SNARES PARTNER WITH SEC1/MUNC18 PROTEINS TO RELEASE NEUROTRANSMITTER AT SYNAPSES

by

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ABSTRACT

Lipid membranes organize eukaryotic cells into functional compartments called organelles. Material is delivered to and from organelles in a regulated fashion. Vesicles bud from a source compartment, move across the cell and fuse with a target membrane. SNARE proteins, with Sec1/Munc18 (SM) proteins, drive the fusion of vesicles with their target by bridging the apposing membranes and forcing them together. The SNARE/SM fusion complex is essential for all vesicle fusion. Each trafficking pathway utilizes a different set of SNARE/SM family members.

In the nervous system the secretory pathway is responsible for the release of neurotransmitters, which pass signals between neurons. The neuronal SNAREs include synaptobrevin, syntaxin, and SNAP-25. However, it is not clear that these are the only SNAREs responsible for neurotransmitter release. In fact countless studies have reported residual neurotransmission in the absence of each of these proteins, raising the question what is the mechanism responsible for residual fusion in neuronal SNARE knockouts?

In Chapter 2, I explore this question by focusing on the neuronal SNARE SNAP-25. We characterize the *snap-25* genetic locus in *C. elegans* and examine the physiology of neurons lacking the SNAP-25 protein. We find that SNAP-25 plays an important role in docking and fusing synaptic vesicles but is not strictly essential for either one. We reveal that the conserved SNARE protein, SNAP-29 is capable of substituting for SNAP-

25 in synaptic vesicle fusion. We demonstrate that the SNAP-29 protein is natively expressed in neurons and localized at synapses. Our observations suggest that the canonical neuronal SNAREs may not act alone in releasing neurotransmitters.

Finally, I explore the mechanism by which the neuronal SM protein (Unc18) facilitates fusion. Unc18 binds SNAREs in three configurations. A binary complex with syntaxin is important for trafficking. At nerve terminals, UNC-18 interacts with an N-terminal peptide on syntaxin and with the SNARE four-helix bundle. Our experiments demonstrate that the N-peptide of syntaxin is a passive tether facilitating Unc18's transition from the binary syntaxin interaction to a direct interaction with the ternary SNARE complex. Future work is required to elucidate the fusogenic properties of Unc18's interaction with the ternary complex.

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CHAPTER 1

INTRODUCTION

Membrane organization requires fusion

Lipid membranes form the outer barrier of eukaryotic cells and divide their contents into compartments called organelles. Each organelle contributes specialized functions to cell viability. The endoplasmic reticulum is a protein factory, the mitochondria a power plant, and the lysosome a recycling center. These functions are interdependent and require material to be shipped in and out of organelles. Lipid bound vesicles do most of the shipping in a cell. They transport cargo by budding from donor compartments and fusing with acceptor membranes. This dissertation explores the molecular nature of vesicle fusion at synapses.

For lipid membranes to function as effective barriers, it is imperative that they resist spontaneous fusion with inappropriate compartments. Indeed, cellular membranes intrinsically repel one another, and protein machinery is used to overcome this resistance. Prior to membrane contact (>2nm), negatively charged phospholipids cause electrostatic repulsion of apposing membranes. At this distance, steric clash of membrane proteins not involved in fusion also deters membrane approach (Chernomordik and Kozlov, 2003; Cohen and Melikyan, 2004). At closer distances (<2 nm), immediately prior to contact, membranes experience a host of repulsive forces collectively termed “hydration forces,”

which resist dehydration of the fluid/lipid interface (Leikin et al., 1993). Specialized fusion proteins provide force to draw apposing membranes together and deform lipids to decrease the contact surface area and lessen “hydration forces” (Chernomordik and Kozlov, 2003; Cohen and Melikyan, 2004).

Proteins mediate the fusion of many different compartments in eukaryotic cells. Vesicle fusion is driven by SNARE proteins, which bridge the two membranes and mechanically induce fusion by conformational change (Broadie et al., 1995; Nickel et al., 1999; Weber et al., 1998). Vesicle trafficking throughout a cell maintains the form and function of the endoplasmic reticulum, Golgi, and lysosome, and thus SNARE proteins are the central players in most membrane dynamics in a cell (Figure 1.1). However, some organelle maintenance occurs by SNARE-independent fusion. In these reactions, alternative players span apposed membranes and provide kinetic energy by conformational change.

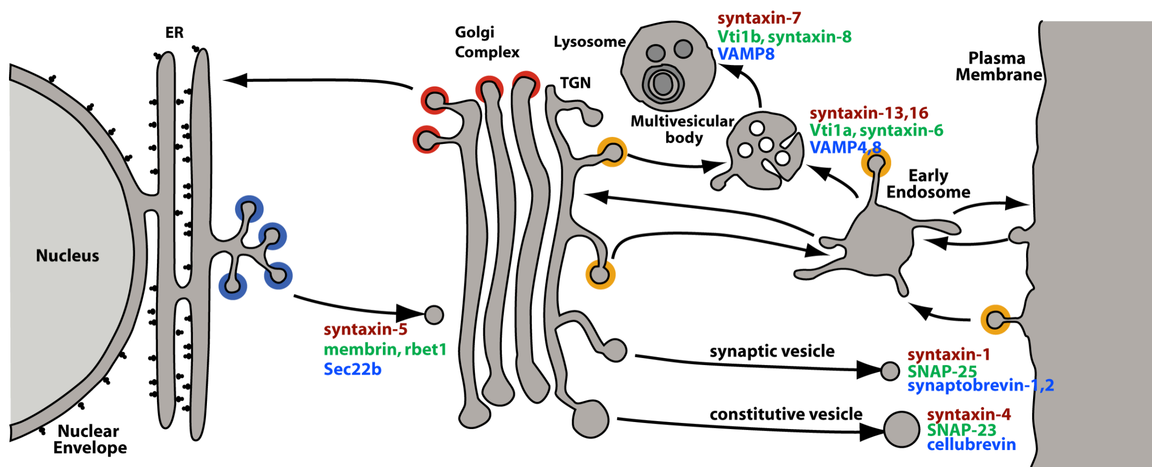

Figure 1.1 Vesicle trafficking is responsible for dynamic membrane remodeling throughout the cell’s endomembrane system. Different SNARE family members mediate vesicle fusion at each location in a cell. Synaptobrevin family members (blue) reside on vesicles and syntaxin (red) and SNAP-25 (green) family members are anchored to target membranes (Adapted from Jahn et al., 2003 and Bonifacino and Glick, 2004).

Most SNARE-independent fusion is driven by membrane spanning GTPases. In mitochondria, inner and outer envelope fusion is mediated by the GTPase Fzo1 (Hales and Fuller, 1997; Hermann et al., 1998; Rapaport et al., 1998). Similar molecules are localized to the outside surface of mitochondria called mitofusins. These molecules bridge the cytosol to execute homotypic fusion via antiparallel coiled coils (Koshiba et al., 2004). Homotypic fusion also occurs in the endoplasmic reticulum, and like the mitochondria relies on a GTPase (atlastin) for forcing membranes together (Hu et al., 2009; Orso et al., 2009).

The task of fusion is a particularly interesting challenge in the case of viral infection of a host cell. This is the only case where the proteins begin on only one side of apposing membranes. Here, the viral fusion proteins are equipped with highly hydrophobic peptides called "fusion peptides," which penetrate the membrane of the host cell. Once both membranes are firmly anchored, the viral fusion machinery undergoes a dramatic conformational change driving the membranes together (Eckert and Kim, 2001). Thus, all known fusion reactions require membrane spanning protein complexes and exothermic conformational changes to overcome the repulsion of membranes.

SNAREs mediate vesicle fusion

Molecular exchange between organelles and with the extracellular milieu relies on transportation by carrier vesicles. These small (30 nm) membrane bound spheres were initially observed in the first electron micrographs of neurons (De Robertis and Bennett, 1954; De Robertis, 1955; Palay, 1954). At the same time, electrophysiological observations of spontaneous endplate potentials at the frog NMJ (neuromuscular

junction) determined that bio-active molecules are released in quantal “packets” (Fatt and Katz, 1952). This work revolutionized our understanding of how a cell secretes neurotransmitters. We now recognize that similar “packets” are responsible for trafficking processes throughout cells. Vesicle biogenesis occurs at the Golgi apparatus. Vesicles divide and mature in order to be delivered to their correct target membrane where fusion ultimately releases their cargo. Vesicles are responsible for modulating receptor residence on the cell surface and secretion of endocrine and exocrine hormones, as well as releasing small molecules such as neurotransmitters.

Much of our understanding of cargo trafficking by vesicles comes from seminal work on the cellular secretion pathway. Two investigators approached this problem in very different ways. Randy Schekman used a forward genetic screen to isolate yeast cells defective in secretion, identifying 23 genes critical for vesicle processing and fusion (Novick et al., 1980). Meanwhile James Rothman’s group developed a cell-free fusion assay, which was inhibited by the compound NEM (N-ethylmaleimide) (Balch et al., 1984; Block et al., 1988). Through careful experimentation they identified NSF (NEM sensitive factor) as the target of NEM (Wilson et al., 1989). This protein in concert with SNAP (Soluble NSF attachment protein), proved important for unwinding and activating a four-protein complex involved in fusion (Söllner et al., 1993b; Mayer et al., 1996; Nichols et al., 1997). The discovery that SNAP binds SNARE proteins gave them their name (SNAP attachment protein receptors) (Söllner et al., 1993a). Through very different approaches, Schekman and Rothman identified SNARE proteins and important accessory factors required for vesicle fusion. Schekman’s work is only a single example of the many studies that identified vesicle trafficking genes by forward genetics. Together, the

combination of genetics and biochemistry has proven a powerful partnership in elucidating the molecules and mechanisms responsible for SNARE mediated fusion.

SNAREs make up a large conserved family of membrane-associated proteins. They are composed exclusively of alpha-helical segments, each protein with a 60–70 amino acid amphipathic helix called the "SNARE motif." SNARE motifs are unstructured in solution, but readily assemble into a parallel four helix coiled-coil with other SNAREs (Fasshauer et al., 1997; Sutton et al., 1998). SNARE proteins are membrane associated. With only a few exceptions, a single SNARE family protein resides on the vesicle and is called the "v-SNARE." The other three SNARE motifs reside on the target membrane and are called "t-SNAREs" (Fasshauer et al., 1998; Kloepper et al., 2007). SNARE motifs zipper together to form a parallel four-helix bundle called the "core complex" (Figure 1.2). The membrane distal N-termini nucleate the complex and wind down towards the C-termini, drawing the apposed membranes together. Following fusion, disassembly is achieved by enzymatic melting via the cofactors NSF and SNAP proteins (Mayer et al., 1996; Nichols et al., 1997).

The SNARE core complex is highly stable and requires boiling with sodium dodecyl sulfate for disassembly (Fasshauer et al., 2002; Hayashi et al., 1994). The stability of SNAREs can be attributed to strong hydrophobic interactions between "layer residues" that run the length of the core complex. One exception occurs halfway down the length of every SNARE motif. Here, invariant charged residues form an ionic interaction in the center of the core complex that has been termed the "0 layer" (Fasshauer et al., 1998). The conservation of the 0 layer residues provides an effective evolutionary categorization for classifying the relatedness of SNAREs in evolution

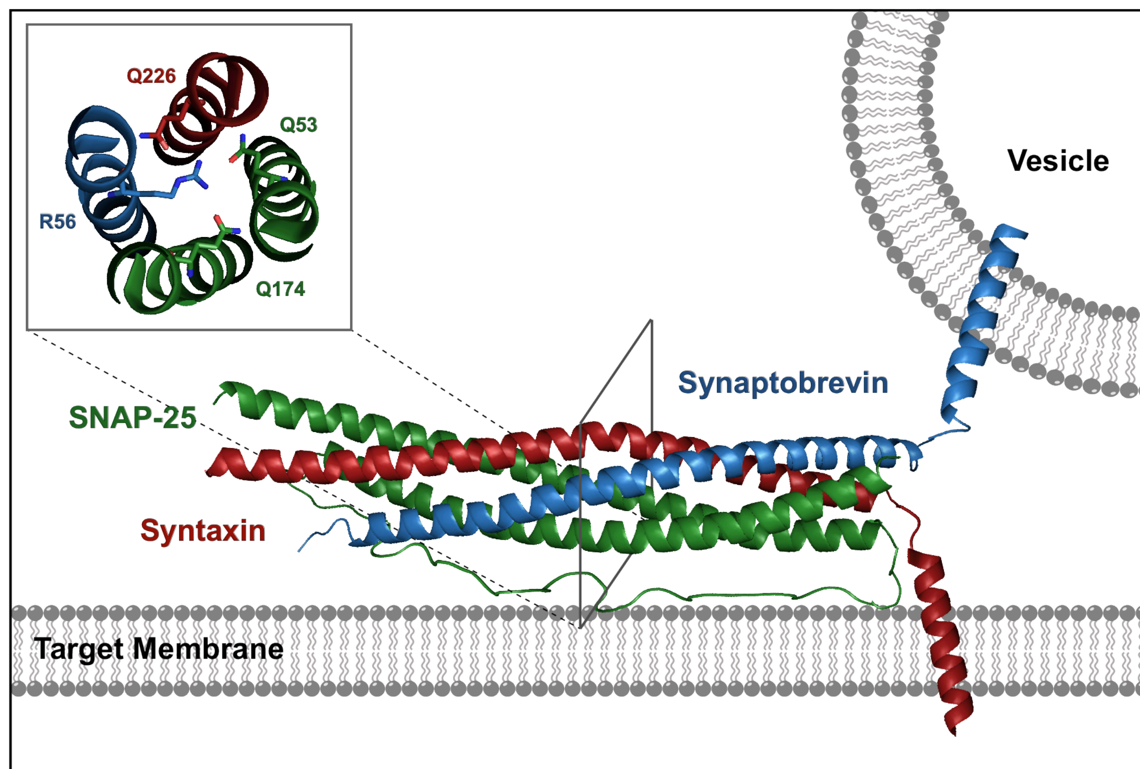

Figure 1.2 The SNARE complex forms a parallel four-helix bundle to bridge the vesicle and target membranes. Synaptobrevin (blue) is anchored to the vesicle by a transmembrane domain. Syntaxin (red) is inserted into the plasma membrane by a transmembrane domain. SNAP-25 (green) is associated with the target membrane by palmitoylation and contributes two alpha-helices to the complex. Inset displays the four residues that define the 0 layer.

(Kloepper et al., 2007). Three of the SNARE motifs contain glutamine residues and are referred to as Qa-, Qb-, and Qc-SNAREs. The fourth SNARE motif (the R-SNARE) has an arginine at the zero-layer, which interacts with all three glutamines (Fasshauer et al., 1998). Despite the conservation, the functional role of the zero-layer remains mysterious. It may keep SNAREs in register or facilitate SNARE disassembly following fusion, but these models have not been supported *in vivo*. (Fasshauer et al., 1998; Hanson et al., 1997; Lauer et al., 2006; Scales et al., 2001).

In summary, SNAREs are believed to be central to all vesicle fusion reactions in cellular trafficking. Four parallel SNARE motifs zipper together, nucleated at their N-termini, to bridge apposing membranes and catalyze fusion.

Neuronal secretion is fast and regulated

The secretion of neurotransmitter at nerve terminals is the most tightly regulated fusion reaction known. At synaptic junctions, a presynaptic depolarization opens voltage-gated calcium channels allowing for small bursts of elevated intracellular calcium. Synaptotagmin binds Ca^{2+} and triggers the full zippering of prenucleated SNARE complexes. Vesicle contents are released, and neurotransmitters diffuse a short distance across the synaptic cleft. Neurotransmitters bind postsynaptic receptors triggering a new electrical signal. Synapses stall fusion in preparation for coordinated transmitter release, which can occur in under a millisecond (Bruns and Jahn, 1995; Schikorski and Stevens, 2001). This regulation involves accessory factors that modify SNARE-mediated fusion.

Three neuronal SNARE proteins are highly conserved across metazoans. The R-SNARE, synaptobrevin or VAMP (Vesicle Associated Membrane Protein), resides on the vesicle membrane, and the Q-SNAREs, syntaxin (Qa) and SNAP-25 (Qbc) are associated with the plasma membrane. SNAP-25 is a particular focus of this dissertation, and I will give it special attention throughout. SNAP-25 is a 206-amino acid 25-kD protein. It is unique among the other SNAREs as it contributes two 70-amino acid SNARE motifs joined by a long flexible linker. SNAP-25 was first identified by Oyler and colleagues where they found SNAP-25 mRNA enriched at presynaptic terminals in the hippocampus (Oyler et al., 1989). Subsequent studies reported that SNAP-25 is expressed in

neuroendocrine cells (Jacobsson et al., 1994) and motor neurons (Jacobsson et al., 1996). SNAP-25 is anchored to the plasma membrane by palmitoylation of a “cysteine quartet” (Gonzalo and Linder, 1998; Gonzalo et al., 1999; Hess et al., 1992; Lane and Liu, 1997). The linker motif immediately adjacent to the palmitoylation residues is thought to be a critical advancement toward fast calcium-evoked transmission (Nagy et al., 2008). Synaptic vesicles proceed through three ordered stages in the release of neurotransmitter into the synaptic cleft (Figure 1.3). (1) Docking is defined ultrastructurally and includes all vesicles contacting the plasma membrane (Hammarlund et al., 2007; Schikorski and Stevens, 2001). (2) Maturation indicates fusion competence. This stage involves vesicle priming, which is defined molecularly as the initial N-terminal nucleation of SNARE proteins (Südhof, 1995). Functionally speaking, “fusion competent” vesicles occupy the RRP (readily releasable pool) (Rosenmund and Stevens, 1996). (3) Fusion occurs in response to calcium influx and is believed to require the previous two steps.

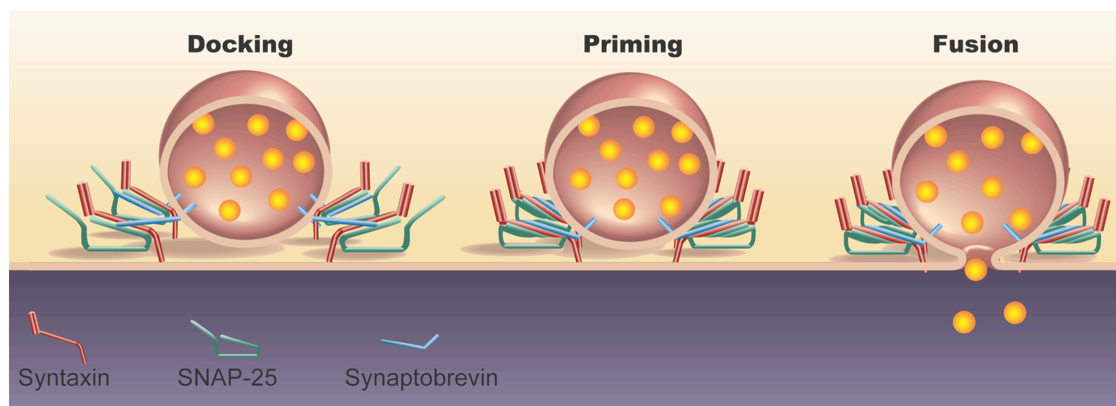

Figure 1.3 Synaptic vesicles proceed through three stages to release neurotransmitter. Vesicle docking is defined by contact between the vesicle and target membrane and likely requires all three SNARE proteins. Priming describes a state in which vesicles are prepared for immediate fusion upon stimulation. In this state, SNAREs zippering has begun, but is not allowed to wind to completion. Fusion occurs when SNAREs zipper completely.

Docking

The original SNARE hypothesis proposed that SNAREs confer localization by docking vesicles at the membrane (Rothman, 1994; Söllner et al., 1993a). This was later dismissed when ablation of syntaxin and synaptobrevin by genetics and clostridial toxins showed no defect in vesicle docking (Broadie et al., 1995; Hunt et al., 1994; Marsal et al., 1997; O'Connor et al., 1997). Instead, these experiments suggested that SNAREs are necessary for the downstream stages of priming and fusion. However, advances in electron microscopy and sample preparation have allowed more stringent criteria for defining docking (Hammarlund et al., 2007). Furthermore, new approaches to identifying "docked" vesicles including a cell-free biochemical assay (Chieregatti, 2004; Chieregatti et al., 2002), TIRF (total internal reflection microscopy) (Wu et al., 2012), and cell-free docking of unilamellar vesicles (Parisotto et al., 2012) have contributed to our perspectives on docking. Unfortunately, the field is still divided as to which molecular players constitute the functional docking machinery.

The question of molecular docking can be divided into two questions: (1) what vesicle molecule(s) are required for docking? and (2) what plasma membrane molecule(s) are required for docking? The evidence from *C. elegans* suggests that synaptobrevin on the vesicle is required for docking, and syntaxin on the plasma membrane is required for docking (Hammarlund et al., 2007, 2008; Palfreyman, 2009). These observations support the original model that SNAREs dock synaptic vesicles. TIRF microscopy on cultured PC12 cells lends further support to this model by demonstrating that all three SNARE proteins are required, and docking appears to rely on trans-SNARE pairing by the traditional zippering model (Wu et al., 2012).

However, other groups report that the vesicle anchor for docking is synaptotagmin. Dense core granules and cell-free unilamellar vesicles appear to dock via t-SNAREs and synaptotagmin. Synaptobrevin is not required, and thus SNARE zippering occurs downstream of docking (De Wit et al., 2009; Mohrmann et al., 2013; Parisotto et al., 2012). This docking appears to require Munc18, which could serve as a membrane anchor on the plasma membrane. A bimolecular syntaxin/Munc18 complex may serve this function, which would suggest that SNAP-25 is dispensable for docking (Verhage and Sørensen, 2008). Alternatively, SNAP-25 may interact directly with rabphilin or synaptotagmin to dock synaptic vesicles (De Wit et al., 2009; Mohrmann et al., 2013; Parisotto et al., 2012; Tsuboi and Fukuda, 2005).

Maturation

Vesicles must pass through a maturation step to become fusion competent. Two parallel lines of research have approached this phenomenon by molecular and functional criteria independently. The definition of vesicle priming arose from a series of experiments in endocrine cells that specifically disrupted SNARE interactions in a manner that assigned N- and C-terminal interactions to SNARE nucleation and fusion respectively (Chen et al., 2001b; Hay and Martin, 1992; Melia et al., 2002; Xu et al., 1999a). The term priming in the strictest sense describes this molecular interaction (Südhof, 1995); however, it is loosely used in the literature to describe fusion competence.

As early as 1961, electrophysiologists recognized the heterogeneity of vesicle release events and referred to different populations of synaptic vesicles as representing

different “pools” (Birks and MacIntosh, 1961). The RRP represents those that are immediately available for fusion by hypertonic conditions, high-frequency electrical stimulation or caged calcium release (Rosenmund and Stevens, 1996; Schneggenburger et al., 1999). Whether this pool represents docked vesicles (Rizzoli and Betz, 2005) or primed vesicles (Becherer and Rettig, 2006) is uncertain and may depend on the model system or even the specific synapse studied.

Fusion

Under the zippering model for fusion, primed SNAREs are arrested in the nucleated form, stalling fusion until initiated by a trigger (Chen et al., 2001a; Fasshauer and Margittai, 2004; Fiebig et al., 1999; Hanson et al., 1997; Hua and Charlton, 1999; Lin and Scheller, 1997; Melia et al., 2002; Pobbati et al., 2006; Sørensen et al., 2006; Xu et al., 1999b). Complexin serves as a brake, blocking the fusion of primed vesicles and accumulating a release-ready reserve (Hobson et al., 2011; Ishizuka et al., 1995; Martin et al., 2011; McMahon et al., 1995; Pabst et al., 2002; Takahashi et al., 1995). UNC-13 localizes primed vesicles near calcium channels and increases Ca^{2+} sensitivity via calmodulin (Hu et al., 2013). Synaptotagmin is believed to be the Ca^{2+} sensor and therefore the trigger for fusion (Fernandez-Chacon et al., 2001; Geppert et al., 1994). However, the precise mechanism by which synaptotagmin initiates fusion is mysterious. It is anchored to synaptic vesicles via a transmembrane domain and contains two C2 Ca^{2+} binding domains. Synaptotagmin’s role as a fusion trigger appears to involve Ca^{2+} dependent interaction with the SNARE proteins and penetration of the synaptic plasma membrane (Bai et al., 2002; Chapman et al., 1995; Fernandez-Chacon et al., 2001;

Gerona et al., 2000; Herrick et al., 2006; Lynch et al., 2007; Rhee et al., 2005). Upon triggering, SNAREs completely zipper to the C-termini, executing fusion upon calcium instruction.

Synaptic vesicle fusion events are classified by the nature of the fusion stimulus (Neher and Sakaba, 2008). At rest, in the absence of a trigger, vesicle fusion is referred to as “spontaneous.” When a depolarization induces Ca^{2+} influx into the cell, fusion events are fast and “synchronous.” Following synchronous fusion, elevated release probability persists as a result of a slower mechanism for Ca^{2+} evoked release—termed “asynchronous release.” Synchronous and asynchronous fusions are considered “evoked” events and require Ca^{2+}. Finally, some synapses are modulated by graded membrane potentials resulting in more gradual Ca^{2+} dynamics. These synapses are referred to as “tonic synapses” in contrast to “phasic” synapses, which respond with synchronized release (Atwood and Karunanithi, 2002; Millar et al., 2005). Tonic synapses include mossy fibers, retinal bipolar cells, and many invertebrate NMJs. SNARE proteins are required for all of these forms of fusion. However, each SNARE family member may interact differently with accessory proteins providing a molecular signature to different vesicle pools (Raingo et al., 2012; Ramirez and Kavalali, 2012; Ramirez et al., 2012). These results are discussed at length when we consider the different SNAREs and potential redundancy at synaptic terminals.

SNAREs: an addressing system for fusion?

The original “SNARE hypothesis” proposed that SNAREs serve as an addressing system for directing trafficking of vesicles to their appropriate destinations (Rothman,

1994). Under this model, each vesicle adorns v-SNAREs that only interact with a single set of cognate t-SNAREs on the appropriate target membrane. "Cognate" SNARE pairing describes the selective nature of SNAREs for a specific set of partners. The simplest test of this model is to survey SNARE proteins for interaction *in vitro*. The results from these experiments are inconsistent with the SNARE hypothesis. Co-immunoprecipitation experiments demonstrate that Golgi and plasma membrane t-SNAREs interact promiscuously with other v-SNAREs (Fasshauer et al., 1999; Tsui and Banfield, 2000). Analysis by circular dichroism spectroscopy suggests that multiple SNARE combinations form thermal-stable complexes with affinities close to those observed with cognate pairs (Scales et al., 2000; Yang et al., 1999). Finally, the ability of SNAREs to fuse artificial liposomes may provide the strictest *in vitro* criteria for specificity. Indeed, most SNARE pairs have proven to be selective by this assay (McNew et al., 2000; Parlati et al., 2002). However, SNAP-47 is capable of replacing SNAP-25 in fusing proteoliposomes (Holt et al., 2006).

The most compelling evidence for SNARE promiscuity comes from studies of genetic null mutations in living organisms or tissues. Null analysis of neuronal SNARE genes rarely results in the complete abrogation of neurotransmission, suggesting that substitution by other SNARE orthologs is sufficient for fusion. The following discussion explores the evidence for redundancy with each of the three canonical neuronal SNAREs.

Synaptobrevin

In flies, synaptobrevin nulls lack evoked release, but some spontaneous fusion persists (Deitcher et al., 1998) In worms, synaptobrevin nulls arrest as larva but exhibit

some locomotion prior to termination (Nonet et al., 1998). Deletion of mouse synaptobrevin-2 reduces neurotransmission. Evoked release is decreased nearly 100-fold, but spontaneous and hypertonic release is only affected 10-fold (Schoch et al., 2001). This has been attributed to cellubrevin in chromaffin cells (Borisovska et al., 2005) suggesting that spontaneous and evoked fusion may be differentially regulated by the R-SNAREs for endocrine fusion. However, cellubrevin does not appear to contribute to synaptic fusion when assayed in hippocampal culture (Deák et al., 2006). Instead, it appears that the Qb SNARE Vti1a drives spontaneous fusion at central synapses (Ramirez et al., 2012), and VAMP-4 may be responsible for maintaining an asynchronous pool in neurons (Raingo et al., 2012).

Syntaxin

In flies and worms, syntaxin is strictly required for neurotransmitter release (Broadie et al., 1995; Hammarlund et al., 2007; Schulze et al., 1995). However, the syntaxin knockout mouse has only subtle defects in neurotransmission (Fujiwara et al., 2006). This is likely due to redundancy from Syntaxin 1B. Acute proteolysis of syntaxin by botulinum toxin reduces neurotransmission to approximately 10% in squid giant synapses (Marsal et al., 1997; O'Connor et al., 1997) and hippocampal culture (De Wit et al., 2006)

SNAP-25

Most agree that SNAP-25 is not strictly required for spontaneous release in mammals (Bronk et al., 2007; Delgado-Martinez et al., 2007; Tafoya et al., 2006;

Washbourne et al., 2002). In fact, Washbourne and colleagues reported higher rates of minis at the diaphragm NMJ, and Delgado and colleagues reason that SNAP-25 may only be required for evoked release. By most reports, SNAP-25 is strictly required for evoked fusion. However, one group observed Ca^{2+} evoked responses in *snap-25* null hippocampal neurons, suggesting that synchronous fusion may be possible in the absence of SNAP-25 (Bronk et al., 2007). Studying SNAP-25 in flies has been hampered by a very closely related SNAP-25 paralog unique to flies called SNAP-24. SNAP-24 almost completely replaces SNAP-25 function in neurons, making flies an unfavorable model for exploring SNAP-25 function (Niemeyer and Schwarz, 2000; Vilinsky et al., 2002).

