A Genetic Analysis of Complexin Function in Neurotransmitter Release and Synaptic Plasticity

by

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B.S. Biological Sciences
Stanford University 2003

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

FEBRUARY 2009

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Submitted to the Department of Biology on February 12, 2009 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

ABSTRACT

Information transfer at neuronal synapses requires rapid fusion of docked synaptic vesicles in response to calcium influx during action potentials. The molecular nature of the fusion clamp machinery that prevents exocytosis of synaptic vesicles in the absence of a calcium signal is still unclear. Here we show that complexin, a small alpha-helical protein that binds fully assembled SNARE complexes, functions as the synaptic vesicle fusion clamp *in vivo*. *Drosophila* has a single complexin homolog that is abundantly expressed in presynaptic nerve terminals. Animals lacking complexin die throughout development, with adult escapers showing severe locomotion defects and a loss of visual function. Electrophysiological analysis at neuromuscular junctions in *complexin* null mutants reveals a dramatic increase in spontaneous synaptic vesicle fusion that is independent of nerve stimulation or extracellular calcium. High frequency stimulation at high calcium concentrations shows that the readily releasable pool in *complexin* mutants is severely depleted. Thus, complexin is required for maintenance of the readily releasable pool of vesicles at the synapse, and without it vesicles exocytose directly after priming. These data indicate that complexin interacts with assembled SNARE complexes to prevent premature vesicle fusion in the absence of calcium entry. In addition, a preliminary analysis of *synaptotagmin 1; complexin* double mutants reveals that the elevated mini frequency in *complexin* single mutants is dependent on synaptotagmin 1. This finding suggests that the dominant function of complexin at the synapse is to prevent synaptotagmin 1 from triggering fusion in the absence of calcium. Further analysis of *synaptotagmin 1*; *complexin* double mutants may reveal new aspects of the mechanism of the calcium-regulated vesicle fusion reaction.

Minis have long been thought to represent background noise at the synapse, but there is now growing evidence that mini frequency is important in synaptic maintenance and plasticity. *Complexin* mutants display a substantial synaptic overgrowth phenotype. We hypothesized that the enhanced mini frequency in *complexin* mutants drives synaptic overgrowth and that complexin is phosphorylated by PKA to regulate mini frequency at *Drosophila* synapses in an activity-dependent retrograde signaling pathway that mediates a large increase in mini frequency and a concomitant induction of synaptic growth. Like *complexin* mutants, a syntaxin mutant with elevated mini frequency also displays enhanced synaptic growth, providing further evidence that an increase in mini frequency drives synaptic plasticity. S126 in complexin is phosphorylated by PKA *in vitro*. Future results may reveal that S126 is phosphorylated by PKA *in vivo* to regulate mini frequency in an activity-dependent manner. These results have the potential to reveal a new role for minis in local synaptic plasticity in response to neuronal activity.

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Chapter 1:Introduction

Molecular Mechanisms of Synaptic Vesicle Exocytosis

Sarah Huntwork-Rodriguez

Synaptic Transmission

The process of synaptic transmission is the basis for neuronal function and is regulated during several types of synaptic plasticity. Proper function of the nervous system requires that synaptic transmission be a rapid and highly regulated process to produce specific neuronal connections and firing patterns. Early work at the neuromuscular junction by Katz and colleagues to gain insight into mechanisms of synaptic transmission showed that action potentials arriving at nerve terminals trigger rapid calcium influx into the presynaptic compartment, promoting release of neurotransmitter into the synaptic cleft (Katz and Miledi 1967; Mulkey and Zucker 1991). It was observed that release of neurotransmitter is quantal, i.e. it occurs in discrete, rather than graded, amounts (Fatt and Katz 1952). This observation was explained by the discovery that quantal transmission occurs by fusion of synaptic vesicles with the presynaptic membrane, releasing neurotransmitters into the synaptic cleft that open ligand-gated ion channels in the postsynaptic membrane (Heuser, Reese et al. 1979). The time period between an action potential reaching a nerve terminal and production of a postsynaptic potential is approximately 200 μsec (Llinas, Steinberg et al. 1981; Cope and Mendell 1982). While there are still many questions about how such a rapid and tightly regulated process can occur, much has been learned over the past two decades about the formation, processing, and recycling of synaptic vesicles at the nerve terminal; the process of membrane fusion itself; and the molecular machinery required for calcium-regulated vesicle fusion.

The Synaptic Vesicle Cycle

Synaptic vesicle exocytosis occurs at specialized electron-dense structures known as active zones, which are directly apposed to postsynaptic densities at the synaptic cleft. Active zones contain the specialized molecular infrastructure for synaptic vesicle exocytosis, including

calcium channels, scaffolding proteins, synaptic vesicles, and vesicle fusion machinery. Many synapses contain only one active zone, whereas other types of synapses contain many active zones. Examples of this second category include the Calyx of Held in the mammalian auditory brainstem or neuromuscular junctions. At the active zone, synaptic vesicles cycle through a series of maturation steps as they are prepared for release, undergo exocytosis, and then are recycled for another round of fusion (**Figure 1**). After being filled with neurotransmitter, synaptic vesicles are targeted to the presynaptic membrane at an active zone, where they dock and undergo priming, in which synaptic vesicles are made competent for fusion. After priming, synaptic vesicles wait for calcium influx through nearby calcium channels, at which point fusion with the presynaptic membrane proceeds. Synaptic vesicles are then recycled for another round of vesicle fusion (Sudhof 2004). Each of these steps are governed by a set of proteins specialized for neurotransmitter release and membrane recycling. For example, targeting and docking are known to involve Rab3, a GTP-binding protein, and RIMs, components of the active zone apparatus that act as Rab effectors at the synapse. Munc18 and munc13 are both absolutely required for synaptic vesicle exocytosis and function in priming. Complexins function in a post-priming step in the last moments before fusion, and synaptotagmin 1 functions as a calcium sensor for neurotransmitter release (Sudhof 2008).

Vesicle pools in synaptic transmission

The available synaptic vesicles at a synapse are divided into separate pools based on the conditions under which they are released. While the terminology for these pools varies from system to system, at least three distinct pools of vesicles exist at most synapses investigated (Rizzoli and Betz 2005). During a single stimulation, or a series of low frequency stimulations, the vesicles that are released come from the Readily Releasable Pool (RRP), which is generally

thought to consist of those vesicles that are docked and primed for release (Schikorski and Stevens 2001). During high frequency stimulation, the RRP is depleted within five to fifteen seconds (Schneggenburger, Meyer et al. 1999; Delgado, Maureira et al. 2000; Richards, Guatimosim et al. 2003). The RRP contains only a small percentage of the total number of vesicles at a synapse (Delgado, Maureira et al. 2000; Schikorski and Stevens 2001; Rizzoli and Betz 2004). During moderate frequency stimulation, vesicles are continuously released from and rapidly recycled into the Recycling Pool (Harata, Ryan et al. 2001; Kuromi and Kidokoro 2003; Richards, Guatimosim et al. 2003), which contains about 10 to 20% of the total vesicles at a synapse (Delgado, Maureira et al. 2000; Harata, Ryan et al. 2001). During high frequency stimulation, the rate of endocytosis and recycling is limiting, and the terminal must therefore mobilize vesicles from the Reserve Pool (RP) to maintain release from the synapse (Heuser and Reese 1973; Kuromi and Kidokoro 2000). The reserve pool is only mobilized at a synapse once the other pools have been depleted and contains 80 to 90% of the total vesicle population at a synapse (Kuromi and Kidokoro 1998; Richards, Guatimosim et al. 2000; Richards, Guatimosim et al. 2003). The RRP and the recycling pool recycle quickly and directly from the plasma membrane, whereas the Reserve Pool recycles more slowly and may rely on endocytic intermediates such as endosomes (Heuser and Reese 1973; Richards, Guatimosim et al. 2000; Richards, Guatimosim et al. 2003; Rizzoli and Betz 2004). While the RRP by necessity must be localized near active zones and the plasma membrane, the recycling pool and the reserve pool are not spatially segregated at synapses and one cannot distinguish the pools visually by EM (Harata, Ryan et al. 2001; de Lange, de Roos et al. 2003; Rizzoli and Betz 2004), although in *Drosophila* neuromuscular junction boutons the reserve pool tends to cluster near the periphery (Kuromi and Kidokoro 1998).

Regulation of mini frequency at synapses

Two modes of release exist at synapses: evoked release, in which synaptic vesicles exocytose in response to an action potential arriving at the nerve terminal, and spontaneous release, in which individual synaptic vesicles fuse with the presynaptic membrane in the absence of an action potential. These spontaneous release events produce miniature postsynaptic potentials, or minis. Evoked responses are well known to be modulated during synaptic plasticity events, as in the case of LTP or LTD. In contrast, minis are generally thought to represent background noise in a synapse. There is a growing range of evidence, however, that spontaneous release frequency is modulated at synapses during synaptic development and plasticity, adding a new layer of regulation of synaptic activity.

When minis were first described in detail by Fatt and Katz at the frog neuromuscular junction, it was noted that frequency of minis at a given NMJ can vary over 1000-fold, while average mini amplitude remains fairly constant (Fatt and Katz 1952) (**Figure 2**). Mini frequency was shown to be highly sensitive to changes in temperature and osmolarity of the bath. For example, exposure of the preparation to a 50% sucrose solution increased mini frequency 45-fold. Some evidence suggests that this potential for variation in mini frequency is utilized by the nervous system. Mini frequency can be enhanced by at least 50-fold by natural products such as forskolin and nicotine in the nerve terminal, as well as by intracellular pathways that increase the concentration of cyclic AMP (Dryden, Singh et al. 1988; Yoshihara, Suzuki et al. 2000), indicating that second messenger cascades can regulate mini frequency.

This regulation is known to be involved in activity-dependent synaptic plasticity at the *Drosophila* neuromuscular junction (Yoshihara, Adolfsen et al. 2005). At embryonic neuromuscular junctions, postsynaptic calcium influx due to high frequency stimulation induces a retrograde signaling mechanism to activate a PKA-dependent increase in cyclic AMP

concentration that enhances mini frequency about 100-fold over a time period of several minutes. This enhancement in mini frequency requires release of postsynaptic vesicles by synaptotagmin 4, which serves as a calcium sensor in this pathway. Synaptic growth is also stimulated in a synapse-specific manner by the action of synaptotagmin 4. Enhancement in mini frequency might therefore be used as a synaptic tag to specifically enhance growth at active synapses.

The *Drosophila* neuromuscular junction is not the only system in which minis have been observed to modulate synaptic function. Minis have been shown to be intimately involved in synaptic maintenance of dendritic spines in the mammalian central nervous system. Dendritic spines are the sites of synaptic input for many types of neurons in the CNS, and dendritic spine density must therefore be maintained in order to receive input from a large number of neurons. Dendritic spine density in pyramidal cells of the CA1 hippocampus layer is highly dependent on minis but not on evoked release (McKinney, Capogna et al. 1999). While blocking action potentials in acute hippocampal slices by application of tetrodotoxin had no effect on dendritic spine density, application of botulinum neurotoxin, which blocks all synaptic transmission, reduced dendritic spine density to about 25% of the density found in untreated neurons. This effect was rescued by external application of AMPA, demonstrating that loss of spontaneous neurotransmitter release leads to a large decrease in dendritic spine density in neurons of the hippocampus.

In addition to dendritic spine density, protein synthesis in dendritic spines is also highly regulated by minis (Sutton, Wall et al. 2004). In hippocampal cultures, inhibition of action potentials by tetrodotoxin had no effect on protein synthesis in dendrites, whereas inhibition of all synaptic transmission produced a large, rapid increase in protein synthesis. Enhancing mini frequency with α-latrotoxin produces a rapid, acute decrease in protein synthesis in dendritic

spines. Minis therefore strongly regulate postsynaptic translation in a frequency-dependent manner. The products of protein synthesis contribute to structural plasticity (Casadio, Martin et al. 1999; Johnson and Ouimet 2004), so regulation of dendritic protein synthesis by mini frequency may provide a mechanism for tight regulation, and rapid recruitment, of plasticity at least in the hippocampus and possibly in other areas of the brain.

In another example of the importance of mini frequency in modulating synaptic function, bursts of enhanced mini frequency lasting several minutes caused by application of nicotine to hippocampal slices produced concomitant bursts of firing in postsynaptic pyramidal neurons (Sharma and Vijayaraghavan 2003). This effect is due to the small but significant increase in membrane potential that a large increase in mini frequency produces. Thus, firing patterns of neurons in the hippocampus could be regulated in part by regulation of mini frequency.

While the dataset supporting the importance of minis in a wide range of neuronal processes is so far small, it is clear that minis are not simply noise. Their modulation can be used as a tool to mark synapses for growth, to maintain synaptic connections, to regulate postsynaptic translation, and to modulate neuronal firing patterns. It is probable that future work will reveal further functions for minis in neurobiological systems, providing a new avenue of regulation of synaptic function and plasticity at synapses.

Membrane Fusion

Synaptic vesicle exocytosis involves the fusion of two lipid bilayers, one in the synaptic vesicle and one in the presynaptic membrane. Membrane fusion is not unique to synaptic vesicle fusion, but is instead a common process central to many cellular functions, including vesicle trafficking both among organelles and from the Golgi apparatus to the cell surface. All membrane fusion events involve the joining of two separate lipid bilayers into one continuous bilayer. Because the

negatively-charged phospholipid head groups of the bilayer must come into close proximity during fusion, and because the hydrophobic core of the membrane must be bent out of its natural shape and come into contact to some degree with water, membrane fusion is an energetically expensive process. While the precise molecular events of membrane fusion have not been conclusively defined, one popular model of membrane fusion is the stalk hypothesis (Jahn, Lang et al. 2003) (**Figure 3**). In this model, two membranes come into close proximity with each other and the two outer layers fuse while the inner layers remain distinct, forming a stalk where contact is initiated. Following stalk formation, stalk expansion may occur, allowing the two inner layers to form a bilayer and producing a hemifused intermediate. Finally, fusion pore opening is initiated from this intermediate by fusion of the two inner layers, the two aqueous compartments can mix freely, and membrane fusion may proceed to completion. Both protein-free bilayers (Chanturiya, Chernomordik et al. 1997; Lee and Lentz 1997; Yang and Huang 2002) and bilayers containing SNARE fusion proteins (Xu, Zhang et al. 2005; Yoon, Okumus et al. 2006) have been shown in *in vitro* assay systems to proceed through this hemifusion intermediate, adding weight to its biological relevance.

Molecular Mechanisms of Intracellular Membrane Fusion

Synaptic vesicle fusion is one example of the many types of intracellular membrane fusion events. Other well-characterized examples include vacuole fusion and trafficking between cisternae in the Golgi apparatus. Synaptic vesicle fusion is highly specialized in several aspects (**Figure 4**). It occurs very rapidly, proceeding to completion in less than a millisecond (Llinas, Steinberg et al. 1981; Cope and Mendell 1982; Sabatini and Regehr 1998), versus seconds to minutes for other trafficking events (Kjeken, Egeberg et al. 2004). It is highly restricted in space to active zones, which typically have an area on the order of 1 μm^2 (Pierce and Lewin 1994),

whereas other fusion events are typically much less restricted. Fusion of synaptic vesicles is elicited by calcium, whereas other types of fusion events are not regulated by calcium. Finally, synaptic vesicles must wait at the plasma membrane for calcium influx to occur and then fuse in a synchronous manner whereas constitutive intracellular vesicles fusion occurs as soon as vesicles reach their target membranes.

While synaptic vesicle exoctyosis is the best characterized example of calcium evoked vesicle fusion, calcium-regulated fusion occurs in several other cases where vesicle fusion must be exquisitely controlled. During fertilization, sperm meeting an egg release an acrosomal granule vesicle located in the head of the sperm that contains enzymes that assist in penetrating the zona pellucida to reach the egg membrane. Acrosomal exocytosis is regulated by extracellular calcium influx as well as release of calcium from the acrosome itself (Darszon, Beltran et al. 2001; De Blas, Michaut et al. 2002). Like acrosomal exocytosis, mast cell activation in the immune system in response to antigen binding releases a vesicular granule in response to extracellular calcium influx and release of intracellular calcium stores (Kim, Eddlestone et al. 1997). Finally, insulin is released from pancreatic beta cells following a rise in intracellular calcium concentration triggered by increased glucose in the blood (Ashcroft, Proks et al. 1994). Besides these examples and synaptic vesicle fusion, other types of vesicular fusion events are not regulated by calcium and occur on a constitutive basis.

How are the specialized properties of synaptic vesicle exocytosis achieved? One hypothesis is that there are distinct molecular mechanisms of fusion between constitutive intracellular vesicle fusion and calcium-regulated vesicle fusion. An alternative hypothesis is that they occur by similar molecular mechanisms but that calcium-regulated fusion is modulated by neuronal proteins that confer the specialized properties seen in synaptic vesicle fusion. A

large body of evidence has demonstrated that a set of evolutionarily conserved proteins function in both constitutive and calcium-regulated fusion, and that neuronal-specific proteins confer calcium sensitivity to synaptic vesicle exocytosis, arguing for the second hypothesis.

The SNARE complex

The neuronal SNARE proteins, syntaxin, SNAP-25, and synaptobrevin, are present presynaptically either in synaptic vesicles (synaptobrevin) or in the plasma membrane (syntaxin and SNAP-25) and play a central role in synaptic transmission (Trimble, Cowan et al. 1988; Baumert, Maycox et al. 1989; Bennett, Calakos et al. 1992). The SNAREs were demonstrated to be necessary for synaptic vesicle fusion by a series of studies showing the three proteins to be the targets of cleavage by the botulinum and tetanus neurotoxins, peptidases that block neurotransmitter release (Schiavo, Benfenati et al. 1992; Blasi, Chapman et al. 1993; Blasi, Chapman et al. 1993). In a parallel set of experiments, NSF (*N*-ethylmaleimide-sensitive factor) was identified as a cytosolic protein necessary in yeast and mammals for all steps of intracellular vesicle trafficking (Novick, Field et al. 1980; Eakle, Bernstein et al. 1988; Wilson, Wilcox et al. 1989; Schekman and Novick 2004). Importantly, the three proteins that are targeted by botulinum and tetanus neurotoxins, syntaxin, SNAP-25, and synaptobrevin, and are thus integral to synaptic vesicle fusion, were found to form a complex that is the receptor for NSF and its binding adaptor protein alpha-SNAP (Soluble NSF Adaptor Protein), both of which are universally involved in vesicle trafficking (Sollner, Whiteheart et al. 1993). This finding strongly suggested that synaptic vesicle exocytosis occurs by a molecular mechanism common to other types of vesicle fusion events. Consistent with this idea, syntaxin, SNAP-25, and synaptobrevin have homologs in yeast that are necessary for membrane trafficking between intracellular compartments (Bennett and Scheller 1993). Because they bind to alpha-SNAP,

syntaxin, SNAP-25, synaptobrevin, and the larger family of homologs involved in vesicle trafficking are called SNAREs, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors. The SNAREs constitute a superfamily of proteins with members in all eukaryotes, including 25 members in *S. cerevisiae*, 36 members in humans, and 54 members in *Arabidopsis thaliana* (Jahn and Scheller 2006).

The SNAREs have been shown to be sufficient to drive fusion of membranes in *in vitro* liposome fusion assays (Weber, Zemelman et al. 1998), and this in combination with the clear necessity of the neuronal SNAREs for neurotransmitter release has led to general agreement that they represent the core machinery that drives fusion of membranes. The domain structures of the neuronal SNAREs and the crystal structure of the assembled SNARE complex are shown in **Figure 5**. All SNAREs contain at least one SNARE motif, a series of 60-70 amino acids arranged in heptad repeats. Syntaxin and synaptobrevin each have one SNARE motif and one transmembrane domain, while SNAP-25 has two SNARE motifs separated by a flexible linker that is palmitoylated to tether the protein to the plasma membrane.

In addition to a transmembrane domain and a SNARE motif, syntaxin has an N-terminal domain, termed the HABC domain, attached to the rest of the protein by a flexible linker (Margittai, Fasshauer et al. 2003). This N-terminal domain contains an anti-parallel three-helix bundle that can bind intramolecularly with the syntaxin SNARE motif in the syntaxin "closed conformation" (Fernandez, Ubach et al. 1998; Lerman, Robblee et al. 2000; Misura, Scheller et al. 2000; Bracher and Weissenhorn 2004). The closed conformation of syntaxin is incompatible with binding to the other SNAREs and may be stabilized by the neuronal protein nSec1/Rop/munc18 (Pevsner, Hsu et al. 1994; Yang, Steegmaier et al. 2000). It is also possible that binding of munc18 to syntaxin is promoted by the closed conformation. Regulation of the

closed and open states of syntaxin appears to be a key point of regulation of docking or priming possibly mediated by munc18 and munc13 (Misura, Gonzalez et al. 2001; Richmond, Weimer et al. 2001; Varoqueaux, Sigler et al. 2002).

The crystal structure of the assembled SNARE complex (Sutton, Fasshauer et al. 1998) shows that the SNAREs bind via coiled-coil interactions of four parallel alpha-helices, two contributed by SNAP-25 and one each contributed by syntaxin and synaptobrevin. While the SNARE motifs of the individual SNAREs are not well structured, binding with the other SNAREs to form a ternary complex drives spontaneous formation of the alpha-helices, releasing free energy. The ternary SNARE complex is remarkably stable, resisting denaturation by sodium dodecyl sulfate (SDS), and impervious to cleavage by the botulinum and tetanus neurotoxins (Hayashi, McMahon et al. 1994). The ternary SNARE complex is unwound in an ATP-dependent mechanism by NSF, a reaction that requires alpha-SNAP for binding of NSF to the SNARE complex (Sollner, Bennett et al. 1993; Hayashi, Yamasaki et al. 1995).

Originally, synaptobrevin and its homologs in other trafficking vesicles were called v-SNAREs, and syntaxin, SNAP-25, and their homologs in target membranes were called t-SNAREs. It was thought that v-SNAREs in the vesicle and t-SNAREs in the target membrane bind, driving fusion. This is a convenient and easily conceptualized model. However, it is somewhat inaccurate because it does not account for homotypic fusion of two similar membranes, two vesicles for example, and because many membranes contain both v- and t-SNAREs. A more relevant classification scheme for SNAREs was developed based on an analysis of the structure of the neuronal SNARE complex and conservation of residues within that structure (Fasshauer, Sutton et al. 1998). Within the four-helix bundle, conserved residues contributed by each of the helices meet in a series of 16 layers to make a hydrophobic core of the

complex. However, the center layer, or 0 layer, contains four charged residues buried within this hydrophobic core, one glutamine from syntaxin, two glutamines from the two helices of SNAP-25 and one arginine from synaptobrevin (Sutton, Fasshauer et al. 1998). This arrangement of charged residues is conserved among all known SNARE complexes and it is therefore thought that functional SNARE complexes always have three glutamines and one arginine at the zero layer, each contributed by one SNARE motif. Thus, the members of the SNARE superfamily have been classified into R-SNAREs and Q-SNAREs. The 3Q:1R rule has been tested in a variety of experimental conditions using site-directed mutagenesis (Katz and Brennwald 2000; Ossig, Schmitt et al. 2000; Graf, Riedel et al. 2005) and has largely been verified by these studies. In the neuronal SNARE complex, synaptobrevin is the R-SNARE and syntaxin and SNAP-25 are Q-SNAREs (Fasshauer, Sutton et al. 1998).

The zipper model of membrane fusion driven by SNAREs

How do the SNAREs drive membrane fusion? While there is still much to be learned about this process, many details have emerged. The "zipper" model of membrane fusion driven by SNAREs holds that SNARE binding begins in *trans*, i.e. with SNAREs on opposing membranes binding at their N termini. More specifically, an acceptor complex consisting of syntaxin bound to SNAP-25 in a 1:1 stoichiometry is formed in the acceptor membrane. This complex is partially helical and its formation is rate-limiting for fusion (Fiebig, Rice et al. 1999; Fasshauer and Margittai 2004). During priming, synaptobrevin binds to the N termini of the acceptor complex SNARE motifs with its N terminus. Binding then proceeds like a zipper from the N termini of the helices to the C termini, with the unstructured SNARE domains spontaneously folding into alpha helices as binding proceeds and the stable four-helix structure forms. It is this spontaneous folding that provides the energy to overcome the energy barrier for lipid fusion. As

the binding reaction proceeds to the C termini of the SNARE complex, the transmembrane domains of the SNAREs, still in separate membranes, are drawn together, pulling the membranes with them until they join. Once this happens, the SNARE complex is in its *cis* formation, with the SNAREs in the same membrane. Finally, alpha-SNAP and NSF bind the *cis*-SNARE complexes and unwind them to free the individual SNAREs for another round of binding and fusion.

Several lines of evidence support the zipper model of SNARE complex function in vesicle fusion. The ternary SNARE complex has its alpha helices arranged in a parallel manner (Sutton, Fasshauer et al. 1998), as is required for zippering from the N to C terminus of the SNARE complex. The formation of the SNARE complex is associated with large structural changes in the individual SNAREs from disordered to highly alpha helical in both the neuronal SNARE complex and a distantly related yeast SNARE complex, and these structural changes are associated with a large release in energy as measured by thermal stability (Fasshauer, Otto et al. 1997; Rice, Brennwald et al. 1997). HSQC NMR spectra of individual yeast SNAREs and the assembled SNARE complex reveal that the N termini of the monomeric SNAREs are structured and alpha helical whereas the C termini are unstructured, while the structure of the assembled SNAREs is almost entirely ordered as a set of alpha helices (Fiebig, Rice et al. 1999). This suggests that binding of the SNARE complex is likely to start at the N termini and proceed to the C termini, which are closest to the vesicles. Indeed, recent *in vitro* analysis of synaptobrevin binding to syntaxin-SNAP-25 acceptor complexes in the presence of N and C terminal truncations of synaptobrevin demonstrated that it is critical for the N termini of the acceptor complex to be available for binding to take place whereas the availability of the C termini is not critical (Pobbati, Stein et al. 2006). Additionally, destabilization of the hydrophobic interactions

in the C-terminal SNARE motifs by mutation of key residues in SNAP-25 selectively prevents synaptic vesicle exocytosis rather than priming. A similar mutational approach in the N terminus or in the middle of the SNARE motifs prevents priming (Sorensen, Wiederhold et al. 2006). Finally, it is clear that the stiffness and length of the linker region between the SNARE motifs and the transmembrane domains of syntaxin and synaptobrevin are critical for vesicle fusion to proceed. With increasing length of this linker region efficiency of fusion decreases (McNew, Weber et al. 1999; Wang, Dulubova et al. 2001), consistent with the idea that the zippering of the SNAREs and drawing together of their transmembrane domains exerts mechanical force on the two membranes to drive fusion. This idea is supported by the finding that the transmembrane domains of syntaxin and synaptobrevin are both necessary for SNARE function in driving fusion (McNew, Weber et al. 2000). Atomic Force Microscopy experiments on fusing liposomes containing SNARE complexes provide evidence that the transmembrane domains of the SNAREs increase tension at the fusion site of two membranes, making deformation of the membranes easier and decreasing the energy barrier to fusion (Abdulreda, Bhalla et al. 2008).

Some debate exists as to whether SNARE complex assembly and membrane fusion occurs in one zippering reaction or if there are intermediates in the reaction. Evidence is mounting that SNARE-driven membrane fusion occurs through a hemifusion intermediate and that the zippering reaction of SNARE binding occurs in a two-step process in which the N termini form a stable complex followed by formation of the C terminal complex, which is less stable. Evidence for the hemifusion intermediate is based on variety of approaches for observing fusion in liposome fusion assays. Monitoring of total lipid mixing and inner leaflet mixing in a fusion assay showed that hemifusion dominated at the beginning of the reaction whereas complete fusion proceeded more slowly (Lu, Zhang et al. 2005; Xu, Zhang et al. 2005), arguing

for a sequential mechanism from hemifusion to full fusion. Observations of individual liposomes fusing using Total Internal Reflection (TIRF) Microscopy clearly demonstrated hemifusion intermediates during fusion events (Yoon, Okumus et al. 2006). Finally, measurement of forces contributing to fusing liposomes containing SNAREs indicated two different stages of fusion, first hemifusion, then proceeding to full fusion (Abdulreda, Bhalla et al. 2008). It has been suggested that the N and C termini of the SNARE complex might bind in separate stages during zippering. Indeed, the two sections show different thermal stabilities upon binding of synaptobrevin (Pobbati, Stein et al. 2006). It is possible therefore that SNARE complex formation can be halted at an intermediate stage during zippering. The authors suggest that this might occur at a point where the force generated by SNARE assembly balances with the repulsive force of fusing lipid membranes. Additional force then might be applied after calcium influx to allow full SNARE assembly and full fusion (Pobbati, Stein et al. 2006).

Summary: Sequence of events in SNARE-driven fusion

Based on the sum of this data, a picture of synaptic vesicle fusion is emerging that proceeds in a step-wise manner. First, an acceptor complex of bound SNAP-25 and syntaxin on the plasma membrane awaits binding by synaptobrevin. At this point, the N termini of the SNARE motifs are alpha helical whereas the C termini close to the transmembrane domain of syntaxin are disordered. During priming, the N terminus of synaptobrevin binds the N terminus of the acceptor complex and zippering partially proceeds, forming fusion-competent vesicles maintained in a hemifused state with the outer leaflets fused but the inner leaflets remaining separate and the SNARE complex in a *trans* formation. Finally, upon influx of calcium following an action potential full fusion proceeds as zippering is completed and the transmembrane domains of the SNARE complex are drawn together, creating tension in the

fusing membranes and pulling the reaction from hemifusion to full fusion with opening of a fusion pore. Presumably additional proteins present at the nerve terminal mediate some of these steps. For example, it is known that synaptotagmin 1 is the calcium sensor for neurotransmitter release that triggers synchronous fusion of synaptic vesicles after an action potential. Details of this reaction are discussed below.

Regulators of Synaptic Vesicle Exocytosis

While the neuronal SNAREs are sufficient to drive fusion, it is clear that additional regulators are present that confer the specialized properties of neurotransmitter release. Synaptic vesicle exocytosis is rapidly triggered by calcium influx, so a calcium sensor must be present that binds calcium and triggers fusion. Additionally, synaptic vesicle exocytosis is distinct from constitutive fusion because synaptic vesicles must wait in a primed, fusion competent state for calcium influx to occur, whereas in constitutive exocytosis vesicles fuse as soon as they are competent to do so. Because calcium-regulated fusion and constitutive fusion occur via a common SNARE-driven mechanism, calcium-regulated exocytosis is thought to require a fusion clamp. A fusion clamp would prevent release prior to calcium influx and allow release after calcium influx, acting to synchronize and speed the fusion process (Bennett and Scheller 1993; Popov and Poo 1993; Sollner, Whiteheart et al. 1993).

Given that the SNAREs drive exocytosis, it is highly probable that any regulators of the late stages of neurotransmitter release, such as a calcium sensor or a fusion clamp, would impinge on the SNARE complex. While there are many different proteins present at nerve terminals, only a small subset bind assembled or partially assembled neuronal SNARE complexes. The functions of these proteins give us insight into the final sequence of synaptic vesicle exocytosis, its regulation by calcium, and the ways that accessory proteins have been

used to confer the special properties of neurotransmitter release. Two proteins that are critical for triggering of exocytosis by calcium are synaptotagmin, a calcium sensor for synchronous neurotransmitter release, and complexin, a neuronally expressed protein that binds the SNARE complex whose function is less well defined.

Synaptotagmin 1

The dependence of quantal release on calcium is highly cooperative with a Hill coefficient of nearly four (Dodge and Rahamimoff 1967). For this reason, it was proposed that a calcium sensor exists at the nerve terminal that binds calcium and triggers release with a similar cooperativity. Synaptotagmin 1 is one of the best-characterized SNARE-interacting proteins in the nerve terminal and is widely acknowledged to be the calcium sensor for neurotransmitter release. It binds calcium at the elevated calcium concentrations of at least 20 μM found at active zones during calcium influx and synaptic vesicle exocytosis (Brose, Petrenko et al. 1992; Llinas, Sugimori et al. 1992; Heidelberger, Heinemann et al. 1994). It is localized to synaptic vesicles at active zones, it can bind up to 5 calcium ions, and it exhibits calcium-dependent phospholipid binding with a Hill coefficient of two to three (Davletov and Sudhof 1993). Therefore, it possesses the properties necessary for a synchronous calcium sensor.

Synaptotagmin 1 is a 65-kDa protein that is part of a large family of proteins with 17 isoforms in humans and 7 isoforms in flies (Adolfsen, Saraswati et al. 2004; Craxton 2007). It consists of a short intralumenal domain, a transmembrane domain, and two calcium-binding C2 domains, C2A and C2B, connected by a flexible linker (Matthew, Tsavaler et al. 1981; Perin, Fried et al. 1990). C2 domains are approximately 130-amino-acid beta sandwich structures with three flexible loops extending from one end, and are found in many proteins where they frequently serve to coordinate calcium binding and lipid binding (Nalefski, Wisner et al. 2001).

The C2 domains in synaptotagmin have been shown to bind calcium when synaptotagmin is in a lipid environment (Brose, Petrenko et al. 1992). Furthermore, the C2 domains of synaptotagmin bind lipid membranes in a calcium-dependent manner (Perin, Fried et al. 1990; Brose, Petrenko et al. 1992; Davletov and Sudhof 1993; Chapman and Jahn 1994; Fernandez, Arac et al. 2001) by inserting their flexible loops into lipid bilayers once calcium binding has occurred (Brose, Petrenko et al. 1992; Davletov and Sudhof 1993; Davis, Bai et al. 1999). This reaction proceeds within the millisecond time frame required for triggering of synaptic vesicle exocytosis (Davis, Bai et al. 1999). The two C2 domains are partially redundant for calcium-dependent lipid binding function (Earles, Bai et al. 2001) and cooperate with each other to induce lipid penetration in response to calcium binding (Bai, Wang et al. 2002). Recent evidence suggests that calcium binding by the C2B domain induces C2B binding to two membranes via basic residues on the surface of the domain, bringing the membranes into close proximity and possibly aiding the SNAREs in bringing the vesicle into close proximity with the plasma membrane (Arac, Chen et al. 2006).

Synaptotagmin interacts with individual SNAREs and the SNARE complex in a calcium-dependent manner (Li, Ullrich et al. 1995; Davis, Bai et al. 1999; Gerona, Larsen et al. 2000). Both C2 domains are required for synaptotagmin binding to t-SNAREs and the assembled SNARE complex (Davis, Bai et al. 1999; Gerona, Larsen et al. 2000). This binding reaction is necessary for calcium-evoked fusion (Gerona, Larsen et al. 2000), but it is unclear what role calcium-dependent binding of synaptotagmin to the SNARE complex might play.

Biochemical evidence indicates that synaptotagmin has many of the properties expected of a calcium sensor for synaptic vesicle exocytosis, including calcium binding in the range of

calcium concentrations found at active zones and calcium-dependent conformational changes such as binding of lipids and binding of the SNARE complex.

Synaptotagmin is necessary for fast, synchronous neurotransmitter release in vivo

In addition to biochemical evidence, there is also substantial genetic evidence for synaptotagmin's role as a calcium sensor in neurotransmitter release. Synaptotagmin knockouts in mice (Geppert, Goda et al. 1994) and in flies (Littleton, Stern et al. 1993; DiAntonio and Schwarz 1994; Littleton, Stern et al. 1994) both show a severe decrease in synchronous fusion, indicating synaptotagmin's importance in fast exocytosis in response to calcium influx. *Synaptotagmin 1* null mutants in *Drosophila* also display an asynchronous phase of vesicle fusion that is suppressed in wild type animals (Yoshihara and Littleton 2002). This asynchronous phase of release has been observed in other systems (Goda and Stevens 1994) and shows fourth-order cooperativity with respect to calcium concentration just as synchronous fusion does, suggesting the presence of a second calcium sensor with low calcium affinity responsible for slow, asynchronous fusion. *Synaptotagmin* mutants in *Drosophila* that are missing the C2B domain but have a fully functional C2A domain show both synchronous and asynchronous phases of release, albeit with a lower cooperativity of release in the synchronous phase. This demonstrates that asynchronous and synchronous release can be genetically separated and further argues for a separate asynchronous calcium sensor whose function is suppressed by synaptotagmin 1 (Yoshihara and Littleton 2002). Most importantly for the hypothesis that synaptotagmin 1 functions as a calcium sensor in neurotransmitter release, when the degree of dependence of lipid binding by synaptotagmin on calcium is changed, there is a concomitant change in the dependence of release on calcium without changing the readily releasable pool or calcium influx (Fernandez-Chacon, Konigstorfer et al. 2001; Yoshihara and Littleton 2002).

This demonstrates synaptotagmin's role in specifically regulating calcium-evoked fusion as opposed to it acting at earlier stages such as docking or priming. Thus, synaptotagmin 1 functions as a calcium sensor for the fast, synchronous phase of release and suppresses asynchronous release in *Drosophila*.

Synaptotagmin knockouts display a large enhancement in mini frequency

In addition to eliminating synchronous neurotransmitter release, early reports from *Drosophila synaptotagmin* knockouts showed an increase in spontaneous release by about two- to six-fold (Littleton, Stern et al. 1993; Broadie, Bellen et al. 1994; DiAntonio and Schwarz 1994). This phenotype was not observed in early studies of mouse *synaptotagmin* null mutants using recordings from autaptic cultured hippocampal neurons (Geppert, Goda et al. 1994). However, a recent study found a five-fold increase in mini frequency compared to wild type in mouse *synaptotagmin 2* loss-of-function mutants at the Calyx of Held and at the NMJ (Pang, Sun et al. 2006). Similarly, when recordings were taken from cultured cortical neurons of *synaptotagmin 1* null mutants, a five-fold increase in mini frequency was observed. In early discussions of synaptotagmin's function at synapses it was proposed that synaptotagmin might be a vesicle fusion clamp based on the facts that synaptotagmin binds both the assembled SNARE complex and lipid membranes, that it is specifically expressed in neurons, and that it is highly conserved (Bennett and Scheller 1993; Popov and Poo 1993; Sollner, Whiteheart et al. 1993). The enhanced mini frequency observed in *Drosophila* mutants bolstered this view (Popov and Poo 1993), but because no enhancement in mini frequency was seen in mammalian mutants the idea fell out of favor. With new evidence, it now seems more likely that synaptotagmin plays some role in vesicle fusion clamping. Indeed, a recent report demonstrates that *in vitro* synaptotagmin can inhibit fusion in the absence of calcium but can also promote fusion in the presence of

calcium (Chicka, Hui et al. 2008). Unfortunately, complexin, another protein implicated clamping synaptic vesicles prior to calcium influx, was not included in the analysis, which might have revealed much about how these two protein function together, if at all, during calcium-evoked fusion.

While the exact molecular sequence of events involved in synaptotagmin's triggering of synaptic vesicle exocytosis is still under debate, it is clear that it functions as a calcium sensor for fast synchronous neurotransmitter release. Key aspects of its function include calcium-dependent binding to phospholipids and to the SNARE complex. Thus, it is likely that upon calcium binding of synaptotagmin, binding to the SNARE complex is enhanced and synaptic vesicles and the plasma membrane are pulled together, activating fusion. There is also evidence that synaptotagmin suppresses fusion in the absence of high calcium concentrations.

Complexin

In addition to synaptotagmin, a second neuronal-specific protein known to bind the SNARE complex and modulate its function is complexin. There is growing evidence that complexin levels are altered in several neurodegenerative diseases and mental disorders, including Parkinson's Disease (Basso, Giraudo et al. 2004), schizophrenia and bipolar disorder (Eastwood and Harrison 2000), Wernicke's encephalopathy (Hazell and Wang 2005), and Huntington's Disease (Morton, Faull et al. 2001). Four isoforms of complexin exist in mammals, and they were first identified as novel proteins that co-immunoprecipitate from rat brain extracts with the SNAREs, synaptotagmin, and N-type calcium channels (Saisu, Ibaraki et al. 1991; McMahon, Missler et al. 1995). Initial analysis showed that complexins are small, highly charged proteins of 15-20 kDa that are well conserved from invertebrates to vertebrates (**Figure 6**) and contain no transmembrane domains. They display high alpha-helical content, and specifically bind the

neuronal SNARE complex with a 1:1 stoichiometry (Ishizuka T., Saisu H. et al. 1995; McMahon, Missler et al. 1995; Pabst, Hazzard et al. 2000).

Phylogenic analysis of the complexin family highlights certain features of complexin's lineage (**Figure 7**). The four mammalian complexins consist of two subfamilies, complexin 1/2 and complexin 3/4. Other vertebrate species with sequence available have homologs of complexin 1/2 but not 3/4, although this might be due to incomplete sequence availability rather than an actual absence of complexins 3 and 4 in these species. The two complexin subfamilies split off from a common ancestor shared with the invertebrate complexins. Invertebrate organisms typically have only one complexin gene, with the exception of C. *elegans* which has two complexin genes (only one is included in the phylogenetic analysis because of incomplete sequence availability).

Expression and localization of mammalian complexins

Mammalian complexins contain between 134 and 160 amino acids. Complexins 1 and 2 are expressed at high levels throughout the brain (Yamada, Saisu et al. 1999). Complexins 3 and 4 are mainly restricted to expression in the retina, with limited expression of complexin 3 in the hippocampus and several other areas of the brain (Reim, Wegmeyer et al. 2005). Immuno-electron microscopy images show that complexin localizes to presynaptic terminals (Takahashi, Yamamoto et al. 1995). Complexins 1 and 2 have distinct but overlapping localization within the mammalian nervous system. Complexin 1 is the main isoform present at neuromuscular junctions, and the two isoforms are differentially expressed in the synaptic layers of the hippocampus (McMahon, Missler et al. 1995). Consistent with this observation, in hippocampal cultures complexins 1 and 2 are both present but generally localize to separate synapses (Ono, Baux et al. 1998). Additionally, complexin 1 has been observed to mainly localize to axo-

somatic synapses while complexin 2 mainly localizes to axo-dendritic synapses (Takahashi, Yamamoto et al. 1995). *In situ* hybridization to complexin 1 and 2 mRNAs and electron microscopy immunohistochemistry demonstrated they are differentially expressed in many areas of the mouse brain including the olfactory bulb, hippocampus, cerebral cortex, pyriform cortex, cerebellum, thalamus, and facial nuclei (Ishizuka, Saisu et al. 1999; Yamada, Saisu et al. 1999). The differential expression of complexins 1 and 2 suggests their distribution is segregated by the circuitry of the neurons in which they are expressed, with complexin 2 involved in circuits of cognition, emotional behavior and control of voluntary movement and complexin 1 involved in circuits of motor learning programs and sensory processing (Freeman and Morton 2004). Complexins 3 and 4 also have differential expression patterns. While both are present in the retina, complexin 3 is highly enriched in cone photoreceptor terminals while complexin 4 is enriched in rod photoreceptor terminals (Reim, Wegmeyer et al. 2005). All four isoforms are to some extent functionally redundant (Xue, Stradomska et al. 2008), but their highly specific differential expression patterns suggest that subtle differences in their function might be used to modulate synaptic function.

In addition to expression in the brain, complexins are also expressed in several other cell types in which vesicle exocytosis is a calcium-regulated event. Complexins 1 and 2 localize to the acrosome in rat sperm (Redecker, Kreutz et al. 2003). Complexin 1 is highly expressed in insulin-secreting β cells of the pancreas (Abderrahmani, Niederhauser et al. 2004), while complexin 2 is highly expressed in mast cells of the immune system (Tadokoro, Nakanishi et al. 2005). Other presynaptic proteins involved in synaptic vesicle exocytosis have been reported to be present in these cell types, suggesting that calcium-regulated fusion in these various settings occurs by a common mechanism.

