Fig 4-14. Synaptotagmin α is required for short-term memory, but not learning. (A) The training and testing protocol used was as previously described in (Quinn et al., 1974). (B) The average learning scores for each genotype were determined immediately and 60 minutes post-training. Twelve learning assays (N =12) were averaged together for both the 0 and 60 minute scores. No statistical difference was observed between S44 and JZ1 when populations were tested immediately after training. However, a significant decrease in 60 minute memory was detected in JZ1 animals compared to the S44 controls. * indicates $P < .005$ using a Student's paired t-test. Error bars are SEM.

There is little debate that Syt 1 is required for fast, synchronous neurotransmitter release at synapses. However it is unknown whether other synaptotagmin isoforms also participate in synaptic vesicle exocytosis, potentially as the asynchronous calcium sensor. Syt 4 and Syt 7 are additional synaptotagmin isoforms highly expressed throughout the nervous system (Adolfsen et al., 2004; Li et al., 1995b) and speculated to regulate synaptic vesicle fusion *in vivo*. Syt 7 localizes to the plasma membrane of PC12 cells when overexpressed , while endogenous Syt 7 has been reported to be concentrated at presynaptic active zones of central synapses, where it is hypothesized to act as a plasma membrane calcium sensor in synaptic vesicle exocytosis (Sugita et al., 2001). Recent biochemical studies have also speculated that the relatively slow dissociation of calcium from the Syt 7 C2 domains could account for why the calcium-dependent asynchronous phase of release is still observed following collapse of the presynatic calcium transient (Hui et al., 2005). In addition to its role as a calcium sensor, different splice variants of Syt 7 differentially target synaptic vesicles to fast or slow recycling pathways when transfected into neurons (Virmani et al., 2003). Similar to Syt 7, several studies have postulated that Syt 4 may function during synaptic vesicle exocytosis. Overexpression of *syt 4* in *Drosophila* motor neurons decreases EJP amplitudes, suggesting Syt 4 is a presynaptic inhibitor of neurotransmitter release (Littleton et al., 1999). Upregulation of *syt 4* in PC12 cells alters fusion pore dynamics (Wang et al., 2001) and has been reported to influence the choice between 'kiss and run' and full fusion (Wang et al., 2003).

Although several functional studies implicate Syt 4 and Syt 7 in synaptic vesicle exocytosis, no studies have investigated their loss-of-function synaptic phenotypes. To more rigorously investigate the role of Syt 4 and Syt 7 in neurotransmitter release, we

generated loss-of-function mutants in *Drosophila* and characterized their synaptic physiology at the neuromuscular junction. Surprisingly, neither null mutations in *syt 4* nor hypomorphic 'knock-down' of *syt 7* in neurons alters basic synaptic transmission at the *Drosophila* NMJ. Furthermore, paired-pulse facilitation is unaltered in *syt 4* and *syt 7* mutants, suggesting that neither of these isoforms is likely to encode the asynchronous calcium sensor. These results are consistent with our earlier *in vivo* localization studies, which indicate that Syt 4 localizes postsynaptically and Syt 7 fails to localize to NMJ synapses in *Drosophila*. If neither Syt 4 nor Syt 7 encodes the asynchronous calcium sensor, it becomes difficult to reconcile how any of the remaining *Drosophila* synaptotagmin isoforms could supply this function. Syts α and β are exclusively expressed in subsets of putative neurosecretory cells and are not expressed in motor neurons where asynchronous release has been measured (Adolfsen et al., 2004). The final two synaptotagmin isoforms encoded in the *Drosophila* genome, Syt 12 and Syt 14, only have 2 and 3 out of the 10 calcium-coordinating aspartic acid residues within their C2 domains, indicating these isoforms most likely function in calcium-independent trafficking pathways. Therefore, our genetic studies in *Drosophila* argue against the idea that another synaptotagmin isoform encodes the asynchronous calcium sensor, making a simplistic 'two synaptotagmin' calcium sensor model of neurotransmitter release unlikely. Without identification of the asynchronous calcium sensor, the exact nature of the calcium sensor(s) responsible for synaptic vesicle fusion remains unsolved.