In addition to SNAP-25, mammals express three related Qbc-SNAREs, SNAP-23, SNAP-29, and SNAP-47. Of these, SNAP-23 is the most carefully studied. Overexpressing SNAP-23 is sufficient to restore tonic fusion in SNAP-25 null neurons (Delgado-Martinez et al., 2007) and chromaffin cells (Sørensen et al., 2003). In both studies, SNAP-23 overexpression produced an electrophysiological phenotype that did not match that of *snap-25* null cells. These observations suggest that SNAP-23 is unlikely responsible for the residual fusion in *snap-25* null cells. The authors of these reports concluded that SNAP-29 or SNAP-47 may drive the residual neurotransmission at snap-25 null synapses, but these speculations have not been tested *in vivo*.

SNAP-29 at synaptic terminals

The Qbc-SNARE, SNAP-29, provides an interesting challenge to the specificity model for SNARE function. SNAP-29 is a close relative to SNAP-25 (32% identical in mammals), but is ubiquitously expressed in all tissues assayed in metazoans (including

brain, heart, kidney, liver, lung, pancreas, placenta, skeletal muscle, spleen, and testis). As a result, SNAP-29 was initially proposed to serve a vital role in cellular trafficking associated with the Golgi and late endosome (Steegmaier et al., 1998; Wong et al., 1999). SNAP-29 resembles SNAP-25 as it contains two SNARE motifs joined by a long unstructured linker. However, unlike SNAP-25, SNAP-29 lacks a membrane binding palmitoylation motif. SNAP-29 is believed to be a cytosolic protein that associates with membranes via protein interaction (Hohenstein and Roche, 2001; Steegmaier et al., 1998; Wong et al., 1999).

SNAP-29 appears to be a promiscuous SNARE interactor. SNAP-29 was first identified by its interaction with Syntaxin-3 in a yeast two-hybrid screen (Steegmaier et al., 1998). Steegmaier et al. further demonstrated that SNAP-29 interacts with many of the Qa family members by *in vitro* binding assays, including Syntaxin 1a, 3, 4, 7, 13, and 17. Others have shown that SNAP-29 binds to the Golgi Syntaxin 6 (Schardt et al., 2009; Wendler et al., 2001; Wong et al., 1999). This interaction is especially provocative; Syntaxin 6 is a Qc-SNARE and is more closely related to the SNAP-25 family than the syntaxin family of proteins. Therefore, the interaction of SNAP-29 with syntaxin 6 is not only promiscuous but homotypic. Furthermore, SNAP-29 forms high affinity ternary SNARE complexes with any combination of one of five R-SNAREs (VAMP 2, 4, 7, 8 or rSec22b) and three Qa-SNAREs (Syntaxin 1a, 4 or 13) (Yang et al., 1999).

The role of SNAP-29 in the nervous system is particularly unclear. SNAP-29 forms ternary complexes with the synaptic Qa-SNARE Syntaxin1A and Synaptobrevin 2 with higher thermal stability than any other SNAP-25 family member (Scales et al., 2000; Yang et al., 1999). SNAP-29 is expressed in central and peripheral neurons and localizes

at presynaptic terminals. Furthermore, SNAP-29 is enriched in synaptic vesicle purifications (Holt et al., 2006; Su et al., 2001). These observations all suggest that SNAP-29 may actively participate in SNARE mediated fusion of synaptic vesicles. Indeed, SNAP-29 can substitute for SNAP-25 in secretion of epinephrine from PC12 cell, although SNAP-23 was reported to be a more effective substitute (Scales et al., 2000).

SNAP-29 has also been implicated in a rather heretical function for a SNARE family member. SNAP-29 was shown to bind the outside of preformed neuronal SNARE complexes, stabilizing the ternary SNARE bundle by competing for the binding site of alpha-SNAP (Su et al., 2001). This group went on to demonstrate that SNAP-29 overexpression consistently decreases evoked release at cultured hippocampal synapses (Pan et al., 2005). However, this defect is relatively subtle. In conclusion, SNAP-29 is a promiscuous SNARE with controversial roles in mediating synaptic vesicle fusion.

UNC-18 is required for fusion

SNARE proteins are often considered the minimal machinery for fusion (Weber et al., 1998). However, all known SNARE interactions are accompanied by the Sec-1/Munc18 (SM) family of proteins, and SM proteins are required for vesicle fusion (Verhage et al., 2000). The mammalian SM proteins responsible for neurotransmission at synapses are known as Munc18, named after the *C. elegans* protein UNC-18 (Hosono et al., 1992). I will use the name Unc18 in reference to synaptic proteins of all species, and refer to the greater protein family as SM proteins. SM proteins have been implicated in the vesicle cycle at docking, priming, and fusion stages (Toonen and Verhage, 2007).

SM proteins are required for trafficking syntaxin. Mutants lacking SM proteins consistently show a two-fold depletion of syntaxin at the plasma membrane in yeast, invertebrates, and mammals (Bryant and James, 2001; Medine et al., 2007; Rowe et al., 2001; Voets et al., 2001; Weimer et al., 2003). Furthermore, multiple groups have demonstrated that Unc18 proteins are required for docking of synaptic vesicles and dense core vesicles (Gulyás-Kovács et al., 2007; Voets et al., 2001; Weimer et al., 2003). However, the docking defect may be an indirect effect via its binding partner syntaxin since recent evidence suggests that syntaxin is required for docking (Hammarlund et al., 2007; Wu et al., 2012). It is possible that Unc18 mutants are defective for docking due to a depletion of membrane bound syntaxin (Gerber et al., 2008; Verhage et al., 2000). Therefore, the role of Unc18 proteins in docking vesicles remains unresolved, but it likely serves a positive role in exocytosis downstream of docking.

Once vesicles move into position at the plasma membrane, syntaxin assumes the open conformation and allows SNARE proteins to form the SNARE complex (Chen et al., 1999, 2001a; Fiebig et al., 1999; Nicholson et al., 1998). It is believed that these “primed” vesicles represent the RRP, which can be measured using electrophysiological methods. Structural experiments with yeast and mammalian protein suggest that Unc18 stabilizes a SNAP-25/syntaxin “acceptor” complex facilitating SNARE priming (Burkhardt et al., 2008; Weninger et al., 2008). Furthermore, in mouse chromaffin cells, different Munc18 variants can rescue priming to different levels, independent of docking (Gulyás-Kovács et al., 2007). These results suggest that Unc18 may prime vesicles once they reach the plasma membrane. However, it is possible that Unc18 serves as an instrument for executing the fusion event itself. By analyzing the kinetics of individual

fusion events in cultured endocrine cells, Fisher *et al*. proposed that Munc18 contributes to the formation of a fusion pore (Fisher et al., 2001). Finally, in support for a role in fusion, pre-incubation of SNAREs with Unc18 increases the rate of fusion in liposome fusion assays (Shen et al., 2007).

Unc18's numerous functions in vesicle exocytosis have been attributed to its multiple SNARE binding modes. Of the Unc18/SNARE interactions, the most thoroughly studied is thought to inhibit SNARE assembly. Unc18 forms a high-affinity clamp on syntaxin, locking it in a “closed” (fusion incompetent) state (Dulubova et al., 1999; Misura et al., 2000). This interaction is necessary for trafficking syntaxin to the plasma membrane (McEwen and Kaplan, 2008; Medine et al., 2007). Once at the synaptic terminal, UNC-13 is required to overcome the closed state of syntaxin and permit SNARE assembly (Richmond et al., 2001).

The high-affinity interaction of Unc18 with syntaxin appears to be a recent evolutionary development, reserved only for neuronal SM proteins. However, two additional binding modes between syntaxin and Unc18 are conserved throughout all SM proteins (Toonen and Verhage, 2007). These interactions occur following the “opening” of syntaxin.

The first mode (the N-peptide interaction) involves the binding of the extreme N-terminus of syntaxin with a hydrophobic pocket in Unc18 (Bracher and Weissenhorn, 2002; Hu et al., 2007; Shen et al., 2007). The N-peptide of syntaxin is required for Unc18 to bind the assembled SNARE complex *in vitro* (Dulubova et al., 2007; Rickman et al., 2007; Shen et al., 2007). Ablation of the N-peptide interaction in transgenic human embryonic kidney cells disrupts secretion (Khvotchev et al., 2007). Furthermore,

introducing a point mutation in syntaxin's N-peptide eliminates Unc18's fusogenic influence on SNARE mediated liposome fusion (Shen et al., 2007). However, these observations do not explain the functional significance of the N-peptide interaction. Some models suggest that it sends an activation signal to Unc18; however, others propose the N-peptide simply tethers Unc18 to SNAREs. In the third chapter, we describe evidence that supports the latter.

In the final SNARE binding mode, Unc18 interacts with the partially assembled trans-SNARE complexes (Dulubova et al., 2007; Khvotchev et al., 2007; Rickman et al., 2007). The structural contacts of this interaction have not yet been determined. The functional significance of Unc-18's interaction with the SNARE complex can be best understood by looking to yeast SM proteins. The yeast SM orthologs do not bind closed syntaxin and only interact with syntaxin's N-peptide and the assembled core complex. These interactions promote fusion and occur downstream of docking (Bracher and Weissenhorn, 2002; Carr et al., 1999; Grote et al., 2000).

Taken together, we favor the following model (Figure 1.4). Unc18 binds closed-syntaxin for trafficking to the synapse. This interaction is supported by the N-peptide. When syntaxin opens to nucleate SNARE priming, the N-peptide holds Unc18 near the complex. Finally, Unc18 binds the trans-SNARE complex with support from the N-peptide to promote vesicle fusion.

Outline of the dissertation

The work presented herein explores two discrete problems that hamper our understanding of SNARE function and vesicles fusion: (1) it is unclear whether the Qbc-

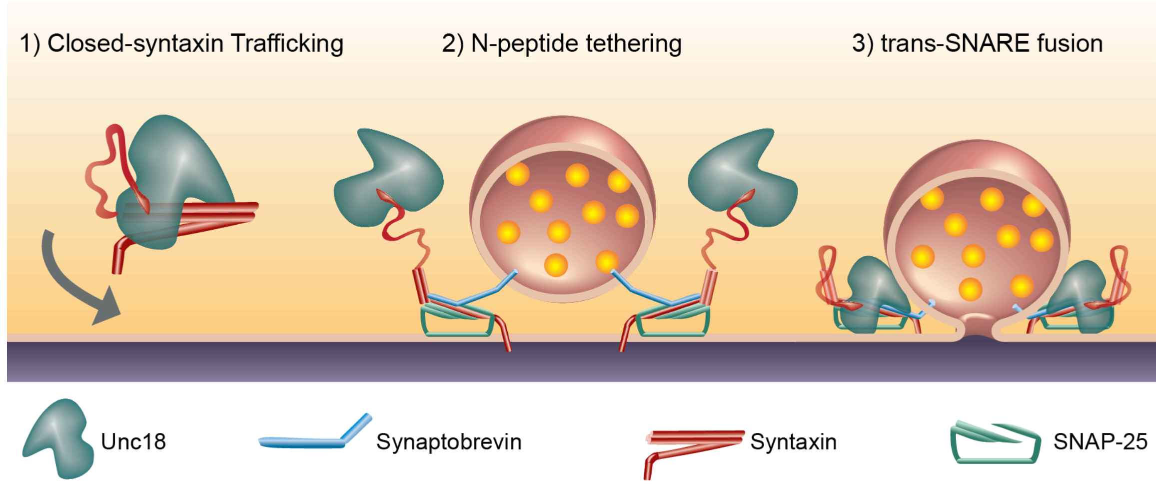

Figure 1.4 A molecular model for the function of Unc18/SNARE interactions. 1) Unc18 and syntaxin cochaperone one another in a binary complex supported by the closed interaction and N-peptide interaction. 2) The N-peptide holds Unc18 near the complex when syntaxin is open. 3) Unc18 binds trans-SNAREs to drive fusion. This interaction is supported by the N-peptide.

SNARE SNAP-25 is strictly required for docking and fusing synaptic vesicles, or if alternative mechanisms or proteins contribute to fusion, and (2) the N-peptide of Unc18 is important for vesicle fusion, but its functional mechanism has been mysterious.

In Chapter 2, I analyze neurosecretion in the absence of the Qbc-SNARE SNAP-25 in *C. elegans*. Our results suggest that SNAP-25 is required for normal docking of synaptic vesicles at the presynaptic plasma membrane and executing efficient fusion. However, significant levels of docking and fusion persist, implicating a SNAP-25 independent mechanism for secretion at the *C. elegans* NMJ. We demonstrate that these fusion reactions require the neuronal R-SNARE synaptobrevin and are thus SNARE-mediated. Furthermore, we show that overexpressing syntaxin and synaptobrevin is not sufficient for increasing fusion. Only overexpression of the Qbc-SNARE SNAP-29 in neurons is sufficient to rescue SNAP-25 null animals and increase fusion.

In the third chapter, I investigate the functional significance of the N-peptide interaction with Unc18. My experiments in *C. elegans*, paired with liposome fusion studies, exclude some of the most provocative models of the N-peptide interaction and demonstrate that this interaction is required for loading Munc18 onto the four-helix SNARE bundle.

Finally, in the fourth chapter, I discuss the implications of this work, the questions it raises, and preliminary results for new lines of investigation.

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CHAPTER 2

SNAP-29 SUBSTITUTES FOR SNAP-25 IN FUSING SYNAPTIC VESICLES

Abstract

SNARE proteins make up the core molecular machine responsible for vesicle fusion. The canonical model for synaptic vesicle fusion suggests that a single set of cognate SNARE proteins, including synaptobrevin, syntaxin, and SNAP-25, drives fusion for neurotransmitter release. In this study, we analyze neurotransmission in *snap-25* null neurons in the nematode *C. elegans*. We report that neurotransmission is strongly depressed, but some productive transmitter secretion remains. Synaptic vesicles dock and fuse in the absence of SNAP-25 protein. These fusion events are calcium sensitive and require the canonical R-SNARE, *snb-1*. Importantly, we demonstrate that neuronal overexpression of *snap-29*, and not the other Qbc-SNARE *aex-4*, is sufficient for rescuing the viability of *snap-25* null animals. Overexpression of *snap-29* restores neurotransmission in these animals by increasing tonic fusion but not evoked neurotransmitter release. We show that SNAP-29 is expressed in *C. elegans* neurons and is localized at synapses. These data are the first to directly implicate SNAP-29 in synaptic vesicle fusion.

Introduction

Vesicle fusion is executed by a conserved family of proteins called SNARE proteins. SNAREs are anchored to apposing membranes and span the cytoplasm to form a parallel four-helix bundle termed the “core complex” (Lin and Scheller, 1997; Sutton et al., 1998). The vesicle contributes a single SNARE motif, which twists together with three helices associated with the target membrane (known as v-SNAREs and t-SNAREs, respectively) (Broadie et al., 1995; Nickel et al., 1999; Weber et al., 1998). The canonical SNARE proteins responsible for fusion of synaptic vesicles include the v-SNARE synaptobrevin and the t-SNAREs syntaxin and SNAP-25. Synaptobrevin and syntaxin are type II transmembrane proteins, each contributing a single helix to the complex. SNAP-25 is unique in that it contributes two parallel SNARE motifs anchored to the plasma membrane by palmitoylation of four cysteine residues in the central linker (Vogel and Roche, 1999). Together these proteins are considered the minimal machinery for fusion as they are capable of fusing liposomes *in vitro* (Weber et al., 1998).

The original SNARE hypothesis postulated that SNAREs are responsible for docking vesicles at the appropriate target membrane (Rothman, 1994). Subsequent studies contradicted this theory (Broadie et al., 1995; Bronk et al., 2007; Hunt et al., 1994; Marsal et al., 1997; O'Connor et al., 1997). However, with more advanced imaging techniques, the consensus is shifting to support the role of SNAREs in docking vesicles (De Wit et al., 2006; Gutierrez et al., 1997; Hammarlund et al., 2007, 2008; Wu et al., 2012). However, all but one of these studies have focused specifically on syntaxin and synaptobrevin, and there is little evidence to directly confirm or deny a role for SNAP-25

in docking vesicles. Therefore, to clearly demonstrate that docking requires the SNARE core-complex, it is important to examine docking at SNAP-25 null synapses.

The canonical synaptic vesicle SNAREs are sufficient for fusion *in vitro* (Weber et al., 1998), but genetic ablation *in vivo* rarely eliminates fusion. In the case of the neuronal t-SNARE SNAP-25, all studies report some degree of spontaneous fusion in its absence (Bronk et al., 2007; Delgado-Martinez et al., 2007; Sørensen et al., 2003; Tafoya et al., 2006; Washbourne et al., 2002). In fact, Delgado-Martinez and colleagues suggest that spontaneous fusion rates in hippocampal culture are the same with and without SNAP-25 when normalized for the density of synapses. Moreover, Washburn *et al.* reported that the spontaneous neurotransmitter release rate at neuromuscular junctions (NMJs) of SNAP-25 knockout mice was higher than at wild-type synapses. Most studies conclude that SNAP-25 is strictly required for evoked fusion; however, one report observed small Ca^{2+}-evoked responses in the absence of SNAP-25 (Bronk et al., 2007).

These studies and others attribute the residual activity at SNARE null synapses to genetic substitution by homologous non-neuronal SNAREs. Mammalian neurons express 3 Qbc homologs, SNAP-23, SNAP-29, and SNAP-47. SNAP-23 appears to be capable of substituting for SNAP-25 to some degree; however, in each report the authors concluded that SNAP-23 cannot account for the residual activity observed in the mouse SNAP-25 knockout (Delgado-Martinez et al., 2007; Scales et al., 2000; Sørensen et al., 2003). The ability of SNAP-29 and SNAP-47 to support synaptic vesicle fusion has not been directly tested. However, SNAP-29 binds syntaxin and synaptobrevin with affinities approaching that of SNAP-25 and better than any other Qbc-SNARE homolog (Yang et al., 1999).

Furthermore, both SNAP-29 and SNAP-47 are enriched on purified synaptic vesicles (Holt et al., 2006; Su et al., 2001; Takamori et al., 2006).

Here, we have tested the requirement of SNAP-25 for neurotransmission at *C. elegans* NMJs. Worms lack SNAP-23 and SNAP-47 and only express the Qbc homolog SNAP-29 in the nervous system. Worms require SNAP-25 to develop beyond the second larval stage (L2), but null larvae are capable of locomotion, suggesting some level of productive neurotransmission. We engineered tissue-specific rescued animals and analyzed vesicle docking and fusion at the NMJ. We observed a reduction in vesicle docking and fusion in null neurons, but residual vesicle docking, tonic fusion, and evoked neurotransmission remain in the absence of SNAP-25. Residual fusion is calcium sensitive and requires the R-SNARE *snb-1*. We found that overexpression of *snap-29* in neurons rescued the viability of *snap-25* null animals and increased tonic neurotransmission. However, evoked fusion was unchanged. We confirmed that SNAP-29 is natively expressed throughout the nervous system of *C. elegans* and report that it is localized to synaptic varicosities.

Our results add to a growing body of evidence across many systems suggesting a SNAP-25 independent mode of synaptic vesicle secretion. We show that SNAP-29 is sufficient for fusion in *C. elegans* and suggest that it likely supplements neurotransmission in other systems.

Results

The *C. elegans snap-25* gene (*ric-4*) encodes the neuronal Qbc-SNARE SNAP-25

Mutations in the *C. elegans snap-25* (*ric-4*) locus were first isolated in screens for animals with reduced neurotransmission (Miller et al., 1996; Nguyen et al., 1995). We report a comprehensive list of *snap-25* alleles, including updated molecular information on published and unpublished isolates (Table S2.1). We have confirmed the presence of two alternatively spliced *snap-25* transcripts by 5' RACE (rapid amplification of cDNA ends). They agree with the EST (expressed sequence tags) data available on wormbase.org and are annotated as *snap-25A* and *snap-25B* (Figure 2.1A). *ok173* (kindly provided by Robert Barsted) and *ox528* (generated in house by mosDel [Frøkjaer-Jensen et al., 2010]) are two novel alleles that delete over 80% of the coding locus. We believe they represent complete nulls. *ox45* is a point mutation in the start codon of *snap-25* exon 1A, which selectively eliminates this isoform. The *ox45* mutation results in dramatically reduced expression of the gene and represents a recessive loss of function hypomorph (M. Nonet, personal communication).

The *C. elegans snap-25* gene encodes a highly conserved member of the neuronal Qbc family of proteins. The *C. elegans* protein is 70% similar to that of the human homolog and the SNARE motifs are particularly well conserved (Qb: 65% identity, 79% similarity; Qc: 58% identity, 82% similarity) (Figure 2.1B). *snap-25* null worms (*ox528* and *ok173*) arrest at the second larval stage (L2). We have fully rescued these animals (Figure 2.1C) by expressing a genomic fragment of *snap-25* under a neuron-specific promoter from the synaptotagmin gene (*Psnt-1*) (Figure S2.1). *C. elegans snap-25* has

previously been reported to be expressed pan-neuronally (Hwang and Lee, 2003). Our rescue data confirms that the lethal phenotype is specific to mutations in the *snap-25* locus and that neurons are the critical tissue for SNAP-25 function.

Although *snap-25* null worms are subviable, the larvae are notably healthier than worms with null mutations in the cognate t-SNARE syntaxin (*unc-64*) (Figure 2.1C). *snap-25* null animals grow larger and are capable of locomotion. Single *snap-25* larva move many millimeters across a plate over the course of 2 days, while *unc-64* larvae are paralyzed upon hatching (Figure 2.1D). Locomotion in the *snap-25* null worms is the first evidence that neurotransmission may not strictly require SNAP-25 protein.

Tissue specific rescue of the *snap-25* null

In order to study *snap-25* null neurons in living animals, we engineered two strains with tissue-specific *snap-25* rescue (Figure S2.1A). Worms only require neurotransmission in acetylcholine neurons of the head for viability. Therefore, we expressed *snap-25* under the vesicular acetylcholine transporter promoter (*Punc-17*) driving *snap-25* expression throughout the cholinergic nervous system. We will refer to this strain as “ACh-only.” These animals are viable and develop to adulthood but lack *snap-25* expression in all GABA neurons. Additionally, we used a modified *Punc-17* promoter lacking an enhancer required for motor neuron expression. We will refer to this strain as “head-only.” The “head-only” strain lacks *snap-25* expression in all motor neurons. Both strains are strongly uncoordinated but appear grossly similar to SNARE mosaics we have engineered for syntaxin (Hammarlund et al., 2007; Rathore et al., 2010) (Figure S2.1B).

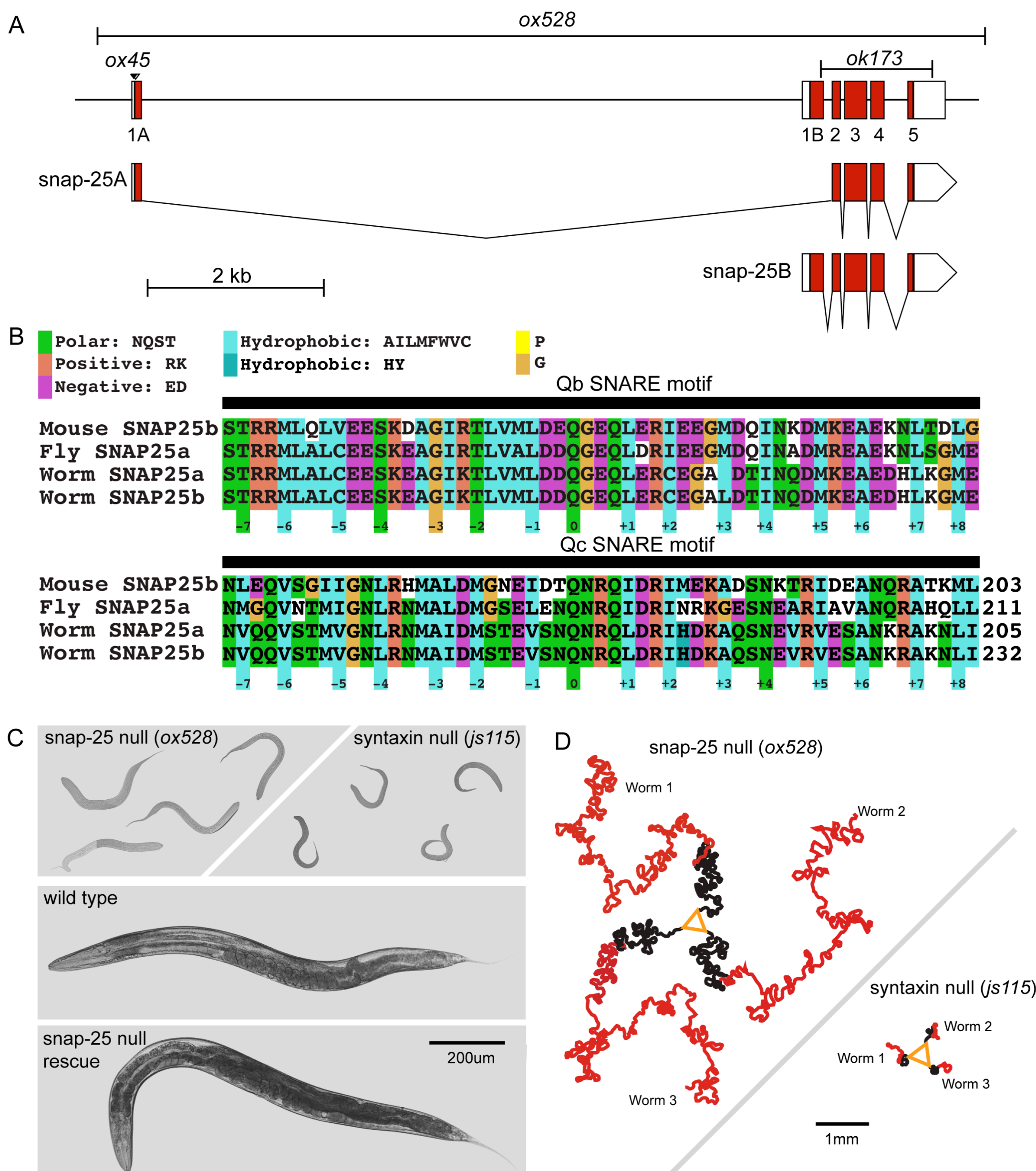


Figure 2.1. *snap-25* encodes a conserved neuronal Qbc-SNARE. (A) The *snap-25* locus is composed of 5 exons with alternative splicing of the first exon resulting in snap-25a and *snap-25b*. Alleles *ok173* and *ox528* delete the genomic region indicated with brackets. Each results in a null allele. *ox45* is a hypomorph selectively deleting *snap-25a* by a point mutation in the start codon. (B) The SNAP-25 SNARE motifs are well conserved. (Worm similarity to mouse Qb/Qc: 86% / 80%. Identity to mouse Qb/Qc: 75% / 53%). Layer residues are indicated by position number. (C) Confocal images depicting strains at terminal stage. *snap-25* (*ox528*) arrests at L2. Syntaxin nulls (*js115*) arrest at L1. A neuronally expressed *snap-25* transgene fully rescues ox528 and animals develop to adulthood. (D) Worm locomotion diagrams demonstrate that *snap-25* nulls are capable of locomotion. Single worms were placed on individual plates. Black lines represent tracks at 24 hrs. Red lines represent tracks at 48 hrs. Tracks were superimposed to start at the corners of the orange triangle for clarity.

Synaptic vesicle docking is decreased in the absence of SNAP-25

We examined the ultrastructure of presynaptic terminals of NMJs and found that SNAP-25 is required for normal docking but not absolutely essential (Figure 2.2). We used the "ACh-only" strain for these experiments, providing an internal control by comparing acetylcholine and GABA synapses. At acetylcholine synapses, the numbers of "total vesicles," "docked vesicles," and "tethered vesicles" were the same between wild-type and "ACh-only" strains (Figure 2.2A–B). This is expected since *snap-25* is expressed in acetylcholine neurons of both of these strains. Only the hypomorph (*ox45*) showed a reduction in docked ACh vesicles (approximately 50%), implicating *snap-25* in docking.

Examining GABA terminals provided more evidence for SNAP-25 mediated docking (Figure 2.2C–D). In the absence of SNAP-25, synapses had a 50% reduction of docked vesicles compared to rescued GABA synapses. The hypomorph (*ox45*) was also defective for docking (approximately 50% less than the wild type). Tethering was normal at GABA synapses in all strains. Finally, we saw a small reduction in the total number of synaptic vesicles in the pan-neuronal rescued strain and hypomorph.

The SNAP-25 null docking data resemble our previous ultrastructural observations of syntaxin null synapses; however, in that case synaptic vesicle docking was almost completely abolished (Hammarlund et al., 2007, 2008). It is possible that syntaxin is able to dock synaptic vesicles without a Qbc-SNARE forming a bridge to molecules on the vesicle prior to interaction with SNAP-25. Alternatively, a homologous Qbc protein may replace SNAP-25, allowing for docking via a noncanonical four-helix bundle.