Complexin knockout mice have strong defects in behavior and synaptic transmission

Knockouts of complexins 1 and 2 in mice separately or as double knockouts demonstrate the necessity of complexin for proper cognitive and nervous system function. Complexin 2 knockouts display significant impairment in long term potentiation (LTP) in both the CA1 and CA3 pyramidal cell layers of the hippocampus, while basic properties of synaptic transmission such as evoked postsynaptic currents are similar to wild type (Takahashi, Ujihara et al. 1999; Huang, Ujihara et al. 2000). This suggests that complexin 2 might be involved in modulating presynaptic neurotransmitter release during synaptic plasticity. Complexin 2 knockouts have approximately normal life spans, living to at least one year of age, but have a range of motor and cognitive impairments that get progressively worse with age, including impaired performance on the rotarod, defects in learning in the Morris water maze, abnormal grooming behavior, and decreased exploratory behavior (Glynn, Bortnick et al. 2003). Complexin 1 knockout mice show a more severe phenotype. They develop a strong ataxia, have sporadic seizures, are infertile, and die within two to four months after birth (Reim, Mansour et al. 2001). Like complexin 2 knockouts, there are no significant changes in basic synaptic transmission properties in hippocampal culture neurons from complexin 1 knockout mice. When these mice are reared on an enhanced feeding regimen that allows them to live a normal life span (>2 years), they display severe defects in motor coordination, including running and swimming, impaired rotarod performance, dystonia, and resting tremors (Glynn, Drew et al. 2005). Higher functions are also impaired, with deficits in grooming, rearing behavior, exploratory behavior, and complex social behavior (Glynn, Drew et al. 2005; Drew, Kyd et al. 2007). Complexin 1/2 double knockouts die within hours after birth and have severe defects in synaptic transmission in cultured hippocampal neurons (Reim, Mansour et al. 2001). The enhanced phenotype observed in double knockouts demonstrates that complexins 1 and 2 are partially functionally redundant.

The structure of complexin and the complexin / SNARE complex

The properties of complexin's structure as an isolated protein and when bound to the SNARE complex have been extensively studied by NMR and X-Ray crystallography (Pabst, Hazzard et al. 2000; Bracher, Kadlec et al. 2002; Chen, Tomchick et al. 2002). Isolated complexin has no tertiary structure but contains a central conserved alpha-helical domain extending from residues 26 to 83. The N and C termini are unstructured and therefore their interactions with the SNARE complex or other binding partners are less well characterized than the central alpha helical portion. X-Ray crystallography was performed using residues 26 to 83 of rat complexin bound to the minimal SNARE complex (the SNARE motifs minus the transmembrane domains and syntaxin's regulatory N terminal domain) (Chen, Tomchick et al. 2002) (**Figure 8**). This showed that complexin binds the groove between syntaxin and synaptobrevin in an antiparallel fashion via coiled-coil interactions. Binding induces an overall increase in alpha helical content in complexin, mainly due to structural changes in the C terminal portion of the 26-83 fragment. Residues 30 to 47 assemble into an alpha helix independently of SNARE binding and this portion has been termed the accessory alpha helix, as it does not directly participate in SNARE binding. Contact between complexin and the SNARE complex is localized to the C terminal end of the complexin fragment between residues 48 and 70, while the N terminus is unbound and extends into solution. Complexin binds syntaxin and synaptobrevin at residues surrounding the SNARE complex zero layer (Bracher, Kadlec et al. 2002). Binding does not induce a large change in the structure of the minimal SNARE complex, but deuterium exchange experiments show that it slightly stabilizes the interface between syntaxin and synaptobrevin (Chen, Tomchick et al. 2002). This suggested that complexin might stabilize the assembled SNARE complex as part of the fusion process, functioning as a "tape" that assists the SNAREs in assembling and driving fusion. In contrast, Bracher et al proposed based on the squid complexin

structure that complexin might function as part of a complex to keep synaptic vesicles in a primed and fusion-ready state for calcium-triggered exocytosis. These opposing views can be seen as a microcosm of the competing views of complexin's function, detailed below.

Complexins 1 and 2 bind the assembled SNARE complex with dissociation constants in the low nanomolar range (Pabst, Margittai et al. 2002). Complexins 3 and 4 are less well characterized with respect to their SNARE complex binding affinities, but complexin 3 has a similar binding affinity to those of complexins 1 and 2, whereas complexin 4 has a much lower SNARE complex binding affinity (Reim, Wegmeyer et al. 2005). Complexin can bind monomeric syntaxin as well as a binary complex of SNAP-25 and syntaxin, but these interactions are much weaker than complexin's binding to the assembled SNARE complex (McMahon, Missler et al. 1995; Weninger, Bowen et al. 2008).

Evolutionarily conserved post-translational modification of complexins

A growing body of evidence indicates that complexins are regulated by two forms of post-translational modification: prenylation and phosphorylation. Prenylation involves covalent addition of a farnesyl (15-carbon) or geranylgeranyl (20-carbon) lipid group to a conserved cysteine residue at or near the C terminus of a protein, proteolysis of the remaining C terminus of the protein, and methylation of the cysteine. A "CAAX box" motif, consisting of a cysteine, two aliphatic residues ("A"), and a fourth variable residue ("X"), encodes the prenylation site, and the X dictates which type of lipid is transferred to the protein (Zhang and Casey 1996). In the case of complexins, the majority of invertebrate isoforms contain CAAX boxes (**Figure 6**), as do mammalian complexins 3 and 4. In addition, mammalian complexins 3 and 4 are known to be farnesylated and this modification is critical for the function of complexin 4 (Reim, Wegmeyer et al. 2005). Farnesylation of invertebrate complexins has not been studied, but all known

invertebrate complexins that have CAAX motifs have glutamines in the X position, indicating that they too are farnesylated. Farnesylation of proteins typically is important for concentration of the protein at the membrane as well as for protein-protein interactions and protein stability (Zhang and Casey 1996). Indeed, farnesylation of mouse complexins 3 and 4 is required for their proper localization to synapses. Because they are distributed mainly to retinal ribbon synapses, it is thought that concentration of complexins 3 and 4 at the membranes of these synapses may contribute to the unique release properties of ribbon synapses, which release vesicles at a rate 30 times that of conventional synapses (Stevens and Tsujimoto 1995; Reim, Wegmeyer et al. 2005), suggesting that the specific properties of the different complexin isoforms might be important in determining important properties of synaptic function in mammals. Complexins 3 and 4 are more related to each other than they are to complexins 1 and 2 (Reim, Wegmeyer et al. 2005), and the fact that invertebrate complexins typically have CAAX motifs suggests that the common ancestor of the vertebrate complexins had such a motif, which was then lost after the split between the complexin 1/2 and complexin 3/4 subfamilies (Brose 2008).

Phosphorylation of mammalian complexins has also been shown to be a potentially important mode of regulation of complexin's function. Complexin 2 isolated from primary cortical cultures from rats is highly phosphorylated on serine 93 (Hill, Callaghan et al. 2006), but it is currently unclear what the functional significance of this modification is. Complexins 1 and 2 are also phosphorylated in rat brains at serine 115. Protein kinase CK2 phosphorylates this site *in vitro*, producing enhanced binding of complexins 1 and 2 to the SNARE complex (Shata, Saisu et al. 2007). Both of these phosphorylation sites are 100% conserved among the mammalian complexin 1/2 subfamily. These data suggest that phosphorylation of complexin

could provide an avenue by which complexin's function is regulated. While the two known mammalian phosphorylation sites are not preserved in invertebrates, the invertebrate complexins contain several conserved residues in the same region of complexin sequence that may be phosphorylated to regulate complexin's function. Which residues of complexin are phosphorylated *in vivo* and the functional consequences of these modifications are exciting areas for future study of complexin, its overall role in neurotransmitter release, and its possible role in synaptic plasticity.

Function of complexin in synaptic vesicle exocytosis and its mechanism of action

The properties of complexin binding to the SNARE complex, along with its strong expression in the brain and low expression in other tissues, suggest that it is an important modulator of SNARE function and neurotransmitter release. A host of genetic, biochemical, and electrophysiological studies confirm this and demonstrate that complexin's binding to the SNARE complex mediates its regulation of calcium-evoked neurotransmitter release in a post-priming step. These same studies provide complementary but often seemingly contradictory evidence for complexin's precise role in neurotransmitter release.

Overexpression studies have concluded that complexin is a negative regulator of neurotransmitter release. Microinjection of complexin II into presynaptic terminals of *Aplysia* buccal ganglia results in a marked decrease in the evoked response, and there is a corresponding increase in evoked responses after injecting anti-complexin antibodies. Two separate studies on PC12 cells showed a decrease in neurotransmitter release when complexin was overexpressed via transfection (Itakura, Misawa et al. 1999; Edwardson, Wang et al. 2003). Similarly, overexpression of complexin in adrenal chromaffin cells by transfection inhibited exocytosis. Overexpression of an R59H mutant complexin, which greatly reduces SNARE complex binding,

did not inhibit exocytosis, suggesting that the overexpression phenotype requires SNARE binding (Archer, Graham et al. 2002). In pancreatic beta cells, which release insulin-containing granules in a calcium- and SNARE-dependent reaction and which therefore express many neuronal-specific proteins, enhanced complexin expression reduces secretion of insulin (Abderrahmani, Niederhauser et al. 2004). Finally, addition of sub-micromolar amounts of complexin to permeabilized sperm severely inhibits acrosomal exocytosis by blocking release at a point where SNAREs are assembled in loose trans complexes. This block is relieved by addition of calcium and the C2B domain of synaptotagmin VI, which is known to be essential for the acrosomal reaction (Roggero, De Blas et al. 2007). Taken together, this evidence suggests that complexin is a negative regulator of synaptic vesicle exocytosis and that it may function by preventing full zippering of the SNARE complex.

However, studies of a mouse complexin 1/2 double knockout animals suggest the opposite (Reim, Mansour et al. 2001). Double knockout mice die within hours after birth. In hippocampal autaptic cultures from these mice, there is a 50% reduction in evoked release compared to wild type cells at 4 mM extracellular calcium with no detectable defect in the readily releasable pool of vesicles, as determined by application of a hypertonic sucrose solution. The readily releasable pool of vesicles corresponds to those vesicles that are docked and primed. Therefore, the defect in these cultured neurons is in an exocytosis step after priming. The authors also observed that while evoked release was smaller than in wild type animals at medium and low levels of external calcium, at high levels of external calcium (>12 mM), the evoked response approached that of wild type cells. Therefore, the double complexin knockout has a calcium sensing defect very similar to that of a synaptotagmin hypomorphic allele, indicating that complexin may promote release during calcium sensing or during fusion after calcium

sensing. While complexins 1 and 2 are the predominant complexins expressed in the brain, there is a limited amount of complexin 3 expressed in the brain as well, potentially confounding these results. However, complexin 1/2/3 triple knockouts have a nearly identical set of synaptic transmission defects as the complexin 1/2 knockout, and complexin 3 can rescue the defects seen in the 1/2 double knockout, indicating that it functions similarly to complexins 1 and 2 (Xue, Stradomska et al. 2008). Complexin 1/2/3 triple knockouts also showed reduced evoked responses and reduced spontaneous postsynaptic current frequency in two central nervous system synapses, GABAergic synapses in the striatum and in brainstem slice recordings from the pre-Bötzinger complex. Complexin 4 can also rescue the defect seen in complexin 1/2 double knockouts, so the possibility still remains that compensatory effects by the final complexin isoform could confound this analysis, although it is not expressed in the central nervous system. Also, because the 1/2 double knockout is lethal within hours after birth in mice, central nervous system synapses are not mature at the time of the recordings done in this study. Thus, *in vivo* recordings from mature synapses containing no complexin have not been completed to date, but the recordings that have been done in mammalian *complexin* knockouts argue for a positive role of complexin in neurotransmitter release at a post-priming step.

Further experiments have explored the mechanism by which complexin regulates the SNARE complex to modulate neurotransmitter release. In cultured cells expressing "flipped" SNAREs, or SNAREs tethered to the cell surface membrane by a glycerophosphatidylinositol (GPI) anchor, addition of any of the mammalian complexins to the medium restricts fusion of cells in a dose-dependent manner (Giraudo, Eng et al. 2006; Giraudo, Garcia-Diaz et al. 2008). At the highest concentration of complexin tested (40 μM), cytoplasmic complexin limits fusion to 50% of the fusion observed with no complexin added. Increasing the local concentration of

complexin at the membrane by anchoring it with a GPI tether decreases fusion to 3% of normal, demonstrating that in *in vitro* systems complexin is able to restrict fusion as it does in the case of overexpression. This block in fusion is relieved by expression of synaptotagmin and physiological levels of calcium. The block holds the SNARE complex in a conformation that is sensitive to Botulinum neurotoxin serotype B (BoNT/B) but insensitive to Tetanus neurotoxin (TeNT). Both of these toxins cleave synaptobrevin at the same site (Schiavo, Benfenati et al. 1992), but because BoNT/B binds synaptobrevin at a membrane-proximal site closer to the C termini of the SNARE complex, whereas TeNT binds it at a membrane-distal site (Pellizzari, Rossetto et al. 1996), and because these toxins can only bind and cleave SNAREs in their uncomplexed form (Hayashi, McMahon et al. 1994; Pellegrini, O'Connor et al. 1994), this indicates that under these experimental conditions complexin holds the SNARE complex in a partially-zippered *trans* conformation (Giraudo, Eng et al. 2006).

These conclusions are supported by the observation that *Drosophila* complexin and mouse complexin 4 prevent fusion of reconstituted liposomes containing SNAREs, and that this block is relieved by addition of the cytoplasmic domains of synaptotagmin and calcium (Schaub, Lu et al. 2006). Additionally, by separately monitoring mixing of the inner leaflets and outer leaflets of liposomes, it was observed that complexin selectively prevents mixing of the inner leaflets of liposomes, indicating that fusion is blocked at a hemifused state in which the outer leaflets of fusing membranes are fused and the inner leaflets are not fused. When synaptotagmin and calcium are added to liposomes blocked at this stage of fusion, rapid and synchronous fusion of liposomes proceeds. This rapid and synchronous fusion is not observed when synaptotagmin is added without calcium or when synaptotagmin and calcium are added to liposomes without complexin (Schaub, Lu et al. 2006).

The information thus far about complexin's molecular mechanism suggests that complexin functions as a vesicle fusion clamp by halting fusion at a hemifused state in which the SNARE complex is incompletely zippered. Synaptotagmin then responds to calcium binding by overcoming this clamping mechanism through unknown mechanisms and triggering synchronous fusion. In this fusion clamp model, complexin limits spontaneous and slow fusion but promotes synchronous and rapid fusion triggered by calcium and synaptotagmin, possibly explaining why some experiments produce results arguing for a positive role in neurotransmitter release while others produce results arguing for a negative role in neurotransmitter release.

The functional interplay between complexin and synaptotagmin in this model is well supported. Complexin is displaced by synaptotagmin from reconstituted SNARE complexes in supported bilayers, and this displacement depends on the concentration of both calcium and synaptotagmin (Tang, Maximov et al. 2006). The range of calcium concentrations required for displacement is within the range found at active zones during calcium influx. Displacement of complexin from SNARE complexes in supported bilayers is eliminated in synaptotagmin C2B domain mutants that have diminished calcium and phospholipid binding (Dai, Shen et al. 2007). Similarly, synaptotagmin VI and calcium release a block by complexin of acrosomal exocytosis in sperm and this effect is eliminated in synaptotagmin VI C2B mutants that have diminished calcium or phospholipid binding (Roggero, De Blas et al. 2007). These results are consistent and suggest that part of the function of synaptotagmin's previously identified calcium-dependent lipid binding function may be to overcome complexin's block on vesicle release. While synaptotagmin clearly displaces complexin from SNARE complexes in a calcium-dependent manner, in some experimental conditions complexin and synaptotagmin can bind simultaneously

to the SNARE complex (McMahon, Missler et al. 1995; Schaub, Lu et al. 2006), so it is unlikely that this effect is achieved by direct competition for a SNARE complex binding site.

That complexin is displaced from SNARE complexes by synaptotagmin in a calcium-dependent manner is clear, but there is considerable controversy over the "fusion clamp" model of complexin's function, in which it is proposed to prevent vesicle fusion in the absence of a calcium signal and promote synchronous fusion of vesicles. A competing model proposes that complexin promotes vesicle fusion by stabilizing ternary SNARE complexes. Both models are supported by a variety of evidence. Recordings from dissociated cortical neurons expressing a complexin-synaptobrevin fusion construct, which increases the local concentration of complexin at sites of release, decreases spontaneous release frequency to about 1/3rd the normal frequency (Tang, Maximov et al. 2006). Evoked responses in neurons expressing this construct are decreased by about 80%. These results argue for an inhibitory role for complexin in release, but it is unclear whether these results represent physiological complexin function because of the nonphysiological nature of the fusion construct. Interestingly, overexpression of the fusion construct specifically inhibits synchronous release mediated by synaptotagmin and does not inhibit asynchronous release, as determined by high frequency stimulation protocols. This phenomenon is similar to recordings from syt1 null mutants, in which synchronous release is abolished but asynchronous release remains (Geppert, Goda et al. 1994). The phenotype observed may be due to the fact that high concentrations of complexin prevent binding of synaptotagmin to SNARE complexes (Tang, Maximov et al. 2006) and is consistent with the idea that complexin bound to the SNARE complex must be removed by synaptotagmin in order to trigger fast synchronous release.

Deletion analysis of mammalian complexins revealed that complexin has dual roles in neurotransmitter release mediated by separate portions of the protein (Xue, Reim et al. 2007) (**Figure 6**). The minimal SNARE binding region of complexin (residues 47-75) is necessary but not sufficient to rescue the complexin 1/2 null phenotype of decreased evoked responses. Therefore, complexin functions through activities of its non-SNARE-binding regions. Residues 47-134 partially rescue the null phenotype, whereas residues 27-134 do not rescue at all. This suggests that the accessory alpha helix (residues 30-47) functions to suppress complexin's positive role in neurotransmitter release. Introduction of a mutation in the accessory alpha helix that inhibits its alpha helical conformation without affecting SNARE complex binding eliminates this activity, indicating that the alpha helical conformation is necessary for the inhibitory function of the accessory alpha helix. Full length complexin rescues the null phenotype, so residues 1-26 are necessary to overcome the inhibitory function of the accessory alpha helix. One way to explain these results is that the accessory alpha helix mediates a clamping function that requires SNARE binding by complexin. The N terminus is then responsible for relieving this block during calcium-evoked exocytosis by an unknown mechanism. This model posits that complexin's overall function is to promote release during priming but the accessory helix blocks release until calcium influx occurs. Promotion of release is proposed to occur via stabilization of *trans* SNARE complexes (Xue, Reim et al. 2007).

Recent observations of individual liposome fusion events using Total Internal Reflection (TIRF) microscopy support complexin's positive role in vesicle fusion (Yoon, Lu et al. 2008). FRET efficiency between vesicles containing acceptor fluorophores and v-SNAREs and vesicles containing donor fluorophores and t-SNAREs was used as a measure of fusion. Within an hour of mixing of the two types of vesicles, only a small population of vesicles containing SNAREs

alone had fused. With complexin present over a range of concentrations up to 20 μM, the majority of vesicles had a FRET efficiency indicative of fusion. Addition of 1mM calcium enhances this effect and speeds up fusion. Within several seconds of calcium addition after incubation with complexin 25% of vesicles underwent fusion. This suggests that complexin binding alone promotes fusion and confers some calcium sensitivity of fusion. Notably, the authors did not include an analysis of complexin's function in the presence of synaptotagmin. It has been reported that synaptotagmin enhances the clamping function of complexin (Giraudo, Eng et al. 2006).

Summary of evidence for complexin's function in the nervous system

Taken together, the available evidence argues for a dual nature of complexin's role in neurotransmitter release. The fact that complexin mouse knockouts show decreased EPSCs demonstrates a positive role for complexin and this is supported by the finding that complexin promotes vesicle fusion in single vesicle fusion assays. Irrespective of the effects of overexpression of complexin, which are subject to caveats regarding their non-physiological nature, significant biochemical and molecular evidence exists to support the idea that complexin prevents vesicle fusion in the absence of calcium and synaptotagmin, and that this block is relieved by synaptotagmin in the presence of calcium. It is possible that complexin stabilizes an intermediate in the SNARE zippering reaction, speeding SNARE formation to that point, but also preventing full zippering until calcium influx occurs. This would have the effect of promoting fusion but also synchronizing it. It is also possible that complexin prevents synaptotagmin from triggering fusion in a calcium independent manner. In any case, one would expect a vesicle fusion clamp to present characteristics of both a negative and a positive regulator of neurotransmitter release as it both suppresses calcium-independent release and

promotes synchronous calcium-dependent release. However, one would also expect knockout of a fusion clamp to produce enhanced spontaneous synaptic vesicle exocytosis, but this has not been observed in the mouse knockouts. It is possible that additional proteins with clamping function exist in mammals, including synaptotagmins. Also, observations from these mouse knockouts have several potential caveats, detailed below.

Caveats of mouse complexin knockout electrophysiology data

While three of the four complexin isoforms have been knocked out in mice, one isoform that is at least partially redundant remains, although this isoform is not known to be expressed in the brain. Also, because mouse knockouts die within hours after birth, recordings from intact animals are necessarily done from immature synapses, which is why most of the analysis was done in autaptic synapses in the microisland hippocampal primary culture system (Reim, Mansour et al. 2001). Although this system has proven a valuable tool for analysis of synaptic function, it is not without its caveats. The number of release sites from a given recording can vary over several orders of magnitude, so the system is less well defined than more standardized models such as the Calyx of Held or neuromuscular junction (NMJ). This necessitates a great deal of information processing for quantification of even basic properties of synaptic function such as mini frequency or evoked currents. Indeed, this has proved problematic in at least one case, in which elevated mini frequency was observed in NMJ recordings from *Drosophila synaptotagmin* null mutants but not in hippocampal cultures from mouse *synaptotagmin* null mutants (Littleton, Stern et al. 1993; Broadie, Bellen et al. 1994; DiAntonio and Schwarz 1994; Geppert, Goda et al. 1994). It was later discovered in *in vivo* recordings from mouse *synaptotagmin 2* mutants and in recordings from primary cortical cultures of *synaptotagmin 1*

knockout mice that in both cases there is indeed a highly elevated mini frequency phenotype (Pang, Sun et al. 2006), in accordance with the data from *Drosophila* neuromuscular junctions.

As an additional caveat of the complexin knockout recordings, in mice complexin is only fully expressed and properly localized to synapses after the animals are several weeks old or in hippocampal cultures after growth for between 16 and 28 days (Ono, Baux et al. 1998; Reim, Wegmeyer et al. 2005). This suggests that there is some type of synaptic maturation or development that occurs and complexin expression and localization is only necessary after that event. Importantly, there is almost no expression of complexins 1, 2, and 3 in wild type mice on postnatal day 0, when recordings from intact triple knockouts or from acute brainstem slices from triple knockouts were done (Reim, Wegmeyer et al. 2005; Xue, Stradomska et al. 2008). Recordings from hippocampal cultures were done after 10-16 days of growth, when complexin is expressed but not yet specifically localized to synapses (Ono, Baux et al. 1998; Reim, Mansour et al. 2001; Xue, Stradomska et al. 2008). Thus, electrophysiology on double and triple complexin knockouts has to date only been done at time points when complexin is normally either not expressed or not properly localized to synapses.

Additional features of complexin's function may be revealed by *in vivo* electrophysiological recordings from a synapse that is highly stereotyped, at a developmental stage in which complexin is normally expressed and properly localized, and in a simpler genetic system where no residual complexin function can interfere with interpretation of the results. For these reasons, we have completed an analysis of complexin function in *Drosophila* at the larval neuromuscular junction. This synapse fits the description above and has revealed significant evidence that complexin functions as a synaptic vesicle fusion clamp *in vivo*.

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Figures

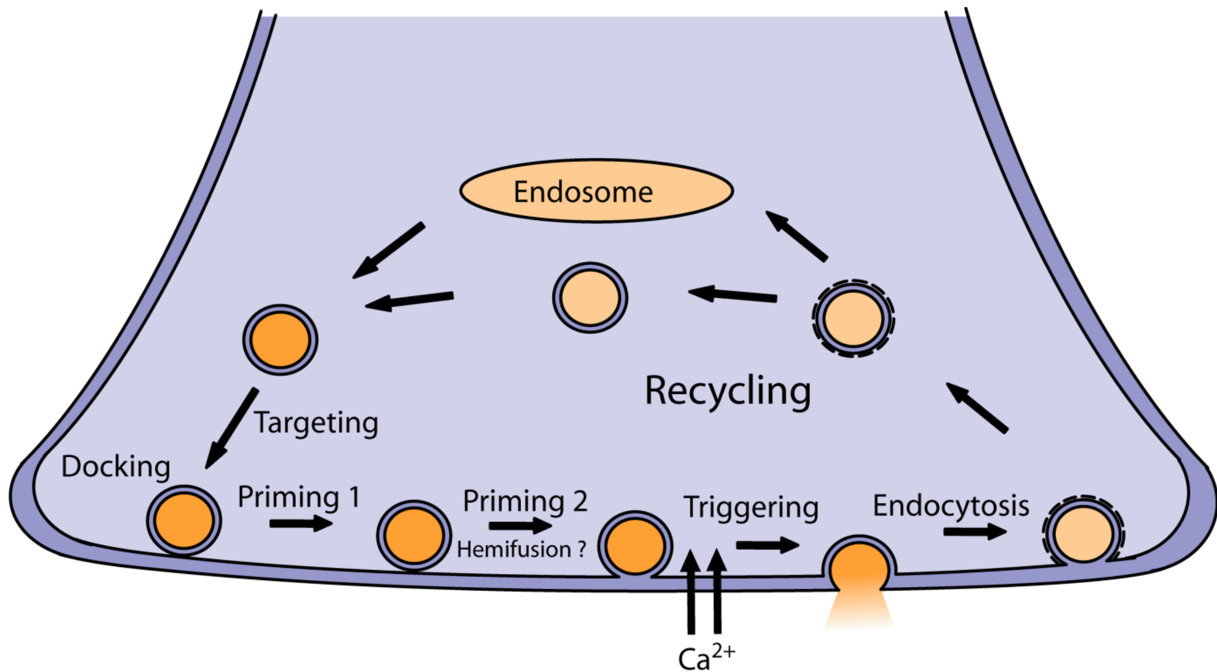

Figure 1. The Synaptic Vesicle Cycle. At presynaptic terminals, synaptic vesicles filled with neurotransmitter (dark orange) are targeted to and dock at the presynaptic membrane. Docking is followed by priming, in which synaptic vesicles are made fusion-competent and may undergo hemifusion. Upon calcium influx, synaptic vesicles fuse and release their neurotransmitter cargo into the synaptic cleft. Synaptic vesicle membrane proteins are then endocytosed and synaptic vesicles are recycled into the vesicle pool either directly or through endosomal intermediates. Black dashes symbolize clathrin.

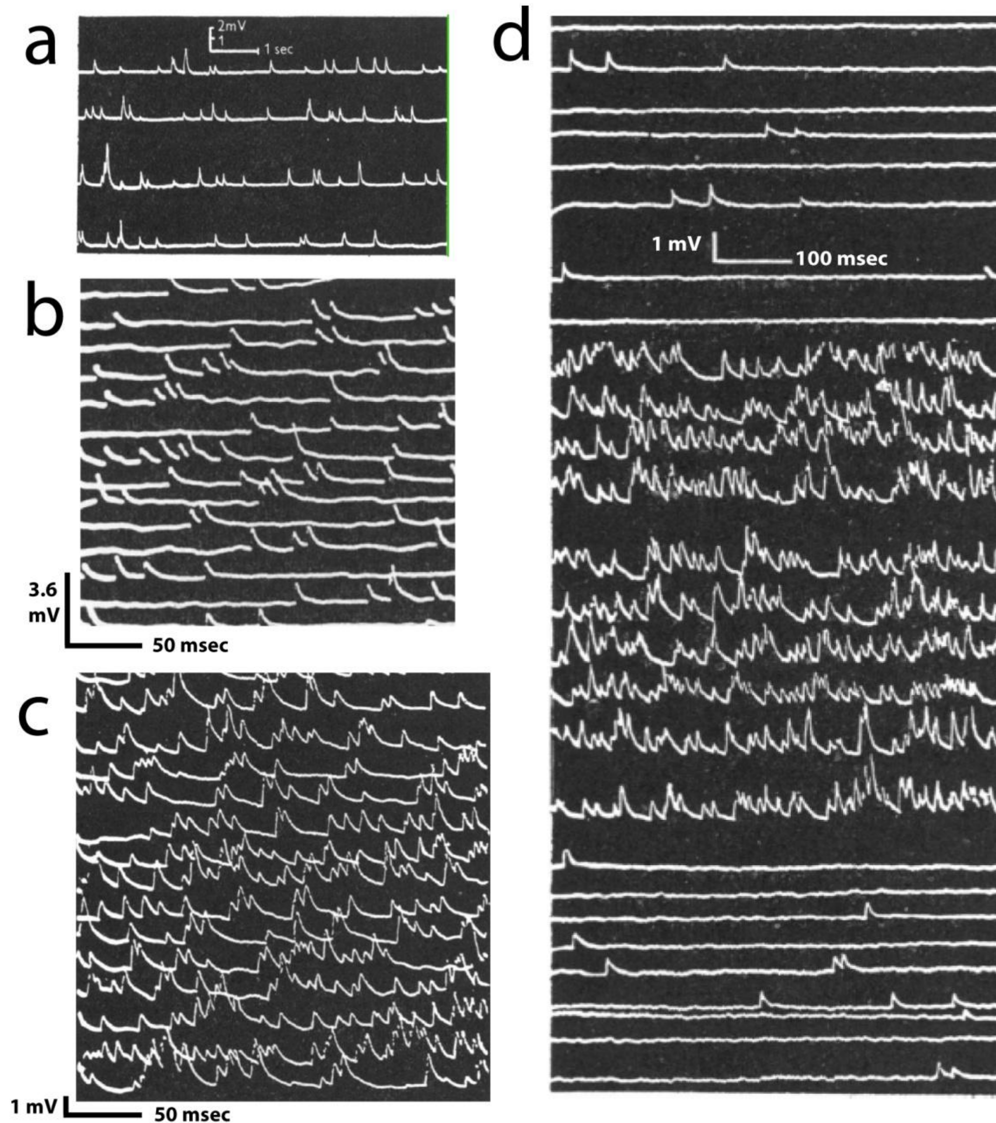

Figure 2. Example figures from Fatt and Katz 1952 showing variability of minis at the frog neuromuscular junction. (**a and b**) Examples of low frequency minis. (**c**) An example of high frequency minis. Recording is from same animal as in **b**. (**d**) Sample recordings showing the effect of high osmotic pressure on mini frequency. Top panel: Minis before application of 50% sucrose solution to muscle. Middle panel: Recordings of minis after application of sucrose solution. Bottom panel: Recordings of minis after washing the sucrose solution from the muscle. Note time and potential scales are not the same for all recordings.

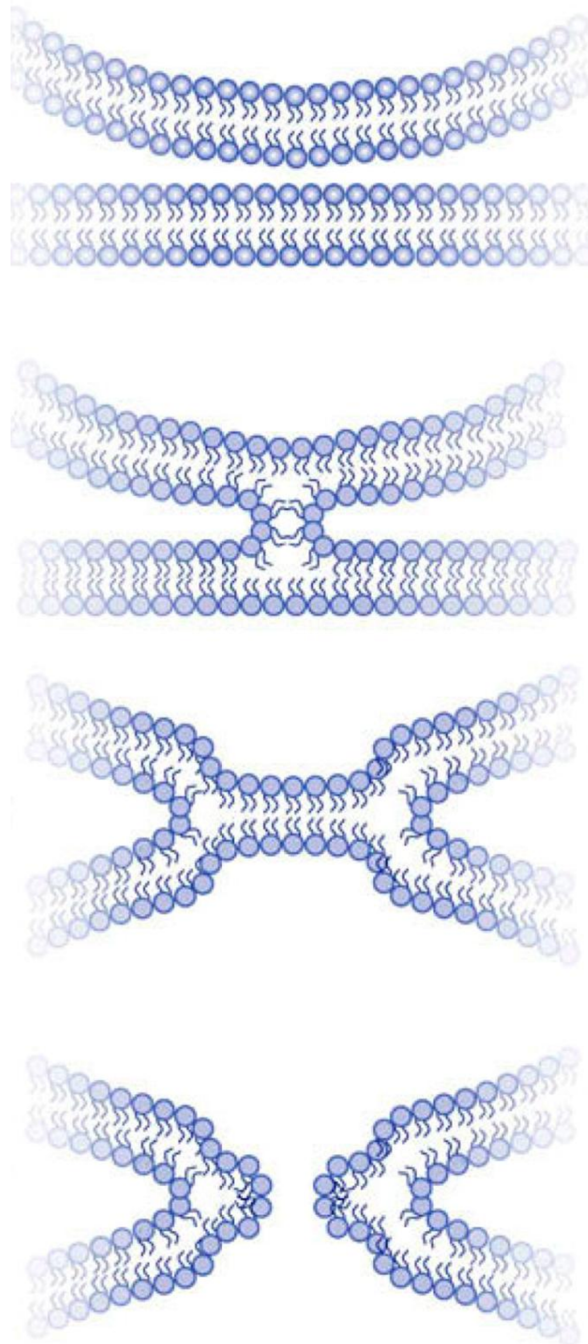

Figure 3. Model of intermediates in membrane fusion from Jackson and Chapman 2008. Two apposed membranes are brought into close proximity and begin fusion, forming a stalk (top two panels). Stalk widening produces a hemifusion intermediate (third panel), followed by fusion pore opening. Modified from Jackson and Chapman, ***Nat Struct Mol Biol*** 2008.

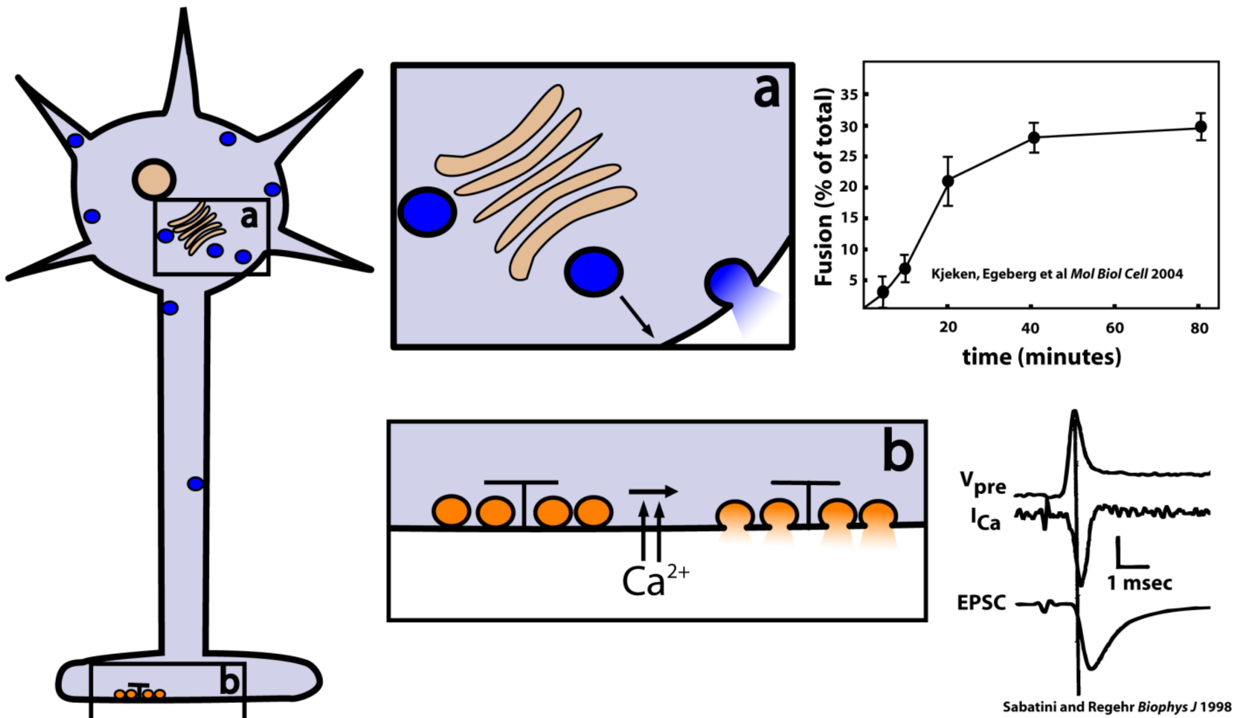

Figure 4. Key differences between calcium evoked synaptic vesicle fusion and constitutive fusion. Left panel: A neuron contains both constitutive vesicle traffic and synaptic vesicle exocytosis. Constitutve vesicle traffic (blue circles) occurs throughout the cell, while synaptic vesicles (orange circles) are localized to active zones. The active zone is depicted with a T bar, the form of the presynaptic density in *Drosophila* neuromuscular junction active zones. (**a and b**) Zoomed-in views of boxed areas in left panel. (**a**) In constitutive vesicle fusion, vesicles constantly traffic between membranes and fuse as soon as they reach their targets. (**b**) In synaptic vesicle exocytosis, synaptic vesicles wait for calcium influx before fusing and fusion is synchronized. Right panel: Representative figures showing the timescales of the two types of fusion. Intracellular vesicle traffic, in this case fusion of endosomes, occurs on timescales of minutes. Synaptic vesicle exocytosis occurs within a millisecond after calcium influx. Representative figures were adapted from Kjeken, Egeberg et al ***Mol Biol Cell*** 2004 and Sabatini and Regehr ***Biophys J*** 1998.

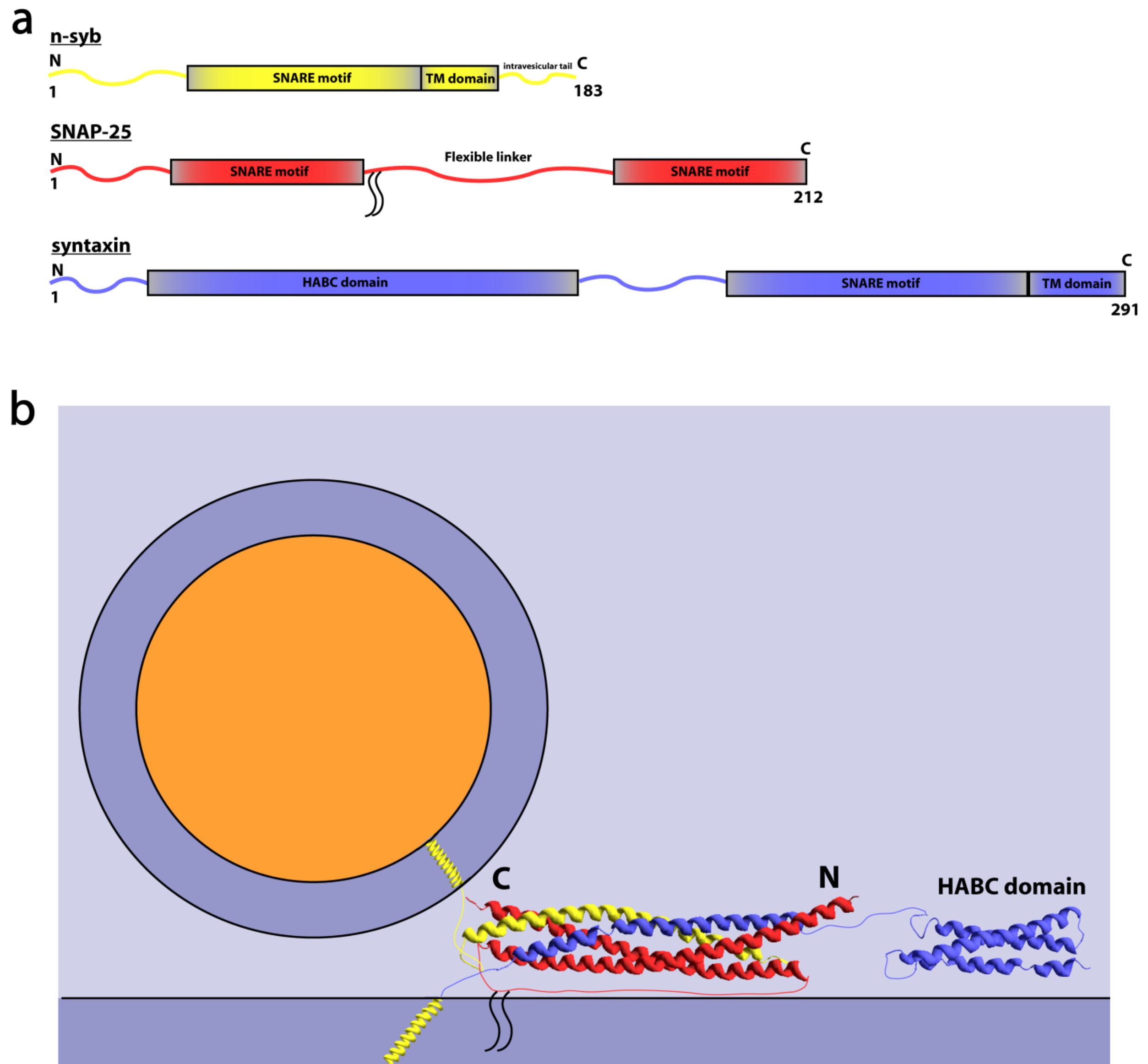

Figure 5. Structure of the neuronal SNAREs. (**a**) Domain structures of the SNAREs. Sequence lengths are based on *Drosophila* protein sequences. Structured domains are shown as boxes. Unstructured sequence is shown as a line. Synaptobrevin (yellow) has one SNARE domain, a transmembrane domain, and short intravesicular tail. SNAP-25 (red) has two SNARE domains joined by a long flexible linker. It is palmitoylated at two cysteines in this linker region (black wavy lines) to tether it to the presynaptic membrane. Syntaxin (blue) has a long N-terminal HABC domain that folds into a series of three alpha helices. Like synaptobrevin, it also has one SNARE domain and one transmembrane domain. (**b**) Three dimensional structure of the SNARE complex. Colors correspond to those in (**a**). Linker regions and transmembrane domains have been added. The N termini of the SNAREs are to the right and the C termini are to the left. Syntaxin is shown in the open conformation. The core SNARE complex structure is from Chen et al ***Neuron*** 2002. The structure of the HABC domain is from Bracher and Weissenhorn ***BMC Struct Biol*** 2004.

