Synaptic Structure and Function at the *Drosophila* Larval Neuromuscular Junction: A Molecular Analysis of Complexin and Radish

By

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ABSTRACT

From yeast to humans, the fusion of vesicles with target membranes is driven by the formation of a parallel four-helix bundle of SNARE proteins that are present on both the vesicular (v-SNAREs) and target plasma membranes (t-SNAREs). The full zippering of this bundle is thought to provide the driving force for membrane fusion. At synapses, vesicle fusion is exquisitely regulated by Ca^{2+} such that neurotransmitter release can occur within 1 ms of an action potential reaching the presynaptic terminal. This feat implies the presence of both a Ca^{2+} sensor and a fusion clamp that prevents vesicles from fusing in the absence of Ca^{2+}. The presynaptic Ca^{2+} sensor for synchronous vesicle release is widely accepted to be Synaptotagmin-1 (Syt1), and there is growing evidence that Complexin (Cpx), which binds to the SNARE complex with high affinity and 1:1 stoichiometry, can act as a vesicle fusion clamp. As suggested by its name, however, Cpx appears to play a more complex role in vesicle release, carrying out different functions in spontaneous vs. evoked fusion events.

Here we show the Drosophila express at least two Cpx isoforms that differ in the C-terminus (Cpx7A and Cpx7B) and can be further regulated by RNA editing and phosphorylation. These isoforms show different effects on spontaneous vs. evoked neurotransmitter release, with Cpx7A being a better fusion clamp and Cpx7B being a better fusion promoter. In addition, these isoforms have different effects on synaptic growth, which may be linked to their effects on synaptic physiology.

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"But there were others who stood out from the conference crowd: young, self-confident individuals whose demeanor suggested they were going places. These were people for whom public speaking seemed to hold no fears. They gave talks that translated each short scientific life into one long success story. They collected new facts like a bee collected pollen. And they had their work routinely published in the distinguished pages of Nature and Science. They came from all corners but were united by a common bond. Who were these people? ... They were the ones who had chosen to work with fruit flies."

-Martin Brooks, Fly

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Chapter 1

A Molecular Understanding of Synaptic Vesicle Exocytosis

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Membrane fusion is an integral part of nearly all cellular processes in eukaryotes. Within cells, proper regulation of fusion allows for communication between membranous organelles and maintenance of functional subcompartments. In addition, external communication is made possible by the fusion of intracellular vesicles with the plasma membrane, exposing vesicular contents to the extracellular space. Nowhere is vesicle fusion more exquisitely regulated than at synapses in the nervous system. The temporal and spatial requirements of communication in the brain are met by cooperation between a number of molecular players acting upon a core machinery that underlies all types of membrane fusion. As this core machinery is thought to default to the "on" state to carry out fusion for basic cellular maintenance, synaptic vesicle fusion has a particular requirement for a "stop" signal to prevent unwanted synaptic transmission and a "go" signal to allow fusion at precisely the correct moment. Studies in a number of systems are now lending more and more support to the idea that these stop/go signals are not traceable to individual proteins but require cooperation between several proteins and, perhaps, even cooperation between multiple domains of the same protein.

The synaptic vesicle cycle and vesicle pools

Before addressing the larger topic of neurotransmitter release, I will begin with a review of synaptic vesicles and quantal neurotransmission at synapses. Fatt and Katz (1952) saw the first hints of quantal neurotransmission at the neuromuscular junction with their observation of spontaneous end plate potentials (EPPs). These spontaneous EPPs appeared to be scaled down versions of evoked EPPs with the

same time course and response to various drugs affecting cholinergic synapses. Thus, the term spontaneous miniature EPPs (mEPPs or, later, "minis") was coined to describe these events. Subsequent work by del Castillo and Katz (1954) showed that as external Ca^{2+} was reduced, the size of evoked EPPs was reduced, eventually reaching the size of the mEPPs. Under these low Ca^{2+} conditions, the amplitude of evoked EPPs varied such that each was an integer multiple of the mEPPs, giving rise to the quantal hypothesis of neurotransmission. Actual visualization of synaptic vesicles, the structural correlates of the "quanta," in the act of releasing neurotransmitters would have to wait nearly two decades. Using freeze-fracture techniques (as opposed to electron microscopy with conventionally fixed tissue), Heuser and co-workers were able observe individual vesicles fusing with the plasma membrane (Heuser and Reese, 1973; Heuser et al., 1974), consistent with the electrophysiological observations of Katz and other researchers.

Packaging neurotransmitters in vesicles comes with a number of important requirements that the neuron must meet. First, there must be a way to fill each vesicle with neurotransmitter, and filled vesicles must be reliably placed at release sites known as active zones and made competent to fuse with the plasma membrane. After fusion, vesicles must be recycled both to provide more vesicles for subsequent rounds of fusion and to prevent unwanted expansion of the plasma membrane. All of these requirements are met by the synaptic vesicle cycle (for review, see Südhof, 2004). The cycle begins when an action potential invades the presynaptic terminal and triggers synaptic vesicle fusion (Katz, 1969). Upon the

arrival of an action potential, voltage-gated Ca^{2+} channels open and cause a local increase in intracellular Ca^{2+} levels that triggers vesicle fusion with the plasma membrane and neurotransmitter release. As described above, even in the absence of an incoming action potential, single synaptic vesicles may spontaneously fuse with the plasma membrane, giving rise to miniature postsynaptic currents (Katz, 1969). Vesicle fusion triggered by Ca^{2+} influx occurs in two phases: a synchronous phase that begins as quickly as 50 μs after Ca^{2+} influx (Sabatini and Regehr, 1996) and an asynchronous phase consisting of an increased rate of vesicle fusion that continues for several hundred milliseconds after Ca^{2+} influx (Barrett and Stevens, 1972; Goda and Stevens, 1994). Following fusion, synaptic vesicles are retrieved (i.e., endocytosed) through multiple pathways that remain the subject of controversy and debate. Capacitance measurements at the calyx of Held have provided an understanding of the time course of synaptic vesicle retrieval (Sun et al., 2002). The rate of retrieval seems dependent on the number of unretrieved vesicles in the plasma membrane. Following spontaneous release events or release triggered by single or low-frequency trains of action potentials, vesicle retrieval occurs on a millisecond timescale. In contrast, release triggered by high-frequency trains of action potentials leads to retrieval on a timescale of seconds. This kinetic data suggests multiple modes of endocytosis, and in fact, these modes were first hypothesized over 35 years ago on the basis of biochemical studies with synaptosomes (Barker et al., 1972) and electron microscopy at the neuromuscular junction (Ceccarelli et al., 1973; Heuser and Reese, 1973). Subsequent studies have

shown that fast modes of retrieval with local recycling and refilling of vesicles predominate at low vesicle release frequencies, whereas slower, clathrin-mediated endocytosis predominates at high vesicle release frequencies (Koenig and Ikeda, 1996; Richards et al., 2000). Following fusion and retrieval, synaptic vesicles must be recycled, refilled, docked at release sites, and primed for another round of fusion. Vesicles can be recycled either locally or through endosomal intermediates. They are then reacidified via a vacuolar proton pump (Maycox et al., 1988), creating an electrochemical gradient that drives neurotransmitter uptake through one of a number of pumps, depending on the neurotransmitter (for review, see Südhof, 2004). The subsequent steps of vesicle "docking" at release sites and "priming" to become fusion competent are poorly understood at the molecular level but are thought to involve a number of the components of the membrane fusion machinery discussed below (for review, see Rizo and Rosenmund, 2008).

Synaptic vesicles in the presynaptic terminal are not all alike and differ in their functional properties and capacity for release (for review, see Rizzoli and Betz, 2005). The definition of vesicle pools has varied over time and between systems, but in general, it is well accepted that there are three distinct vesicle pools: the readily releasable pool, the recycling pool, and the reserve pool. The readily releasable pool consists of vesicles that are already docked at the active zone and primed for fusion immediately upon stimulation. Depletion of this pool occurs quickly with brief application of high frequency stimulation (Elmqvist and Quastel, 1965; Schneggenburger et al., 1999; Delgado et al., 2000; Richards et al., 2003),

depolarization (Neves and Lagnado, 1999), or hypertonic solution (Rosenmund and Stevens, 1996). The recycling pool is thought to sustain release at more moderate levels of stimulation (i.e., those that are more likely to be encountered physiologically). During such stimulation, this pool is recruited and refilled with newly recycled vesicles (Harata et al., 2001; Kuromi and Kidokoro, 2003; Richards et al., 2003). The majority of synaptic vesicles, however, are held in the reserve pool. This pool is seemingly only recruited upon intense stimulation (for review, see Rizzoli and Betz, 2005). Kuromi and Kidokoro (1998) used the *Drosophila* temperature-sensitive dynamin mutant *shibire*, which lacks endocytosis above the restrictive temperature of 34°C, to show that vesicles were recruited from the reserve pool during stimulation at 34°C once the recycling pool was depleted, but not during stimulation at room temperature. These findings are consistent with work at the frog NMJ showing that reserve pool vesicles are almost never recruited until the recycling pool has been depleted (Richards et al., 2000; Richards et al., 2003).

Some studies have suggested that the different properties of evoked vs. spontaneous fusion events can be explained by the use of different vesicle populations. Recent data, however, has shown that both modes of release rely on the same vesicle pool. When the quantal hypothesis of neurotransmitter release was first postulated (del Castillo and Katz, 1954), it was assumed that the same quanta (or vesicles) were released during spontaneous and evoked fusion events. This assumption was maintained over several decades. More recently, work at hippocampal synapses showed that vesicles labeled with the styryl dye AM1-44

during spontaneous endocytosis are more likely to be released spontaneously than vesicles labeled following a stimulus. In addition, blocking vesicle refilling with the vacuolar ATPase inhibitor folimycin preferentially depletes neurotransmitter from spontaneously fusing vesicles (Sara et al., 2005). Subsequent studies found differential release properties (Mathew et al., 2008) and localization (Chung et al., 2010) of vesicles loaded with styryl dyes by different protocols (e.g., spontaneous vs. stimulus-driven). In addition, labeling vesicles with a biotinylated version of the synaptic vesicle protein synaptobrevin (biosyn) has suggested that separate vesicle pools supply different modes of release (Fredj and Burrone, 2009). On the other hand, separately labeling vesicles endocytosed spontaneously and in response to stimulation with spectrally separable styryl dyes has shown that both populations are released at the same rate following trains of action potentials (Groemer and Klingauf, 2007). To address this controversy, Wilhelm et al. (2010) tested all possible combination of styryl dye loading and unloading (evoked/evoked, evoked/spontaneous, spontaneous/evoked, and spontaneous/spontaneous) in four different preparation (the NMJs of mice, frogs, and *Drosophila* third-instar larvae and cultured hippocampal neurons) and found similar levels of dye unloading (i.e., vesicle fusion) under all conditions, consistent with the same pool of vesicles supplying both spontaneous and evoked fusion. Similar results have been observed with biotinylated antibodies against the lumenal portion of the synaptic vesicle protein synaptotagmin and with synaptopHluorin, a pH-sensitive GFP attached to the lumenal portion of synaptobrevin. Thus, while there may be some conditions and

protocols in which vesicles are preferentially recruited for spontaneous or evoked fusion, there does not appear to be precise separation between vesicles for each release mode.

The core fusion machinery: SNARE proteins

Early studies of vesicle fusion *in vitro* led to the purification of NSF (N-ethylmaleimide–sensitive factor) and SNAP (soluble NSF attachment protein), both of which were found to be necessary for vesicle fusion (Malhotra et al., 1988; Wilson et al., 1989; Clary et al., 1990). Subsequently, the SNARE proteins were identified on the basis of their ability to bind to NSF and SNAP (thus the name SNAp REceptor) (Sollner et al., 1993). At synapses, there are two SNARE proteins associated with the plasma membrane, the target-SNAREs (t-SNAREs) SNAP-25 and syntaxin, and one SNARE protein associated with the vesicle membrane, the vesicle-SNARE (v-SNARE) synaptobrevin. The clostridial neurotoxins botulinum and tetanus toxins, which cleave the SNAREs and block neurotransmission (Blasi et al., 1993a; Blasi et al., 1993b; Schiavo et al., 1993), highlight the central role of the SNAREs in synaptic vesicle fusion. Homologs of the synaptic SNAREs are expressed in different cellular compartments, such that a limited number of SNARE combinations allows for some measure of fusion specificity (Fukuda et al., 2000; McNew et al., 2000; Parlati et al., 2000).

The individual SNARE proteins have unremarkable structure on their own but zipper together into a highly stable four-helix coiled-coil bundle (known as a trans-SNARE complex or a SNAREpin) with syntaxin and synaptobrevin each contributing

one alpha helix and SNAP-25 contributing two (Sutton et al., 1998). The orientation of SNAREs in the vesicle and target membranes is such that zippering of the SNAREpin brings these membranes into close apposition, the first step in fusion. The length of the linker between the transmembrane portion of the SNAREs and the SNAREpin is critical for fusion. Increasing the length of this linker has been shown to inhibit fusion, likely by eliminating close membrane apposition (McNew et al., 1999; Deák et al., 2006). The energy of SNAREpin zippering is thought to provide the driving force to overcome the energy barrier of membrane fusion, which is quite high given the necessary mixing of polar head groups and hydrophobic tails of phospholipids. Each zippered SNAREpin provides on the order of 35 k_BT of energy (Li et al., 2007), and the activation energy of membrane fusion has been estimated to be on the order of 40-140 k_BT (Cohen et al., 2004). Therefore, at least two to three SNARE complexes would be needed to provide the requisite energy for membrane fusion. Current estimates indicate that three to ten SNAREpins are required to achieve proper fusion kinetics (Mohrmann et al., 2010; Karatekin et al., 2010).

After membrane fusion, the SNAREs are held in what is called the cis-SNARE complex and must be released and recycled to participate in subsequent rounds of vesicle fusion. NSF is a hexameric ATPase that can indirectly bind to SNARE proteins via the adapter protein SNAP (Sollner et al., 1993). The cis-SNARE complex is disassembled by NSF (Mayer et al., 1996), releasing the individual SNARE proteins. The t-SNAREs and v-SNAREs must then be sorted and separated so that t-SNAREs remain at release sites, while v-SNAREs are endocytosed and

incorporated into newly formed synaptic vesicles. The mechanisms underlying these trafficking events remain unclear.

SM (Sec1/Munc18-like) proteins: SNARE chaperones

When the synaptic SM protein Munc-18 was found to bind to the synaptic t-SNARE syntaxin-1 (Hata et al., 1993), its potential role in membrane fusion was unknown. In addition to its SNARE domain, syntaxin contains an Habc domain consisting of three alpha helices that fold back onto the SNARE domain and holds syntaxin in a "closed" state in which it cannot interact with other members of the SNARE complex (Fernandez et al., 1998; Misura et al., 2000). Munc-18 binds to the four-helix bundle formed between the Habc domain and the SNARE domain of syntaxin, maintaining the "closed" state (Dulubova et al., 1999). Given this configuration, Munc-18 was originally hypothesized to be a negative regulator of vesicle fusion; however, studies in multiple systems have identified SM proteins as positive regulators of fusion (for example, see Brenner, 1974; Novick et al., 1980). In fact, genetic deletion of Munc-18 inhibits vesicle fusion even more than deletion of the v-SNARE synaptobrevin (Verhage et al., 2000), suggesting a critical role of Munc-18 in promoting fusion. The potential mechanistic basis of this role was provided by additional structural studies showing an interaction between Munc-18 and the N-terminus of syntaxin that would allow Munc-18 to bind to the four-helix bundle of the SNARE complex instead of "closed" syntaxin (Dulubova et al., 2002; Yamaguchi et al., 2002). This interaction between Munc-18 and the SNARE complex may stabilize the zippering SNAREpin and promote fusion by some as yet

undetermined mechanism. Together, SNARE proteins and SM proteins constitute the core machinery for all modes of membrane fusion.

Munc13 and RIM1α: "Priming" the release machinery

The events leading up to neurotransmitter release that make vesicles competent to fuse with the plasma membrane in response to an incoming action potential are grouped together under the somewhat nebulous umbrella "priming." Among the proteins thought to be important for priming, Munc13-1 and RIM1α are particularly noteworthy given the dramatic effects of their absence on neurotransmitter release. In mice, the deletion of both Munc13-1 and Munc13-2 eliminates both spontaneous and evoked neurotransmitter release (Varoqueaux et al., 2002). Notably, this phenotype can be rescued by expression of a minimal region of Munc13-1 termed the MUN domain, highlighting the key role of this region in the function of the protein (Basu et al., 2005). On the other hand, deletion of RIM1α in mice has much milder effects on neurotransmitter release than deletion of Munc13 (Schoch et al., 2002), although several forms of synaptic plasticity are disrupted in RIM1α mutants (reviewed in Rizo and Rosenmund, 2008). Interestingly, in *C. elegans* the synaptic defects in null mutants of the Munc13 homolog unc-13 and the RIM1α homolog unc-10 can be rescued by a syntaxin mutation that maintains the protein in an "open" conformation (Richmond et al., 2001). These findings suggest that Munc13 and RIM1α both act to convert syntaxin from a "closed" to an "open" state, making it available for SNARE complex formation. In fact, a direct interaction has been demonstrated between the N-termini of Munc13-1 and RIM1α, and

disrupting this interaction results in defects in vesicle priming (Betz et al., 2001). In addition, a tripartite complex can be formed between Munc13-1, RIM1α, and Rab3A (Dulubova et al, 2005), which is itself required for multiple forms of synaptic plasticity. The precise function of this complex is unclear, but it may regulate spatial or temporal aspects of Munc13-1 and RIM1α function (Dulubova et al, 2005).

Synaptotagmin: The Ca^{2+} sensor

Given its low intracellular concentration at baseline (in the submicromolar range), Ca^{2+} is an ideal second messenger and plays a critical role in multiple cellular processes ranging from signal transduction to muscle contraction. In the nervous system, Ca^{2+} influx determines the timing of neurotransmitter release from presynaptic terminals, and synaptotagmin is widely regarded to be the Ca^{2+} sensor that triggers evoked neurotransmitter release. Synaptotagmin is a synaptic vesicle protein with a single membrane-spanning domain and two Ca^{2+}-binding C_2 domains, C_2A and C_2B (Perin et al., 1991). It was first hypothesized to be the Ca^{2+} sensor for fast neurotransmitter release based on its localization and Ca^{2+} and phospholipid binding properties (Brose et al., 1992) but has since been shown to be involved in several aspects of the synaptic vesicle cycle, including docking and endocytosis (reviewed in Chapman, 2008). Early genetic knockout studies of synaptotagmin-1 in *Drosophila*, *C. elegans*, and mice showed impairment of synaptic transmission, but different conclusions were drawn as to whether synaptotagmin was the Ca^{2+} sensor for fast neurotransmitter release (reviewed in Koh and Bellen, 2003). The critical piece of evidence in support of synaptotagmin-1 as the Ca^{2+} sensor for fast

neurotransmitter release came from studies showing that a point mutation in synaptotagmin-1 that reduced its Ca^{2+} affinity caused a concurrent reduction in the Ca^{2+} sensitivity of neurotransmitter release (Fernandez-Chacon et al., 2001). Synaptotagmin can bind simultaneously to Ca^{2+}, plasma membrane phospholipids, and membrane-bound SNARE complexes (Dai et al., 2007), putting it in an ideal position to translate Ca^{2+} binding into membrane fusion.

A role for synaptotagmin-1 as a vesicle fusion clamp has also been proposed. In support of this idea, several groups have reported an increase in the frequency of spontaneous vesicle fusion events at the *Drosophila* larval NMJ in synaptotagmin-1 null animals (Littleton et al., 1994; DiAntonio et al., 1994; Mackler et al., 2002). In synaptotagmin knockout mice, although no increase in the frequency of spontaneous fusion was reported in hippocampal neurons grown on microislands (Geppert et al., 1994), a subsequent study of cultured cortical neurons did find such an increase (Pang et al., 2006). A model is now emerging in which apo-synaptotagmin-1 (i.e., synaptotagmin-1 not bound to Ca^{2+}) acts as a fusion clamp and, upon binding Ca^{2+}, subsequently promotes fusion. This idea has been supported by data from liposome fusion assays (Chicka et al., 2009).

Complexin: The fusion clamp that promotes fusion?

Of all the proteins involved in neurotransmitter release, perhaps none has been more controversial in recent years than complexin. Seemingly divergent results in different experimental systems have given rise to two camps: those who view complexin as a fusion clamp and those who consider its function to be promoting

fusion. Most recently, a more nuanced view of complexin has emerged in which it exerts different effects on different modes of vesicle fusion, but several unresolved issues still remain.

Identifying complexins and early functional studies

Complexin was originally isolated from rat brain homogenates based on its ability to bind to the SNARE complex (McMahon et al., 1995). Subsequent X-ray crystallography studies showed a complexin fragment bound by its central helix to the groove between synaptobrevin and syntaxin in the assembled SNARE complex (Chen et al., 2002; Bracher et al., 2002). The structure of the far N-terminus and C-terminal half of complexin, however, remains a mystery. In mammals, complexins 1 and 2 were the first to be characterized (McMahon et al., 1995), followed nearly a decade later by complexins 3 and 4 (Reim et al., 2005). All complexin isoforms are small, highly charged proteins, and complexin 2 belongs to the uncommon class of proteins that show 100% amino acid identity between mice, rats, and humans, suggesting a critical functional role (McMahon et al., 1995). Complexin 1 and 2 transcripts are highly enriched in the brain, and their protein products can be found in overlapping yet distinct subdomains. In addition, complexin 1 is expressed in the testis, and complexin 2 is present at low levels in all tissues (McMahon et al., 1995). In contrast, complexins 3 and 4, which share only 24-28% amino acid identity with complexins 1 and 2, are concentrated at ribbon synapses in the retina (Reim et al., 2005). In fact, complexin 4 expression appears to be entirely restricted to the retina, whereas complexin 3 is also expressed elsewhere throughout the brain, particularly

in the hippocampus and cerebellum (Reim et al., 2005). Interestingly, complexins 3 and 4 share a C-terminal CAAX-box motif known to be a consensus sequence for post-translational farnesylation (for review, see Zhang and Casey, 1996). This motif is absent from complexins 1 and 2, but present in complexin isoforms from a number of other species, including *Drosophila*, and may represent a functional specialization necessary at the unique retinal ribbon synapse where complexins 3 and 4 are expressed (Reim et al., 2005).