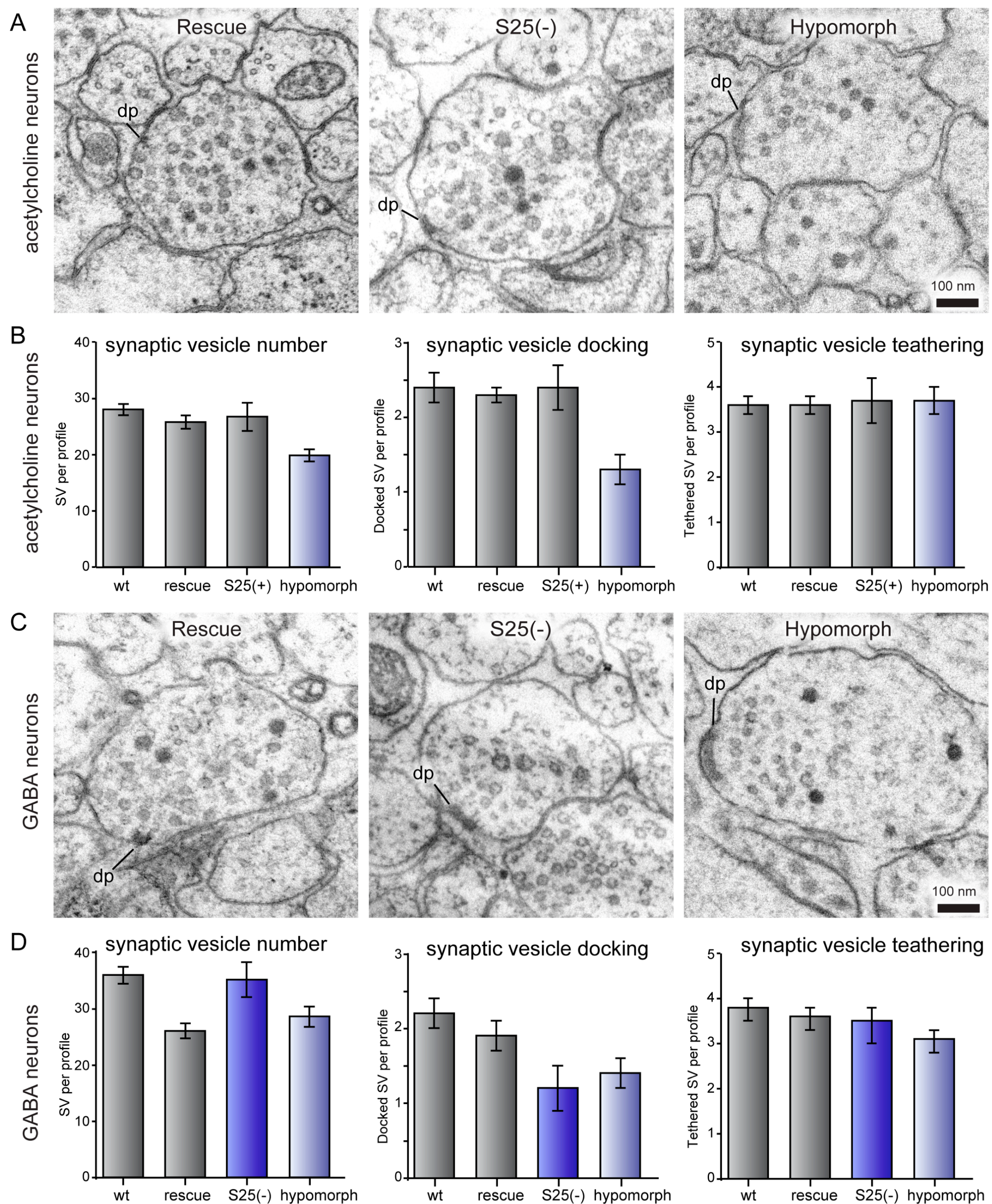


Figure 2.2 Synaptic vesicle docking is reduced at *snap-25* null synapses. (A) Electron microscopy of GABA neuromuscular junctions in ventral nerve cords. Panels 1 and 2 show synapses of *ox528* rescued with the wild-type and "ACh-only" transgenes respectively. The third panel displays a GABA terminal from the hypomorph (*ox45*). Dense projections are labeled (dp). Scale bar: 100 nm. (B) Quantification of the total vesicles/profile, docked vesicles/profile and tethered vesicles/profile. Docking is reduced in the hypomorph only (50%). (C) Representative micrographs of acetylcholine terminals as described in A. (D) Quantification as in B. Vesicle docking is reduced 50% in the *snap-25* null and hypomorph terminals, compared to the rescue and wild type respectively.

Synaptic vesicle fusion is reduced at SNAP-25 null NMJs

We recorded miniature postsynaptic currents at *C. elegans* NMJs. We found that the frequency of tonic fusion events (minis) was significantly reduced (15% of wild type), but not completely abolished (Figures 2.3A and 2.3C). This important result indicates that residual vesicle fusion remains in the absence of SNAP-25. SNAP-25 independent minis were indistinguishable in kinetics and amplitude from those at wild-type synapses and pan-neuronal expression of *snap-25* fully rescued the frequency defect. The *snap-25* hypomorph produced intermediate activity. This observation is consistent with the many accounts of SNAP-25 independent spontaneous fusion reported (Bronk et al., 2007; Delgado-Martinez et al., 2007; Tafoya et al., 2006; Vilinsky et al., 2002; Washbourne et al., 2002; Sørensen et al., 2003). In addition, Ca^{2+} evoked synchronous fusion also remained in the absence of SNAP-25 (Figures 2.3B and 2.3C). The mean amplitude of Channelrhodopsin-2 (ChR2) mediated evoked currents from *snap-25* null synapses was 74% lower than that of wild-type synapses. Neuronal expression of *snap-25* fully rescued evoked release. The observation of SNAP-25 independent evoked release was surprising, as most studies report that SNAP-25 is strictly required for Ca^{2+} evoked fusion (Delgado-Martinez et al., 2007; Sørensen et al., 2003; Tafoya et al., 2006; Washbourne et al., 2002). However, our observations are consistent with those of Bronk and colleagues, who reported small evoked currents in hippocampal cultures (Bronk et al., 2007).

To test whether the reduced function of *snap-25* synapses was specific to vesicle secretion and not a secondary consequence of nervous system development, we analyzed gross neuronal architecture, synapse density, and postsynaptic responses (Figure S2.2). We found that GABA neurons appeared normal in *snap-25* null (*ox528*) larvae and adult

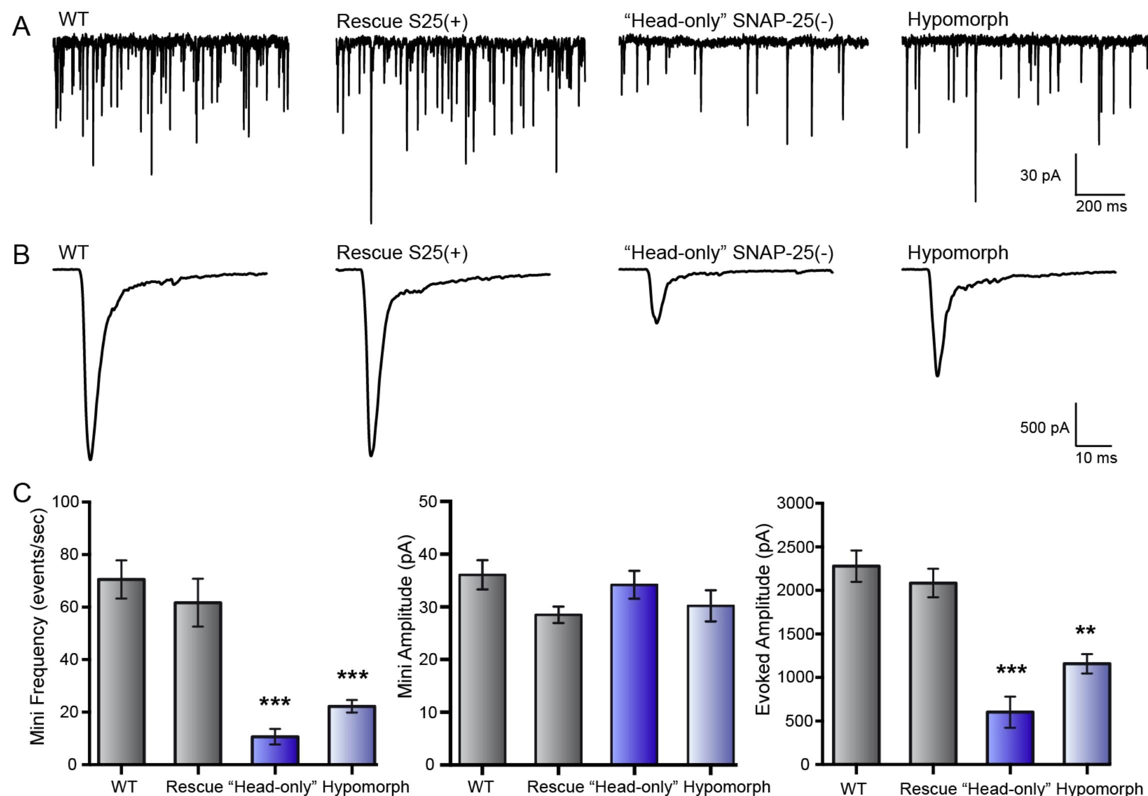


Figure 2.3 Tonic mini rates are reduced in *snap-25* null neurons. (A) Representative traces of miniature currents recorded from the *C. elegans* neuromuscular junctions. The wild type is compared with the pan-neuronal and "head-only" rescued null (*ox528*) as well as the hypomorph (*ox45*). (B) Representative traces of channelrhodopsin-2 evoked post-synaptic currents from strains described in A. (C) Quantification of the mini frequency, mini amplitude, and evoked amplitude. Pan-neuronal expression of *snap-25* (*Psnt-1::snap-25*) rescued the mini frequency of the null (*ox528*) (Rescue, 61.7 ± 9.1 minis/sec; n = 8 vs. "head-only," 10.8 ± 2.7 minis/sec; n = 10 ; p<0.0001) The average rate of fusion at rescued synapses (*Psnt-1::snap-25*) was not significantly different from the rate at wild-type synapses (70.5 ± 7.2 minis/s; n = 14). The average rate of fusion measured from the hypomorph (*ox45*) (22.2 ± 2.4 minis/s; n = 8) was significantly lower than that measured from the wild type (p<0.0001). Mini amplitude was statistically equivalent across all strains. *Psnt-1::snap-25* rescued the ChR2 evoked amplitude of the null (*ox528*) (Rescue, 2084 ± 164 pA; n = 9 vs. "head-only," 600 ± 180; n = 10 ; p<0.0001). The average evoked amplitude recorded from rescued synapses was not significantly different from that recorded at wild-type synapses (2279 ± 179 pA; n = 9). The average evoked amplitude measured in the hypomorph (*ox45*) (1158 ± 111; n = 8) was significantly lower than that measured in the wild type (p = 0.0001). Significance calculated by one-way ANOVA with Bonferroni correction for multiple comparisons. * p<0.05, ** p<0.01, *** p<0.001.

"head-only" animals. The density of GABA synapses in "head-only" animals was indistinguishable from wild-type and rescued strains, as assayed by a SYD-2::GFP (alpha liprin, which marks presynaptic dense projections). Finally exogenous application of GABA induced postsynaptic currents that were indistinguishable between "head-only" (*ok173*) and wild-type strains.

Substitution by SNAP-29 bypasses the requirement for SNAP-25

Most studies have concluded that homologous substitution accounts for SNAP-25 independent fusion at null synapses. However, before advancing to this conclusion, we considered a broad spectrum of possible models to explain residual fusion at the *C. elegans* NMJ: (1) unintended expression of *snap-25* in cholinergic motor neurons of the "head-only" strain, (2) SNARE independent fusion, (3) fusion by syntaxin/synaptobrevin binary complexes, and (4) substitution by homologous Qbc-SNAREs.

Our tissue-specific rescue strategy required that we engineer animals that only express *snap-25* in cholinergic head neurons. We used a previously defined promoter to exclusively express *snap-25* in the head; no fusion is observed when the syntaxin null (*js115*) is rescued by expression with the Punc-17 "head-only" promoter (Hammarlund et al., 2007; Rathore et al., 2010). However, it is formally possible that our transgene expresses some *snap-25* in cholinergic motor neurons, causing low-level SNAP-25 dependent fusion at "head-only" NMJs. To test this, we selectively blocked ACh receptors by applying the drug d-tubocurare (dTBC) to our recording bath. If low-level expression accounted for residual current, we would expect that all of the current would

be eliminated when "head-only" animals were bathed in dTBC. Instead, we found that applying dTBC decreased tonic release frequency by approximately 50% in the "head-only" strain and wild-type controls (Figures 2.4A and 2.4B). Similar results were obtained in recordings from the equivalent *ok173* null strains. Furthermore, the drug was 100% effective at eliminating ACh activity, as all minis were gone when applied to a GABA receptor null (*unc-49*) (Figure S2.3). Therefore, a significant amount of current at "head-only" NMJs is due to GABA neurotransmission. It is very unlikely that inadvertent *snap-25* expression in cholinergic neurons is responsible for fusion in this strain.

SNARE independent vesicle fusion has never been demonstrated *in vivo*, but we considered it a formal possibility. We engineered a worm strain with targeted degradation of the v-SNARE *snb-1*. We constitutively expressed the zinc endopeptidase tetanus toxin light chain (TeTx) in GABA neurons under the promoter from the vesicular GABA transporter (*Punc-47*). TeTx specifically cleaves synaptobrevin with high efficacy. If the residual fusion in "head-only" animals requires synaptobrevin, we predicted that GABA mediated current would be eliminated. We found that cutting synaptobrevin with TeTx strongly reduced mini rates in the wild type (66% decrease) and nearly abolished tonic fusion events at SNAP-25 null synapses (90% decrease) (Figures 2.4C and 2.4D). Furthermore, the application of dTBC completely eliminated fusion in both strains. Once again, dTBC was used to eliminate acetylcholine current and isolate GABA specific vesicle fusion. Our results confirm that all GABA neurotransmission requires synaptobrevin in wild-type and "head-only" strains. Furthermore, the fact that P*unc-47*::TeTx reduced minis by 90% in the "head-only" strain suggests that the majority of the SNAP-25 independent minis are from GABA synapses. Most importantly, residual

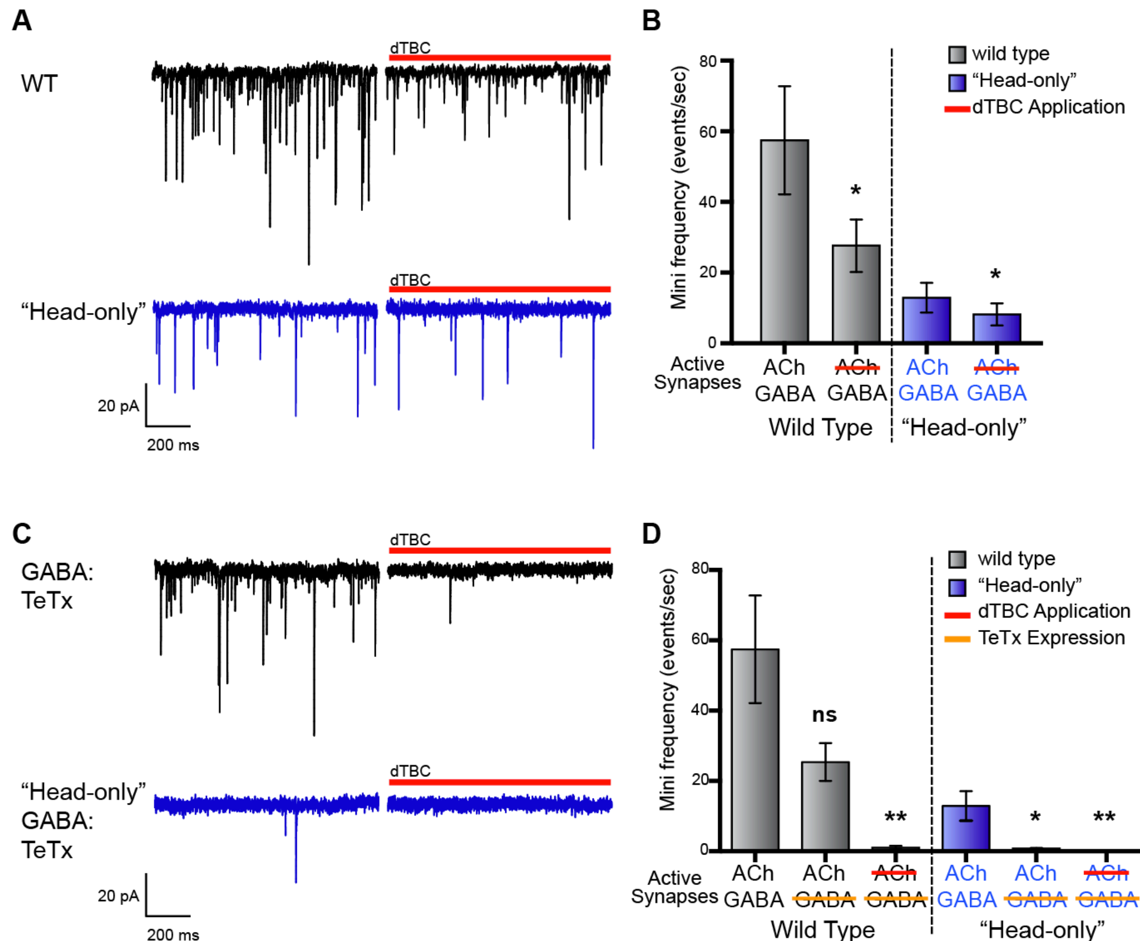


Figure 2.4 *snap-25* independent minis are predominantly GABAergic and require the neuronal R-SNARE *snb-1*. (A) Representative miniature currents. The wild type is compared with the "head-only" rescued null (*ox528*). dTBC was applied directly to NMJs by perfusion of the recording chamber. Traces represent activity after stabilization (>60 sec dTBC application). (B) Quantification of average min frequencies before and after drug application. The frequency of minis at wild-type synapses (57.4 ± 15.3 minis/sec; n = 7) decreased by 48% with the application of dTBC (27.6 ± 7.4 minis/sec; n = 7; p = 0.0216). Likewise, SNAP-25 independent minis in the "head-only" strain (12.9 ± 4.2 minis/sec; n = 7) decreased in frequency by 37% with dTBC application (8.1 ± 3.1 minis/sec; n = 7; p = 0.0182). (C) Representative minis in strains expressing GABA::TeTx in the wild-type compared to "head-only" genetic backgrounds. (D) Quantification of the average mini frequencies before and after dTBC application. Expression of TeTx in GABA neurons of the wild type caused a nonsignificant decrease in mini frequency (56%; wt: 57.4 ± 15.3 minis/sec; n = 7 vs. TeTx: 25.4 ± 5.4 minis/sec; n = 8; p = 0.0574). Applying dTBC nearly abolished fusion (1.1 ± 0.5 mins/sec; n = 8; p = 0.0026). Expressing GABA::TeTx in "head-only" animals decreased mini frequency ("head-only": 12.9 ± 4.2 minis/sec; n = 7 vs. "head-only" + TeTx: 0.77 ± 0.18 minis/sec; n = 6; p = 0.0225). The addition of dTBC eliminated fusion (0.07 ± 0.07 minis/sec; n=6; p= 0.0023). Significance of TeTx expression calculated by unpaired t-test. Significance of dTBC application calculated by paired t-test. * p<0.05, ** p<0.01

minis are SNARE mediated.

Is it possible that SNARE mediated fusion can occur via syntaxin and synaptobrevin alone? In fact, neuronal syntaxin and synaptobrevin interact in a binary coiled-coil with a force that may contribute to vesicle fusion (Liu et al., 2006, 2009, 2011). Furthermore, the binary complex is sufficient *in vitro* for driving fusion of liposomes and native vesicles with planar lipid bilayers (Bowen et al., 2004; McNally et al., 2004; Liu, 2005; Woodbury and Rognlien, 2000) but not in liposome mixtures (Schuette, 2004; Tucker et al., 2004). Therefore, binary fusion driven by syntaxin and synaptobrevin is supported by considerable evidence, but remains untested *in vivo*.

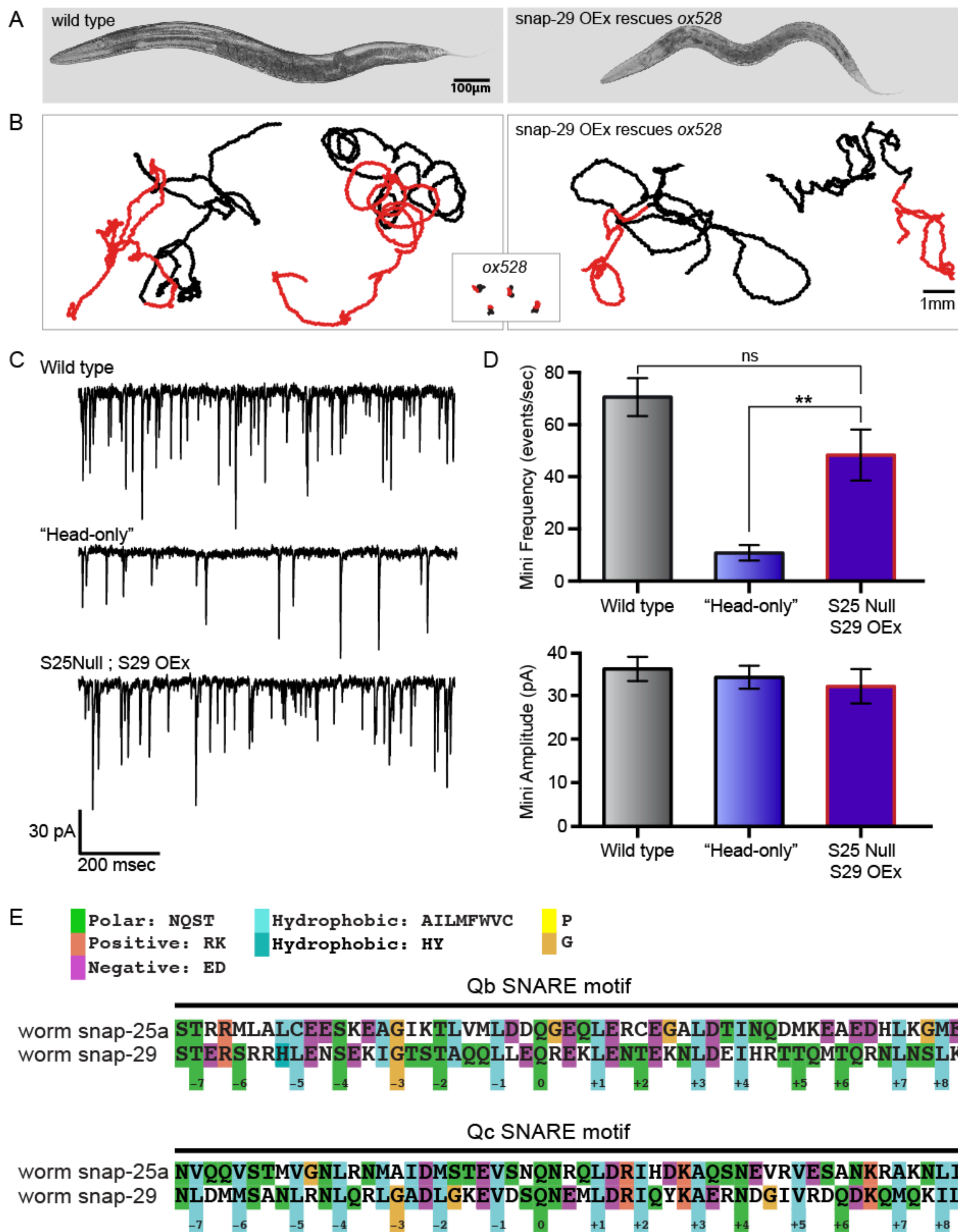
To test the binary fusion model and homologous substitution model, we reasoned that overexpression of the alternative fusion components may bypass the requirement for SNAP-25. First, we overexpressed worm syntaxin and synaptobrevin homologs (*unc-64* and *snb-1*). Transgenic extrachromosomal arrays were generated by microinjection of *unc-64* and *snb-1* under pan-neuronal expression (*Psnt-1*). These arrays rescued syntaxin (*js115*) and synaptobrevin (*js124*) null animals, confirming that the transgenes are functional. However, when injected into the balanced *snap-25* null (*ox528*/*oxTi417*), we found no increase in the fitness of arrested *snap-25* null larvae.

Finally, we considered that one or more *snap-25* homologs might be capable of synaptic vesicle fusion. Worms express two alternative Qbc-SNAREs, *aex-4* and *snap-29*. Both have comparable identity to *snap-25* at the whole protein level (22%), but the SNARE motifs of SNAP-29 have significantly higher identity (approximately 30%) (Table S2.1). We overexpressed *aex-4* and *snap-29* in the neurons of *snap-25 (ox528)* animals from multicopy extrachromosomal arrays. Pan-neuronal (*Prab-3*) *aex-4*

expression gave no fitness advantage. *Prab-3::snap-29* overexpression (S29-OEx) rescued viability of *snap-25 (ox528)* null animals. Rescued animals are smaller than the wild type, but grow to adulthood and produce moderate brood sizes (Figure 2.5A). S29-OEx dramatically increased mobility. Age matched (first-larval stage) animals with S29-OEx move many millimeters per hour—similar to locomotion rates of the wild type. In contrast, the *snap-25* null moved very little during a 2-hour assay (Figure2.5B).

Does S29-OEx increase fusion at synapses? We recorded minis from neuromuscular junctions of *snap-25* null animals rescued with S29-OEx and found that synaptic vesicle fusion occurred at an average rate of 48 events/sec, 70% of wild-type activity (Figures 2.5C and 2.5D). The fusion was significantly more active than that observed at "head-only" SNAP-25 null synapses. Therefore, S29-OEx is correlated with an increase in mini frequency above *snap-25* null synapse rates. Minis were indistinguishable from the wild type in kinetics and amplitude. These data suggest that SNAP-29 may be substituting for SNAP-25 in neurotransmitter release. An alignment of SNAP-25 with SNAP-29 SNARE motifs shows some divergence, yet the hydrophobic "layer-residues" responsible for SNARE pairing are highly conserved, suggesting that substitution may be possible (Figure 2.5C). To test the relevance of this result to synaptic physiology we proposed the following criteria: (1) SNAP-29 must be natively expressed in neurons and present at synapses, (2) SNAP-29 must be sufficient for increased vesicle fusion in genetically paired experiments, and (3) SNAP-29 must be required for normal vesicle fusion.

Figure 2.5 Neuronal overexpression of *snap-29* rescues viability, locomotion, and minis in snap-25 null worms. (A) Confocal images depicting strains at terminal stage. The wild type is shown in comparison to the *snap-25* null (*ox528*) rescued by overexpression of *Prab-3::snap-29* from an extrachromosomal array. Animals grow to adulthood, but are uncoordinated and smaller than the wild type. (B) Locomotion diagrams demonstrate that neuronal *snap-29* overexpression rescues the locomotion phenotype of snap-25 null (*ox528*) larvae (L1). Rescued animals move across plates at near wild-type rates. Eight L1 animals of each genotype were placed on single plates. Black lines indicate tracks after 30 min. Red lines indicate tracks from 30 to 60 min. Patterns with the broadest distribution were selected from each strain. (C) Representative mini recordings. (D) Quantification of mini frequency and amplitude. Overexpression of SNAP-29 in the *snap-25* null (*ox525*) resulted in a strain with mini rates near that of the wild type (wild type, 70.54 ± 7.2 minis/sec; n = 14 vs. *ox528* ; S29 OEx, 48.33 ± 9.8 minis/sec; n = 6 ; p = 0.0981). The frequency of minis in this strain was significantly greater than the "head-only" rescued strain (10.8 ± 2.7 minis/sec; n = 10 ; p<0.0056). Mini amplitude was equivalent in all three strains. (E) The SNAP-29 SNARE motifs are aligned with those from SNAP-25. The amino acid sequence has considerable divergence, however hydrophobic "layer residues" are preferentially conserved (Indicated by numbers). Significance calculated by one-way ANOVA with Bonferroni correction for multiple comparisons. ** p<0.01


A
wild type
100µm
snap-29 OEx rescues ox528
B
snap-29 OEx rescues ox528
ox528
1mm
C
Wild type
"Head-only"
S25Null ; S29 OEx
30 pA
200 msec
D
ns
**
Mini Frequency (events/sec)
Wild type
"Head-only"
S25 Null S29 OEx
Mini Amplitude (pA)
E
Polar: NQST
Positive: RK
Negative: ED
Hydrophobic: AILMFWVC
Hydrophobic: HY
P
G
Qb SNARE motif
worm snap-25a STRRMLALCEESKEAGIKTLVMLDDQGEQLERCEGALDTINQDMKEAEDHLKGME
worm snap-29 STERSRRHLENSEKIGTSTAQQLLEQREKLENTEKNLDEIHRTTQMTQRNLNSLK
Qc SNARE motif
worm snap-25a NVQQVSTMVGNLRNMAIDMSTEVSNQNRQLDRIHDKAQSNEVRVESANKRAKNLI
worm snap-29 NLDMMSANLRNLQRLGADLGKEVDSQNEMLDRIQYKAERNDGIVRDQDKQMQKIL

SNAP-29 is expressed in *C. elegans* neurons and localized to synaptic varicosities

Previous work has demonstrated that *snap-29* is expressed ubiquitously in worms and mammals (Sato et al., 2011; Steegmaier et al., 1998). We wanted to examine the native expression pattern of SNAP-29 in the *C. elegans* nervous system. We used fosmid recombineering to make a translational GFP fusion of SNAP-29 in its native genomic context. The resulting fosmid contained 25 kilobases of 3-prime sequence followed by GFP::SNAP-29 and 8 kilobases of 5-prime genomic DNA. Transgenic animals expressing this fosmid presented with diffuse fluorescence in many tissues including the intestine, muscle, coelomocytes, and neurons. In order to examine the nervous system expression alone, we took advantage of the fact that neurons in *C. elegans* are the only tissue that is insensitive to RNAi. We grew these animals on anti-GFP feeding RNAi and selectively knocked down fluorescence in non-neuronal cells. The resulting images show pan-neuronal expression of SNAP-29 from its native genomic locus (Figure 2.6A).

snap-29 is strongly expressed in motor neurons of the dorsal and ventral cord. In order to test the localization of SNAP-29 protein in neurons, we engineered transgenic animals with *GFP::snap-29* expressed under the GABA specific promoter (*Punc-47*). Images of the ventral nerve cord of these animals demonstrate that GFP::SNAP-29 is punctate and co-localizes with the synaptic vesicle marker tagRFP::SNB-1 (Figure 2.6B). It is worth noting that SNAP-25 localization is not restricted to synapses to the same extent (Figure 2.6C). In conclusion, SNAP-29 is expressed and localized appropriately for synaptic vesicle fusion.