If neither Syt 4 nor Syt 7 participates in synaptic vesicle exocytosis *in vivo*, what are their endogenous functions? We have addressed this question with respect to Syt 4, utilizing physiological recordings at the embryonic NMJ. High frequency stimulation of

the embryonic NMJ dramatically increases the frequency of miniature EJCs (minis), triggering a form of acute plasticity we have termed high frequency-induced miniature release (HFMR). Chelating postsynaptic calcium reduces HFMR to below detection levels, suggesting the large increase in presynaptic mini frequency depends on postsynaptic calcium levels. Additionally, *syt 4* null mutants completely lack the HFMR response, while postsynaptic, but not presynaptic, expression of a *syt 4* transgene can rescue HFMR at *syt 4* null NMJs. These experiments indicate Syt 4 may regulate the calcium-dependent exocytosis of retrograde signals from the muscle which cause dramatic changes in presynaptic release properties. Indeed, postsynaptic Ca^{2+}-dependent vesicle fusion has been identified at mammalian hippocampal synapses and is required for synaptic potentiation (Lledo et al., 1998; Maletic-Savatic and Malinow, 1998). Our data suggest the Syt 4 family (which includes two isoforms in mammals, Syt 4 and Syt 11) functions as postsynaptic Ca^{2+} sensors for retrograde signaling. Candidate retrograde signals include neurotransmitters, neuropeptides, cell adhesion proteins, or classical neuromodulators like catecholamines. We also considered the possibility that Syt 4-containing postsynaptic vesicles may transport synaptic glutamate receptors (Malinow et al., 2000). However, GluR immunoreactivity does not colocalize with Syt 4 immunopositive postsynaptic vesicles (Adolfsen et al., 2004) and the size of miniature EJCs is normal in *syt 4* mutants (data not shown), suggesting that trafficking of glutamate receptors by Syt 4 is unlikely at the *Drosophila* NMJ. We have provided numerous lines of evidence that Syt 4 functions within the postsynaptic compartment. First, the majority of Syt 4-specific immunostaining resides within the muscle, rather than the presynaptic bouton, when motor terminals are stained with a Syt 4 antibody (Chapter 2). Second,

only postsynaptic expression of *syt 4* can rescue the HFMR defects measured in *syt 4* null mutants (Chapter 4) and, finally, pHluorin-Syt 4 fusion proteins are efficiently trafficked to microdomains of the postsynaptic plasma membrane of larval NMJ boutons, when expressed using the muscle-specific Gal4 driver, MHC-Gal4 (Chapter 4). Future genetic and physiological experiments on several candidate molecules will be informative in identifying the nature of the HFMR retrograde signal.

Apart from Syt 1 and Syt 4, we postulate Syt α functions in an additional vesicle trafficking pathway in neurons, perhaps dense-core vesicle exocytosis. Syt α is expressed in several subsets of neurons within the *Drosophila* CNS, including those that profusely innervate the mushroom bodies, and localizes to neuropeptide-releasing cells in the periphery (Chapter 2). Syt α null mutants are viable and have specific defects in short term memory, but not learning. One intriguing possibility is that Syt α may regulate the exocytosis of the memory neuropeptide, *amnesiac*, since *syt α* null mutants phenocopy *amnesiac* loss-of-function mutations. Further rescue experiments using the *syt α* transgene and several sets of neuronal Gal4 drivers will allow us to determine where *syt α* is required for normal memory retention. If the *amnesiac* hypothesis is correct, we expect that expression of the *syt α* transgene with dorsal paired medial neuron (DPM)-specific Gal4 drivers should rescue the memory defect in *syt α* null animals, as amnesiac was previously shown to function within these neurons (Waddell et al., 2000).

In summary, molecular genetic characterization of the *Drosophila* synaptotagmin family has provided a number of interesting insights into basic synaptotagmin biology and provided a solid foundation for exploring how different regulated exocytosis pathways function within the nervous system. These studies suggest synaptotagmins

function in separate subcellular trafficking pathways, with Syt 1 being the only isoform involved in synaptic vesicle fusion. Furthermore, they imply it is unlikely that another synaptotagmin isoform is responsible for the residual calcium-sensing that occurs at Syt 1-deficient synapses. We have provided compelling evidence that Syt 4 functions in a postsynaptic trafficking pathway important in the release of unknown retrograde signals that can modulate presynaptic release properties, and Syt α is required for olfactory memory, possibly by regulating the secretion of the *amnesiac* neuropeptide from dense-core vesicles. The functions of the remaining *Drosophila* synaptotagmin isoforms are still uncertain, but future genetic studies in both vertebrate and invertebrate model systems should continue to uncover the diversity of regulated exocytosis pathways within neurons.

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