The earliest functional studies of complexins were carried out using *Aplysia* buccal ganglia, which have large cell bodies conducive to microinjection of compounds that can easily diffuse to synapses and affect synaptic transmission. In this preparation, presynaptic injection of a monoclonal antibody against complexin 2 leads to an increase in the amplitude of postsynaptic IPSCs in response to single evoked presynaptic action potentials. In contrast, injection of recombinant complexin 2 leads to a decrease in IPSC amplitude (Ono et al., 1998). These effects of complexin 2 injection are the opposite of those observed for α-SNAP injection. In fact, the effect of complexin 2 injection can be reversed by α-SNAP injection and vice versa (Ono et al., 1998). These findings suggest that complexin 2 and α-SNAP have opposing effects on the same substrate and that complexin 2 has an overall effect of inhibiting neurotransmitter release. Perhaps no subsequent studies have equaled these early experiments in terms of acute intervention, which is necessary to avoid the complication of compensatory mechanisms. Antibody injection,

however, is a somewhat crude method of protein inactivation and can have unintended effects.

Additional evidence for complexin function has come from studies in PC12 cells in which acetylcholine release from small synaptic vesicles is inhibited by overexpression of either complexin 1 or 2 (Itakura et al., 1999). Furthermore, complexin 2 overexpression in adrenal chromaffin cells leads to a reduction in the number of evoked exocytotic events and a reduction in the amount of catecholamines released during a single exocytotic event as measured by carbon fiber amperometry. Taken together, the early studies of complexin function favor the idea that complexin is a negative regulator of transmitter release.

In vitro fusion assays of complexin function

In vitro systems have the advantage of well-defined components, allowing the effects of individual components and interactions between components to be evaluated with a precision that is difficult using *in vivo* systems. Two types of *in vitro* approaches have been used to study complexin function: lipid mixing by liposome fusion (Weber et al., 1998) and cell fusion by flipped SNAREs (Hu et al., 2003). Lipid mixing by liposome fusion involves the generation of a population of v-SNARE liposomes containing synaptobrevin and a population of t-SNARE liposomes containing syntaxin and SNAP-25. In addition, the lipid component of the v-SNARE liposomes is labeled with a quenched mixture of fluorophores, such that when a v-SNARE liposome fuses with a t-SNARE liposome, lipid mixing dilutes and unquenches the fluorophores, and the increase in fluorescence can be used to track

fusion kinetics. As an added level of analysis, pretreating the v-SNARE liposomes with the reducing agent sodium dithionite permanently quenches the fluorophores in the outer leaflet, allowing inner and outer leaflet mixing of the lipid bilayers to be measured separately. On the other hand, cell fusion with flipped SNAREs involves transfection of COS cells with constructs expressing a v-SNARE or both t-SNAREs in a "flipped" orientation with their SNARE domains located extracellularly. The v-SNARE cells are additionally labeled with cytosolic RFP, and the t-SNARE cells are labeled with nuclear CFP, allowing fusion to be tracked by identifying cells with cyan nuclei in a red cytosol. Unlike liposomes in which the orientation of the incorporated SNARE proteins is stochastic, cells expressing flipped SNAREs allow precise control over SNARE orientation and placement in the membrane.

A liposome fusion assay was used by Schaub et al. (2006) to study the effects of complexin and synaptotagmin on SNARE-mediated fusion. Using mouse complexin 4 and *Drosophila* complexin 7A (both of which contain the C-terminal farnesylation motif), they found that both complexins greatly inhibited liposome fusion driven by the neuronal SNAREs syntaxin-1A, SNAP25b, and synaptobrevin-2. In fact, fusion was arrested at the hemifusion step at which the outer leaflet lipids had mixed, but the inner leaflet lipids had not. The addition of synaptotagmin and Ca^{2+} relieved this hemifusion arrest and allowed full liposome fusion to proceed rapidly. These results support a model in which complexin blocks neuronal SNARE-mediated fusion, while synaptotagmin removes this block in the presence of Ca^{2+}. Notably, liposome fusion studies do not recapitulate the membrane curvature of a

synaptic vesicle fusing with an essentially planar plasma membrane, and it is unclear whether *Drosophila* complexin 7A interacts differently with mouse SNARE proteins than with endogenous *Drosophila* SNAREs. At the same time, Giraudo et al. (2006) used cell fusion by flipped SNAREs to show that both soluble and membrane-anchored mouse complexin 1 could robustly block cell fusion and that this block could be overcome by release of the complexin 1 anchor and the addition of synaptotagmin and Ca^{2+}. These results were essentially identical to those of Schaub et al. (2006) and further strengthened the idea of complexin as a negative regulator of SNARE-mediated fusion.

In subsequent work, Malsam et al. (2009) used a liposome fusion assay to study all four mouse complexins along with *Drosophila* complexin 7A and found that mouse complexins 1 and 2 actually promoted liposome fusion, whereas mouse complexins 3 and 4 and *Drosophila* complexin 7A (all of which contain the C-terminal farnesylation motif) all inhibited liposome fusion. Interestingly, when the C-terminus of complexin 1 was transferred to complexin 3, this complexin 3/1 chimeric protein promoted liposome fusion. These data suggest that the effects of complexin on membrane fusion are isoform-specific and may be critically dependent on the presence or absence of a C-terminal farnesylation motif. Furthermore, these results apparently contradict the earlier cell fusion study of Giraudo et al. (2006) in terms of the role of complexin 1. In that study, however, complexin 1 was most potent as an inhibitor of cell fusion when it contained a C-terminal GPI anchor, again highlighting the potential importance C-terminal farnesylation for complexin function.

Reductionist systems such as liposome fusion and cell fusion by flipped SNAREs, however, are by their very nature artificial, and results obtained in such systems must be corroborated by *in vivo* evidence.

Complexin knock-out/down studies

A complexin 2 knockout (KO) mouse was the first reported genetic disruption of complexin (Takahashi et al., 1999). Given the 100% amino acid conservation of complexin 2 among mice, rats, and humans, it was somewhat surprising that these mice showed normal hippocampal synaptic transmission and short-term plasticity as measured by paired-pulse facilitation. Long-term potentiation (LTP) was reduced, however, in both CA1 and CA3. To avoid compensation by complexin 1, double knockout (DKO) mice for complexins 1 and 2 were generated, and their synaptic transmission properties were studied in hippocampal autapses (Reim et al., 2001). The DKO mice showed a dramatic reduction in evoked EPSC amplitude, but no change in the rate of spontaneous fusion events or the amplitude of mEPSCs. The size of the readily-releasable vesicle pool in the DKO mice was also unchanged relative to controls, suggesting that complexin acts to promote release at a post-priming step. In support of this idea, the DKO mice also showed initial facilitation in response to a train of action potentials, indicating a reduced release probability, and reduced Ca^{2+} sensitivity of neurotransmitter release. These results are apparently at odds with earlier *in vitro* studies and cell/liposome fusion assays, all of which suggested a negative role of complexin in neurotransmitter release. This difference could have been due to the nonphysiological nature of the *in vitro* studies or possible

compensation by complexin 3, which is also expressed throughout the brain, in the DKO mice.

Analysis of the *Drosophila* knockout of complexin added to the controversy surrounding complexin function (Huntwork and Littleton, 2007). *Drosophila* has only a single complexin gene, eliminating the possibility of compensation by other isoforms and simplifying the phenotypic analysis. Synaptic transmission was evaluated at the third-instar larval neuromuscular junction (NMJ) of complexin knockout animals, revealing a dramatic increase (>20-fold) in the frequency of spontaneous vesicle fusion events. Even after taking into account the 64% increase in the number of release sites in the knockout animals, this change in mini frequency represents a substantial increase in the probability of spontaneous vesicle fusion. This finding was perhaps surprising given the lack of any effect on spontaneous fusion events observed in the complexin 1/2 DKO mice (Reim et al., 2001). On the other hand, evoked neurotransmitter release was reduced at elevated Ca^{2+} levels (>0.25 mM) but remained unchanged at lower Ca^{2+} levels, indicating a reduction in the Ca^{2+} sensitivity of neurotransmitter release similar to that seen in the complexin 1/2 DKO mice (Reim et al., 2001). The spontaneous fusion phenotype of the *Drosophila* complexin knockout is highly indicative of a role for complexin as a vesicle fusion clamp, but it is unclear why a similar phenotype was not observed in mice. Again, it is possible that compensation by complexin 3 is to blame, but it could also be that cultured hippocampal autapses are functionally different from the larval NMJ. Regardless, analysis of the *Drosophila* complexin knockout represents one of

the first indications that complexin might have different effects on two different modes of vesicle release: spontaneous and evoked. This idea was further supported by data from *C. elegans* complexin-1 knockout animals, which showed a 3-fold increase in the rate of tonic fusion at a graded synapse, but a 10-fold decrease in the amplitude of evoked fusion events (Martin et al., 2011; Hobson et al., 2011).

The generation of complexin 1/2/3 triple knockout (TKO) mice finally addressed the issue of potential compensation by complexin 3 in the DKO mice but increased the growing controversy surrounding complexin function. Using autaptic hippocampal cultures, Xue et al. (2008) reported a decrease in evoked EPSC amplitude and in the Ca^{2+} sensitivity of neurotransmitter release in the TKO mice, similar to that seen in the DKO mice (Reim et al., 2001); however, they also reported a ~30% decrease in spontaneous EPSC frequency in both the DKO and TKO mice. Notably, the original report on the DKO mice did not find any effect on spontaneous EPSCs using the same autaptic culture system (Reim et al., 2001). This decrease in spontaneous EPSC frequency in the TKO and DKO mice was also observed at inhibitory and excitatory synapses in brainstem slices of the pre-Bötzinger complex. The size of the readily-releasable vesicle pool was unchanged in the DKO and TKO mice relative to controls; thus, the reduction in both evoked EPSC amplitude and spontaneous ESPC frequency indicates a decrease in the probability of vesicle release. It remains unclear why there is an apparent difference in the effects of complexin on vesicle fusion between mice and flies. Compensation by other members of the synaptic vesicle release machinery could be at play in both systems,

and it is possible that autaptic synapses do not behave as endogenous synaptic connections. There is agreement between flies and mice, however, in the apparent effects of complexin on evoked neurotransmitter release, namely, to increase the Ca^{2+} sensitivity of release and the probability of vesicle fusion.

Acute complexin knockdown studies have also been carried out using short hairpin RNA (shRNA), making developmental compensation by other complexins or members of the vesicle release machinery less likely. Maximov et al. (2009) used a lentiviral expression system to express an shRNA targeting both complexins 1 and 2 in cultured cortical neurons from mice and found a 3- to 4-fold increase in the frequency of spontaneous EPSCs and a 3- to 4-fold decrease in the amplitude of evoked EPSCs, similar to – although less severe than – the phenotypes observed at the *Drosophila* NMJ (Huntwork and Littleton, 2007). It is unclear why the mouse knockout and knockdown studies of complexin function came to such different conclusions regarding the role of complexin in spontaneous vesicle fusion. It may be due to differences between hippocampal autapses and cultured cortical neurons. Alternatively, the acute nature of the shRNA knockdown may have avoided compensation by other members of the vesicle release machinery or the knockdown itself may have been incomplete.

Complexin domain studies

With so many studies showing different effects of complexin on different kinds of vesicle release (spontaneous vs. evoked) in different species and different synapses, studies of complexin subdomains have become necessary to clarify its

seemingly multiple roles. The field has generally looked at complexin as having four distinct domains, the far N-terminus (~30 residues) followed by the accessory helix (~20 residues), the central helix (~20 residues), and the C-terminus (~65 residues). The central helix is clearly required to bind to the SNARE complex as suggested by crystal structures (Chen et al., 2002; Bracher et al., 2002), and constructs that lack this domain or key binding residues within it fail to rescue complexin knockout or knockdown synapses (Xue et al., 2007; Maximov et al., 2009; Martin et al., 2011) and fail to clamp cell fusion by flipped SNAREs as observed with wild type (WT) complexin (Giraudo et al., 2008). Binding to the SNARE complex alone, however, does not appear to be sufficient to recapitulate complexin function (Xue et al., 2007).

The N-terminus and accessory helix, meanwhile, seem to promote and clamp fusion, respectively, and balance one another in the context of full length complexin. When both are absent, only mild effects on complexin function are observed in mouse hippocampal autaptic cultures, whereas complexin constructs lacking only the N-terminus (residues 1-27) lead to a full loss-of-function phenotype, suggesting that the N-terminus promotes neurotransmitter release (Xue et al., 2007). Even constructs lacking fewer N-terminal residues (1-15 and 1-7) fail to rescue the complexin null phenotype in autaptic cultures (Xue et al., 2010). Interestingly, how much of the N-terminus is deleted appears to influence complexin function in *C. elegans*. When only the first 15 residues of the N-terminus of complexin-1 were deleted (equivalent to mouse residues 1-14), the frequency of spontaneous vesicle fusion was greatly reduced and the amplitude of evoked EPSCs was actually

increased at the NMJ, suggesting that the N-terminus promotes spontaneous release but inhibits evoked release (Hobson et al., 2011). Meanwhile, when the first 22 residues were deleted (equivalent to mouse residues 1-21), there was little effect on aldicarb sensitivity (a measure of acetyl choline release) or locomotion (Martin et al., 2011). These studies could indicate differences between hippocampal autapses and the *C. elegans* NMJ or that the N-terminus of complexin has multiple roles in neurotransmitter release. There is some structural basis for the hypothesis that the N-terminus promotes neurotransmitter release. Xue et al. (2010) used NMR spectroscopy to identify residues of the N-terminus of complexin that interact with the membrane-proximal C-terminus of the SNARE complex, perhaps influencing the force transfer from the SNARE complex that drives membrane fusion. There is also evidence to suggest how the accessory helix clamps fusion. Giraudo et al. (2009) noted that the accessory helix of complexin has substantial similarity to synaptobrevin and might replace synaptobrevin in the SNARE complex, blocking full zippering and thus clamping fusion. In fact, introducing mutations into complexin to increase its similarity to synaptobrevin also increased its clamping function in cell fusion assays (Giraudo et al., 2009).

The C-terminus of complexin is the most mysterious domain in terms of both structure and function. In hippocampal autapses, a complexin construct lacking the C-terminus was fully able to rescue the complexin null phenotypes (Xue et al., 2007), suggesting either that the C-terminus is not critical for complexin function or that the C-terminus harbors balanced, opposing functions similar to those described for the

N-terminus and accessory helix. Constructs lacking the entire C-terminus fail to clamp cell-cell fusion by flipped SNAREs as well as full length complexin, predicting a negative influence of the C-terminus on vesicle release (Giraudo et al., 2008). In *C. elegans*, meanwhile, a complexin construct lacking the C-terminus failed to rescue the aldicarb sensitivity of complexin-1 null animals and only partially rescued their locomotor defects, indicating an important role of the C-terminus in inhibiting neurotransmitter release at the NMJ (Martin et al., 2011), consistent with findings from cell fusion assays. Interestingly, rat complexin 1 can be phosphorylated both *in vitro* (by protein kinase CK2) and *in vivo* at serine-115 in the C-terminus, and this phosphorylated form of complexin binds to the assembled SNARE complex with greater affinity than unphosphorylated complexin (Shata et al., 2007). In addition, phosphomimetic mutations in serine-115 reduce the ability of complexin 1 to promote liposome fusion (Malsam et al., 2009). Furthermore, the CK2 inhibitor 5,6-dichlorobenzimidazole riboside causes a nearly 100-fold increase in the frequency of spontaneous neurotransmitter release at the frog NMJ (Rizzoli and Betz, 2002). These findings provide a biochemical basis for how the C-terminus of complexin may influence synaptic vesicle release and suggest that phosphorylation of complexin by CK2 may increase its ability to inhibit spontaneous neurotransmitter release.

Cross-species studies of Complexin

As the field has trended toward viewing complexin as a protein of domains with distinct inhibitory and facilitatory functions in neurotransmitter release, an idea has emerged that perhaps the differences in phenotype between the mouse and

Drosophila complexin KO animals could be explained by different weighting of these domains, such that *Drosophila* complexin functions more like a clamp overall, whereas mouse complexins have more facilitatory roles (Xue et al., 2009). In support of this idea, *Drosophila* complexin 7A nearly abolished both spontaneous and evoked neurotransmitter release when expressed at complexin TKO hippocampal autapses and severely reduced EPSC amplitude and mEPSC frequency when overexpressed at WT hippocampal autapses (Xue et al., 2009). In contrast, overexpression of mouse complexin 1 at WT hippocampal autapses was previously shown to have no effect on neurotransmitter release (Xue et al., 2007). Reverse experiments have also been done involving the expression of mouse complexins at the *Drosophila* third instar larval NMJ. In complexin null larvae, mouse complexins 1, 2, and 3 were not able to fully rescue the increased frequency of spontaneous neurotransmitter release but actually rescued the decreased amplitude of evoked EPSCs to beyond WT levels (Xue et al., 2009; Cho et al., 2010). On the other hand, mouse complexin 4 was better able to rescue the increased frequency of spontaneous neurotransmitter release, despite being expressed at lower than WT levels, but only rescued the amplitude of evoked EPSCs to WT levels (Cho et al., 2010).

Studies have also been performed using chimeric constructs of mouse and *Drosophila* complexins. Removal of the entire C-terminus of *Drosophila* complexin 7A or simply mutating its farnesylation motif blocked its ability to inhibit release in autaptic cultures, and only its ability to inhibit spontaneous release was restored

when the C-terminus of mouse complexin 1 was substituted for the *Drosophila* complexin 7A C-terminus (Xue et al., 2009). When only the farnesylation motif of *Drosophila* complexin 7A was replaced with the final four residues of mouse complexin 1, this chimeric construct was unable to inhibit the increased frequency of spontaneous neurotransmitter release in complexin null animals at the *Drosophila* NMJ but increased evoked ESPC amplitudes to greater than WT levels (Cho et al., 2010). Similar substitution with the entire C-terminus of mouse complexin 3, which contains a farnesylation motif like that of *Drosophila* complexin 7A, resulted in promotion of both spontaneous and evoked neurotransmitter release in hippocampal autapses (Xue et al., 2009). Clearly, the mere presence of a farnesylation motif is not enough to restore the inhibitory functions of the C-terminus of *Drosophila* complexin 7A. Similar studies have been done with substitutions of the *Drosophila* complexin 7A N-terminus and accessory helix. When both of these domains were replaced with the analogous mouse complexin 1 sequences, the ability of *Drosophila* complexin 7A to inhibit both evoked and spontaneous neurotransmitter release was somewhat reduced in hippocampal autapses; however, substituting only the far N-terminus had no effect (Xue et al., 2009), suggesting that the accessory helix of *Drosophila* complexin 7A has an inhibitory effect on neurotransmitter release stronger than that mediated by the accessory helix of mouse complexin 1 (Xue et al., 2007). These studies indicate that the "weighted domains" model of complexin function across species may explain some of the differences observed between mouse and *Drosophila* KO animals, but all cross-species experiments are hindered

by the fact that proteins may not behave normally when placed in the cellular milieu of another species. This point is especially critical for protein-protein interactions such as those between complexin and the SNARE complex.

Complexin/synaptotagmin/SNARE biochemistry

Fast neurotransmitter release in response to Ca^{2+} influx clearly requires both complexin and synaptotagmin acting on the SNARE complex, and both of these proteins seem to have both facilitatory and inhibitory roles in membrane fusion. The mechanism by which these two proteins work in concert to orchestrate synaptic vesicle fusion, however, remains unclear. Complexins 1 and 2 were identified on the basis of their ability to bind the assembled SNARE complex, and early biochemical studies showed that they also bind to isolated syntaxin, but not synaptobrevin or SNAP-25, with lower affinity than to the assembled complex (McMahon et al., 1995). These binding characteristics are similar to those of Munc-18 and synaptotagmin. Furthermore, both complexins and synaptotagmin compete with α-SNAP, but not with each other, for syntaxin binding (McMahon et al., 1995), consistent with the idea that complexin and synaptotagmin act at a post-priming step in membrane fusion, separate from α-SNAP (Söllner et al., 1993). Subsequent studies have shown that complexin can bind to the SNAP-25:syntaxin acceptor complex on target membranes, although with lower affinity than to the fully assembled SNARE complex (Guan et al., 2008; Weninger et al., 2008). This interaction with the SNAP-25:syntaxin acceptor would be critical for complexin to function as a clamp, such that it is already in place before synaptobrevin arrives and full SNARE zippering and

membrane fusion commence. Similarly, synaptotagmin can bind the SNAP-25:syntaxin acceptor complex in addition to isolated SNAP-25 and syntaxin (Rickman et al., 2004; Bhalla et al., 2006; Dai et al., 2007; Lynch et al., 2007). The proposed mechanisms for the interplay between complexin, synaptotagmin, and the SNARE complex have revolved around whether complexin and synaptotagmin can bind to the SNARE complex simultaneously. The earliest models favored sequential binding in which complexin acts to promote the assembly of SNARE complexes but blocks full fusion until it is displaced by Ca^{2+}-synaptotagmin (Tang et al., 2006; Giraudo et al., 2006; Schaub et al., 2006; Dai et al., 2007; Giraudo et al., 2008). Evidence for this model comes from the observation that complexin is able to displace assembled SNARE complexes from GST-synaptotagmin in GST-pull down assays and able to displace synaptotagmin from purified native SNARE complexes. More pertinently, synaptotagmin is able to displace complexin from membrane-embedded SNARE complexes in the presence of Ca^{2+} (Tang et al., 2006). Furthermore, synaptotagmin is able to relieve the complexin fusion clamp when added to liposome (Schaub et al., 2006) and cell fusion assays (Giraudo et al., 2006). This sequential model was challenged, however, by recent evidence that complexin and synaptotagmin can bind to assembled SNARE complexes concurrently (Chicka et al., 2009). In coflotation assays with liposomes lacking phosphatidyl serine, which binds synaptotagmin with high-affinity, synaptotagmin was able to bind to assembled SNARE complexes already saturated with complexin. In fact, neither synaptotagmin nor complexin affected the extent of binding of the

other to assembled SNARE complexes over the range of concentrations tested. Furthermore, Chicka et al. (2009) showed that apo-synaptotagmin blocked fusion in a liposome fusion assay and that this block occluded any additional block by the addition of complexin. These results were interpreted to indicate that apo-synaptotagmin acts as a fusion clamp early in SNARE assembly, whereas complexin acts as a clamp later in SNARE assembly. The precise localization of complexin, however, and the timing of its arrival during SNARE assembly are unknown, and it remains possible that complexin is present on SNAP-25:syntaxin acceptor complexes and acts as a fusion clamp early in SNARE assembly. Live imaging in PC12 cells has shown that the fluorescent signal of GFP-tagged complexin 2 peaks at sites of exocytosis with the onset of neuropeptide Y release from secretory granules and then disappears by lateral spreading, presumably due to diffusion of cis-SNARE complexes away from release sites (An et al., 2010). Interestingly, complexin appears absent from non-fusing secretory granules in this system, calling into question its role as a fusion clamp. Increasing levels of complexin, however, progressively reduce the frequency of fusion events, indicating some negative role for complexin in exocytosis from secretory granules.