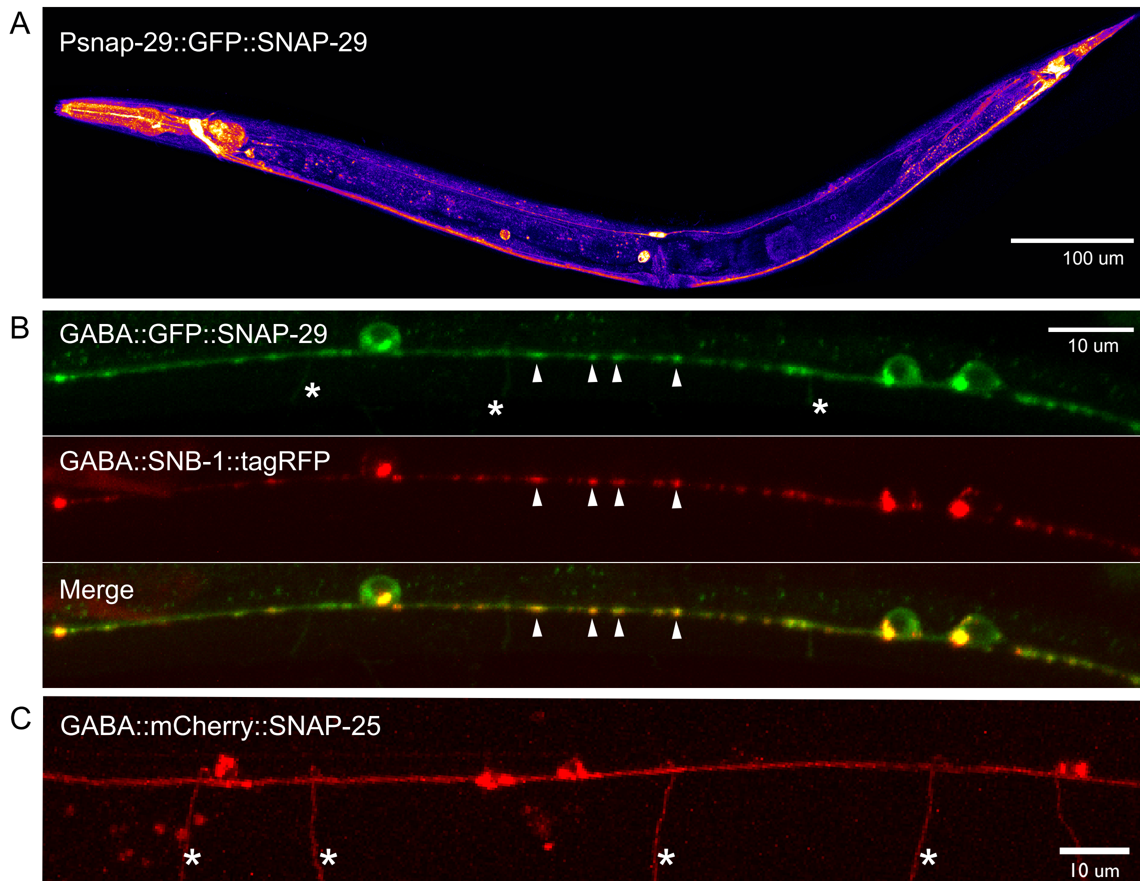


Figure 2.6 SNAP-29 is expressed in *C. elegans* neurons and localizes to synapses. (A) The native *snap-29* locus (33kb) was recombineered to include a translational fusion with GFP and expressed from an extrachromosomal array. Non-neuronal expression is knocked down by anti-GFP feeding RNAi. GFP::SNAP-29 is visible throughout the nervous system in a young adult hermaphrodite. The worm is oriented rostral left and dorsal up. (B) Three panels display the ventral nerve cord of a single young hermaphrodite expressing fluorescent protein-fusions in the GABA nervous system (*Punc-47*). GABA expression allows visualization of individual synapses. In the top panel, GFP::SNAP-29 appears punctate in the cord. Arrowheads indicate select puncta. Very faint expression can be seen in axon commissures (*). In the middle panel, SNB-1::tagRFP marks synapses. Select puncta are indicated with arrowheads. The bottom panel displays a merged image. GFP::SNAP-29 puncta colocalize with SNB-1::tagRFP. (C) The expression of mCherry::SNAP-25 in GABA neurons (*Punc-47*) is diffuse. No puncta are distinguishable, and the protein is present in single axon commissures (*).

SNAP-29 is sufficient for tonic currents but not synchronous fusion

Does S29-OEx directly stimulate synaptic vesicle fusion? We overexpressed *snap-29* under the pan-neuronal promoter P*rab-3* in wild-type animals, “head-only” transgenics, and the hypomorph (*ox45*). We found that mini rates were significantly higher with S29-OEx at *snap-25* null and hypomorph synapses; however, wild-type mini rates were unchanged (Figures 2.7A and 2.7B). Next we tested whether SNAP-29 effects evoked fusion. We observed no change in the evoked current with or without S29-OEx at *snap-25* null synapses. Remarkably, S29-OEx decreased the amplitude of evoked currents in the wild type and hypomorph (Figures 2.7C and 2.7D). This observation suggests that SNAP-29 is incapable of evoked fusion and may compete with SNAP-25 by committing vesicles to the synchronous fusion pool.

Does SNAP-29 play a role in neurotransmission at normal synapses? Mini rates at 5 mM Ca^{2+} showed no increase with S29-OEx. It is possible that fusion is exhausted in these conditions. Therefore, we recorded tonic currents at multiple Ca^{2+} concentrations. We found that at lower calcium concentrations, S29-OEx resulted in an elevation of mini rates above wild-type frequencies (Figure 2.8). However, in the wild-type background these results fall short of statistical significance. We have recently found that perfusing multiple calcium solutions to generate paired recordings is a better approach. These experiments are ongoing, but already provide more compelling evidence that SNAP-29 can participate in fusion at wild-type synapses. These data also provide evidence that SNAP-25 independent minis are sensitive to Ca^{2+}. Once again, the recording strategy lacks statistical leverage, but our revised approach will better address this issue.

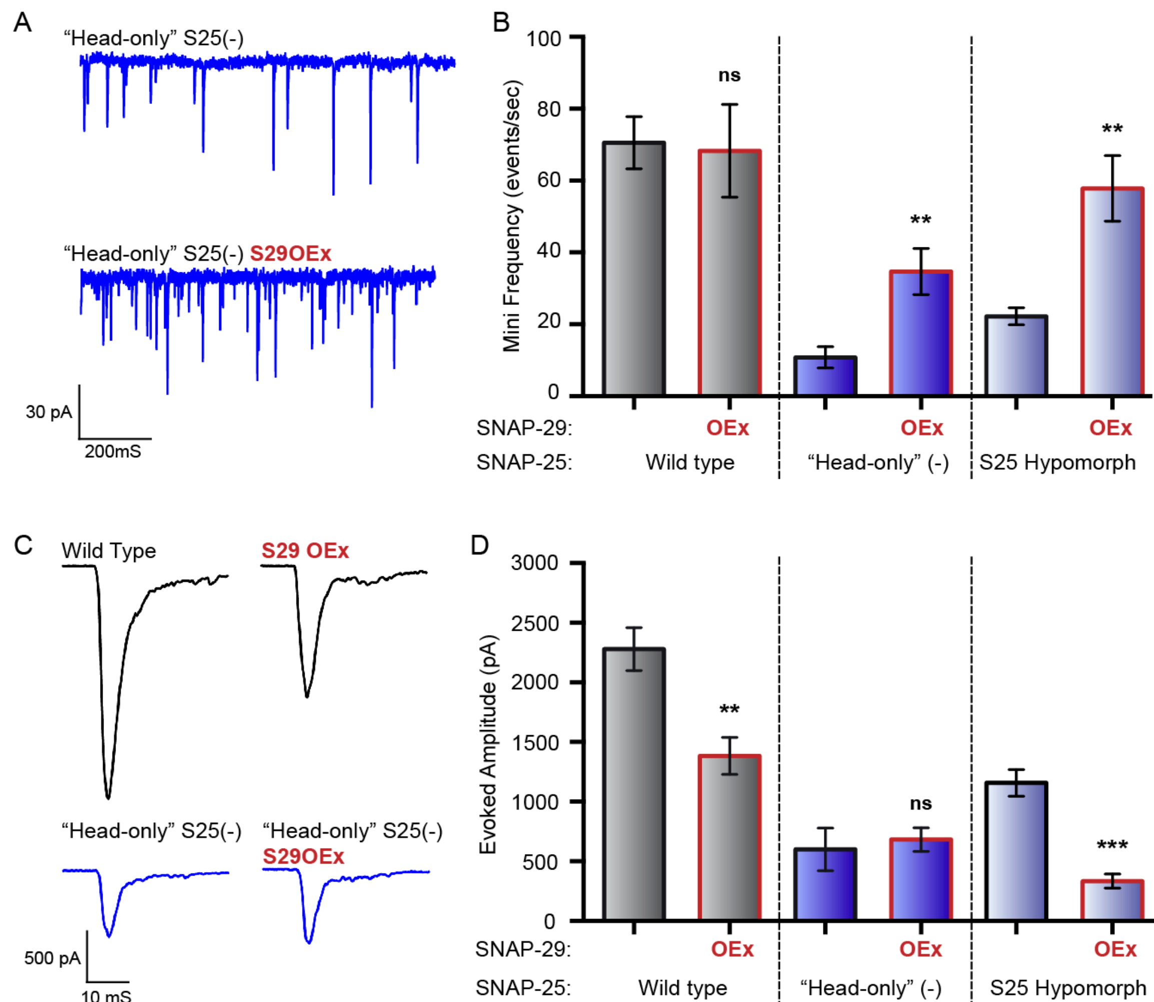


Figure 2.7 Overexpression of neuronal *snap-29* increases tonic fusion rates and decreases evoked fusion amplitude. (A) Representative miniature currents from the "head-only" strain with and without overexpression of *Prab-3::snap-29*. (B) Quantification of the tonic mini rate. The wild-type mini frequency (70.5 ± 7.2 minis/s; n = 14) was unchanged with S29-OEx (68.3 ± 12.9 minis/s; n = 8). S29-OEx approximately tripled the rate of minis in the absence of SNAP-25 ("head-only": 10.8 ± 3.0 minis/s; n = 10 vs. "head-only"+S29OEx: 34.7 ± 6.4 minis/s; n = 8; p = 0.0023) and in the hypomorph (*ox45*: 22.3 ± 2.4 minis/s; n = 8 vs. *ox45*+S29OEx: 57.8 ± 9.1 minis/s; n = 7; p=0.0015). (C) Representative ChR2 evoked currents in wild-type and null synapses with and without S29-OEx. (D) Quantification of the evoked fusion amplitude. S29-OEx caused a significant decrease in the evoked amplitude in the wild type (2279 ± 179 pA; n = 9 vs. 1383 ± 154 pA; n = 7; p = 0.0026) and the hypomorph (1158 ± 111 pA; n = 8 vs. 335 ± 58 pA; n = 7; p < 0.0001). S29-OEx had no effect on the "head-only" rescued strain. Significance of S29-OEx calculated by unpaired t-test. * p<0.05, ** p<0.01, *** p<0.001.

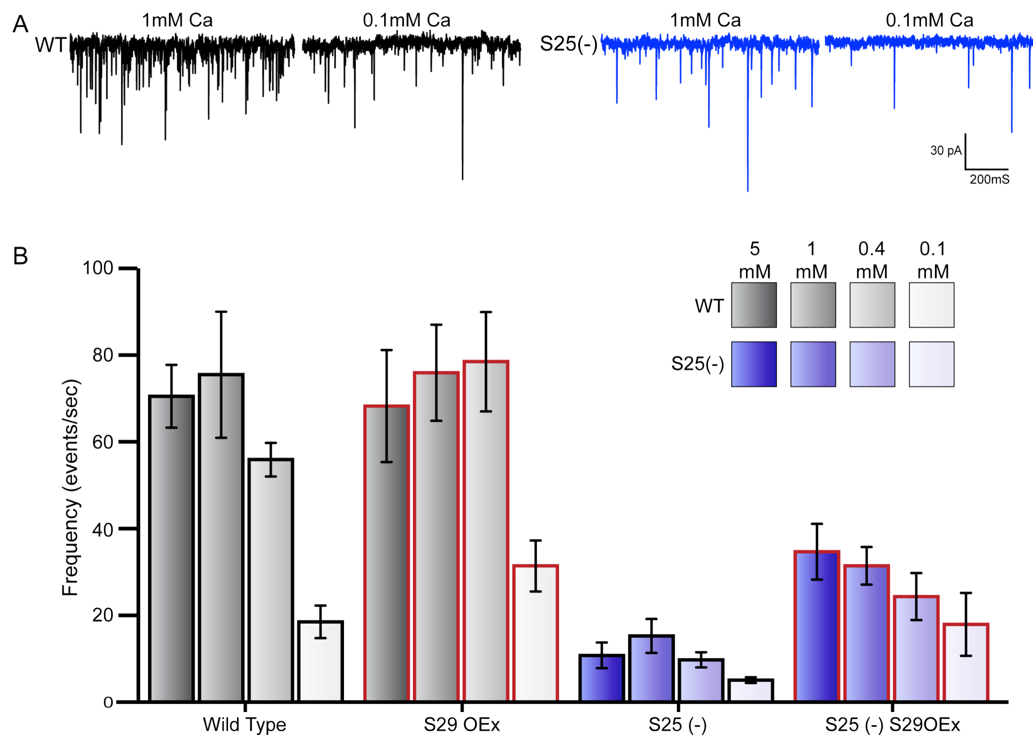


Figure 2.8 *snap-29* overexpression does not significantly increase the tonic fusion rate of fusion at wild-type synapses. (A) Representative miniature currents from the wild-type and *ox528* “head-only” strain at 1 mM and 0.1 mM extracellular Ca^{2+} (B) Quantification of the tonic mini rate at decreasing calcium concentrations. Reducing the extracellular $[Ca^{2+}]$ decreased mini rates in all strains but only to a significant degree when reduced to 0.1mM. Low extracellular $[Ca^{2+}]$ revealed a small but insignificant increase in fusion rates due to S29-OEx at wild-type synapses. S29-OEx approximately doubled the fusion rate at all Ca^{2+} concentrations in the “head-only” *ox528* strain.

Is SNAP-29 required for normal fusion rates at wild-type and SNAP-25 null synapses?

Thus far we have demonstrated that SNAP-29 is sufficient for synaptic vesicle fusion. In addition, it is important to distinguish whether or not SNAP-29 is necessary for normal fusion rates at wild-type and *snap-25* null synapses. In order to address this, we must knock-out *snap-29*; however, this experiment presents particularly difficult challenges. *snap-29* null alleles are cell-lethal, eliminating the possibility of chronic *snap-29* loss-of function strains. An effective alternative would be to knock down SNAP-

29 protein by acute means. With this strategy, we might directly test the requirement of SNAP-29 for normal vesicle fusion in the *snap-29* "head-only" and wild-type animals. We are currently exploring multiple recently published methods for acute protein degradation of SNAP-29 (Cho et al., 2013; Iwamoto et al., 2010; Neklesa et al., 2011).

Discussion

The results presented here demonstrate that synaptic vesicle docking and fusion is decreased but not eliminated in the absence of SNAP-25 at *C. elegans* NMJs. We have characterized the *C. elegans snap-25* locus and described the morphological and functional consequences of *snap-25* mutations. SNAP-25 is required for survival, but nulls are motile, suggesting some residual neurotransmission. *snap-25* mutant synapses are defective in docking clear-core vesicles compared to rescued animals (50%). Tonic fusion is reduced (85%), and evoked fusion is reduced (75%); however, both forms of exocytosis remains. We find that neuronal S29-OEx rescues *snap-25* null animals. Finally, S29-OEx in neurons supports tonic but not evoked fusion.

The role of SNARE proteins in docking synaptic vesicles has a long contentious history. However, evidence is mounting that SNAREs are required for docking (De Wit et al., 2006; Gutierrez et al., 1997; Sutton et al., 1998; Wu et al., 2012). A null mutation in *C. elegans* syntaxin (*unc-64*), which lacks a functional homolog, completely eliminates docking of synaptic vesicles (Broadie et al., 1995; Hammarlund et al., 2007) and dense core vesicles (Hammarlund et al., 2008; Vogel and Roche, 1999). We show that SNAP-25 hypomorphic and null alleles decrease docking by 50%. Although docking is not eliminated, it is clear that SNAP-25 plays an important function in docking some

population of synaptic vesicles. Since syntaxin is strictly required for docking at these synapses, it is possible that syntaxin engages in docking by two mechanisms—SNAP-25 dependent and independent. The second mechanism may involve UNC-18. However, given the evidence for SNARE-mediated docking (De Wit et al., 2006; Hammarlund et al., 2007, 2008; Wu et al., 2012), we speculate that that the remaining docking observed in this study is likely due to substitution by SNAP-29.

SNAP-25 independent spontaneous fusion is consistently observed across taxa and cell types. To date, six studies have reported spontaneous release in the absence of SNAP-25 (Bronk et al., 2007; Delgado-Martinez et al., 2007; Sørensen et al., 2003; Tafoya et al., 2006; Washbourne et al., 2002). Most have attributed this to nonphysiologically relevant substitution by another protein, but it remains unclear what other SNARE protein(s) are substituting for SNAP-25 and whether this substitution serves a physiological function. In addition, evoked release has been observed at *snap-25* null hippocampal synapses, but responses are infrequent and very small (Bronk et al., 2007). We confirm that SNAP-25 independent evoked release occurs at the *C. elegans* NMJs, adding support to the observations of Bronk *et al*., which until now stood alone.

In this investigation we consider SNAP-25 independent fusion with open minds, taking into consideration rather heretical models for residual activity. In particular, we consider that fusion may occur by a syntaxin/synaptobrevin binary complex. A significant body of literature supports the notion that syntaxin and synaptobrevin interact in a binary complex and fuse liposomes *in vitro* (Bowen et al., 2004; Laage et al., 2000; Liu, 2005; Liu et al., 2006, 2009, 2011; Margittai et al., 1999; McNally et al., 2004; Miller et al., 1996; Nguyen et al., 1995; Pevsner et al., 1994; Woodbury and Rognlien,

2000; Yang et al., 2008; Yersin et al., 2003). Still, no experiments have tested this model *in vivo*. The experiments reported here involved overexpression of the *C. elegans* syntaxin and synaptobrevin genes. The fact that these transgenes did not bypass a requirement for SNAP-25 is not conclusive evidence against binary fusion. However, we expect that the residual fusion is a result of SNAP-29 substitution. If knocking down SNAP-29 protein by acute degradation eliminates fusion, we will be more comfortable dismissing the binary fusion model completely.

We demonstrate that SNAP-29 is capable of facilitating synaptic vesicle fusion *in vivo*. We confirm that *snap-29* is expressed ubiquitously in *C. elegans* including throughout the nervous system. Furthermore, we show that GFP::SNAP-29 is localized at presynaptic terminals in *C. elegans,* supporting reports of synaptic localization at hippocampal synapses (Su et al., 2001). Notably, multiple studies have shown that SNAP-29 copurifies with synaptic vesicles (Holt et al., 2006; Su et al., 2001).

This study is the first to directly show that SNAP-29 is sufficient for increasing tonic fusion in wild-type and *snap-25* null neurons. Moreover, overexpressing SNAP-29 in neurons rescues the viability of *snap-25* null animals. This observation is consistent with the fact that tonic fusion is the most important form of neurotransmitter release at the *C. elegans* neuromuscular junction. These findings are consistent with published reports on mammalian SNAP-29. SNAP-29 binds syntaxin and synaptobrevin with remarkable affinity. In fact, mammalian SNAP-29 is more stable in complex with synaptobrevin and syntaxin than SNAP-23 (Yang et al., 1999). Indeed, the addition of SNAP-29 protein is capable of increasing the fusion of epinephrine filled vesicles from PC12 cells, although not as well as SNAP-23 (Scales et al., 2000).

We report that SNAP-29 does not support synchronous fusion of synaptic vesicles; on the contrary, SNAP-29 significantly decreases evoked amplitude when snap-25 is expressed at native levels (in the wild type) or reduced levels (in the hypomorph). We suggest a competition-model to reconcile the positive effect SNAP-29 has on tonic fusion with the negative effect it has on synchronous fusion. SNARE complexes formed with SNAP-29 may commit synaptic vesicles to the tonic pool, decreasing the number of vesicles available for synchronous fusion. This would explain why evoked fusion is unchanged with overexpression of SNAP-29 in the absence of SNAP-25 but decreased in the presence of SNAP-25. However, it is possible that SNAP-29 directly interacts with canonical SNARE complexes to decrease evoked fusion. In fact, SNAP-29 has been reported to decrease SNARE recycling by competing with alpha-SNAP in cultured neurons (Pan et al., 2005; Su et al., 2001). However, this study only reports a defect in evoked currents with repetitive stimulation. Furthermore, it is difficult to reconcile their model with the fusogenic properties we report here.

In conclusion, this study provides an additional example of SNAP-25 independent fusion at synapses. We speculate that this dependable alternative to the canonical SNARE mediated release may have some functional role at native synapses. We demonstrate that SNAP-29 is effective at fusing synaptic vesicles; however, we have not yet proven it is required for normal fusion.

Methods

Strains

The wild-type *C. elegans* strain was Bristol N2. All strains were maintained at 22 °C on standard nematode growth medium plates seeded with the bacterial strain OP50. Strains used in this study are summarized in Table S2.3

Plasmids and genetics

snap-25 was rescued by the constructs summarized in Figure S2.1. To build the rescuing construct, the native *snap-25* locus was amplified in fragments and cloned into Gateway ENTRY vectors. We were unable to amplify across a 3.5 kb region of the first intron and thus omitted that region. The ENTRY clones included *Psnt-1::snap-25*(exon1) [4-1]; *snap-25genomic*_stop [1-2]; *snap-25*_3'UTR [2-3], and the resulting expression clone was *Psnt-1::snap-25*(minigene)::*snap-25*_UTR. A similar strategy was use to build the tissue specific rescuing constructs; however, in this case we elected to exclude all *snap-25* specific regulatory elements. We amplified *snap-25* cDNA from a worm cDNA library. The resulting expression clone was *Punc-17* or *Punc-17*Δ::*snap-25*cDNA::*let-858*UTR. All constructs were built with this strategy. All transgenes, except those listed as "overexpression," were expressed as MosSCIs at the specified chromosomal locus. Overexpression of *snap-29*, *aex-4,* and syntaxin/synaptobrevin was achieved by injecting 25 ng/ul of the expression clone(s) and 2 ng/ul Pmyo-2::mCherry, diluted in 1 kb promega ladder for stuffer.

Imaging

Worms were immobilized by 25 mM sodium azide and imaged on a confocal microscope (Pascal LSM5; Carl Zeiss Inc.) with a plan-Neofluar 40× 1.3-numerical aperture oil objective (Carl Zeiss). Images of agar and food in the background were removed using Photoshop (Adobe Systems) for clarity.

Electrophysiology

Electrophysiological recordings were performed as previously described (Richmond and Jorgensen, 1999; Richmond et al., 1999) with minor adjustments. Briefly, the animals were immobilized with cyanoacrylic glue (Gluture; WPI, Inc.), and a lateral incision was made to expose the ventral medial body muscles. The preparation was treated with collagenase (type IV; Sigma-Aldrich) for 15 s at a concentration of 0.5 mg/mL. The muscle was voltage-clamped using the whole-cell configuration at a holding potential of −60 mV. All recordings were performed at 21 °C using an EPC-9 patch-clamp amplifier (HEKA) run on an ITC-16 interface (HEKA). Data were acquired using Pulse software (HEKA). Data analysis and graph preparation were performed using Pulsefit (HEKA), Mini Analysis (Synaptosoft), and Stata64 (Stata Co.). Bar graph data are presented as the mean ± standard error of the mean.

Electron microscopy

Electron microscopy and synaptic morphometry were performed as previously described (Watanabe et al., 2013). Briefly, 10 young adults from each genotype were frozen in parallel using a high-pressure freezer (HPM 010, Bal-Tec). The frozen samples

were transferred into vials containing 1% osmium tetroxide (EMS), 1% glutaraldehyde (EMS), 1% milliQ water, and anhydrous acetone. Following the freeze substitution and fixation, the samples were embedded into epon-araldite plastic (Ted Pella). 250–300 contiguous sections were cut and mounted onto formvar-coated single-slot grids and imaged using a transmission electron microscope (H-7100, Hitachi) equipped with a digital camera (SC100, Gatan). Synaptic vesicles, dense projections, and plasma membrane were traced in imageJ using a pen tablet (21UX, Wacom), and their x- and y-coordinates were exported as text files. The number of vesicles and distance from vesicles to dense projections or plasma membrane were calculated using Matlab scripts we developed (Watanabe, Davis, and Jorgensen, unpublished). We defined a synapse as profiles containing a dense projection in this study. Docked vesicles are those that are in the physical contact with membrane. Tethered vesicles are those that are close (within 30 nm) but are not in contact with membrane.

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Supporting Information

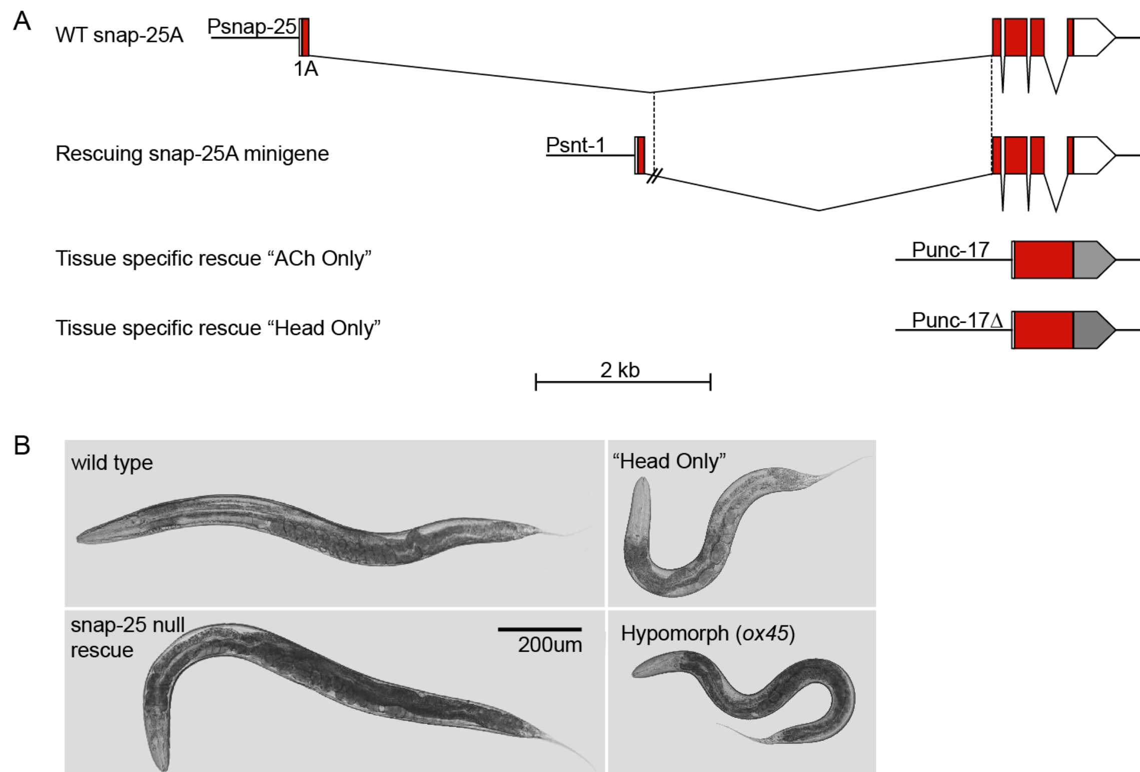

Figure S2.1. Transgene design and rescue. (A) *snap-25* transgenes are aligned to the relevant exon structure in the wild-type locus. The rescuing construct was made with the Psnt-1 promoter the wild-type gene. A 3.5 kb region of the first intron was omitted. Tissue specific strains were made with *Punc-17* variants followed by *snap-25* cDNA and the *let-858* 3’UTR. (B) Confocal images depict representative terminal stage animals. The wild-type transgene fully rescues the *ox528* null. The “head-only” transgene rescues viability, but animals are small and uncoordinated resembling the hypomorph (*ox45*).

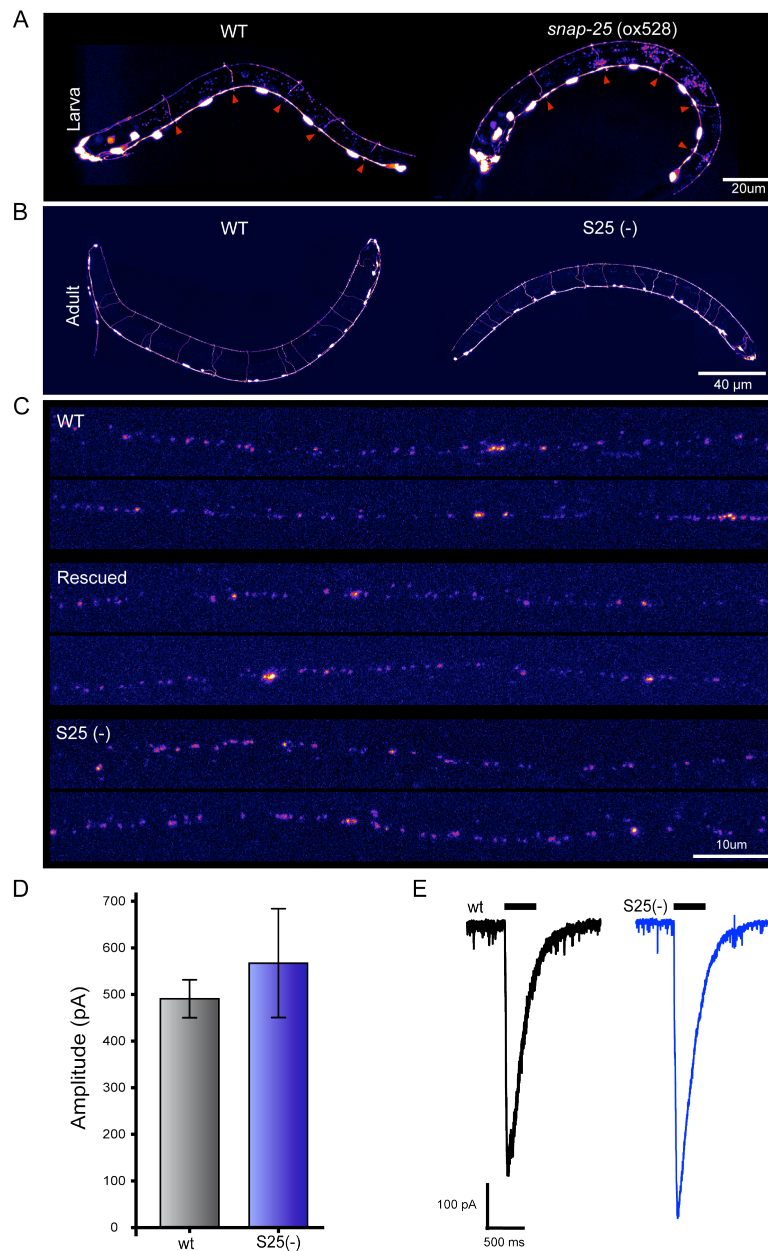

Figure S2.2. The nervous system develops normally in the absence of SNAP-25. (A) Soluble GFP is expressed in the GABA nervous system (*Punc-47*) of homozygous wild type and *snap-25* null (*ox528*) larvae (L1). Neurons in the wild type look indistinguishable from those in the null. (B) Punc-47::GFP is expressed in the wild-type and the "head-only" strains. The gross morphology of the GABA nervous system is the same. (C) Alpha liprin is expressed in GABA neurons (*Punc-47::syd-2*::GFP) and marks dense projections. The synapse number and distribution in the rescued null and "head-only" strains look indistinguishable from the wild type. (D–E) Quantification and representative traces of postsynaptic responses to exogenous GABA. The post synaptic response to GABA from the "head-only" strain (*ok173*) is similar to that of the wild type.