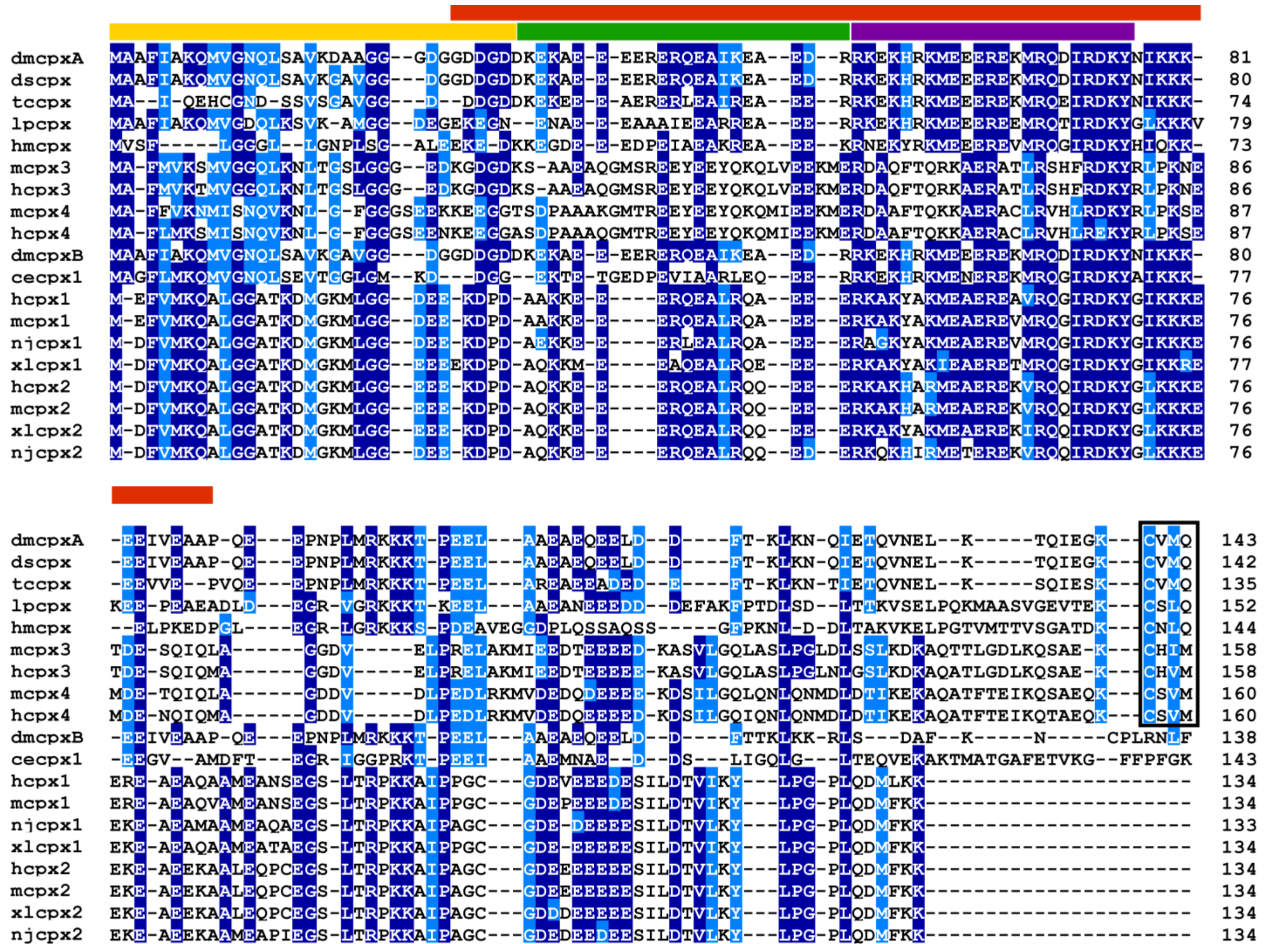

Figure 6. Alignment of complexins from vertebrate and invertebrate species. Primary sequences of complexins were aligned for maximal homology using CLC Bio Sequence Analysis software. Residues that are identical in at least 50% of species are highlighted in dark blue. Similar residues are highlighted in light blue. Similarity groups: F,Y,W; I,L,V,M; H,R,K; D,E; G,A; T,S; N,Q. The colored bars highlight important fragments of the sequence. Red bar: Fragment crystallized in rat complexin structure (Chen et al ***Neuron*** 2002). Purple bar: Central alpha helix responsible for binding to the SNARE complex. Green bar: accessory alpha helix. Yellow bar: N terminal 26 residues proposed to overcome the inhibitory function of the accessory alpha helix (Xue et al ***Nat Struct Mol Biol*** 2007). Black box: CAAX motif found in complexins from invertebrate species and mammalian complexins 3 and 4. Abbreviations: **dm**, *Drosophila melanogaster*; **ds**, *Drosophila simulans;* **tc**, *Tribolium castaneum*; **lp**, *Loligo pealei*; **hm**, *Hirudo medicinalis*; **m**, mouse (*Mus musculus*); **h**, human (*Homo sapiens*); **ce**, *Caenorhabditis elegans*; **nj**, *Narke japonica*; **xl**, *Xenopus laevis*. Two splice isoforms of complexin (dmcpxA and dmcpxB) aexpressed in *D. melanogaster* are shown. They differ in their C terminal sequence.

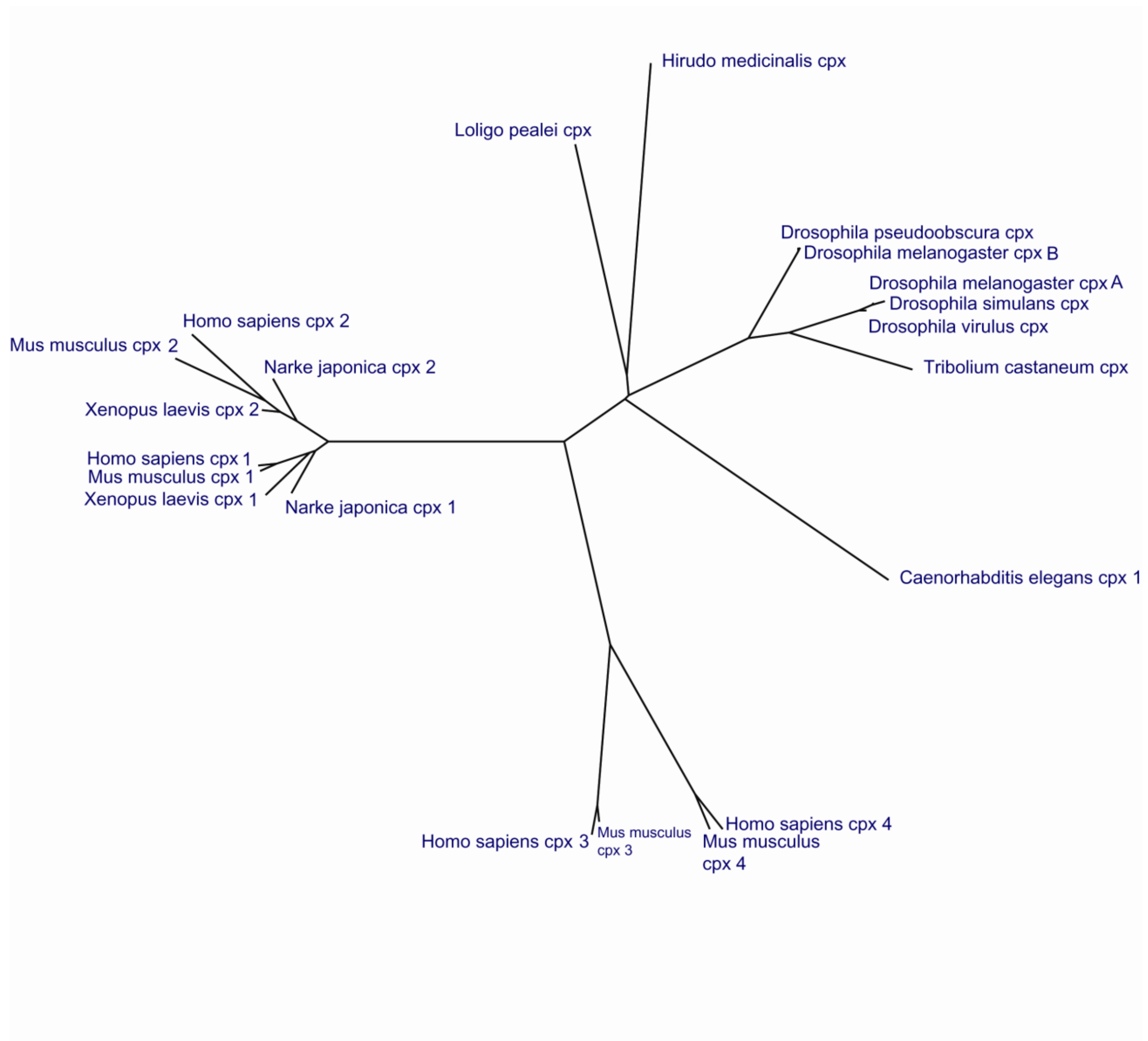

Figure 7. Phylogenic tree showing evolutionary relationships of vertebrate and invertebrate complexins. Mammalian complexins 3 and 4 form a subfamily separate from the complexin 1 and 2 subfamily.

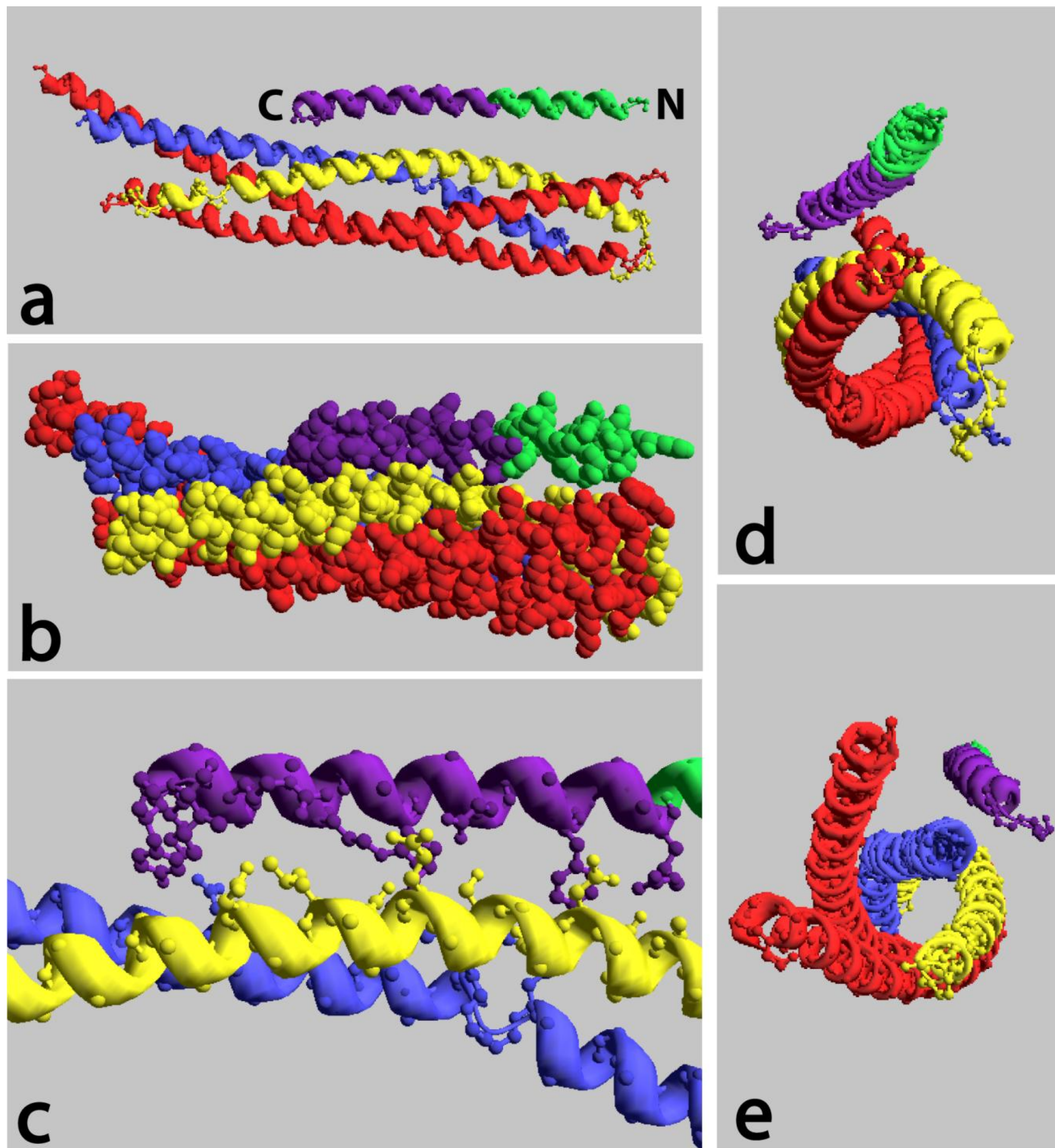

Figure 8. Three dimensional structure of complexin bound to the SNARE complex (Chen et al *Neuron* 2002). All panels: Green = complexin accessory alpha helix (residues 32-47); Purple = Complexin central alpha helix (Residues 48-72); Blue = syntaxin SNARE motif; Yellow = synaptobrevin SNARE motif; Red = SNAP-25 SNARE motifs. For complexin, color scheme corresponds to colored bars in **Figure 6**. For SNAREs, color scheme corresponds to colors in **Figure 5**. (**a-c**) N termini of SNAREs are to the left and C termini are to the right. Transmembrane domains and synaptic vesicle would be to the right. (**a**) Ribbon diagram showing alpha helical structures and coiled-coil interactions. (**b**) Space filling model showing interaction of complexin central alpha helix with the groove between syntaxin and synaptobrevin. (**c**) Close-up of binding site of complexin. For the sake of clarity, SNAP-25 helices are not shown as they do not participate in binding. (**d and e**) Ribbon diagrams showing structure from SNARE C terminal and SNARE N terminal sides, respectively.

Chapter 2

A Complexin Fusion Clamp Regulates Spontaneous Release and Synaptic Growth

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The majority of the work described in these studies was performed by Sarah Huntwork-Rodriguez. Fractionation of head extracts was performed by Bill Adolfsen. The blot of head extract fractions was performed by Robin Stevens. EM was performed by Claire Haueter and Hugo Bellen. Electrophysiology was performed in large part by J. Troy Littleton. Voltage clamp electrophysiology was performed by Ramon Jorquera. Parts of this work have been published in *Nature Neuroscience*, 2007 Oct; 10(10):1235-7.

Introduction

Intracellular vesicular trafficking is ubiquitous among eukaryotes, with vesicle fusion driven by assembly of the SNARE (soluble N-ethlymaleimide-sensitive factor attachment protein receptor) complex (Sollner, Whiteheart et al. 1993). Vesicle fusion occurs when vesicle-bound SNAREs bind with SNAREs in the target membrane via coiled-coil interactions (Sutton, Fasshauer et al. 1998), bringing the membranes into close proximity and releasing enough free energy to overcome the energy barrier imposed by lipid fusion (Fasshauer, Otto et al. 1997; Rice, Brennwald et al. 1997). While most intracellular trafficking events occur constitutively and on a time scale of minutes, synaptic vesicles must wait in a fusion-competent state before fusing, and synaptic vesicle exocytosis in response to an action potential occurs synchronously within approximately 200 μsec of calcium influx (Llinas, Steinberg et al. 1981; Cope and Mendell 1982). Despite these vast differences in kinetics and regulation, synaptic vesicle exocytosis and intracellular vesicular trafficking both occur via a common SNARE-driven mechanism (Bennett and Scheller 1993). It is therefore thought that neuronal regulatory proteins have evolved that impinge on SNARE function to allow synaptic vesicles to wait for calcium influx before fusing, to trigger fusion in the presence of calcium, and to speed the kinetics of fusion (Wojcik and Brose 2007). A thorough understanding of the molecular events leading up to fusion requires identification of these regulators and dissection of the molecular mechanisms by which they regulate the fusion process. An effective strategy to achieve this goal has been to identify conserved neuron-specific proteins that bind the assembled SNARE complex and then to study functions of these proteins using genetic and biochemical methods. Indeed, synaptotagmin 1 was identified as the calcium sensor for neurotransmitter release using this strategy.

While the calcium sensor for neurotransmitter release has been identified, the identity of a vesicle fusion clamp that would prevent exocytosis in absence of a calcium influx has yet to be

conclusively found. Ever since it was demonstrated that synaptic vesicle exocytosis and intracellular membrane trafficking occur via a common SNARE-driven mechanism (Bennett and Scheller 1993), it has been thought that such a regulator must exist because intracellular vesicle fusion events are constitutive, whereas synaptic vesicles wait in a fusion-competent state before synaptic vesicle exocytosis is triggered by calcium influx (Bennett and Scheller 1993; Popov and Poo 1993; Sollner, Whiteheart et al. 1993). Indeed, recent evidence demonstrates that in a cell-free assay using purified synaptic vesicles and proteoliposomes, fusion proceeds constitutively, suggesting the presence of cytoplasmic regulators that serve the fusion clamp function (Holt, Riedel et al. 2008). Because synaptic vesicle exocytosis is an essential process, and because a fusion clamp would regulate the last stages of fusion, one would expect a fusion clamp to be conserved, expressed specifically in neurons, and to function by acting on the SNARE complex.

Complexins are small, conserved, neuron-specific cytosolic proteins that interact with assembled SNARE complexes with a 1:1 stoichiometry (McMahon, Missler et al. 1995; Chen, Tomchick et al. 2002). Alterations in complexin levels in humans have been implicated in a host of neurological diseases ranging from schizophrenia (Lee, Song et al. 2005; Sawada, Barr et al. 2005) to Parkinson's and Huntington's Disease (Basso, Giraudo et al. 2004; DiProspero, Chen et al. 2004; Zabel, Sagi et al. 2006), suggesting complexin dysfunction may contribute to several human neuropathologies. When either two (Reim, Mansour et al. 2001) or three (Xue, Stradomska et al. 2008) of the four mouse complexin genes are removed, the animals die within hours after birth. Evoked neurotransmitter release is decreased in both hippocampal autaptic cultures and in the brainstems of knockout mice (Reim, Mansour et al. 2001; Xue, Stradomska et al. 2008), while the readily releasable pool of vesicles remains unchanged. This suggests that complexin serves a positive, post-priming role in neurotransmitter release. However, recent

biochemical evidence demonstrates that complexin can reduce SNARE-mediated fusion *in vitro* in cell-cell (Giraudo, Eng et al. 2006) or liposome (Schaub, Lu et al. 2006) fusion assays, largely because of a block in inner-leaflet mixing (Schaub, Lu et al. 2006) and a concomitant block of full zippering of the SNARE complex (Giraudo, Eng et al. 2006), suggesting that complexin may function as a clamp to stabilize a hemifusion intermediate formed by the SNARE complex.

In addition to preventing fusion of synaptic vesicles in the absence of calcium, a vesicle fusion clamp must be relieved by calcium in order to allow release to proceed. Indeed, *in vitro,* the fusion block imposed by complexin can be rapidly eliminated by a combination of calcium and synaptotagmin to trigger fusion (Giraudo, Eng et al. 2006; Schaub, Lu et al. 2006). Synaptotagmin rapidly triggers fusion *in vitro* in the presence of complexin and calcium by displacing complexin from SNARE complexes in a calcium-dependent manner (Tang, Maximov et al. 2006). Thus, biochemically, complexin has the properties necessary to function as a fusion clamp, but the electrophysiology data from complexin knockout mice are inconsistent with this conclusion because knockout of a vesicle fusion clamp would be expected to dramatically enhance spontaneous release of synaptic vesicles and this has not been observed (Reim, Mansour et al. 2001; Xue, Stradomska et al. 2008).

The mouse electrophysiology data have several caveats, however. First, there are four complexins (Reim, Wegmeyer et al. 2005) in mammals and only three have been knocked out (Xue, Stradomska et al. 2008), leaving the possibility of compensatory effects by the fourth isoform. This compensatory effect is unlikely to occur, however, because complexin 4 is only expressed in the retina (Reim, Wegmeyer et al. 2005) and not in the central nervous system. Second, and more importantly, the recordings from brainstem slices and striatum of complexin 1/2/3 triple knockout mice were done, by necessity, on postnatal day 0, when synapses may not

have fully developed and when complexin expression in the central nervous system has not yet begun in wild type mice (Reim, Wegmeyer et al. 2005). Complexin is not fully expressed in the central nervous system of mammals until they are several weeks old (Ono, Baux et al. 1998; Reim, Wegmeyer et al. 2005). Additionally, in hippocampal cultures where much of the analysis of the synaptic transmission defect in complexin 1/2 knockouts was done, complexin is not properly localized to synapses until after day 16 of growth (Ono, Baux et al. 1998), while recordings were done on cultures between 10 and 16 days old (Reim, Mansour et al. 2001; Xue, Stradomska et al. 2008). Finally, recent evidence suggests that an enhanced rate of spontaneous release can be missed by recordings from autaptic hippocampal cultures. *Synaptotagmin* knockouts in *Drosophila* were reported in the early 1990's to have a spontaneous release frequency two to six times the frequency observed in wild type animals (Littleton, Stern et al. 1993; Broadie, Bellen et al. 1994; DiAntonio and Schwarz 1994). This was not thought to be significant because no such increase in mini frequency was observed in autaptic hippocampal recordings from syt1 knockout mice (Geppert, Goda et al. 1994). Recently however, recordings from the Calyx of Held, the neuromuscular junction, and cortical cultures from *synaptotagmin* mouse knockouts showed an increase mini frequency about five times that of the wild type mini frequency (Pang, Sun et al. 2006).

Thus, a greater understanding of complexin's function in synaptic vesicle exocytosis may be gained by an electrophysiological analysis of *complexin* knockouts at a highly stereotyped, mature synapse in a simple genetic background where interpretation of the results are not confounded by the presence of additional complexins. The larval neuromuscular junction in *Drosophila melanogaster* is ideal for such an analysis. We therefore undertook a study of complexin function in this system and found significant evidence that complexin does indeed

function as a vesicle fusion clamp *in vivo*. Additionally, because there is a massive enhancement in mini frequency in *complexin* mutants, we were able to observe the effects of enhanced mini frequency on synaptic plasticity at the neuromuscular junction. Our observations add strong evidence to the growing range of evidence that suggests that minis are not just background noise at synapses but may play a significant role in synaptic plasticity and information storage.

Experimental Procedures

Drosophila Genetics and Generation of a complexin Null Mutant

Drosophila were cultured on standard medium at 22°C. The *Drosophila* genome encodes a single gene located on the 3rd chromosome at cytological interval 82A1 encoding the complexin protein (also known as synaphin) (Tokumaru, Umayahara et al. 2001), which has been previously demonstrated to bind assembled SNARE complexes and reduce SNARE-mediated fusion *in vitro* (Schaub, Lu et al. 2006). The locus spans 22.5 kb and contains eight exons, with the start codon for the ORF located within the third exon. A second uncharacterized gene, CG9780, is contained within intron three of the complexin locus. A null mutant in complexin (cpx^{SH1}) was generated by imprecise excision of PKG030233 (Bellen, Levis et al. 2004), a P-element insertion located 2.7 kb 5' of the primary *complexin* translation start site in the first intron of the gene. KG03023 flies are viable and fertile and do not disrupt complexin expression. Using PCR analysis of 600 *white*$^{-}$ excision events, we isolated a 17 kb intragenic deletion within complexin (cpx^{SH1}) that removed exons 2-4, which contain the start codon and most of the coding region for the protein. To confirm that this *complexin* allele was a null, we performed a western analysis on protein extracts from control adults, cpx^{SH1} heterozygotes, and cpx^{SH1} homozygotes. Complexin immunoreactivity showed reduced protein levels in heterozygotes compared to controls, and absent protein in homozygotes. In addition, immunocytochemistry on

control and *cpx*^{SH1} homozygous larvae with anti-complexin antisera confirmed that the *cpx* mutant completely removed expression of the protein. Homozygous *cpx*^{SH1} mutants and *cpx*^{SH1}/*Df(3R)ED5021* (Ryder, Blows et al. 2004) (a deletion removing *complexin* that spans the cytological interval 81F6-82A5) animals showed identical phenotypes, indicating *cpx*^{SH1} behaves as a null mutation. Df(3R)ED5021 was used in trans to *cpx*^{SH1} to remove complexin in physiology experiments. Primers used to define the cpx deletion included:

<u>PCR across the P element insertion site</u>

5' primer: GCTATGTGCTTCCGGTCCCGTCTTC

3' primer: GAACGGCCCGAGCTCGAAAGCACGACTG

<u>Product 1</u>

5' primer: GCTATGTGCTTCCGGTCCCGTCTTC

3' primer: GTCCTGGCGGTCCTGCAATCTTGCC

<u>Product 2</u>

5' primer: CGAGAATTTTCGGACGGCGCTAAAG

3' primer: GAACGGCCCGAGCTCGAAAGCACGACTG

<u>Product 3</u>

5' primer: CCGAATGCCACGACCAAGAAAG

3' primer: CTGCAGCATCTGTCGAGCGTG

<u>Product 4</u>

5' primer: GTGTCATGCAGTGATATGTCAGTCACC

3' primer: GCATTCGAGTGTCATCGTGCCGATTC

<u>Product 5</u>

5’ primer: GCAGGCCAGCATTCCCTATC

3’ primer: CGCTATTCATGGTCTAGTCATTTGAGCC

<u>Product 6</u>

5’ primer: CTCGGCAGGCGCTCTGTCAGCGTATCG

3’ primer: GTCCATTCCGGTAGCCACATTGGCTG

To confirm that defects in our mutant analysis were due to complexin function in the nervous system, we took advantage of the UAS/GAL4 (Brand and Perrimon 1993) system to selectively drive transgene expression within neurons. We subcloned a *complexin* cDNA encoding complexin 7B that spans the ORF into the pUAST vector and generated transgenic flies expressing *UAS-complexin* by standard injection techniques.

Protein Expression and Antibody Production

Complexin was amplified by PCR from a *Drosophila* cDNA library and subcloned into pGEX-2T (GE Healthcare). Recombinant complexin fused with GST was expressed in *E. coli* (BL21) and purified using glutathione-sepharose 4B (Amersham Biosciences). GST was cleaved from complexin by incubation with thrombin and further purified by anion exchange chromatography on a MonoQ 5/5 column (Amersham Biosciences). Peak fractions were concentrated and further purified by gel filtration on a Superose 12 10/30 column (Amersham Biosciences) in 1X TS (100 mM Tris base, 100 mM NaCl, pH 7.4). Sample loading and elution was performed on an AKTA FPLC (Amersham Biosciences). Polyclonal antibodies to complexin were generated in rabbits (Invitrogen).

Immunostaining

3rd instar larvae were reared at 22°C and dissected in HL3.1 (Stewart, Atwood et al. 1994) (70 mM NaCl, 5mM KCl, 10 mM $NaHCO_3$, 4mM $MgCl_2 \cdot 6H_2O$, 5 mM Trehalose, 115 mM Sucrose, 5 mM HEPES, pH 7.2). Following washes, larvae were incubated with primary antibody overnight at 4 °C, incubated with secondary antibodies for four hours at room temperature, and mounted in 80% glycerol for imaging. The dilutions of primary antibodies were as follows: complexin (1:500), anti-HRP (1:10,000), anti-nc82 (1:50), anti-Dlg (1:1000). The nc82 antibody against Bruchpilot developed by Erich Bruchner and the 4F3 antibody against Dlg developed by Corey Goodman were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Secondary antibodies from Jackson ImmunoResearch Laboratories were used at a dilution of 1:250 and were as follows: Cy2-conjugated Goat anti-Rabbit, Cy2-conjugated Goat anti-Mouse, Rhodamine Red-conjugated Goat anti-Mouse, and Rhodamine Red-conjugated Goat anti-Rabbit. Visualization was performed under light microscopy using a 40x oil-immersion lens. Images were taken with confocal microscopy (Axoplan 2; Carl Zeiss MicroImaging, Inc.) using PASCAL software (Carl Zeiss MicroImaging, Inc.). Immunoreactivity for complexin was abolished in null mutants lacking the protein, confirming the antisera we generated specifically cross react with complexin.

Western Blots

Western blots were performed using standard laboratory procedures by probing nitrocellulose membranes with a variety of antibodies. Blocking was performed in a solution containing four parts TBS (10 mM Tris Base pH 7.5, 150 mM NaCl) to one part Odyssey Blocking Buffer (LI-COR Biosciences). Antibody incubations were performed in a solution containing four parts

TBST (1X TBS with 1% Tween-20) to one part Odyssey Blocking Buffer. Antiserum dilutions were: complexin, 1:10,000; Syt 1, 1:10,000; arginine kinase, 1:10,000; α-SNAP 1:1,000; syntaxin, 1:1,000; synaptobrevin, 1:1,000; SNAP-25, 1:1,000; CSP, 1:200; Rab3, 1:500; Rop, 1:500. The probes were detected using Alexa Fluor 680-conjugated goat anti-rabbit IgG at a dilution of 1:3000 (Invitrogen Molecular Probes) and IRDye 800-conjugated goat anti-mouse IgG at a dilution of 1:10,000. Visualization was done using the LI-COR Odyssey Imaging System (LI-COR Biosciences).

Electrophysiology

Electrophysiology under current clamp at 3rd instar NMJs was performed as previously described (Rieckhof, Yoshihara et al. 2003) at the indicated extracellular calcium concentrations in HL3.1 saline at muscle fiber 6 of segments A3 to A5. Elevated temperature recordings were done at 32ºC.

Electroretinograms: ERGs were performed at room temperature on adult flies aged two to seven days. The flies were briefly anesthetized on ice and immobilized in clay. A sharp glass electrode was placed in the eye and a reference electrode was placed in the thorax, allowing analysis of the on- and off-transients that represent normal synaptic communication between photoreceptors and second order neurons in the visual system. No defects were observed on photoreceptor depolarization in response to a light pulse, suggesting specific defects in synaptic communication as opposed to membrane excitability.

ERGs and current clamp recordings were done using an Axoclamp-2B amplifier (Axon) and digitized with an Instrunet Model 100 digitizer at 10 kHz and analyzed with Superscope 3.0

software (GW Instruments). Mini frequency was quantified in *complexin* mutants by counting discernable individual peaks. This is certainly an underestimate of mini frequency in the mutant, as the massive increase in spontaneous exocytosis leads to an incredible number of fusion events that are tightly packed, with compound fusion events on top of each other, generating underestimates of the total rate in the mutant due to a failure to clearly separate individual events.

Electrophysiology under voltage clamp: Postsynaptic currents were recorded at segments A3 of ventral longitudinal muscle 6 in third instar larvae using two-electrode voltage clamp (OC-725, Warner Instruments, Hamden, CT) at -80 mV holding potential in a modified HL3 solution containing (in mM) 10 $NaHCO_3$; 5 KCl; 4 $MgCl_2$; 5 HEPES; 70 NaCl; 5 Threalose;115 Sucrose, pH 7.2. The Ca^{2+} concentration was adjusted to a final concentration required in each condition. Data acquisition and analysis were done using Axoscope 9.0 and Clampfit 9.0 software (Axon Instruments, Foster City, CA), respectively. Quantal content of nerve-evoked responses was estimated by dividing the current integral of individual nerve-evoked currents by the integral of the current evoked by the release of an individual quantum, as described (Acharya, Labarca et al. 1998; Delgado, Maureira et al. 2000). For stimulation, nerves were cut close to the ventral ganglia and sucked into the stimulating pipette. Nerve stimulation was applied at the indicated frequencies using a programmable stimulator Master-8 (A.M.P.I., Jerusalem, Israel).

Electrophysiology Quantification

Genotypes used for physiological analysis shown in **Figure 5** are: control (w^-; *precise excision/precise excision*); cpx (w^-; cpx^{SH1}/cpx^{SH1}); rescue (w^-, *UAS-cpx/C155elav-Gal4;*cpx^{SH1}/cpx^{SH1}) and cpx over deficiency (w^-; cpx^{SH1}/*Df(3R)ED5021*). For **Figure 5** and

Figure 6, cpx^{SH1} homozygotes (Student t-Test, ** = P<0.0001) and cpx^{SH1}/*deficiency* larvae (Student t-Test, ** = P<0.0001) have mini frequencies >20-fold higher than controls. Data used in the analysis include: Control (Mini Frequency (MF)=3.14±2.9 Hz; Resting Potential (RP)=-51.1±1.1; n=18); cpx^{SH1} (MF=67.0±4.8 Hz; RP=-48.3±0.9, n=27); cpx^{SH1}/*deficiency* (MF=58.3±2.3 Hz; RP=-48.6±1.3, n=18); Rescue (MF=2.2±0.2, RP=-54.5±1.8, n=25). **Figure 8** summarizes average EJP amplitude (± s.e.m.) versus extracellular calcium concentration in cpx^{SH1} (closed circles) and precise excision (open squares) larvae. At low calcium concentrations (0.15 mM and 0.2 mM), there is no statistically significant difference between mutants and controls. cpx^{SH1} larvae have significantly lower EJPs at higher calcium concentrations of 0.4 mM (Student t-Test, ** = P<0.001) and 1 mM (Student t-Test, ** = P<0.01) compared to precise excision controls. Data used in the analysis include: 0.15 mM calcium (Control EJP=9.1±0.9 mV, RP=-57.4±1.7, n=17; cpx^{SH1} EJP=9.8±0.9 mV, RP=-47.4±1.1, n=11); 0.2 mM calcium (Control EJP=15.5±3.1 mV; Resting Potential (RP)=-51.1±1.1; n=10), cpx^{SH1} (EJP=13.5±0.8 mV; RP=-48.3±0.9, n=18), Rescue (EJP=15.9±2.2 mV, RP=-54.5±1.8, n=8); 0.4 mM calcium (Control EJP=28.2±3.1 mV, RP=-54.7±1.5, n=9; cpx^{SH1} EJP=19.6±0.9 mV, RP=-56.6±1.2, n=15); 1.0 mM calcium (Control EJP=33.1±2.2 mV, RP=-58.7±1.8, n=14; cpx^{SH1} EJP=25.9±1.2 mV, RP=-60.2±2.6, n=9). Comparing mini frequency across a range of extracellular calcium, **Figure 6** shows that cpx^{SH1} mutants show statistically significant (Student t-Test) elevated spontaneous release at 0 mM (** = P<0.0001), 0.15 mM (** = P<0.0001), 0.2mM (** = P<0.0001), 0.4 mM (** = P<0.0001) and 1.0 mM (** = P<0.0001) extracellular calcium compared to controls. Data used in the analysis include: cpx^{SH1} – 0 mM calcium (MF=83.2 ± 6.5 Hz, n=10), 0.15 mM (MF=80.8 ± 9.2 Hz, n=6), 0.2 mM (MF=66.9 ± 4.8 Hz, n=27), 0.4 mM (MF=75.9 ± 2.3 Hz, n=15) and 1.0 mM (MF=66.35 ± 2.5 Hz, n=10); precise excision control - 0

mM calcium (MF=2.86 ± 0.2 Hz, n=19), 0.15 mM (MF=2.7 ± 0.5 Hz, n=7), 0.2 mM (MF=3.1 ± 0.3 Hz, n=18), 0.4 mM (MF=3.9 ± 0.6 Hz, n=5) and 1.0 mM (MF=4.0 ± 0.4 Hz, n=16). **Figure 10** shows that complexin overexpression decreases mini frequency and increases evoked response amplitude. Genotypes used in this analysis are: complexin overexpression (*elav*-GAL4; UAS-*complexin*), no driver control (UAS-*complexin*), and driver alone control (*elav*-Gal4). Recording were done in 0.2 mM extracellular calcium. Animals overexpressing complexin have a >2.8-fold reduction in mini frequency compared with controls (Student t-Test, ** = P<0.0001 compared to UAS-*complexin*, ** = P<0.0001 compared to *elav*-GAL4). Data used in the analysis include: *elav*-GAL4; UAS-*complexin* (Mini Frequency (MF)=0.9 ± 0.09 Hz; n=32), *elav*-GAL4 (MF=2.8 ± 0.2 Hz; n=27), UAS-*complexin* (MF=2.7 ± 0.3 Hz; n=16). Overexpression of complexin results in a statistically significant enhancement in average EJP amplitude versus controls (Student t-Test, ** = P<0.0001 compared to UAS-*complexin*; ** = P<0.003 compared to *elav*-GAL4). Data used in the analysis include: *elav*-GAL4; UAS-*complexin* (EJP=23.3 ± 1.4 mV; Resting Potential (RP)=-60.4 ± 1.6; n=18); UAS-*complexin* (EJP=12.5 ± 1 mV; RP=-60.5 ± 3, n=8); *elav*-GAL4 (EJP=17.2 ± 1.5 mV, RP=-58.2 ± 1.3, n=24). Genotypes used in analysis in **Figure 11** are: complexin overexpression (ov cpx) (*elav*-GAL4; UAS-*complexin*); cpx (*w; cpx^{SH1}/cpx^{SH1}*); control (*w; precise excision/precise excision*).

Morphological Quantification

To quantify NMJ bouton number, anti-Synaptotagmin 1 (Syt 1) stained varicosities were counted at muscle fiber 6/7 of segments A3 and A4 and at muscle fiber 4 of segment A3. The number of Syt 1-positive varicosities was statistically increased compared to controls at muscle fiber 6/7 of segment A3 (Student t-Test, ** = P<0.0001). Data used in the varicosity number analysis include: Control (muscle (m) 6/7 segment A3: bouton #=76.3±2.8, n=32); cpx^{SH1} (m6/7 segment

A3: bouton # =140.6±4.9, n=35); Rescue (m6/7 segment A3: bouton # =73.9±4.2; n=16). For active zone quantification, antisera against the active-zone protein bruchpilot (nc82) was used and nc82 positive puncta were counted from confocal stacks taken through muscle fiber 6/7 at segment A3 as previously described (Dickman, Lu et al. 2006). The number of nc82-positive active zones was statistically increased compared to controls (Student t-Test, ** = $P<0.0002$). Data used in the active zone analysis include: Control precise excision: active zone # per m6/7 synapse = 990.4 ± 102.1, n= 12; cpx^{SH1}: active zone # per m6/7 synapse = 1620.5 ± 111.5, n=11.

Adult Locomotion Analysis

Adult climbing assays and spontaneous locomotion were performed on flies aged 6-7 days as previously described (Wang, Saraswati et al. 2004). Briefly, climbing assays were done on flies aged 6-7 days, and the time required to climb 5 cm in a plastic vial was recorded, with a cutoff of 15 min. Spontaneous locomotion assays were done by introducing 6- to 7-day-old flies into a chamber and quantifying the number of times the fly crossed a line bisecting the chamber in a four minute period.

Gradient Centrifugation

Isolation of Canton-S head homogenates was performed as described previously (Littleton, Serano et al. 1999). A post-nuclear extract was layered onto a 10–30% sucrose gradient and centrifuged at 50,000 RPM for 1 h in a NVT65 rotor (Beckman Coulter). 1-ml fractions were collected beginning from the bottom of the gradient and proceeding to the top. After collection, fractions were mixed with an equal volume of 2x SDS-PAGE loading buffer and probed by Western analysis.

Transmission Electron Microscopy

EM supplies were obtained from Electron Microscopy Sciences. Wandering third-instar larvae were dissected in HL3 (Stewart, Atwood et al. 1994) and fixed in 0.13 M Cacodylate buffer containing 4% paraformaldahyde and 1% glutaraldehyde (pH 7.2). Samples were secondarily fixed in 1% OsO_4 in 0.13 M cacodylate buffer containing 0.26 M sucrose (pH 7.2), stained en bloc with 2% uranyl acetate, dehydrated, and embedded in Scipoxy 812 resin (Energy Beam Sciences). Serial sections (65–75 nm) were collected on nickel grids and stained with 1% uranyl acetate followed by Reynold's lead citrate. Data were captured with an electron microscope (model JEM-1010; JEOL), and images were processed in Photoshop 7 (Adobe) and assembled in CorelDRAW 12 (Corel).

Vesicle density is defined as the number of vesicles/bouton midline cross-sectional area in μm^2. Major vesicle axis is defined as the longest line bisecting a vesicle. Cisternae density is defined as the number of cisternae / bouton midline cross-section area in μm^2. For quantification of vesicle density, major vesicle axis, and cisternae density, vesicles were counted from 12 boutons from 3 different animals per genotype. Major vesicle axes were counted from 100 vesicles at random per bouton. For quantification of the number of vesicles within a 250nm radius of the active zone and the number of docked vesicles, 10 active zones from 3 different animals per genotype were counted.

Results

Features of the Drosophila complexin genomic locus and complexin isoforms

The *Drosophila melanogaster* genome has a single gene encoding the complexin protein (Tokumaru, Umayahara et al. 2001; Smith, Shu et al. 2007; Wilson, Goodman et al. 2008) (**Figure 1a**). The genomic locus is predicted to encode fourteen different transcripts produced

by alternative splicing, most of them differing in the first exon, which contains a large portion of the 5' untranslated region (UTR) (exons are shown by red bars in **Figure 1a**). Alternative splicing of the first exon is well supported by sequences of expressed sequence tags (ESTs) that correspond to sequences in the alternate first exons (see numbers below exons in **Figure 1a**). In general, the 5' UTR of mRNA sequences can mediate regulation of both translation initiation and mRNA stability (Pickering and Willis 2005), so the wide variety of 5' UTRs in complexin transcripts suggests that the timing, level, and/or location of complexin expression is tightly regulated in *Drosophila.*

The *complexin* locus may encode up to six different protein sequences. Two complexin isoforms, complexin 7a and complexin 7b (cpx7a and cpx7b in **Figure 1**), are encoded by transcripts that were isolated by PCR from a cDNA library generated from adult flies. These two isoforms mainly differ in their C termini, encoded by two alternate seventh exons, 7a and 7b. The coding sequence for cpx7a is contained in exons 3, 4b, 5, 6, and 7a. Complexin 7a contains a conserved CAAX box motif (black box in **Figure 1b**) that, in mammalian complexins 3 and 4, is farnesylated for enhanced localization of complexin to the plasma membrane (Reim, Wegmeyer et al. 2005). Presumably farneslyation of *Drosophila* complexin 7a also occurs, although this has not been definitely shown. Complexin 7b does not contain an N terminal CAAX box motif. This motif is clearly not universally necessary for complexin function, since complexins 1 and 2 do not have a CAAX box motif but are still functional. It is more likely that the CAAX box is used as it is in mammals to affect the subcellular localization of complexin. There is no support for expression of complexin 7b by ESTs, but it was the first *Drosophila* complexin reported in the literature (Pabst, Hazzard et al. 2000; Tokumaru, Umayahara et al. 2001) and in this study it was isolated from a *Drosophila* cDNA library by PCR, so it is

expressed under at least some circumstances. Also, the unique exon 7b is highly conserved among 10 related *Drosophila* species.

Nine of the fourteen transcripts predicted by Flybase (the primary database of *Drosophila* genomic data) encode a single sequence, complexin A (cpxA in **Figure 1b**), which is named after the Flybase annotation of the transcript encoding it. Expression of complexin A is well supported by available EST sequences. The coding region for this isoform of complexin is contained in exons 3, 5, 6, and 7a. CpxA is nearly identical to cpx7a with the exception of five amino acids (19-23) near the N terminus that are encoded by exon 3 in cpxA and exon 4b in cpx7a. Alternative splicing of exon 4 may produce several additional complexin isoforms, although support for the expression of these isoforms by ESTs is much weaker than for complexin A. Isoform R (named for the encoding transcript) is produced by splicing of exon 4a and is identical to complexin A except in its N terminal thirteen residues. Isoform L is encoded by exon 4c and again differs from complexin 7a only its N terminal residues. Two additional exons, 4d and 4e, are predicted to produce an identical complexin sequence, cpxJ/K, that is truncated in the SNARE binding region of complexin. This region is widely reported to be necessary for complexin's function (Pabst, Hazzard et al. 2000; Chen, Tomchick et al. 2002; Xue, Reim et al. 2007), so it is unlikely that if cpxJ/K is expressed it is functional. All *Drosophila* complexin transcripts contain exons five and six, which encode the highly conserved accessory and central alpha helices in complexin (green and purple bars in **Figure 1b**). Exon 3 contains the primary *complexin* translation start site (ATG in **Figure 1a**). Exons 4a, 4c, and exon 5 contain additional potential translation start sites.

The C terminus of complexin is regulated by both post-transcriptional and post-translational modifications. Farnesylation of complexin at the C terminus has already been

discussed. Additionally, in *Drosophila* the transcripts encoding complexin 7a have been shown to be modified by RNA editing to produce two amino acid changes: I124M and N129 to S, G, or D (orange boxes in **Figure 1b**) (Hoopengardner, Bhalla et al. 2003). Editing of RNAs occurs by deamination of adenosine to inosine, which is read as a guanosine by the ribosome. Only a small subset of transcripts are known to be RNA edited, and nearly all encode proteins involved in rapid neurotransmission (Hoopengardner, Bhalla et al. 2003). The functional consequences of these modifications are currently unclear.