Summary

The precise timing of neurotransmitter release in response to an arriving action potential is the key feature of synapses that allows for communication and signaling in the nervous system. It is well accepted that neurotransmitters are stored in and released from synaptic vesicles at active zones and that the SNARE complex

makes up the core machinery mediating membrane fusion between vesicles and the plasma membrane. The myriad of proteins known to contribute to this process, however, is continually growing, and each seems to play a number roles. As more details emerge about the precise mechanism of neurotransmitter release, it is important not to perseverate on labels such as "docking protein," "fusion clamp," or "Ca^{2+} sensor" as we have already seen how even individual domains of a single protein can have multiple, or even opposing, functions. The importance of studying protein functions in multiple systems is also evident from the studies outlined above. For example, interactions between complexins, synaptotagmin, and SNARE proteins that were not evident in solution became clear once the proteins were studied in their more natural environment in reconstituted membranes, and the seemingly opposed phenotypes of complexin KO animals in different systems have fueled detailed studies of individual domains and mutations that have taught us a great deal about the biology of complexin and neurotransmitter release.

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Chapter 2

Complexin expression analysis

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The majority of the work described in this chapter was performed by Lauren Buhl. In situ hybridization was performed by Dina Volfson and J. Troy Littleton. Dina Volfson assisted in the generation of the recombineered transgenic lines.

Introduction

Complexins are key players in the control of fast synchronous neurotransmitter release, which underlies the bulk of synaptic communication in the nervous system. In conjunction with a number of other mediators, particularly the vesicle-bound calcium sensor synaptotagmin, complexins modulate synaptic vesicle fusion with the plasma membrane via binding to the trimeric SNARE complex, which underlies all forms of membrane fusion. In mammals, four complexin genes with different, yet partially overlapping, expression patterns combine to meet the diverse requirements of synapses throughout the nervous system. Understanding the factors that regulate complexin expression in the nervous system is particularly critical given that numerous psychiatric and neurological disorders show increases or decreases in complexin expression, including schizophrenia, Huntington's disease, Parkinson's disease, Alzheimer's disease, depression, and bipolar disorder based on data from patients and post-mortem tissues, and traumatic brain injury, Wernicke's encephalopathy, fetal alcohol syndrome, alcoholism, addiction, and ischemia/reperfusion injury based on animal models (for review, see Brose, 2008). In addition, several single nucleotide polymorphisms in complexin 2 have been associated with cognition in schizophrenic patients (Begemann et al., 2010). To date, however, no mutations in complexin have been linked to any disorder, and it is unclear if the alterations in complexin expression in the disorders listed above are a direct cause of dysfunction or a result of other changes in the brain. In *Drosophila*, only a single complexin gene must serve the entire animal, but my work has shown

that this single gene is quite complex in terms of its expression via both alternative splicing and RNA editing, perhaps providing the diversity needed to serve synaptic transmission at a broad range of synapses.

Results

cDNA analysis

Unlike mammals, which have four complexin genes, there is only a single complexin gene in *Drosophila*, and early studies with this gene focused on a single mRNA species (Huntwork and Littleton, 2007). By analyzing EST databases, however, it is clear that multiple mRNA species are expressed from the complexin locus (Fig. 1A). There are eight exons in complexin, several of which may be alternatively spliced. The most extensive alternative splicing occurs in exon 1, resulting in 13 different 5' untranslated regions (UTRs), the function of which remains unknown. The *Drosophila* database FlyBase (http://www.flybase.org) lists a total of 21 predicted mRNA isoforms (most of which differ only in the 5' UTR) with varying levels of certainty. To clarify which of these isoforms is actually expressed and to determine the relative expression of each, I carried out sequence analysis of individual cDNAs of the complexin protein-coding region isolated by reverse transcription of total mRNA and PCR amplification. The final coding exon of *Drosophila* complexin (exon 7) has two splice variants, exon 7A and exon 7B; therefore, I amplified cDNAs from the beginning of the protein-coding region to the stop codons in either exon 7A or 7B. There were three splice variants that utilized exon 7A in a total of 50 sequenced cDNAs and two splice variants that utilized exon

7B in a total of 20 sequenced cDNAs. All of this variation involved usage of exons 4 and 5. Based on EST databases, there are three predicted options for exon 4 (4.1, 4.2, and 4.3) and two predicted options for exon 5 (5.1 and 5.2). Among the splice variants that utilized exon 7A, all of them either used exon 4.2, which encodes only four amino acids, or skipped exon 4 altogether. All of those that used exon 4.2 also used exon 5.2, which begins three amino acid later than exon 5.1, and those that skipped exon 4 used either exon 5.1 or 5.2. As a percentage of the total sequenced cDNAs, the three complexin mRNA species utilizing exon 7A were as follows: 3-4.2-5.2 = 64%, 3-5.1 = 32%, and 3-5.2 = 4%. Among the splice variants that utilized exon 7B, all of them either used exon 4.2 or skipped exon 4 altogether, similar to the variants utilizing exon 7A. When exon 4.2 was used, exon 5.2 was also used, and when exon 4 was skipped, exon 5.1 was used. As a percentage of the total sequenced cDNAs, the two complexin mRNA species utilizing exon 7B were as follows: 3-5.1 = 84% and 3-4.2-5.2 = 16%. In subsequent studies of the C-terminus of complexin herein, Cpx7A and Cpx7B refer to the most common isoforms utilizing exon 7A (3-4.2-5.2) and exon 7B (3-5.1), respectively.

The alternative splicing in exons 4 and 5 leads to changes in the complexin protein sequence in the N-terminus, near the beginning of the accessory helix. Likely the most significant difference between splice isoforms in this region is the use of a negatively charged aspartate or a small, uncharged glycine at position 19: Cpx7A has an aspartate at this position, whereas Cpx7B has a glycine. This region is not well conserved with mammalian complexins, but mouse complexins 3 and 4 have a

glycine and phenylalanine, respectively, at this position, whereas mouse complexins 1 and 2 both have a positively charge lysine. The presence of a negatively charged aspartate in Cpx7A compared to a positively charged lysine in mouse complexins 1 and 2 might be expected to lead to functional differences between these isoforms. Position 19, however, is not one of the residues in the N-terminus previously identified as being critical for interactions with the SNARE complex thought to promote vesicle fusion (Xue et al., 2010).

Alternative splicing of exon 7 causes the most drastic changes in the complexin protein sequence. Exon 7A encodes the final 24 residues of the protein, whereas exon 7B encodes only 20 residues. These regions have very little homology with each other or with complexins from other species (Fig. 1B). Interestingly, exon 7A contains a C-terminal CAAX box known to be a farnesylation motif, but this motif is absent from exon 7B. Mammalian complexins 3 and 4 also share this motif, whereas complexins 1 and 2 do not. Presumably, this motif mediates interactions with the vesicle or plasma membrane or with other proteins.

There is also some variation in the 3' UTR of complexin. Transcripts utilizing exon 7A also contain exon 8A, which makes up the majority of the 3' UTR and contains multiple binding sites for the translational regulator Pumilio (Gerber et al., 2006). These sites may affect the abundance of Cpx7A at the protein level. Transcripts utilizing exon 7B do not contain exon 8A and lack any known motifs in the 3' UTR. This difference could indicate differential regulation of Cpx7A and Cpx7B.

Complexin 7A is the predominant isoform in Drosophila

Given that alternative splicing of exon 7 in *Drosophila* complexin causes the most extensive sequence variation of any splicing event, I carried out quantitative RT-PCR (qRT-PCR) to determine the relative abundance of mRNAs using exon 7A and exon 7B. I designed primers to amplify only those transcripts using exon 7A or exon 7B and compared RT-PCR amplification of total RNA samples prepared from flies to a standard curve of Cpx7A or Cpx7B mRNA prepared *in vitro*. In mRNA samples prepared from wild type (WT) adults, Cpx7A was ~1,000-fold more abundant than Cpx7B. Similar results were seen with mRNA samples prepared from WT third-instar larvae, suggesting the relative expression of exon 7A vs. exon 7B is not developmentally regulated (Fig 2A).

I also examined whether the relative abundance of exon 7A or exon 7B was activity-dependent using a previously reported approach (Guan et al., 2005). In short, total RNA samples were prepared from the temperature-sensitive mutants *paralytic*^{TS1} (para^{TS1}) and *seizure*^{TS1} (sei^{TS1}) following different heat shock protocols (acute and chronic) to mimic neuronal hypoactivity and hyperactivity, respectively. RT-PCR amplification of each sample was quantified relative to an internal control (Act88F). The levels of transcripts utilizing exon 7A and exon 7B were mildly activity-dependent but always remained within 40% of wild-type levels. At baseline before heat shock, both Cpx7A and Cpx7B levels were elevated in sei^{TS1} and reduced in para^{TS1} compared to WT adults. In sei^{TS1} mutants, both exon 7A and exon 7B levels dropped with either heat-shock protocol, whereas in para^{TS1} mutants, these levels

remained essentially unchanged (Fig. 2B). Cpx heterozygous animals show a 50% decrease in Cpx protein levels but lack any behavioral or synaptic growth phenotypes (see Chapter 3); thus, it is unlikely that these minor changes in transcript levels would have a substantial effect on overall function of the flies, but they may have important effects on synaptic physiology.

In situ hybridization

To further evaluate the expression of Cpx7A and Cpx7B in intact animals, we carried out in situ hybridization analysis of late-stage embryos. Antisense hybridization probes specific for Cpx7A and Cpx7B were designed from the 3' UTRs of exon 7A and exon 7B, respectively. Both Cpx7A and Cpx7B showed clear expression in the developing central nervous system and ventral nerve cord (Fig. 2C). Cpx7A expression appeared to be much stronger than that of Cpx7B, consistent with the qRT-PCR results; however, this assay was not quantitative. No staining was evident when using sense hybridization probes, suggesting specificity of the observed expression patterns.

Specific antibodies

Although it seems clear that Cpx7A is the predominant complexin isoform at the mRNA level, I generated specific antibodies against Cpx7A and Cpx7B to test whether this findings holds true at the protein level and to observe potential differences in localization of the proteins. Specific antiserum was generated in rabbits immunized with peptides encoded by exon 7A or exon 7B and then purified on columns containing immobilized full-length Cpx7A or Cpx7B, respectively. These

antibodies were exquisitely specific for recombinant Cpx7A or Cpx7B expressed as GST-fusion proteins. Unfortunately, they did not recognize any complexin protein on western blots prepared from *Drosophila* adult head extracts (Fig. 3A). Similar results were observed for antibody dilutions ranging from 1:50 to 1:1,000 and for extracts treated with proteinase inhibitors (data not shown). These antibodies were also tested by immunohistochemistry on fixed third-instar larval fillets. Again no staining was apparent at the NMJ in tissues fixed with paraformaldehyde (data not shown), unlike the robust NMJ staining observed with the pan-Complexin antibody (Huntwork and Littleton, 2007). Similar results were seen for samples fixed with methanol and with Bouin's fixative (data not shown).

NMJ expression of Cpx7A and Cpx7B transgenes

In lieu of direct observation of Cpx7A and Cpx7B protein expression in third-instar larvae, I evaluated the expression of Cpx7A and Cpx7B transgenes using the Gal4/UAS system (Brand and Perrimon, 1993). I generated transgenic *Drosophila* expressing Cpx7A or Cpx7B under the control of the UAS promoter. When driven pan-neuronally with elav^{C155}-Gal4 in a complexin null background (cpx^{SH1}), both Cpx7A and Cpx7B were robustly expressed at the NMJ (Fig. 3B), similar to immunostaining observed with the pan-Complexin antibody. The halo-shaped distribution of both transgenes was reminiscent of synaptic vesicle-associated proteins such as synaptotagmin (Littleton et al., 1993) and synaptogyrin (Robin Stevens, personal communication). Interestingly, both transgenes appeared to be preferentially expressed in type 1b terminals compared to smaller type 1s terminals,

although expression was clearly present in both types of terminals. The electrophysiological properties of type 1b and 1s terminals are distinct (Lnenicka and Keshishian, 2000), and differential levels of complexin would be an intriguing explanation for these properties. A preference for type 1b terminals, however, has not been observed with the pan-complexin antibody (Huntwork and Littleton, 2007) and may be an artifact of the Gal4/UAS expression system.

Recombineering

Given the limitations of the Gal4/UAS system – namely, that it is rare to find a Gal4 driver that faithfully recapitulates the endogenous expression of a given transgene – I turned to a bacterial artificial chromosome (BAC) transgenesis approach using the P[acman] vector, which allows for germline transformation of DNA fragments > 100 kb (Venken et al., 2006). The large capacity of this vector allows for the transformation of entire genetic loci, including introns and upstream and downstream regulatory elements, allowing the expression of multiple isoforms (unlike the Gal4/UAS system) under endogenous controls. In addition, the P[acman] vector is equipped for recombineering to modify DNA inserts and for ϕC31 integrase-mediated targeted transformation into the *Drosophila* genome. Venken et al. (2007) generated a P[acman] library covering most of the *Drosophila* genome, and I chose a clone containing a ~78 kb region surrounding the complexin locus and including adjacent genes on either side. From this starting point, I used recombineering with galK substitution (Warming et al., 2005) to individually mutate the splice acceptor sites for exon 7A and exon 7B. All three of these BACs (P[acman]-Cpx (unmodified),

P[acman]-CpxΔ7A, and P[acman]-CpxΔ7B) were then trimmed using gap repair (see Methods) to contain only the complexin locus and surrounding sequences up to the preceding and following genes. Transgenic animals were generated from each trimmed BAC and crossed into the complexin null background such that all complexin expression was driven from the P[acman] insertion. Using this approach, transgenic animals were successfully generated for P[acman]-Cpx and P[acman]-CpxΔ7B.

RNA editing

In addition to alternative splicing, complexin expression in *Drosophila* can also be regulated post-transcriptionally by RNA editing. Hoopengardner et al. (2003) used a comparative genomics approach with 18 *Drosophila* species to identify targets of the RNA editing enzymes adenosine deaminases acting on RNA (ADARs) and found three sites of RNA editing within exon 7A of complexin: position 375, which encodes isoleucine (Ile) residue 125, and positions 388 and 389, which encode asparagine (Asn) residue 130. RNA editing at position 375 changes Ile-125 to methionine (Met), and RNA editing at positions 388 and 389 could change Asn-130 to aspartate (Asp), serine (Ser), or glycine (Gly). The amino acid possibilities at residue 130 are intriguing as Asp is phosphomimetic and Ser is phosphocompetent, raising the possibility that phosphorylation at this site may regulate complexin function.

The ADAR enzymes require a double-stranded RNA substrate, which is usually provided by an imperfect duplex in the pre-mRNA formed by base pairing

between the exon containing the adenosine to be edited and an intronic region called the editing site complementary sequence (ECS) (Higuchi et al., 1993). To further investigate the occurrence of RNA editing in exon 7A, I compared complexin intronic sequences from 12 *Drosophila* species to identify highly conserved regions that could function as the ECS. A region of near perfect conservation was found in the final ~65 bp of intron 6 extending through the first ~88 bp of exon 7A (Fig. 4A). The mfold algorithm for prediction of RNA secondary structure (Zuker, 2003) predicted that this region folds into an extended, imperfect RNA duplex (Fig. 4B), satisfying the requirements for an ADAR substrate.

To further investigate the extent of RNA editing, I sequenced individual cDNA segments of the editing region amplified by RT-PCR. Interestingly, editing was never observed at the second and third sites (A388, A389) unless editing had also occurred at the first site (A375). According to the mfold algorithm, editing at A375 actually extends the length of the imperfect RNA duplex (Fig. 4C), perhaps making it a more stable or attractive substrate for ADAR to carry out editing at sites A388 and A389. At these final two sites, editing was either observed at A389, giving rise to a Ser at residue 130, or at both A388 and A389, giving rise to a Gly at residue 130, but never at A388 alone. In summary, four RNA editing isoforms of exon 7A can be observed *in vivo*: Ile125/Asn130 (the unedited form), Met125/Asn130, Met125/Ser130, and Met125/Gly130. As a percentage of 96 individual cDNAs sequenced, each isoform was as follows: Ile125/Asn130 = 61.5%, Met 125/Asn130 = 7.3%, Met125/Ser130 = 10.4%, and Met125/Gly130 = 20.8%.

Phylogenetic analysis

The SNARE proteins that make up the basal machinery for membrane fusion are conserved from ancient eukaryotes such as yeast all the way to humans. Complexin and other genes involved in calcium-mediated vesicle fusion at synapses, however, arose more recently in evolution. Complexin has eumetazoan origins as homologous sequences can be found in the genomes of the placozoan *Trichoplax adhaerens* (Srivastava et al., 2008) and the cnidarian *Nematostella vectensis* (Putnam et al., 2007), but not of the demosponge *Amphimedon queenslandica* (Srivastava et al., 2010). Interestingly, the vesicular calcium sensor synaptotagmin 1 appears to have arisen before complexin as a homologous sequence is present in the *Amphimedon queenslandica* genome. The closest relative to *Drosophila* complexin is in the squid *Loligo pealei*, followed by two complexin genes in the nematode *C. elegans*. Sequence alignment of complexins from a number of species (Fig. 1B) shows highly conserved regions, including some perfectly conserved residues, in the accessory helix and SNARE-binding central helix, whereas the N- and C-termini are less well conserved. The far C-terminus is particularly poorly conserved, with the exception of a C-terminal farnesylation motif (-CAAX) shared by mouse complexins 3 and 4, *Drosophila* complexin 7A, and squid complexin.

Discussion

Gaining a better understanding of the factors that regulate complexin expression is an important goal given the many neurological conditions and disease in which complexin expression is altered. In *Drosophila*, a number of potential

regulatory factors are present, the first being alternative splicing of 13 different 5' UTRs. UTRs have to shown to affect a number of processes in the post-transcriptional regulation of gene expression, including transport out of the nucleus, translation efficiency, subcellular localization, and stability (van der Velden and Thomas, 1999; Jansen, 2001; Bashirullah et al., 2001). It remains unknown whether any of the 5' UTRs in *Drosophila* complexin affect any of these processes, but preliminary experiments using 5' rapid amplification of cDNA ends (5' RACE) suggest that Cpx7A and Cpx7B transcript utilize different 5' UTRs (data not shown), which could lead to the observed differences in expression levels. On the other end of the complexin locus, the 3' UTR of Cpx7A is notable for the Pumilio binding elements located in exon 8A (Gerber et al., 2006). Pumilio is the founding member of the conserved Pumilio-Fem 3-binding factor (Puf) family of RNA-binding proteins that typically repress gene expression by affecting mRNA stability and translation efficiency (Wickens et al., 2002). Pumilio is critical for pattern formation during early embryonic development (for review, see Kuersten and Goodwin, 2003), but a number of studies have also identified important roles for Pumilio in nervous system development and function. Multiple alleles of Pumilio have effects on long-term memory formation in *Drosophila* (Dubnau et al., 2003). Pumilio is also important for proper dendritic branching of *Drosophila* larval peripheral sensory neurons, particularly higher order dendrite branches (Ye at al., 2004). Several nervous system targets of Pumilio have been identified, including the voltage-gated sodium channel Para (Mee et al., 2004; Murano et al., 2008) and the postsynaptic scaffolding protein

Discs-large (Chen et al., 2008). At the *Drosophila* larval NMJ, Pumilio represses expression of the postsynaptic glutamate receptor subunit GluRIIA (Menon et al., 2004), which allows more current to flow through the channel in response to neurotransmitter release compared to the GluRIIB subunit (DiAntonio et al., 1999; Sigrist et al., 2002). The observation of Pumilio binding sites in the 3' UTR of Cpx7A, but not Cpx7B, raises the possibility that the predominance of Cpx7A at the mRNA level may not be maintained at the protein level. The development of isoform specific antibodies for use in western blot analysis and immunohistochemistry will be necessary to address this point.

The presence of a C-terminal farnesylation motif in Cpx7A, but not Cpx7B, is another element that may differentiate the subcellular localization of these isoforms. Among the four mammalian complexin genes, complexins 3 and 4 share this C-terminal farnesylation motif (Reim et al., 2005), whereas complexins 1 and 2 do not (McMahon et al., 1995). In HEK293 cells, the farnesylation motif on complexins 3 and 4 causes them to show a different expression pattern than complexins 1 and 2. Whereas complexins 1 and 2 are diffusely distributed in the cytoplasm, complexins 3 and 4 are mostly associated with the plasma membrane and present in granular structures in the cytosol. Furthermore, mutation of the C-terminal farnesylation motif of complexins 3 and 4 results in an expression pattern similar to that of complexins 1 and 2 (Reim et al., 2005). Somewhat surprisingly, the expression patterns of Cpx7A and Cpx7B transgenes were identical at the larval NMJ by immunohistochemistry. There are several explanations for why differential localization was not observed for

these isoforms, unlike that observed for the mammalian complexins. 1) Whereas farnesylation of mammalian complexins 3 and 4 has been demonstrated *in vitro* using a radio-labeled prenyl precursor (Reim et al., 2005), no such result has been reported for *Drosophila* Cpx7A, raising the possibility that it is not truly farnesylated. Mutation of the Cpx7A farnesylation motif, however, greatly reduces the apparent clamping function of the WT protein at hippocampal autapses (Xue et al., 2009) and the *Drosophila* NMJ (Cho et al., 2010). These results argue that farnesylation is indeed important for the function of Cpx7A. 2) The differential expression patterns of complexins 1 and 2 vs. complexins 3 and 4 in HEK293 cells could an artifact of the *in vitro* system or the GFP tag used to visualize the proteins. Untagged versions of the proteins, however, still showed differential localization, with complexins 1 and 2 in the soluble fraction and complexins 3 and 4 in the membrane fraction following cell homogenization and centrifugation (Reim et al., 2005). 3) The resolution of immunohistochemistry observed by confocal microscopy may be insufficient to identify differential subcellular localization of Cpx7A and Cpx7B at the larval NMJ. The greater resolution and detail provided by immuno-electron microscopy may be able to distinguish the localization of Cpx7A in the vesicular and/or plasma membrane vs. a more cytosolic localization of Cpx7B. 4) Finally, the association of Cpx7A with the vesicular and/or plasma membrane may be dynamic, similar to the synaptic vesicle association and dissociation of Rab3 that parallels synaptic vesicle exo- and endocytosis (Fischer von Mollard et al., 1991).