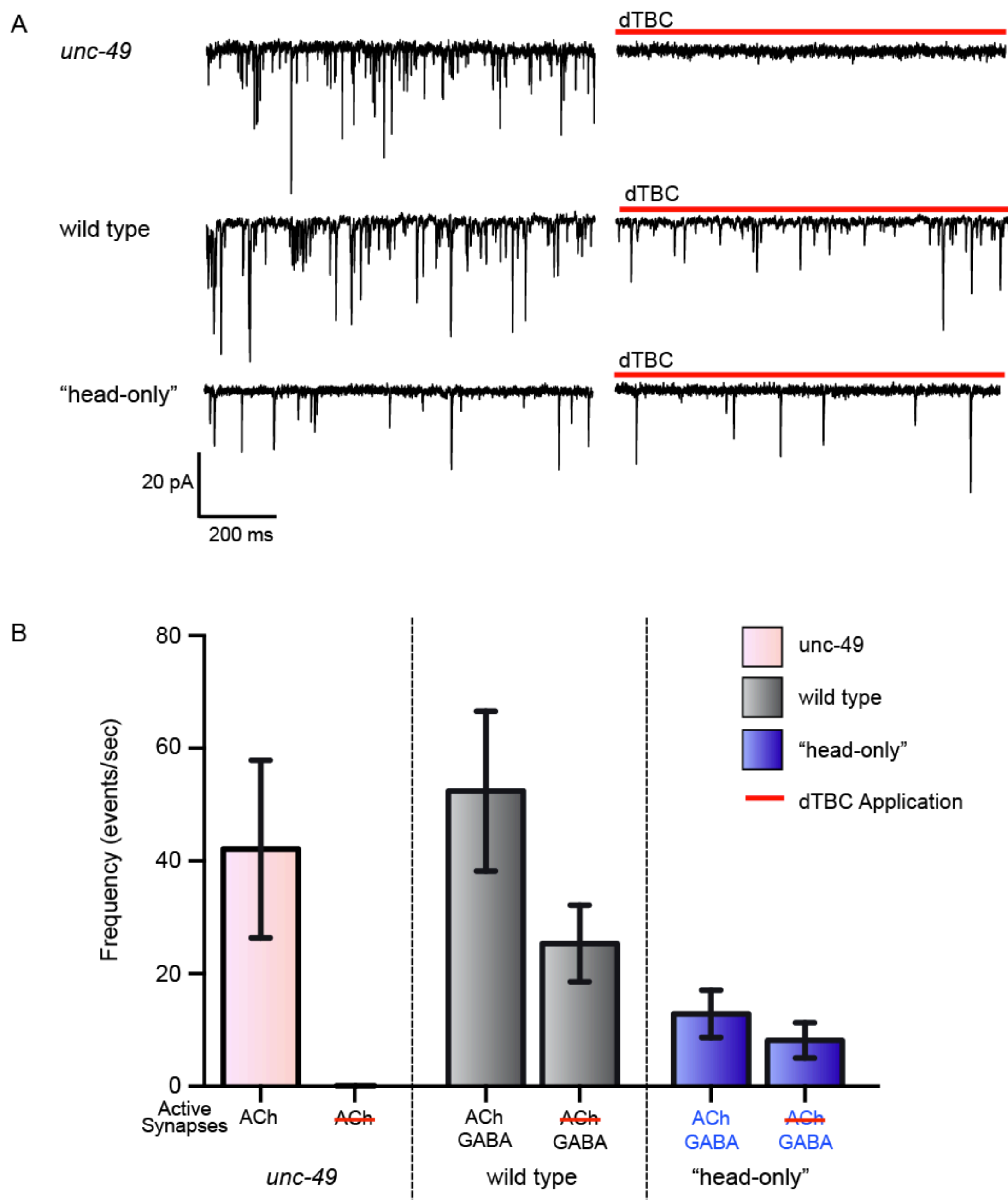

Figure S2.3. *snap-25 (ok173)* independent minis are predominantly GABAergic. (A) Representative miniature currents. The GABA receptor null (unc-49) is compared with the wild type and the "head-only" rescued null (*ok173*). dTBC was applied directly to NMJs by perfusion of the recording chamber. Traces represent activity after stabilization (>60 sec dTBC application). (B) Quantification of average min frequencies before and after drug application. Application of dTBC completely eliminated fusion at unc-49 synapses (42.1 minis/s; n = 8 vs. 0.07 minis/s; n = 8) The frequency of minis at wild-type synapses (52.4 minis/s; n = 8) decreased by 48% with the application of dTBC (25.4 minis/s; n = 8). Likewise, SNAP-25 independent minis in the "head-only" (*ok173*) strain (10.3 minis/s; n = 5) decreased in frequency by 40% with dTBC application (6.2 minis/s; n = 5).

Table S2.1. Qbc-SNARE protein identity in *C. elegans*

SNAP-25	Homologs	Identity (%)
Full protein sequence	Qbc-SNAREs	
SNAP-25b	SNAP-29	22
SNAP-25b	AEX-4	22
SNAP-25a	SNAP-29	19
SNAP-25a	AEX-4	20
SNARE motif only (Qb/c)	Qbc-SNAREs	
SNAP-25aQb	SNAP-29Qb	30
SNAP-25aQb	AEX-4Qb	23
SNAP-25aQc	SNAP-29Qc	32
SNAP-25aQc	AEX-4Qc	31
SNARE motif only (Qb)	Qb-SNAREs	
SNAP-25Qb	GOS-28	12
SNAP-25Qb	MEMB-1	14
SNAP-25Qb	MEMB-2	10
SNAP-25Qb	SEC-20	13
SNAP-25Qb	VTI-1	19
SNARE motif only (Qc)	Qc-SNAREs	
SNAP-25Qc	NBET-1	27
SNAP-25Qc	SYX-6	25
SNAP-25Qc	USE-1	7

Table S2.2 *snap-25* alleles

Mis-sense mutations	Change in SNAP-25A	Change in SNAP-25B	Flanking Sequence
ox45	M1K	(n/a)	TGAACGGTCAGACAACAAGCAATAA [T>A] GTCAGGAGATGATGATATTCCAGAG
ys7	G43A	G70A	CTTTGCGAAGAAAGTAAAGAGGCTG [G>C] AATCAAAACTTTGGTTATGCTCGAT
md2112	C60Y	C87Y	TGGTTTTCAGAACAACTTGAGCGTT [G>A] CGAAGGTGCTCTTGATACAATCAAT
js20	K94R	K121R	GGTTTGTGTGTATTGCCATGGAACA [A>G] GACCGATGACTTTGAAAAGACGGAA
Deletions	**Size**	**Exons Affected**	**Flanking Sequence**
ok173	1251 bp	exons 1B-5	TGAGCTTAAAGGGCTTAATGTGGGA [Δ1251 bp] TAGCTGAAACTTGAAACACCTGGTA
gk312	1379 bp	exon 1A	AAAAAGTACCTTTTTCGAAAAAAAT [Δ1379 bp] TTTTTCTCGTTCACTCACGGGGAAA
gk333	869 bp	exon 1A	ACAGGTGCCTTCTCATTTCCCATTT [Δ869 bp] GAATGTTCTGATTTTTCTACACTCA
gk322	882 bp	exon 1A	TTTCACCATTTTTTCATCTTAAAAA [Δ882 bp] TACGCCAAAATAGTCCCCGCCTTTT
ox528	*10743* bp	exons 1A-5	CGAAGGAAATACGGGATAAGGCGA [Δ10743 bp] GGTGTATTGGAAATACAAACACTT
Tc1 Insertions		**Exon Affected**	**Flanking Sequence**
md1088		exon 3	CATCGATGGGACCTCAAGGAGGATA [Tc1] TATTACCAAGTAAGTTTTATTAGAA
md1136		exon 3	CATCGATGGGACCTCAAGGAGGATA [Tc1] TATTACCAAGTAAGTTTTATTAGAA
md1152		exon 3	CATCGATGGGACCTCAAGGAGGATA [Tc1] TATTACCAAGTAAGTTTTATTAGAA
md1192		exon 3	CATCGATGGGACCTCAAGGAGGATA [Tc1] TATTACCAAGTAAGTTTTATTAGAA

Table S2.3 *C. elegans* strains

Strain	Genotype
N2	*wild type*
EG5425	*oxIs364[Punc-17::ChR2::mCherry] X*
NM2715	*jsIs826[Punc-47::TeTxLC::GFP] X*
EG8160	*oxEx1986[Prab-3::snap-29 ; Pmyo-2::mCherry]*
EG8164	*oxIs364[Punc-17::ChR2::mCherry] X ; oxEx1990[Prab-3::snap-29 ; Pmyo-2::mCherry]*
EG1306	*oxIs12[Punc-47:GFP, lin-15+] X ; lin-15(n765ts) X*
EG6497	oxSi302[Punc-47::syd-2::tagRFP-T unc-119(+)] IV
EG45	*snap-25 (ox45) V*
EG8133	*snap-25 (ox45) V ; oxIs364[Punc-17::ChR2::mCherry] X*
EG8163	*snap-25 (ox45) V ; oxEx1989[Prab-3::snap-29 ; Pmyo-2::mCherry]*
EG8167	*snap-25 (ox45) V ; oxIs364[Punc-17::ChR2::mCherry] X ; oxEx1993[Prab-3::snap-29 ; Pmyo-2::mCherry]*
EG7757	oxSi652[Punc-17::snap-25a(cDNA); unc-119+; 5605] II ; snap-25 (ox528 [Prps-27::neoR]) V
EG7759	oxSi498[Punc-17(deltaCord):: snap-25acDNA; CBunc-119+ 10882] IV ; snap-25 (ox528 [Prps-27::neoR]) V
EG8018	oxSi649[Psnt-1:: snap-25minigene; unc-119+] II ; oxSi302[Punc-47::syd-2::tagRFP-T unc-119(+)] IV ; snap-25(ox528 [Prps-27::neoR]) V
EG8019	oxSi652[Punc-17:: snap-25a(cDNA); unc-119+] II ; oxSi302[Punc-47::syd-2::tagRFP-T unc-119(+)] IV ; snap-25(ox528 [Prps-27::neoR]) V
EG8020	oxSi649[Psnt-1::snap-25minigene; unc-119+] II ; snap-25(ox528 [Prps-27::neoR]) V ; *oxIs364[Punc-17::ChR2::mCherry]* X
EG8036	oxSi498[CBunc-119;Punc-17(deltaCord):: snap-25a(cDNA), 10882] IV ; snap-25(ox528 [Prps-27::neoR]) V ; *oxIs364[Punc-17::ChR2::mCherry] X*
EG8161	oxSi649[Psnt-1::snap-25minigene; unc-119+] II ; snap-25 (ox528 [Prps-27::neoR]) ; oxEx1987[Prab-3::snap-29 ; Pmyo-2::mCherry]
EG8162	oxSi498[CBunc-119;Punc-17(deltaCord)::snap-25a(cDNA), 10882] IV ; snap-25(ox528 [Prps-27::neoR]) ; oxEx1988[Prab-3::snap-29 ; Pmyo-2::mCherry]
EG8165	oxSi649[Psnt-1::snap-25minigene; unc-119+] II ; snap-25 (ox528 [Prps-27::neoR]) V ; *oxIs364[Punc-17::ChR2::mCherry] X* ; oxEx1991[Prab-3::snap-29 ; Pmyo-2::mCherry]
EG8166	oxSi498[CBunc-119;Punc-17(deltaCord)::snap-25a(cDNA)] IV ; snap-25(ox528 [Prps-27::neoR])V ; *oxIs364[Punc-17::ChR2::mCherry] X* ; oxEx1992[Prab-3::snap-29 ; Pmyo-2::mCherry]
EG8181	oxSi498[CBunc-119;Punc-17(deltaCord)::snap-25a(cDNA)] IV ; snap-25(ox528 [Prps-27::neoR]) V ; oxEx2001[Prab-3::aex-4 ; Pmyo-2::mCherry]

Table S2.3 Continued

Strain	Genotype
EG8187	oxSi498[CBunc-119;Punc-17(deltaCord)::snap-25acDNA, 10882] IV ; snap-25 (ox528 [Prps-27::neoR]) V ; jsIs826[punc-47::TeTxLC::GFP] X
EG8230	snt-1(md290) II ; oxSi498[CBunc-119;Punc-17(deltaCord)::snap-25acDNA, 10882] IV ; snap-25 (ox528 [Prps-27::neoR]) V
EG8269	snap-25 (ox528 [Prps-27::neoR]) / oxTi417[Peft-3:mCherry:tbb-2UTR] V
EG8270	snap-25 (ox528 [Prps-27::neoR]) / oxTi417[Peft-3:mCherry:tbb-2UTR] V ; oxIs12[Punc-47:GFP, lin-15+] X
EG8271	snap-25 (ox528 [Prps-27::neoR]) V ; oxEx2011[Prab-3::snap-29 ; Pmyo-2::mCherry]
EG5567	oxIs554[Punc-17::snap-25a(cDNA)::let858] IV ; snap-25(ok173) V
EG6891	unc-119(ed3) III ; oxSi498[CBunc-119;Punc-17(deltaCord)::snap-25acDNA, 10882] IV ; snap-25(ok173) V

CHAPTER 3

SYNTAXIN N-TERMINAL PEPTIDE MOTIF IS AN INITIATION FACTOR FOR THE ASSEMBLY OF THE SNARE-SEC1/MUNC18 MEMBRANE FUSION COMPLEX

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FEATURE ARTICLE

Syntaxin N-terminal peptide motif is an initiation factor for the assembly of the SNARE–Sec1/Munc18 membrane fusion complex

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Intracellular membrane fusion is mediated by the concerted action of *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and Sec1/Munc18 (SM) proteins. During fusion, SM proteins bind the N-terminal peptide (N-peptide) motif of the SNARE subunit syntaxin, but the function of this interaction is unknown. Here, using FRET-based biochemical reconstitution and *Caenorhabditis elegans* genetics, we show that the N-peptide of syntaxin-1 recruits the SM protein Munc18-1/nSec1 to the SNARE bundle, facilitating their assembly into a fusion-competent complex. The recruitment is achieved through physical tethering rather than allosteric activation of Munc18-1. Consistent with the recruitment role, the N-peptide is not spatially constrained along syntaxin-1, and it is functional when translocated to another SNARE subunit SNAP-25 or even when simply anchored in the target membrane. The N-peptide function is restricted to an early initiation stage of the fusion reaction. After association, Munc18-1 and the SNARE bundle together drive membrane merging without further involving the N-peptide. Thus, the syntaxin N-peptide is an initiation factor for the assembly of the SNARE-SM membrane fusion complex.

Intracellular membrane fusion is the basis of a wide range of fundamental biological processes, including organelle maintenance, hormone secretion, and inside–outside distribution of receptors and transporters. The merging of intracellular membrane bilayers is mediated by a fusion complex comprised of SNAREs and Sec1/Munc18 (SM) proteins (1). The core of the fusion machinery is the trans-SNARE complex (SNAREpin) formed by the pairing of the vesicle-rooted SNARE (v-SNARE) with the target membrane-associated SNAREs (t-SNAREs) (2–5). N- to C-terminal zippering of the trans-SNARE complex brings two membranes into close apposition and helps to overcome the energy barrier for fusion (6–10). SM proteins are soluble factors of 60–70 kDa that directly interact with their cognate trans-SNARE complexes to promote the speed and specificity of a fusion reaction (11–14).

Each fusion pathway in the cell requires a specific subset of SNAREs and SM proteins (15). The most intensely studied form of intracellular membrane fusion is calcium-triggered neurotransmitter release at the chemical synapse, which serves as the brain's major form of cell–cell communication (15–19). Neurotransmitter secretion is mediated by the fusion of exocytic vesicles with the plasma membrane and requires the v-SNARE vesicle-associated membrane protein 2 (VAMP2; also known as synaptobrevin-2), the t-SNAREs syntaxin-1 and soluble *N*-ethylmaleimide-sensitive factor attachment protein (SNAP)-25, and the SM protein Munc18-1/nSec1 (UNC-18 in nematodes and ROP in flies) (20–28).

The interaction between SNAREs and SM proteins involves multiple binding modes. The primary target of SM proteins is believed to be the four-helix SNARE bundle (29–31). Assembled from the SNARE motifs and the transmembrane domains of t- and v-SNAREs (4, 5), the SNARE bundle is the principle driving force for membrane fusion. Although individual SNARE subunits exhibit heterogeneous conformations, the four-helix structure of assembled SNARE bundles is universal across pathways or species (15, 32).

A second SM protein binding target is the N-terminal peptide motif (N-peptide) of the t-SNARE subunit syntaxin. The N-peptide, located at the extreme N terminus of syntaxin, is characterized by two or three charged residues followed by a hydrophobic leucine or phenylalanine residue. The hydrophobic residues insert into a peripheral pocket on the cognate SM protein (Fig. 1 *A* and *B*) (33, 34). First shown in the Golgi and endocytic SNAREs (35, 36), the N-peptide binding mode was later found to be widespread among SM–syntaxin pairs (37–40). Functionally, the four-helix SNARE bundle and the syntaxin-1 N-peptide constitute a minimal complement for Munc18-1 binding and activation, whereas the rest of the SNARE sequences, including the syntaxin-1 Habc domain, are dispensable (31). However, it remains unknown how the short N-peptide motif acts in concert with Munc18-1 and the SNARE bundle to drive fusion.

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Several models could explain the role of the N-peptide in synaptic vesicle fusion. First, the N-peptide may provide an oriented binding surface to stabilize an otherwise low-affinity interaction between Munc18-1 and the SNARE complex. Second, the N-peptide could allosterically activate the SM protein. Third, conversely, the SM protein could allosterically activate a conformational change in syntaxin. Fourth, the N-peptide may simply recruit the SM protein to its cognate SNARE complex (13, 29, 41–43). Here, we tested these models in reconstituted fusion assays and then confirmed our conclusions with genetic analysis of *Caenorhabditis elegans* exocytosis in vivo. We found that the N-peptide physically recruits Munc18-1 to the SNARE bundle to facilitate their assembly. After association, Munc18-1 and the SNARE bundle together drive the merging of membrane bilayers without further involvement of the N-peptide. We conclude that the N-peptide acts as an initiation factor for the assembly of the fusion-competent complex.

Results

Spacing Between the N-Peptide and the SNARE Motifs Is Flexible. To determine the function of the syntaxin N-peptide in membrane fusion, we took advantage of a FRET-based reconstituted liposome fusion assay that recapitulates SNARE–Munc18-1–dependent synaptic vesicle fusion (13). Neuronal SNAREs—syntaxin-1, SNAP-25, and VAMP2—were reconstituted into lip-

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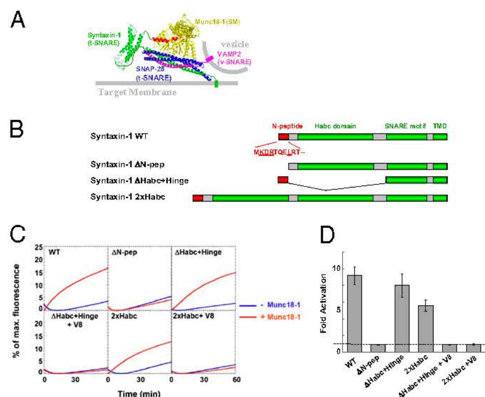

Fig. 1. The spacing between the N-peptide and the SNARE motifs is flexible. (*A*) Model of the SNARE–SM fusion complex. The SM protein Munc18-1 binds to both the SNARE bundle and the N-peptide motif of syntaxin-1. Modeled from the atomic structures of the SNARE core bundle (4, 5), the Habc domain of syntaxin-1 and the SM–N-peptide complex (33, 34). The model is intended to depict the two primary modes of SM–SNARE interaction. Yellow, Munc18-1 (SM protein); green, syntaxin-1 (t-SNARE heavy chain); blue, SNAP-25 (t-SNARE light chains, only the SNARE motifs are shown); pink, VAMP2 (v-SNARE); red, the N-peptide of syntaxin. The structures were edited in PyMol (DeLano Scientific LLC). (*B*) Diagrams of WT syntaxin-1, a ΔN-peptide syntaxin-1 mutant, a ΔHabc+Hinge syntaxin-1 mutant in which the spacing sequence between the N-peptide and the SNARE motif (amino acids 21–194) was removed, and a 2×Habc syntaxin mutant in which a second copy of the Habc domain (amino acids 27–146) was inserted into syntaxin-1. The first 10 aa of the N-peptide sequence are shown with the characteristic residues underlined. TMD, transmembrane domain. (*C*) Fusion of the t-SNARE liposomes containing WT or mutant syntaxin-1 with the VAMP2 or VAMP8 (V8) liposomes in the absence or presence of 5 μM Munc18-1. The slight fluorescence decrease at the beginning of the basal reaction is caused by the temperature change. (*D*) Fold increase in the initial fusion rates of the reactions in *C*. The dashed line indicates the basal fusion level (with no Munc18-1 activation). Error bars indicate SD.

osomes at physiologically relevant surface densities. WT t- and v-SNAREs drove a slow basal fusion reaction that was strongly accelerated by Munc18-1 (approximately ninefold increase in initial rate) (Fig. 1 *C* and *D*). As previously observed (13), deletion of the N-peptide motif from syntaxin-1 selectively eliminated the activation of fusion by Munc18-1 without affecting the basal fusion rate (Fig. 1 *B*–*D*).

The N-peptide and the four-helix SNARE bundle comprise a minimal complement for Munc18-1 binding and activation (31). How are these two Munc18-1 binding modes coordinated? It is possible that the N-peptide and the SNARE bundle bind simultaneously to Munc18-1 such that both of the interactions contribute to the overall stability of the complex. In agreement with this model, the SNARE–Munc18-1 binding affinity is significantly reduced when either the N-peptide or the SNARE bundle binding is disrupted (13, 44, 45).

To test the concurrent binding model, we examined whether Munc18-1 can activate conformationally constrained SNARE mutants that do not allow Munc18-1 to simultaneously grasp both the N-peptide and the SNARE bundle. Because the N-peptide and the SNARE bundle are recognized by different interfaces of Munc18-1, molecular modeling shows that a flexible hinge is required for Munc18-1 to engage in simultaneous binding (Fig. 1*A*). Previously, we found that deletion of an Habc-containing region (amino acids 34–171) from syntaxin-1 had no effect on Munc18-1 activation of fusion (31). The ΔHabc syntaxin-1 mutant, however, retains a flexible hinge of 27 residues (amino acids 21–33 and 172–185). Here, we removed the remaining flexible sequence to obtain a ΔHabc+Hinge syntaxin mutant (Δ21–194) that, structurally, is unlikely to satisfy both binding modes at one time (Fig. 1 *A* and *B*). If a concurrent binding mechanism is involved, we expect that SNARE complexes containing this ΔHabc+Hinge syntaxin-1 mutant would not be activated by Munc18-1. Surprisingly, when reconstituted into liposomes, the ΔHabc+Hinge SNARE mutant drove a basal fusion reaction that was activated by Munc18-1 to a level comparable with that of WT SNAREs (Fig. 1 *C* and *D*), suggesting that a flexible hinge is not required. When VAMP2 was substituted with VAMP8/endobrevin, a noncognate v-SNARE isoform involved in lysosomal/late endosomal fusion (46), Munc18-1 stimulation was abolished (Fig. 1 *C* and *D*). This v-SNARE selectivity implies that the SNARE complexes containing the ΔHabc+Hinge mutant are regulated by Munc18-1 through the same mechanism as WT SNAREs rather than introducing a novel fusogenic mechanism independent of SNARE complex formation.

Increasing the spacing between the N-peptide and the SNARE motif of syntaxin-1 does not disrupt Munc18-1 stimulation of fusion either. We inserted a second copy of the three-helix Habc domain (amino acids 27–146) into WT syntaxin-1 such that the hinge between the N-peptide and the SNARE motif was doubled in length (from ∼9 to ∼18 nm) (Fig. 1*B*). Duplication of the Habc domain is expected to generate substantial molecular crowding between the N-peptide and the SNARE bundle and would likely alter the cooperative binding. However, we found that the fusion reaction mediated by this 2×Habc SNARE mutant was still robustly activated by Munc18-1 (Fig. 1 *C* and *D*). Again, when VAMP2 was substituted with the noncognate v-SNARE VAMP8, Munc18-1 acceleration of fusion was eliminated (Fig. 1 *C* and *D*).

Importantly, all of the SNARE pairs tested here elicited comparable basal fusion reactions (Fig. 1*C*), implying that the SNARE bundle assembly remained intact. Thus, the position of the N-peptide on syntaxin is flexible. This is incompatible with the coincident binding model, which predicts a conformationally constrained configuration of the SNARE–Munc18-1 complex. Rather, our data suggest that the N-peptide motif and the SNARE bundle bind to Munc18-1 consecutively en route to fusion.

N-Peptide Is Fully Functional When Translocated to SNAP-25. The spatial flexibility of the N-peptide along the length of syntaxin suggests that Munc18-1 does not allosterically modulate syntaxin upon binding. To test this directly, we fused the N-peptide motif to the N terminus of SNAP-25 and coreconstituted this N-peptide–SNAP-25 chimera with a syntaxin ΔN mutant (lacking the N terminus of syntaxin-1) into liposomes (Fig. 2 *A* and *B*). Strikingly, whereas the SNAREs containing the syntaxin ΔN mutant were not activated by Munc18-1, the addition of the N-peptide motif to SNAP-25 fully restored Munc18-1 stimulation (Fig. 2 *B*–*D*). Substitution of VAMP2 with the noncognate v-SNARE VAMP8 resulted in complete loss of Munc18-1 activation (Fig. 2 *B*–*D*). Thus, the N-peptide motif functions equally well on either subunit of the t-SNARE complex. These data are consistent with the spatial flexibility of the N-peptide on syntaxin-1 and further support that the N-peptide and SNARE bundle bind to Munc18-1 consecutively in the fusion reaction.

N-Peptide Is Dispensable After Munc18-1 Is Loaded onto the SNARE Complex. How can the N-peptide regulate fusion with such spatial flexibility? The simplest explanation is that the N-peptide merely initiates the SNARE–Munc18-1 assembly process, with no involvement in subsequent fusion steps. We reasoned that, if the N-peptide only acts at an early stage of the fusion reaction, it would not be needed after the SNARE–Munc18-1 fusion complex is formed. To test this possibility, we introduced a Tobacco Etch Virus (TEV) protease cleavage site between the N-peptide and the SNARE motif of syntaxin-1 to obtain a syntaxin-1 TEV variant (Fig. 3*A*). When reconstituted into liposomes, syntaxin-1 TEV paired with SNAP-25 to elicit a basal fusion reaction that was fully activated by Munc18-1 (Fig. 3 *B*–*D*). TEV protease, a highly active

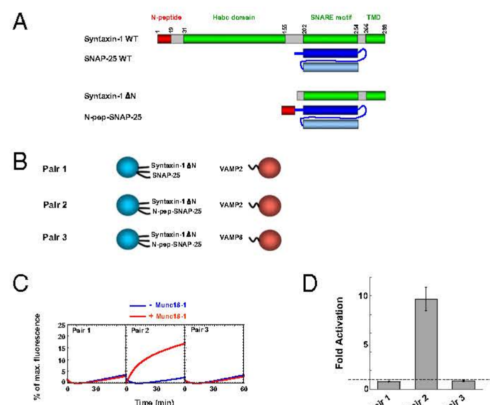

Fig. 2. The N-peptide is fully functional when translocated to SNAP-25. (*A*) Diagrams of WT syntaxin-1, a ΔN syntaxin-1 mutant that lacks the N-terminal domain (amino acids 1–150), WT SNAP-25, and an N-peptide–SNAP-25 (N-pep-SNAP-25) chimera in which the syntaxin N-peptide motif (amino acids 1–30) was fused to the N terminus of SNAP-25. (*B*) Illustrations of the liposome fusion pairs. (*C*) Fusion of the WT or mutant t-SNARE liposomes with the VAMP2 or VAMP8 (V8) liposomes in the absence or presence of 5 μM Munc18-1. (*D*) Fold increase in the initial fusion rates of the reactions in *C*. The dashed line indicates the basal fusion level (with no Munc18-1 activation). Error bars indicate SD.

cysteine protease, completely removed the N-peptide motif from syntaxin-1 TEV during 1 h of digestion at 4 °C (Fig. S1*A*). As expected, when the N-peptide was deleted before the mixing of the t-SNARE liposomes with Munc18-1 and the v-SNARE liposomes, the fusion reaction was not stimulated by Munc18-1 (Fig. 3 *B–D*).