Mammalian complexins have been shown to be phosphorylated at two residues in the C terminus, Serine 93 and Serine 115 (Hill, Callaghan et al. 2006; Shata, Saisu et al. 2007). It is not clear what purpose this phosphorylation serves, but these amino acids are highly conserved among mammalian complexins 1 and 2. While these phosphorylation sites are not conserved in invertebrate species, several other predicted phosphorylation sites in the C terminus are conserved among invertebrates (**Figure 1b**), including predicted phosphorylation sites for PKA, PKC, and CaM kinase II (Obenauer, Cantley et al. 2003; Blom, Sicheritz-Ponten et al. 2004).

Subcellular localization of *Drosophila* complexin

To characterize the subcellular localization of complexin, we generated antisera to recombinant *Drosophila* complexin 7b that recognized a 16-kDa protein in brain extracts and is enriched in both CNS and peripheral synapses (**Figure 2**). The antisera generated recognize both complexin 7a and 7b *in vitro.* Co-staining for complexin and the pan-neuronal marker horseradish peroxidase shows high levels of localization of complexin at nerve terminals of the NMJ (small arrowhead in **Figure 2a**), with very little localization to axons (large arrowhead in **Figure 2a**). Co-staining for complexin and the postsynaptic marker discs large (Dlg) (**Figure 2b-d**) displays non-overlapping staining of the two proteins, demonstrating that complexin is localized

presynaptically. Complexin is localized to both type 1b and 1s synapses at the NMJ (**Figure 2c**), which are two of the major types of glutamatergic synapses at *Drosophila* larval NMJs. Co-staining for complexin and the active-zone protein bruchpilot / nc82 demonstrates that complexin is expressed diffusely in presynaptic terminals (**Figure 2e-f**) and is not specifically localized to active zones. To characterize the compartmental localization of complexin, velocity gradient subcellular fractionation experiments using 10–30% sucrose gradients were performed on extracts from wild type adult fly heads (**Figure 2g**) (Adolfsen, Saraswati et al. 2004). Subcellular compartments were identified in fractions using the compartmental markers ROP (plasma membrane and cytosol) (Salzberg, Cohen et al. 1993), HRS (endosomes) (Lloyd, Atkinson et al. 2002) and synaptogyrin (synaptic vesicles) (Robin Stevens, personal communication). While complexin partitions to all fractions, it is most concentrated in the cytosolic fractions, similar to the distribution of Rop. There is also some enhanced concentration in the synaptic vesicle fractions. This experiment suggests that complexin is mainly localized to the cytosol, but further experiments with a larger number of compartmental markers would be warranted to confirm this conclusion. *Drosophila* complexin is localized presynaptically at nerve terminals, and is possibly a cytosolic protein, consistent with complexin functioning in regulation of neurotransmitter release.

Generation of a *Drosophila complexin* null mutant

To determine the *in vivo* function of complexin, we generated the first complete knockout of complexin in any organism. A 17-kb intragenic deletion within *complexin* (cpx^{SH1}) that removed most of the coding region was generated by imprecise excision of a P element located in the first intron of the *complexin* gene (**Figure 3a-c**). A precise excision lacking any deletion was also generated, and served as a control for genetic backgrounds in most experiments. Imprecise

excision of the P element was confirmed by a PCR across the P element insertion site in genomic DNA from wild type (*white*), *precise excision*, and cpx^{SH1} homozygote adult fly heads (**Figure 3b** and orange bar in **3a**). The boundaries of the deletion were mapped by PCR of six 500 base-pair products at locations upstream and downstream of the P element insertion site (**Figure 3c** and green bars in **3a**). The deletion extends from the P element insertion site in the first intron to somewhere between exons 7a and 8 (purple bar and dashes in **Figure 3a**), removing the primary translation start site, the additional putative translation start sites, and nearly all of the coding region of *complexin*. Western analysis (**Figure 3d**) confirmed the loss of expression of the complexin protein in cpx^{SH1} homozygotes, and reduced expression in heterozygotes compared with *white* controls. Immunocytochemistry (**Figure 3e**) at third-instar larval NMJs using antibodies to complexin and the neuronal marker HRP revealed that, while neuronal connectivity with muscles remains in cpx^{SH1} mutants, there is no evidence of expression of complexin in the mutant, further confirming that it is a null allele. Expression levels of many synaptic proteins, including arginine kinase, α-SNAP, syntaxin, synaptobrevin, SNAP-25, syt 1, cysteine string protein, Rab3, and Rop (sec1/unc-18) do not change in *complexin* mutants (**Figure 3f**).

Null mutants lacking complexin display several striking phenotypes. The cpx^{SH1} allele is semilethal, with most homozygous animals dying before adult eclosion. Escaper adults are infertile and show severe motor defects, moving visibly slower than controls, frequently falling to the bottom of the vial, and having difficulty righting themselves after falling. Quantification of the motor defect in *complexin* mutants using a wall climbing assay (**Figure 4a**) and a spontaneous locomotion assay (**Figure 4b**) confirms these observations, with *complexin* mutants taking 69.9 times longer on average to climb a vial and crossing a chamber a factor of 33.9 times fewer than their wild type counterparts. To determine whether these phenotypes might result

from defects in synaptic transmission, electroretinograms detecting synaptic transmission in the second order neurons of the retina were performed on controls and null mutants. These demonstrated that *complexin* null mutants lack the on- and off-transients that represent normal synaptic transmission in response to light pulses in the visual system (arrows in **Figure 4c**), suggesting that *cpx*^{SH1} mutants have a synaptic transmission defect. Pan-neuronal expression of a *UAS-complexin7b* transgene with the *elav*-GAL4 neuronal driver rescues the reduced viability, motor defects, and abnormal synaptic transmission of *cpx*^{SH1} mutants (**Figure 4a,c**), demonstrating that these phenotypes are due to lack of complexin expression and not to second-site mutations.

Electrophysiological characterization of the *Drosophila complexin* null mutant *cpx*^{SH1}

To analyze the role of complexin in neurotransmitter release, we performed electrophysiological recordings at *Drosophila* third-instar larval abdominal muscle 6 synapses. *cpx*^{SH1} mutants displayed a profound enhancement in the frequency of miniature excitatory junctional potentials (mEJPs or minis) (**Figure 5a**). By expanding the time scale of the recordings and attempting to distinguish individual minis (arrows in **Figure 5b**), we quantified the frequency of minis in control and mutant larvae. Mini frequency was enhanced by at least 20 fold at *cpx*^{SH1} mutant synapses (67 ± 4.8 Hz compared to 3.1 ± 0.3 Hz at control synapses), demonstrating continuous exocytosis of synaptic vesicles in the absence of any stimulation (**Figure 5c**). This is likely to be an underestimate of the true mini frequency in *cpx*^{SH1} mutants because it is difficult to separate individual minis in the mutant due to their high frequency. *cpx*^{SH1} heterozygous with a deficiency that removes *complexin* (cpx/Df in **Figure 5c**) also displays a massively enhanced mini frequency (58.3 ± 2.3 Hz). Neuronal expression of a *UAS-complexin7B* transgene in a

homozygous null mutant background rescued the enhanced frequency of spontaneous release (2.2 ± 0.2 Hz) (**Figure 5**).

There are several possible explanations for the enhanced mini frequency observed in *complexin* mutants. One is that complexin functions as a vesicle fusion clamp, and without it synaptic vesicles are released immediately after priming. Other explanations are that the minis result from aberrant influx of calcium into the nerve terminal, that neurotransmitter is released from the nerve terminal extravesicularly, that a morphological defect results from removal of complexin that causes enhanced spontaneous release, or that there is a postsynaptic defect in *complexin* mutants, an obvious example of which would be aberrant opening of glutamate receptors in the muscle. We performed a series of experiments to distinguish among these possibilities.

To confirm that the increase in miniature postsynaptic potentials resulted from increased spontaneous vesicle fusion, as opposed to non-vesicular dumping of glutamate at the synapse or to a postsynaptic defect, we made use of a temperature-sensitive allele of dynamin ($shibire^{TS1}$) that blocks endocytosis at 32 ºC (Koenig and Ikeda 1989). High-frequency stimulation at 32 ºC depletes the synaptic vesicle pool in *shibire* mutants. If the enhanced mini frequency observed in cpx^{SH1} mutants is due to vesicular release from the presynaptic terminal, high frequency stimulation would be predicted to eliminate the enhanced spontaneous vesicle fusion in double mutants. Indeed, after a 5-minute 10-Hz stimulation train at 32ºC in $shibire^{TS1}$; cpx^{SH1} double mutants, which abolished evoked release, the elevated mini frequency observed at permissive temperatures was eliminated (**Figure 6a**). To determine whether the enhanced mini frequency observed in *complexin* mutants is due to aberrant influx of calcium into the nerve terminal, we quantified mini frequency at control and mutant NMJs over a range of extracellular calcium

concentrations from 0mM to 1mM. The frequency of spontaneous release in *cpx*^{SH1} mutants remained strongly elevated across the range of calcium concentrations, including 0 mM (**Figure 6b**), ruling out enhanced calcium influx into the presynaptic nerve terminal as the cause for the enhanced spontaneous release. Thus, the enhanced mini frequency in *complexin* null mutants is due to a large increase in the frequency of spontaneous, calcium-independent release of synaptic vesicles from presynaptic nerve terminals.

These observations suggest that complexin functions as the synaptic vesicle fusion clamp *in vivo*, and with its loss, synaptic vesicles continuously fuse in the absence of stimulation. Another possible explanation for the enhanced mini frequency in *complexin* mutants is that synapses in the mutants have a severe morphological defect that causes enhanced mini frequency. By analyzing synaptic structure, we were able to determine whether this might be the case. This experiment also provided us an opportunity to determine the consequences of increased mini frequency on synaptic growth. To analyze synaptic structure, we counted synaptic varicosities in age-matched control and *cpx*^{SH1} third-instar NMJs at three different muscle segments. *cpx*^{SH1} mutants showed a two-fold overproliferation of boutons at each muscle examined (**Figure 7a-d**), and neuronal expression of a *complexin* transgene in the mutant background reverted the synaptic overgrowth phenotype. Overgrowth of NMJs resulted in a concomitant increase in the total number of active zones present at synapses, with 64% more active zones at muscle 6/7 segment A3 in *complexin* mutants (**Figure 7e,g**). There is almost no difference in the number of active zones per bouton between wild type (13.0 active zones / bouton) and mutant (11.53 active zones / bouton) synapses (**Figure 7f**), demonstrating that the increase in the total number of active zones in *complexin* mutants is due to the increase in bouton number. These data eliminate any structural considerations that could account for the enhanced

minis, as the 64% increase in the number of active zones is insufficient by far to explain the >20-fold increase in spontaneous release. Based on this data, we conclude that complexin functions as a vesicle fusion clamp *in vivo*.

Loss of the synaptic vesicle fusion clamp in complexin mutants resulted in a defect in evoked excitatory junctional potentials (EJPs) at the NMJ (**Figure 8**). In high extracellular calcium concentrations of 1.0 mM and 0.4 mM, evoked responses in cpx^{SH1} mutants after nerve stimulation were significantly reduced compared with controls, whereas in low extracellular calcium concentrations of 0.2 mM and 0.15 mM we observed no difference. Because *complexin* mutant synapses showed a 64% increase in active zone number this indicates that neuromuscular junction synapses lacking complexin had a reduction in evoked fusion events per active zone that was exacerbated at high calcium levels, where large numbers of synchronous synaptic vesicle fusion events are required. This suggests that with loss of the vesicle fusion clamp in *complexin* mutants and uncontrolled release of synaptic vesicles, there is a defect in the number of vesicles available for release during evoked responses. Such a defect might result from fewer docked vesicles or fewer primed vesicles.

To determine whether there are ultrastructural defects in *complexin* mutants that could account for the observed electrophysiological phenotypes, we performed electron microscopy (EM) to image the ultrastructure of mutant and control type 1 NMJ synapses (**Figure 9**). Analysis of images of boutons in control and mutant larvae (**Figure 9a,b**), showed no significant differences in the number of synaptic vesicles per bouton (**Figure 9c**) or the size of synaptic vesicles, as measured by the average length of the major axis of synaptic vesicles (**Figure 9d**). There were also no significant differences in the number of vesicles within 250 nm of active zones (presynaptic density in **Figure 9f** and **g**) or the number of docked vesicles (**Figure 9h** and

i). Thus, the defect in evoked potentials in *complexin* mutants is unlikely to result from a decrease in the number of docked vesicles. The only difference in ultrastructure between mutant and control observed was a significant increase in the number of cisternae per bouton in *complexin* mutants (**Figure 9e**). Cisternae are large, clear vesicles resulting from bulk endocytosis at the synapse that are precursors for synaptic vesicle formation (Koenig and Ikeda 1989). They can be found in small numbers in wild type synapses (Zhang, Koh et al. 1998), but are observed in greater numbers when large numbers of vesicles must be made, as in the case of *shibire* mutants after depletion of vesicles from the nerve terminal (Koenig and Ikeda 1989). This phenotype is consistent with the massive increase in mini frequency observed in *complexin* mutants and suggests a mechanism by which the vesicle pool might be maintained when the rate of exocytosis is so elevated.

Ultrastructural analysis indicates that *complexin* mutants do not have a depletion in docked vesicles. It is therefore likely that the reduced EJP amplitude observed at high calcium concentrations in the mutant is due to depletion of the readily releasable pool of vesicles. If this is the case, the function of the fusion clamp can be understood to be prevention of spontaneous release of vesicles in the readily releasable pool, which consists of those vesicles that are docked and primed, conserving them for evoked release. Overexpression of complexin would then be predicted to decrease spontaneous release frequency at the NMJ and enhance the size of the readily releasable pool of vesicles, causing an increased amplitude of evoked responses. To test this, a *UAS-complexin7b* transgene was overexpressed pan-neuronally with the driver *elav-Gal4* (**Figure 10**). Immunohistochemistry at NMJs of *precise excision*, rescue (*elav-Gal4,UAS-cpx7B;cpx*^{SH1}*/cpx*^{SH1}), and overexpression (*elav-Gal4;UAS-cpx7b/UAS-cpx7b*) larvae demonstrates that the overexpressed complexin is localized to NMJs (**Figure 10a**). Western

blots on adult heads shows levels of complexin several times higher in overexpression flies compared to control flies (**Figure 10b**). Overexpression of complexin in larvae significantly decreases mini frequency by more than 2.8-fold at NMJs compared to controls (**Figure 10c,e**) (complexin overexpression mini frequency = 0.9 ± 0.09 Hz, *elav-GAL4* mini frequency = 2.8 ± 0.2 Hz; n=27, *UAS-complexin* mini frequency = 2.7 ± 0.3 Hz, P < 0.0001.). Overexpression of complexin also significantly enhances the average amplitude of evoked potentials by 36.2% (**Figure 10d,f**) (*elav*-GAL4; UAS-*complexin* EJP=23.3 ± 1.4 mV, *UAS-complexin* EJP=12.5 ± 1 mV, *elav-GAL4* EJP=17.2 ± 1.5 mV, P < 0.0001). These observations support the idea that complexin functions to keep primed vesicles from fusing prematurely, holding them for evoked release. In addition, these observations suggest that complexin does not saturate SNARE complexes at the NMJ because overexpression of complexin is able to decrease mini frequency from that observed at wild type synapses. If normal complexin expression levels were sufficient to saturate SNARE complex binding at the synapse, overexpression of complexin would be predicted to have little or no effect on mini frequency.

To further test the idea that complexin prevents vesicles in the readily releasable pool (RRP) from fusing prematurely, we tested the effect of high frequency stimulation (HFS) on evoked responses in control, mutant, and overexpression larvae under voltage clamp (**Figure 11**). Short term synaptic depression due to (HFS) at high calcium concentrations results from rapid depletion of the RRP. If *complexin* mutants have a depleted RRP, this will be reflected in a decrease in depression compared to wild type during HFS because the initial train of evoked responses will have low amplitudes at the outset, while the recycling pool should not be affected. Under voltage clamp, cpx^{SH1} displays a large increase in mini frequency compared to controls, just as it does under current clamp (**Figure 11a**). During a HFS of 10Hz stimulation for 150

seconds at 2.0 mM extracellular calcium, control responses rapidly undergo depression as the RRP depletes (**Figure 11b**). After depletion of the RRP, the recycling pool sustains evoked responses. In *complexin* mutants, initial responses have smaller amplitudes compared to the control and the initial depression phase is specifically abolished, leaving the sustained response of the recycling pool intact. In contrast, overexpression of complexin increases the depression effect, with increased initial responses, indicating that in overexpression larvae the RRP size is enhanced.

Calcium influx into nerve terminals triggers synaptic vesicle exocytosis in a fast synchronous phase of release and a slow, asynchronous phase (**Figure 11c**, left panel). The two phases have distinct kinetics of release and only synchronous release requires calcium sensing by synaptotagmin 1 (Geppert, Goda et al. 1994; Yoshihara and Littleton 2002). In *complexin* mutants, as in *syt1* mutants, the synchronous phase is specifically affected. When EPSCs are normalized to the number of active zones present at NMJs in control and mutant larvae, the amplitude of the synchronous phase of release in *complexin* mutants is reduced compared to controls, while the kinetics of asynchronous release are unaffected (**Figure 11c**, right panel). In contrast, overexpression of complexin reduces the asynchronous phase of release. Taken together, these data suggest that complexin assists in synchronizing release by holding vesicles in the readily releasable pool of vesicles, preventing them from fusing prematurely with the presynaptic membrane.

Discussion

Our analysis demonstrates that the interaction of complexin with the SNARE complex provides a molecular fusion clamp to prevent spontaneous release from the readily releasable pool of vesicles at synapses in the absence of stimulation. These *in vivo* observations match well with

the predicted fusion clamp model based on the *in vitro* reduction of SNARE-mediated fusion by complexin (Giraudo, Eng et al. 2006; Schaub, Lu et al. 2006). Although analysis of cultured hippocampal autaptic neurons lacking three of the four mouse complexin genes (Reim, Wegmeyer et al. 2005) showed reduced neurotransmitter release (Reim, Mansour et al. 2001) (Xue, Stradomska et al. 2008), which matches the observations in *Drosophila*, mini frequency in the mouse system was reported to be unchanged. It is unclear what underlies the discrepancy in mini frequency between models, but one potential explanation is that in mouse either complexin 4 or other unknown components of the fusion clamp machinery compensate for the loss of complexin. Another potential explanation is that recordings from mice or hippocampal cultures from mice were done at P0 or days 10 to 16 of growth, when complexin is either not expressed or is not localized to synapses in wild type cells (Ono, Baux et al. 1998; Reim, Wegmeyer et al. 2005). It is possible that this reflects a maturation process at synapses and only after this is complete is complexin required. If this is the case, recording from synapses early in development may not reveal all aspects of complexin's function at the synapse. In this study, we have generated the first complete knockout of *complexin* in any organism and recorded from mutant synapses that are developmentally mature and highly stereotyped, revealing a new aspect of complexin's function *in vivo* that provides a fusion clamp mechanism for synaptic vesicle exocytosis.

It has long been known that minis represent single vesicle fusion events, but their underlying function has remained unclear. Our results unexpectedly uncovered a marked effect on synaptic growth at the *Drosophila* NMJ in *complexin* mutants. Although previous studies in hyperexcitable *Drosophila* mutants have demonstrated that increased neuronal activity promotes synaptic growth (Guan, Saraswati et al. 2005), the contributions of spontaneous versus evoked

signaling are unknown. Activity-dependent retrograde signaling by the postsynaptic calcium sensor Synaptotagmin 4 has also been shown to transiently increase mini frequency 100-fold and trigger enhanced synaptic growth at *Drosophila* embryonic NMJs (Yoshihara, Adolfsen et al. 2005). It is tempting to speculate that regulation of complexin during retrograde signaling underlies activity-dependent enhancement of spontaneous fusion and synaptic growth. There is at present little evidence that the increased minis cause the morphological overgrowth, and further studies will be required to dissect the mechanism by which *complexin* mutants enhance synaptic growth. Recent work in mammals suggests that spontaneous release can regulate dendritic spine morphogenesis (McKinney, Capogna et al. 1999) and dendritic protein synthesis (Sutton, Wall et al. 2004). Complexin dysfunction has also been implicated in human diseases, including schizophrenia (Sawada, Barr et al. 2005), indicating that abnormal spontaneous fusion may contribute to certain neurological diseases.

Given that complexin and the synaptic vesicle calcium sensor Synaptotagmin 1 compete for binding to SNARE complexes (Tang, Maximov et al. 2006), an attractive model for synaptic vesicle exocytosis is the following: During priming, complexin stabilizes a hemifused intermediate by preventing full zippering of the SNARE complex, by preventing synaptotagmin 1 from triggering vesicle fusion, or possibly via both mechanisms. Upon calcium activation of synaptotagmin 1, the clamp is relieved, perhaps by removal of complexin from the SNARE complex. Synaptotagmin 1 is then free to drive fusion and synaptic vesicles proceed from a hemifused state to full fusion. Our results demonstrate that, together with synaptotagmin 1, complexin functions in vesicles fusion by acting as a key neuronal modulator of the SNARE complex that adapts the ubiquitous membrane trafficking machinery for synaptic vesicle fusion.

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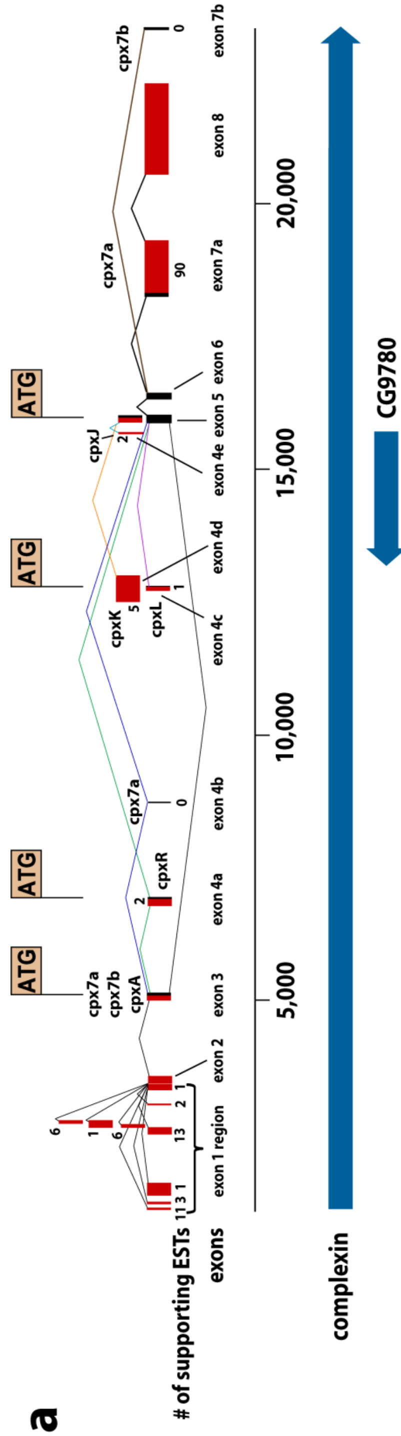
Figures



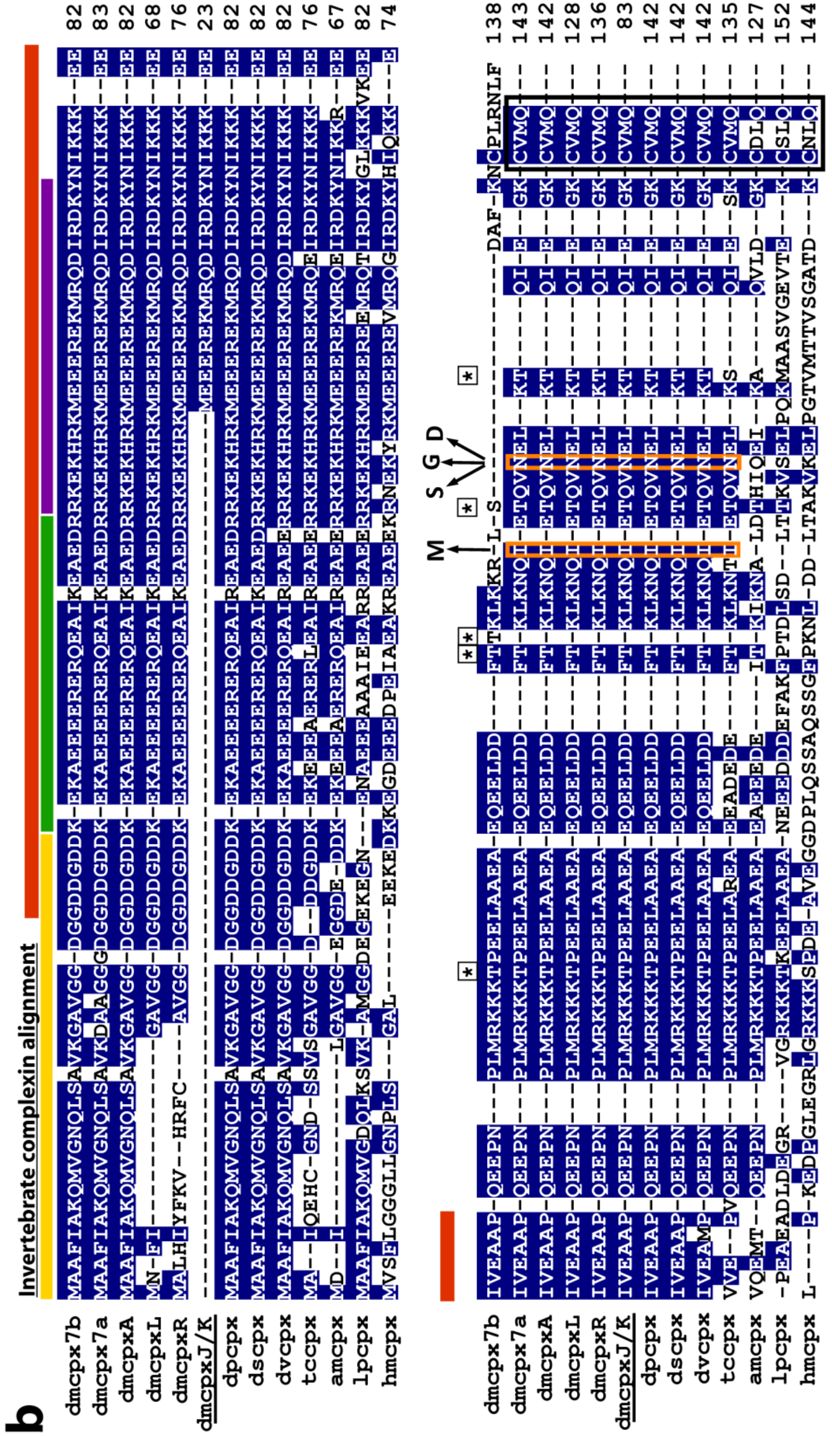


Figure 1. Features of the *Drosophila complexin* locus and its protein products. (**a**) *Drosophila complexin* is diagrammed. Red and black bars represent exons. Black portions of exons indicate protein coding regions, while red portions indicate untranslated regions. Lines connecting exons represent splice junctions. Exon numbers are labeled below diagrammed exons. Exons 1, 4, and 7 are predicted to be alternatively spliced. Exon 1 alternative splicing produces many different 5' UTRs, suggesting tight regulation of complexin expression. Exon 4 alternative splice junctions are shown in several different colors for the sake of clarity. Exon 4 alternative splicing is predicted to produce several protein sequence variants differing in the N terminal region of complexin (panel **b**). Exon 7 alternative splicing produces two sequence variants, "cpx7a" and "cpx7b", that mainly differ in their C terminal sequences. Two versions of exon 5 are shown that are identical in sequence, but the top exon 5 highlights the putative translation start site for cpxJ/K (panel **b**). For alternatively spliced exons, the number of ESTs whose sequences map to those exons is indicated to show the extent to which the predicted splice variants have been experimentally verified. Protein sequence complexin A is produced by the majority of predicted transcripts and is most supported by EST data. However, only transcripts encoding complexins 7a and 7b have been isolated from cDNA libraries. The primary translation start site (ATG) for the locus is present in exon 3, but there are 3 additional predicted alternative start sites. CG9780 (blue arrow pointing left) is a gene of unknown function located in the intron between exons 4 and 5. Transcript sequences, EST sequences, translation start site predictions, and annotations are based on the sequence of the *Drosophila melanogaster* genome revision 5.1 and were downloaded from Flybase (http://flybase.org) (Smith et al ***Science*** 2007; Wilson et al ***Nucleic Acids Research*** 2008) and analyzed using CLC Bio DNA Analysis software. (**b**) Alignment of six *Drosophila melanogaster* complexins (7a, 7b, A, L, R, and J/K) with complexins from other invertebrate species. The six *D. melanogaster* complexin isoforms are separated from the other invertebrate complexins by a black line in the alignment. The alternative exons that encode the different complexin isoforms are labeled in panel **a** according to the isoform they encode. cpxA, cpxL, cpxR, and cpxJ/K are named for the annotations of the transcripts that produce them and differ from dmcpx7a in their N terminal sequence. Complexins L, R, and J/K are encoded by alternate exons 4c, 4a, and 4e and 4d, respectively. cpxJ/K is encoded by two different fourth exons (4e and 4d) differing in their untranslated regions that have identical coding sequences with the putative translation start site in exon 5. cpxJ/K is an N-terminal truncation of cpx7a. Complexins 7a and 7b have distinct C termini encoded by exons 7a and 7b. Only cpx7a and cpx7b have been definitively shown to be expressed *in vivo*. Primary sequences of complexins were aligned for maximal homology using CLC Bio Analysis software. Residues that are identical in at least 50% of sequences are highlighted in dark blue. The colored bars highlight important fragments of the sequence. Red bar: Fragment crystallized in rat complexin structure (Chen et al ***Neuron*** 2002). Purple bar: Central alpha helix responsible for binding to the SNARE complex. Green bar: accessory alpha helix. Yellow bar: N terminal 26 residues proposed to overcome the inhibitory function of the accessory alpha helix (Xue et al ***Nat Struct Mol Biol*** 2007). Black box: CAAX motif found in complexins from invertebrate species and mammalian complexins 3 and 4. Abbreviations: dm, *Drosophila melanogaster*; dp, *Drosophila pseudoobscura*; ds, *Drosophila simulans*; dv, *Drosophila virilis*; tc, *Tribolium castaneum*; lp, *Loligo pealei*; am, *Apis mellifera;* hm, *Hirudo medicinalis*. mRNAs that encode cpx7a, cpxA, cpxL, cpxR, and cpxJ/K are edited at cpx7a sites I124 to M and N129 to serine, glycine, or aspartic acid (highlighted by orange boxes) (Hoopengardner et al *Science* 2003). Complexin is predicted to be phosphorylated at several sites (*). Phosphorylation predictions were implemented using two phosphorylation prediction programs, NetphosK (http://www.cbs.dtu.dk/services/NetPhosK/) (Blom et al ***Proteomics*** 2004) and Scansite (http://scansite.mit.edu) (Obenauer et al ***Nucleic Acids Res*** 2003). Predicted phosphorylation sites and corresponding kinases marked by asterisks from left to right: T101, PKA and PKC; T118 and T119, PKC; T126, casein kinase 2; S126, PKC and PKA. Numbering is relative to dmcpx7b if site is present in dmcpx7b; otherwise numbering is relative to dmcpx7a.

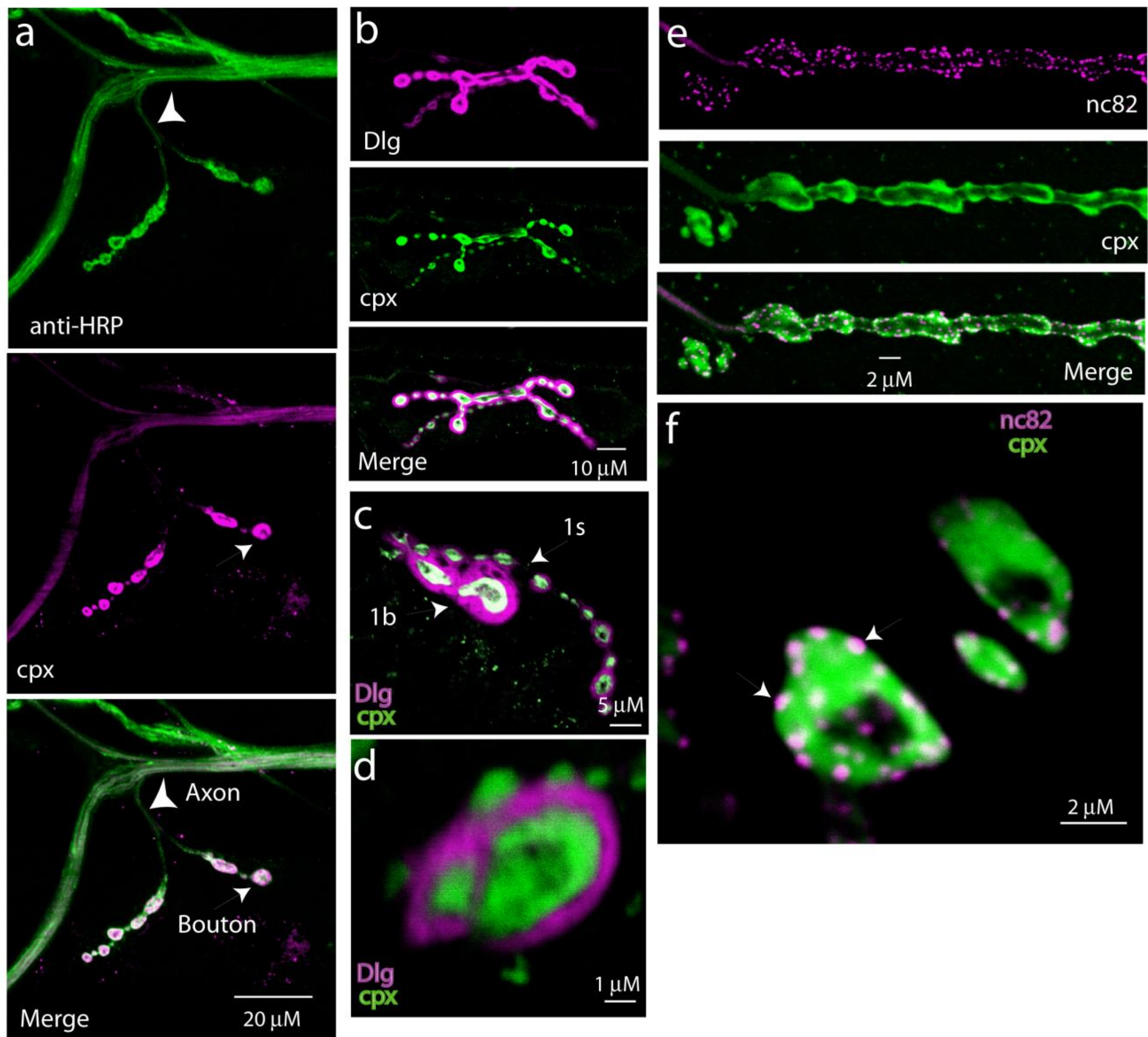

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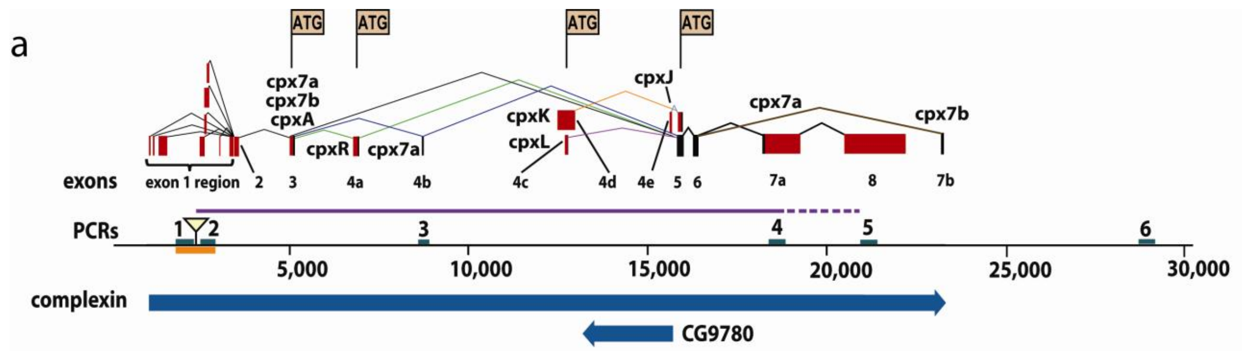
Figure 2. Subcellular localization of Drosophila complexin. (**a-f**) Third instar larval NMJs stained with antisera against complexin and (**a**) the neuronal tissue marker Horseradish Peroxidase (HRP), (**b-d**) The postsynaptic marker Discs large (Dlg), and (**e-f**) the active zone marker Bruchpilot/nc82. (**a**) Complexin (magenta) is enriched in nerve terminals. HRP (green) shows immunoreactivity to both axons (arrowhead) and nerve terminals. Complexin immunoreactivity is low in axons but high in nerve terminals. (**b**) Dlg staining (magenta) and complexin staining at the NMJ do not overlap, showing that complexin is expressed presynaptically. (**c**) Complexin is expressed at two of the major types of excitatory glutamatergic synapes at NMJs, types 1b and 1s. (**d**) Magnified view of complexin and dlg costaining showing complexin's presynaptic expression. (**e-f**) Images of nc82 (magenta) and complexin (green) at (**e**) an NMJ or (**f**) individual NMJ boutons. Complexin is not enriched at active zones but is present throughout the nerve terminal. (**g**) Complexin primarily localizes to cytosolic fractions on a sucrose gradient of post-nuclear adult fly head extracts. Post-nuclear fractions of Canton S head extracts were separated on 10-30% sucrose gradients. Isolated fractions were probed for subcellular markers by Western analysis, including antisera against ROP / unc18, which localizes to the plasma membrane (left-most fractions). Synaptic vesicle fractions were identified using the synaptogyrin antibody, cytosolic fractions were indicated by immunostaining for ROP, and endosomal fractions by staining for HRS (Lloyd et al. ***Cell*** 2002). The last collected fraction (right-most lane) often contained contaminants from the residual membrane debris extracted from the tube sides in the final step.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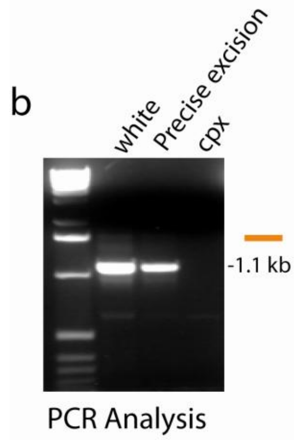
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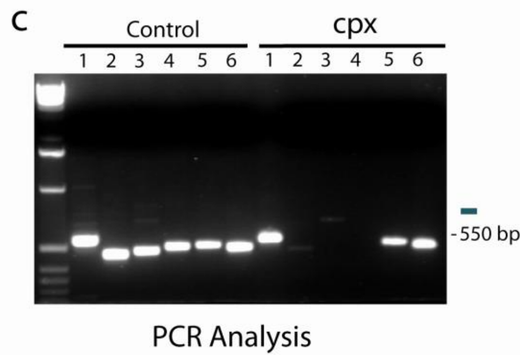
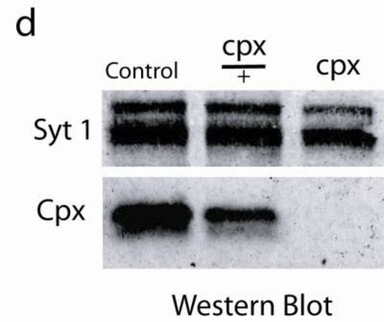
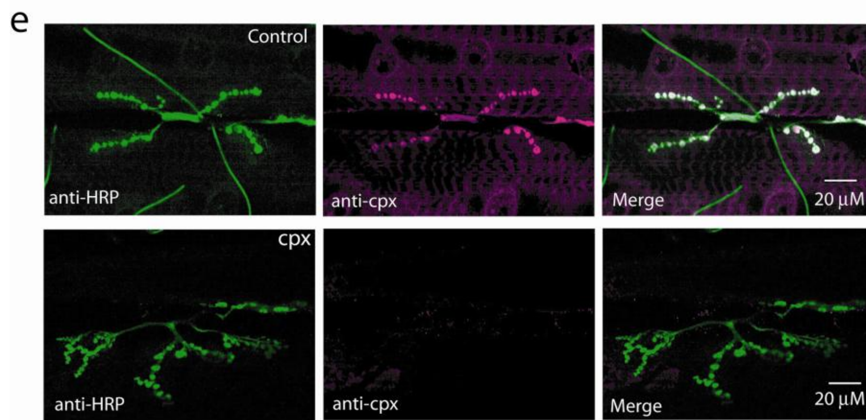
white
Precise excision
cpx
-1.1 kb
PCR Analysis


c
Control
cpx
1 2 3 4 5 6 1 2 3 4 5 6
-550 bp
PCR Analysis


d
Control
cpx/+
cpx
Syt 1
Cpx
Western Blot


e
Control
anti-HRP
anti-cpx
Merge
20 μM
cpx
anti-HRP
anti-cpx
Merge
20 μM

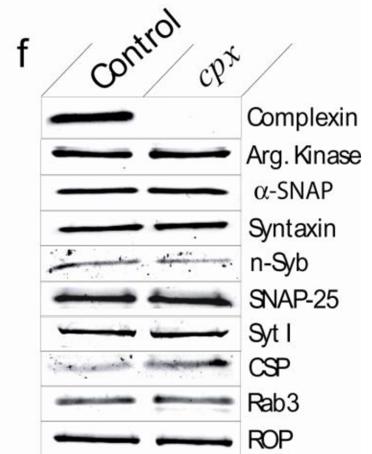

f
Control
cpx
Complexin
Arg. Kinase
α-SNAP
Syntaxin
n-Syb
SNAP-25
Syt I
CSP
Rab3
ROP

Figure 3. Generation of a *complexin* null mutant in *Drosophila*. (**a**) The complexin genomic locus is diagrammed. A deletion mutant was produced by imprecise excision of a P-element (yellow triangle) located in the first intron of complexin, 2.7 kb upstream of the primary translation start site (ATG) in exon 3. Orange and green bars represent diagnostic PCR products used in deletion mapping. (**b**) PCR spanning the P-element insertion site (orange bar in panel **a**) on genomic DNA from *white* (positive control), *precise excision*, and cpx^{SH1} flies reveals lack of the genomic PCR product in the null mutant. (**c**) PCR mapping of ~500 base pair stretches of genomic DNA at defined locations upstream and downstream of the P-element in *white* (control) and cpx^{SH1} animals reveals an intragenic *complexin* deletion removing the region between the P element insertion site in the first exon to a location between exons 7a and 8 defined by PCR products 2-4. Numbers refer to green bars in panel **a**. Purple bar in panel **a** shows extent of deletion in cpx^{SH1} mutants. (**d**) Western analysis of control, heterozygotes and cpx^{SH1} mutants with an anti-complexin antibody shows reduced expression in heterozygotes and loss of the protein in the null mutant. Syt 1 immunoreactivity (top lanes) was used as a loading control. (**e**) Immunohistochemistry at larval NMJs confirms the absence of complexin in the cpx^{SH1} mutant compared to control. Anti-HRP (green) was used to mark axons and varicosities at the NMJ. Complexin staining in the *white* control (top panels) is present, but is absent in the mutant (bottom panels). (**f**) Expression levels of many synaptic proteins are not altered in *complexin* mutants compared to *precise excision* controls. Proteins analyzed by Western Blot include: arg. kinase (arginine kinase), α-SNAP (soluble N-ethylmaleimide-sensitive fusion protein (NSF)-attachment protein), syntaxin, n-syb (synaptobrevin); SNAP-25 (synaptosome-associated protein of 25000 daltons), syt1 (synaptotagmin 1); CSP (cysteine string protein); Rab3; Rop (sec1/unc-18).