The apparently increased expression of Cpx7A and Cpx7B transgenes in type 1b boutons compared to type 1s boutons at muscles 6/7 in third-instar larvae is intriguing. With the exception of anterior segments, muscles 6/7 received innervation from two axons: axon 1, which terminates in larger type 1b boutons, axon 2, which terminates in smaller type 1s boutons (Atwood et al., 1993). In focal recordings, the EPSPs elicited from type 1s boutons are actually larger than those from type 1b boutons, although there is considerable overlap between the two (Kurdyak et al., 1994; Lnenicka and Keshishian, 2000). Furthermore, type 1b terminals show facilitation in response to prolonged repetitive stimulation, whereas type 1s terminals show synaptic depression, suggesting that the probability of neurotransmitter release is higher at type 1s boutons at baseline (Lnenicka and Keshishian, 2000). These morphological and physiological properties have led to the suggestion that type 1b and 1s boutons correspond to the tonic and phasic motor axons on crustaceans (Atwood, 1976; Kurdyak et al., 1994). It is interesting to consider that the differences in neurotransmission between type 1b and 1s terminals – and their crustacean correlates – may be due to differences in complexin levels. Given that this staining pattern is not observed with the pan-complexin antibody in WT animals, however, it may be an artifact of the pan-neuronal elav^{C155}-Gal4 driver. Other neuronal drivers should be used to test this possibility.

RNA editing of exon 7A adds yet another level of complexity to the expression of Cpx7A (Bass et al., 1988; Wagner et al., 1989). ADAR enzymes catalyze the deamination of adenosine to inosine in mRNA substrates. This inosine is then read

at a guanine by the translational machinery (Basilio et al., 1962). In mice, loss-of-function of ADAR2, which is predominantly expressed in the brain, results in severe epileptic seizures, and these mice die shortly after birth. Interestingly, this phenotype can be rescued by expressing a constitutively edited form of the AMPA-type glutamate receptor subunit GluR2 (Higuchi et al., 2000). In *Drosophila*, deletion of the single ADAR gene results in flies that are WT in appearance but have multiple behavioral deficits, including severe incoordination, temperature-sensitive paralysis, seizures, and a complete lack of courtship displays and mating (Palladino et al., 2000). The preponderence of nervous system phenotypes observed in ADAR mutant animals suggests that many of the targets of RNA editing function in the nervous system. In mammals, only a handful of these targets have been somewhat serendipitously discovered, most notably the GluR-B subunit of AMPA receptors, which is edited from glutamine to arginine in nearly 100% of transcripts (Seeburg et al., 1998). In *Drosophila*, comparative genomics between several species has allowed for the identification of ~50 neuronal mRNAs that are edited at one or more sites (Hoopengardner et al., 2003). These transcripts include the voltage-gated sodium channel Para (Hanrahan et al., 2000; Reenan et al., 2000), voltage-gated calcium channels (Smith et al., 1998), voltage-gated potassium channels (Ingleby et al., 2009), ligand-gated ion channel subunits, and components of the neurotransmitter release machinery, including synaptotagmin, UNC-13, and complexin. A neurotransmitter release phenotype in an ADAR mutant animal has not

been reported to date but would shed light on the functional importance of RNA editing for communication within the nervous system.

As noted above, a C-terminal farnesylation motif in *Drosophila* complexin 7A is also found in mammalian complexins 3 and 4 and in squid complexin; however, it is unclear if alternative splicing and RNA editing are used to diversify complexin expression in other species. Among the mammalian complexins, alternative splicing is only predicted for human complexin 2 within the 5' UTR. The effects of this splicing are unknown but presumably involve mRNA stability, localization, or translation efficiency. Interestingly, the 5' UTR of complexin 2 also contains a microRNA binding site that is conserved among mammals, and a single nucleotide polymorphism in this site is associated with reduced complexin 2 protein levels in peripheral blood mononuclear cells in humans (Begemann et al., 2010). RNA editing has also not been reported outside of *Drosophila*, but the comparative genomics approach used to identify the RNA editing sites in *Drosophila* Cpx7A (Hoopengardner et al., 2003) has not been applied to other species in which RNA editing sites are typically stumbled upon fortuitously. In C. elegans, my own analysis of the single intron in complexin-1 did not detect any regions of near perfect conservation – analogous to that in intron 6 of *Drosophila* complexin – among the five *Caenorhabditis* species with available genome sequences. This finding, however, does not rule out the possibility of RNA editing of complexin-1 in *C. elegans*. To properly evaluate RNA editing of complexin in any species, individual cDNA sequences should be compared to genomic sequences to detect A-to-G

changes indicative of an editing site, just as I report here for *Drosophila* Cpx7A. Similar cDNA analysis, including 5'- and 3'-RACE to evaluate the UTRs, should also be used to identify any currently unknown alternative splicing of complexins in other species.

Methods

cDNA analysis

Total RNA was extracted from ten adult Canton S flies using an RNeasy Mini Kit (Qiagen) and treated with DNase I (Ambion) according to the manufacturers' instructions. DNase I was then removed from the sample with the RNeasy Mini Kit. Single stranded cDNA was synthesized in a total volume of 20 µL from 1 µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. PCR was carried out by standard procedures using 2 µL of the above reverse transcription reaction as a template and the following primers for cDNA analysis (Cpx7A: forward 5'-CACCATGGCGGCCTTCATAGCTAAG-3' and reverse 5'-TCACTGCATGACACATTTTCCC-3'; Cpx7B: forward 5'-CACCATGGCGGCCTTCATAGCTAAG-3' and reverse 5'-TCAGAACAAGTTTCTCAAAGGACAAT-3') and for RNA editing analysis (forward 5'-CGCCGAAGCGGAGCAGGAAGA-3' and reverse 5'-TCACCGCCTCGTTCGGATTTTGAT-3'). PCR products were separated and visualized on a 1.2% agarose gel containing ethidium bromide. The bands corresponding to Cpx7A (~436 bp) and Cpx7B (~421 bp) were excised, and the DNA

was purified using a QIAquick Gel Extraction Kit (Qiagen). 4 μL of the purified DNA was used for cloning into the pCR-Blunt II-TOPO vector using a TOPO Cloning Kit according to the manufacturer's protocol (Invitrogen). DNA was isolated from individual transformants and sequences from the T7 promoter at the MIT Biopolymers Lab using an Applied Biosystems Model 3730 capillary DNA sequencer with the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems). Sequence analysis was carried out using the Lasergene software suite (DNASTAR).

Quantitative RT-PCR (qRT-PCR)

qRT-PCR was carried out using an Applied Biosystems 7300 Real-Time PCR System. Total RNA extraction and single stranded cDNA synthesis were carried out as described above for cDNA analysis. PCR was carried out in triplicate for each of two independent total RNA samples per genotype in optical 96-well plates (Applied Biosystems). The reaction mixtures were as follows: 25 μL of 2x QuantiTect SYBR Green PCR Master Mix (Qiagen), 300 nM forward primer, 300 nM reverse primer, and 5 μL of single stranded cDNA (see above) in a total volume of 50 μL. The thermal cycling conditions were 15 min at 95°C, followed by 40 cycles of 15 sec at 94°C, 30 sec at 54°C, and 30 sec at 72°C. A final dissociation step was carried out to evaluate product integrity, and reaction samples were run on a 1.2% agarose gel and stained with ethidium bromide. The primer sequences were as follows: Act88F (actin) forward 5'-ACTTCTGCTGGAAGGTGGAC-3' and reverse 5'-ATCCGCAAGGATCTGTATGC-3', Cpx7A forward 5'-CCCCCAAGAAGAGCCCAATC-3' and reverse 5'-

CACTGCATGACACATTTTCCCTCTAT-3', and Cpx7B forward 5'-CGCCGAAGCGGAGCAGGAAGAG-3' and reverse 5'-GGGCGTGCTGGTGTGGGTGTCT-3'.

In situ hybridization

Sense and antisense RNA probes labeled with digoxigenin (Roche) were generated by *in vitro* transcription from the first 500 bp in the 3' UTRs of both Cpx7A and Cpx7B. In situ hybridization was carried out as described previously (Tautz and Pfeifle, 1989)

Generation of Cpx7A- and Cpx7B-specific antibodies

Exon 7A and exon 7B were subcloned into the pGEX-5X-1 vector (GE Healthcare Life Sciences), expressed, and purified as GST-fusion proteins The GST tag was then cleaved using the Factor Xa Cleavage Capture Kit (EMD Chemicals, USA). The resulting exon 7A and exon 7B proteins were then used to immunize rabbits (ProSci, Inc., Poway, CA). The resulting immune sera were purified using full-length Cpx7A or Cpx7B protein, respectively, immobilized on HiTrap™ NHS-activated HP columns, and fractions containing the purified antibodies were stored in PBS (pH 7.4) at 4°C.

Immunohistochemistry

Immunostaining was performed on wandering third-instar larvae at room temperature. Third-instar larvae were dissected in *Drosophila* HL3.1 physiological saline and fixed in 4% formaldehyde for 30 min before staining with anti-Complexin antiserum at 1:500 or goat anti-HRP antiserum conjugated to DyLight 549 (Jackson

ImmunoResearch). Immunoreactive proteins were visualized on a Zeiss Pascal confocal microscope.

Recombineering

The P[acman] BAC CH321-65L02 (Venken et al., 2009), which contains the entire complexin locus and several surrounding genes, was used as the starting point for recombineering. The splice acceptor sites of exon 7A and exon 7B were individually mutated from AG to CT using *galK* selection as described previously (Warming et al., 2005). Briefly, a 1390-bp region surrounding the splice acceptor site of exon 7A and a 1430-bp region surround the splice acceptor site of exon 7B were amplified from CH321-65L02 by PCR and cloned into pCR-Blunt II-TOPO (Invitrogen). The splice acceptor sites were then mutated from AG to CT using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's protocol. A GalK cassette with ~500-bp ends homologous to the ends of the 1390-bp region for exon 7A and the 1430-bp region for exon 7B was generated by a three-way PCR ligation reaction. These GalK cassettes were individually introduced into CH321-65L02 in the recombineering E. coli strain SW102, which lacks the GalK gene and contains a heat shock-inducible λ prophage carrying the exo, bet and gam genes necessary for homologous recombination; non-heat shocked SW102 cells were used as a negative control. Before plating, cells were washed twice in M9 salts to remove all traces of rich media and then plated on M63 minimal medium agar containing 0.2% galactose as a carbon source and 25 μg/mL chloramphenicol for BAC selection. Surviving clones were restruck onto

MacConkey agar with 0.2% galactose and 25 μg/mL chloramphenicol to confirm galactose fermentation. The mutated 1390-bp fragment surrounding exon 7A and 1430-bp fragment surrounding exon 7B were then introduced into the CH321-65L02 BACs carrying GalK at the respective positions in SW102 cells as described above. After two washes in M9 salts, cells were plated on M63 minimal medium agar containing 0.2% glycerol as a carbon source, 0.2% 2-deoxy-galactose as negative selection for cells still carrying GalK, and 25 μg/mL chloramphenicol for BAC selection. All products were confirmed by PCR analysis and DNA sequencing.

The complexin locus from CH321-65L02 and the two splice acceptor mutants was then transferred to the ampicillin-resistant attB-P[acman]-Ap^R using gap repair. Two ~500-bp regions (homology arms) immediately downstream of the gene preceding complexin and immediately upstream of the gene following complexin were cloned in tandem into attB-P[acman]-Ap^R by a three-way ligation such that a Pac1 restriction site separated the two. This targeting vector was then linearized by digestion with Pac1, creating the "gap." The complexin locus from CH321-65L02 and the two splice acceptor mutants was introduced into the linearized vector by homologous recombination in SW102 cells. Cells were plated on LB agar medium containing 50 μg/mL ampicillin for BAC selection. The three vectors generated in attB-P[acman]-Ap^R were termed P[acman]-Cpx (unmutated), P[acman]-CpxΔ7A (exon 7A splice acceptor site mutated), and P[acman]-CpxΔ7B (exon 7B splice acceptor site mutated). All products were confirmed by PCR analysis and DNA sequencing and then injected into *Drosophila* embryos to generate transgenic

animals with a targeted insertion at the AttP40 site on the second chromosome (Genetic Services, Inc., Cambridge, MA).

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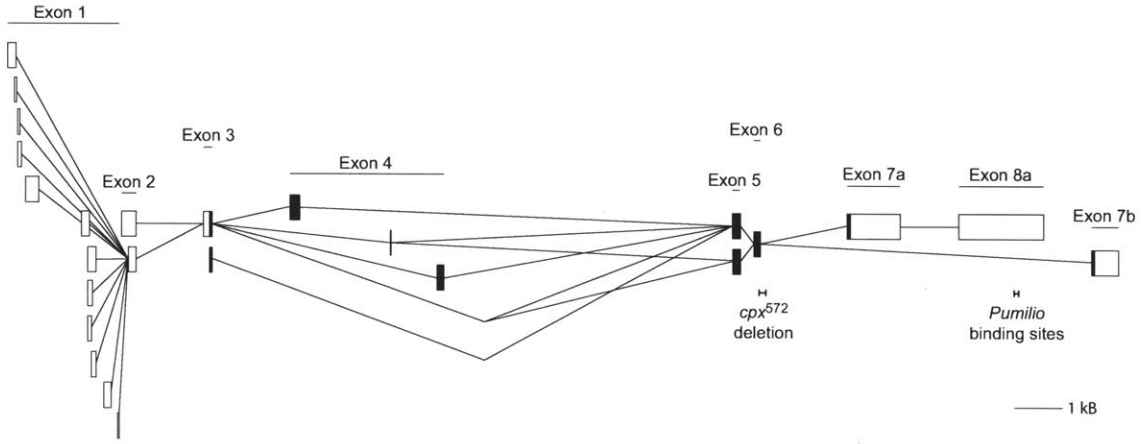
Figures

Figure 1. **The *Drosophila* complexin locus.** A) Schematic illustrating the *Drosophila* complexin locus and potential splicing events. Untranslated regions are shown as white boxes, and protein-coding regions are shown as black boxes. The deletion in cpx572 and the Pumilio binding sites in exon 8A are shown. B) Protein sequence alignment of *Drosophila* Cpx7A (dmCpx7A) and Cpx7B (dmCpx7B); squid Cpx (lpCpx); *C. elegans* Cpx-1 (ceCpx1) and Cpx-2 (ceCpx2); mouse Cpx-1 (mmCpx1), Cpx-2 (mmCpx2), Cpx-3 (mmCpx3), and Cpx-4 (mmCpx4); Nematostella vectensis Cpx (nmCpx); and Trichoplax adhaerens Cpx (taCpx). Alignment was generated using ClustalW.

A


Exon 1
Exon 2
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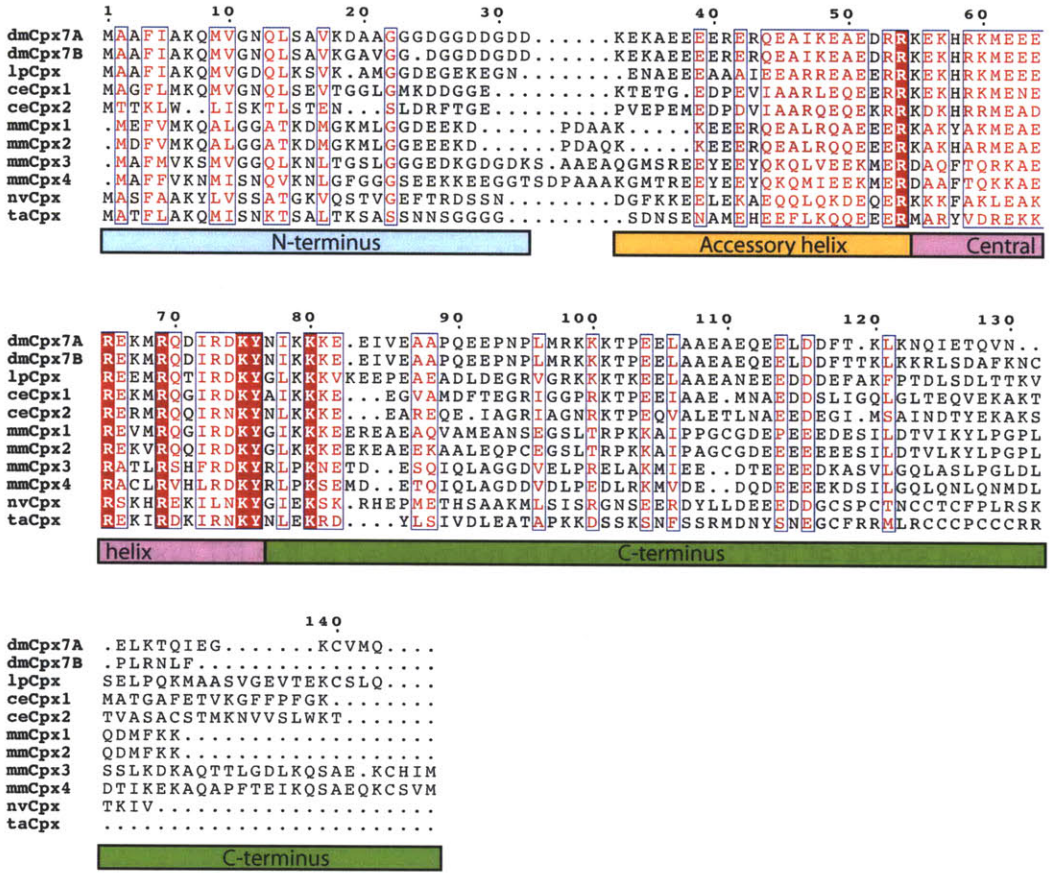
Exon 5
Exon 6
Exon 7a
Exon 8a
Exon 7b
cpx572 deletion
Pumilio binding sites
1 kB

B


dmCpx7A
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Accessory helix
Central
helix
C-terminus

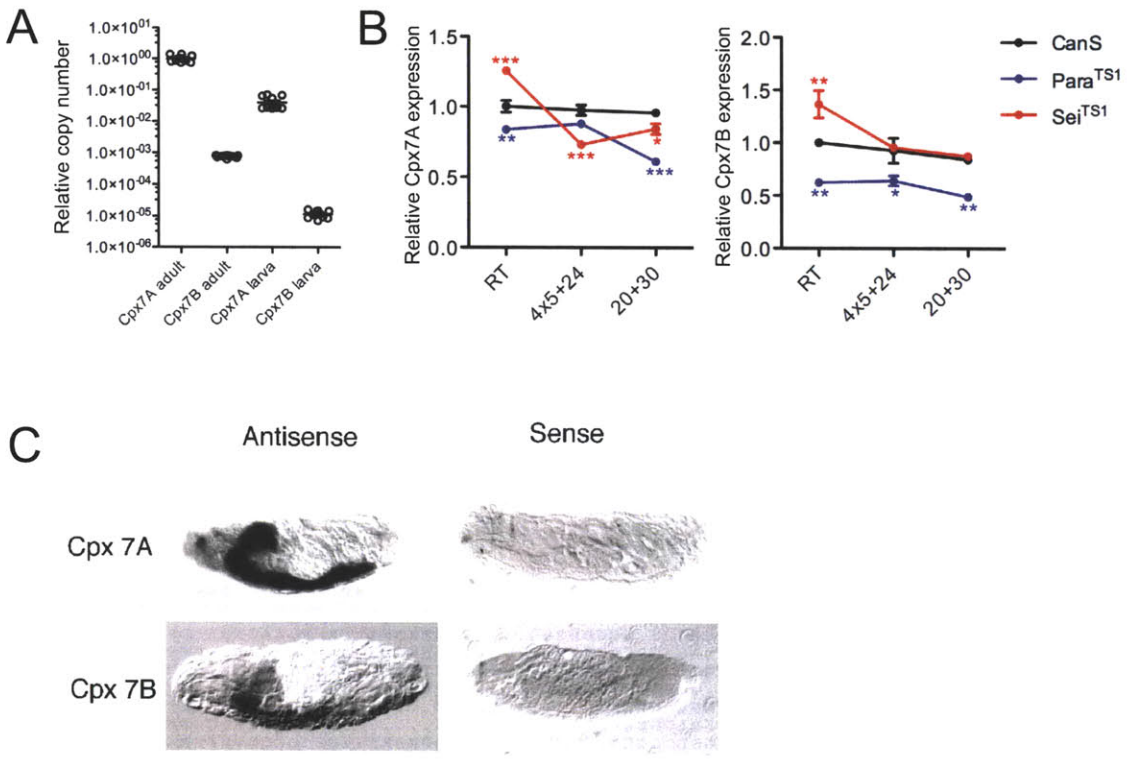

Figure 2. **Complexin mRNA expression.** A) qRT-PCR expression data for Cpx7A and Cpx7B from wandering third-instar larvae and whole adults. Absolute quantification was done using a standard curve of Cpx7A or Cpx7B mRNA. Error bars show SEM. B) Cpx7A and Cpx7B expression in whole adults of the indicated genotypes is shown relative to actin for three treatment conditions: room temperature (RT), 24-hr recovery following four separate 5-min heat shock pulses at 38°C spaced 1 hr apart (4x5+24), and 30-min recovery following a 20-min heat shock at 38°C. Expression is normalized to the RT condition. Error bars show SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. C) In situ hybridization with antisense probes for Cpx7A and Cpx7B shows strong expression of both transcripts in the developing nervous system. No staining was observed with sense probes.

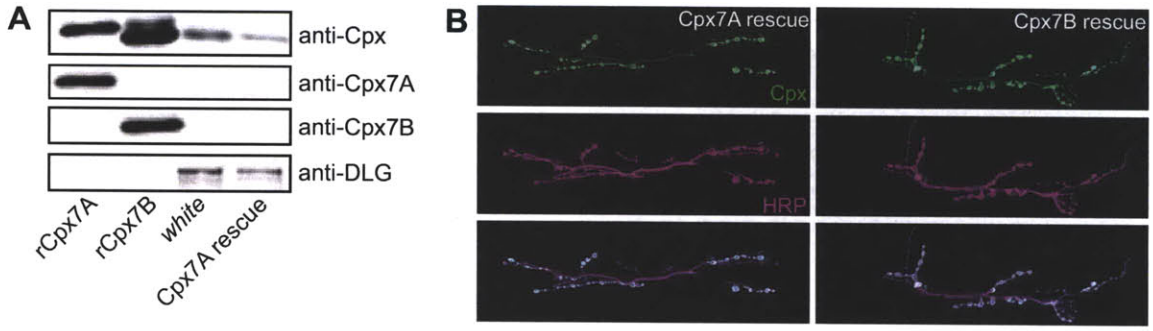

Figure 3. **Complexin protein expression.** A) Western blot using a pan-Complexin antibody (anti-Cpx), specific antibodies against Cpx7A (anti-Cpx7A) and Cpx7B (anti-Cpx7B), and anti-DLG as a loading control. Lanes 1 and 2 contain recombinant Cpx7A (rCpx7A) and Cpx7B (rCpx7B), respectively. Lanes 3 and 4 contain protein extracts from adult heads of *white* and Cpx7A rescue (elav^{C155}-Gal4;;cpx^{SH1},UAS-Cpx7A) animals, respectively. Note the specificity of anti-Cpx7A and anti-Cpx7B for their respective recombinant proteins, but the absence of any Cpx band detected by these specific antibodies in the head extracts. B) Immunostaining of muscle 6/7 of wandering third-instar larvae with anti-Cpx and anti-HRP. The bottom row shows an overlay. Cpx7A rescue (elav^{C155}-Gal4;;cpx^{SH1},UAS-Cpx7A) and Cpx7B rescue (elav^{C155}-Gal4;;cpx^{SH1},UAS-Cpx7B) animals are shown. Note the apparent increase in anti-Cpx staining in type 1b terminals vs. type 1s terminals.