Next, we incubated the TEV-cleavable t-SNARE liposomes with Munc18-1 and the v-SNARE liposomes for 1 h at 4 °C, which allowed the fusion complexes to assemble and accumulate without progressing to drive membrane merging (13). Then, the N-peptide motif was removed from the SNAREs by TEV protease digestion, also carried out at 4 °C. When the temperature was elevated to 37 °C, we found that the fusion reaction was fully activated by Munc18-1, although the N-peptide was absent from the SNARE liposomes (Fig. 3 *B–D*). Complete proteolysis of the t-SNARE liposomes was confirmed by SDS/PAGE and Coomassie blue staining (Fig. S1*A*). To preclude the possibility that a small fraction of the SNARE complexes was protected from TEV cleavage by Munc18-1 binding, we also examined SNARE digestion in detergent micelles. In solution, the formation of the SNARE–Munc18-1 complex also requires the N-peptide motif, and importantly, all SNARE molecules are bound to Munc18-1 (13, 45). We found that the TEV protease completely cleaved syntaxin-1 TEV in the presence of Munc18-1 (Fig. S1*B*), indicating that Munc18-1 binding does not hinder the proteolysis of the N-peptide.

These results suggest that, although critical to the assembly of Munc18-1 with the SNAREs, the N-peptide function is restricted to an early initiation stage of the fusion reaction. After association, Munc18-1 and the SNARE bundle together drive membrane merging without further participation of the N-peptide. This finding is consistent with our observation that Munc18-1 consecutively binds the N-peptide and the SNARE bundle during fusion, and it agrees with a previous model that the SNARE bundle constitutes the primary target of Munc18-1 (47). Thus, the syntaxin N-peptide serves as an initiation factor for the formation of the fusion-competent complex.

N-Peptide Is Not an Allosteric Activator of Munc18-1. How does the N-peptide initiate the fusion complex assembly? It is possible that the N-peptide induces a transient conformational change in Munc18-1; for example, the central cavity domain could become receptive to interactions with the SNARE bundle. This positive cooperative mechanism is similar to the allosteric activation of enzymes as described by the Monod–Wyman–Changeux theory

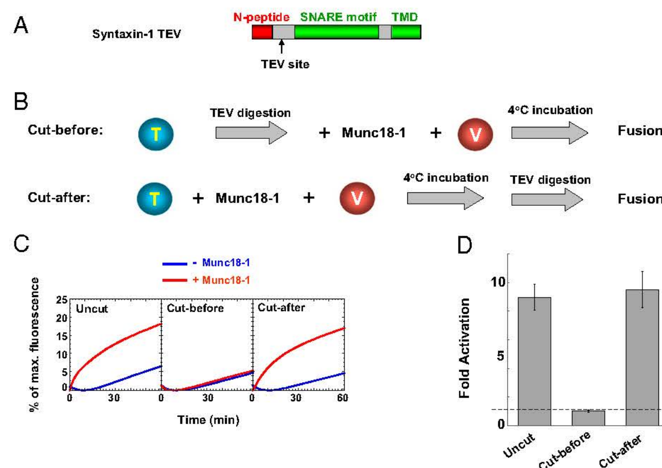

Fig. 3. The N-peptide is dispensable after Munc18-1 is loaded onto the SNARE complex. (*A*) Diagram of the syntaxin-1 TEV variant, in which the spacing sequence (amino acids 31–194) between the N-peptide motif and the SNARE motif was replaced with a TEV cleavage site (ENLYFQG). (*B*) Illustrations of the fusion procedures. (*C*) Fusion of the indicated t- and v-SNARE liposomes in the absence or presence of 5 μM Munc18-1. (*D*) Fold increase in the initial fusion rates of the reactions in *C*. The dashed line indicates the basal fusion level (with no Munc18-1 activation). Error bars indicate SD.

(48). Alternatively, the N-peptide may physically recruit Munc18-1 to facilitate its association with the metastable SNARE bundle.

If the N-peptide binding activates Munc18-1 through an allosteric conformational change, then it should still be capable of binding Munc18-1 and promoting fusion when disconnected from the SNARE bundle. However, we found that a soluble N-peptide fragment (amino acids 1–45) failed to support the enhancement of fusion by Munc18-1. No activation of the fusion reaction was observed, even when the N-peptide fragment was added at a 20-fold molar excess to Munc18-1 (Fig. S2 *A* and *B*). Moreover, the soluble N-peptide fragment had little effect on Munc18-1 stimulation of WT SNAREs (Fig. S2 *A* and *B*). These negative results are likely due to the intrinsically low binding affinity between Munc18-1 and the soluble N-peptide fragment (13, 44). To augment the association, we next engineered an autoregulatory Munc18-1 variant in which the N-peptide motif is directly fused to the N terminus of Munc18-1 through a flexible hinge (Fig. 4*A*). If Munc18-1 function involves allosteric conformational activation, the intramolecular N-peptide is expected to lock Munc18-1 in a constitutively on state, even in the absence of a syntaxin-linked N-peptide. However, we found that the ectopic N-peptide failed to restore Munc18-1 activation to the SNARE complexes containing the syntaxin-1 ΔN-peptide mutant (Fig. 4 *B* and *C*). Unexpectedly, when added to the fusion reaction of WT SNAREs, the N-peptide–Munc18-1 molecule was completely incapable of stimulating fusion (Fig. 4 *B* and *C*). This suggests that the ectopic N-peptide motif acts as a dominant negative inhibitor by competing with the native syntaxin-linked N-peptide for Munc18-1 binding. To rule out the possibility that the N-peptide linkage causes Munc18-1 misfolding, we next examined the ability of the N-peptide–Munc18-1 variant to bind the closed syntaxin monomer, a specialized binding mode that does not critically depend on the N-peptide (44). We found that both WT Munc18-1 and the N-peptide–linked Munc18-1 variant bound equally well to the syntaxin-1 monomer, implying that the addition of an ectopic N-peptide motif does not alter the overall structure of Munc18-1 (Fig. S3).

These results show that the N-peptide is not functional when disconnected from the SNARE membranes, although it remains bound to Munc18-1. Thus, the N-peptide does not promote SNARE–Munc18-1 association through allosteric activation of Munc18-1. Rather, our data support a model whereby the N-peptide physically recruits Munc18-1 to the SNARE bundle to initiate their assembly.

Membrane-Anchored N-Peptide Can Act *in Trans* to Recruit Munc18-1 and Activate Membrane Fusion. We reasoned that, if the role of the N-peptide is simply to recruit Munc18-1, localizing it on the membrane surface near the SNARE bundle (but with no direct connection) would also facilitate SNARE–Munc18-1 association. To test this hypothesis, we designed a split syntaxin system in which the N terminus (N-peptide + Habc) and the C terminus (SNARE motif) of syntaxin-1 are present on separate molecules—in essence, severing the head from the trunk (Fig. 5 *A* and *B*). To maintain the spacing, the head fragment (containing the N-peptide and the Habc domain) was fused to a generic α-helix derived from the bacterial protein TolA and anchored to the lipid bilayer through the transmembrane segment of syntaxin-1 (Fig. 5*A*). Next, the head and trunk fragments of syntaxin-1 were independently reconstituted into liposomes with SNAP-25. As expected, the N-terminal head fragment supported neither basal fusion nor Munc18-1 activation because of a lack of the SNARE motif (Fig. 5 *C* and *D*). The trunk fragment (containing the syntaxin-1 SNARE motif), however, supported basal levels of fusion, but the fusion was not stimulated by Munc18-1 (Fig. 5 *C* and *D*). When both the head and trunk fragments of syntaxin-1 were reconstituted into the same liposomes with SNAP-25 (at a 1:1:1 molar ratio), basal fusion was observed in the absence of Munc18-1 (Fig. 5 *B* and *C*). Strikingly, the fusion reaction mediated by this split syntaxin pair was robustly activated by Munc18-1 (Fig. 5 *C* and *D*). These data suggest that the two syntaxin fragments reconstituted WT syntaxin-1 activity.

Therefore, the membrane-anchored N-peptide, although disconnected from the SNARE motifs, can act *in trans* to promote

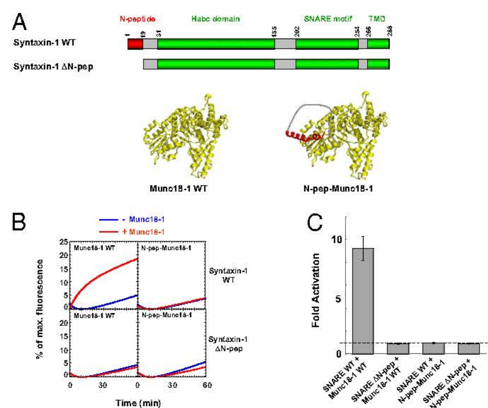

Fig. 4. The N-peptide is not an allosteric activator of Munc18-1. (*A Upper*) Diagrams of WT and ΔN-peptide (ΔN-pep) syntaxin-1 molecules. (*Lower*) Structural models of WT Munc18-1 and the N-peptide–Munc18-1 (N-pep-Munc18-1) variant in which the syntaxin N-peptide motif (red) is fused to the N terminus of Munc18-1 (yellow) through a flexible hinge (gray). The hinge contains 10 glycine residues and an HA epitope (YPYDVPDYA). (*B*) Fusion of the indicated t- and v-SNARE liposomes in the absence or presence of 5 μM WT or N-peptide–linked Munc18-1. (*C*) Fold increase in the initial fusion rates of the reactions in *B*. The dashed line indicates the basal fusion level (with no Munc18-1 activation). Error bars indicate SD.

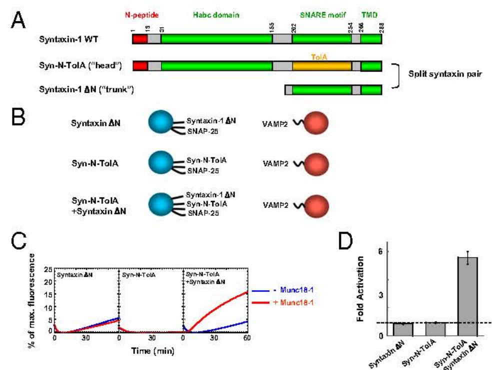

Fig. 5. Membrane-anchored N-peptide can act *in trans* to recruit Munc18-1 and activate fusion. (*A*) Diagrams of WT syntaxin-1 and a split syntaxin pair. (*B*) Illustrations of the liposome fusion pairs. The syn-N-TolA (head) chimera was created by replacing the SNARE motif of syntaxin-1 with a generic α-helix derived from the bacterial protein TolA. (*C*) Fusion of the indicated t- and v-SNARE liposomes in the absence or presence of 5 μM Munc18-1. (*D*) Fold increase in the initial fusion rates of the reactions in *C*. The dashed line indicates the basal fusion level (with no Munc18-1 activation). Error bars indicate SD.

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membrane fusion. These data further support that the N-peptide physically recruits Munc18-1 to the SNARE bundle.

Split Syntaxin Pair Mediates Synaptic Vesicle Fusion in Vivo. To test our results in an intact physiological system, we examined synaptic vesicle fusion at the neuromuscular junction in the nematode *C. elegans*. The synaptic fusion machinery in nematodes is conserved with that of mammals, requiring both syntaxin/UNC-64 and the SM protein Munc18-1/UNC-18 (23, 49). Moreover, worms with N-peptide mutations exhibit uncoordinated phenotypes (38, 39), similar to the UNC-18 mutant animals (23). Syntaxin null animals (*js115*) arrest at the first larval (L1) stage immediately after hatching (Fig. 6*B*) (50, 51). The null phenotype was fully rescued by expressing a WT syntaxin transgene under its native promoter (Fig. 6*B*). To test whether the N-peptide of nematode syntaxin can regulate vesicle fusion when detached from the SNARE motif, we engineered two transgenic strains expressing either the N-terminal head (N-peptide + Habc) or the trunk (SNARE motif) fragment of syntaxin in the null background (Fig. 6*A* and Figs. S4 and S5). Consistent with the in vitro reconstitution data, neither of the transgenes rescued the syntaxin null phenotype (Fig. 6*B*). However, when both the head and trunk fragments of syntaxin were coexpressed in the null background, the transgenic animals grew to full size and exhibited functional, although uncoordinated, locomotion (Fig. 6*B*).

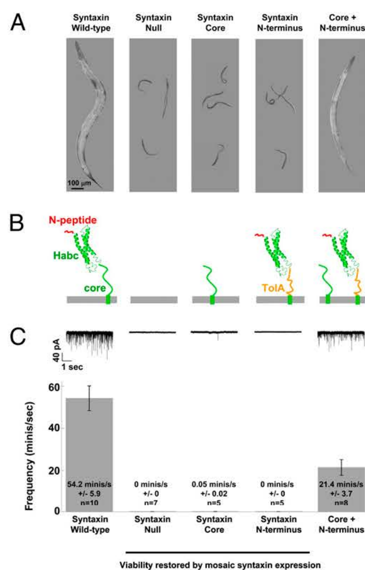

Fig. 6. The split syntaxin pair mediates synaptic vesicle fusion in vivo. (*A*) Confocal images depicting age-matched null and syntaxin-rescued animals. Syntaxin null animals arrest at the L1 larval stage. The WT syntaxin transgene fully restored viability, coordination, and health. By contrast, neither of the split syntaxin fragments rescued the null phenotype. However, when expressed together, the split syntaxin transgenes (N terminus + SNARE motif) fully rescued animal viability. It should be noted that, although the split syntaxin transgenic animals grew more slowly than WT transgenic animals, they eventually reached full size. (*B*) Diagrams of WT syntaxin/UNC-64 and the split syntaxin pair that were expressed in syntaxin null *C. elegans*. The diagrams are arranged to correspond with the data in *A* and *C*. (*C Upper*) Representative traces of miniature currents recorded from the *C. elegans* neuromuscular junction. (*Lower*) Quantification of the miniature current frequency. The WT transgene rescued the syntaxin null phenotype (54.2 ± 5.9 minis/s; n = 10). To restore viability of arrested animals and allow for electrophysiological recording from adult animals, syntaxin was selectively expressed in the brain neurons (mosaic rescue). Syntaxin null synapses of motor neurons were completely devoid of spontaneous vesicle fusion (0 minis/s; n = 7). Neither the syntaxin SNARE motif (0.05 ± 0.02 minis/s; n = 5) nor the N terminus (0 minis/s; n = 5) of syntaxin restored the fusion. However, when the split syntaxin pair was expressed in the null background, miniature rate was restored to ~40% of WT level (21.4 events/s ± 3.74; n = 8). Error bars represent the SEM.

To quantify exocytosis, we examined endogenous rates of synaptic vesicle fusion at the neuromuscular junction by using whole-cell patch-clamp recording. Each recorded miniature current (mini) represents a single vesicle fusion event. Because neither the head nor the trunk fragment of syntaxin rescued the null mutant, we recorded from a mosaic strain in which syntaxin is expressed only in the worm brain but not in the motor neurons. These mosaic animals survive to adulthood but exhibit no minis at the neuromuscular junction (Fig. 6*C*) (49). Introduction of the WT (full-length) syntaxin transgene restored the endogenous synaptic release (54.2 minis/s) (Fig. 6*C*). As expected, the N-terminal head fragment alone was incapable of driving vesicle fusion (0 minis/s). Similarly, minis were rarely recorded (0.05 minis/s) from synapses expressing just the trunk fragment (the SNARE motif of syntaxin). When both fragments were expressed together, however, synaptic release was restored (21.4 minis/s) (Fig. 6*C*). The amplitude of the miniature currents was indistinguishable between the WT and the split syntaxin transgenes (Fig. S6). Thus, the split syntaxin pair can mediate synaptic vesicle fusion in vivo.

These in vivo observations correlate well with our reconstitution data and establish that the membrane-tethered N-peptide can act *in trans* to recruit Munc18-1 and promote membrane fusion. Because the N-peptide is detached from the SNARE bundle in this split syntaxin arrangement, these data further support that the N-peptide function is limited to an early stage of the reaction. This complementary line of evidence is important, because in the TEV experiment (Fig. 3), although the N-peptide was efficiently cleaved by the TEV protease, it was not possible to determine if all syntaxin molecules had been cleaved in the reactions.

Together, these results show that the N-peptide directly recruits Munc18-1 to the SNARE bundle to initiate its assembly into the fusion complex.

Discussion

Syntaxin-1 N-Peptide Is an Initiation Factor for the Assembly of the SNARE–Munc18-1 Membrane Fusion Complex. In this work, we show that the syntaxin N-peptide acts as an initiation factor for the assembly of the membrane fusion complex. Our data suggest a model in which the fusion reaction of synaptic exocytosis involves three sequential steps: (*i*) the soluble Munc18-1 protein binds the N-peptide motif of syntaxin-1 and is recruited to the zippering SNARE bundle, (*ii*) Munc18-1 assembles with the SNARE bundle to form a fusion-competent complex, and (*iii*) Munc18-1 and the SNARE bundle together drive the merging of membrane bilayers without further participation of the N-peptide (Fig. 7).

The N-peptide initiates the assembly reaction by physically recruiting Munc18-1 to the four-helix SNARE bundle. The recruitment needs a physical connection between the N-peptide and the SNARE bundle. The connection can be either proteinaceous (through covalent attachments between the N-peptide and the SNARE motifs) or membranous (by localizing the N-peptide on the same membrane as the SNARE motifs). Physical recruitment is known to dramatically enhance the ability of a regulatory factor to interact with the metastable SNARE bundle. For instance, the fusion inhibitor complexin completely arrests a fusion reaction only when brought to the proximity of the fusion site through a direct linkage (52, 53). Interestingly, complexin remains associated with SNAREs even when the linkage is removed (53), reminiscent of the full capacity of the SNARE-bound Munc18-1 to stimulate fusion after N-peptide proteolysis. We found that the N-peptide does not function as a soluble fragment or when

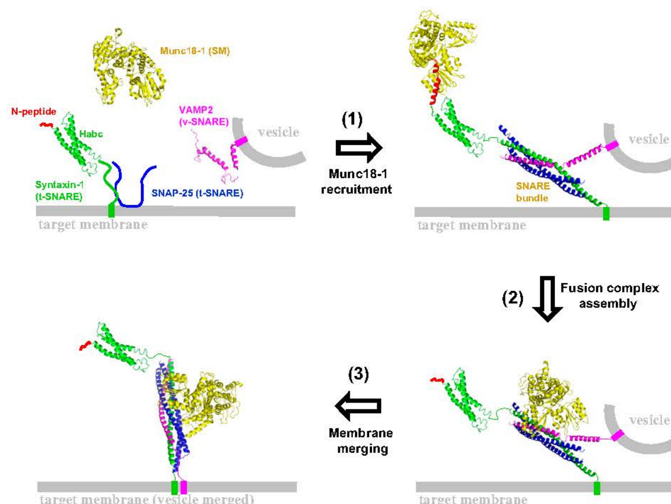

Fig. 7. Model of the syntaxin N-peptide function in membrane fusion. During fusion, the SM protein is first recruited by the N-peptide to the vicinity of the zippering SNARE bundle (step 1). This recruitment promotes the downstream formation of the SNARE–SM fusion complex (step 2). The merging of two membrane bilayers is mediated by the fusion complex comprised of the SM protein and the SNARE bundle without further involving the N-peptide (step 3). This model is based on our data of functional reconstitution and genetic analysis. Future binding and structural studies will provide further details of the recruitment pathway. Images were modeled from the atomic structures of the SNARE core bundle (4, 5), the Habc domain of syntaxin-1 (74), the SM–N-peptide complex (33, 34), and unpaired VAMP2 (75). Yellow, Munc18-1 (SM protein); green, syntaxin-1 (t-SNARE heavy chain); blue, SNAP-25 (t-SNARE light chains, only the SNARE motifs are shown); pink, VAMP2 (v-SNARE); red, N-peptide. Structures were edited in PyMol. In the SNARE bundle, the C-terminal part of VAMP2 helix was pulled away from the t-SNAREs to reflect the partially zippered status of the trans-SNARE complex.

ectopically fused to Munc18-1, indicating that allosteric activation of Munc18-1 conformation cannot account for the positive role of the N-peptide in fusion. However, it remains possible that the N-peptide function involves both physical recruitment and allosteric activation of Munc18-1 function.

In addition to its conserved binding to the SNARE complex, Munc18-1 can also interact with the closed syntaxin-1 monomer, which is formed when syntaxin's Habc domain folds back onto its own SNARE motif (54). Munc18-1 binding locks syntaxin-1 in the closed state that is incompatible with SNARE complex zippering (44, 55, 56). It has been hypothesized that SM proteins promote membrane fusion by regulating the closed to open conformational transition of syntaxin (33, 44, 57). However, we find that a syntaxin mutant lacking the entire N terminus, including the Habc domain, fully supports SNARE–Munc18-1–dependent membrane fusion when the N-peptide is translocated to SNAP-25. This provides definitive evidence that the open/closed conformational cycle of syntaxin-1 is not required for Munc18-1 activation of fusion. Furthermore, the N-peptide is able to regulate fusion even when it is completely detached from the SNARE bundle, showing that Munc18-1 binding is unlikely to transduce conformational changes through the intact syntaxin-1 molecule. Our findings are also in agreement with previous studies in which the binary syntaxin–Munc18-1 interaction was weakened by point mutations (56, 58, 59). Thus, despite its importance in fine tuning the efficiency of synaptic release (56), binding to the closed syntaxin monomer is dispensable for the conserved positive function of Munc18-1 in vesicle fusion.

General Role of the N-Peptide Binding Mode in Intracellular Membrane Fusion. In contrast to its essential roles in metazoan membrane transport, the N-peptide motif of syntaxin seems to be dispensable for many yeast fusion pathways under normal growth conditions (60, 61). Moreover, the N-peptide binding mode is entirely absent in the yeast SM proteins Sec1p and Vps33p (62–64). At first glance, these functional discrepancies conflict with the initiation factor model suggested here. However, given a closer look, a pattern emerges, where the affinity of an SM–SNARE pair seems to be inversely proportional to the requirement for the N-peptide. For instance, compared with the N-peptide–dependent Munc18-1 molecule, the yeast endocytic SM protein Vps45p seems to have evolved sufficiently high affinity for its cognate SNARE bundle (57, 60). As a result, an initiation factor (the N-peptide) is likely dispensable for the assembly of the yeast endocytic fusion complex.

In certain fusion reactions, it is possible that SM proteins are recruited to the SNARE bundles through alternative routes. A group of membrane transport factors, including Mso1 and Rabs, are known to interact with both SNAREs and SM proteins (63, 65–67). These SM-interacting factors may play alternative/compensatory roles in initiating the fusion complex formation when the N-peptide binding mode is lacking or inhibited. Intriguingly, Mso1 occupies the same binding site on SM proteins as the N-peptide and has been postulated to mimic the N-peptide in facilitating membrane fusion (66). Functional compensation by alternative initiation factors may explain the discrepancies over the observed consequences of N-peptide disruption in vesicle fusion (61, 68, 69). Regardless of the SM recruitment mechanism, ultimately, the merging of membrane bilayers is driven by a conserved fusion complex comprised of the four-helix SNARE bundle and its cognate SM protein.

Methods

Proteoliposome Reconstitution. All lipids were obtained from Avanti Polar Lipids. For t-SNARE reconstitution, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoserine (POPS), and cholesterol were mixed in a molar ratio of 60:20:10:10. For v-SNARE reconstitution, POPC, POPE, POPS, cholesterol, *N*-(7-nitro-2,1,3-benzoxadiazole-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine (NBD-DPPE), and *N*-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidylethanolamine (rhodamine-DPPE) were mixed at a molar ratio of 60:17:10:10:1.5:1.5. SNARE proteoliposomes were prepared by detergent dilution and isolated on a Nycodenz density gradient flotation (31). Complete detergent removal was achieved by overnight dialysis of the samples in Novagen dialysis tubes against the reconstitution buffer (25 mM Hepes, pH 7.4, 100 mM KCl, 10% glycerol, 1 mM DTT). SNARE proteins were kept at physiologically relevant surface densities, with protein to lipid ratios of 1:200 for v-SNAREs, similar to VAMP2 densities reported for native synaptic vesicles (70), and 1:500 for t-SNARE liposomes. This reconstitution procedure is known to yield homogenous populations of proteoliposomes that exhibit similar fusion properties as native membranes (70, 71).

All SNARE mutants were reconstituted into liposomes at the same molar densities as WT SNAREs. The diameters of our WT t- and v-SNARE liposomes were 93.3 ± 12.0 nm (polydispersity = 11.8 ± 3.2%) and 79.9 ± 3.6 nm (polydispersity = 10.9 ± 2.9%), respectively, as determined by dynamic light

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scattering. Reconstituted liposomes were routinely monitored by EM with negative staining.

Liposome Fusion Assay. Fusion reactions and data analysis were performed as previously described (31). A standard fusion reaction contained 45 μL unlabeled t-SNARE liposomes and 5 μL labeled v-SNARE liposomes, and it was conducted in a 96-well Nunc plate at 37 °C. Fusion was followed by measuring the increase in 7-nitrobenzo-2-oxa-1,3-diazole fluorescence at 538 nm (excitation = 460 nm) every 2 min in a BioTek Synergy HT microplate reader. At the end of the reaction, 10 μL 2.5% dodecyl-maltoside were added to the liposomes. Fusion data were presented as the percentage of maximum fluorescence change. To assess the regulatory activity of Munc18-1, v- and t-SNARE liposomes were incubated with or without 5 μM Munc18-1 on ice for 1 h before the temperature was elevated to 37 °C to initiate fusion. The maximum fusion rate within the first 20 min of liposome fusion was used to represent the initial rate of a fusion reaction. Full accounting of statistical significance was included for each figure based on at least three independent experiments. Munc18-1 dose dependence and requirement for preincubation were routinely tested for SNARE mutants as previously described (13, 31). Identical Munc18-1 activation was observed when the fusion data were presented as either percentage of maximum fluorescence or rounds of fusion (31). The correlation between fluorescence increase and rounds of fusion can be calculated by measuring the fluorescence signals of donor liposomes that mimic the lipid compositions expected of liposome products before fusion and after one round, two rounds, or three rounds of fusion (72). For reference, one round of fusion is approximately equivalent to 25% of maximum fluorescence (72, 73). It should be noted that, because we have not examined content mixing of the liposomes, membrane fusion in our experiments means lipid mixing of the liposomes.

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Supporting Information

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SI Methods

Protein Expression and Purification. Recombinant t- and v-SNARE proteins were expressed and purified as previously described (1, 2). The t-SNARE complex was composed of untagged rat syntaxin-1 and mouse SNAP-25 with an N-terminal His_6 tag. The v-SNARE proteins had no extra residues left after the tags were removed. Recombinant untagged Munc18-1 protein was produced in *Escherichia coli* as previously described (3). SNARE and Munc18-1 mutants were generated by site-directed mutagenesis or standard molecular cloning, and they were purified similarly to WT proteins. SNAREs were stored in a buffer containing 25 mM Hepes (pH 7.4), 400 mM KCl, 1% n-octyl-β-D-glucoside (OG), 10% glycerol, and 0.5 mM Tris(2-carboxyethyl)phosphine (TCEP). Soluble factors were stored in the protein binding buffer (25 mM Hepes, pH 7.4, 150 mM KCl, 10% glycerol, 0.5 mM TCEP).

Protein Interactions in a Copurification Assay. SNARE–Munc18-1 interactions were probed in a copurification assay in which the bacterial lysate containing His_6-small ubiquitin-like modifier (SUMO)-Munc18-1 was mixed with the lysate containing the GST-tagged syntaxin-1 cytoplasmic domain (amino acids 1–262). All binding assays were carried out in the protein binding buffer (25 mM Hepes, pH 7.4, 150 mM KCl, 10% glycerol, 0.5 mM TCEP). Nickel agarose resin (Qiagen) was added to the lysate to isolate His_6-SUMO-Munc18-1 and associated proteins. After washing three times with the protein binding buffer, the protein complexes bound to the beads were resolved on SDS/PAGE and stained by Coomassie blue.

C. elegans **Strains and Genetics.** ***Strains.*** The WT *C. elegans* strain was Bristol N2. All strains were maintained at 22 °C on standard nematode growth medium plates seeded with the bacterial strain OP50. Strains used in this study are summarized in Table S1.

Plasmids. The WT syntaxin gene is contained in pTX21 (Fig. S4*A*) (gift from Mike Nonet, Washington University, St. Louis) (4). Microinjection of the plasmid into the syntaxin null strain NM979 (20 ng/μL with P*unc-122::GFP* and pLitmus28) yielded *oxEx263*, which was X-ray–integrated (*oxIs33*).

To test multiple amino terminal truncations of syntaxin (syntaxin trunk SNARE motif), we made a construct containing the endogenous promoter and the start codon followed by the 3′ half of syntaxin starting with exon 6 (pMH420) (Fig. S5*B*). A fragment was amplified from pTX21 containing the SalI site upstream of the native start and appending SphI after the start codon with a 4-bp spacer (oligos, syx5, and syx3). This fragment was cloned into pTX21 using the endogenous SalI and NheI sites. To make the construct expressing the SNARE motif of syntaxin (trunk), two oligos (syx181upper and syx181lower) were hybridized, phosphorylated, and cloned into pMH420 with SphI and NheI sites. A clone containing a single insert was isolated (pMH424) (Fig. S5*C*). Microinjection into NM979 (5 ng/uL with *Pmyo-2::GFP* and *lin-15*) yielded *oxEx497*, which was X-ray–integrated and outcrossed (*oxIs154*). Animals were crossed into EG3278 to generate mosaics.

To make a construct with a TolA helix in place of the SNARE motif, we first deleted the SNARE motif and substituted SphI and KpnI sites (pMH429). We amplified a fragment downstream of the SNARE motif flanked by PfoI and NsiI sites introducing internal SphI and KpnI sites (oligos: snaredel long 5′ and snaredel 3′). This fragment was ligated into pTX21 at the endogenous PfoI and NsiI sites (pMH429). Next, the TolA helix was amplified from TolAII pET14b (gift from Michael Kay, University of Utah, Salt Lake City; oligos: TolA5′ and TolA3′). The fragment was ligated into pMH429 at the SphI and KpnI sites (pMH437) (Fig. S4*C*). Microinjection of pMH437 into NM979 (100 ng/uL with *Pexp-1::GFP* and *lin-15*) yielded *oxEx565*, which was X-ray–integrated and outcrossed (*oxIs236*). Animals were crossed into EG3278 to generate mosaics. Sequences of oligonucleotide primers used in this study are listed in Table S2.