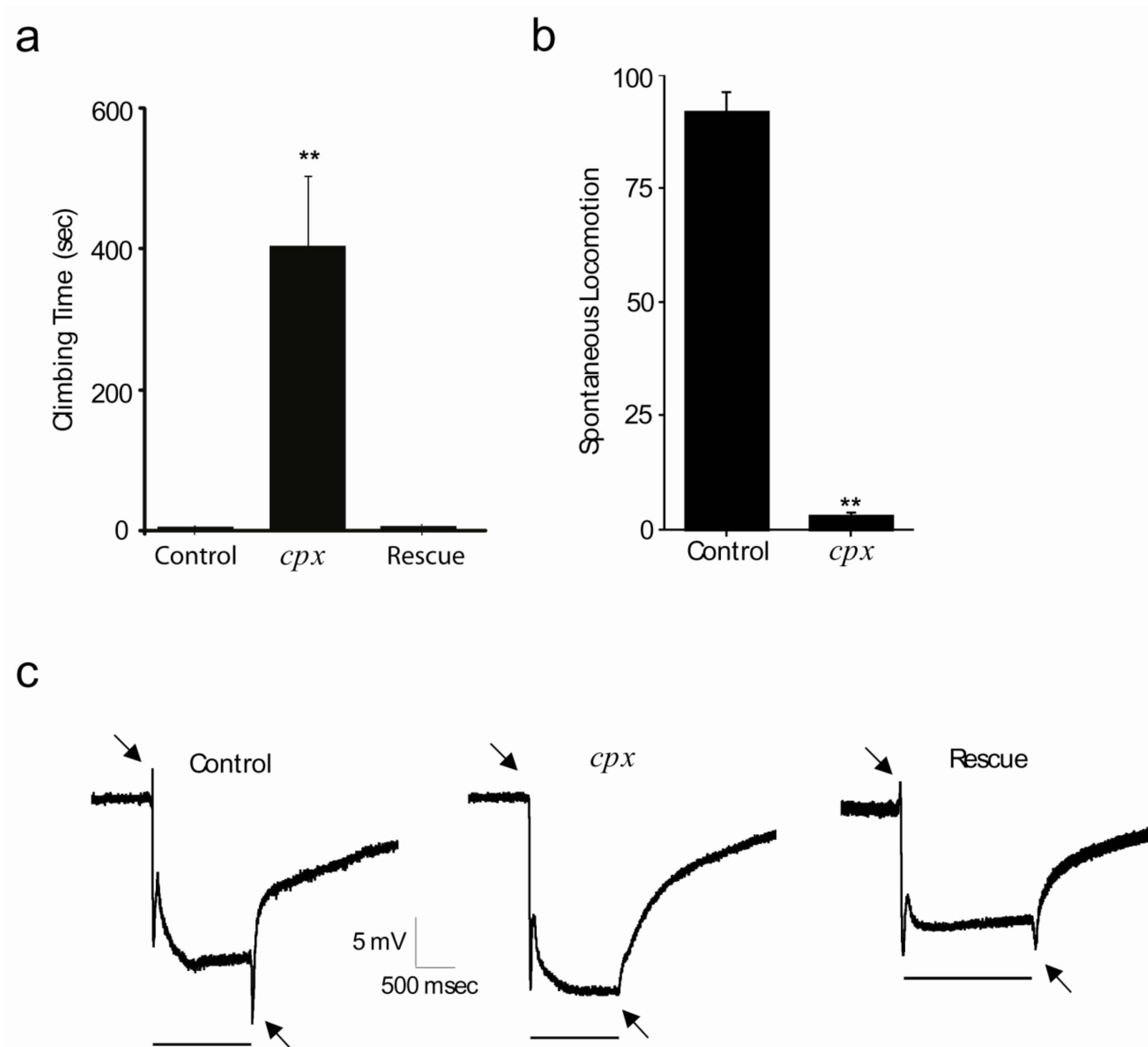


Figure 4. *Complexin* null mutants have severe defects in motor coordination and synaptic transmission. (**a**) cpx^{SH1} escaper adults show severe motor defects compared to *precise excision* controls and rescue animals in a wall climbing assay. The numbers of animals assayed were: *precise excision*, n=15; *cpxSH1*, n=7; rescue (*w-, UAS-cpx/C155elav-Gal4; cpx^{SH1} / cpx^{SH1}*), n=14. (**b**) cpx^{SH1} escaper adults are deficient in spontaneous locomotion, crossing an enclosure significantly fewer times than controls. The numbers of animals assayed were: *precise excision*, n=10; cpx^{SH1}, n=7. Error bars for **a** and **b** are s.e.m. and statistical significance was determined by the Mann-Whitney U test (** = P<0.0001). (**c**) On/off transients (arrows) in electroretinogram recordings are absent in cpx^{SH1} mutants, compared with control *white* lines and rescue animals, indicating a disruption of synaptic transmission between photoreceptors and second order neurons in the visual system.

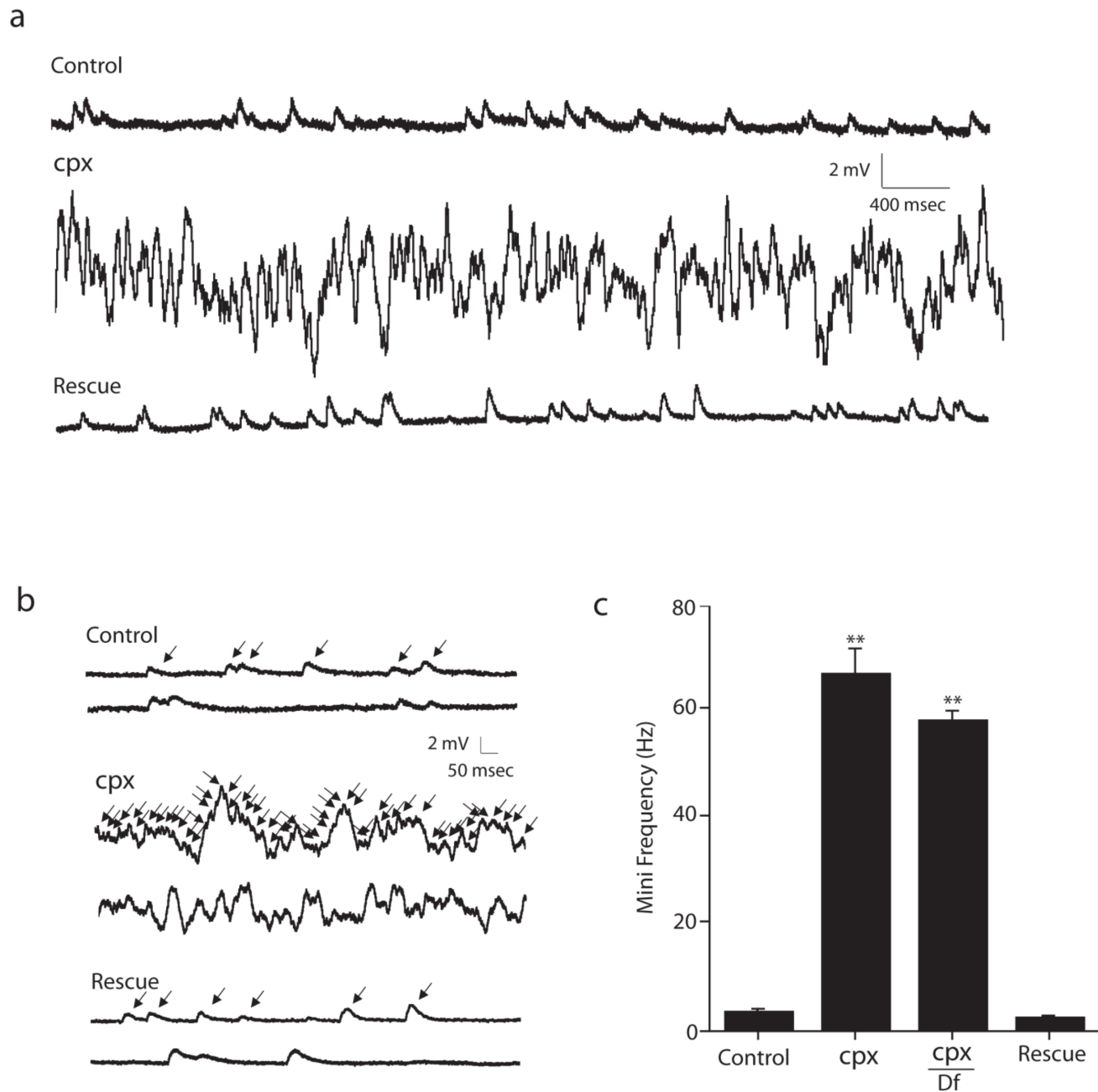
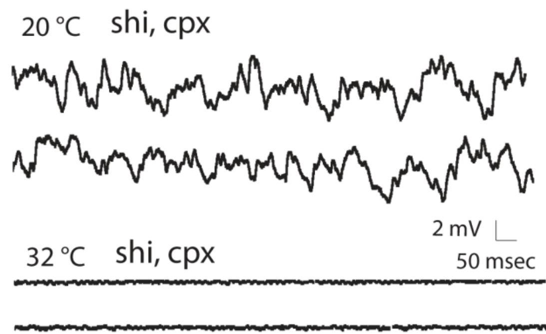


Figure 5. *Complexin* mutants show a massive increase in mini frequency at third-instar larval NMJs. (**a-c**) The genotypes analyzed are: control (*w-; precise excision/precise excision*); cpx (*w-; cpx^{SH1}/ cpx^{SH1}*); rescue (*w-, UAS-cpx/C155elav-Gal4; cpx^{SH1}/ cpx^{SH1}*) and cpx/Df (*w-; cpx^{SH1}/Df(3R)ED5021*). (**a**) Sample traces of mEJPs in *precise excision*, *cpx^{SH1}* and rescue larvae. *cpx^{SH1}* mutants show massive spontaneous exocytosis compared to controls. (**b**) Sample traces with expanded timescale showing individual minis labeled with arrows in the top trace from the indicated genotypes. (**c**) Quantification of average mini frequency (Hz ± s.e.m.) in *precise excision*, *cpx^{SH1}*, *cpx^{SH1}/Df*, and rescue animals in 0.2 mM extracellular calcium. *cpx^{SH1}* homozygotes (Student t-Test, ** = P<0.0001) and *cpx^{SH1}/Df* larvae (Student t-Test, ** = P<0.0001) have mini frequencies >20-fold higher than controls. Data used in the analysis include: Control (Mini Frequency (MF)=3.14±2.9 Hz; RP=-51.1±1.1; n=18), *cpx^{SH1}* (MF=67.0±4.8 Hz; RP=-48.3±0.9, n=27), *cpx^{SH1}*/Df (MF=58.3±2.3 Hz; RP=-48.6±1.3, n=18), Rescue (MF=2.2±0.2, RP=-54.5±1.8, n=25).


a
20 °C shi, cpx
2 mV
50 msec
32 °C shi, cpx

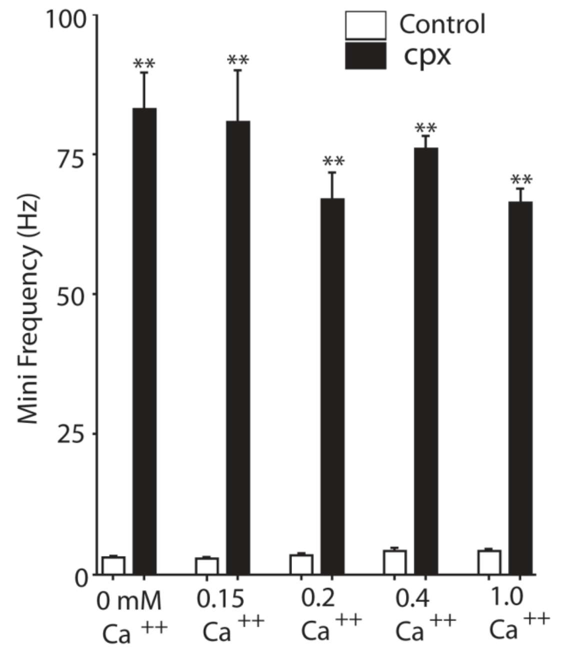
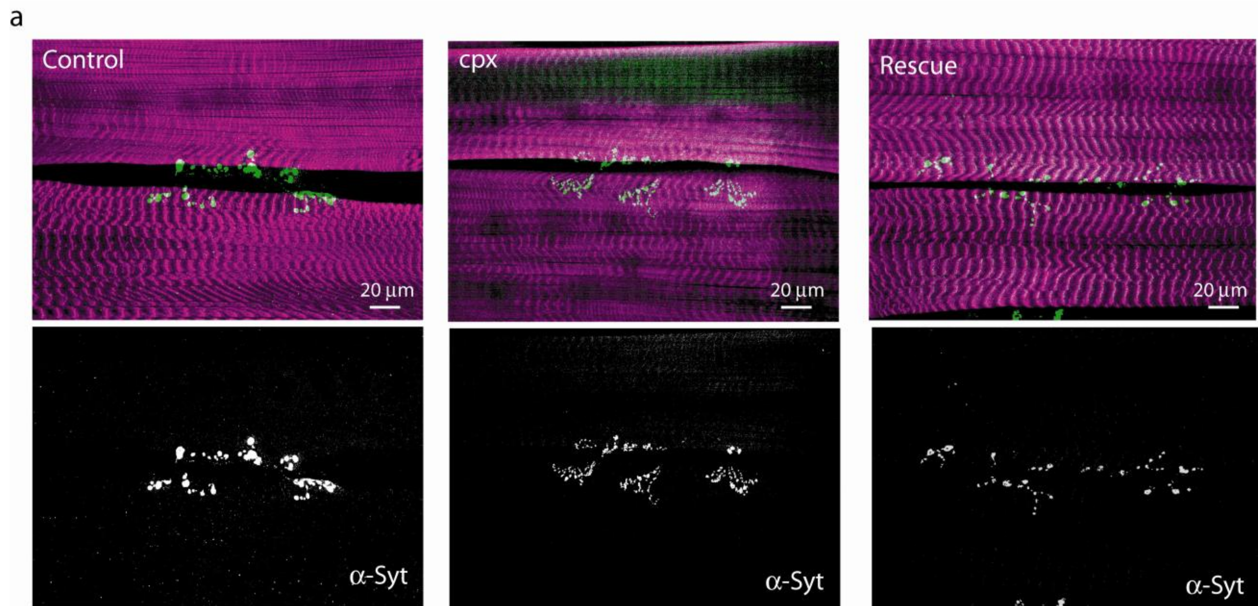
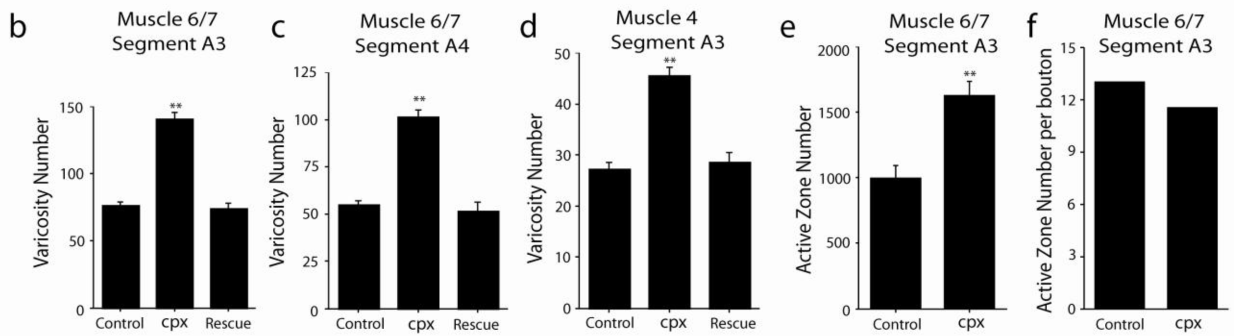

b
Control
cpx
Mini Frequency (Hz)
100
75
50
25
0
**
0 mM Ca ++
0.15 Ca ++
0.2 Ca ++
0.4 Ca ++
1.0 Ca ++

Figure 6. Enhanced mini frequency in *complexin* mutants is due to spontaneous vesicle release from the presynaptic terminal and does not require calcium influx. (**a**) Recordings at 20°C and at 32°C (following a 5 min 10 Hz stimulation) in $shibire^{TS1}$; cpx^{SH1} double mutants. In the double mutant, no minis are observed at the restrictive temperature after stimulation, demonstrating that the mEJPs observed in the mutant are due to spontaneous synaptic vesicle release from the presynaptic terminal. (**b**) cpx^{SH1} mutants show statistically similar (Student t-Test) elevated spontaneous release at 0 mM (** = P<0.0001), 0.15 mM (** = P<0.0001), 0.2 mM (** = P<0.0001), 0.4 mM (** = P<0.0001) and 1.0 mM (** = P<0.0001) extracellular calcium compared to controls. Data used in the analysis include: cpx^{SH1} - 0 mM calcium (MF=83.2 ± 6.5 Hz, n=10), 0.15 mM (MF=80.8 ± 9.2 Hz, n=6), 0.2 mM (MF=66.9 ± 4.8 Hz, n=27), 0.4 mM (MF=75.9 ± 2.3 Hz, n=15) and 1.0 mM (MF=66.35 ± 2.5 Hz, n=10); *precise excision* control - 0 mM calcium (MF=2.86 ± 0.2 Hz, n=19), 0.15 mM (MF=2.7 ± 0.5 Hz, n=7), 0.2 mM (MF=3.1 ± 0.3 Hz, n=18), 0.4 mM (MF=3.9 ± 0.6 Hz, n=5) and 1.0 mM (MF=4.0 ± 0.4 Hz, n=16).


a
Control
cpx
Rescue
20 µm
20 µm
20 µm
α-Syt
α-Syt
α-Syt


b
Muscle 6/7
Segment A3
Varicosity Number
150
100
50
0
**
Control
cpx
Rescue
c
Muscle 6/7
Segment A4
Varicosity Number
125
100
75
50
25
0
**
Control
cpx
Rescue
d
Muscle 4
Segment A3
Varicosity Number
50
40
30
20
10
0
**
Control
cpx
Rescue
e
Muscle 6/7
Segment A3
Active Zone Number
2000
1500
1000
500
0
**
Control
cpx
f
Muscle 6/7
Segment A3
Active Zone Number per bouton
15
12
9
6
3
0
Control
cpx

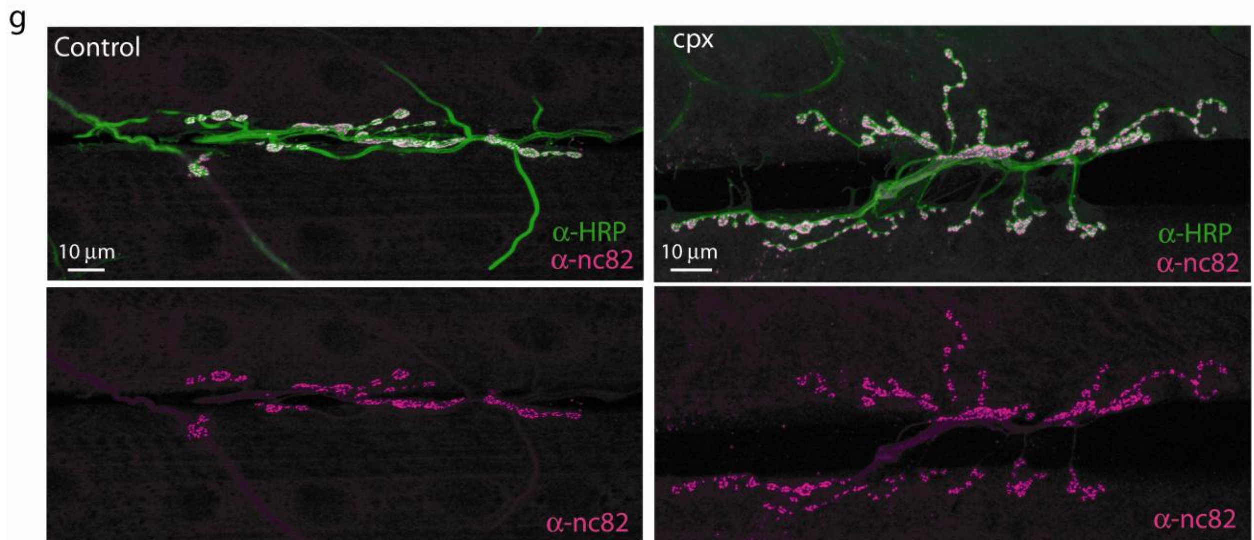
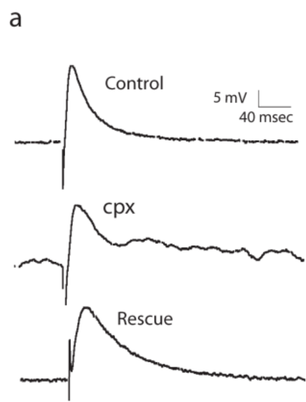
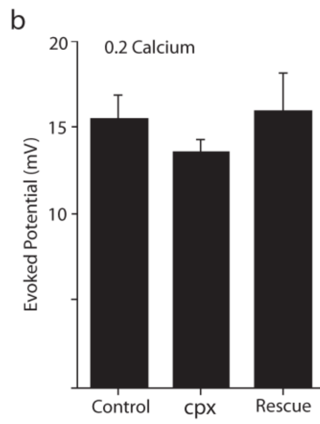

g
Control
cpx
α-HRP
α-nc82
α-HRP
α-nc82
10 µm
10 µm
α-nc82
α-nc82

Figure 7. Quantification of synaptic overgrowth at *complexin* mutant NMJs. (**a**) Anti-syt 1 staining of third-instar larval muscle 6/7 NMJs from segment A3 showing synaptic overgrowth in cpx^{SH1} compared to *precise excision* and rescue animals. Top panels: green = Syt 1 to label varicosities; magenta = Texas-Red phalloidin to label muscle actin. Bottom panels: Syt 1 staining alone. (**b-d**) Quantification of synaptic overgrowth ± s.e.m. at three NMJs in *precise excision*, cpx^{SH1}, and rescue animals. The number of Syt 1-positive varicosities in cpx^{SH1} mutants was statistically increased compared to controls at (**b**) muscle fiber 6/7 of segment A3 (Student t-Test, ** = P<0.0001), (**c**) muscle fiber 6/7 of segment A4 (Student t-Test, ** = P<0.0001), and (**d**) muscle fiber 4 of segment A3 (Student t-Test, ** = P<0.0001). Data used in the analysis include: Control (muscle (m) 6/7 segment A3: bouton # = 76.3±2.8, n=32; 6/7 segment A4: bouton # = 54.8±2.2; m4 segment A3: bouton # =27.2±1.4; n=32), cpx^{SH1} (m6/7 segment A3: bouton # = 140.6±4.9, n=35; m6/7 segment A4: bouton # =101.6±3.5; m4 segment A3: bouton # =45.6±1.6; n=35), Rescue (m6/7 segment A3: bouton # = 73.9±4.2, n=16; m6/7 segment A4: bouton # =51.4±4.9; m4 segment A3: bouton # =28.5±2.0; n=16). (**e**) Quantification of active zone number ± s.e.m. at muscle 6/7 NMJs of segment A3. **P=0.01. Data used in the active zone analysis include: Control *precise excision*: active zone # per m6/7 synapse = 990.4 ± 102.1, n= 12; cpx^{SH1}: active zone # per m6/7 synapse = 1620.5 ± 111.5, n=11. (**f**) Number of active zones per bouton at muscle 6/7 NMJs of segment A3. Values were generated by dividing the mean number of active zones in a given genotype by the mean number of boutons. Data used in the analysis include: Control *precise excision*: mean number of active zones per m6/7 synapse = 990.4, mean number of boutons per m6/7 synapse = 76.3, number of active zones per bouton = 13.0; cpx^{SH1}: mean number of active zones per m6/7 synapse = 1620.5 mean number of boutons per m6/7 synapse = 140.6, number of active zones per bouton = 11.53. (**g**) Staining of third-instar larval muscle 6/7 NMJs from segment A3 with antibodies to horseradish peroxidase (HRP) and bruchpilot (nc82), showing increased active zone number and enhanced synaptic proliferation in cpx^{SH1} mutants.


a
Control
5 mV
40 msec
cpx
Rescue


b
0.2 Calcium
Evoked Potential (mV)
20
15
10
Control
cpx
Rescue

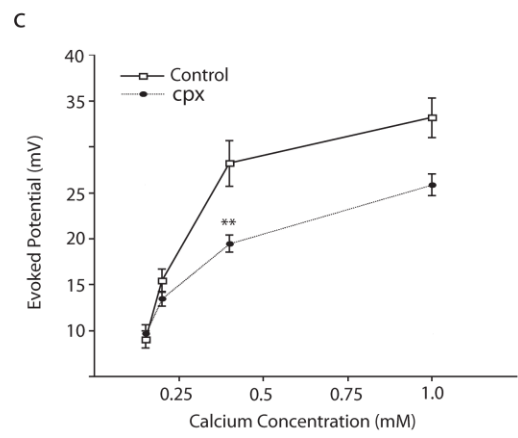
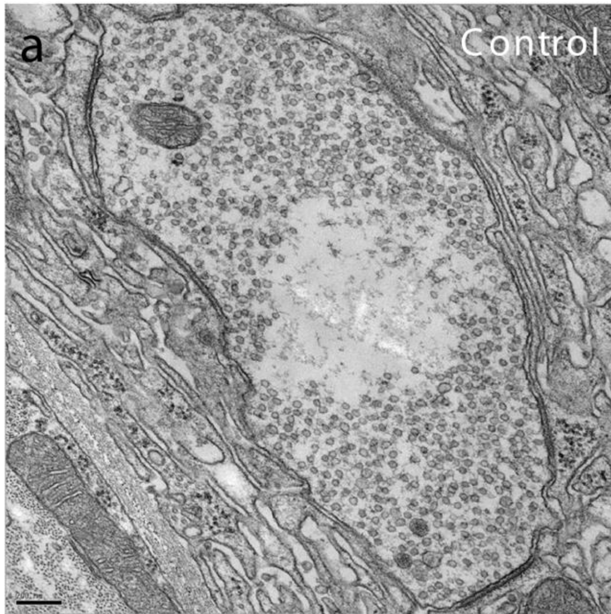
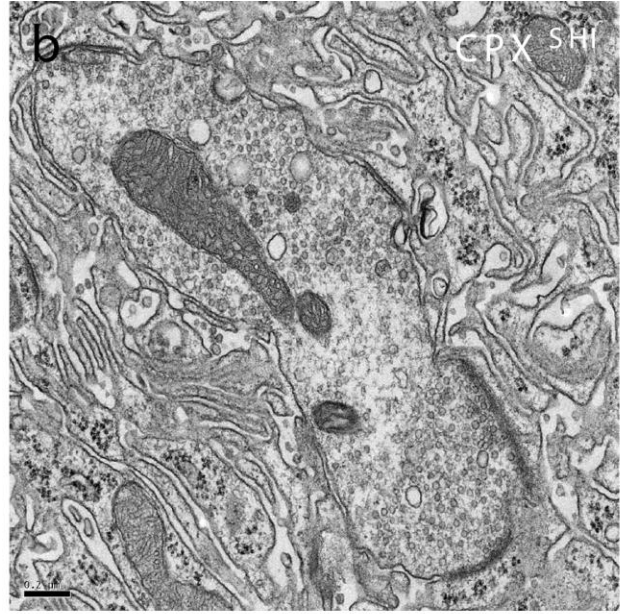
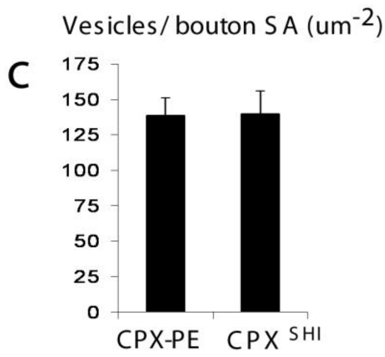
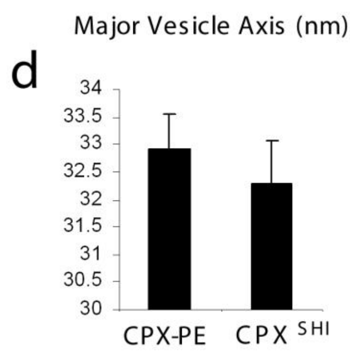

c
Control
cpx
Evoked Potential (mV)
40
35
25
20
15
10
**
0.25
0.5
0.75
1.0
Calcium Concentration (mM)

Figure 8. Complexin mutants have significant defects in EJP amplitude at elevated calcium concentrations. (**a**) Sample traces of EJPs from *precise excision*, cpx^{SH1} and rescue larvae at 0.2 mM extracellular calcium. (**b**) Quantification of evoked response ± s.e.m. at 0.2 mM extracellular calcium. cpx^{SH1} mutants show no statistically significant difference (Student t-Test) in average EJP amplitude at this calcium concentration. Data used in the analysis include: Control (EJP=15.5±3.1 mV; Resting Potential (RP)=-51.1±1.1; n=10), cpx^{SH1} (EJP=13.5±0.8 mV; RP=-48.3±0.9, n=18), Rescue (EJP=15.9±2.2 mV, RP=-54.5±1.8, n=8). (**c**) Summary of average EJP amplitude (± s.e.m.) versus extracellular calcium concentration in cpx^{SH1} (closed circles) and *precise excision* (open squares) larvae. At low calcium concentrations (0.15 mM and 0.2 mM), there is no statistically significant difference between mutants and controls. cpx^{SH1} larvae have significantly lower EJPs at higher calcium concentrations of 0.4 mM (Student t-Test, ** = P<0.001) and 1 mM (Student t-Test, ** = P<0.01) compared to *precise excision* controls. Data used in the analysis include: 0.15 mM calcium (Control EJP=9.1±0.9 mV, RP=-57.4±1.7, n=17; cpx^{SH1} EJP=9.8±0.9 mV, RP=-47.4±1.1, n=11); 0.2 mM calcium (see B); 0.4 mM calcium (Control EJP=28.2±3.1 mV, RP=-54.7±1.5, n=9; cpx^{SH1} EJP=19.6±0.9 mV, RP=-56.6±1.2, n=15); 1.0 mM calcium (Control EJP=33.1±2.2 mV, RP=-58.7±1.8, n=14; cpx^{SH1} EJP=25.9±1.2 mV, RP=-60.2±2.6, n=9).

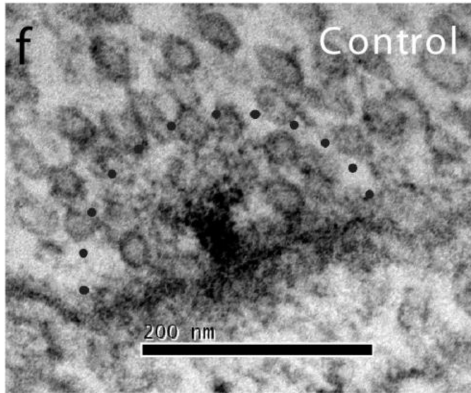

a
Control

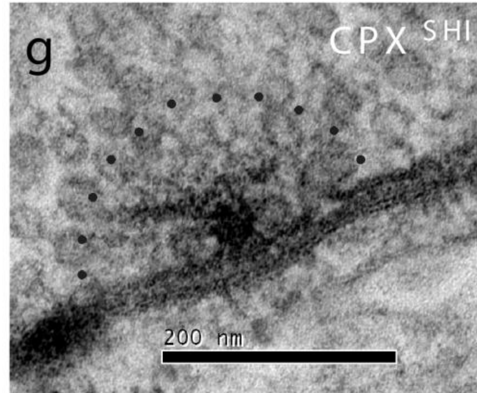

b
CPX SHI

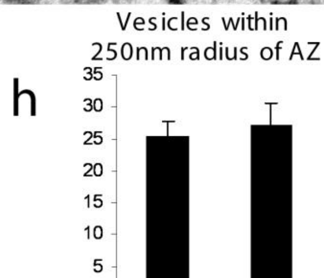

Vesicles/ bouton S A (um-2)
c
175
150
125
100
75
50
25
0
CPX-PE
CPX SHI


Major Vesicle Axis (nm)
d
34
33.5
33
32.5
32
31.5
31
30.5
30
CPX-PE
CPX SHI

Cisternae/bouton S A (um-2)
e
2.5
2
1.5
1
0.5
0
CPX-PE
CPX SHI


f
Control
200 nm


g
CPX SHI
200 nm


Vesicles within
250nm radius of AZ
h
35
30
25
20
15
10
5

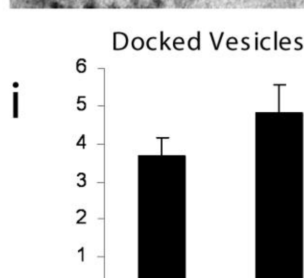
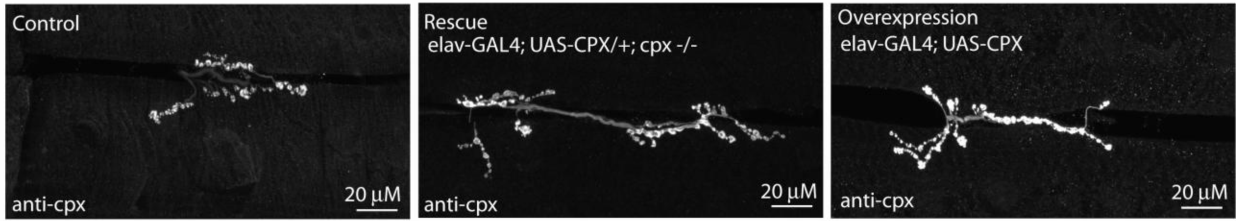
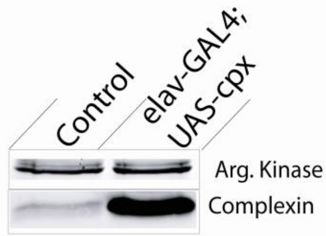
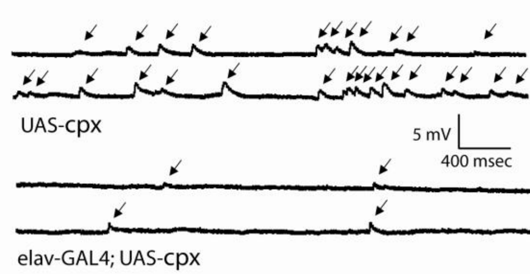
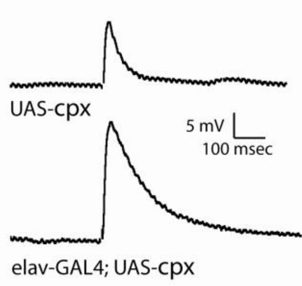

Docked Vesicles
i
6
5
4
3
2
1

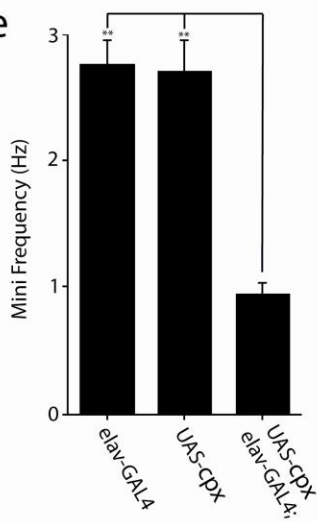
Figure 9. Ultrastructural analysis of complexin mutants. (**a** and **b**) Electron Micrographs of Type I boutons of *complexin precise excision* controls (**a**) and *complexin* null mutants (**b**). (**c-e**) Quantification of vesicle density (**c**), major vesicle axis (**d**), and cisternae density (**e**) in control and *complexin* mutant boutons. Vesicles were counted from 12 boutons from 3 different animals per genotype. The vesicle densities between two genotypes are not significantly different (P>0.05). Major vesicle axes were counted from 100 vesicles at random per bouton. Vesicle axes were measured from 12 boutons from 3 animals per genotype. The major vesicle axes between the two genotypes are not significantly different (P>0.05). Cisternae were counted from at least 12 boutons from at least 3 animals per genotype. Cisternae densities in the different genotypes are significantly different (P<0.05). Error bars indicate s.e.m. (**f** and **g**). Electron micrographs of active zones in control (**f**) and *complexin* mutant (**g**). (**h-i**). Quantification of vesicles within 250nm radius of the active zone and docked vesicles. Vesicles (**h**) and docked vesicles (**i**) within 250 nm radius from the T bar were counted from 10 active zones from 3 different animals per genotype. Average number of vesicles and docked vesicles within the 250nm radius from the T bar between the two genotypes are not significantly different (P>0.05). Error bars indicate s.e.m.


a
Control
anti-cpx
20 μM
Rescue
elav-GAL4; UAS-CPX/+; cpx -/-
anti-cpx
20 μM
Overexpression
elav-GAL4; UAS-CPX
anti-cpx
20 μM


b
Control
elav-GAL4; UAS-cpx
Arg. Kinase
Complexin


c
UAS-cpx
5 mV
400 msec
elav-GAL4; UAS-cpx


d
UAS-cpx
5 mV
100 msec
elav-GAL4; UAS-cpx


e
Mini Frequency (Hz)
elav-GAL4
UAS-cpx
elav-GAL4; UAS-cpx

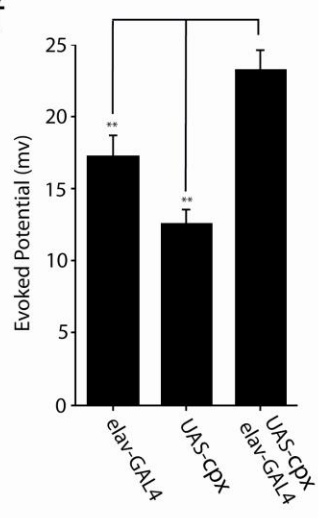
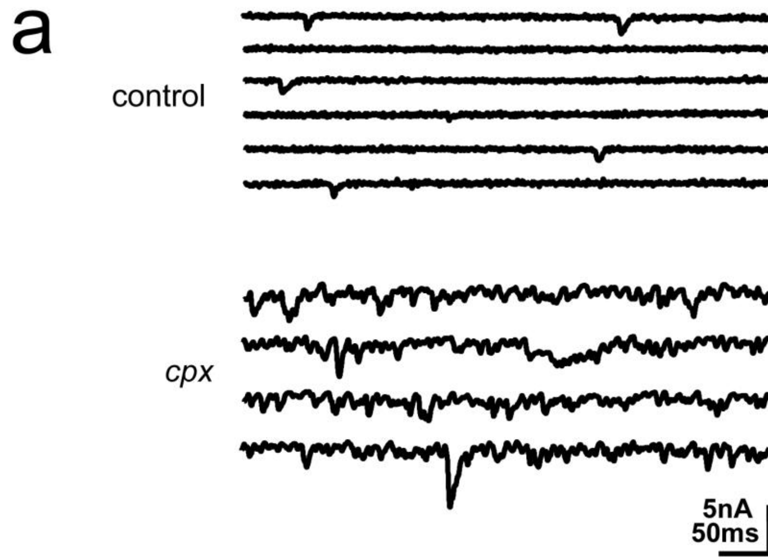

f
Evoked Potential (mv)
elav-GAL4
UAS-cpx
elav-GAL4 UAS-cpx

Figure 10. Complexin overexpression reduces spontaneous mini frequency and enhances evoked release. (**a**) Complexin staining at NMJs in control (*precise excision*), rescue (*elav-Gal4,UAS-cpx; cpx^{SH1}/cpx^{SH1}*), and overexpression (*elav-Gal4; UAS-cpx*) larvae shows that overexpressed complexin is localized to NMJs. (**b**) A Western blot of protein extracts from adult heads in control and overexpression flies shows a several-fold overexpression of complexin compared to the control. A blot for arginine kinase serves as an internal loading control. (**c**) Sample traces of mEJPs in 3rd instar larvae overexpressing complexin (*elav-GAL4; UAS-complexin*) versus control animals (*UAS-complexin*; no driver) at muscle 6 NMJs. Individual minis are labeled with arrows in traces from each genotype. (**d**) Sample traces of EJPs from 3rd instar larvae overexpressing complexin (*elav-GAL4; UAS-complexin*) versus control animals (*UAS-complexin*; no driver) at muscle 6 NMJs in 0.2 mM extracellular calcium. (**e**) Quantification of average mini frequency (Hz ±SEM) in larvae overexpressing complexin (*elav-GAL4; UAS-complexin*) versus control animals (*UAS-complexin or elav-GAL4*) in 0.2 mM extracellular calcium. Animals overexpressing complexin have a >2.8-fold reduction in mini frequency compared with controls (Student t-Test, ** = P<0.0001 compared to *UAS-complexin*, ** = P<0.0001 compared to *elav-GAL4*). Data used in the analysis include: *elav-GAL4; UAS-complexin* (Mini Frequency (MF)=0.9 ± 0.09 Hz; n=32), *elav-GAL4* (MF=2.8 ± 0.2 Hz; n=27), *UAS-complexin* (MF=2.7 ± 0.3 Hz; n=16). (**f**) Quantification of evoked response ± SEM at 0.2 mM extracellular calcium. Overexpression of complexin results in a statistically significant enhancement in average EJP amplitude versus controls (Student t-Test, ** = P<0.0001 compared to *UAS-complexin*; ** = P<0.003 compared to *elav-GAL4*). Data used in the analysis include: *elav-GAL4; UAS-complexin* (EJP=23.3 ± 1.4 mV; Resting Potential (RP)=-60.4 ± 1.6; n=18); *UAS-complexin* (EJP=12.5 ± 1 mV; RP=-60.5 ± 3, n=8); *elav-GAL4* (EJP=17.2 ± 1.5 mV, RP=-58.2 ± 1.3, n=24).


a
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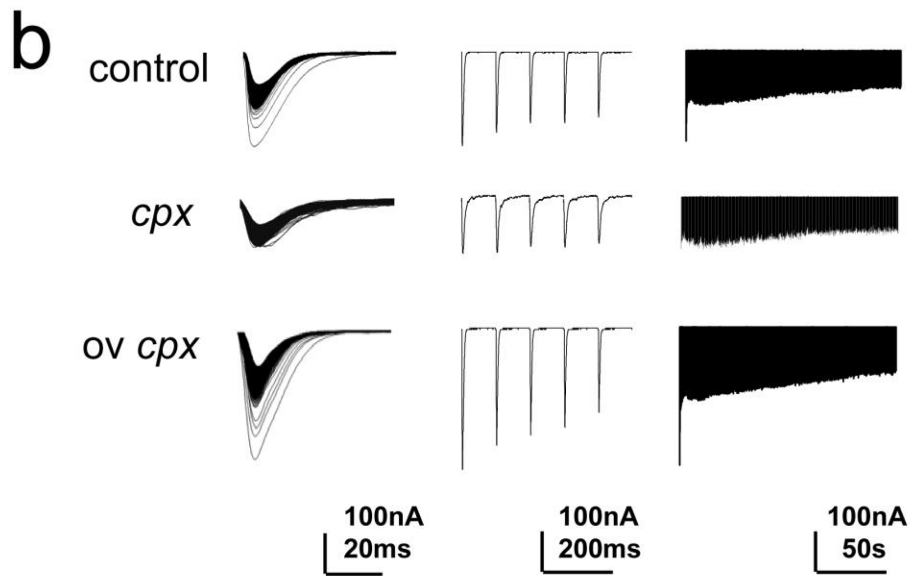
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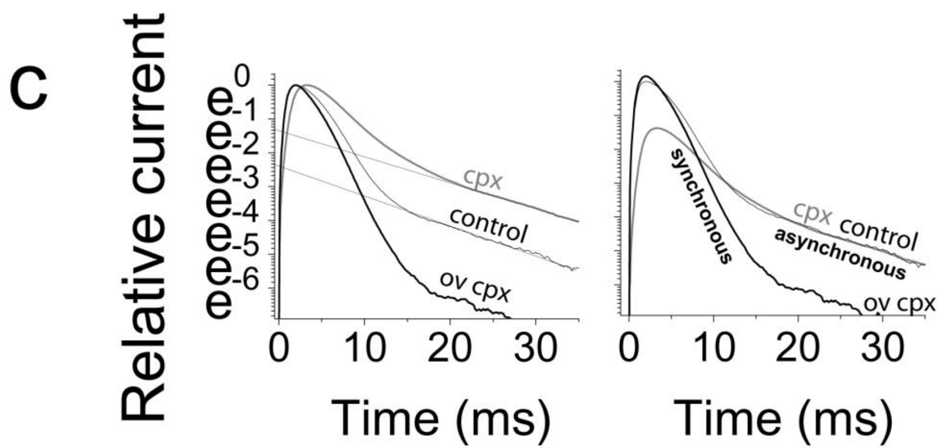
c
Relative current
0
-1
-2
-3
-4
-5
-6
cpx
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ov cpx
synchronous
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10
20
30
Time (ms)
Time (ms)

Figure 11. Complexin is required for maintenance of a readily releasable pool and synchronizes synaptic vesicle exocytosis. (**a**) cpx^{SH1} mutants show enhanced spontaneous release under voltage clamp, the same phenotype seen under current clamp. Shown are recordings of postsynaptic currents in control and cpx synapses in the presence of 0.1 mM external Ca^{2+}. (**b**) Complexin regulates the early phase of short-term synaptic plasticity. Representative nerve evoked EPSCs elicited during 150s of 10Hz stimulation in control, cpx^{SH1} (cpx) and complexin overexpression (ov cpx). The left panel shows the total events aligned at the time of the nerve stimulus. The right panel shown continuous recording in control, cpx and ov cpx. The central panel displays five stimuli of the train in each condition. Stimulus artifact was removed digitally for clarity. The behavior observed in the EPSCs during high frequency stimulation was reproduced in 3 different larvae tested for each phenotype. The external Ca^{2+} concentration in the bath solution was 2.0 mM. (**c**) Complexin modulates synchronous evoked release. Absolute EPSCs are plotted in a semi logarithmic graph to investigate their components. The fast and slow components are easily distinguished during the first 15 and last 20 ms respectively. Normalized currents by the average number of active zones in each genotype (990.4 in control and 1620.5 for cpx^{SH1}) are displayed in the right panel assuming the same number of active zones in complexin overexpression and controls. As can be seen, the asynchronous component in cpx^{SH1} is the same as in control synapses and dramatically diminished in ov cpx, while the synchronous component in cpx^{SH1} is reduced. The external calcium concentration in the bath solution was 2.0 mM.