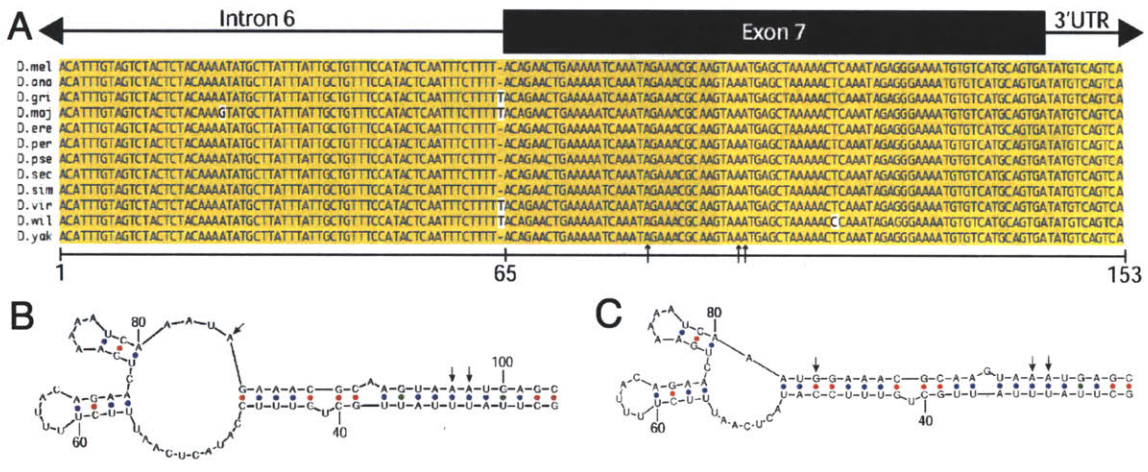

Figure 4. **RNA editing in Cpx7A.** A) Genomic DNA sequence alignment of 12 *Drosophila* species showing near perfect conservation from the end of intron 6 through the beginning of exon 7. Alignment was generated using ClustalW. B) Predicted RNA folding (mfold) of the region shown in A. C) Predicted RNA folding of the region shown in A following RNA editing at site A375. Arrows indicate sites where RNA editing can occur.

Chapter 3

Functional characterization of the Complexin C-terminus

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The majority of the work described in this chapter was performed by Lauren Buhl. Sarah Huntwork-Rodriguez generated the Cpx^{572} mutant. Voltage clamp electrophysiology was performed by Ramon Jorquera. Generation and analysis of phospho-incompetent Complexin transgenic animals was performed by Richard Cho and Dina Volfson.

Introduction

Complexin regulates a late step in synaptic vesicle fusion, but it is still debated whether complexin acts as positive or negative regulator of neurotransmitter release (see Chapter 1). Genetic knock out (KO) studies, particularly in mice and *Drosophila*, have reached different conclusions about the role of complexin in neurotransmitter release. Cultured neurons from complexin triple KO mice show a decreased rate of spontaneous neurotransmitter release events (i.e., minis) and a decreased amplitude of evoked EPSCs compared to controls, favoring a positive role for complexin in vesicle fusion (Xue et al., 2008). On the other hand, *Drosophila* complexin KO animals show a dramatically elevated mini frequency and a decreased amplitude of evoked EPSCs at the larval neuromuscular junction (NMJ) at elevated Ca^{2+} concentrations, favoring a role for complexin as a fusion clamp (Huntwork and Littleton, 2007).

An emerging view is that individual domains of complexin perform different roles during the multistep process of vesicle fusion: the N-terminus appears to promote fusion and balance the negative influence of the accessory helix (Xue et al., 2007), whereas the central helix is critical for SNARE complex binding (Chen et al., 2002; Bracher et al., 2002), and the C-terminus appears to inhibit vesicle fusion (Giraudo et al., 2008; Martin et al., 2011). Of these domains, the C-terminus is the least conserved (see Chapter 2) and has been perhaps the least studied. In *Drosophila*, alternative splicing and RNA editing combine to generate multiple

different complexin C-termini, making it an ideal system in which to study the functions of this domain.

Drosophila complexin mutants also show overgrowth of synaptic terminals at the larval NMJ, suggesting that their defects in synaptic transmission, particularly the elevated mini frequency, may lead to changed in synaptic morphology. Although many pathways that regulate synaptic growth at the larval NMJ have been described, it is unknown how these pathways are affected by changes in mini frequency.

Here, we demonstrate that the C-terminus is important for complexin protein trafficking and stability and that two complexin isoforms, Cpx7A and Cpx7B, with different C-termini have different effects on evoked and spontaneous neurotransmitter release and on synaptic growth. These data are among the first to show that the opposing effects of complexin on evoked and spontaneous neurotransmitter release may be mediated by the C-terminus. We further show that the synaptic overgrowth observed in complexin null larvae can be suppressed by mutations that inhibit known synaptic growth pathways. Finally, we show that Cpx7A is a phosphorylation target of PKA *in vitro* and that phosphorylation of complexin is necessary for certain forms of short-term plasticity.

Results

cpx null mutants show synaptic overgrowth

In addition to a > 20-fold increase in mini frequency at the larval NMJ in cpx^{SH1} animals, Huntwork and Littleton (2007) also reported an 84.4% increase in

the number of synaptic boutons, suggesting that the increase in mini frequency may be driving synaptic growth. I carried out a more in depth analysis of this synaptic overgrowth phenotype using a variety of different allelic combination and RNAi. In cpx^{SH1} homozygotes, I observed only a 53.6% increase in bouton number compared to a precise excision control strain (Fig. 1A-B). This increase in bouton number was not observed in cpx^{SH1} heterozygotes, despite a 50% reduction in complexin protein levels. The reasons for the rather large discrepancy between my observations and those reported by Huntwork and Littleton (2007) are unclear but may include the fact that I used an anti-horse radish peroxidase (HRP) antibody to label synaptic boutons, whereas Huntwork and Littleton (2007) used an anti-synaptotagmin-1 antibody, which has previously been noted to increase the number of type I boutons observed relative to anti-HRP staining (Lnenicka and Keshishian, 2000).

As an additional approach, I evaluated cpx null mutants using different allelic combinations with the deficiency strains cpxΔ1 and cpxΔ2 (gift from James McNew), which were generated using the FLP/FRT system (Parks et al., 2004) and lack only the complexin locus. Evaluating these alleles in combination with cpx^{SH1} eliminates the influence of recessive mutations present in any of the strains and allows for more robust analysis of synaptic growth in cpx null larvae. Compared to controls, both cpx^{SH1}/cpxΔ1 and cpx^{SH1}/cpxΔ2 animals showed a 22% increase in bouton number (Fig. 1C-D), confirming the presence of synaptic overgrowth in cpx null mutants, albeit to a smaller degree than previously reported (Huntwork and Littleton, 2007). Given that unhealthy *Drosophila* strains such as cpx^{SH1} will often acquire second-site

mutations that mask their initially observed phenotypes (J. Troy Littleton, personal communication), I backcrossed the cpx^{SH1} line to the Canton S (CS) wild type (WT) strain for five generations and rebalanced two independent "cantonized" lines (cpx^{SH1-5} and cpx^{SH1-6}) over TM6B in a white^{1118} (w^{1118}) background. Compared to w^{1118} controls, neither w;;cpx^{SH1-5} nor w;;cpx^{SH1-6} larvae showed a significant increase in bouton number (Fig. 1E-F). When evaluated in combination with cpxΔ1, however, both cpx^{SH1-5}/cpxΔ1 and cpx^{SH1-6}/cpxΔ1 animals showed a ~30% increase in bouton number at muscle 6/7 (Fig. 1G-H), but not at muscle 4 (Fig. 1I-J), compared to cpxΔ1 heterozygous controls.

In order to further characterize synaptic growth in complexin null larvae, I utilized two different UAS-Cpx RNAi lines (Vienna *Drosophila* RNAi Center). When driven with a pan-neuronal $elav^{C155}$-Gal4 driver along with UAS-Dicer2, both RNAi lines completely inhibit complexin protein expression, making it undetectable on western blots of adult head extracts (data not shown). As the cpx^{SH1} allele also deletes a second gene (CG9780) and microRNA (mir-929) nested within the complexin locus, an RNAi approach is particularly advantageous in that it selectively eliminates complexin protein expression. I observed a 42% increase in bouton number for both of the Cpx RNAi lines driven with $elav^{C155}$-Gal4 along with UAS-Dicer2 compared to the undriven Cpx RNAi lines alone (Fig. 1K-L). Driving UAS-Dicer2 alone with $elav^{C155}$-Gal4, however, resulted in an 23% increase in bouton number compared to control animals, suggesting the presence of endogenous RNAi targets involved in synaptic growth at the larval NMJ (Fig. 1K-L). This finding

complicates analysis of the Cpx RNAi results. In fact, the difference in synaptic growth between elavC155-Gal4 driving Cpx RNAi together with UAS-Dicer2 vs. driving UAS-Dicer2 alone was not significant.

Suppression of synaptic overgrowth in cpx null mutants

It has been well described how retrograde TGFβ signaling regulates synaptic growth at the *Drosophila* larval NMJ, and mutations that reduce this signaling decrease bouton number (Aberle et al., 2002; Marqués et al., 2002; McCabe et al., 2003). To test whether the synaptic overgrowth observed in cpx^{SH1} larvae requires TFGβ signaling, I generated double mutants for complexin (cpx^{SH1}) and the type-II BMP receptor wishful thinking (wit), which is expressed on motor neuron terminals and required to transmit retrograde BMP signals from the muscle to the motor neuron. Wit single mutants showed a significant reduction in bouton number relative to controls as previously reported (Marqués et al., 2002), and the synaptic growth of cpx,wit double mutants was indistinguishable from that of the wit single mutants (Fig. 2A-B). These findings indicate that TGFβ signaling is necessary for the synaptic overgrowth observed in cpx^{SH1} mutant larvae and further show that this overgrowth is a regulatable process, not simply an artifact due to a drastically elevated mini frequency.

Synaptotagmin-4 (syt-4) is known to be required postsynaptically for certain types of synaptic plasticity (Yoshihara et al., 2005), and the $syt\text{-}4^{BA1}$ null mutant is known to suppress synaptic overgrowth at the larval NMJ in certain hyperactivity mutants (Barber et al., 2009). I generated double mutants for $syt\text{-}4^{BA1}$ and cpx^{SH1}

and found that these double mutant larvae had fewer boutons than cpx^{SH1} single mutants, but more boutons than syt-4^{BA1} single mutants (Fig. 2C-D). These findings indicate that syt-4 is required for some, but not all, of the synaptic overgrowth observed in cpx^{SH1} mutant larvae, again showing that this overgrowth is regulatable by known synaptic growth signaling pathways. Interestingly, *syntaxin*$^{3-69}$ (syx^{3-69}) mutants, which have an increased mini frequency and synaptic overgrowth qualitatively similar to cpx null mutants, showed complete suppression of their synaptic overgrowth in a syt-4 null background (Fig. 2E-F).

The *paralytic* (para) locus encodes a voltage-gated sodium channel involved in action potential conduction, and temperature-sensitive para mutants are paralyze above the restrictive temperature due to action potential failure (Siddiqi and Benzer, 1976; Wu and Ganetzky, 1980). Interestingly, even at room temperature, $para^{TS1}$ was able to suppress the synaptic overgrowth of cpx^{SH1} mutants (Fig. 2G-H). The $para^{TS1}$ mutation would not be expected to alter the increased mini frequency of cpx^{SH1} mutants; therefore, its suppression of the cpx^{SH1} overgrowth phenotype may be related to altered action potential conduction. It has been reported, however, that $para^{TS1}$ mutants do not show conduction abnormalities at room temperature (Siddiqi and Benzer, 1976; Wu and Ganetzky, 1980). It is possible that cpx^{SH1} mutants have increased action potential propagation to the NMJ that drives synaptic growth and cannot be sustained in the presence of the $para^{TS1}$ mutation, even at room temperature.

The far C-terminus of complexin is necessary for protein stability and localization

In an EMS screen to identify additional complexin alleles in *Drosophila*, cpx^{572} was identified as a likely loss-of-function mutation. Sequence analysis revealed that cpx^{572} contains a small deletion near the end of exon 6, leading to a premature stop codon and essentially deleting exon 7 (see Chapter 2, Fig. 1A). Similar to the cpx^{SH1} null allele, cpx^{572} is semi-lethal, and the adult escapers are severely uncoordinated and ataxic. This behavioral phenotype highlights the importance of the far C-terminus in complexin function. I evaluated the complexin protein levels in cpx^{572} adult head extracts and found that while the truncated protein product was present, it was only expressed at < 20% of WT levels (Fig. 3A), suggesting that the far C-terminus is important for complexin protein stability. I also evaluated complexin expression at the third-instar larval NMJ in cpx^{572} animals and found that while some expression could be detected within motor axons, there was no expression observed at synaptic boutons (Fig. 3B), even at increased laser power by confocal microscopy. This finding suggests that the far C-terminus is important for complexin localization, although Cpx7A and Cpx7B transgenes that differ entirely in the far C-terminus show similar expression patterns at the larval NMJ (see Chapter 2). Given the lack of complexin expression at synaptic terminals, it is not surprising that cpx^{572} larvae also show a dramatic increase in mini frequency at the NMJ (Fig. 3C), similar to that reported for cpx^{SH1} null larvae (Huntwork and Littleton, 2007). It is difficult to ascertain the effects of the cpx^{572} mutation on neurotransmitter release at the larval NMJ, however, without proper localization to synaptic terminals. Interestingly, I did not observe hyperproliferation of synaptic bouton at the larval NMJ in cpx^{572} animals

(Fig. 3D), unlike the reported overgrowth phenotype of cpx^{SH1} animals. This separation of increased neurotransmitter release and synaptic growth in cpx^{572} animals is intriguing and may indicate that complexin regulates synaptic growth separately from its role in neurotransmitter release at synaptic terminals.

Cpx7A and Cpx7B differentially rescue synaptic overgrowth and synaptic transmission defects in cpx null mutants

Alternative splicing of the complexin pre-mRNA generates isoforms with two alternative C-termini termed Cpx7A and Cpx7B (see Chapter 2). To evaluate the existence of any functional differences between these two isoforms, I tested the ability of Cpx7A and Cpx7B transgenes expressed pan-neuronally to rescue the phenotypes of cpx null mutants in terms of locomotor behavior, synaptic morphology, and synaptic transmission. Both Cpx7A and Cpx7B were equally able to rescue the profound locomotor deficits of complexin mutant animals in a climbing assay, although not to control levels (Fig. 4A), suggesting that multiple isoforms are required to rescue this behavior or that the Gal4/UAS system cannot fully recapitulate endogenous complexin expression patterns. Interestingly, whereas Cpx7A was able to rescue the synaptic overgrowth of complexin mutant animals, Cpx7B was not able to do so (Fig. 4B). Cpx7A and Cpx7B also differed in their abilities to rescue the synaptic transmission defects of complexin null animals. Cpx7A rescued both mini frequency and the amplitude of evoked EPSCs back to control levels. In contrast, Cpx7B only partially rescued mini frequency but over-rescued the amplitude of evoked EPSCs to nearly twice control levels (Fig. 4C-D).

Thus, Cpx7A has a greater ability to clamp spontaneous vesicle fusion, whereas Cpx7B has a greater ability to promote evoked vesicle fusion. There have been a number of reports that complexin has different effects on spontaneous vs. evoked neurotransmitter release (Huntwork and Littleton, 2007; Maximov et al., 2009; Cho et al., 2010; Hobson et al., 2011; Martin et al., 2011), but these data are among the first to suggest that the C-terminus might mediate these differences.

Complexin kinase assays

It has been suggested that phosphorylation of presynaptic targets by cyclic AMP-dependent protein kinase A (PKA) underlies some forms of short-term synaptic plasticity at the *Drosophila* NMJ such as the increased mini frequency observed following high-frequency stimulation of the motor neuron (Yoshihara et al., 2005). Given the increased mini frequency observed at the larval NMJ in cpx null mutants, complexin is a likely candidate to be a target of PKA phosphorylation mediating this form of plasticity. I scanned the sequences of Cpx7A and Cpx7B for predicted PKA phosphorylation sites (MetaPredPS) and found one site in Cpx7A (Thr-102) and two sites in Cpx7B (Thr-101 and Ser-126), all of which are located in the C-terminus. To evaluate whether any of these sites could be phosphorylated by PKA, I carried out an *in vitro* kinase assay comparing WT Cpx7A and Cpx7B with mutated versions of the proteins in which each kinase site was individually altered to a phospho-incompetent alanine residue (Fig. 5A-B). Based on this approach, only Ser-126 of Cpx7B was robustly phosphorylated by PKA as nearly all of this phosphorylation was lost in the Ser-126-Ala mutant (Fig. 5B). In addition to PKA, other kinases are

also predicted to phosphorylated Cpx7A and Cpx7B, including protein kinase C (PKC) at Thr-102 of Cpx7A and at Thr-101 and Ser-126 of Cpx7B, cyclin-dependent kinase (CDK) at Thr-102 of Cpx7B and Thr-101 of Cpx7B, and protein kinase CK2 at Ser-126 of Cpx7B (MetaPredPS). Using the same *in vitro* assay with these kinases, I found that PKC phosphorylates both Cpx7A and Cpx7B, although not at Thr-102 or Thr-101, respectively, as the phosphorylation was unaltered when these residues were mutated to become alanines (Fig. 5C). Both CK2 and CDK-5, a member of the CDK family with known roles in the nervous system (for review, see Lai and Ip, 2009), failed to phosphorylate either Cpx7A or Cpx7B; however, an appropriate positive control was not identified for either kinase so it is likely that these assays were not optimized. As described in Chapter 2, RNA editing of Cpx7A can create a potential phosphorylation site in the Asn-130-Ser isoform. This serine is predicted to be a phosphorylation target of Ca^{2+}/calmodulin-dependent protein kinase II (CamKII), which has a number of known roles in the nervous system (for review, see Wayman et al., 2008). An *in vitro* kinase assay with CamKII and both the WT and Asn-130-Ser isoform of Cpx7A gave variable results, with CamKII phosphorylating both isoforms. In some experiments, the phosphorylation was slightly increased in the Asn-130-Ser isoform (Fig. 5D). I also attempted to identify phosphorylated residues in complexin derived from protein extracts of adult heads using immunoprecipitation followed by mass spectrometry. This approach did not identify any phosphorylated residues, but it is still possible that complexin is phosphorylated at low levels or in an activity-dependent manner.

Complexin phosphorylation is important for synaptic plasticity

To evaluate the importance of phosphorylation for complexin function *in vivo*, we generated transgenic animals expressing phospho-incompentent versions of Cpx7A or Cpx7B (termed $Cpx7A^{5P}$ and $Cpx7B^{5P}$, respectively) in which all potential Ser and Thr phosphorylation sites were mutated to Ala (Ser-15, Thr-102, Thr-120, Thr-127, and Thr-134 for $Cpx7A^{5P}$, and Ser-15, Thr-101, Thr-119, Thr-120, and Ser-126 for $Cpx7B^{5P}$). When expressed pan-neuronally in a cpx null background, both of these transgenes behaved similarly to their WT counterparts in terms of their effects on mini frequency, the amplitude of evoked EJCs, and bouton number (Fig. 6A-C), suggesting that phosphorylation is not critical for complexin function at baseline. Unlike their WT counterparts, however, larvae expressing these phospho-incompetent transgenes did not exhibit activity-dependent enhancement of mini frequency following high-frequency stimulation at the NMJ (Fig. 6D), suggesting that complexin phosphorylation is necessary for some forms of short-term synaptic plasticity.

Discussion

Cpx null mutants show synaptic overgrowth

It is well established that synaptic activity can drive synaptic growth, but the role of minis in this process is unknown. In addition to a dramatic increase in mini frequency, complexin null mutants show an increased number of synaptic terminals at the larval NMJ (Huntwork and Littleton, 2007). My findings confirm this synaptic overgrowth, although to a much smaller degree than previously reported, and they

suggest that the synaptic growth assay is highly sensitive to genetic background. In *C. elegans*, complexin mutants do not show an increased number of synaptic terminals at the NMJ (Hobson et al., 2011; Martin et al., 2011), but complexin 2 has been linked to synaptic growth in rat cerebellar cortex (Yang et al., 2009). Complexin 2 is a target of the transcription factor NeuroD, and the E3 ligase Cdc20/APC targets NeuroD for proteasomal degradation, decreasing complexin 2 levels and driving presynaptic differentiation. Although the increased mini frequency at the *Drosophila* larval NMJ in complexin null mutants could be driving synaptic overgrowth, it is actually quite difficult to separate the effects of minis vs. action potential-induced neurotransmitter release. $Syx^{3\text{-}69}$ mutants have a point mutation in the Habc domain that leads to paralysis at elevated temperature and an increase in mini frequency at room temperature (Littleton et al., 1998). I found that these mutants also showed synaptic overgrowth (Fig. 2E-F), suggesting that minis can drive synaptic growth. In contrast, the lack of synaptic overgrowth in cpx^{572} mutants (Fig. 3D) despite a dramatic increase in mini frequency (Fig. 3C) suggests that minis do not drive synaptic growth in all circumstances.