Imaging. Animals were synchronized by collecting embryos and allowing them to mature for 3 d. Worms were immobilized by 25 mM sodium azide and imaged on a confocal microscope (Pascal LSM5; Carl Zeiss Inc.) with a plan-Neofluar 40× 1.3-numerical aperture oil objective (Carl Zeiss). Images of agar and food in the background were removed using Photoshop (Adobe Systems) for clarity.

Electrophysiology. Electrophysiological recordings were performed as previously described (5, 6) with minor adjustments. Briefly, the animals were immobilized with cyanoacrylic glue (Gluture; WPI, Inc.), and a lateral incision was made to expose the ventral medial body muscles. The preparation was treated with collagenase (type IV; Sigma-Aldrich) for 15 s at a concentration of 0.5 mg/mL. The muscle was voltage-clamped using the whole-cell configuration at a holding potential of −60 mV. All recordings were performed at 21 °C using an EPC-9 patch-clamp amplifier (HEKA) run on an ITC-16 interface (HEKA). Data were acquired using Pulse software (HEKA). Data analysis and graph preparation were performed using Pulsefit (HEKA), Mini Analysis (Synaptosoft), and Stata64 (Stata Co.). Bar graph data are presented as the mean ± SEM.

1. Shen J, Rathore SS, Khandan L, Rothman JE (2010) SNARE bundle and syntaxin Npeptide constitute a minimal complement for Munc18-1 activation of membrane fusion. *J Cell Biol* 190:55–63.
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4. Saifee O, Wei L, Nonet ML (1998) The Caenorhabditis elegans unc-64 locus encodes a syntaxin that interacts genetically with synaptobrevin. *Mol Biol Cell* 9:1235–1252.
5. Richmond JE, Davis WS, Jorgensen EM (1999) UNC-13 is required for synaptic vesicle fusion in *C. elegans*. *Nat Neurosci* 2:959–964.
6. Richmond JE, Jorgensen EM (1999) One GABA and two acetylcholine receptors function at the *C. elegans* neuromuscular junction. *Nat Neurosci* 2:791–797.

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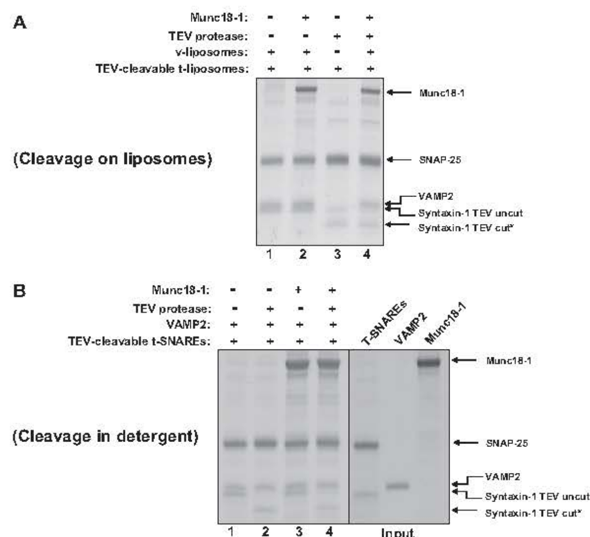

Fig. S1. Tobacco Etch Virus (TEV) digestion of the syntaxin-1 TEV variant. (*A*) Target membrane-associated SNARE (t-SNARE) and vesicle-rooted SNARE (v-SNARE) liposomes were incubated with Munc18-1 and the TEV protease as indicated. After 1 h of TEV proteolysis at 4 °C, the samples were resolved on SDS/PAGE and stained with Coomassie blue. Note that approximately one-third of syntaxin-1 TEV molecules faced the luminal side of the liposomes and thus, were not cleaved. The same intensity of the cleaved fragments in lanes 3 and 4 indicates that the presence of Munc18-1 did not affect t-SNARE liposome proteolysis. (*B*) t- and v-SNARE proteins were mixed with Munc18-1 and the TEV protease as indicated. After 1 h of incubation at 4 °C, the samples were resolved on SDS/PAGE and stained with Coomassie blue. Asterisk indicates that only the C-terminal cleavage fragment was evident on the gel, whereas the N-terminal fragment (20 aa) was too small to resolve.

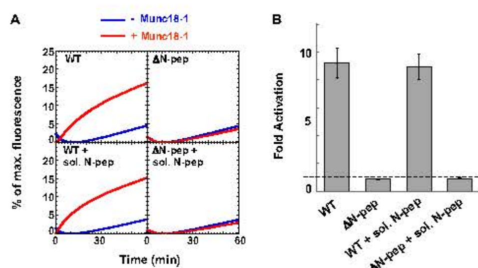

Fig. S2. The soluble N-terminal peptide motif (N-peptide) fragment (sol. N-pep) does not stimulate the fusion reaction. (*A*) Fusion of the indicated t- and v-SNARE liposomes in the absence or presence of 5 μM Munc18-1. The soluble N-peptide fragment (amino acids 1–45) was added at 100 μM. (*B*) Fold increase in the initial fusion rates of the reactions in *A*. The dashed line indicates the basal fusion level (with no Munc18-1 activation). Error bars indicate SD.

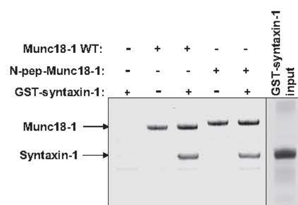

Fig. S3. The N-peptide–linked Munc18-1 variant binds to monomeric syntaxin-1 equally as well as WT Munc18-1. Nickel agarose resin was used to isolate His_6-small ubiquitin-like modifier-Munc18-1 and associated proteins in a copurification assay. The protein complexes (on the left) and the input GST–syntaxin-1 (on the right) were resolved by SDS/PAGE and stained with Coomassie blue.

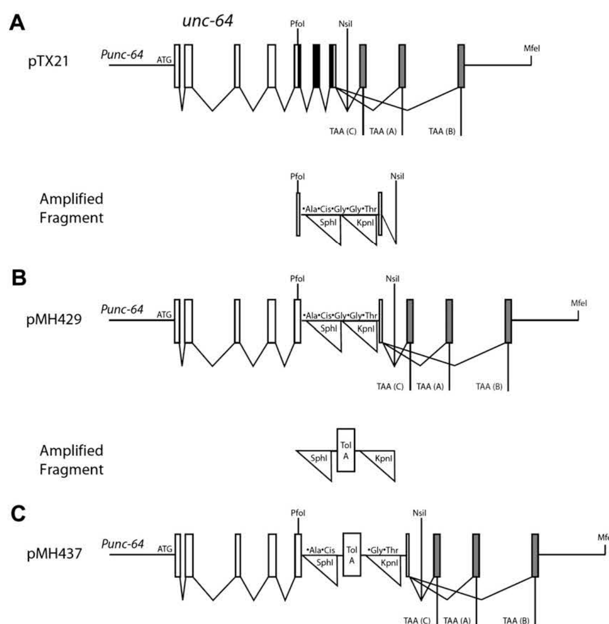

Fig. S4. Generation of the *Caenorhabditis elegans* construct expressing the syntaxin/*unc-64* N-terminal head fragment. (*A*) The plasmid pTX21 (gift of M. Nonet, Washington University, St. Louis) contains the *unc-64* gene. The 3′ end of the gene contains three splice variants that encode alternative transmembrane domains (gray exons), which produce transcripts UNC-64C, UNC-64A, and UNC-64B. (*B*) The SNARE motif (black) was removed, and restriction sites SphI and KpnI were inserted in its place (pMH429). (*C*) An exogenous α-helix was amplified from bacterial TolA (with flanking SphI and KpnI restriction sites; gift of M. Kay, University of Utah, Salt Lake City) and ligated into pMH424 (pMH437).

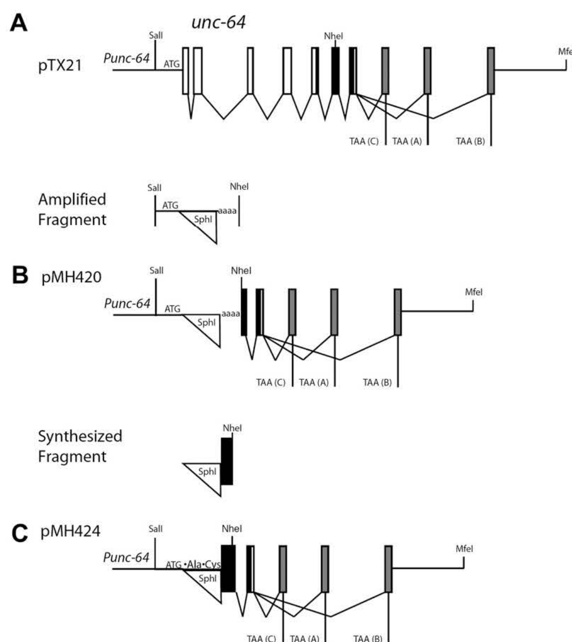

Fig. S5. Generation of the *C. elegans* construct expressing the syntaxin/*unc-64* trunk SNARE motif fragment. (*A*) The WT *unc-64* locus was expressed from pTX21. The SNARE motif is shaded black. (*B*) A truncated *unc-64* construct lacking the N-peptide, Habc, and part of the SNARE motif was built by replacing this region with an SphI restriction site (pMH420). The construct was used as a cassette to add back different segments of the amino terminus of syntaxin. (*C*) A fragment was synthesized and inserted that fuses part of exon 5 to exon 6 such that the N terminus of the protein begins with the SNARE motif starting at residue 181 (pMH424). Note that this SNARE motif construct still produces all three transmembrane variants.

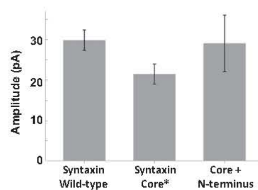

Fig. S6. The mean amplitude of miniature currents was indistinguishable among WT, split syntaxin, and the SNARE motif-only transgenic animals ($P > 0.05$; two-tailed *t* test with Bonferroni correction). Transgenic animals expressing only the N terminus of syntaxin exhibit no spontaneous fusion and thus, were not included. Error bars represent the SEM.

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Table S1. List of *C. elegans* strains

Strain	Genotype
NM979	*unc-64(js115)/ bli-5(e518)*
EG2466	*unc-64(js115); oxIs33[unc-64(+);Punc-122::GFP]*
EG3278	*unc-64(js115); oxEx536[Punc-17::unc-64(+); Pglr-1::unc-64(+); Punc-122::GFP; lin-15(+)]*
EG3279	*unc-64(js115); oxIs154[Punc-64:UNC-64(SNARE motif); Pmyo-2::GFP; lin-15(+)]; oxEx536[Punc-17::UNC-64(+); Pglr-1::unc-64(+); Punc-122::GFP; lin-15(+)]*
EG4039	*unc-64(js115); oxIs236[Punc-64:UNC-64(N-terminal) Pexp-1::GFP; lin-15(+)]; oxEx536[Punc-17::UNC-64(+); Pglr-1::UNC-64(+); Punc-122::GFP; lin-15(+)]*
EG6154	*unc-64(js115); oxIs236[Punc-64:UNC-64(N-terminal); Pexp-1::GFP; lin-15(+)]; oxIs154[Punc-64:UNC-64(SNARE motif); Pmyo-2::GFP; lin-15(+)]*

Table S2. Oligonucleotide primer sequences for *C. elegans* constructs

Name	Sequence
syx5	caaaaaaggcctagtctagtc
syx3	gctagcttttgcatgccatgttgttgttcctgttg
syx181upper	catcatcacagatacccaacaggcaaaacaaacg
syx181lower	ctagcgtttgttttgcctgttgggtatctgtgatgatgcatg
snaredel long 5′ oligo	tccgggagtatttacacaagcatgcggtggtacca gggttcgtttttttacgtttttgg
snaredel 3′ oligo	acgcaatttgtctaccgtatacc
TolA5′	gcatgcggtggttcgtccatcgacg
TolA3′	ggtaccctcggcctgttttgcggc

CHAPTER 4

PERSPECTIVES

Summary

SNARE proteins in conjunction with SM proteins are responsible for vesicle fusion throughout all eukaryotic cells. This dissertation describes my research using the model organism *C. elegans* to explore some of the central mysteries of synaptic vesicle exocytosis. My results from analyzing SNAP-25 null synapses are consistent with traditional SNARE-mediated fusion models. SNAP-25 is a central player in exocytosis and important for docking synaptic vesicles and executing fusion. However, we report for the first time that SNAP-29 is capable of substituting for SNAP-25 in tonic but not evoked neurotransmission. These data add to mounting evidence that alternative SNARE interactions may be responsible for specific forms of neurotransmitter release from neurons.

The role of SM proteins in mediating exocytosis is poorly understood. The N-terminus of syntaxin binds Unc18 proteins and is required for fusion, but the mechanical significance of this interaction has been unclear. We used an *in vitro* fusion assay to test the requirements of the Unc18/N-peptide interaction. We found that the N-peptide interaction is not responsible for passing a catalytic message between syntaxin and Unc18. Rather, it serves as a passive tether to keep Unc18 near the fusion complex. Using

chimeric proteins, we provided further support for this model at the neuromuscular junction in *C. elegans*.

My work on SNAP-25 and Unc18 is presented in Chapters 2 and 3 as experimental narratives with conclusive evidence. In this final chapter, I highlight observations that lack clear understanding and discuss the models and experiments that we are considering to resolve these mysteries. First, I summarize our understanding of unc-18 function and present the key challenges that we face in unveiling its role in fusion. Then, I expand on the snap-25 null experiments to discuss how the lack of specificity might contribute to SNARE mediated fusion at the synapse. Finally, I describe preliminary results that suggest a novel role for SNAREs in vesicle recycling at synapses.

Mechanics of the SNARE/Unc18 machine

SNARE mediated fusion has an appealing aesthetic: the winding of helices draws membranes together and forces fusion. However, this is an overly simplified view of the fusion apparatus. The SM proteins accompany every SNARE mediated fusion reaction explored (Carr and Rizo, 2010; Rizo and Südhof, 2012; Südhof and Rothman, 2009; Toonen and Verhage, 2007). Furthermore, ablation of SM proteins eliminates fusion including that of neuronal secretion (De Wit et al., 2009; Verhage et al., 2000; Weimer et al., 2003). At one time SNAREs were considered the “minimal machinery” for fusion (Weber et al., 1998); however, that has now been revised to include SM proteins since the addition of SM proteins accelerates the rate of fusion twenty-fold (Shen et al., 2007). Therefore, SM proteins are now considered obligate SNARE partners in driving vesicle fusion. Still, the biophysical mechanism of Unc18 function remains mysterious.

The functional role of SM proteins in facilitating fusion has largely been clouded by their diverse and divergent modes of interaction with SNARE proteins. The yeast secretory SM protein Sec1 binds the SNARE four-helix bundle but lacks an interaction with the Habc domain or N-peptide (Carr et al., 1999). In contrast, the ER/Golgi SM proteins Sly1 and Vps45 were first reported to exclusively interact with the N-peptide of syntaxin (Bracher and Weissenhorn, 2002; Dulubova et al., 2002; Yamaguchi et al., 2002). However, another study shows Sly1 binds the ternary SNARE complex (Peng and Gallwitz, 2002). The neuronal SM protein Munc18 binds syntaxin in the closed state with very high affinity (Misura et al., 2002), largely masking the interactions with both the N-peptide and the four-helix SNARE complex (Burkhardt et al., 2008; Dulubova et al., 2007; Khvotchev et al., 2007; Shen et al., 2007), but despite this confusing history, there now appears to be a unified acceptance that most SM proteins share the N-peptide and SNARE complex interactions (Rizo and Südhof, 2012; Südhof and Rothman, 2009). The interaction with closed syntaxin, despite being high affinity, appears to be a late evolutionary addition to neuronal SM proteins.

My work on Unc18, as described in Chapter 3, demonstrated that the syntaxin N-peptide is necessary for the transition from a binary interaction with closed-syntaxin to association with the four-helix bundle. We were able to rule out more elaborate models involving conformational coupling or allosteric modulation as observed with Sly1 (Arac et al., 2005). Instead, the N-peptide appears to serve as a tether, keeping Unc18 near the fusion apparatus in transition from the closed interaction to direct association with the SNARE core complex. It is possible that this interaction has evolved to deal with a very crowded molecular environment. Many proteins bind the SNAREs complex including

synaptotagmin, complexin, tomosyn, Munc13, and others. It seems likely that the high affinity binary interaction with syntaxin draws Unc18 to sites of fusion. When syntaxin opens and Unc18 is released, the N-peptide tethers Unc18 to the complex. This in turn allows the low affinity SNARE interaction to take place at the time of fusion.

How does Unc18 binding to the core complex stimulate fusion? Multiple models attempt to address this question (Figure 4.1). Many believe that SM proteins facilitate efficient trans-SNARE zippering. Consistent with this, SM proteins bind to trans-SNAREs incorporated in liposomes, but poorly bind cis-SNARE complexes (Shen et al., 2007). This model is further supported by the dramatic increase in SNARE-mediated fusion of liposomes with the addition of Munc18. This stimulation occurs with SNAREs and SM proteins alone and require no other molecular factors (Shen et al., 2007).

In a second model, some argue that SM proteins displace a fusion-inhibitor such as complexin. Indeed, Munc18 has been implicated in binding to the same residues as complexin (Chen et al., 2002; Shen et al., 2007). Alternatively, SM proteins may guide the tethering HOPS (homotypic fusion and vacuole sorting) complex and protect the assembled SNAREs from melting by NSF (Collins et al., 2005; Starai et al., 2008). Removing a negative factor is an appealing model and could be applied to other molecules. However, this mechanism cannot stand alone, as such regulators are not included in liposome fusion assays.

In a third model, some have speculated that SM proteins may serve as bulky substrates, which prevent SNARE transmembrane domains from drifting into the fusion stalk (Dulubova et al., 2007; Rizo et al., 2006). Two surfaces of Unc18 are rich in basic residues that may in fact interact with the vesicle and plasma membranes (Rizo and

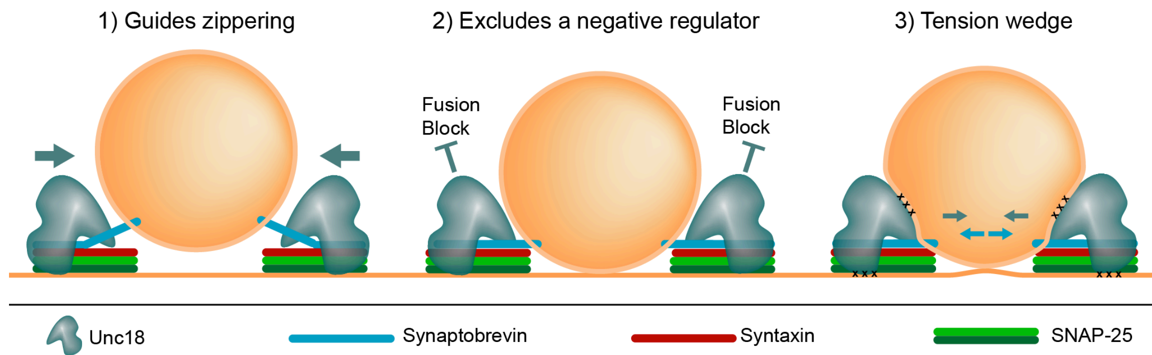

Figure 4.1 Three models for the function of Unc18's interaction with the SNARE core complex. (1) Unc18 may assist in fusion by facilitating SNARE-zippering. (2) Unc18 may provide a bulky substrate holding SNAREs away from the fusion pore. This may involve interaction directly with the apposed membranes and could provide antagonizing force on membranes inducing curvature. (3) Unc18 may protect the SNAR bundle from unwinding by the ATPase NSF. Unc18 is colored in teal. SNAREs are represented as simple bars: synaptobrevin (blue), syntaxin (red), and SNAP-25 (dark and light green).

Südhof, 2012). Given membrane contact and rotational freedom around the SNARE complex, one can even imagine that SM proteins might be driven towards the fusion pore as the SNARE complex twists—like a nut on a screw. This highly speculative model is attractive, as it would result in increased curvature on both membranes. These models are not mutually exclusive, and each should be considered and tested independently.

An important step towards testing these models will be defining the interaction interface of Unc18 with the trans-SNARE protein complex. Such a structural picture will reveal its Unc18's orientation relative to the vesicle and plasma membrane as well as other SNARE regulatory proteins. However, this is a difficult task, as SNARE proteins must be anchored in apposing membranes to maintain the trans configuration. Two groups have made inroads into this problem. In one study, investigators made targeted mutations to Munc18 that they predicted to specifically disrupt core complex binding (Deák et al., 2009). They reported two mutations that specifically disrupted SNARE core

complex binding without changing the affinity of Munc18 with closed-syntaxin. Remarkably, they found that the rescue of neurotransmission correlated with the mutants' ability to bind SNARE complexes.

In a second study, investigators focused on the yeast SM protein Sec1, which only binds the trans SNARE complex. They randomly mutagenize Sec1 and looked for yeast clones with temperature sensitive growth defects. Many of the mutations isolated specifically disrupt Sec1 binding to the SNARE bundle (Hashizume et al., 2009). The mutations from these studies identified residues in neighboring grooves on the respective SM proteins (Figure 4.2). Determining where these residues map onto the SNARE bundle would provide powerful information for testing the molecular role of Unc18 in fusion.

In an effort to identify the SNARE residues that interact with these Unc18 amino acids, we are conducting high-throughput suppressor screens in *C. elegans*. We have selected a subset of the mutations identified by Deák and Hashizume that involve charge reversals. These mutations are expected to have strong negative consequences on binding that could be repaired by a compensatory mutation on their interaction partners. We have engineered six of these mutations into the *C. elegans* genome. The resulting animals are uncoordinated and grow at slower rates than the wild type. We will expose large populations of these animals to mutagen, screen for healthier animals, and sequence the SNARE loci for compensatory mutations. We have recently had success with this approach, determining that many amino acid contacts formed in a protein complex including the bar domain FCHO and adaptor protein AP2 (Hollopeter, unpublished). The Unc18 suppressor screens are especially appealing as they may reveal important residues that interact at different stages in the fusion process.

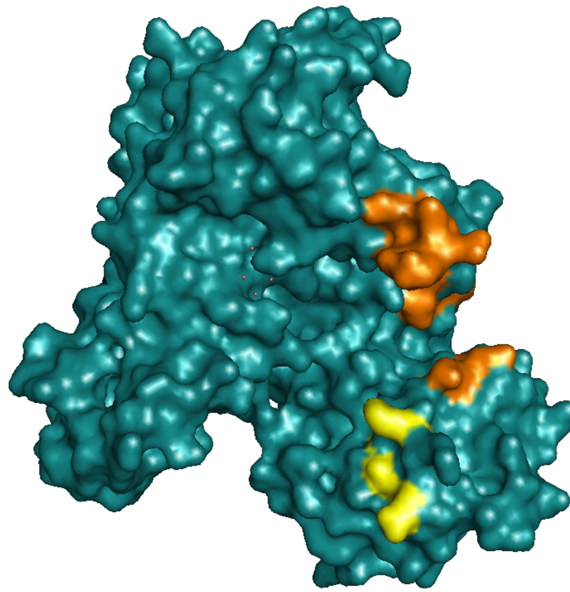

Figure 4.2 The surface of Munc18 as extracted from the binary complex with syntaxin (PDB 3c98). Two groups have identified surface residues on SM proteins that bind the SNARE core complex. Select residues identified in a temperature sensitive Sec1 screen are colored in orange (Hashizume et al., 2009). The three residues designed to disrupt binding of Munc18 to the SNARE complex are colored in yellow (Deák et al., 2009). We have made the equivalent mutations in C. elegans.

SNAREs and specificity

Different SNAREs are selectively expressed in subsets of cells, localized to specific membranes, and have varying degrees of affinity for other SNARE partners. These characteristics have largely informed our views of the division of labor amongst the SNARE family of proteins. However, functional differences lie beyond the resolution of cellular localization and biochemical crosstalk between noncognate SNAREs suggests that they do not simply interact with a single set of partners. Therefore, defining the precise functional role of each SNARE protein at the synapse requires genetic perturbations paired with electrophysiological characterization.

SNARE proteins may be differentially associated with specific forms of neurotransmitter release. The canonical neuronal SNAREs, syntaxin, synaptobrevin, and SNAP-25 are necessary for normal levels of vesicle fusion. However, as discussed in Chapter 1, these SNAREs appear to be more strictly required for evoked fusion than for spontaneous release (Deitcher et al., 1998; Delgado-Martinez et al., 2007; Schoch et al., 2001; Washbourne et al., 2002). Our results from SNAP-25 null neurons in *C. elegans* are consistent with this pattern. These observations beg the question, which other SNAREs are supplementing fusion at the synapse? We provide the first report of SNAP-29 serving as a functional Qbc SNARE for neurotransmitter release. Overexpression of SNAP-29 substitutes for SNAP-25 to the extent that rescued animals are highly motile and have near normal rates of tonic vesicle fusion. Interestingly, SNAP-29 overexpression has no effect on evoked fusion. In a similar manner, the Qb SNARE VtiI preferentially supports spontaneous release in hippocampal culture (Ramirez et al., 2012). The effect is mild in the synaptobrevin null background but appears dramatic in the synaptobrevin 2 knockouts. In addition, the R-SNARE VAMP4 is preferentially associated with the asynchronous release mechanism (Raingo et al., 2012).

The molecular preference of SNARE proteins for spontaneous or evoked fusion may be associated with separate populations of vesicles. Large populations of synaptic vesicles fill each nerve terminal. However, upon stimulation, only a fraction of vesicles take up external tracers. These actively recycled vesicles are referred to as the “Recycling pool” (Fernandez-Alfonso and Ryan, 2008; Harata et al., 2001; Rizzoli and Betz, 2005a). The population of vesicles that does not respond to even intense stimulation paradigms make up the “Resting Pool” (Fernandez-Alfonso and Ryan, 2008).

Vesicle pools are attractive candidates to explain a different source of evoked and spontaneous neurotransmitter release. Indeed, the recycling pool disproportionately takes up external tracers under stimulated conditions, and the resting pool internalizes markers at rest (Chung et al., 2010; Fredj et al., 2009; Mathew et al., 2008; Sara et al., 2005). However, these results have been refuted in other studies (Groemer and Klingauf, 2007; Hua et al., 2010; Wilhelm et al., 2010). Nonetheless, it appears as though these pools each have a unique molecular identity as all look the same morphologically and are evenly distributed throughout synaptic terminals (Rizzoli and Betz, 2005b). Both VtiI and VAMP7 are found disproportionately on resting-pool vesicles. Optically tracking fusion with pH sensitive proteins demonstrates that they fuse spontaneously and do not contribute to evoked release (Hua et al., 2011; Ramirez et al., 2012).

These observations suggest that neuronal SNAREs have evolved a division of labor that specifies different modalities of neurotransmitter release. Our work now implicates SNAP-29 in spontaneous neurotransmission, but not evoked. Our report of coordinated locomotion in animals with SNAP-29 and no SNAP-25, demonstrates that these fusion events are regulated. However, we have yet to clearly define SNAP-29 neurotransmission in *C. elegans*. First, we need to determine if the SNAP-29 mediated minis depend on synaptobrevin or if they are recruiting an alternative R-SNARE. To this end, we will cross Punc-47::TeTx into SNAP-29 overexpression strains. Second, we will use acute protein degradation to test if SNAP-29 is required for fusion in wild-type animals. These experiments will selectively eliminate the population of fusion events under native control of SNAP-29.

Finally, the SNAP-25 independent fusion in the "head-only" transgenics cannot be completely explained by SNAP-29. We see no evidence for SNAP-29 evoked neurotransmission, yet some evoked current remains in the absence of SNAP-25. Therefore, we are considering overexpression and knock-down of other candidate SNAREs involved in synchronous release. Ultimately, with the help of others, we hope to clearly define the functional repertoire of each SNARE protein at the synapse.

A role for SNAREs in synaptic vesicle recycling

The recycling of vesicle proteins and lipids is an essential aspect of maintaining a functional synapse distant from the cell soma (Figure 4.3). Following the full collapse of a vesicle into the plasma membrane, vesicle-specific lipids and proteins are gathered and marked by adaptor proteins. AP-2 recruits clathrin, which forms a uniform spherical coat internalizing a nascent vesicle (Saheki and De Camilli, 2012). Clathrin-mediated endocytosis assembles a complete vesicle with all of the necessary machinery required for transmitter refilling and fusion. However, this process is relatively slow proceeding with a time constant of approximately 15 seconds (Granseth et al., 2006; Balaji and Ryan, 2007).