Chapter 3

A Functional Analysis of Complexin's Role in Exocytosis and Synaptic Plasticity

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The majority of the work described in these studies was performed by Sarah Huntwork-Rodriguez. Lauren Barr and Dina Volfson performed the *in vitro* kinase assays. Amanda Hartman and Krys Foster assisted in the mutagenesis screen. Electrophysiology was performed in large part by J. Troy Littleton.

Introduction

The role of minis in modulation of synaptic function and synaptic growth

Since Fatt and Katz published the first seminal papers describing synaptic transmission at the neuromuscular junction (Fatt and Katz 1951; Fatt and Katz 1952), it has been known that neurotransmitter release occurs in discrete packets, or quanta. Quantal release occurs in two different modes at the synapse: individual quanta can spontaneously release from the synapse to elicit miniature postsynaptic potentials (minis), or multiple quanta can release synchronously in response to an action potential, producing an evoked postsynaptic potential.

Evoked responses are well known to be altered during synaptic plasticity, as in the case of LTP or LTD. In contrast, minis have long been thought to represent background noise at synapses. This perception has recently begun to change due to studies demonstrating that mini frequency can play critical roles in maintaining dendritic spine density in CA1 pyramidal cells in the hippocampus (McKinney, Capogna et al. 1999), in suppressing dendritic protein synthesis (Sutton, Wall et al. 2004), and in regulation of spike frequency in pyramidal cells of the hippocampus in response to nicotine application (Sharma and Vijayaraghavan 2003).

In addition to these examples of the roles that minis may play in the mammalian central nervous system, mini frequency at the *Drosophila* embryonic neuromuscular junction (NMJ) is upregulated by over 100-fold after high frequency stimulation (Yoshihara, Adolfsen et al. 2005) (**Figure 1**). This upregulation of mini frequency lasts for several minutes and requires the function of a pathway that is activated by postsynaptic calcium influx through glutamate receptors in the muscle. This calcium influx is sensed by synaptotagmin 4 in the postsynaptic compartment, triggering postsynaptic fusion of vesicles and releasing an unknown retrograde signal into the synaptic cleft. Retrograde signaling upregulates cAMP in the presynaptic compartment, activating Protein Kinase A (PKA), which in turn acts on an unknown downstream

effector to enhance mini frequency. In addition to regulating mini frequency at the NMJ, this pathway is critical for normal synaptic growth, and overexpression of synaptotagmin 4 or a constitutively active form of PKA causes synaptic overgrowth at third-instar larval NMJs. It is currently unclear how upregulation of mini frequency may be tied to activity-dependent synaptic growth, but one attractive model is that enhanced mini frequency "tags" a synapse for plasticity, allowing local regulation of synaptic morphology.

In the *complexin* null mutant, we had the opportunity to study a situation where there was a large enhancement in mini frequency with largely normal evoked responses. Interestingly, we observed a large and significant increase in synaptic plasticity at larval NMJs, suggesting that the increase in minis is driving synaptic plasticity. It is also possible that the increased synaptic growth observed is due to a developmental defect specific to *complexin* mutants or to compensation for the reduced EJP amplitudes observed at *complexin* mutant NMJs. The fact that *complexin* null mutants display a large enhancement in mini frequency with a concomitant increase in synaptic growth suggests it could be modulated *in vivo* to cause the PKA-dependent rise in mini frequency observed at embryonic NMJs, possibly by being a phosphorylation target of PKA. Complexin is phosphorylated *in vivo* in mammalian systems (Hill, Callaghan et al. 2006; Shata, Saisu et al. 2007), and there are two conserved potential phosphorylation sites in the C terminal portion of *Drosophila* that might be phosphorylated by PKA, T101 and S126. Here, we test the hypotheses that modulation of mini frequency contributes to synaptic plasticity and that complexin is the downstream target of PKA to drive synaptic growth at the larval NMJ.

Synaptotagmin / complexin dynamics during neurotransmitter release

The idea that complexin functions as a vesicle fusion clamp at the nerve terminal is supported both by our results at the *Drosophila* NMJ and by biochemical evidence. Mechanistically, some

biochemical evidence suggests that complexin functions at least in part by preventing full zippering of the SNAREs (Giraudo, Eng et al. 2006; Roggero, De Blas et al. 2007). Additionally, several recent studies have demonstrated that synaptotagmin and complexin function together during the last moments before fusion to trigger synchronous release (Giraudo, Eng et al. 2006; Tang, Maximov et al. 2006; Roggero, De Blas et al. 2007). These studies suggest a model of complexin function in which complexin prevents fusion of vesicles until calcium influx occurs, at which point calcium binding by synaptotagmin allows it to compete with complexin for SNARE binding, creating a switch mechanism during synchronous evoked fusion (Tang, Maximov et al. 2006). Given that synaptotagmin and complexin are both intimately involved in regulating synaptic vesicle exocytosis during the last few moments before fusion, a complete understanding of the process of synaptic vesicle exocytosis inherently requires an understanding of how these two proteins function together in triggering evoked neurotransmitter release.

Two potential models for how complexin and synaptotagmin may function in vesicle exocytosis are shown in **Figure 2**. In the first (left panel in **Figure 2**), complexin functions independently of synaptotagmin, preventing vesicle fusion by its action on the SNARE complex until synaptotagmin binds calcium and triggers fusion. In the second (right panel in **Figure 2**), complexin functions by preventing synaptotagmin from triggering release until calcium influx occurs. There is biochemical evidence supporting both models. For example, *in vitro,* complexin appears to prevent full zippering of SNAREs until synaptotagmin binds calcium, which then releases the block on fusion. This argues for the first model. On the other hand, *in vitro* liposome reconstitution experiments incorporating the SNAREs and v-SNARE-bound synaptotagmin have demonstrated that synaptotagmin can increase the rate of fusion in the

absence of calcium, suggesting that synaptotagmin has some calcium-independent fusogenic activity (Mahal, Sequeira et al. 2002). If this represents *in vivo* activity of synaptotagmin, a mechanism would be required to prevent synaptotagmin from triggering fusion in the absence of calcium, arguing for the second model. The two models are not mutually exclusive and could both occur at the synapse. Here, we begin to investigate the dynamics of complexin-synaptotagmin function in synaptic vesicle exocytosis genetically by making a series of *synaptotagmin 1; complexin* double mutants designed to test the two models.

Experimental Procedures

Drosophila Genetics

Drosophila were cultured on standard medium at 22°C. Putative *complexin* mutants were generated by feeding ethane methylsulfonate (EMS) to Canton S males as described (Lewis and Bacher 1968). These males were crossed to Tm3Sb,Ser/Tm6, a line containing two 3rd chromosome balancers. Individual males or virgin female progeny with the Sb, Ser dominant markers (CS*/Tm3Sb,Ser) were crossed to cpx^{SH1}/Tm3Sb. Chromosomes that failed to complement cpx^{SH1} were recovered to establish CS*/Tm3Sb strains. Individual strains were recrossed to cpx^{SH1} to confirm non-complementation. Dominance or recessiveness of the phenotypes of the EMS mutants was determined by crossing the mutant lines to Canton S and visually inspecting the progeny for the mutant phenotypes. Complexin protein expression in the mutants was determined, where applicable, by Western blots on adult head extracts. Line #2016 contains an uncharacterized null allele of *complexin* and a second-site lethal mutation. Line #572 contains a hypomorphic allele of complexin resulting in reduced protein expression, truncation of the protein at position 114, and point mutation of residues 110-114. For electrophysiology studies of mutant line # 572, recordings were done on *572/Df(3R)ED5021*

larvae. Df(3R)ED5021 is a deletion removing *complexin* that spans the cytological interval 81F6-82A5 (Ryder, Blows et al. 2004). Other mutant lines have not been fully characterized.

For rescue studies, we took advantage of the UAS/GAL4 (Brand and Perrimon 1993) system to selectively drive transgene expression within neurons in the *complexin* null mutant background. Point mutants UAS-complexin 7b T101A, UAS-complexin 7b T101D, and UAS-complexin 7b R64H were produced by site directed mutagenesis of pSH3A, a pUAST vector containing a subcloned *complexin* cDNA encoding complexin 7B. Site directed mutagenesis was carried out using the QuikChange XL Site Directed Mutagenesis kit (Stratagene). For RNAi of complexin and synaptotagmin 1, UAS-cpxRNAi and UAS-syt1RNAi lines were obtained from the Vienna Drosophila RNAi Center (Dietzl, Chen et al. 2007) (http://www.vdrc.at, stock numbers 21477 and 8876, respectively).

syt1 single mutants and *syt1; cpx* double mutants were cultured by crossing flies of desired genotypes and allowing females to lay eggs on grape juice plates (2.2% agar, 1.2% sucrose, 25% grape juice concentrate, 0.2% Tegosept, 0.5% 1X Penicillin/Streptomycin antibiotic mix (Invitrogen catalog number 15070-063)) at 25 °C for 24 hours. 1st instar single or double mutants were transferred to new grape juice plates with yeast paste and kept at 25°C in a humid chamber until the third-instar stage was reached, followed by electrophysiology and immunohistochemistry.

Protein Expression

Complexin 7a and 7b were amplified by PCR from a *Drosophila* cDNA library and subcloned into pGEX-2T and pGEX-5X-1, respectively (GE Healthcare). Recombinant complexin fused

with GST was expressed in *E. coli* (BL21) and purified using glutathione-sepharose 4B (Amersham Biosciences). GST was cleaved from complexin by incubation with thrombin.

In Vitro Kinase Assays

10 µg of complexin fused with GST was incubated with 1 µl PKA (New England Biolabs, 2,500 Units/ µl) in kinase buffer (30 mM HEPES [pH 7.2], 10 mM $MgCl_2$, 5 mM $MnCl_2$, 1 mM DTT) supplemented with 100 µM cold ATP and 5 µCi [^{32}P]γATP (Perkin Elmer) in a final volume of 35 µl for 45 min on ice. The reaction was terminated by the addition of 35 µl of 2× sample buffer. Samples were separated by SDS-PAGE and [^{32}P]γATP incorporation was assessed by autoradiography.

Immunostaining

3rd instar larvae were reared at 22°C and dissected in HL3.1 (Stewart, Atwood et al. 1994) (70 mM NaCl, 5mM KCl, 10 mM $NaHCO_3$, 4mM $MgCl_2 \cdot 6H_2O$, 5 mM Trehalose, 115 mM Sucrose, 5 mM HEPES, pH 7.2). Following washes, larvae were incubated with primary antibody overnight at 4 °C, incubated with secondary antibodies for four hours at room temperature, and mounted in 80% glycerol for imaging. The dilutions of primary antibodies were as follows: complexin (1:500), anti-Dlg (1:1000). The 4F3 antibody against Dlg developed by Corey Goodman was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. Secondary antibodies from Jackson ImmunoResearch Laboratories were used at a dilution of 1:250 and were as follows: Cy2-conjugated Goat anti-Mouse and Rhodamine Red-conjugated Goat anti-Rabbit. Visualization was performed under light microscopy using a 40x oil-immersion lens. Images were taken with confocal microscopy

(Axoplan 2; Carl Zeiss MicroImaging, Inc.) using PASCAL software (Carl Zeiss MicroImaging, Inc.).

SNARE Complex Binding Assays

GST-fused proteins were expressed in BL21 cells and purified using glutathione-sepharose beads (Amersham Biosciences). 5 mL of Canton S flies were frozen in liquid nitrogen and vortexed. Heads were isolated using a sieve, homogenized on ice in homogenate buffer plus protease inhibitors (1X TS, 0.2mM EGTA, 1mM PMSF, 2μg/mL aprotinin, 1μg/mL leupeptin, and 1 μg/mL pepstatin), and solubilized in homogenate buffer with 1.25% Triton X-100 for 30 min. Following solubilization, cell debris and head cuticle were removed by centrifugation in a microcentrifuge at maximum speed for 10 min. 200 μL head extract was then added to each tube containing the desired concentration of beads plus homogenate buffer and 1.25% Triton X-100 and incubated with shaking at 4 °C for two hours. For T101 binding assays, heat shocked samples were incubated at 100 °C for 3 min and Protein Kinase A inhibitor amide 6-22 (EMD Biosciences) was added to samples at a concentration of 68 nM. Beads were washed 5 times in cold 1X TS, boiled in 2X reducing sample buffer, and analyzed by Western blotting.

Western Blots

Western blots were performed using standard laboratory procedures. Blocking of nitrocellulose membranes was performed in a solution containing four parts TBS (10 mM Tris Base pH 7.5, 150 mM NaCl) to one part Odyssey Blocking Buffer (LI-COR Biosciences). Antibody incubations were performed in a solution containing four parts TBST (1X TBS with 1% Tween-20) to one part Odyssey Blocking Buffer. Antiserum dilutions were: complexin, 1:10,000; arginine kinase, 1:10,000; syntaxin, 1:1,000; synaptobrevin, 1:1,000; anti-GST 1:10,000. The probes were detected using Alexa Fluor 680-conjugated goat anti-rabbit IgG at a dilution of

1:3000 (Invitrogen Molecular Probes) and IRDye 800-conjugated goat anti-mouse IgG at a dilution of 1:10,000. Visualization was done using the LI-COR Odyssey Imaging System (LI-COR Biosciences).

Electrophysiology

Electrophysiology at 3rd instar NMJs was performed as previously described (Rieckhof, Yoshihara et al. 2003) at the indicated extracellular calcium concentrations in HL3.1 saline at muscle fiber 6 of segments A3 to A5. Recordings were done using an Axoclamp-2B amplifier (Axon) and digitized with an Instrunet Model 100 digitizer at 10 kHz and analyzed with Superscope 3.0 software (GW Instruments). Mini frequency was quantified in *complexin* and *synaptotagmin* mutants by counting discernable individual peaks.

Electrophysiology Quantification

Genotypes used for physiological analysis shown in **Figure 9** are: control (w^-; *precise excision/precise excision*); cpx (w^-; cpx^{SH1}/cpx^{SH1}); syt 1; cpx (*w-; $syt1^{AD4}/syt1^{N13}$*; cpx^{SH1}/cpx^{SH1}); and syt 1 (*w-; $syt1^{AD4}/syt1^{N13}$*). cpx^{SH1} homozygotes have mini frequencies >20-fold higher than controls and ~10-fold higher than syt 1; cpx double mutants (Student t-Test, ** = P<0.0001). syt 1; cpx mutants have a mean mini frequency significantly higher than controls (Student t-test, ** = P<0.0001). Data used in the analysis include: Control (Mini Frequency (MF)=3.14±0.3 Hz; n=18); cpx^{SH1} (MF=66.9±4.8 Hz; n=27); syt 1; cpx (MF=6.57±0.44 Hz, n=7); syt 1 (MF=3.98±0.35 Hz, n=9). Genotypes used for physiological analysis shown in **Figure 11** are: control (w^-; *precise excision/precise excision*); cpx (w^-; cpx^{SH1}/cpx^{SH1}); and 572/Df (*572/Df3R5021*). Data used in the analysis include: Control (Mini Frequency (MF)=3.14±0.3 Hz; n=18); cpx^{SH1} (MF=66.9±4.8 Hz; n=27); 572/Df (MF=79.75±2.69, n=6).

Morphological Quantification

To quantify NMJ bouton number, anti-complexin or anti-HRP stained varicosities were counted at muscle fiber 6/7 of segments A3 and A4. For *syx*$^{3\text{-}69}$, the number of cpx-positive varicosities was statistically increased compared to controls at muscle fiber 6/7 of segment A3 (Student t-Test, ** = P=0.0004). Data used in the varicosity number analysis include: Control (Canton S) (bouton # =71.44±4.45, n=9); *syx*$^{3\text{-}69}$ (bouton # =128.8±11.8, n=10). For *para*^{ts1}*; cpx*^{SH1} double mutant analysis, the number of HRP-stained boutons in *cpx*^{SH1} was statistically increased compared to *precise excision* controls and *para*^{ts1}*; cpx*^{SH1} double mutants at m6/7 segments A3 and A4 (Student t-Test, * = P <.05, ** = P < .001). Data used in the *para*^{ts1}*; cpx*^{SH1} double mutant analysis include: Precise excision (m6/7 segment A3 bouton # = 64.125±3.36, n=8; m6/7 segment A4 bouton # = 44.36±3.0, n=13), *cpx*^{SH1} (m6/7 segment A3 bouton # = 103.5±15.98, n=4; m6/7 segment A4 bouton # = 83.0±8.66, n=6), *para*^{ts1}*; cpx*^{SH1} (m6/7 segment A3 bouton # = 73.5±8.75, n=6; m6/7 segment A4 bouton # = 56.9±4.64, n=9).

Adult Locomotion Analysis

Adult climbing assays were performed on flies aged 6-7 days as previously described (Wang, Saraswati et al. 2004). Climbing assays were done on flies aged 6-7 days, and the time required to climb 5 cm in a plastic vial was recorded, with a cutoff of 15 min.

Results

Enhanced mini frequency in a temperature sensitive allele of syntaxin drives synaptic overgrowth

To investigate the role that mini frequency plays in synaptic plasticity, we took advantage of a temperature sensitive allele of syntaxin, *syx*$^{3\text{-}69}$, that was previously identified in a screen for temperature sensitive paralytic mutants (Littleton, Chapman et al. 1998). This mutant is the only other *Drosophila* mutant known to display a greatly enhanced mini frequency on the same scale

as *complexin* mutants (**Figure 3a**), with a mini frequency approximately 7-fold higher than wild type flies (Lagow, Bao et al. 2007). If enhanced mini frequency can drive synaptic growth, one would expect that, like *complexin* mutants, $syx^{3\text{-}69}$ mutants would display enhanced synaptic growth. Indeed, bouton counting at $syx^{3\text{-}69}$ larval NMJs shows that there is a large and significant increase in the number of varicosities over wild type controls (**Figure 3b**). Adult $syx^{3\text{-}69}$ flies have normal evoked potentials (Lagow, Bao et al. 2007), eliminating the possibility of compensatory effects causing enhanced synaptic overgrowth that could potentially confound the interpretation of the overgrowth in *complexin* mutants.

The enhanced mini frequency phenotype observed in $syx^{3\text{-}69}$ mutants led us to hypothesize that the mutation in these mutants impairs the ability of complexin to bind syntaxin, producing a phenotype similar to that of *complexin* mutants. $syx^{3\text{-}69}$ mutants contain a T254I point mutation that reportedly alters SNARE complex formation *in vitro*. Molecular modeling suggests that this alteration is caused by a stronger zippering reaction in the mutant, with subsequent enhancement in mini frequency. Analysis of the three dimensional structure of the squid complexin-SNARE complex (**Figure 3c**) (Bracher, Kadlec et al. 2002) shows that T254 in syntaxin is located in the C-terminal portion of the SNARE domain of syntaxin, placing it in close proximity to the transmembrane domains of the SNAREs during the fusion reaction. Further analysis has shown that this residue is on a hydrophobic surface of syntaxin in the +7 layer (Lagow, Bao et al. 2007). The closest side chain in proximity to T254 is K79 of SNAP-25, and their Van der Waals surfaces are close enough to suggest that these two residues bind during SNARE complex formation (**Figure 3c**). This does not explain why the T254I mutation would enhance SNARE zippering, since the putative binding partner of T254 is a charged lysine residue. In any case, T254 is far from the binding site of complexin with the SNARE complex, although this does not

necessarily preclude an effect on complexin binding, which could occur indirectly through changes in the coiled-coil interactions of the SNARE complex or through interactions with complexin outside the fragment of complexin that was crystallized.

To test whether syx^{3-69} alters binding of complexin with syntaxin, we used recombinant, GST-fused wild type syntaxin and mutant syx^{3-69} to perform binding assays with native complexin from adult fly head protein extracts (**Figure 3d**). Protein extracts were incubated with GST-fused syntaxins bound to glutathione beads over a range of GST-syntaxin protein concentrations from 0.0125 mg/mL to .4 mg/mL. No major changes in the complexin binding affinity of syx^{3-69} were observed, suggesting that the syx^{3-69} T254I mutation does not alter the complexin – syntaxin binding affinity. However, this assay does not test binding of complexin with the assembled SNARE complex, it only tests binding of monomeric syntaxin, potentially confounding interpretation of the results. To definitively determine whether complexin's SNARE complex binding affinity is altered in syx^{3-69} mutants, the best experiment would be to perform the head extract binding assay using recombinant complexin to bind assembled SNARE complexes in the fly CNS. This would test binding of assembled SNARE complexes as opposed to monomeric syntaxin. Based on these results, it is unlikely that the large enhancement in mini frequency in syx^{3-69} is due to a change in syntaxin's complexin binding affinity. However, the large increase in the number of varicosities at syx^{3-69} NMJs strongly supports the idea that minis can drive synaptic growth.

Enhancement of synaptic growth in *complexin* mutants is suppressed by decreased neuronal activity

In order to determine how evoked potentials and minis might interact at the NMJ to regulate synaptic morphology and synaptic growth, we crossed the cpx^{SH1} allele with a temperature sensitive allele of the major sodium channel in the nervous system, $para^{TS1}$ (Loughney, Kreber et

al. 1989), to make a double mutant. *para*^{TS1} mutants paralyze and action potentials cease at the restrictive temperature of 37 °C (Wu and Ganetzky 1980; Ganetzky 1984). *para*^{TS1}*;cpx*^{SH1} double mutants display similar behavioral defects to *complexin* null mutants alone. Interestingly, even when larvae are grown at the permissive temperature of 25 °C, *para*^{TS1} suppresses the synaptic overgrowth phenotype of *cpx*^{SH1} mutants (**Figure 4**). This effect is observed at two different body wall muscle segments (6/7 A3 and A4). While the average number of boutons at *para*^{TS1}*;cpx*^{SH1} double mutant synapses is higher on average than *precise excision* controls, this difference is not significant. Interpretation of these results would benefit from two improvements to the experiment. First, varicosity number can depend to some extent on the genetic background of the animals, and the *precise excision* control is in a *white-* background, while the two mutant lines are in a *white+* background. For proper comparison, especially comparison of the double mutant to the control, the precise excision control should be put into the same red-eyed background that the mutants are in. Secondly, it is unclear from this experiment whether the overgrowth phenotype of *complexin* mutants is completely suppressed by *para*^{TS1} or whether there is still some overgrowth. To determine this, bouton counts of *para*^{TS1} larvae grown at 25 °C should be completed. Thus, preliminary experiments demonstrate that decreased neuronal activity suppresses the synaptic overgrowth phenotype observed in *complexin* mutants, but the extent of this effect is currently unclear and requires further experiments.

The role of complexin phosphorylation in regulating mini frequency and synaptic growth

Studies at *Drosophila* embryonic NMJs have revealed that an activity-dependent signaling cascade requiring postsynaptic synaptotagmin 4 and presynaptic PKA function regulates synaptic growth and mini frequency (Yoshihara, Adolfsen et al. 2005) (**Figure 1**). In response to high

frequency stimulation, mini frequency at embryonic NMJs is increased by over 100-fold, and the pathway required for this upregulation is also required for normal synaptic plasticity. Given that *complexin* null mutants display large increases in both mini frequency and synaptic proliferation, and given that there are several predicted phosphorylation sites in the C terminus of complexin, we hypothesized that complexin is phosphorylated by PKA in this pathway to mediate the increase in mini frequency (**Figure 5a**). According to our hypothesis, prior to high frequency stimulation (left panel in **Figure 5a**), complexin is bound to SNARE complexes and fully functional, producing a low mini frequency state. During high frequency stimulation (middle panel), complexin is phosphorylated by PKA in the presynaptic terminal in response to a synaptotagmin-4–dependent retrograde signal from the muscle. Because of this phosphorylation, complexin's vesicle fusion clamp function is impaired, possibly because of decreased binding affinity for the SNARE complex. This would produce a high mini frequency state (right panel).

In order to test this hypothesis, we first wanted to determine whether complexin can be phosphorylated *in vitro* by PKA, and if so, at which sites. One attractive candidate site for PKA phosphorylation is T101 (orange box in **Figure 5b**). T101 is the third residue in a four-residue consensus PKA phosphorylation motif, KKTP, that is 100% conserved in all invertebrate complexins examined so far and is present in all isoforms of *Drosophila* complexin. To determine whether this residue is phosphorylated by PKA *in vitro*, we mutated T101 in complexin 7b to a phospho-incompetent alanine residue, and performed kinase assays on GST-tagged recombinant mutant and wild type complexins (**Figure 5c**). Expressed recombinant proteins were incubated with PKA and ATP-γ-^{32}P, reactions were stopped with sample buffer, and proteins were separated by SDS-PAGE. Phosphorylation of complexin was detected by autoradiography. Phosphorylation of complexin was observed, whereas no phosphorylation

could be detected in GST alone controls or samples with no enzyme added. A positive control reaction with the kinase Cdk5 was successful, although it did not produce phosphorylated protein at the high level that the PKA / complexin reaction did. Thus, complexin 7b is highly phosphorylated *in vitro* by PKA. However, the T101A mutant was also highly phosphorylated by PKA, demonstrating that, at least *in vitro,* T101 is not the site of phosphorylation by PKA.

To determine whether phosphorylation of the T101 site might change complexin's SNARE complex binding affinity, we performed SNARE binding assays using recombinant wild type complexin 7b and two point mutants, complexin T101A (phospho-incompetent), and complexin T101D (phospho-mimetic) (**Figure 5d**). A third point mutant, complexin R64H, had previously been reported to be unable to bind SNARE complexes in mammalian systems (Archer, Graham et al. 2002) and was therefore used as a negative control for SNARE binding. To detect SNARE binding, recombinant proteins fused to GST and bound to glutathione beads were incubated with adult fly head protein extracts. To detect both monomeric syntaxin binding and SNARE complex binding, recombinant proteins were also incubated with head extracts that had been heat-shocked to melt SNARE complexes into their monomeric components. Under these conditions, complexin should only detectably bind syntaxin since that is the only monomeric binding partner it interacts with. Finally, to control for the possibility that PKA in the head extracts might phosphorylate wild type complexin or the complexin point mutants during the incubation step, a PKA inhibitor cocktail was added to head extracts to determine whether blocking phosphorylation of complexin by PKA in the test tube might cause a detectable change in SNARE complex binding affinity. Under all conditions tested, there was no decrease in binding affinity of the T101A and T101D point mutants for syntaxin, and if anything both showed a slight increase in binding affinity (bottom blot, **Figure 5d**). A GST alone control did

not detectably bind syntaxin, and as predicted the R64H mutant did not bind syntaxin either. No detectable synaptobrevin was bound under any conditions, so the binding assay failed to detect SNARE complex binding and instead measured only monomeric syntaxin binding. It is unclear why complexin failed to bind assembled SNARE complexes in protein extracts because SNARE complexes are remarkably stable. They are resistant to proteolysis and are highly thermally stable (Hayashi, McMahon et al. 1994). Still, a negative control with very weak or nonexistent SNARE complex binding did not bind any monomeric syntaxin, demonstrating that a change in binding affinity for the SNARE complex will in all probability be reflected in a change in binding affinity for monomeric syntaxin. Thus, it is likely based on these results that it is not T101 that is phosphorylated by PKA. Consistent with this conclusion is the fact that pan-neuronal expression of UAS-T101A and UAS-T101D point mutants in the *complexin* null mutant background fully rescues the lethality and behavioral defects of *complexin* null mutants.

Our results thus far show that complexin 7b is highly phosphorylated by PKA *in vitro* but not at the T101 position. In addition to T101, S126 in complexin 7b is predicted to be phosphorylated by PKA. We therefore performed a PKA phosphorylation assay using wild type complexin 7b and an S126A point mutant (**Figure 6a**). This mutation removed nearly all of the phosphorylation of complexin 7b, demonstrating that S126 is the major phosphorylation target of PKA *in vitro*.

The S126 residue is only present in complexin 7b and is not present in complexin 7a or any of the other splice isoforms of complexin. We therefore performed a kinase assay to determine whether complexin 7a is phosphorylated by PKA (**Figure 6b**). We observed a small amount of phosphorylation of complexin 7a, but not nearly to the extent that complexin 7b is phosphorylated. In fact, complexin 7a phosphorylation occurs at about the same level as the

complexin 7b S126A mutant, suggesting that complexin 7a lacks a major PKA phosphorylation site.

In addition to PKA, complexin is also predicted to be phosphorylated by PKC at T101. We therefore tested whether it is phosphorylated by PKC *in vitro* (**Figure 6c**). Phosphorylation of both complexin 7a and 7b by PKC occurred at very low levels and this phosphorylation was not eliminated by mutation of T101 to alanine, so this site is not phosphorylated by PKC *in vitro*. Thus, the only major phosphorylation target in complexin detected thus far is S126, which is highly phosphorylated by PKA *in vitro*. Further studies will determine whether phosphorylation of this site occurs *in vivo* and whether phosphorylation is required for establishing and storing memories in *Drosophila* or for normal synaptic development.

R64H point mutants in complexin rescue behavioral defects in *complexin* null mutants

We generated a point mutant in complexin 7b, R64H, that in mammalian complexins has been reported to be deficient in SNARE binding (Archer, Graham et al. 2002) and does not detectably bind syntaxin in a head extract binding assay (**Figure 5d**). Point mutants in complexin lacking SNARE binding activity have been used to demonstrate that the proposed functions of complexin require SNARE binding. These experiments have established that any effects of genetic or biochemical manipulations in experiments are specific to complexin's normal *in vivo* function via SNARE binding and are not non-specific artifacts of the experimental system (Archer, Graham et al. 2002; Xue, Reim et al. 2007; Giraudo, Garcia-Diaz et al. 2008; Yoon, Lu et al. 2008). Following this line of reasoning, we decided to test whether the R64H mutant can rescue the behavioral defects observed in *complexin* mutants. R64 in *Drosophila* complexin corresponds to R59 in mammalian complexins and is a 100% conserved residue from invertebrates to vertebrates located in the central alpha helix of complexin responsible for

SNARE binding (orange box in **Figure 7a**). It interacts via hydrogen bonds with syntaxin residue N57 to mediate binding to the SNARE complex (**Figure 7b**). In order to determine whether the R64H point mutant is capable of rescuing the behavioral defect in *complexin* null mutants, we expressed a wild type complexin transgene and two different R64H point mutant transgenes (R64H 2C and R64H 3A) pan-neuronally in the null mutant background (**Figure 7c**). Western blotting showed that the rescuing transgenes are expressed in adult fly heads at levels equal to or greater than a driver alone control (C155) or a *precise excision* control.

To our surprise, R64H point mutants rescued the behavioral defect to a large degree. This rescue was quantified in a wall climbing assay (**Figure 7d**). *Precise excision* flies climb the wall of a vial quickly and with very little variation in the length of time required (2.0 ± 0.174 sec.) In comparison, *complexin* null mutants can take up to 15 minutes to climb a vial and are highly variable in how quickly they climb (405 ± 97.8 sec.) Complexin 7b largely rescues the null motor defect, but complexin 7b rescue animals still take significantly longer to climb the vial and display greater variability than *precise excision* flies (5.43 ± 0.53 sec., $P < 0.0000005$ compared to *precise excision*, $P < 0.000005$ compared to *complexin* null mutants). This is not surprising given that there are several predicted complexin splice variants differing in both protein sequence and 5' UTRs, so rescuing with one variant pan-neuronally likely does not fully reproduce the expression pattern of complexin in the nervous system. While R64H rescues did take nearly three times longer on average than wild type rescues to climb the vial (14.33 ± 0.667 and 14.83 ± 3.40 sec., $P < 0.005$), indicating some function is indeed lost in this point mutant, complexin function for the most part remains when climbing capability is compared with null mutants. Recently it has been reported that R59 point mutants in mammalian complexins retain some SNARE binding capability (Xue, Reim et al. 2007). To completely abolish SNARE

binding, several pairs of double point mutants can be employed, all of which alter highly conserved residues in the central alpha helix of complexin responsible for SNARE binding (Xue, Reim et al. 2007). The mostly likely explanation for our results is that, like in mammalian complexins, R64H mutants in *Drosophila* retain some SNARE binding ability, although it was not detected in our binding assay. For this reason, we are generating double point mutants in complexin that completely eliminate SNARE binding in order to more definitively demonstrate that complexin's native function *in vivo* is as a vesicle fusion clamp for neurotransmitter release.

Double mutant analysis of complexin / synaptotagmin 1 dynamics during neurotransmitter release

Synaptotagmin 1 and complexin both function during the moments before neurotransmitter release to synchronize calcium-triggered exocytosis. A complete understanding of the molecular events leading up to neurotransmitter release will require an understanding of how these two proteins work together during synchronous release. We propose two possible models for how complexin may function as a vesicle fusion clamp (**Figure 8a**). One possibility is that complexin prevents fusion of synaptic vesicles by directly inhibiting the fusion activity of the SNARE complex (left panel). This activity would be independent of synaptotagmin function. The second possibility is that complexin functions by preventing synaptotagmin from triggering vesicle fusion in the absence of calcium (right panel). In both cases, synaptotagmin triggers fusion after calcium influx by removing complexin from the SNARE complex. These two models might be distinguished by testing the properties of a double mutant of *synaptotagmin* and *complexin*. In the first model, the enhanced mini frequency observed in complexin mutants would persist in a *complexin; synaptotagmin* double mutant. In the second model, the enhanced mini frequency would depend on the presence of synaptotagmin and would be eliminated in a *complexin; synaptotagmin* double mutant. In order to test these two models, we made a series of

complexin; synaptotagmin double mutants utilizing a variety of *synaptotagmin* alleles (summarized in **Figure 8b** and diagrammed in **Figure 8c**). In the double null mutant, homozygous *cpx*^{SH1} is crossed with two different null alleles of *synaptotagmin*, *syt1*^{N13} (Littleton, Stern et al. 1994) and *syt1*^{AD4} (DiAntonio, Parfitt et al. 1993; DiAntonio and Schwarz 1994). In addition, the *complexin* null allele was crossed with several different types of *synaptotagmin* hypomorphic alleles. s*yt1*^{T41} has a wild type protein sequence but very low expression of synaptotagmin (Littleton, Stern et al. 1993). *syt1*^{AD1} contains a stop codon between the two C2 domains of synaptotagmin that completely removes the C2B domain (DiAntonio and Schwarz 1994) and eliminates the fourth-order cooperativity of synchronous release (Yoshihara and Littleton 2002). *syt1*^{AD3} contains a Y364N point mutation that disrupts calcium-dependent conformation changes of synaptotagmin and displays very low amplitude EJPs but leaves calcium cooperativity intact (DiAntonio and Schwarz 1994; Yoshihara and Littleton 2002). In addition to using these well characterized alleles of *synaptotagmin* and *complexin*, we also employed fly lines containing transgenic RNAi expression constructs against both *synaptotagmin* and *complexin*. By using these different alleles, we hope to gain mechanistic insight into how complexin and synaptotagmin function together in neurotransmitter release.

Although *synaptotagmin 1* single mutants under normal growth conditions die during or before the first-instar stage, they can be grown to the third-instar stage for electrophysiological recordings fairly easily by careful culturing on grape juice-agar medium. We used the same culturing technique to attempt to grow *synaptotagmin; complexin* double mutants to the third-instar stage for analysis. Of the different double mutant stocks generated, we have thus far attempted to culture three combinations to third-instar stage for electrophysiology experiments: *syt1*^{N13}/*syt1*^{AD4}; *cpx*^{SH1}/*cpx*^{SH1} (double null), *syt1*^{T41}/*syt1*^{AD1}; *cpx*^{SH1}/*cpx*^{SH1} (*synaptotagmin*

hypomorphic alleles with *complexin* null allele), and *C155, UAS-dicer;UAS-syt1RNAi/+; cpx^{SH1}/cpx^{SH1}* (*synaptotagmin* RNAi expressed pan-neuronally with *complexin* null allele). We found that even when these double mutants were cultured on grape juice plates, the vast majority of animals died before reaching the third-instar stage. This was true for all three genotypes tried. In only one case have we been able to successfully grow a double mutant to third instar stage. Staining this double null mutant for complexin and Dlg confirmed it was a *complexin* mutant (**Figure 9a**). The motor behavior of the larva (uncoordination and body wall contraction waves lasting three to four seconds) was very similar to that of *synaptotagmin 1* single mutants. In addition, evoked responses from the double mutant showed no synchronous release with highly elevated asynchronous release, typical of *synaptotagmin 1* null mutants (Yoshihara and Littleton 2002) (**Figure 9b**, left panel). Finally, the double mutant displayed a high degree of paired pulse facilitation, again typical of a *synaptotagmin 1* null mutant (**Figure 9b**, right panel) (Saraswati, Adolfsen et al. 2007). Thus, all indications are that this larva was in fact a double mutant.

Recordings of minis from the double mutant revealed an almost complete suppression of the mini frequency phenotype observed in *complexin* null mutants alone (**Figure 9c**). *Complexin* null mutants have an average mini frequency of 66.9 Hz, while the double mutant had an average mini frequency of 6.6 Hz. This is still significantly elevated compared to the control frequency of 3.1 Hz. This may be because *syt 1* mutants alone have been reported to have an elevated mini frequency (Littleton, Stern et al. 1993; Broadie, Bellen et al. 1994; DiAntonio and Schwarz 1994), although in our hands during these experiments *syt 1* null mutants alone did not show a significantly elevated mini frequency, or because complexin has some synaptotagmin-independent function (i.e. the first model of complexin function in **Figure 8a**). This evidence argues that complexin functions in neurotransmitter release in part to block synaptotagmin from

triggering fusion in the absence of calcium influx. Without complexin present at the synapse, synaptotagmin's basal calcium-independent function may drive fusion of primed vesicles without restriction, producing the enhanced mini frequency phenotype observed at *complexin* null mutant synapses. Confirmation of this suppression effect awaits further experimental results from more double mutant animals.

An EMS screen to identify new alleles of complexin

To elucidate the *in vivo* function of a given protein, random mutation of that protein has often proved useful. An important example of this is synaptotagmin, whose function as a calcium sensor for neurotransmitter release was in part established using alleles of synaptotagmin generated by random EMS mutagenesis (DiAntonio, Parfitt et al. 1993; Littleton, Stern et al. 1993; Broadie, Bellen et al. 1994; DiAntonio and Schwarz 1994; Littleton, Stern et al. 1994). Complexin function has been investigated in depth by generating null alleles (Reim, Mansour et al. 2001; Xue, Stradomska et al. 2008), targeted mutation of residues in the binding site of complexin (Xue, Reim et al. 2007), and deletion analysis (Xue, Reim et al. 2007). However, unbiased, random mutation of *complexin* has so far not been utilized to study complexin's *in vivo* function. To this end, we generated new *complexin* alleles by mutation of wild type flies with the chemical mutagen ethane methyl sulfonate (EMS). Nearly 5,000 mutagenized lines were screened for loss-of-function *complexin* alleles by non-complementation of the cpx^{SH1} lethality and motor defect phenotypes (**Figure 10a**). Lines of flies failing to fully complement cpx^{SH1} were established and subjected to verification of the phenotype observed in primary screening. Phenotypes of the mutants identified are shown in (**Figure 10b**). Sequencing and Western blotting of the mutants is ongoing, but three mutants immediately stood out as potential *complexin* mutants. Line #570 has a motor defect reminiscent of *complexin* null mutants, both as

a homozygous stock and heterozygous with *cpx*^{SH1}. 570 flies move slowly, tend to remain on the bottom of the vial (bottom-dwelling), and are slightly uncoordinated. The 570 phenotype is semi-dominant, with a less dramatic phenotype observable when heterozygous with wild type. Thus far sequencing of 570 has not revealed a mutation in *complexin*, but complexin is expressed at wild type levels in 570 (**Figure 11a**, left panel), so if a *complexin* mutation is found in 570 mutants, it is likely to be in the coding sequence.

Line #2016 has a motor and lethality defect identical to *complexin* null mutants, and 2016/*cpx*^{SH1} flies show no expression of complexin by Western blot (**Figure 11a**, right panel), indicating that line #2016 contains a new null allele of *complexin*. However, 2016 is homozygous lethal and is also lethal when heterozygous with a deficiency uncovering *complexin*. In both cases, lethality occurs at a stage prior to the wandering third-instar larval stage. This lethality is not consistent with the *complexin* null phenotype, which is semi-lethal with most animals dying during the pupal stage. While EMS mutagenesis for the most part produces point mutations, it can also cause deletions. We therefore suspected that there might be a large deletion in 2016 that removes a part of complexin and a neighboring gene, thus causing the lethality phenotype. To investigate whether there is a deletion in *complexin* in line 2016, a mapping PCR was performed on genomic DNA isolated from 2016/*cpx*^{SH1} adults. PCR products 1-6 from the PCR originally performed to map the *cpx*^{SH1} deletion were successfully polymerized from 2016/ *cpx*^{SH1} gDNA (**Figure 11b**). Since products two through four cannot be polymerized from *cpx*^{SH1} gDNA, we know from this experiment that 2016 at least does not contain a large deletion in the region of the genomic DNA corresponding to PCR products two through four, which includes all of the coding sequence for complexin except the 7b exon. Thus, rather than 2016 containing a large deletion in *complexin* and a neighboring gene, it is more likely that it

contains both a mutation in *complexin* resulting in loss of expression of the complexin protein and a second site mutation in a necessary gene uncovered by the deficiency. If this mutant is to be pursued, it should be backcrossed to wild type to remove the second site lethal.