Synaptic overgrowth in cpx null mutants requires known growth pathways

Given the presence of both a dramatic increase in mini frequency and synaptic overgrowth in *cpx* null mutants, I was interested in determining whether this growth was dependent on known signaling pathways or represented a separate mechanism. The synaptic overgrowth observed in cpx^{SH1} null mutants was fully suppressed by null mutations in the type II BMP receptor wit and partially

suppressed by a null mutation in the post-synaptic vesicle protein syt-4. Wit is present presynaptically at motor neuron terminals where it forms a heterodimer with the type I BMP receptor *thick veins* (tkv) and acts as a receptor for the retrograde messenger *glass bottom boat* (gbb). This signaling complex is then internalized and eventually acts through a number of pathways to promote synaptic growth and maturation (Marques, 2005). In contrast, the signaling pathway for syt-4 is less well established. Syt-4 is required for the synaptic growth observed in response to increased temperature, and a null mutation in syt-4 (syt-4^{BA1}) is able to suppress temperature-dependent synaptic overgrowth in *seizure* mutants (Barber et al., 2009). It is interesting that syt-4^{BA1} was fully able to suppress the synaptic overgrowth observed in syx^{3-69} mutants but only partially able to suppress the synaptic overgrowth observed in cpx^{SH1} mutants. This finding suggests that something more than just an increased mini frequency in responsible for driving synaptic growth in cpx null mutants.

Cpx7A and Cpx7B have divergent effects on synaptic transmission

It has previously been reported that complexin has different effects on evoked vs. spontaneous neurotransmitter release, tending promote evoked release and inhibit spontaneous release (Huntwork and Littleton, 2007; Maximov et al., 2009; Cho et al., 2010; Hobson et al., 2011; Martin et al., 2011), although this is not the case in all systems (Xue et al., 2007; Xue et al., 2008; Xue et al., 2009; Xue et al., 2010). Here, we show that *Drosophila* Cpx7A and Cpx7B also have different effects on these two modes of release, with Cpx7A showing a greater ability to inhibit

spontaneous release and Cpx7B showing a greater ability to promote evoked release to even greater than control levels. The reasons for these differences are unclear. The far C-termini of Cpx7A and Cpx7B share essentially no homology, and the most concrete difference between the two is the presence of a C-terminal farnesylation motif in Cpx7A that is absent from Cpx7B. Farnesylation of Cpx7A could anchor it in either the vesicle or plasma membrane and effectively increase the local concentration of Cpx7A at sites of vesicle fusion. If promoting vesicle fusion in response to an action potential and clamping vesicle fusion in the absence of such signals required different numbers of complexin molecules, a difference in local concentration could explain the functional differences between Cpx7A and Cpx7B observed here. For example, clamping vesicle fusion in the absence of an incoming action potential might require a complexin molecule to be present on every SNARE complex connecting the vesicle and plasma membranes, whereas promoting fusion might require a complexin molecule on only a few SNARE complexes.

Cpx7A, but not Cpx7B, rescues synaptic overgrowth in complexin mutants

Along with their different effects on evoked vs. spontaneous neurotransmitter release, Cpx7A and Cpx7B also showed differences in their effects on synaptic growth: Cpx7A was able to rescue the synaptic overgrowth of complexin mutants, whereas Cpx7B was not. Larvae expressing Cpx7B showed an elevated mini frequency and an elevated amplitude of evoked EPSCs compared to larvae expressing Cpx7A, and either of these factors could contribute to the failure of Cpx7B to rescue the synaptic overgrowth of complexin mutants. Given that the

cpx^{572} mutants showed normal synaptic growth, however, even with a dramatically increased mini frequency, it is more likely that synaptic overgrowth observed in larvae expressing Cpx7B is due to the increased amplitude of their evoked EPSCs. Alternatively, the differences in synaptic growth between larvae expressing Cpx7A vs. Cpx7B could be unrelated to synaptic transmission and indicate a separate role for the C-terminus of complexin.

Complexin phosphorylation is necessary for short-term synaptic plasticity

Certain forms of patterned synaptic activity have the ability to modify subsequent synaptic transmission, and this phenomenon is termed synaptic plasticity. One form of short term synaptic plasticity in *Drosophila* is an increase in mini frequency following trains of high-frequency stimulation at the NMJ (Yoshihara et al., 2005). This form of plasticity requires post-synaptic expression of syt-4, which presumably mediates release of an unknown retrograde messenger that leads to signaling events in the presynaptic terminal and modifies the vesicle fusion machinery. Furthermore, the effects of high-frequency stimulation can be mimicked by presynaptic application of forskolin, an activator of adenylyl cyclase and the downstream kinase PKA (Yoshihara et al., 2005). Given that complexin mutants show a phenotype similar to presynaptic forskolin application, it is possible that complexin may be a target of PKA and that this phosphorylation event underlies the effects of both high-frequency stimulation and forskolin application.

Here, I show that Cpx7A can be phosphorylated *in vitro* by PKA at Ser-126 in the C-terminus. The only previous report of complexin phosphorylation is of mouse

complexin 1 being phosphorylated by protein kinase CK2 at Ser-115 in the C-terminus (Shata et al., 2007), increasing its affinity for the SNARE complex and decreasing its ability to promote liposome fusion *in vitro* (Malsam et al., 2009). Although there is essentially no homology between this region of mouse complexin 1 and *Drosophila* Cpx7A, these phosphorylation events do occur in similar domains of the protein and could have similar effects. Interestingly, the CK2 inhibitor 5,6-dichlorobenzimidazole riboside causes a nearly 100-fold increase in mini frequency at the frog NMJ (Rizzoli and Betz, 2002), similar to that observed with forskolin at the *Drosophila* NMJ (Yoshihara et al., 2005). In addition to the demonstrated phosphorylation of Cpx7A by PKA, both Cpx7A and Cpx7B contain predicted CK2 phosphorylation sites, raising the possibility that both PKA and CK2 might regulate complexin function to mediate their effect on synaptic transmission. Unfortunately, the *in vitro* kinase assay used here was not optimized for CK2; thus, it is unclear if CK2 actually phosphorylates Cpx7A or Cpx7B.

We further show that complexin phosphorylation is not required for proper synaptic transmission and growth at baseline but is required to increase mini frequency following high-frequency stimulation at the larval NMJ, given the absence of this form of synaptic plasticity in animals expressing phospho-incompetent forms of Cpx7A or Cpx7B. Further studies will be necessary to determine the contributions of individual phosphorylation sites and different kinases to overall complexin function *in vivo*.

Methods

Drosophila genetics

Drosophila were cultured on standard medium at 22°C. The cpx^{572} mutant was identified in a standard EMS mutagenesis screen of the 3rd chromosome based on failure to complement the cpx^{SH1} allele.

Immunohistochemical and western blot analysis

Anti-complexin antiserum (Huntwork and Littleton, 2007) was used at 1:500 for immunohistochemistry and 1:1,000 for western blots and detected using goat anti-rabbit antiserum conjugated to Alexa Fluor 546 (Invitrogen, #A-11035) for immunohistochemistry and Alexa Fluor 680 (Invitrogen, #A-21076) for western blots. Western blot analysis was done using a LICOR Odyssey infrared scanner (LICOR).

Synaptic growth assay

Low density *Drosophila* cultures were set up between 3-5 adults males and 3-5 virgin females allowed to lay eggs for 24 hours. Immunostaining was performed on wandering third-instar larvae at room temperature. Third-instar larvae were dissected in *Drosophila* HL3.1 physiological saline and fixed in 4% formaldehyde for 30 min before staining with goat anti-HRP antiserum conjugated to DyLight 549 (Jackson ImmunoResearch) at 1:500. Immunoreactivity was visualized on a Zeiss Pascal confocal microscope. Confocal images were quantified for varicosity number with blinding to genotype. All error measurements are SEM. Comparisons between groups were made using one-way analysis of variance followed by Tukey's post-test

to compare pairs of groups. Statistical analysis was done used GraphPad Prism software.

In vitro kinase assays

In vitro Cpx kinase assays were performed using the catalytic subunit of PKA, PKCα, CamKII, CDK5, and protein kinase CK2 (all from New England Biolabs), according to the manufacturer's instructions, with the addition of [g-32P]ATP (Perkin Elmer). Approximately 10mg of purified GST-fusion protein was used per reaction and incubated with 2500 units of recombinant kinase. Reaction products were separated by SDS-PAGE, and the gels were stained with Bio-Safe Coomassie (Bio-Rad), dried, and exposed to autoradiography film for 30 min to 24 hr at room temperature.

Immunoprecipitation/Mass spectrometry

Adult head extracts were prepared from WT CS flies by grinding in lysis buffer containing PhosStop phosphatase inhibitors (Roche) and Complete protease inhibitors (Roche). Extracts were then centrifuge to remove debris and incubated overnight at 4°C with a slurry of protein A sepharose beads (GE Healthcare) bound to anti-Cpx. The beads were then washed in lysis buffer and boiled to separate any bound proteins. The samples were then centrifuged to pellet the beads, and the supernatant was run on a 10-20% acrylamide gel (Bio-Rad). The gel was then fixed in 40% methanol/10% acetic acid and stained with Bio-Safe Coomassie (Bio-Rad). The ~18-kD band corresponding to Cpx was excised and submitted to Ion Trap

LCMS with a Thermo Electron Model LTQ Ion Trap mass spectrometer connected to an Agilent Model 1100 Nanoflow HPLC system (MIT Biopolymers Laboratory).

Electrophysiology

Excitatory junctional potentials (EJPs) were recorded in current clamp mode as described previously (Rieckhof et al., 2003) at the NMJs of ventral longitudinal muscle 6 in segments A3 to A5 of wandering third-instar larvae at the indicated concentrations of extracellular Ca^{2+}. Excitatory postsynaptic currents (EPSCs) were recorded using two-electrode voltage clamp (OC-725, Warner Instruments, Hamden, CT) at the NMJs of ventral longitudinal muscle 6 in segment A3 of wandering third-instar larvae using a holding potential of -80 mV and modified HL3 solution containing (in mM) 10 NaHCO3, 5 KCl, 4 MgCl2, 5 HEPES, 70 NaCl, 5 trehalose, and 115 sucrose at pH 7.2. Data acquisition and analysis were done using Axoscope 9.0 and Clampfit 9.0 software (Axon Instruments, Foster City, CA), respectively. For stimulation, segmental nerves were cut as close as possible to the ventral nerve cord 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).

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Figures

Figure 1. **Complexin null mutants show synaptic overgrowth.** Quantification of NMJ bouton number at muscles 6/7 (except I and J, which show muscle 4) in segment A3 of wandering third-instar larvae. Immunostaining was done with anti-HRP. Representative images are shown to the right of each graph in the same order as the bars on the graph. Error bars show SEM. P-values for Student's t-test or one-way ANOVA with Tukey's post hoc test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Average values (±SEM) are as follows: A) cpx^{Pre} = 68.93 ± 2.577 and cpx^{SH1} = 105.9 ± 2.173; C) cpx^{Pre} = 77.78 ± 4.663, $cpx^{SH1}/cpx\Delta1$ = 94.67 ± 4.876, and $cpx^{SH1}/cpx\Delta2$ = 95.17 ± 3.361; E) control (w^{1118}) = 89.73 ± 3.639, $cpx^{SH1\text{-}5}$ = 89.08 ± 3.957, and $cpx^{SH1\text{-}6}$ = 92.13 ± 3.386; G) $cpx\Delta1/+$ = 75.94 ± 2.068, $cpx^{SH1\text{-}5}/cpx\Delta1$ = 98.00 ± 2.559, and $cpx^{SH1\text{-}6}/cpx\Delta1$ = 100.3 ± 2.396; I) $cpx\Delta1/+$ = 28.47 ± 1.564, $cpx^{SH1\text{-}5}/cpx\Delta1$ = 30.65 ± 1.210, and $cpx^{SH1\text{-}6}/cpx\Delta1$ = 33.25 ± 1.726; K) CpxRNAi = 82.14 ± 2.805, C155>Dic2 = 101.3 ± 5.138, and C155>Dic2,CpxRNAi = 116.9 ± 4.050.

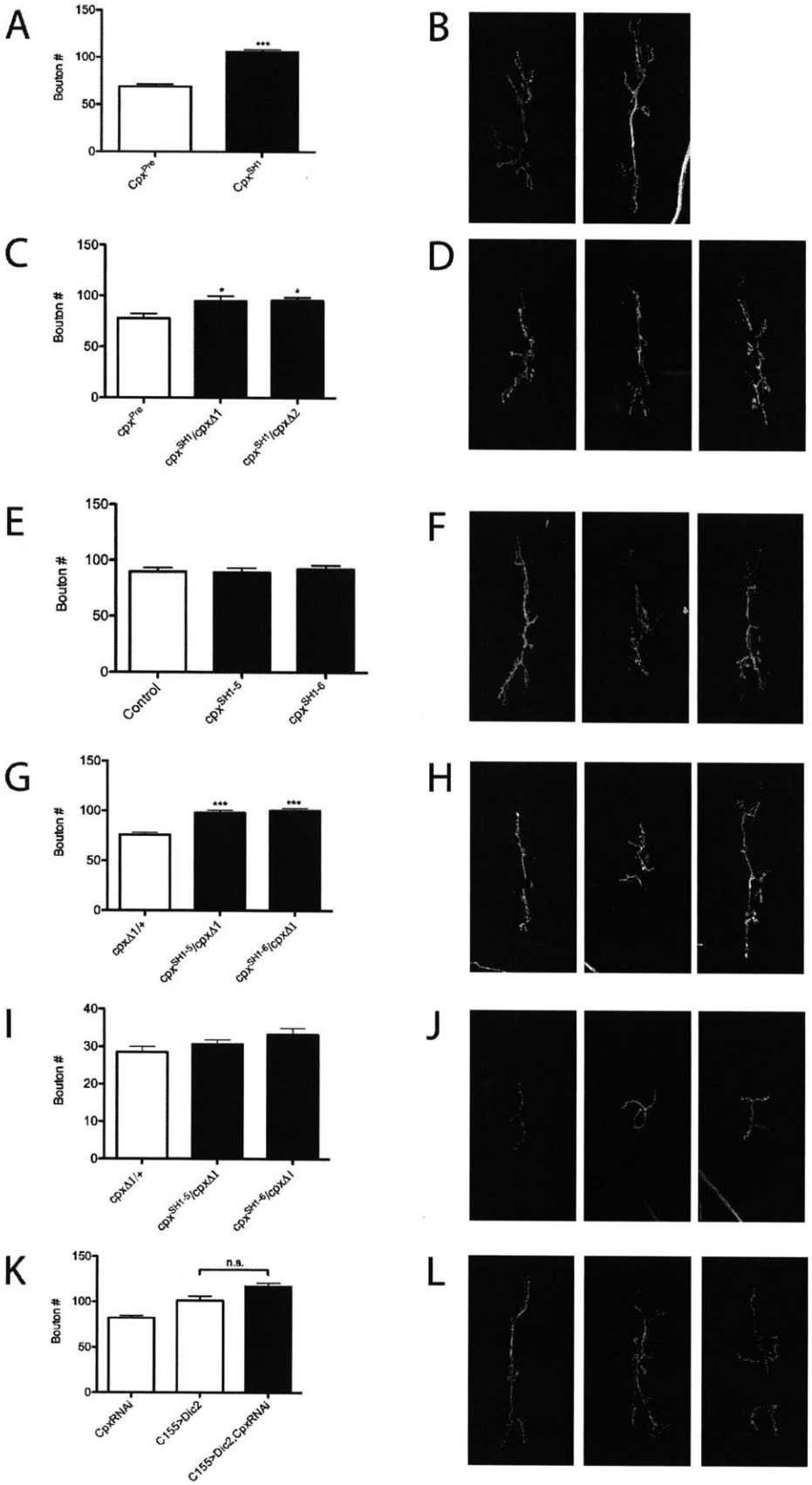
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Figure 2. **Suppression of synaptic overgrowth in cpx^{SH1} mutants.** Quantification of NMJ bouton number at muscles 6/7 in segment A3 of wandering third-instar larvae. Representative images are shown to the right of each graph. Immunostaining was done with anti-HRP. Error bars show SEM. P-values for one-way ANOVA with Tukey's post hoc test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Average values (±SEM) are as follows: A) $wit^{A12/B11}$ = 54.00 ± 2.113, cpx^{SH1} = 106.3 ± 4.245, and cpx^{SH1},$wit^{A12/B11}$ = 47.75 ± 2.73; C) cpx^{Pre} = 77.78 ± 4.663, cpx^{SH1} = 106.3 ± 4.245, $syt4^{Pre}$ = 83.60 ± 3.174, $syt4^{BA1}$ = 77.54 ± 4.034, and cpx^{SH1},$syt4^{BA1}$ = 92.63 ± 2.468; E) control (w^{1118}) = 87.75 ± 3.940, $syt4^{BA1}$ = 83.29 ± 4.121, syx^{3-69} = 124.8 ± 4.394, and $syt4^{BA1}$,syx^{3-69} = 80.17 ± 2.330; G) cpx^{Pre} = 77.78 ± 4.663, cpx^{SH1} = 106.3 ± 4.245, $para^{TS1}$;;cpx^{SH1} = 69.09 ± 2.567.

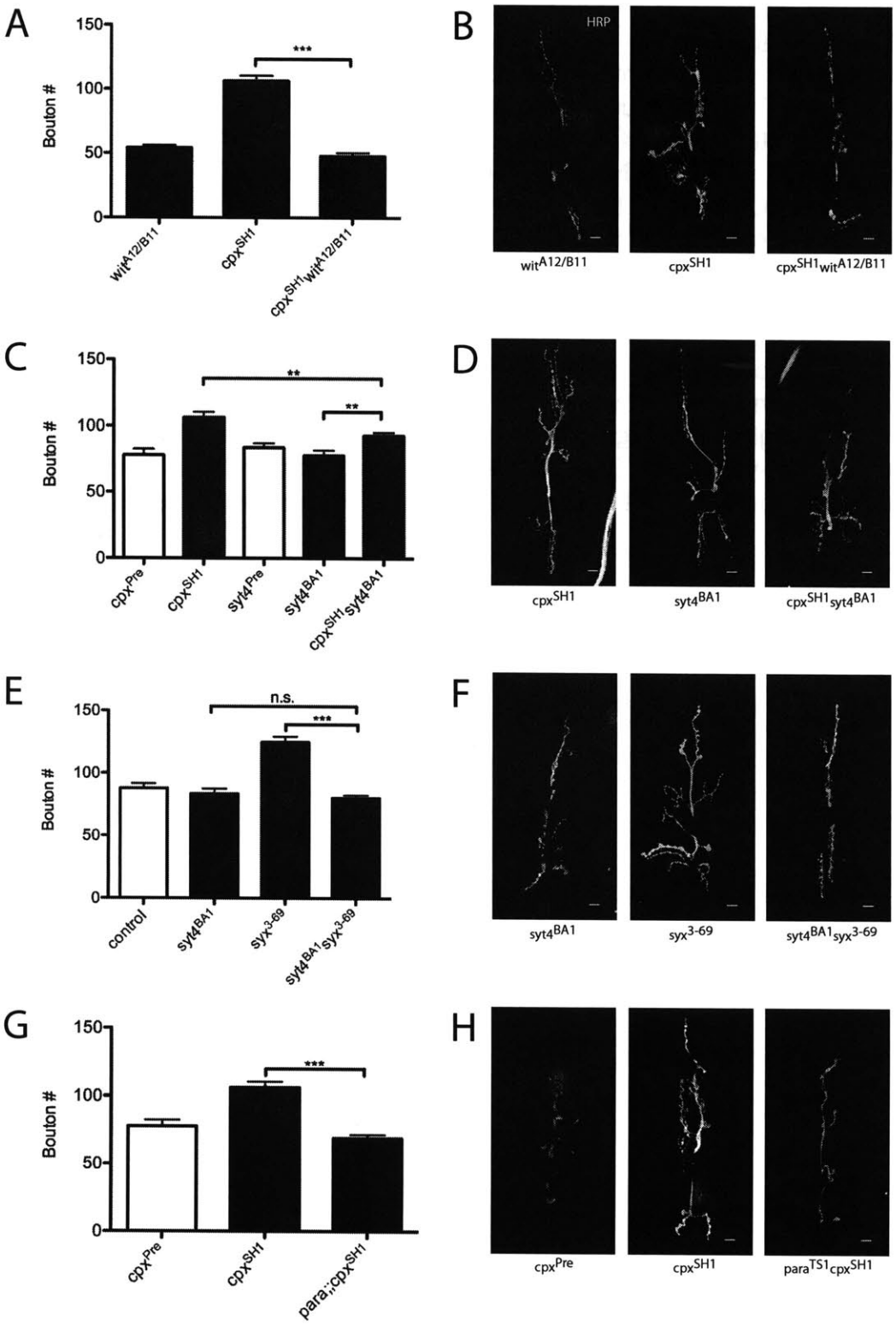
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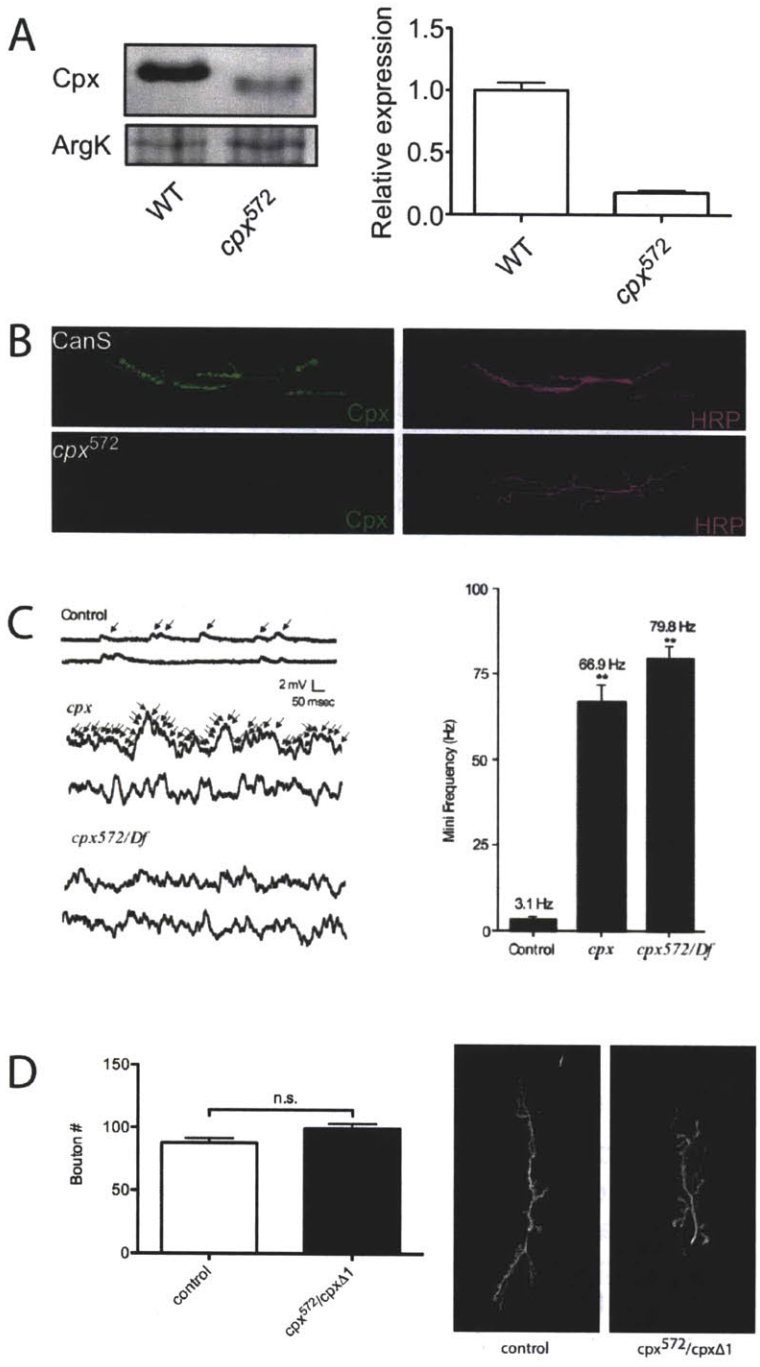
Figure 3. **cpx^{572}**. A) Western blot analysis of protein extracts from adult heads of the indicated genotypes using anti-Cpx and anti-arginine kinase (anti-ArgK) as a loading control. The graph to the right quantifies Cpx protein levels relative to ArgK and normalized to WT. B) Immunohistochemistry of the NMJ on muscles 6/7 of wandering third-instar larvae of the indicated genotypes (control - CanS (Canton S)) using anti-Cpx and anti-HRP. Note the lack of Cpx expression in synaptic terminals of cpx^{572} mutants. C) Sample traces and quantification of mini frequency in control (precise excision), cpx (cpx^{SH1}/cpx^{SH1}), and cpx^{572} (cpx^{572}/DfED5021) larvae. cpx^{572} mutants have 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); cpx^{572}/Df (MF = 79.75 ± 2.69, n=6). Error bars show SEM. D) Quantification of NMJ bouton number at muscles 6/7 in segment A3 of wandering third-instar larvae. Representative images are shown to the right of each graph. Immunostaining was done with anti-HRP. Error bars show SEM. P-values for Student's t-test: not significant (n.s.). Average values (±SEM) are as follows: control (w^{1118}) = 87.75 ± 3.940 and $cpx^{572}/cpx\Delta1$ = 99.57 ± 3.897.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cpx572
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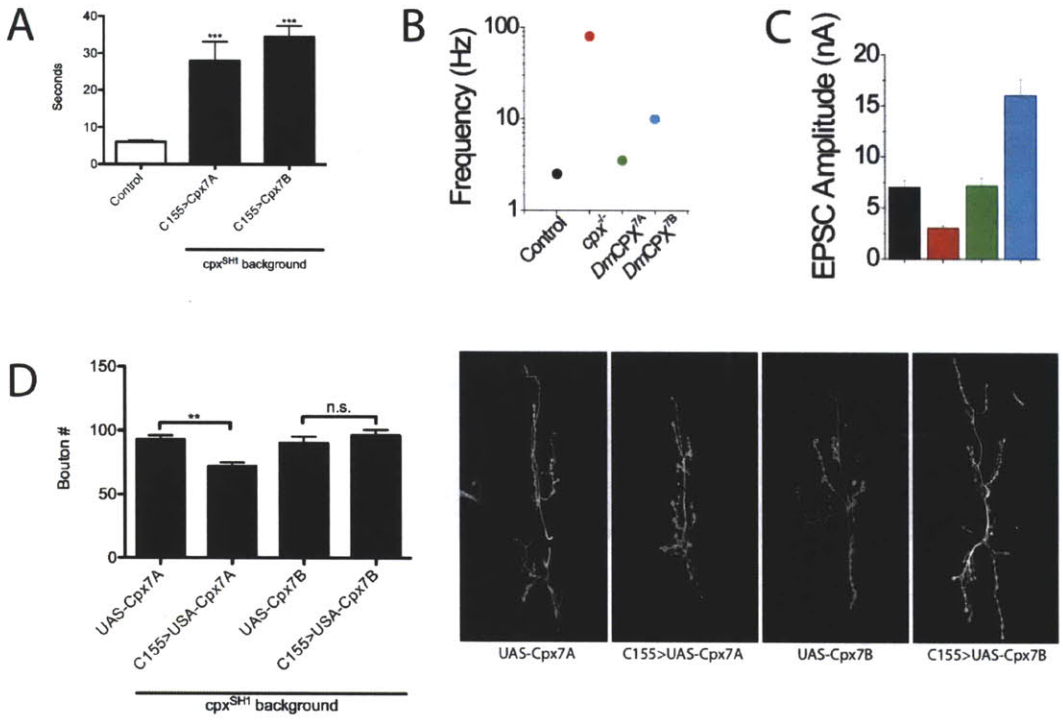