Neurons are capable of exocytosis at alarming rates, necessitating a rapid mechanism to reclaim vesicle material, maintain cell morphology, and clear sites of fusion. Under conditions of rapid exocytosis (up to 500 Hz at the calix of held synapse), large folds of membrane are internalized by ultrafast bulk-endocytosis (Cousin, 2009; Smith et al., 2008) (Figure 4.3). This form of endocytosis has received less attention; however, it has been observed in diverse cell types including the worm and frog

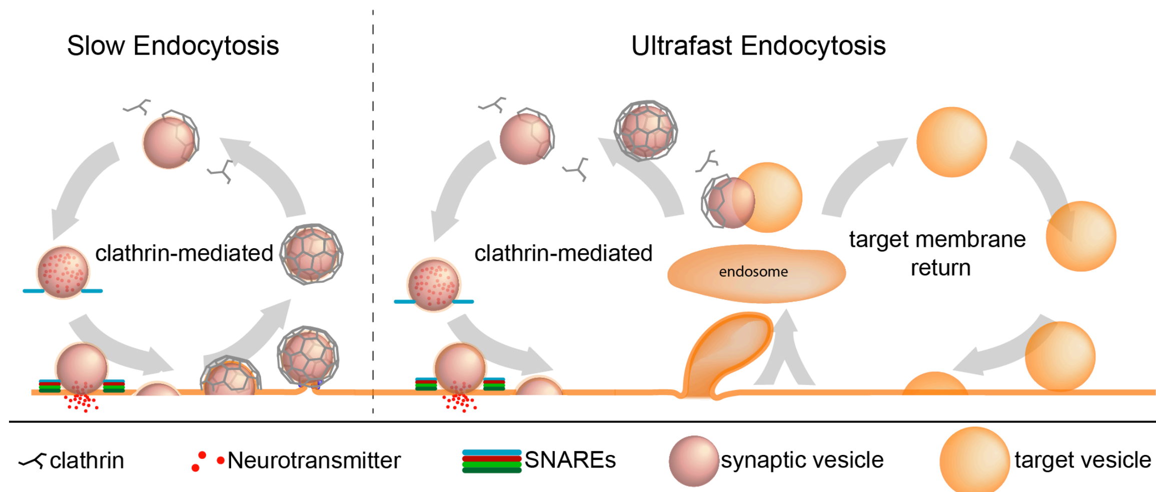

Figure 4.3 The synaptic vesicle cycle proceeds by slow clathrin-mediated endocytosis and ultra-fast bulk-endocytosis. Slow endocytosis is driven by the vesicle coat clathrin, which produces vesicles with selective protein constituents. Ultrafast endocytosis occurs under intense stimulation and results in large endosomes. These endosomes are resolved by clathrin-mediated budding. It is not known if target membrane is recycled back to the plasma membrane.

neuromuscular junctions (Kittelmann et al., 2013; Miller and Heuser, 1984; Richards et al., 2000; Watanabe et al., 2013), retinal bipolar cells (Holt et al., 2003), and mammalian central synapses (Clayton et al., 2010; Watanabe, in press). Synaptic vesicle biogenesis occurs from endosomes by a clathrin-mediated process but is thought to rely on the alternative adaptor proteins AP-3 and AP-1 (Blumstein et al., 2001; Faundez et al., 1998; Glyvuk et al., 2010).

How do SNARE proteins participate in vesicles recycling? Their role in clathrin-mediated endocytosis has been carefully considered. Following fusion, “spent” Cis-SNAREs are disassembled on the plasma membrane (Littleton et al., 2001) (Figure 4.4). The adaptor protein AP180 and accessory factor CALM recognize synaptobrevin and recruit it to zones of retrieval (Dittman and Kaplan, 2006; Koo et al., 2011; Nonet et al., 1999; Zhang et al., 1998). Clathrin binds AP180 in association with AP2, internalizing a

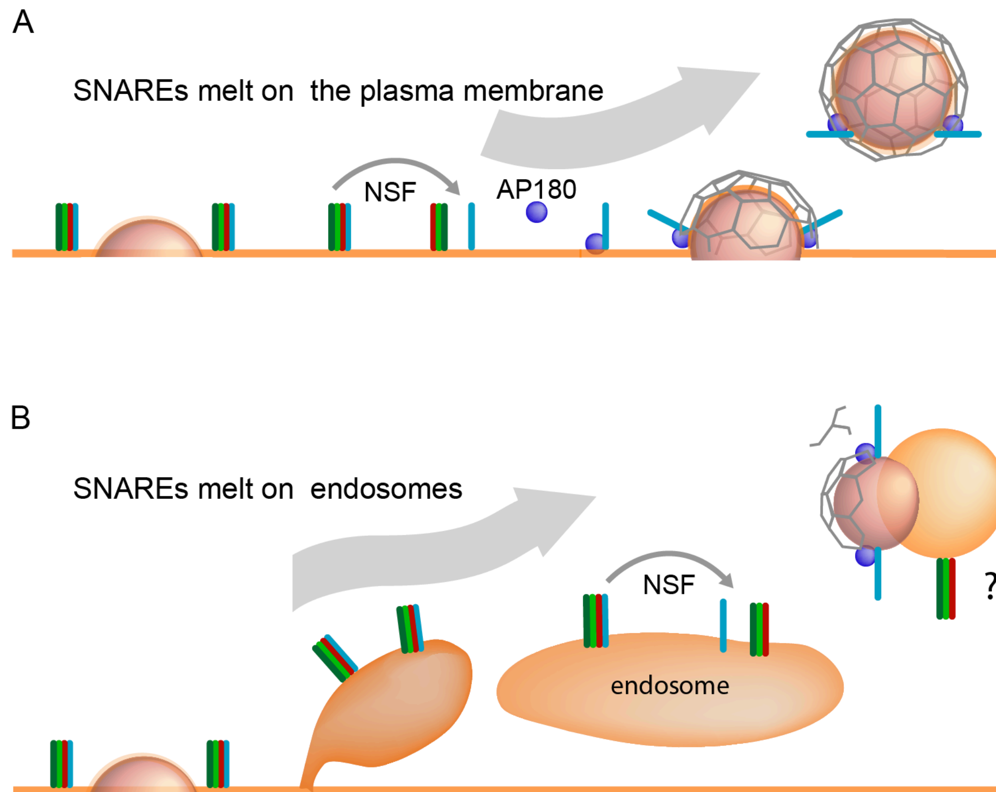

Figure 4.4 SNARE melting and vesicle resolution. Following vesicle fusion, SNARE proteins are dissociated or "melted" by the ATPase NSF. This may occur on the plasma membrane (A) or endosomal compartments (B).

nascent vesicle (Traub, 2003). This model is elegant in its simplicity, producing vesicles with a select population of v-SNAREs.

In contrast, little is know about the path SNAREs take through endosome intermediates. Bulk-endocytosis occurs very rapidly and appears to involve a passive collection of membrane at adherence junctions (Watanabe et al., 2013). Therefore, it seems unlikely that proteins are sorted prior to internalization. In turn, endosomes may be rich in plasma membrane constituents. Syntaxin and SNAP-25 may reside on endosomes as ternary complexes with synaptobrevin or monomeric proteins. The machinery for melting SNAREs is soluble and found throughout soluble and membrane fractions in

neurons (Burgalossi et al., 2010). Although there is no direct evidence for cis-SNARE melting on endosomes, such a mechanism is likely. This would free up synaptobrevin to follow the classical clathrin mediated budding process and result in a population of vesicles rich in target proteins. This "branching" of the synaptic vesicle cycle is speculative, but provides the most parsimonious explanation for resolving unsorted endosomes. Furthermore, this population of vesicles may contribute to the "resting pool" of vesicles that does not respond to stimulation.

Through our studies of SNAP-25 null synapses, we have made a series of observations that implicate SNAP-25 in synaptic vesicle recycling. The first clue came from experiments with synaptic markers. While synapse density appears normal in the absence of SNAP-25 (Figure S2.2C), we found that fluorescent markers targeted to synaptic vesicles revealed abnormal synaptic morphology in SNAP-25 mutants (Figure 4.5). Two fluorescently labeled vesicle markers (UNC-47 and SNG-1) display swollen elongated puncta in GABA neurons in the snap-25 "head-only" and hypomorph strains. Diffusion of synaptic vesicle markers is often attributed to defects in synaptic vesicle endocytosis (Dittman and Kaplan, 2006). Might SNAP-25 be required for internalizing synaptic vesicles? In fact, one group has recently implicated SNAP-25 and syntaxin in synaptic vesicle endocytosis at hippocampal synapses (Zhang et al., 2013) and at calyx of Held synapses (Xu et al., 2013). We reasoned that a defect in endocytosis would result in an increase in the surface residence of the pH sensitive vesicle marker SNB-1::pHuorin. (Dittman and Kaplan, 2006). However, instead we observed a decrease in the fraction of synaptobrevin on the plasma membrane at SNAP-25 null synapses (Figure 4.6). These data indicate that the membrane internalization process is functional in the absence of

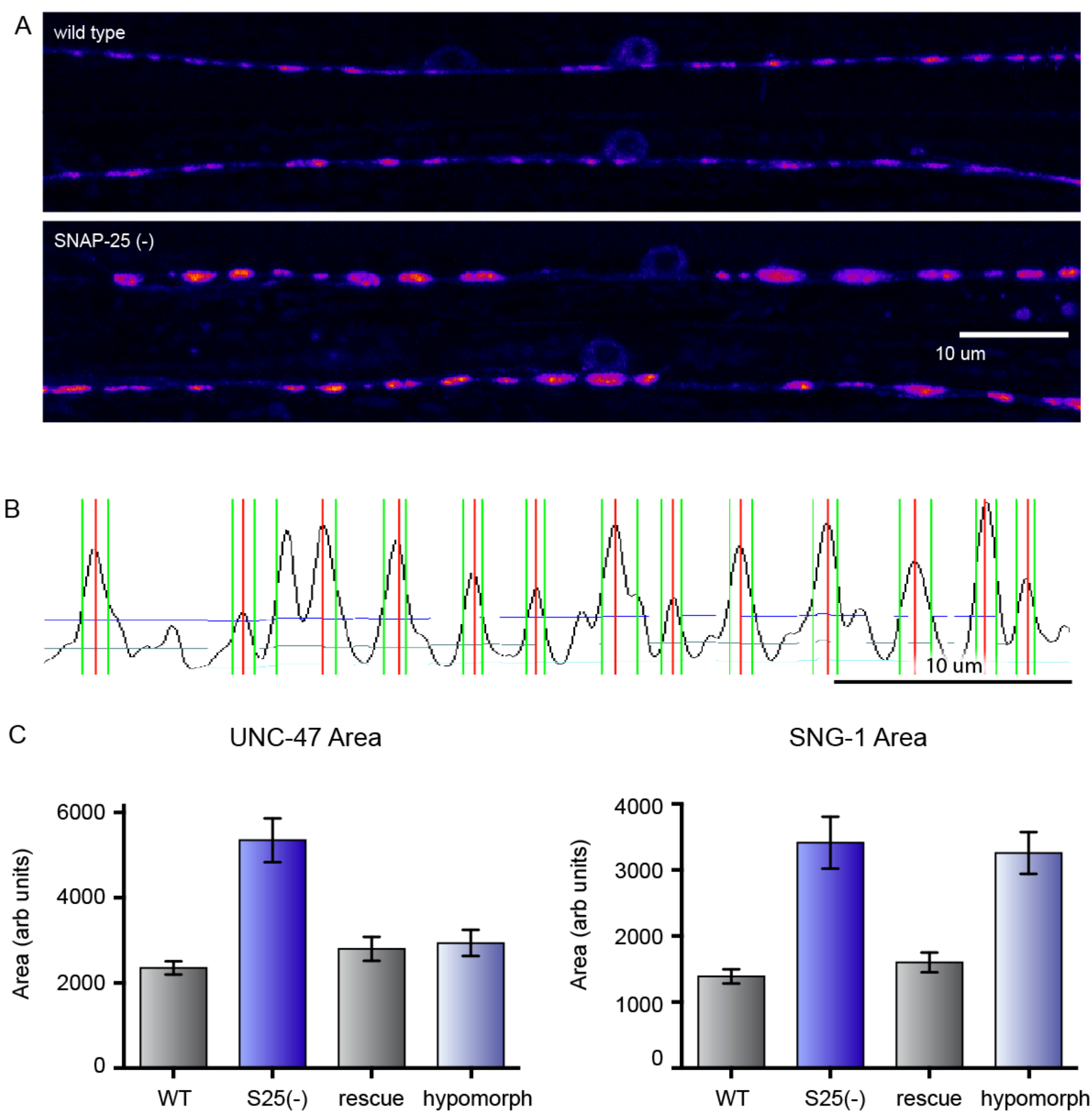

Figure 4.5 Presynaptic morphology is abnormal in the absence of SNAP-25. (A) The vesicular GABA transporter is fused to GFP marking vesicles at presynaptic motor neuron terminals (Punc-47::unc-47::GFP). Two representative images of the ventral nerve cord of wild-type animals are compared to images from "head-only" animals. SNAP-25 null terminals display broader, more robust fluorescence (B) A representative line scan mapping the intensity of pixels/distance. Pucta are defined as maxima (red) above 25% of the local dynamic range (blue), between points that drop below 10% of the local dynamic range (gray). Green lines mark the width at 50% maximum value. (C) The average "area" under the curve for any puncta as defined in B. SNAP-25 null synapses have significantly more robust puncta than the wild type (UNC-47::GFP). Both the SNAP-25 null (*ox528*) and hypomorph (*ox45*) have more robust puncta than the wild type with the synaptic marker SNG-1::mCherry.

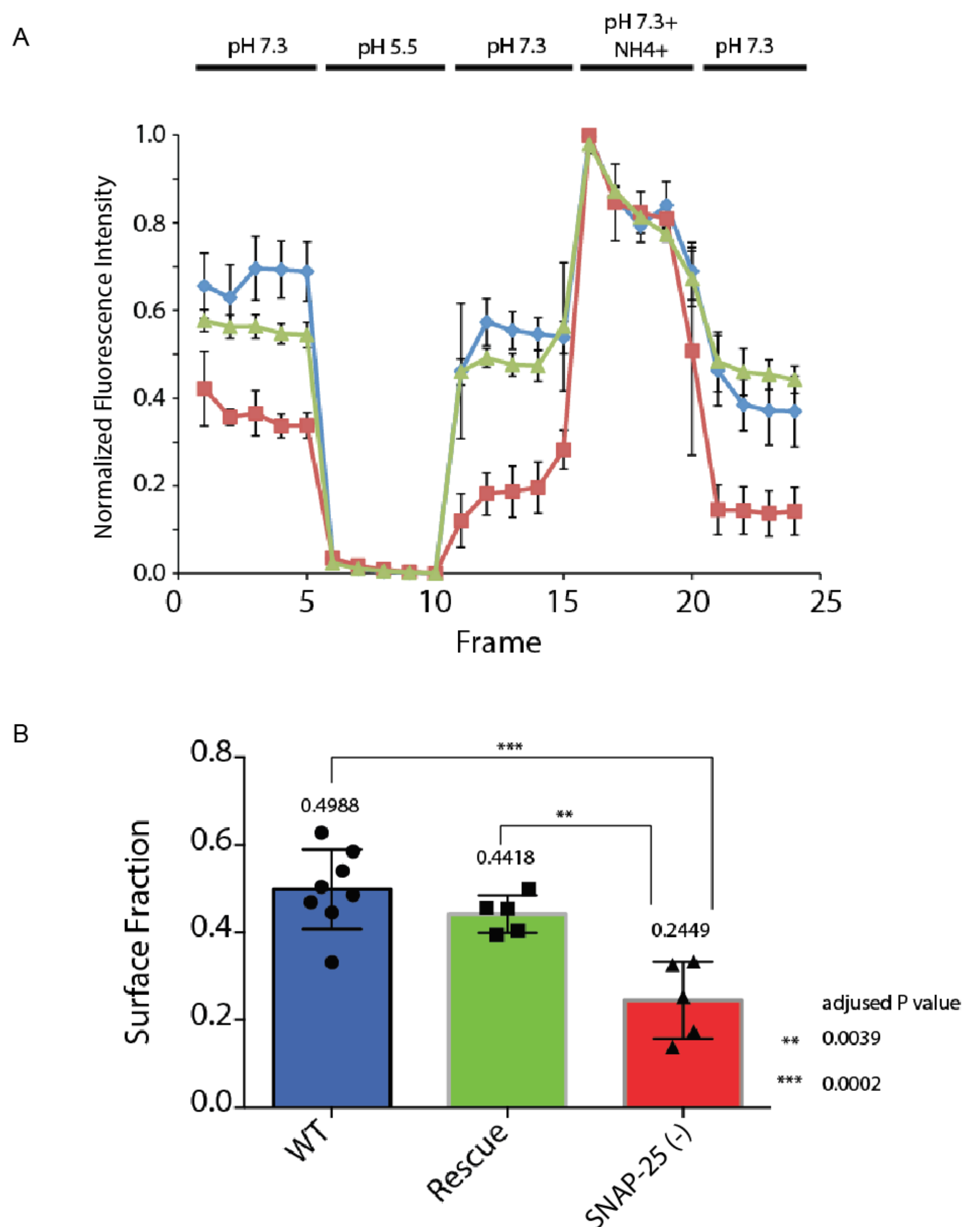

Figure 4.6 Synaptobrevin::pHluorin surface residence is decreased in the absence of SNAP-25. (A) Worms expressing pan-neuronal snb-1::pHluorin were dissected and imaged under different bath conditions. Fluorescence intensity was normalized to the high and low values of each series. Wild-type (blue) and rescued (green) neurons responded to pH in a similar manner. The "head-only" strain (red) displayed low fluorescence intensity until basic conditions were co-applied with $NH4^+$, which exposes vesicle lumen to the bath conditions. (B) Quantification of the surface fraction of snb-1::pHluorin. Average fluorescence intensity at pH7.3 (surface value) was divided by the intensity at pH 7.3 + $NH4^+$ (total value). The "head-only" strain had significantly less snb-1 on the surface than the wild type or the rescue.

SNAP-25 but suggest that vesicles and vesicle proteins are more abundant and diffusely localized in mutant strains.

At first glance, these observations are in conflict with our ultrastructural analysis of *ox528* and *ox45* mutant synapses. As presented in Chapter 2, we saw no increase in the number of synaptic vesicles/profile in SNAP-25 mutants. However, when we analyzed the length of synaptic regions by EM, we found that both *snap-25* null and hypomorph synapses were significantly longer (Figure 4.7A), indicating that vesicles are spilling out beyond the normal confines of the synapse. Furthermore, we observed a significant increase in the number of large synaptic vesicles in *snap-25* mutant synapses (Figure 4.7B). Increases in large vesicle number have been observed in many endocytosis mutants including AP180, AP2, and dynamin (Gu et al., 2013; Nonet et al., 1999; Watanabe et al., 2013), as well as in unpublished cases with synaptojanin and endophilin (E. M. Jorgensen, personal communication).

Finally, we note anecdotal observations of large endosomal structures in *snap-25* mutants. We saw an increased frequency of large clear vacuoles or endosomes in both the hypomorph (*ox45*) as well as the null allele (*ok173*) (Figure 4.7C). Abnormalities were particularly common in *ok173*; however, these data are tenuous as we were unable to rescue the defect. Taken together our fluorescent imaging and ultrastructural observations suggests that SNAP-25 null synapses have a defect in the synaptic vesicle cycle. Membrane is internalized as vesicles or possibly through bulk endocytosis. These structures are acidified as evident by SNB-1::pHluorin assays. However, the total vesicle numbers are increased and expand a greater distance from the dense projection.

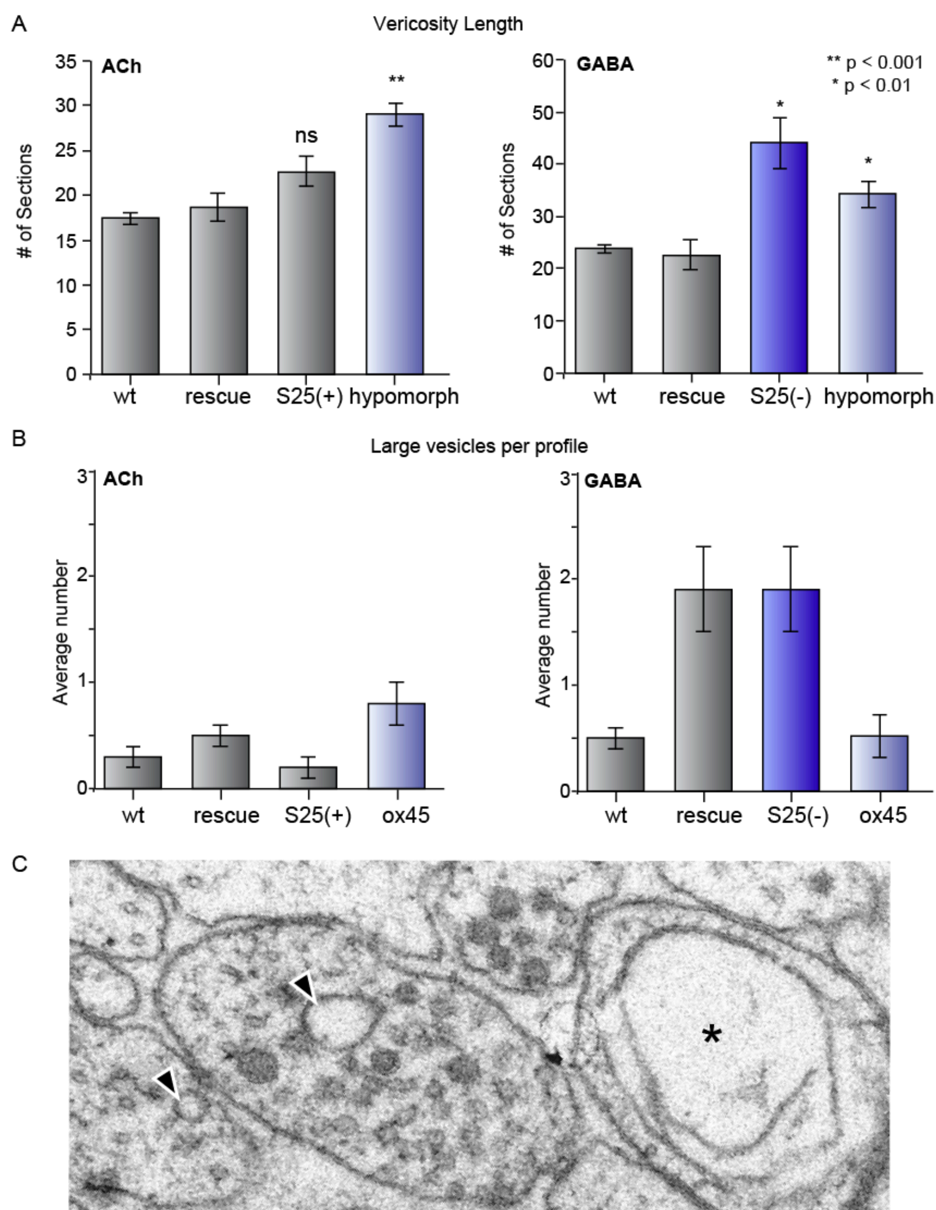

Figure 4.7 Ultrastructural morphology of SNAP-25 null synapses suggests a recycling defect. (A) Quantification of the average length of acetylcholine and GABA synaptic varicosities. In cholinergic neurons the wild-type, rescue, and "ACh-only" strains displayed equivalent terminal length. The hypomorph (*ox45*) had significantly longer varicosities. At GABA synapses, the wild-type and rescue strains had statistically the same synapse length. The SNAP-25 null and hypomorph synapses were nearly twice as long. (B) Quantification of the number of large vesicles per profile in acetylcholine and GABA neurons. At acetylcholine terminals, the hypomorph had twice as many large vesicles compared to the wild type. At GABA terminals, both the rescued strain and the "ACh-only" strain show a dramatic increase in large vesicles. The lack of rescue here is disconcerting. (C) A single section from the ventral nerve cord of the hypomorph strain. Two large vesicles are seen in neighboring synapses (arrowheads). One very large vacuole fills a third neuron (*).

These phenotypes appear unique to SNAP-25. Full reconstructions of syntaxin null synapses show no change in vesicle number or size, and endosome abnormalities have not been observed (Hammarlund et al., 2007). However, it is important to consider that syntaxin null synapses are completely incapable of fusion. These phenotypes may be a result of vesicle recycling following fusion and therefore may be masked in the syntaxin null. Therefore, we are considering the role of both syntaxin and SNAP-25 in vesicle recycling.

The mechanism responsible for these defects remains mysterious. At some level, this phenotype requires a homeostatic response such that the cell allocates more membrane and vesicle proteins to axon terminals. However, we expect this is a secondary defect and not a direct result of SNAP-25's absence. Therefore, we are considering two apposing models to explain the increase in vesicles and endosomes at SNAP-25 null synapses. (1) SNAP-25 is required for the recycling of synaptic vesicles. In this model, synaptic vesicles are internalized, but they lack the appropriate molecular identity to fuse or signal to the cell body to stop making synaptic vesicles. (2) SNAP-25 is required for the recycling of target membrane vesicles. In this model, SNAP-25 is required for returning plasma membrane components to the cell surface after internalization.

SNAP-25: required for the recycling of synaptic vesicles?

Target-SNARE proteins are not traditionally believed to be important for synaptic vesicle recycling. However, both syntaxin and SNAP-25 are found on purified synaptic vesicles (Takamori et al., 2006), and both t-SNAREs have recently been implicated in rapid and slow endocytosis (Xu et al., 2013; Zhang et al., 2013). Furthermore, SNAP-25

binds the endocytosis protein intersectin as well as dynamin in a complex with syntaxin (Okamoto et al., 1999; Peters et al., 2004). Finally, the SNAP-25 chaperone cysteine string protein alpha is tightly associated with synaptic vesicles and is found in complex with the endocytosis protein Hsc70. Therefore, there is ample indirect evidence linking SNAP-25 to endocytosis.

Our data suggest that vesicles are internalized and acidified in the absence of SNAP-25. However, for some reason the cell produces an abundance of vesicles overfilling the nerve terminals. This is not observed in syntaxin nulls (Hammarlund et al., 2007) nor has it been reported in other synaptic vesicle fusion mutants including unc-18, unc-13, or synaptobrevin. It is therefore possible that SNAP-25 is required for establishing the proper identity of a synaptic vesicle. This could result from a requirement of SNAP-25 for sorting vesicle lipids or proteins prior to internalization and may involve adaptors like intersectin or dynamin. This model is relatively vague and difficult to test. However, one prediction is that the vesicle constituents would differ in synapses lacking SNAP-25. We are currently developing a strategy to isolate synaptic vesicles from *C. elegans*. If effective, we will be able to do comparative proteomics on synaptic vesicles isolated from *C. elegans* and with and without native SNAP-25 expression.

SNAP-25: required for the recycling of target-membrane vesicles?

The large number of vesicles accumulating at SNAP-25 null terminals may not be synaptic vesicles. Instead, they may represent a population of "target vesicles" incapable of fusion with the plasma membrane. Such fusion events are important during

development for axon outgrowth and transmembrane protein trafficking. However our data and others suggest that SNAP-25 is not required for these developmental processes (Figure S2.2) (Washbourne et al., 2002). Instead these vesicles may be a byproduct of endocytosis. If t-SNAREs and other plasma membrane proteins are internalized during endocytosis as discussed above, there must be a mechanism for returning them to the plasma membrane.

In Figure 4.8 I present three possible models for SNARE mediated target-membrane return. Only the second model is consistent with the SNAP-25 null phenotype, and I will narrate it in detail here. Following selective clathrin-mediated resolution of synaptic vesicles, target membrane components are concentrated on vesicles. These vesicles for all intents and purposes have plasma membrane identity including the t-SNAREs syntaxin and SNAP-25. Synaptobrevin has a high surface residence at nerve terminals and binds the t-SNAREs in an “upside-down” ternary SNARE complex. This mechanism may be in place to deal with periods of rapid release. However, even in the absence of rapid release, some target vesicle return may be at play. In the absence of SNAP-25, these vesicles may accumulate at synapses as dead-end products explaining the ultrastructural phenotype we have observed at mutant synapse.

The most important prediction of this model is that t-SNAREs are internalized at synaptic terminals. Furthermore, intense stimulation, or the chronic absence of SNAP-25 would be expected to increase the accumulation of syntaxin inside the cell. We are currently testing these predictions. We have designed pH sensitive syntaxin reporter proteins (syntaxin::pHluorin) to determine the surface residence of syntaxin at synapses. Furthermore, since presynaptic terminals appear so large in SNAP-25 mutants, we expect

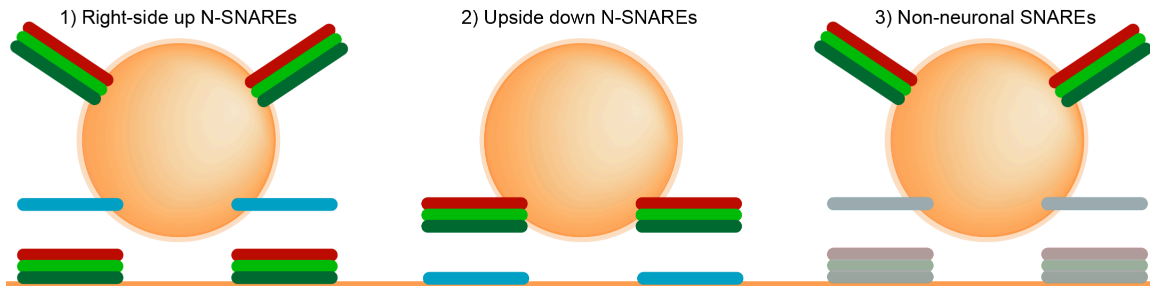

Figure 4.8 The fusion of "target" vesicles must occur by one of three possibilities. (1) Synaptobrevin drives canonical fusion. In effect, plasma membrane components could "hitchhike" on synaptic vesicles and return to the plasma membrane without a dedicated pathway. (2) SNAP-25 and syntaxin pair on vesicles and fuse with synaptobrevin residing on the plasma membrane via an "upside-down" SNARE configuration. (3) An alternative set of SNARE proteins resides at the synapse to drive these fusion events. This model seems unlikely as it necessitates a new set of three or four cognate SNARE proteins that are recycled in parallel with the neuronal SNAREs.

that we may be able to determine whether syntaxin resides inside terminals with traditional confocal imaging. However, we are also pursuing fluorescent EM experiments to test the localization of syntaxin and SNAP-25 on endosomes in normal conditions and following stimulation. These experiments will allow us to query the participation of SNAREs in the synaptic vesicle cycle of wild type and *snap-25* mutant strains.

Conclusions

In summary, this work demonstrates that SNAP-25 is involved in docking and fusion of synaptic vesicles. An alternative SNARE interaction involving synaptobrevin drives SNAP-25 independent fusion. SNAP-29 is capable of fusing synaptic vesicles, but the requirement of SNAP-29 for normal levels of exocytosis remains untested. Unc18 is also required for fusion, but the mechanism of action remains mysterious. Our work shows that the N-peptide of syntaxin binds to Unc18 in order to load it onto the SNARE

core complex. Following complete fusion, SNAP-25 may play a unique role in the recycling of synaptic vesicles or plasma membrane components.

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