Line #572 shows a motor defect very similar to the *complexin* null mutant phenotype but less severe. The animals move more quickly and are more coordinated than *complexin* null mutants. This phenotype is recessive to wild type and the phenotypes of 572 homozygotes and 572/cpx^{SH1} are identical. The 572 allele shows no evidence of the lethality observed in *complexin* null mutants. Western analysis of 572 head extracts showed that line 572 expresses a truncated form of complexin that has very low expression (**Figure 11a**, middle panel), making it very likely that line 572 contains a mutation in the *complexin* coding sequence. Indeed, sequencing of 572 *complexin* revealed a small deletion removing the last 27 nucleotides of exon 5 as well as 108 nucleotides in the adjacent intron, plus an insertion of 6 nucleotides in this same location. Presumably this deletion prevents splicing of the intron between exons 5 and 6, resulting in read-through into the intron during translation. If this is the case, then the resulting mutant protein would have amino acids 110 to 114 mutated from the wild type sequence EQEEL to VYSIL, followed by a stop codon, truncating the mutated protein to a sequence 28 amino acids shorter than the wild type protein (**Figure 11c**). Reduced expression of the mutated protein was confirmed by immunohistochemistry at the larval NMJ (**Figure 11d**), with complexin staining much dimmer at the mutant synapse. Imaging of the mutant synapse with high laser power to brighten the image revealed that the remaining complexin at the synapse is distributed in puncta, rather than being diffusely distributed in the bouton as wild type complexin is. Complexin staining in *complexin* null mutants frequently shows background staining in the axon

but never shows residual staining in NMJ boutons, so the axonal staining in the higher laser power image of the 572 mutant NMJ is probably background staining.

The phenotype of line 572 and its reduced complexin expression demonstrate that this line contains a hypomorphic allele of *complexin*. Consistent with this conclusion, recordings of minis from larval NMJs of 572 mutants reveal an extremely high mini frequency, just as is observed in *complexin* null mutants. It is impossible to separate the effects of reduced expression of the mutant protein and truncation of the protein in 572 mutants, so this allele will likely not be useful for structure-function studies of complexin. However, it may be useful for genetic studies where an allele of *complexin* less severe than the null is needed. Indeed, given the difficulty in obtaining *syt 1;cpx* double null mutants, it may be more productive to attempt to culture *syt 1 null;* cpx^{572} double mutants than to continue trying to culture a double null mutant. In addition to the three EMS mutants described, further analysis of the remaining mutants may reveal new alleles of complexin useful for structure-function studies.

Discussion

Regulation of mini frequency and synaptic plasticity by complexin

Since the time of Katz, it has been known that individual quanta occasionally release from the synaptic terminal in the absence of an action potential, producing miniature postsynaptic potentials, or minis. Katz noted that mini frequency at the frog NMJ can vary over 1000-fold (Fatt and Katz 1952), but until recently it has been thought that minis represent background noise at the synapse. Recent evidence, however, suggests that mini frequency is used as information at the synapse to maintain dendritic spine density (McKinney, Capogna et al. 1999), to suppress dendritic protein synthesis (Sutton, Wall et al. 2004), and possibly to alter spike frequency of pyramidal neurons in the hippocampus (Sharma and Vijayaraghavan 2003). In *Drosophila*, mini

frequency is upregulated by over 100-fold for several minutes after high frequency stimulation (Yoshihara, Adolfsen et al. 2005). This upregulation requires function of a PKA- and synaptotagmin-4- dependent pathway that also drives synaptic growth. Based on the synaptic growth phenotype of *complexin* null mutants, we hypothesized that enhanced mini frequency can drive synaptic growth and that complexin is the downstream target of PKA that regulates mini frequency in this pathway.

Consistent with the idea that enhanced mini frequency drives synaptic growth, *syntaxin*$^{3-69}$ mutants, which have an elevated mini frequency similar to that observed in *complexin* null mutants, display synaptic overgrowth. We have identified a PKA phosphorylation site in complexin 7b, S126, that is highly phosphorylated by PKA *in vitro*. In order to determine whether phosphorylation of this residue impacts synaptic growth *in vivo*, we plan to make transgenic S126A and S126D point mutants and test whether they rescue the synaptic overgrowth phenotype observed in *complexin* null mutants. If S126 phosphorylation drives synaptic growth, then S126A would be predicted to suppress the *complexin* null overgrowth phenotype, while S126D (a phosphomimetic mutant) would not be predicted to rescue, and would even be predicted to drive overgrowth when overexpressed.

Based on the fact that there are no expressed sequence tags (ESTs) that correspond to the unique exon in complexin 7b, complexin 7b is not highly expressed in adult flies or larval imaginal discs (tissues that most of the ESTs from *Drosophila melanogaster* were generated from). It is possible that complexin 7b is expressed in small subsets of cells in the adult, or that it is only expressed at specific times during development. This suggests that if complexin 7b were responsible for regulating mini frequency in the PKA-dependent plasticity model, this type of plasticity would not be active at all synapses, but instead would be restricted to specific cells

or specific times in development. Aside from this explanation it is possible that the *in vitro* phosphorylation assays do not accurately reproduce what is happening *in vivo,* or that complexin is downstream of PKA in the pathway and regulates mini frequency, but complexin is not directly phosphorylated by PKA. One way to test whether complexin is downstream of synaptotagmin 4 and PKA in the plasticity pathway is to make double mutants of *complexin* and the other components of the pathway for epistasis tests. Experiments of this nature are ongoing, but our preliminary evidence suggests that mini frequency can contribute to synaptic plasticity, potentially unveiling a new method for information storage in the nervous system.

Regulation of neurotransmitter release by synaptotagmin 1 and complexin

Because complexin and synaptotagmin 1 function just before and during the calcium-triggering step of synchronous neurotransmitter release, a complete understanding of the molecular events of synaptic vesicle exocytosis requires knowledge of how these two proteins work together. Most studies thus far have examined function of the two proteins as separate entities. We propose two possible pathways, based on available biochemical evidence, by which complexin might function as a vesicle fusion clamp to synchronize vesicle exocytosis in conjunction with synaptotagmin (**Figure 2**). In the first model, complexin blocks fusion by preventing vesicles themselves from fusing, while in the second complexin blocks fusion by preventing synaptotagmin from triggering fusion in the absence of calcium. The second model requires that synaptotagmin have the ability to trigger fusion without calcium influx, and indeed there is some evidence to support this (Mahal, Sequeira et al. 2002). In order to test our two models, we have made a series of *synaptotagmin 1; complexin* double mutants and have begun to attempt to culture them for electrophysiological studies. Preliminary evidence shows that *synaptotagmin* null alleles suppress the enhanced mini frequency phenotype of the *complexin* null allele. This

result would support the second model of complexin function, in which complexin prevents synaptotagmin from triggering fusion in the absence of calcium. However, this result must be thoroughly verified before a firm conclusion can be made.

Unfortunately, the *synaptotagmin; complexin* double mutant combination is highly lethal and it has therefore been difficult to culture double mutants to the third-instar stage. However, there are several more allele combinations that we have not yet tried to culture and that may produce more viable larvae. If the remaining double mutants are lethal as well, there are several potential routes around this problem. If the elevated mini frequency observed in *complexin* mutants is dependent on synaptotagmin function, it is possible that larvae homozygous for the *complexin* null allele and heterozygous for a *synaptotagmin* null allele would show decreased mini frequency. These larvae could be cultured with relative ease but may only show a modest decrease in mini frequency that may not be detectable because of the difficulty in accurately quantifying mini frequency in *complexin* null mutants. A second possibility is to drive synaptotagmin and complexin RNAi constructs only in motor neurons instead of pan-neuronally. This may enhance viability while still allowing study of the double mutant phenotype at NMJs. Finally, *synaptotagmin 1; cpx^{572}* double mutants may be more viable than *synaptotagmin 1; cpx^{SH1}* double mutants given that there is very little lethality associated with the *cpx^{572}* allele. Should any of these approaches work, these double mutant studies have the potential to reveal fundamental aspects of how synaptotagmin and complexin function together at the synapse to trigger synchronous fusion of synaptic vesicles.

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Figures

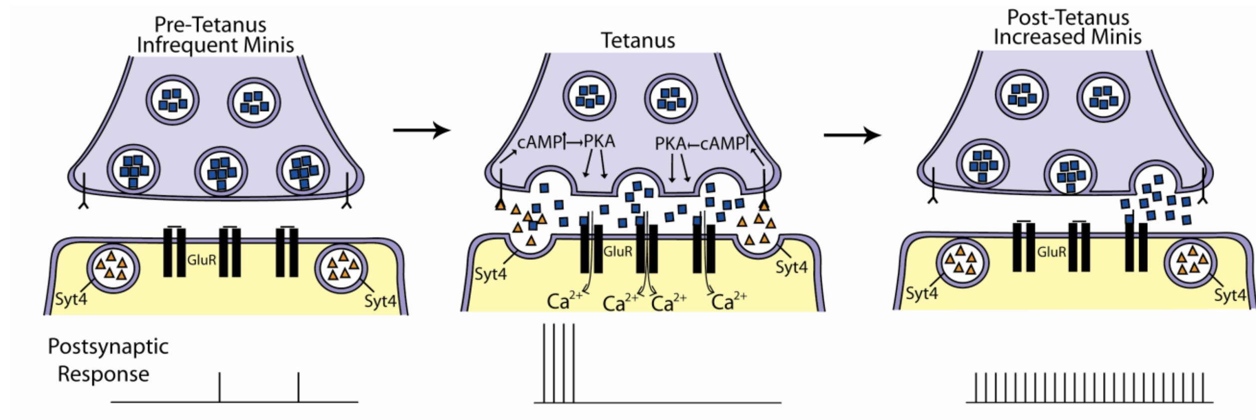


Figure 1. Model of regulation of mini frequency by a synaptotagmin-4- and PKA-dependent signaling cascade adapted from Yoshihara et al, *Science* 2005. Before tetanic stimulation, the synapse is in a low mini frequency state. During tetanic stimulation, calcium influx into the post-synaptic compartment (yellow) triggers synaptotagmin-4- (Syt4)-dependent release of postsynaptic vesicles containing an unknown retrograde signal (orange triangles) into the synaptic cleft. The retrograde signaling protein is bound by presynaptic receptors, triggering a presynaptic increase in cyclic AMP concentration and activating PKA. PKA then acts on downstream effectors to increase mini frequency and synaptic growth. For several minutes after tetanic stimulation a high-mini-frequency state persists. Blue Squares = Neurotransmitter.

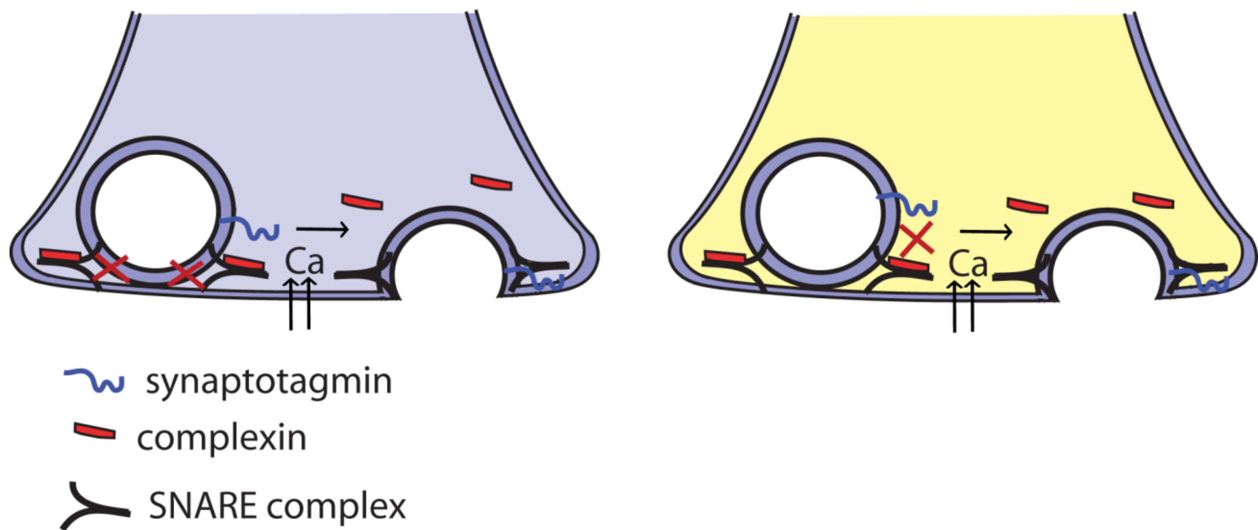


Figure 2. Two models of complexin's fusion clamp function at the synapse. In model 1 (left panel), complexin (red rectangle) prevents vesicle fusion by blocking full SNARE zippering. After calcium influx synaptotagmin (blue line) removes complexin from the SNARE complex and triggers vesicle fusion. In model 2 (right panel), complexin blocks fusion by preventing synaptotagmin from triggering release prior to calcium influx. After calcium influx, synaptotagmin removes complexin from the SNARE complex and triggers fusion. The two different models are not necessarily mutually exclusive and may be distinguished by an electrophysiological analysis of *synaptotagmin 1; complexin* double mutants.

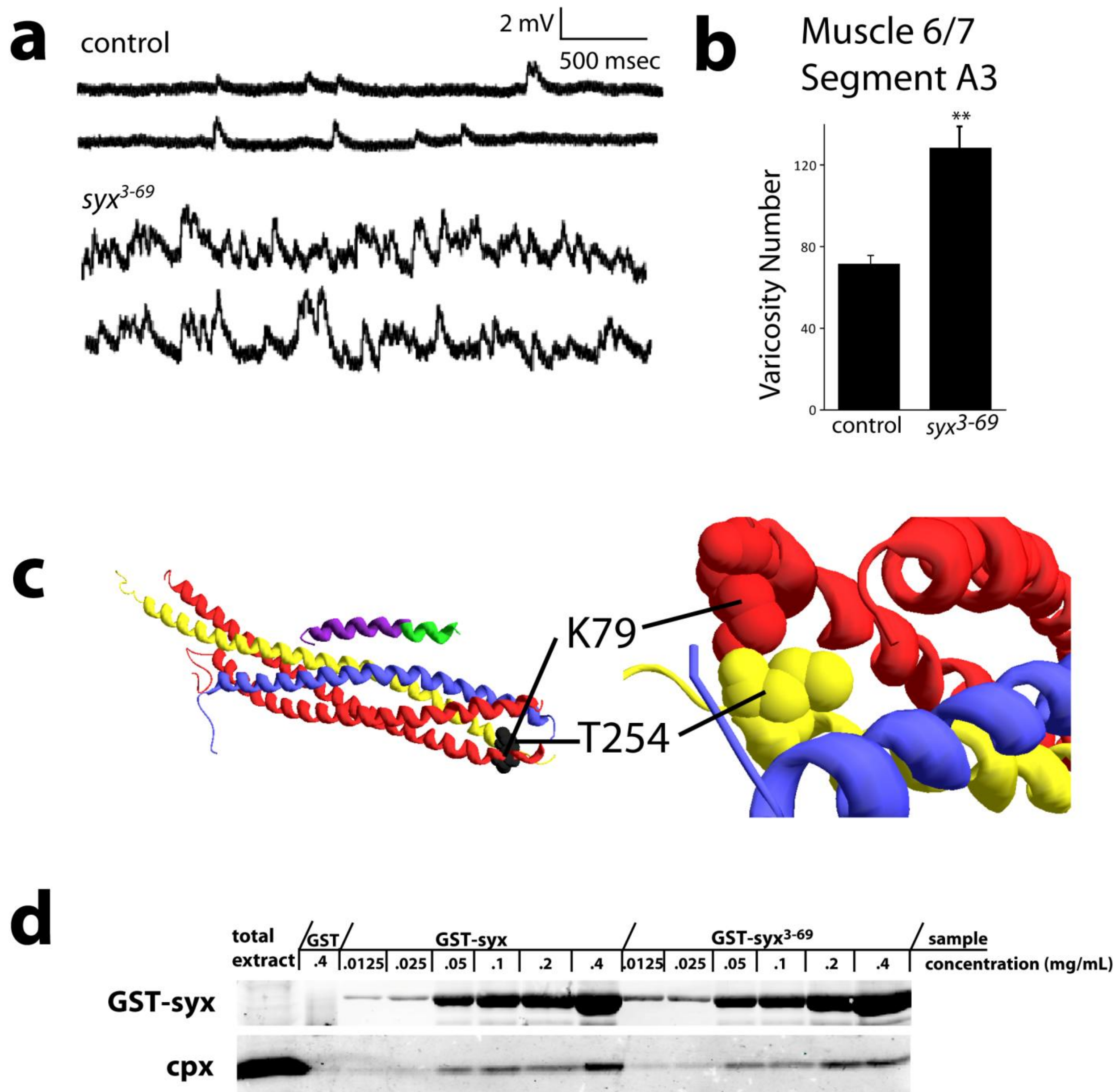
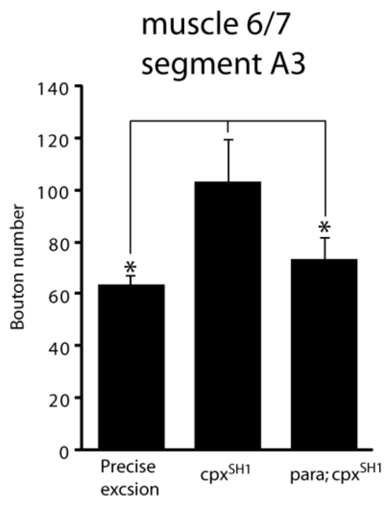


Figure 3. *syx*$^{3\text{-}69}$, a temperature sensitive paralytic mutant with elevated mini frequency, displays synaptic overgrowth similar to that observed in *complexin* mutants. (**a**) Mini recordings from control and *syx*$^{3\text{-}69}$ mutants in 0.2 mM calcium showing the elevated mini frequency phenotype. (**b**) Varicosity number at muscle 6/7 in CS and *syx*$^{3\text{-}69}$ mutants. *syx*$^{3\text{-}69}$ shows a highly significant increase in varicosity number compared to CS (** = P=0.0004). Data used in the analysis include: Control (Canton S) (bouton # =71.44±4.45, n=9); *syx*$^{3\text{-}69}$ (bouton # =128.8±11.8, n=10). (**c**) Location of the T254 amino acid on the coiled-coil structure of syntaxin. T254 in syntaxin (yellow) and its binding partner in SNAP-25 (red), K79, are shown in black in the overall structure (left panel). (Right panel) a close-up view of T254 and K79. (**d**) Assays of complexin binding by syntaxin and syntaxin$^{3\text{-}69}$ across a range of syntaxin concentrations shows no major change in complexin binding affinity in the mutated protein. Error bars are ± s.e.m.


muscle 6/7
segment A3
Bouton number
140
120
100
80
60
40
20
0
*
*
Precise
excsion
cpx^SH1
para; cpx^SH1

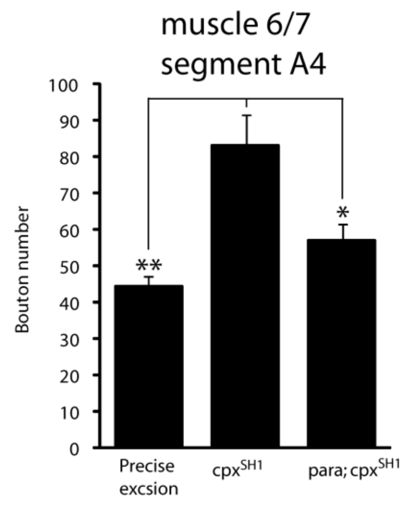
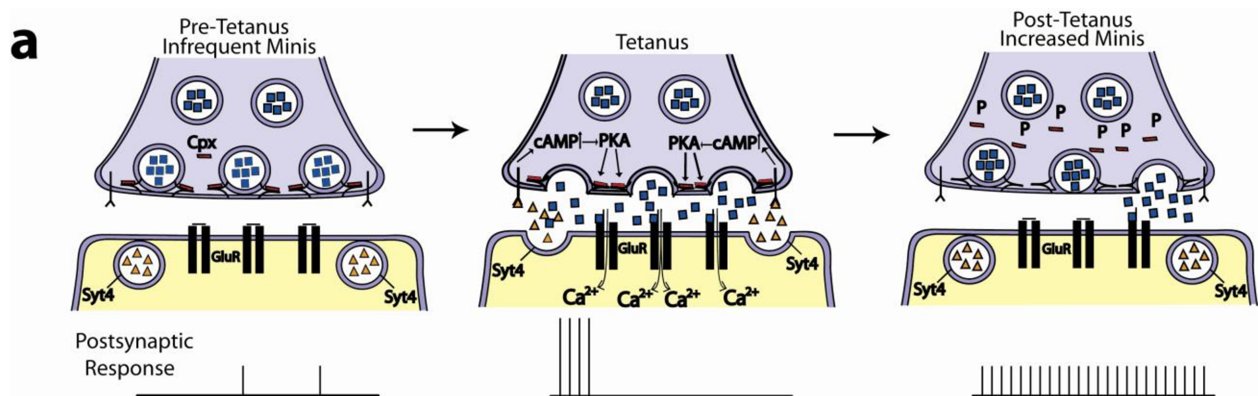
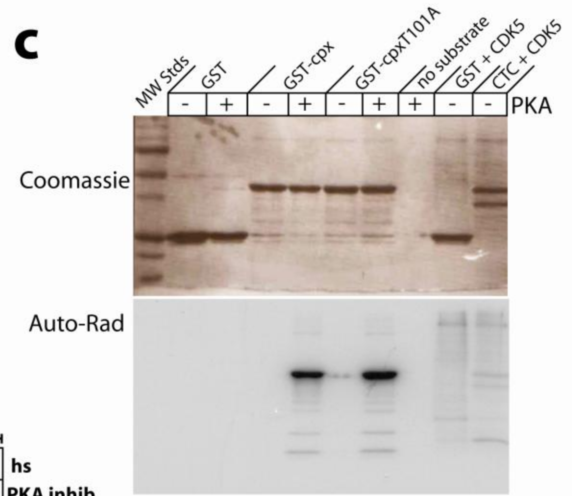

muscle 6/7
segment A4
Bouton number
100
90
80
70
60
50
40
30
20
10
0
**
*
Precise
excsion
cpx^SH1
para; cpx^SH1

Figure 4. Decreased neuronal activity partially suppresses the *complexin* null mutant synaptic overgrowth phenotype. *cpx*^{SH1} mutants display significant overgrowth at muscle fiber 6/7 segments A3 and A4 compared to *precise excision* controls and *para*^{ts1}; *cpx*^{SH1} double mutants (Student t-Test, * = $P < .05$, ** = $P < .001$). Data used in the analysis include: Precise excision (m6/7 segment A3 bouton # = 64.125±3.36, n=8; m6/7 segment A4 bouton # = 44.36±3.0, n=13), *cpx*^{SH1} (m6/7 segment A3 bouton # = 103.5±15.98, n=4; m6/7 segment A4 bouton # = 83.0±8.66, n=6), *para*^{ts1}; *cpx*^{SH1} (m6/7 segment A3 bouton # = 73.5±8.75, n=6; m6/7 segment A4 bouton # = 56.9±4.64, n=9). Error bars are ± s.e.m.



b

	*
dmcpx7b	PLMRKKKTPEELAAEA
dmcpx7a	PLMRKKKTPEELAAEA
dmcpxL	PLMRKKKTPEELAAEA
dmcpxR	PLMRKKKTPEELAAEA
dmcpxJ/K	PLMRKKKTPEELAAEA
dpcpx	PLMRKKKTPEELAAEA
dscpx	PLMRKKKTPEELAAEA
dvcpx	PLMRKKKTPEELAAEA
tccpx	PLMRKKKTPEELAREA
amcpx	PLMRKKKTPEELAAEA
lpcpx	-VGRKKKTKEELAAEA
hmcpx	RLGRKKKSPDE-AVEG



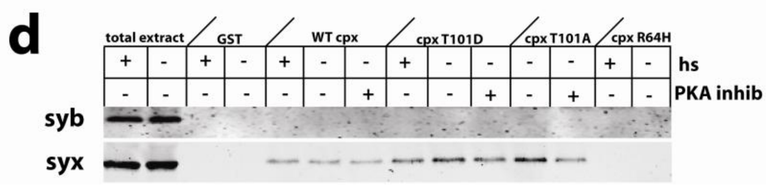


Figure 5. Regulation of complexin by PKA phosphorylation. (**a**) Model of hypothesized regulation of complexin by PKA kinase activity to increase mini frequency at the synapse in response to high frequency stimulation (tetanus). The model is based on **Figure 1** and Yoshihara et al, ***Science*** 2005 and posits that complexin is phosphorylated by PKA in response to activation of the activity-regulated retrograde signaling pathway (middle panel), leading to decreased complexin function, possibly by lowering complexin's SNARE complex binding affinity (right panel). This in turn leads to enhanced mini frequency. (**b**) A putative PKA phosphorylation site in complexin, T101 (* and orange box), is highly conserved throughout invertebrate species and is present in all complexin isoforms. (**c**) Complexin 7b is highly phosphorylated by PKA *in vitro*. GST-fused complexins were incubated with γ-^{32}PATP with or without PKA. Both wild type complexin 7b and a complexin 7b T101A point mutant were phosphorylated by PKA *in vitro*, demonstrating that complexin 7b is phosphorylated by PKA but not at T101. GST alone controls (left lanes) were not phosphorylated, demonstrating that phosphorylation was specific to complexin. A positive control, CTC with the kinase CDK5 was successfully phosphorylated (right lanes). (**d**) Phosphomimetic (T101D) and phosphoincompetent (T101A) point mutants in complexin do not produce major changes in complexin's syntaxin binding affinity. GST-fused complexins were incubated with head extract that either had or had not been heat-shocked (hs) to distinguish between monomeric syntaxin binding and SNARE complex binding. A PKA inhibitor was added to some tubes to control for the possibility that PKA might phosphorylate complexin in the test tube after homogenizing. In all cases, no synaptobrevin binding was detected, indicating that the syntaxin binding observed reflects monomeric syntaxin binding and not SNARE complex binding. No syntaxin binding by the GST control is observed, indicating that the syntaxin binding is specific to complexin. A point mutant in the central alpha helix in complexin responsible for SNARE binding (R64H) does not bind syntaxin in this assay. Abbreviations: **dm**, *Drosophila melanogaster*; **dp**, *Drosophila pseudoobscura;* **ds**, *Drosophila simulans;* **dv**, *Drosophila virilis;* **tc**, *Tribolium castaneum*; **am**, *Apis mellifera*; **lp**, *loligo pealei*; **hm**, *hirudo medicinalis.*

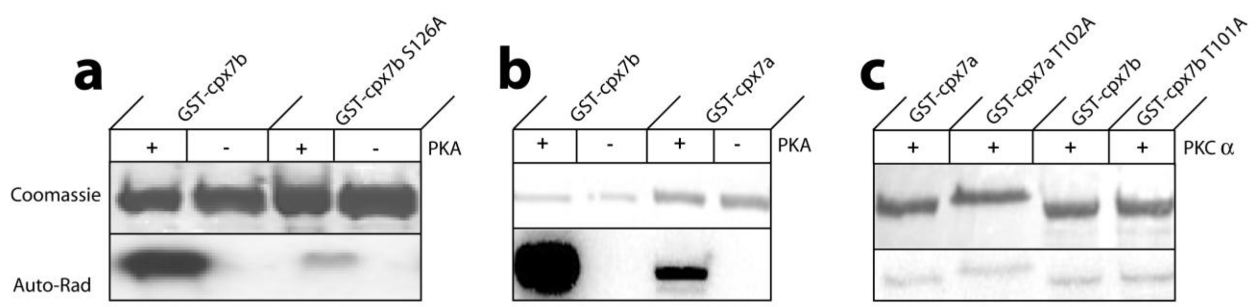
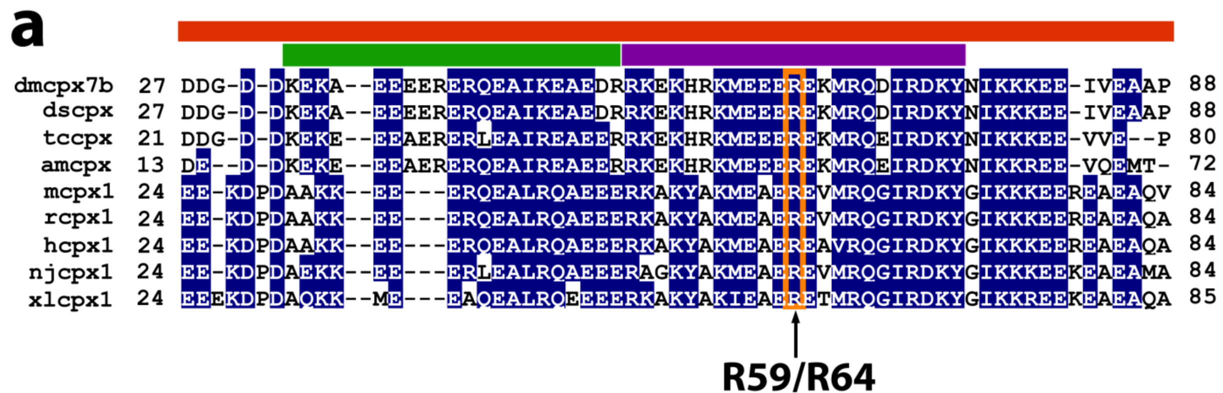
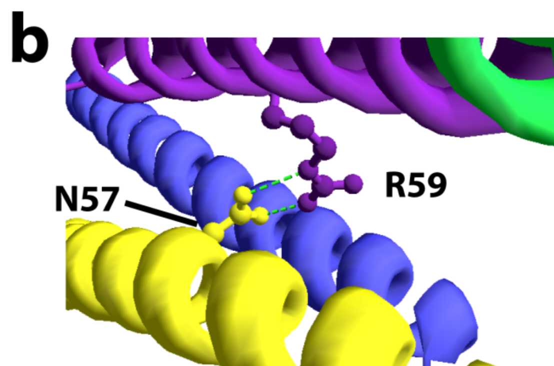
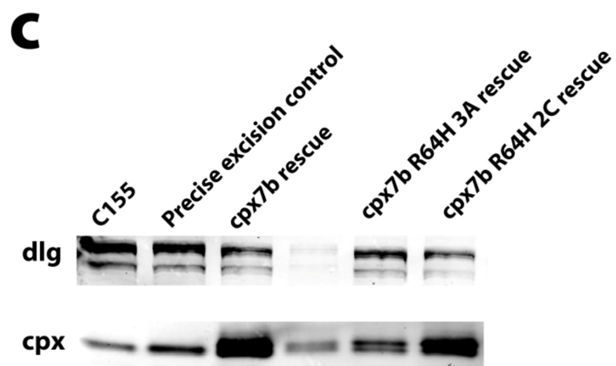

a
GST-cpx7b
GST-cpx7b S126A
+
-
+
-
PKA
Coomassie
Auto-Rad
b
GST-cpx7b
GST-cpx7a
+
-
+
-
PKA
c
GST-cpx7a
GST-cpx7a T102A
GST-cpx7b
GST-cpx7b T101A
+
+
+
+
PKC α

Figure 6. S126 in complexin 7b is the major phosphorylation target of PKA *in vitro*. (**a**) A complexin 7b S126A point mutant is not phosphorylated by PKA *in vitro*, demonstrating that this residue is a target for phosphorylation by PKA. (**b**) Complexin 7a is only phosphorylated at a low level by PKA *in vitro*, suggesting that it is not a target for PKA kinase activity (**c**) Complexin is not phosphorylated by PKC *in vitro*. Mutating complexin 7b T101 or the corresponding residue in complexin 7a, T102, to alanine does not remove the small amount of PKC phosphorylation that does occur, indicating these residues are not targets of PKC *in vitro*.


a
dmcpx7b 27 DDG-D-DKEKA--EEEERERQEAIKEAEDRRKEKHRKMEEEREKMRQDIRDKYNIKKKEE-IVEAAP 88
dscpx 27 DDG-D-DKEKA--EEEERERQEAIKEAEDRRKEKHRKMEEEREKMRQDIRDKYNIKKKEE-IVEAAP 88
tccpx 21 DDG-D-DKEKE--EEAERERLEAIREAEERRKEKHRKMEEEREKMRQEIRDKYNIKKKEE-VVE--P 80
amcpx 13 DE--D-DKEKE--EEAERERQEAIREAEERRKEKHRKMEEEREKMRQEIRDKYNIKKREE-VQEMT- 72
mcpx1 24 EE-KDPDAAKK--EE---ERQEALRQAEEERKAKYAKMEAEREVMRQGIRDKYGIKKKEEREAEAQV 84
rcpx1 24 EE-KDPDAAKK--EE---ERQEALRQAEEERKAKYAKMEAEREVMRQGIRDKYGIKKKEEREAEAQA 84
hcpx1 24 EE-KDPDAAKK--EE---ERQEALRQAEEERKAKYAKMEAEREAVRQGIRDKYGIKKKEEREAEAQA 84
njcpx1 24 EE-KDPDAEKK--EE---ERLEALRQAEEERAGKYAKMEAEREVMRQGIRDKYGIKKKEEKEAEAMA 84
xlcpx1 24 EEEKDPDAQKK--ME---EAQEALRQEEEERKAKYAKIEAERETMRQGIRDKYGIKKREEKEAEAQA 85
R59/R64


b
N57
R59


c
C155
Precise excision control
cpx7b rescue
cpx7b R64H 3A rescue
cpx7b R64H 2C rescue
dlg
cpx

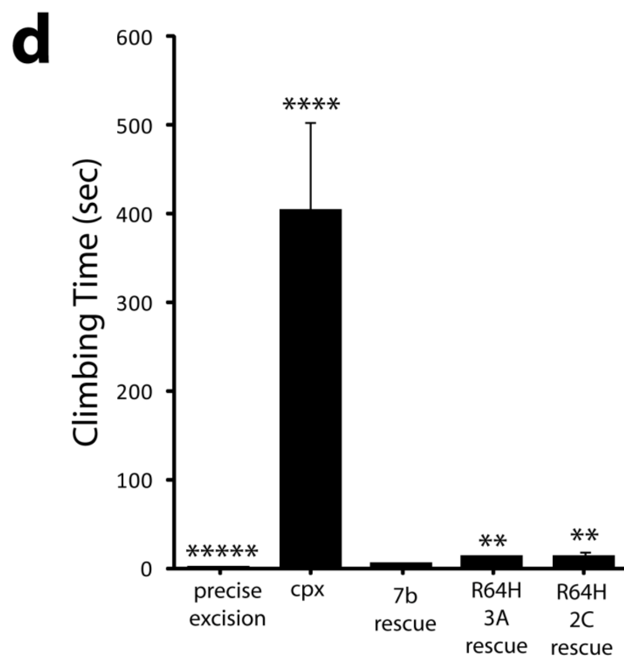
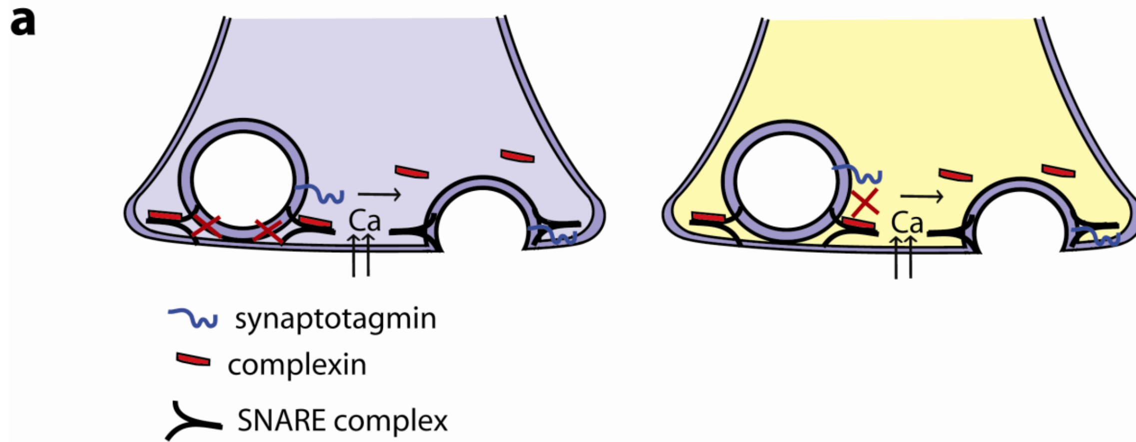

d
Climbing Time (sec)
600
500
400
300
200
100
0
*****
****
**
**
precise excision
cpx
7b rescue
R64H 3A rescue
R64H 2C rescue

Figure 7. *Drosophila* R64H mutants rescue the behavioral defect of *complexin* null mutants, suggesting that R64H retains some SNARE binding activity. (**a**) Alignment of invertebrate and vertebrate complexins showing 100% conservation of R64 (R59 in mammalian complexins). R64 is located in the central alpha helix responsible for binding to the SNARE complex (purple bar). Green bar: accessory alpha helix. Red bar: fragment of complexin crystallized in Chen et al, ***Neuron*** 2002. (**b**) Close-up view showing R64 in complexin (purple) hydrogen bonding (green dashes) with N57 in syntaxin (yellow). Synaptobrevin is shown in blue and SNAP-25 is not pictured for the sake of clarity. (**c**) Western blot showing transgenic complexin expression in a wild type complexin 7b line and two independent complexin 7b R64H lines (3A and 2C). Expression is compared to a driver alone control (C155) and a *precise excision* control. (**d**) Climbing assays demonstrate that R64H point mutants partially rescue the motor defect of *complexin* null mutants. For rescue, transgenic complexin constructs were expressed pan-neuronally in a *complexin* null mutant background. Wild type complexin 7b rescues the phenotype almost completely (7b rescue), although there is still a significant difference in climbing time between *precise excision* and 7b rescue. The R64H rescues took three times longer on average to climb the vial, but still rescued the null phenotype for the most part. Data used in the analysis include: Precise excision (***** = P < 0.0000005, Climbing time = 2.0 ± 0.174 sec., n = 12); cpx (*w;*cpx^{SH1}/cpx^{SH1}) (**** = P< 0.000005, Climbing time = 405.14 sec. ± 97.8 sec., n = 7); 7b rescue (*C155,UAS-cpx7b;* cpx^{SH1}/cpx^{SH1}) (Climbing time = 5.43 ± 0.58 sec., n = 14); R64H3A rescue (*C155; UAS-cpx7b R64H3A,*cpx^{SH1}/cpx^{SH1}) (** = P< 0. 005, Climbing time = 14.33 sec. ± 0.667 sec., n = 3); R64H2C rescue (*C155; UAS-cpx7b R64H2C/+;*cpx^{SH1}/cpx^{SH1}) (** = P< 0. 005, Climbing time = 14.83 sec. ± 3.40 sec., n = 6). Error bars are ± s.e.m. All P values are relative to 7b rescue. Abbreviations: **dm**, *Drosophila melanogaster*; **ds**, *Drosophila simulans;* **tc**, *Tribolium castaneum*; **am**, *Apis mellifera*; **m**, mouse; **r**, rat; **h**, human; **nj**, *Narke japonica*; **xl**, *Xenopus laevis*.