Figure 4. **Cpx7A and Cpx7B rescue different aspect of the cpx null phenotype.** A) Climbing times for a 5-cm vial are reported. Note that neither Cpx7A or Cpx7B driven with elavC155-Gal4 were able to fully rescue the cpx null phenotype. Average times (in sec) were as follows: control (elavC155-Gal4) = 6.00 ± 0.378, C155>Cpx7A (elav^{C155}-Gal4;;cpx^{SH1},UAS-Cpx7A) = 28.00 ± 5.148, and C155>Cpx7B (elav^{C155}-Gal4;;cpx^{SH1},UAS-Cpx7B) = 34.45 ± 2.934. B) Average mini frequencies recorded under voltage clamp in 0.2 mM Ca^{2+} are shown for the indicated genotypes. Control (black, cpx precise excision), cpx-/- (red, cpx^{SH1}), DmCPX^{7A} (green, elav^{C155}-Gal4;;cpx^{SH1},UAS-Cpx7A), and DmCPX^{7B} (cyan, elav^{C155}-Gal4;;cpx^{SH1},UAS-Cpx7B). C) EPSC amplitudes recorded under voltage clamp in 0.2 mM Ca^{2+} are shown for the same genotypes as is B (same color scheme). Error bars show SEM. D) Quantification of NMJ bouton number at muscles 6/7 in segment A3 of wandering third-instar larvae. Representative images are shown to the right of each graph. Immunostaining was done with anti-HRP. Error bars show SEM. P-values for one-way ANOVA and Tukey's post hoc test: **$p < 0.01$; n.s. (not significant). Average values (±SEM) are as follows: UAS-Cpx7A (w;;cpxSH1,UAS-Cpx7A) = 92.62 ± 3.567, C155>UAS-Cpx7A = 71.56 ± 3.307, UAS-Cpx7B (w;;cpxSH1,UAS-Cpx7B) = 89.91 ± 5.171, and C155>Cpx7B = 96.11 ± 4.486.

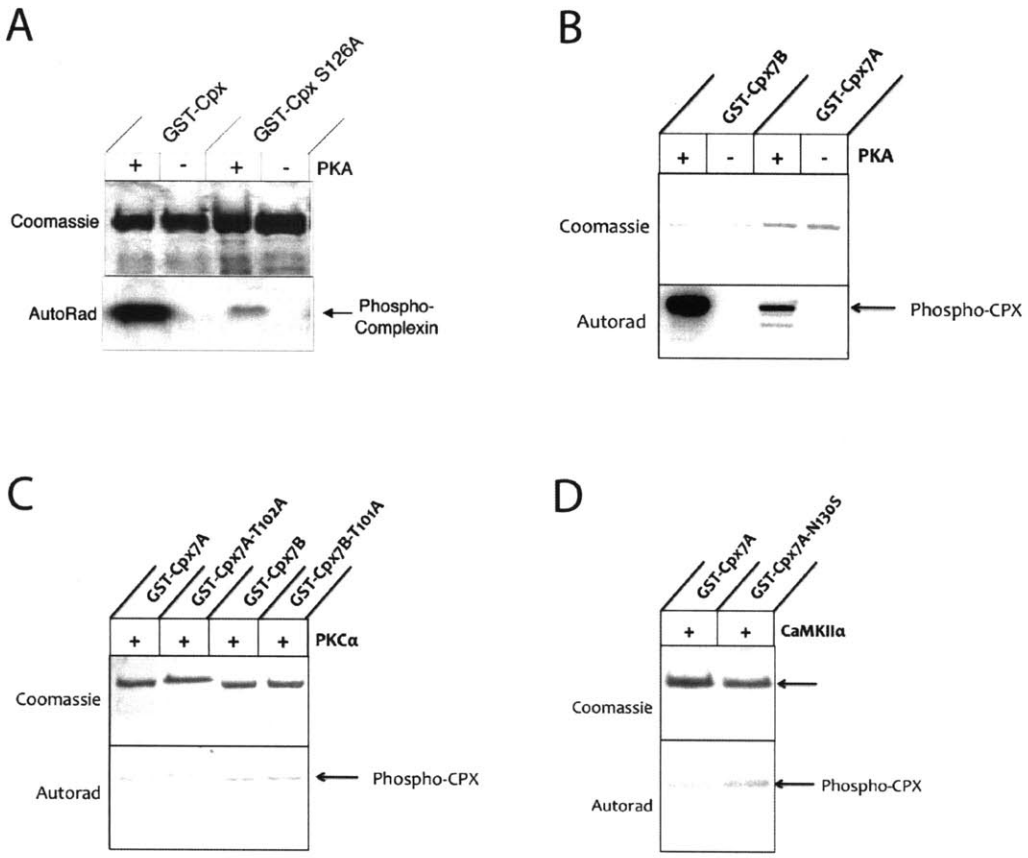
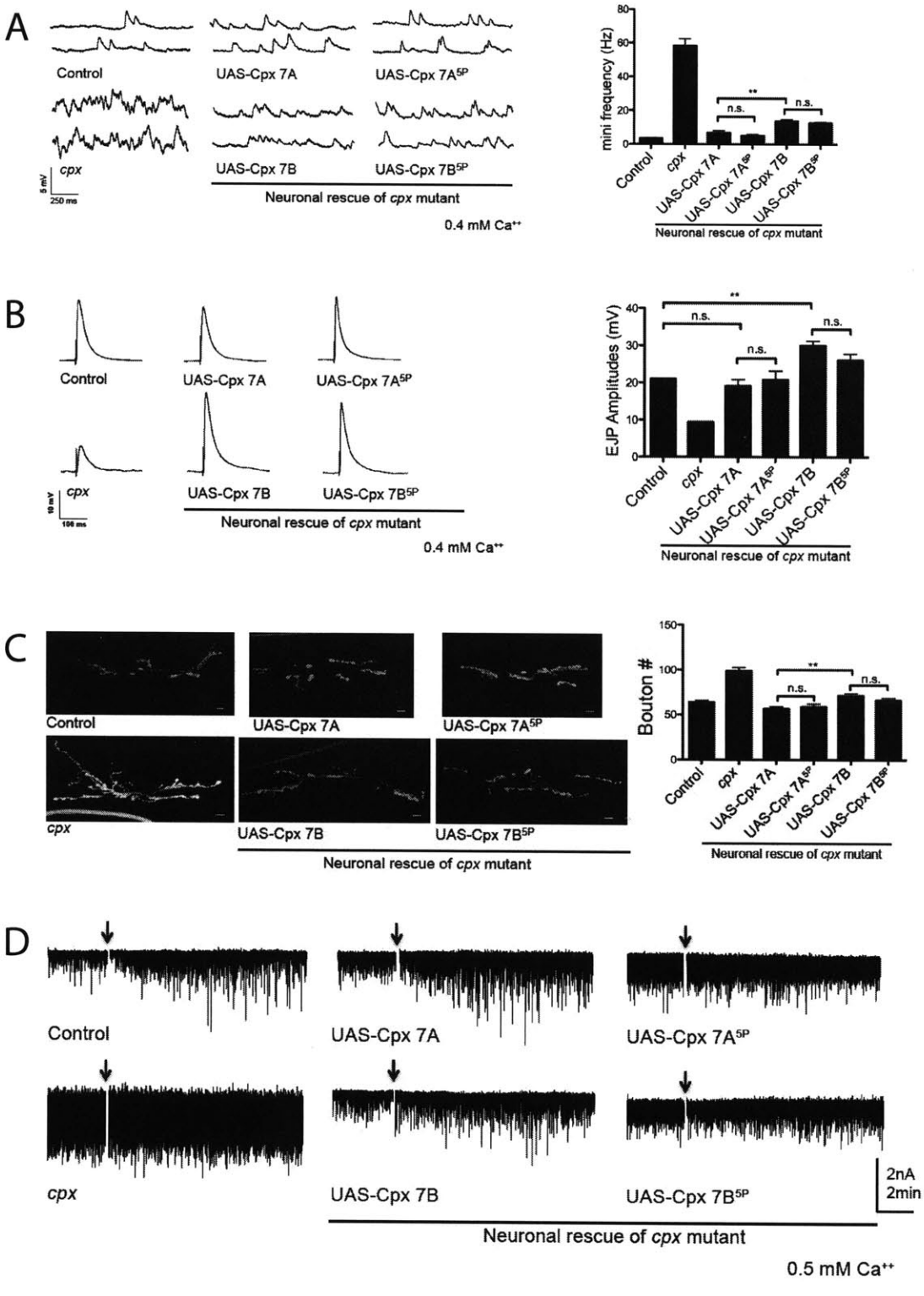


Figure 5. **Complexin phosphorylation in vitro.** Phosphorylation of different Cpx isoforms and mutants by (A-B) the catalytic subunit of PKA, (C) PKCα, and (D) CamKII. Ten micrograms of recombinant GST alone (green arrowhead) or GST–Cpx was incubated with 0.5 mg of each kinase in the presence of [g32P]ATP. Coomassie staining of the SDS-PAGE gel is shown in the top panels, and the bottom panels show the autoradiographs.

Figure 6. **Functional consequences of Cpx phosphorylation.** A) Representative traces of mEJCs recorded in 0.4 mM Ca^{2+} in current clamp mode at muscle 6 of wandering third-instar larvae. For neuronal rescues, the indicated transgenes were driven with $elav^{C155}$-Gal4 in a cpx^{SH1} background. Quantification of mEJP frequency for each genotype is shown in the graph to the right. Error bars in all panels show SEM. Note that the phospho-incompetent Cpx transgenes (5P) rescue mEJP frequency similarly to their WT counterparts. B) Representative traces of evoked EJCs recorded in 0.4 mM Ca^{2+} in current clamp mode at muscle 6 of wandering third-instar larvae. EJP amplitude for each genotypes is shown in the graph to the right. Note that the phospho-incompetent Cpx transgenes (5P) rescue EJC amplitude similarly to their WT counterparts. C) Quantification of NMJ bouton number at muscles 6/7 in segment A3 of wandering third-instar larvae. Representative images are shown to the left of the graph. Immunostaining was done with anti-HRP. Error bars show SEM. P-values for one-way ANOVA followed by Tukey's post hoc test: $^{**}p < 0.01$; not significant (n.s.). D) Representative traces of mEJCs recorded in voltage clamp mode before and after high-frequency stimulation (indicated by arrows). Note that the phospho-incompetent Cpx transgenes fail to show an increased mEJC frequency following high-frequency stimulation.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mini frequency (Hz)
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Chapter 4

Radish

Lauren K. Buhl[1], Zhuo Guan[1], and J. Troy Littleton[1]

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The majority of the work described in this chapter was performed by Lauren Buhl. Radish localization studies were performed by Zhuo Guan and J. Troy Littleton. Parts of this work have been published in *Learning and Memory*, 2011; 18: 191-206.

Introduction

Great strides have been made in recent decades to define the cellular processes underlying memory consolidation. It has long been known that following acquisition of knowledge through some form of training, nascent memories exist in a labile state that is susceptible to disruption by a broad range of interventions (e.g., head trauma, electroconvulsive shock, anesthetics) that alter patterns of neural activity (James, 1890). Over time, however, memories are consolidated and become less and less sensitive to such interventions. More specific approaches to memory disruption have taken advantage of chemicals such as actinomycin D and cycloheximide that disrupt the synthesis of new mRNA transcripts and new proteins, respectively (Flexner et al., 1963; Agranoff, 1967; Barondes, 1970; Springer and Agranoff, 1976). Blocking these cellular processes has seemingly little effect on initial memory formation but prevents persistence of memory beyond a few hours after training. The generation of lasting long-term memory, therefore, appears to involve transit through a labile state and consolidation into a more durable form that requires the synthesis of new mRNA transcripts and proteins.

Drosophila has proven to be an ideal model system in which to identify the molecular players in memory formation. Pioneering work by Quinn et al. (1974) allowed for large-scale forward genetic screens of performance on an olfactory discrimination assay using an operant conditioning paradigm. Tully and Quinn (1985) later introduced an olfactory discrimination assay using a classical conditioning paradigm, and both assays have led to the identification of numerous genes involved

in learning and memory (for review, see Davis, 2005). Memory formation in *Drosophila* has been defined behaviorally in three distinct phases: short-term memory (STM) lasting on the order of hours; anesthesia-resistant memory (ARM), a form of long-term memory (LTM) lasting on the order of days; and long-lasting long-term memory (LLTM) lasting on the order of days to a week (Tully et al., 1994). Initially after training, memory consists of both STM, which is sensitive to interventions that disrupt neural activity (e.g., electrical shock and cold shock), and ARM, which is insensitive to these interventions (Quinn and Dudai, 1976). Subsequently, memory consists of a combination of protein synthesis-dependent LLTM and ARM, depending on the training protocol. Multiple training sessions conducted one after another without a break ("massed" training) only generate ARM, whereas sessions with breaks in between ("spaced" training) generate both ARM and LLTM (Tully et al., 1994).

In *Drosophila*, cAMP levels are critical for the formation of STM. In fact, two of the original STM mutants, *dunce* and *rutabaga*, actually have opposing effects on cAMP. *Dunce* encodes a phosphodiesterase that acts to reduce the level of cAMP (Dudai et al., 1976; Byers et al., 1981; Chen et al., 1986), whereas *rutabaga* encodes an adenylyl cylcase that converts ATP into cAMP (Livingstone et al., 1984; Levin et al., 1992). In addition, the middle term memory mutant *amnesiac* encodes a neuropeptide with homology to pituitary adenylyl cyclase activating peptide in mammals and increased cAMP production in *Drosophila* (Quinn et al., 1979; Feany and Quinn, 1995; Moore et al, 1998; Waddell et al, 2000). cAMP activates cAMP-

dependent protein kinase (PKA), which phosphorylates downstream targets that may be important for STM. The catalytic subunit of PKA acts locally by phosphorylating nearby ion channels (Klein et al., 1982) and also translocates to the nucleus where it phosphorylates the transcriptional activator CREB-1 (cAMP-responsive element binding protein) (Kaang et al., 1993; Bartsch et al., 1998) and the transcriptional repressor CREB-2 (Bartsch et al., 1995; Lee et al., 2003). CREB-1 phosphorylation leads to the activation of immediate early genes such as the CAAT box enhancer binding protein (ApC/EBP) (Alberini et al., 1994; Lee et al., 2001) that regulate downstream gene expression.

Central among the genes important for LTM in *Drosophila* are the *radish* gene product, which underlies ARM (Folkers et al., 1993), and CREB, which is necessary for the formation of LLTM (Yin et al., 1994). In fact, these two gene products appear to define LLTM and ARM as two separable components of LTM (Yin et al., 1994). Radish mutant flies show normal initial learning in the QHB assay, but their performance decays to baseline over the course of eight hours. When tested specifically for ARM by using a cold-shock two hours after training and then testing one hour later, these mutants display a complete lack of ARM. Their protein synthesis-dependent LLTM, however, remains intact (Folkers et al., 1993). Studies of CREB function in *Drosophila* have taken advantage of a dominant negative isoform of this dimeric transcription factor. Flies expressing this isoform are able to generate ARM but completely lack LLTM. Furthermore, when this isoform is expressed in radish mutants, all LTM is lost, suggesting that ARM and LLTM as

defined by radish and CREB, respectively, are the predominant components of LTM (Yin et al., 1994).

As the only molecular link to understanding ARM, the radish gene product has been a subject of great interest. Folkers et al. (2006) used positional cloning to identify the radish gene product as CG15720 (hereafter referred to as Radish), and comparative sequencing indicated an early stop codon resulting in a small C-terminal truncation in radish mutant flies. Restoration of Radish expression using a heat shock-regulated transgene rescued the memory performance defects in radish mutant flies, and western blot analysis showed abundant expression of Radish upon heat shock in lines expressing the transgene. Both of these points suggest that the mutation in CG15720 is in fact causative of the radish mutant phenotype.

The Radish protein has orthologs in the mosquito Anopheles gambiae (66% identity) and the honey bee Apis mellifera (45% identity) but lacks substantial similarity to any proteins of known function. This lack of sequence homology, however, does not necessarily indicate a lack of functional homology in vertebrates and mammals. Among mammalian proteins, Radish shows the closest homology to Arg/Ser-rich splicing factors, and Radish itself is Arg/Ser-rich (14% Arg and 15% Ser) (Folkers et al., 2006). Radish functioning as a splicing factory would appear contrary to the finding that ARM is insensitive to inhibitors of protein synthesis. In those experiments, however, protein synthesis was only reduced by about 50% using cycloheximide (Tully et al., 1994); therefore, ARM may simply be less sensitive than LLTM to protein synthesis inhibition.

The radish locus encodes a 583-residue protein that lacks any predicted functional domains but contains 23 predicted PKA phosphorylation sites, 2 predicted PKC sites, and 5 predicted bipartite nuclear localization sites that overlap with a number of the PKA sites (Folkers et al., 2006). These PKA sites may represent a link between STM pathways known to involve PKA and the pathway leading to ARM. Furthermore, a large-scale yeast-two-hybrid screen identified the small GTPase Rac1 as a binding partner for Radish (Formstecher et al., 2005), perhaps indicating a role of Radish in actin dynamics in the nervous system. Immunohistochemistry shows strong Radish expression in all lobes of the mushroom body, a structure known to be important for olfactory conditioning, in addition to expression in the ellipsoid body and central complex (Folkers et al., 2006).

Given the lack of any striking hints regarding the molecular function of Radish, I undertook a yeast-two-hybrid screen to identify Radish-interacting proteins and perhaps shed light on the role of Radish and the identity of other molecular components of ARM. In addition, I evaluated the localization of Radish and its role at the larval neuromuscular junction (NMJ), reasoning that the defect in central nervous system (CNS) plasticity that blocks the formation of ARM in radish flies may also be evident as a defect in peripheral nervous system (PNS) plasticity. Finally, given the potential role of PKA as a link between STM and ARM, I investigated the ability of PKA to phosphorylate Radish *in vitro*.