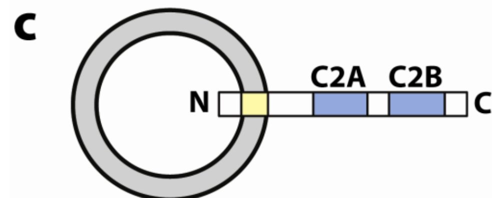
a



b **Summary of syt1;cpx double mutant experiments**

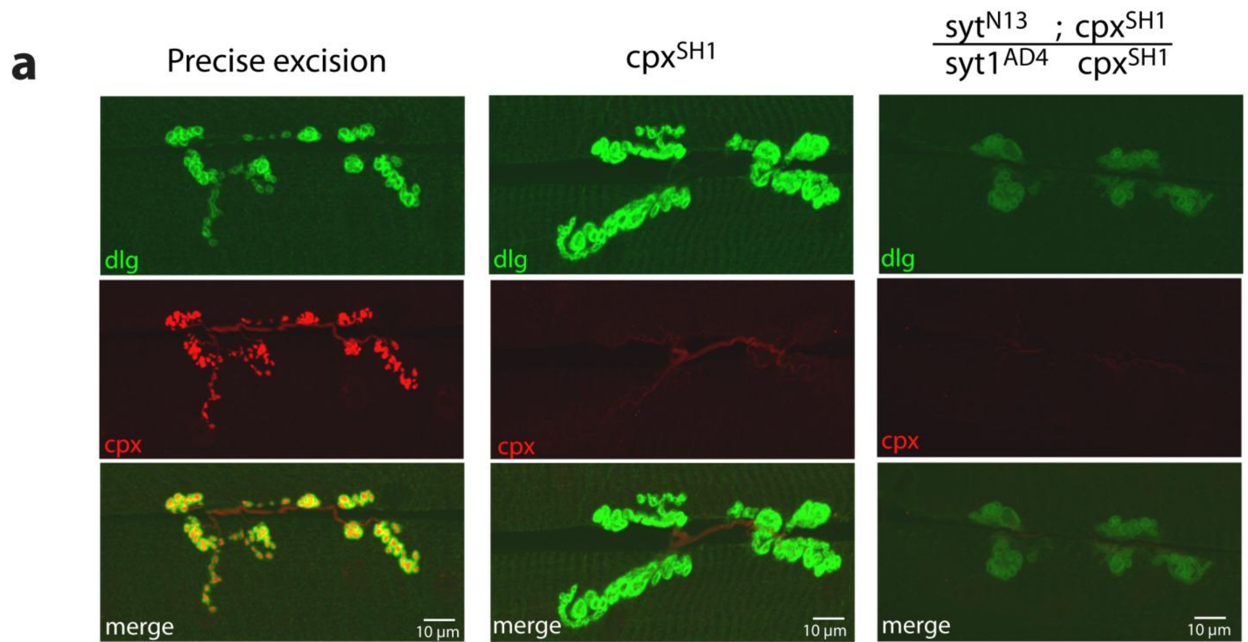
Synaptotagmin;complexin double mutant genotype	Synaptotagmin alleles	Complexin alleles	Result
$syt1^{N13}/syt1^{AD4}; cpx^{SH1}/cpx^{SH1}$	Null	Null	Severe lethality
$syt1^{T41}/syt1^{AD3}; cpx^{SH1}/cpx^{SH1}$	Hypomorph/Point mutant	Null	Severe lethality
$syt1^{T41}/syt1^{AD1}; cpx^{SH1}/cpx^{SH1}$	Hypomorph/Truncation	Null	N/A
C155,UAS-dicer; UAS-syt1RNAi; cpx^{SH1}/cpx^{SH1}	RNAi	Null	Severe lethality
C155,UAS-dicer;$syt1^{N13}/syt1^{AD4}$*; UAS-cpxRNAi*	Null	RNAi	N/A
C155,UAS-dicer;UAS-syt1RNAi;UAS-cpxRNAi	RNAi	RNAi	N/A

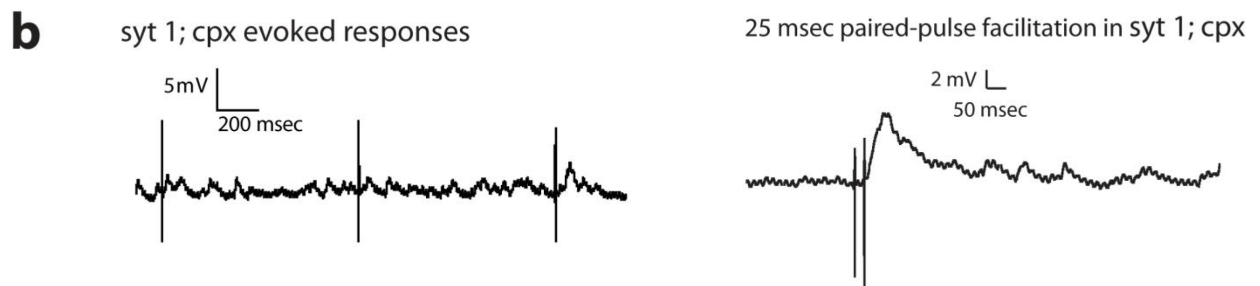
c



Allele	Domain Structure
N13	
AD4	* Stop
AD1	* Stop
AD3	* Y364N

Figure 8. Summary of *synaptotagmin* and *complexin* mutant alleles used to test the two proposed models of complexin's function. (**a**) Two models of complexin's fusion clamp function at the synapse. In model 1 (left panel), complexin (red rectangle) prevents vesicle fusion by blocking full SNARE zippering. After calcium influx synaptotagmin (blue line) removes complexin from the SNARE complex and triggers vesicle fusion. In model 2 (right panel), complexin blocks fusion by preventing synaptotagmin from triggering release prior to calcium influx. After calcium influx, synaptotagmin removes complexin from the SNARE complex and triggers fusion. The two different models are not necessarily mutually exclusive and may be distinguished by an electrophysiological analysis of *synaptotagmin 1; complexin* double mutants. (**b**) Summary table indicating genotypes of double mutant lines, what types of alleles are present in each strain, and the resulting lethality phenotype, if applicable. (**c**) Illustration of the domain structures of the N13, AD4, AD1, and AD3 mutant alleles of *synaptotagmin 1*. Adapted from DiAntonio et al, ***Neuron*** 1994 and Littleton et al ***PNAS*** 1994.


a
Precise excision
cpx^{SH1}
$\frac{syt^{N13}}{syt1^{AD4}}$; $\frac{cpx^{SH1}}{cpx^{SH1}}$
dlg
cpx
merge
10 µm


b
syt 1; cpx evoked responses
5mV
200 msec
25 msec paired-pulse facilitation in syt 1; cpx
2 mV
50 msec

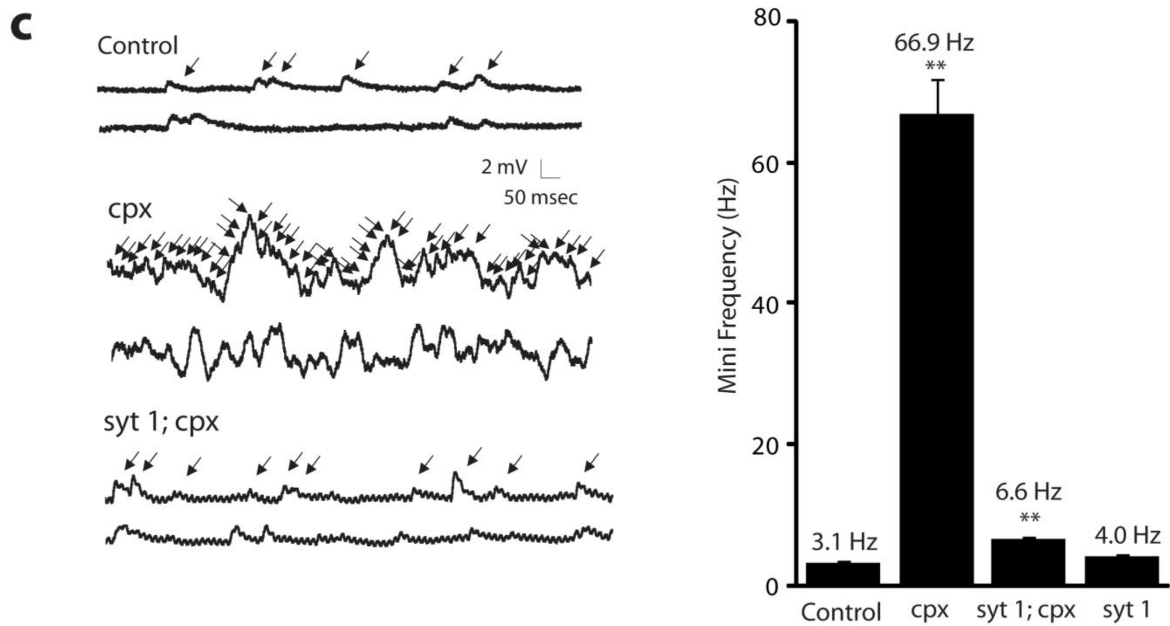
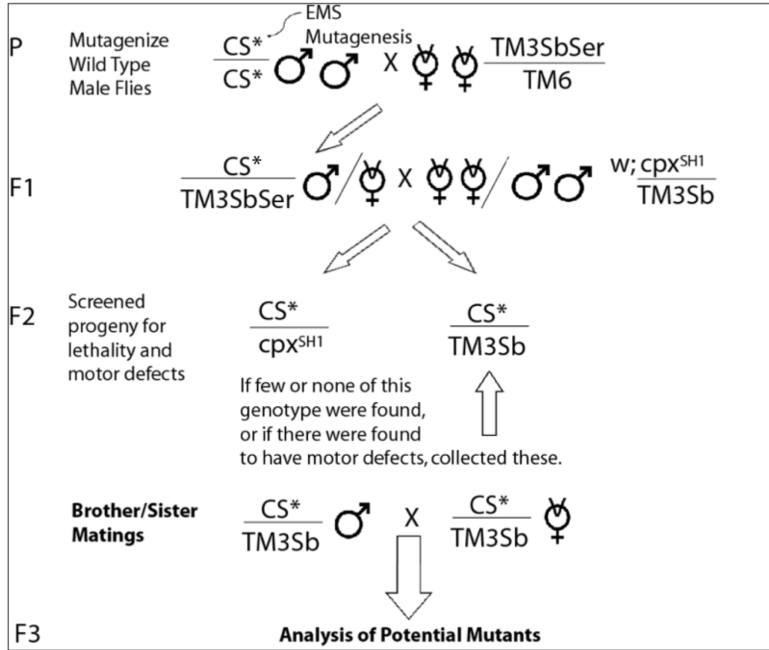

c
Control
cpx
syt 1; cpx
2 mV
50 msec
Mini Frequency (Hz)
80
60
40
20
0
3.1 Hz
66.9 Hz
**
6.6 Hz
**
4.0 Hz
Control
cpx
syt 1; cpx
syt 1

Figure 9. A *synaptotagmin; complexin* double mutant suppresses the elevated mini frequency phenotype seen in *complexin* single mutants. (**a**) Images of *precise excision*, cpx^{SH1}, and $syt^{N13}/syt1^{AD4}$; cpx^{SH1}/cpx^{SH1} NMJs stained with antisera against dlg (green) and complexin (red). Although the double mutant synapse is photobleached, it does not show any complexin staining. (**b**) Sample traces showing evoked responses (left panel) and paired pulse facilitation (right panel) from the double mutant in 0.2 mM extracellular calcium. Evoked responses show only asynchronous release, typical of *synaptotagmin* null mutants. Paired-pulse facilitation is enhanced in the double mutant, again typical of a *synaptotagmin* null mutant. Based on this evidence and the stainings in panel **a**, we concluded that this larva was in fact a double mutant. (**c**) Sample traces (left panel) and quantification (right panel) of minis in a *precise excision* control, *complexin* null mutant, and *syt 1; cpx* double mutant. Recordings were taken in 0.2 mM extracellular calcium. Arrows point to individual minis. Quantification of mini frequency in *syt 1* mutants ($syt^{N13}/syt1^{AD4}$) is also shown in the right panel. Mini frequency in the double mutant is 1/10th that of *complexin* mutants (** = P < 0.0001). The double mutant mini frequency is significantly elevated compared to both the *precise excision* control and *syt 1* single mutants (** = P < .0001). Data used in the analysis include: Control (Mini Frequency (MF)=3.14±0.3 Hz; n=18); cpx^{SH1} (MF=67.0±4.8 Hz; n=27); syt 1; cpx (MF=6.57±0.44 Hz, n=7); syt 1 (MF=3.98±0.35 Hz, n=9). Error bars are ± s.e.m.

a

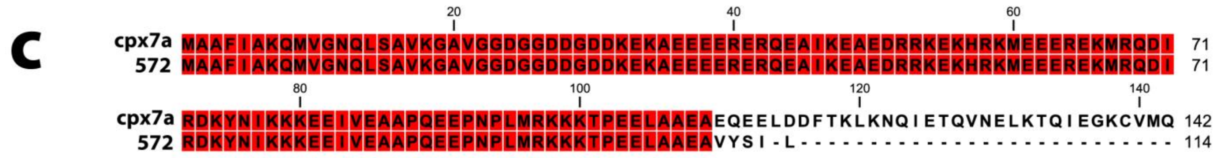


b

Line Number	Phenotype over *cpx* Null	Dominant / Recessive	Homozygous Phenotype	Cpx Expression Level	Sequence Data
107	• Lethal	Recessive	Lethal		
570	• Slow-moving • Bottom Dwelling • Uncoordinated	Semi-dominant	Same as 570/cpx^{SH1}	Wildtype	
572	• Similar to complexin, but move more quickly and with more coordination • Held-out wings	Recessive	Same as 572/cpx^{SH1}	Decreased	Small deletion / protein truncated
573	• Lethal	Recessive	Lethal		
1060	• Lethal	Recessive	Lethal		
1677	• Lethal	Recessive	Lethal		
1755	• Shock sensitive • Unable to fly • Bottom dwelling	Semi-dominant	Lethal		
1934	• Lethal	Recessive	Lethal		
2016	• Phenocopy of cpx^{SH1}	Recessive	lethal	No expression	

Figure 10. Summary of EMS mutagenesis screen for loss of function alleles of *complexin*. (**a**) Diagram of the screen. Male Canton S adults were fed EMS and crossed to Tm3Sb,Ser/Tm6, a line containing two 3rd chromosome balancers. Individual males or virgin female progeny with the Sb and Ser dominant markers (CS*/Tm3Sb,Ser) were crossed to cpx^{SH1}/Tm3Sb. Chromosomes that failed to complement cpx^{SH1} were recovered to establish CS*/Tm3Sb strains (brother/sister matings). Individual strains were recrossed to cpx^{SH1} to confirm non-complementation and were then analyzed by Western blotting and sequencing. (**b**) Table showing available information about mutants recovered from the screen. Line #572 contains a small deletion in the *complexin* coding sequence resulting in a truncated form of the protein. Line #2016 does not express complexin and therefore represents a novel null allele of *complexin*. Sequencing of line #570 has so far not shown any mutation in the *complexin* coding sequence, but the 570 phenotype is remiscent of the *complexin* null phenotype and suggests 570 may contain a loss of function allele of complexin.

a
CS
570
570
CS
572
572
cpx^{SH1}
+
2016
cpx^{SH1}
control
cpx
b
1 2 3 4 5 6
2016 gDNA PCR
cpx^{SH1}


c
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572 MAAFIAKQMVGNQLSAVKGAVGGDGGDDGDDKEKAEEEERERQEAIKEAEDRRKEKHRKMEEEREKMRQDI 71
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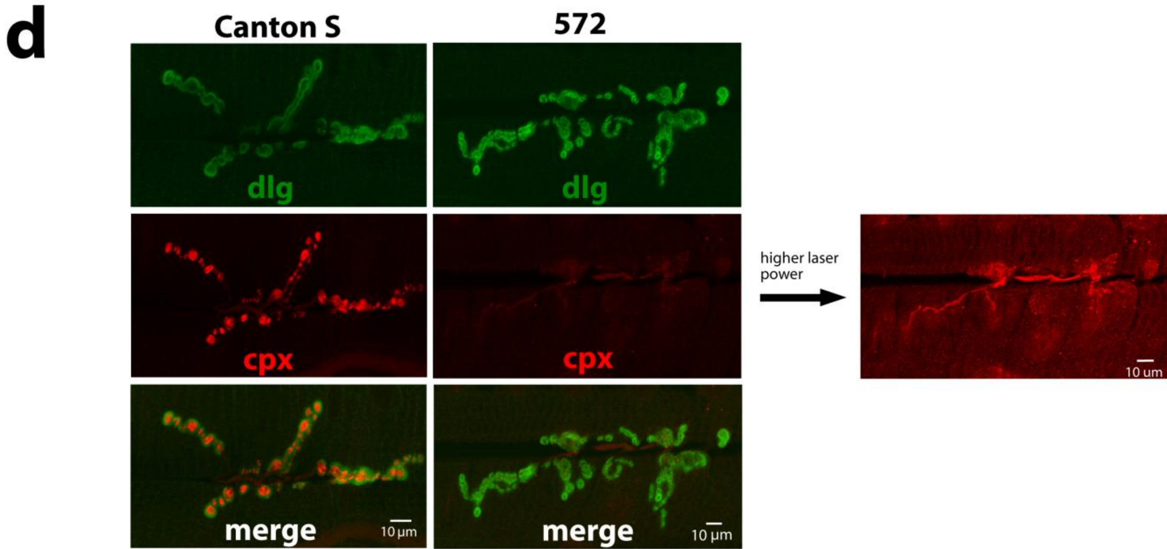
572 RDKYNIKKKEEIVEAAPQEEPNPLMRKKKTPEELAAEAVYSI-L 114


d
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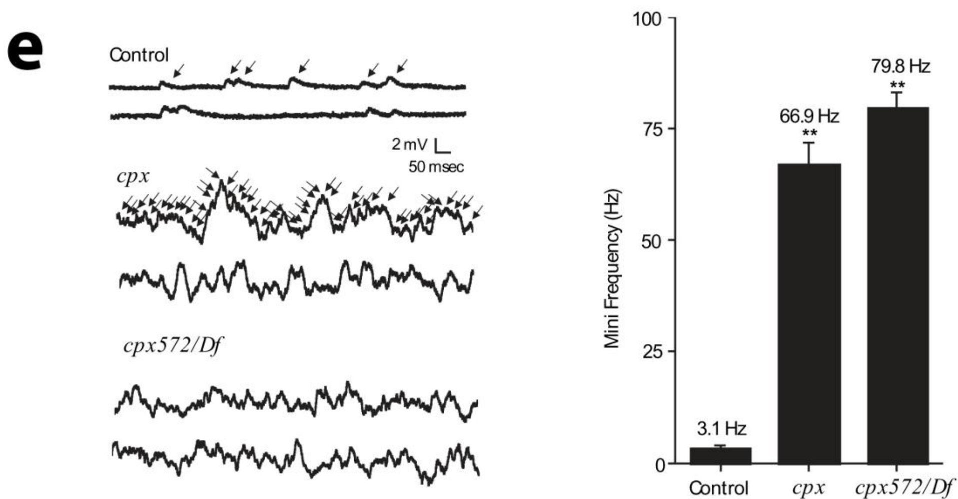
e
Control
2 mV
50 msec
cpx
cpx572/Df
Mini Frequency (Hz)
3.1 Hz
66.9 Hz
79.8 Hz
**
Control
cpx
cpx572/Df

Figure 11. Analysis of mutants generated by non-complementation screening for novel *complexin* alleles. (**a**) Western blots showing complexin expression in three mutant lines, 570, 572, and 2016. 570 has wild type expression. 572 has reduced complexin expression and expresses complexin with a lower molecular weight than wild type, suggesting a truncated protein. 2016 does not express complexin. (**b**) Mapping PCRs showing products 1 through 6 are polymerized from 2016/cpx^{SH1} gDNA. This mapping PCR is the same used to map the deletion in cpx^{SH1}. cpx^{SH1} contains a deletion spanning products two through four, which covers almost all the coding sequence of *complexin*. Thus, line #2016 does not contain a large deletion in the coding sequence of *complexin*. (**c**) Alignment of wild type complexin 7a and the sequence of complexin expressed by line #572. The mutant protein has point mutations at positions 110 to 114, and is truncated by 28 residues at the C terminus. (**d**) Immunohistochemistry showing decreased expression of complexin in line #572 NMJs. Staining was carried out using antiseria against dlg (green) and complexin (cpx, red). Imaging of the 572 NMJ using higher laser power shows that the remaining complexin is localized in puncta at the synapse instead of being diffusely distributed as wild type complexin is. Thus, line #572 contains a hypomorphic allele of *complexin*. (**d**) Sample traces and quantification of mini frequency in control (*precise excision*), cpx (cpx^{SH1}/cpx^{SH1}), and 572 (*572/DfED5021*) larvae. Line number 572 has an elevated mini frequency similar to that of *complexin* null mutants (** = $P < 0.0001$). Data used in the analysis include: Control (Mini Frequency (MF)=3.14±0.3 Hz; n=18); cpx^{SH1} (MF=66.9±4.8 Hz; n=27); 572/Df (MF=79.75±2.69, n=6). Error bars are ± s.e.m.

Chapter 4
Conclusions and Future Directions

Sarah Huntwork-Rodriguez

Summary and Conclusions: Complexin's Role as a Vesicle Fusion Clamp

Complexin Functions as a Vesicle Fusion Clamp in Synaptic Vesicle Exocytosis

Synaptic vesicle exocytosis occurs within 200 μsec of calcium influx and thus is the most rapid and exquisitely controlled vesicle fusion reaction known (Llinas, Steinberg et al. 1981; Cope and Mendell 1982). The synaptic vesicle exocytosis reaction occurs via the same SNARE-driven mechanism as constitutive intracellular vesicle fusion reactions that occur on much slower timescales (Bennett and Scheller 1993). Animals have therefore developed a number of neuron-specific regulatory proteins that adapt vesicle fusion to the highly specialized process observed at synapses.

Because vesicle fusion is constitutive in most cellular fusion reactions but calcium-regulated and synchronous in the nervous system, it has long been hypothesized that a vesicle fusion clamp functions at the synapse to synchronize fusion and maintain vesicles in a fusion-ready state by preventing fusion until calcium influx occurs (Bennett and Scheller 1993; Popov and Poo 1993; Sollner, Whiteheart et al. 1993). However, the identity of the vesicle fusion clamp has remained elusive. Several groups have proposed that complexin functions as a vesicle fusion clamp based on X-Ray crystallography data (Bracher, Kadlec et al. 2002) and *in vitro* fusion assays (Giraudo, Eng et al. 2006; Schaub, Lu et al. 2006), but this idea was not supported by electrophysiology in mouse complexin 1/2 double knockouts (Reim, Mansour et al. 2001) or 1/2/3 triple knockouts (Xue, Stradomska et al. 2008).

These electrophysiology data have several caveats. The *in vitro* recordings were done in the hippocampal neuron micro –island culture system, in which recordings are taken from autapses that have a highly variable number of synaptic contacts ranging over several orders of magnitude (Reim, Mansour et al. 2001; Xue, Stradomska et al. 2008). In the case of synaptotagmin 1, this has resulted in missing an enhanced mini frequency phenotype in mouse

knockouts, in which it was recently revealed from recordings from cortical cultures that there is an increase in mini frequency (Pang, Sun et al. 2006) despite early reports from hippocampal autaptic cultures that no such enhancement in mini frequency existed (Geppert, Goda et al. 1994). An enhancement in mini frequency had been reported from *Drosophila synaptotagmin* knockouts earlier (Littleton, Stern et al. 1993; Broadie, Bellen et al. 1994; DiAntonio and Schwarz 1994), but it was discounted because the enhanced mini frequency phenotype was not observed in mammals.

The third-instar larval NMJ in *Drosophila* offers the opportunity to observe complexin function at mature synapses with a stereotypical pattern of innervation. We therefore generated the first complete *complexin* knockout in any organism. Using recordings from *Drosophila* third-instar larval neuromuscular junctions, we have demonstrated that *complexin* knockouts display a highly elevated spontaneous release frequency at least 20 times greater than controls. These release events do not require calcium influx into the nerve terminal, as they persist in 0 mM extracellular calcium. This phenotype is exactly what would be expected from knockout of the vesicle fusion clamp for neurotransmitter release, and it is therefore highly probable that complexin functions as a vesicle fusion clamp *in vivo*. One argument that could be made is that mammalian and *Drosophila* complexins serve different functions at the synapse, thus accounting for the differences in electrophysiology data. However, recent preliminary data indicate that mammalian complexins rescue the enhanced mini frequency phenotype in *Drosophila complexin* knockouts, although confirmation of this result requires further analysis and quantification (personal communication, Richard Cho). This information indicates that mammalian complexins serve the same *in vivo* function as *Drosophila* complexins, though it does not discount the

possibility that mammalian complexins have evolved new functions not found in the fly homolog.

Complexin maintains the readily releasable pool of vesicles at the synapse

Evoked potentials in *complexin* mutants were found to have normal amplitudes on average at low extracellular calcium concentrations but lower amplitudes at high extracellular calcium concentrations of 0.4 and 1.0 mM. This suggests that mutant synapses have a defect in the size of vesicle population available for evoked responses. Using electron microscopy to examine the ultrastructure of *complexin* mutant synapses, we found no significant change in the number of vesicles in the synapse or the number of docked vesicles at active zones. The only difference between *complexin* mutant synapses and wild type synapses found was an increased number of cisternal structures, which result from bulk endocytosis and are synaptic vesicle precursors (Koenig and Ikeda 1989). This suggests that increased bulk endocytosis is one mechanism by which *complexin* mutants maintain the vesicle population at the synapse despite the constant high rate of exocytosis. Synaptic vesicle recycling through endocytic intermediates such as cisternae is thought to be a slow process requiring several minutes (Richards, Guatimosim et al. 2000) that is responsible for maintaining the reserve pool, so the enhancement in the number of cisternae may indicate increased mobilization of vesicles from the reserve pool in *complexin* mutants.

Ultrastructural analysis did not reveal a depletion of vesicles that would explain the reduced EJP amplitude, so we performed high frequency stimulation under voltage clamp at the NMJ to determine whether the increase in mini frequency in *complexin* mutants results in a depletion of the readily releasable pool of vesicles (RRP). At wild type and complexin overexpression synapses, high frequency stimulation at 2.0 mM extracellular calcium results in

rapid depression as the readily releasable pool of vesicles is depleted, followed by a sustained phase in which the evoked response is maintained at a constant but lower amplitude, reflecting rapid recycling at the synapse. In *complexin* mutants, the depression phase of high frequency stimulation is eliminated because there is a lower amplitude initial response and only the second phase of sustained, lower evoked responses remains, indicating that the readily releasable pool of vesicles is severely depleted or eliminated, while recycling mechanisms remain intact. Thus, complexin is required for maintenance of the readily releasable pool of vesicles, and in the absence of complexin, vesicles release once they are primed.

Complexin specifically regulates synchronous release

Evoked responses consist of two kinetically distinct phases of release. Synchronous release is driven by the calcium sensor synaptotagmin 1 and is defined by rapid rise and fall times due to synchronous release of vesicles. Asynchronous release is defined by a slower response and a long sustained phase of asynchronous vesicle fusion driven by an unknown calcium sensor. A comparison of evoked responses normalized to the number of release sites from wild type, *complexin* mutant, and complexin overexpression flies showed that *complexin* null mutants have no deficiency in asynchronous release but decreased synchronous release. Overexpression of complexin increases the amplitude of evoked responses while decreasing the duration of the asynchronous response. This indicates that by overexpressing complexin, vesicles are more likely to fuse during the synchronous release phase. Given that complexin is required for maintenance of the readily releasable pool of vesicles, it is possible that complexin overexpression pushes vesicles into the RRP, decreasing the asynchronous release phase and increasing synchronous release. This hypothesis supposes that asynchronous release does not utilize the RRP.

An EMS screen to generate new alleles of complexin

In order to further elucidate the mechanism by which complexin functions as a vesicle fusion clamp and to discover how its structure relates to that function, we screened for new loss-of-function *complexin* alleles using EMS mutagenesis followed by a screen for non-complementation of cpx^{SH1}. Sequencing of the mutants generated is not complete, but two new loss-of-function alleles have been confirmed. Line #2016 does not express complexin, indicating that it contains a novel *complexin* null allele. Line #572 contains a small deletion in *complexin* resulting in point mutations in the C terminus and truncation of the protein. This truncated mutant complexin is expressed at low levels, and line #572 therefore contains a hypomorphic allele of *complexin*. Neither of these confirmed *complexin* mutants are particularly useful for structure-function studies of complexin, but 572 will be useful for genetic studies of complexin function. Continued sequencing of the remaining mutants holds promise for discovery of novel *complexin* alleles useful for structure-function studies.

synaptotagmin 1; complexin double mutant analysis suggests complexin functions to prevent synaptotagmin 1 from triggering exocytosis prior to calcium influx

In order to explain the *complexin* mutant phenotype of a >20-fold increase in mini frequency, we proposed two possible models for how complexin may function. First, complexin may prevent fusion by preventing full zippering of the SNAREs. Second, complexin may prevent fusion by inhibiting synaptotagmin from triggering release prior to calcium influx. Some biochemical data suggests that complexin can arrest zippering of the SNAREs (Giraudo, Eng et al. 2006; Roggero, De Blas et al. 2007), supporting the first model. On the other hand, it has been shown that under some experimental conditions synaptotagmin is capable of driving fusion asynchronously and at a low level in the absence of calcium (Mahal, Sequeira et al. 2002). This information supports

the second model because a mechanism would be required *in vivo* to prevent synaptotagmin from triggering release in the absence of calcium at the synapse.

In order to test our two models, we have made several lines to generate a variety of combinations of loss-of-function alleles of *synaptotagmin 1* and *complexin*. While the double mutant is lethal prior to the third-instar stage, we have recorded from one double null mutant escaper larva. Recordings from this animal showed an almost complete suppression of the enhanced mini frequency phenotype of *complexin* single mutants, demonstrating that, for the most part, the minis in *complexin* mutants require synaptotagmin 1 function. This suggests that the second model of complexin function may predominate at synapses.

If this result is verified by recordings from additional double mutants, it implies that complexin's function as a vesicle fusion clamp is not so much to hold back vesicle fusion but instead to synchronize it. Without the positive force of synaptotagmin in fusion, *complexin* mutants do not have a highly elevated mini frequency, suggesting that the neuronal SNARE complex itself cannot drive the high rate of fusion in *complexin* mutants. Synchronous fusion must be tightly temporally regulated, requiring multiple layers of regulation, both positive and negative. The double mutant data generated thus far imply that complexin's function in the nervous system is to synchronize evoked fusion and harness the positive fusogenic force of synaptotagmin for evoked release.

Some evidence indicates that synaptotagmin itself may serve as a vesicle fusion clamp *in vivo* (Littleton, Stern et al. 1993; Broadie, Bellen et al. 1994; DiAntonio and Schwarz 1994; Littleton, Stern et al. 1994; Pang, Sun et al. 2006) and *in vitro* (Chicka, Hui et al. 2008), and that complexin can stimulate vesicle fusion *in vitro* (Yoon, Lu et al. 2008). However, both the *in vitro* studies cited examined synaptotagmin or complexin function alone, and a detailed

understanding these proteins' functions can be more clearly gained by study of their functional dynamics together in vesicle fusion since their mechanisms of action appear to be intimately connected. Still, electrophysiology in *synaptotagmin 1* knockout flies and mice reveals an enhanced mini frequency, suggesting a negative function for synaptotagmin in neurotransmitter release. Thus, synaptotagmin's function in neurotransmitter release may be more complex than the above model suggests. Further analysis of double mutants with the range of *synaptotagmin* alleles available may reveal additional features of how synaptotagmin and complexin function together in neurotransmitter release.

Future Directions: Analysis of Complexin's Function at the Synapse

Mammalian complexin rescue experiments

Because of the differences between the electrophysiology results in *Drosophila* and those in mice, the chief controversy surrounding complexin will continue to be whether it functions as a vesicle fusion clamp or whether it functions in priming as a positive regulator of neurotransmitter release as proposed by those groups involved in electrophysiological analysis of the mouse *complexin* knockouts (Reim, Mansour et al. 2001; Xue, Stradomska et al. 2008). In the end, it may be that complexin plays both positive and negative roles at the synapse and that it is more than simply a fusion clamp. However, our data clearly demonstrate with a very dramatic knockout phenotype that one major role that complexin plays is a vesicle fusion clamp. We must therefore take steps to determine whether the vesicle fusion clamp function is specific to invertebrates or flies or whether it also applies to mammalian complexins as well. The biochemical data already support the idea that the clamping function of complexin is conserved across evolution, as both *Drosophila* complexin and mouse complexin 4 halt liposome fusion at a hemifused state (Schaub, Lu et al. 2006). In general, homologous proteins of different species

serve similar functions in the cell, so it would be quite surprising if fly complexin and mouse complexin had different functions.

For our part, we need to determine whether mammalian complexins rescue the complexin knockout phenotype both behaviorally and electrophysiologically. Early results indicate that both the behavior and mini frequency are partially rescued by mammalian complexins, but this result must be quantified and experimentally verified (personal communication, Richard Cho). A partial rescue is probably the best that can be achieved given the evolutionary distance between the two animals. Once the amount of behavioral and electrophysiological rescue in these animals is quantified, it would be helpful to do SNARE binding assays testing how well mammalian complexins bind *Drosophila* SNARE complex. It is likely that they would not bind as well as *Drosophila* complexin binds, which could easily explain why rescue is partial and not full. Rescue of the *Drosophila complexin* phenotype by mammalian complexins would show that mouse complexin retains the ability to clamp fusion at *Drosophila* NMJs.

In mammalian complexins, it has been found that the N terminus of complexin suppresses a negative regulatory function of the accessory alpha helix (Xue, Reim et al. 2007) leading to the proposal that the accessory alpha helix mediates a clamping function and that this function is suppressed by the N terminus. It was suggested that because the N terminus of complexin does not show much sequence conservation between *Drosophila* and mammals, the N terminus in *Drosophila* does not suppress the clamping function of the accessory alpha helix and this might explain why complexin seems to serve two different functions in the two species. It seems more likely that the N terminus is not well conserved because it does not directly mediate binding to the SNARE complex, but nonetheless this hypothesis should be tested. This could be accomplished by making chimeric proteins with different combinations of the fly and mouse N

termini, accessory alpha helix, and central alpha helix, followed by experiments to determine which chimeras rescue the enhanced mini frequency phenotype. If, for example, the chimera combining the mouse N terminus with the fly accessory alpha helix, central alpha helix, and C terminus rescues the mini frequency phenotype, this would indicate that mouse and fly N termini have similar functions.

Rescue of the *complexin* knockout with complexin mutants lacking SNARE binding

Point mutants in complexin that cannot bind the SNARE complex are generally used in the literature as important negative controls to demonstrate that experimental results represent the *in vivo* function of complexin. In this same line of reasoning, we attempted to rescue the *complexin* null phenotype with a point mutant that supposedly did not bind the SNARE complex. To our surprise, this point mutant for the most part rescued the null behavioral phenotype. It was later revealed that the R64H mutation leaves some SNARE complex binding intact, whereas several double point mutants in key amino acids of the binding domain completely eliminate SNARE binding by complexin (Xue, Reim et al. 2007). We are therefore in the process of generating transgenic lines with a double mutant that abolishes SNARE binding. Given that complexin requires SNARE binding for its activity, this mutant is predicted to be unable to rescue the behavioral and electrophysiological phenotypes of the fly *complexin* knockout.

Detailed analysis of splice isoform expression in *Drosophila*

There are at least five predicted splice isoforms encoding different protein sequences in *Drosophila complexin*: complexin 7a, complexin 7b, complexin A, complexin R, complexin L, and complexin J/K. Two of these isoforms, 7a and 7b, are known to be expressed in *Drosophila* because they were successfully isolated from a cDNA library. 7a and 7b have distinct phosphorylation properties, indicating that in at least one case alternative splicing produces

complexins that could have distinct functions or regulation. It is unknown whether the other four isoforms are expressed, but it is possible that they are found during specific developmental stages or in specific cell types. As a first step, RT-PCR using splice-isoform-specific primers should be performed on embryos, larvae, and adult flies to determine whether the predicted transcripts are expressed in any of these stages. For those isoforms that are shown to be expressed by RT-PCR, i*n situ* hybridizations should also be performed using isoform-specific probes to determine whether they are expressed in specific tissues. In addition, we are in the process of developing antibodies specific to complexins 7a and 7b, which will help to determine the stage and location of expression of these two splice isoforms.

Notably, several of the predicted splice isoforms (7a, A, R, L, J/K) differ in their N terminal sequences, and the N terminus of complexin is thought to regulate complexin's clamping function (Xue, Reim et al. 2007). If these different isoforms are found to be expressed, it is possible that they may confer specific functional properties to complexin. This could be tested by the degree of rescue of the enhanced mini frequency phenotype of *complexin* mutants by the different isoforms, as well as a more detailed analysis of depression during high frequency stimulation and effects on asynchronous release. It is possible that this set of experiments could reveal novel mechanisms developed by *Drosophila* to subtly modulate complexin for cell-type specific or developmental-stage-specific function.

***synaptotagmin*; *complexin* double mutant analysis**

Preliminary results from a *syt 1;cpx* double mutant suggest that the spontaneous release events observed in *complexin* single mutants are dependent on synaptotagmin, which would indicate that complexin's function as a vesicle fusion clamp is partly to prevent synaptotagmin from triggering release prior to calcium influx. In order to verify these results, additional double

mutants must be cultured to the third-instar stage. While this has proved difficult with the three allele combinations we have attempted to culture so far, three additional combinations of *synaptotagmin 1* and *complexin* alleles have been generated that we have not yet tried to culture to the third-instar stage. The alleles include transgenic complexin and synaptotagmin RNAi expression constructs and *synaptotagmin* hypomorphs. It is possible that one of these combinations will allow viability to the third-instar stage. If not, one strategy to circumvent this impasse would be to drive RNAi constructs against both complexin and synaptotagmin in motor neurons and not pan-neuronally, potentially enhancing viability. A second strategy would be to make a *syt 1 null*; cpx^{572} double mutant, which may enhance viability compared to the double null mutant combination.

In addition to generating double mutants in *complexin* and *synaptotagmin*, we have also begun studies to determine the effects of double overexpression of synaptotagmin and complexin. While this approach is subject to more caveats than double mutant analysis because overexpression inherently involves non-physiological levels of protein expression, it is also more likely to produce meaningful results quickly. We know that overexpression of complexin enhances the amplitude of the evoked response and decreases the duration of the asynchronous release phase, while overexpression of synaptotagmin has not been reported to have any physiological effects. We have determined that complexin maintains the readily releasable pool and specifically regulates synchronous release, so a large overexpression of complexin may make synaptotagmin limiting for release of vesicles clamped by complexin. If so, co-overexpression of synaptotagmin 1 and complexin may result in a large increase in EJP amplitude greater than that observed when complexin alone is overexpressed. This experiment

along with the double mutant analysis experiments has the potential to reveal new details of the mechanism of calcium-regulated synaptic vesicle exocytosis at the synapse.

Summary and Conclusions: The Role of Minis in Synaptic Plasticity

Complexin mutants display a severe synaptic overgrowth phenotype

Minis have long been thought to represent background noise at the synapse, but recent evidence suggests that mini frequency is used at synapses for a variety of functions, including maintenance of dendritic spine density (McKinney, Capogna et al. 1999) and regulation of dendritic protein synthesis (Sutton, Wall et al. 2004). At *Drosophila* NMJs, mini frequency is increased by over 100-fold in response to high frequency stimulation by a PKA-dependent retrograde signaling pathway that is required for normal synaptic growth (Yoshihara, Adolfsen et al. 2005). In the *complexin* mutant we were able to observe the effects of a large increase in mini frequency on synaptic plasticity. Based on the number of varicosities present at mutant and control synapses, this results in a large enhancement in synaptic growth at the NMJ. We therefore hypothesized that increased mini frequency enhances synaptic plasticity and that complexin is the downstream target of PKA that is regulated to produce a 100-fold increase in mini frequency at the NMJ after high frequency stimulation. Consistent with this idea, *Drosophila* complexin has several possible phosphorylation targets in its C terminus and mouse complexin is known to be phosphorylated both *in vitro* and *in vivo* (Hill, Callaghan et al. 2006; Shata, Saisu et al. 2007). Mutating these phosphorylation sites and testing their ability to rescue the mini frequency enhancement and altered synaptic growth phenotypes should begin to reveal whether phosphorylation modulates this plasticity pathway.

Enhanced mini frequency in a syntaxin mutant results in synaptic overgrowth

In order to test the idea that increased mini frequency drives synaptic plasticity at the NMJ, we utilized the syntaxin mutant *syx*$^{3\text{-}69}$, the only other *Drosophila* mutant known to have an elevated mini frequency on the same scale as *complexin* mutants. Like *complexin* mutants, *syx*$^{3\text{-}69}$ larvae also display an increased number of varicosities at the NMJ. This demonstrates that the synaptic overgrowth phenotype is not specific to *complexin* mutants but can be observed in a second mutant with a large enhancement in mini frequency.

Because of the enhanced mini frequency phenotype of *syx*$^{3\text{-}69}$ we hypothesized that the point mutation in *syx*$^{3\text{-}69}$, T254I, decreases complexin binding to the SNARE complex. While *in vitro* complexin binding assays disproved this hypothesis, one other remote possibility that has not been tested is that *syx*$^{3\text{-}69}$ has decreased complexin expression that causes the mini phenotype. It is more likely that the enhanced mini frequency in *syx*$^{3\text{-}69}$ is independent of complexin, but this possibility should be tested. The *syx*$^{3\text{-}69}$ mutant may in fact generate unstable SNARE complexes that are more likely to be fusogenic with or without complexin. It will be interesting to test if complexin overexpression can suppress the increased mini frequency and the enhanced synaptic growth in the mutant.

Complexin 7b residue S126 is the major phosphorylation target of PKA *in vitro*

Our hypothesis that complexin is the target of PKA phosphorylation at the synapse to regulate mini frequency led us to search for a target residue in complexin that could be phosphorylated by PKA. Because of sequence conservation and because it was highly predicted to be phosphorylated by PKA, the most likely candidate was T101, but *in vitro* phosphorylation assays strongly suggest this residue is not the target of PKA phosphorylation. Instead, S126, a residue specific to complexin 7b, is highly phosphorylated by PKA *in vitro*. Additionally, phosphoincompetent and phosphomimetic point mutations at T101 do not detectably change the

binding affinity of complexin for the SNARE complex. Although binding affinity is not the only way that complexin function could be modulated, it is one obvious way, so there is no evidence from this experiment to suggest that T101 phosphorylation can modulate complexin function.

Judging by the fact that no ESTs support expression of exon 7b, complexin 7b expression may be restricted to a small subset of cell types or expressed at low abundance in all neurons. If it is indeed complexin 7b that is specifically phosphorylated by PKA to regulate mini frequency, it may be that the retrograde signaling pathway induced by high frequency stimulation is not active throughout the nervous system, but instead is active at specific sites. For example, it could occur specifically in neurons of the mushroom body for learning, or it could occur at specific developmental stages where synaptic growth is particularly pronounced. The fact that complexin 7b appears to be the major isoform of complexin phosphorylated by PKA reinforces the importance of determining when and where complexin 7a and 7b are expressed.

Future Directions: Regulation of Mini Frequency and Synaptic Plasticity by Complexin

Generation of S126 point mutants to determine effects on SNARE binding and synaptic plasticity

If complexin 7b S126 is the *in vivo* target of phosphorylation of PKA in the retrograde signaling pathway, then phosphomimetic and phosphoincompetent point mutants in S126 (S126D and S126A, respectively) would be predicted to have dramatic effects on the synaptic overgrowth phenotype of *complexin* null mutants. Our hypothesis is that complexin is inactivated by phosphorylation, possibly by decreasing complexin's SNARE complex binding affinity, so S126D mutants should not rescue the enhanced mini frequency of *complexin* knockouts and should not rescue synaptic overgrowth. S126A would be predicted to rescue both the enhanced mini frequency and synaptic overgrowth phenotypes. Additionally, overexpression of S126A

may produce synaptic undergrowth because, according to our hypothesis, it would never be inactivated by phosphorylation, thus suppressing activity-dependent increases in mini frequency during development of the NMJ. All of these predictions can be tested by making transgenic complexin 7b S126A and S126D point mutants and determining the mean number of varicosities at NMJs of rescue and overexpression animals. If a change is observed, *in vitro* SNARE binding assays should be performed on the point mutants to determine whether the mutations alter complexin's SNARE binding affinity. Also, given that complexin 7a is not phosphorylated by PKA *in vitro*, rescue of the *complexin* null mutant with complexin 7a may also produce synaptic undergrowth because complexin 7a should be unable to participate in the activity-dependent increase in mini frequency. Finally, complexin 7a, complexin 7b, complexin 7b S126A, and complexin 7b S126D rescues should all be analyzed electrophysiologically to determine whether they can produce activity-dependent changes in mini frequency. Based on our model, complexin 7b rescues would be able to, while complexin 7a and complexin 7b S126A would not be able to. S126D rescues would have an increased mini frequency independent of neuronal activity.

Double mutant analysis to determine whether complexin is downstream of PKA and synaptotagmin 4 in the retrograde signaling pathway

A *synaptotagmin 4* null allele, *syt4*^{BA1}, suppresses activity-induced synaptic overgrowth observed in temperature-sensitive seizure mutants in *Drosophila* (personal communication, Cindy Barber). Thus, synaptotagmin 4 is required for synaptic plasticity driven by neuronal activity. This is consistent with the following model of synaptic plasticity established by Yoshihara et al, 2005: 1. High frequency neuronal firing causes postsynaptic calcium influx through glutamate receptors. 2. This calcium influx is sensed by synaptotagmin 4, triggering release of postsynaptic vesicles containing a retrograde signaling molecule. 3. The retrograde signal is bound by receptors in the presynaptic terminal that activate a pathway responsible for synthesizing cAMP. 4. Increased

cAMP in the presynaptic terminal activates PKA, which acts on downstream effectors to increase mini frequency and induce synaptic growth.

If complexin regulation by PKA and subsequent upregulation of mini frequency is the endpoint of this retrograde signaling pathway, then unlike the overgrowth observed in seizure mutants, the overgrowth phenotype of *complexin* mutants would not be suppressed by $syt4^{BA1}$. The PKA loss-of-function mutant $DC0^{B3}$ (Lane and Kalderon 1993) would also be predicted to be unable to suppress the overgrowth observed in *complexin* mutants. Thus, two critical experiments are to make cpx^{SH1}; $syt4^{BA1}$ and cpx^{SH1}; $DC0^{B3}$ double mutants and determine whether the mean varicosity numbers at NMJs of these double mutants are significantly lower than cpx^{SH1} single mutants. Because $syx^{3\text{-}69}$ mutants also display enhanced mini frequency and enhanced synaptic growth, similar experiments should be performed on $syx^{3\text{-}69}$; $syt4^{BA1}$ and $syx^{3\text{-}69}$; $DC0^{B3}$ double mutants.

The hebbian theory of synaptic plasticity states that repeated stimulation of a postsynaptic cell by an innervating axon produces a synapse-specific increase in synaptic efficacy (Hebb 1949). This synapse-specific plasticity is thought to require a retrograde signal from the postsynaptic to the presynaptic cell that acts locally to specifically strengthen the firing synapse. Induction of high mini frequency by regulation of complexin in response to a retrograde signal provides a mechanism to specifically mark a synapse for structural plasticity and enhanced synaptic efficacy. Such a mechanism could be used during development as neuronal connections elaborate and strengthen or during learning and memory to specifically strengthen synapses for memory storage. Thus, investigation of the role of complexin in regulating mini frequency and synaptic plasticity has the potential to help establish a novel function for minis at the synapse in synapse-specific plasticity, neuronal development, and learning and memory.

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