Results

Radish regulates synaptic morphology and localizes to both the cytoplasm and nucleus

The *Drosophila* larval NMJ has become a popular system in which to study synaptic growth and plasticity due to its ease of accessibility and well-characterized innervation pattern. Given that radish mutants are defective in memory consolidation, a process involving synaptic plasticity in the CNS, they might also show defects in synaptic plasticity and growth in the PNS. Compared with Canton S (CS) wild type control animals, rsh^1 mutants displayed a 21% increase in varicosity number ($P < 0.001$) and a 15% decrease in innervation length ($P < 0.05$), resulting in a compact innervation pattern at the larval NMJ (Fig. 1B-D). These results indicate that rsh^1 mutants have altered axonal branching and synaptic growth.

To analyze the cellular compartment in which the Radish protein may function to regulate ARM and synaptic morphology, I generated Radish anti-serum against a predicted immunogenic region of the protein (iRsh, Ala-383 to Pro-484). I also generated Radish transgenes tagged with eGFP at the N-terminus (UAS-eGPF:Rsh) to evaluate Radish localization in transgenic animals. Previous analysis of Radish immunostaining revealed expression in the calyx, peduncle, and lobes of the mushroom bodies (Folkers et al. 2006), suggesting localization to synapses. Furthermore, the presence of multiple NLSs within the protein suggests Radish might also act in the nucleus. Unfortunately, both the previously published Radish antiserum (Folkers et al., 2006) and my newly generated iRsh antiserum failed to

identify any Radish expression in larval preparations or on western blots. Transgenic animals expressing UAS-eGFP:Rsh in larval salivary glands showed prominent nuclear accumulation of eGFP:Rsh, overlapping with DAPI staining (Fig. 1E-F). Weaker staining in the cytoplasm of the salivary gland cells was also observed. Similarly, robust nuclear accumulation of eGFP:Rsh was observed when driven with the muscle driver mef2-Gal4 (Fig. 1G). When the pan-neuronal elav^{C155}-Gal4 driver was used to express UAS-eGFP:Rsh in the brain, immunostaining was observed in the cytoplasm of neuronal cell bodies and in larval axons (Fig. 1H). Immunostaining was not readily apparent in presynaptic terminals. These data suggest that Radish can localize to both the cytoplasm and nucleus depending on cell type.

Given its weak homology to Ser/Arg-rich splicing factors and the overlap of multiple NLSs with PKA sites, it is tempting to hypothesize that Radish may shuttle in and out of the nucleus in a PKA-dependent manner. Based on the predicted PKA sites contained in the Radish protein, I assessed whether Radish could be phosphorylated by PKA *in vitro*. Full-length Radish proved difficult to purify as a recombinant protein. To overcome these difficulties in protein stability, I generated a recombinant GST:iRsh fusion protein from the same 102-residue fragment used to generate the Radish antiserum. This fragment is predicted to be surface exposed and contains six predicted PKA sites. I assayed phosphorylation of this fragment in *in vitro* assays with recombinant PKA. GST:iRsh underwent PKA-dependent phosphorylation, whereas GST alone was not phosphorylated by PKA, suggesting that Radish function and localization may be regulated through cAMP/PKA signaling.

Yeast two-hybrid screen

To gain insight into the molecular function of Radish, I conducted a yeast two-hybrid screen to identify Radish interactors and other potential players in ARM formation. Full-length Radish was used as bait to screen $1.5x10^6$ transformants of a cDNA library generated from whole adult *Drosophila* (Clontech). Twenty-three clones were identified as Radish interactors, representing 0.0015% of the entire library. Of the genes identified, none had a clear association with learning and memory based on previous work, but a number of them were intriguing nonetheless. A clone of CG2982 was pulled out five times. The predicted gene product of CG2982 contains a Jumanji C domain, which is often present in histone demethylases (for review, see Clissold and Ponting, 2001), consistent with the nuclear localization of Radish. A clone of Pellino, a binding partner of the Ser/Thr kinase Pelle involved in Toll signaling (Grosshans et al., 1999), was pulled out three times. Pellino also possesses E3 ligase activity via its RING domain (Schauvliege et al., 2006), suggesting a possible role in proteasomal degradation. Two clones of proteasomal subunits, Pros26 and Pros28.1, were also pulled out. Mutations that affect proteasomal degradation are known to alter synaptic transmission and plasticity at the *Drosophila* larval NMJ (Speese et al., 2003; Haas et al., 2007). Five members of the Jonah family of proteinases were pulled out: Jon25Bi, Jon25Biii, Jon99Cii, Jon99Ciii, and Jon99Fi (Carlson and Hogness, 1985). Interestingly, Jon99Cii undergoes circadian fluctuations in mRNA levels and is present at reduced levels in dunce mutants (Yun and Davis, 1989). Finally, a clone of CG8216 was pulled out

once. The predicted gene product of CG8216 contains a paired domain, which is important for DNA binding, and is enriched 32.5 times in the brain relative to the whole fly (http://www.flyatlas.org). A biding interaction between CG8216 and Radish is consistent with the nuclear localization of Radish and a role for Radish in the CNS.

A previous large-scale yeast two-hybrid screen identified the small GTPase Rac1 as a Radish interactor (Formstecher et al., 2005). Interestingly, the interaction involved the far C-terminus of Radish, the very region that is truncated in radish mutants, suggesting that disruption of this interaction could mediate the defects in ARM observed in these animals.

Discussion

Drosophila has proven to be a valuable genetic system to identify the molecular players underlying learning and memory formation, and the precise functions of these proteins at synapses in the CNS and PNS is an ongoing area of research. I used the well-characterized larval NMJ preparation as a model glutamatergic synapse to study the role of Radish in synaptic morphology. Radish mutants displayed an increased number of synaptic boutons, but a decreased innervation length along the muscle, resulting in a compact innervation pattern compared to controls. This pattern stands in contrast to the STM mutants rutabaga, which shows a decrease number of synaptic boutons and a decreased innervation length along the muscle, and dunce, which shows an increased number of synaptic boutons and a wild type innervation length along the muscle (Guan et al., 2011).

These specific changes in synaptic morphology may contribute to the specific behavioral deficits in each of these mutants.

Attempts to define the transition from the short-term plasticity events underlying STM to the formation of consolidated memories have highlighted the central role of cAMP. Perhaps no STM process has been better defined at the molecular level than sensitization of the siphon withdrawal reflex in Aplysia. Repeated conditioned stimuli signal through GPCRs in the presynaptic sensory neuron to increase levels of cAMP, activating PKA (Cedar and Schwartz, 1972; Brunelli et al., 1976). PKA is then able to phosphorylate a potassium channel (Klein et al., 1982), resulting in prolonged Ca^{2+} influx and increased neurotransmitter release (Klein and Kandel, 1980). Meanwhile, the transcription factor CREB, another target of PKA, plays a central role in LLTM in Aplysia. Robust training or application of the neuromodulator serotonin leads to translocation of the catalytic subunit of PKA to the nucleus, where it can activate the transcription factor CREB-1 and inhibit the transcriptional repressor CREB-2 (Bartsch et al., 1995). This process leads to the generation of specific mRNA transcripts that can be transported to and captured by activated synapses. Subsequent local protein synthesis and growth of these synaptic connections is thought to underlie the formation of LTM in this system (Dash et al. 1990; Bailey et al. 1992; Kaang et al. 1993; Casadio et al. 1999). The importance of PKA for memory formation is also evident in other systems. *Drosophila* expressing inducible inhibitors of PKA show memory defects (Drain et al., 1991), and PKA is necessary for the proper induction of LTP in the Schaffer collateral pathway and for

hippocampal-dependent memory tasks (Huang et al., 1995; Roberson and Sweatt, 1996; Abel et al., 1997). All of these studies place cAMP/PKA at the transition from short-term plasticity to memory consolidation.

Radish mutant flies lack ARM, a component of LTM, but maintain protein synthesis-dependent LLTM (Tully et al., 1994). Given this phenotype and the multiple PKA phosphorylation sites in the Radish protein, Radish may be another key player in the transition from short-term plasticity events to the formation of consolidated memories. Unfortunately, few clues as to its precise cellular function can be gleaned from primary sequence analysis, and Radish has no clear orthologs beyond a handful of other insect species (Folkers et al., 2006). By utilizing transgenic animals expressing eGFP-tagged Radish, it is clear that Radish can localize to both the nucleus and the cytoplasm depending on cell type, with robust nuclear expression observed in salivary glands and muscle cells and robust expression in cell bodies observed in the CNS. Given the overlap between several NLS and PKA sites in the Radish protein and my confirmation that a surface-exposed fragment of Radish can be phosphorylated by recombinant PKA *in vitro*, it would be interesting to see if the nuclear vs. cytosolic localization of Radish depends on its PKA phosphorylation status. Furthermore, the time course of ARM makes a role for Radish in the nucleus an attractive hypothesis.

Despite the fact that I was unable to detect eGFP:Rsh expression at NMJ synapses in transgenic animals, *radish* mutants showed altered synaptic connectivity compared to controls. Specifically, they displayed an increased number

of synaptic boutons with a decreased innervation length along the muscle, resulting in a more compact innervation pattern than control animals. This pattern is distinct from that observed in *dunce* and *rutabaga* mutants that have altered cAMP levels (Guan et al., 2011). The mechanism underlying this change in synaptic connectivity is unclear, but given the lack of Radish expression at NMJ synapses, it likely involves secondary effects to the loss of Radish in a compartment other than the synapse. The reported interaction of Radish with the small GTPase Rac1 (Formstecher et al., 2005) suggests that cytoskeletal changes may disrupt synapse formation or stability in *radish* mutants. Other Rac1 interactors such as the kinase PAK1 (Hayashi et al., 2002) and the Fragile-X Mental Retardation protein (Schenck et al., 2003; Hayashi et al., 2007) have been linked to changes in synaptic and behavioral plasticity in mammals. Furthermore, Rac1 function has recently been implicated in the act of forgetting in *Drosophila* (Shuai et al., 2010). Although a robust interaction between Radish and Rac1 was not apparent in our yeast two-hybrid system or in GST pull-down assays, such an interaction may be transient or dependent on other factors in the cellular milieu.

I identified a number of potential Radish-interacting proteins in my yeast two-hybrid screen, but none of them has a clear role in learning and memory reported in the literature to date. A handful of interactors (Pellino, Pros26, and Pros 28.1) pointed to a connection between Radish and proteasomal degradation. The ubiquitin-proteasome system (UPS) has been shown to regulate presynaptic release probability in *Drosophila* by affecting the levels of dUNC13 (Speese et al., 2003),

and postsynaptic disruption of UPS function has been reported to cause altered glutamate receptor abundance (Haas et al., 2007). Such changes in synaptic physiology may contribute to the memory defects observed in *radish* mutants and could secondarily lead to the changes in synaptic connectivity I observed at the larval NMJ. Other Radish interactors identified in my yeast two-hybrid screen are currently uncharacterized, including CG2982 and CG8216. CG2982 is intriguing given its Jumanji C domain, which is often present in histone demethylases (Clissold and Ponting, 2001), suggesting a role for Radish in the nucleus. In contrast, CG8216 lacks any clear functional domains, but its transcripts are enriched 32.5 times in the brain relative to the whole fly (http://www.flyatlas.org). Both of these genes deserve further study to evaluate their roles in memory formation and synaptic structure and function.

In short, the precise function of Radish remains somewhat mysterious. I was able to establish the localization of eGFP-tagged Radish in transgenic animals and identified unique defects in synaptic connectivity in *radish* mutants. Primary sequence analysis of the Radish protein, however, yielded few clues as to its function, and none of the interactors identified in my yeast two-hybrid screen has a clearly defined role in learning and memory. Regardless, a number of these interactors are intriguing and deserve further investigation. Loss-of-function studies testing ARM and synaptic structure and function could provide important insight into the role of Radish in the nervous system. Furthermore, although I was able to show that a surface-exposed fragment of Radish can be phosphorylated by recombinant

PKA *in vitro*, the role of PKA phosphorylation of Radish *in vivo* remains untested. Purification and mass spectrometry analysis of eGFP-tagged Radish from fly extracts could identify key phosphorylated residues *in vivo*. Subsequent mutation of these residues to phosphoincompetent amino acids would not only test the role of phosphorylation in Radish trafficking between the nucleus and cytosol but also shed light on the connection between Radish and PKA and, in turn, the connection between short-term plasticity and long lasting forms of memory storage.

Methods

Drosophila genetics

Drosophila were cultured on standard medium at 22°C. The rsh^1 mutants were outcrossed into the Canton S (CS) genetic background, with CS serving as a control for experimental manipulations.

eGFP-tagged and HA-tagged transgenic lines

Full-length Radish was cloned into pUAST in-frame with an N-terminal eGFP tag or HA tag. Each construct was injected into embryos to generate multiple independent transgenic lines that were subsequently crossed to $elav^{C155}$-Gal4 and mef2-Gal4 driver lines and screened for expression of eGFP.

Radish antibody generation

Residues 383-484 were identified as the most immunogenic fragment of Radish using the Jameson-Wolf antigenicity index. This fragment, termed iRsh (immunogenic Rsh), was subcloned into pGEX-5X-1, expressed, and purified as a GST fusion protein. GST was then cleaved from iRsh using a Factor Xa Cleavage

Capture Kit (Novagen). Anti-iRsh antiserum was generated in rabbits immunized with the iRsh peptide (ProSci Inc.).

Immunohistochemical and western blot analysis

Anti-GFP antibodies (Invitrogen, #A11120) were used at 1:5,000 and detected using goat anti-mouse antiserum conjugated to IRDye800 (Rockland, #610-132-121). Anti-Complexin (Huntwork and Littleton 2007) antiserum was used at 1:1,000 and detected using goat anti-rabbit antiserum conjugated to Alexa Fluor 680 (Invitrogen, #A21076). Cell nuclei were detected with SYTO Orange (Molecular Probes) at 1:1000. Western blot analysis was done using a LICOR Odyssey infrared scanner (LICOR).

Yeast two-hybrid screen

Yeast two-hybrid assays were conducted using the Matchmaker system (Clontech, Mountain View, CA). Full-length Radish was cloned into pGBKT7, transformed into yeast strain AH109, and used to screen $1.5x10^6$ transformants of a pACT2 Matchmaker library generated from whole *Drosophila* adult cDNA (Clontech) for growth on minimal media lacking leucine, tryptophan, histidine, and adenine. DNA from colonies passing this screen was purified and retransformed into AH109 yeast cells carrying plasmid pGBKT7-rsh. Directed two-hybrid tests were conducted with pGBKT7-rsh and Rac1 in pAct2 (Clontech). Yeast cells containing both prey and bait constructs were selected on minimal media lacking leucine and tryptophan, then restruck on plates additionally lacking histidine and adenine. Yeast colonies were scored for growth after 3 d at 30°C.

NMJ morphological analysis

Immunostaining was performed on wandering third-instar larvae at room temperature. Third-instar larvae were dissected in *Drosophila* HL3.1 physiological saline and fixed in 4% formaldehyde for 30 min before staining anti-Complexin antiserum at 1:500 or goat anti-HRP antiserum conjugated to DyLight 549 (Jackson ImmunoResearch). Immunoreactive proteins were visualized on a Zeiss Pascal confocal microscope. Rhodamine-phalloidin (Invitrogen) was used at 1:250 to stain muscles for surface area measurements. Confocal images were quantified for varicosity number, synapse branch number (branches with at least five boutons per branch), innervation length along the muscle (measured as the distance between the most distal boutons on muscles 6 and 7), muscle length, and muscle width. All error measurements are SEM.

PKA phosphorylation assay

The Radish kinase assay was performed in triplicate using the catalytic subunit of PKA (New England Biolabs) according to the manufacturer's instructions, with the addition of [gamma-^{32}P]ATP (Perkin Elmer). Approximately 10 mg of purified GST-fusion protein was used per reaction and incubated with 2,500 units of recombinant PKA. Reaction products were separated by SDS-PAGE, and the gels were stained with Bio-Safe Coomassie (Bio-Rad), dried, and exposed to autoradiography film for 30 min at room temperature.

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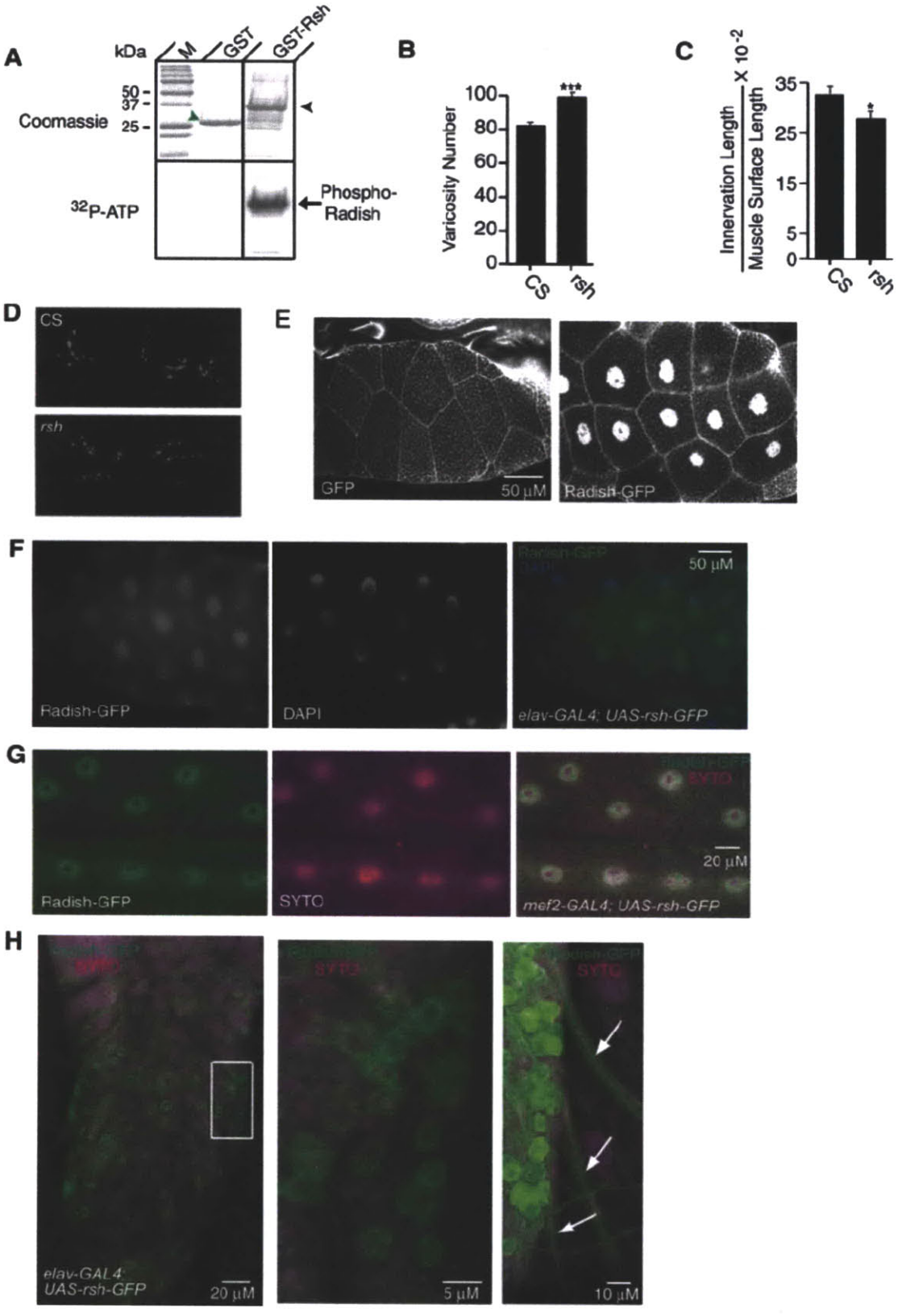
Figures

Figure 1. **Characterization of Radish function and localization.** (A) Phosphorylation of a Radish protein fragment (amino acids 383–484) by the catalytic subunit of PKA. Ten micrograms of recombinant GST alone (green arrowhead) or GST–Radish (black arrowhead) was incubated with 0.5 mg of the PKA catalytic subunit in the presence of [g32P]ATP. Coomassie staining of the SDS-PAGE gel is shown at top, and the bottom panel shows the autoradiograph. (B) Quantification of average varicosity number at muscles 6/7 NMJs in segment A3 in third-instar CS or rsh^1 larvae. Average varicosity number was significantly increased in rsh^1 (99.2 ± 3.2, n = 14) compared with CS (82.2 ± 2.1, n = 11). (***) P-value of Student's t-test (P, 0.001). Error bars represent SEM. (C) Quantification of average innervation length normalized to muscle surface length at muscles 6/7 NMJs in segments A3–A5 in third-instar CS or rsh^1 larvae. Innervation length was significantly decreased in rsh^1 mutants (0.27 ± 0.014, n = 39) compared with CS (0.36 ± 0.017, n = 27). (*) P-value of Student's t-test (P, 0.05). Error bars represent SEM. (D) Immunocytochemistry with anti-Complexin antiserum on muscles 6/7 NMJs in segment A3 of third-instar larvae. (E,F) Expression of UAS-rsh-GFP by $elav^{C155}$-GAL4 in third-instar larvae. (E) Radish–GFP was concentrated in the nucleus of salivary gland cells compared with GFP alone. (F) Colocalization of Radish–GFP and DAPI in the nucleus of salivary gland cells. (G) Radish–GFP was concentrated in the nucleus of muscle cells when driven by the Mef2-GAL4 driver. Nuclei were counterstained with SYTO orange (shown in magenta). (H) Radish–GFP localized to neuronal cell bodies in the ventral nerve cord and axons (arrows) in third-instar larvae. (Middle) A magnified view of the framed area in the left panel. (Right) Radish–GFP in nerve bundles (arrows) exiting the ventral nerve cord. Nuclei were counterstained with SYTO orange (shown in magenta).


A
kDa
M
GST
GST-Rsh
50
37
25
Coomassie
32P-ATP
Phospho-
Radish
B
Varicosity Number
100
80
60
40
20
0
***
CS
rsh
C
Innervation Length / Muscle Surface Length X 10-2
35
25
15
5
0
*
CS
rsh
D
CS
rsh
E
GFP
50 µM
Radish-GFP
F
Radish-GFP
DAPI
50 µM
elav-GAL4; UAS-rsh-GFP
G
Radish-GFP
SYTO
20 µM
mef2-GAL4; UAS-rsh-GFP
H
elav-GAL4
UAS-rsh-GFP
20 µM
SYTO
5 µM
SYTO
10 µM

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