Retrograde Regulation of Activity-Dependent Synaptic Growth by Synaptotagmin 4

by

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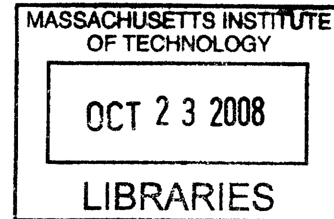
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Cynthia Fuentes Barber

Submitted to the Department of Biology October 15, 2008 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology

ABSTRACT

Ca^{2+} influx into pre- and post-synaptic compartments during neuronal activity is a key mediator of neurotransmitter release and synaptic plasticity. Although the role of presynaptic Ca^{2+} in triggering vesicle fusion is established, molecular mechanisms that underlie responses to postsynaptic Ca^{2+} remain unknown. The Synaptotagmin family of Ca^{2+} sensors includes several evolutionarily conserved neuronal isoforms that are predicted to regulate vesicle fusion, including the synaptic vesicle Ca^{2+} sensor Synaptotagmin 1. Recently, the Synaptotagmin 4 (Syt 4) isoform was localized to postsynaptic vesicles at *Drosophila* neuromuscular junctions (NMJs), suggesting a role in Ca^{2+}-dependent release of retrograde signals. Here we demonstrate that fusion-competent Syt 4 vesicles localize postsynaptically in *Drosophila* central nervous system (CNS) neurons, suggesting Syt 4 may be a general regulator of postsynaptic vesicle trafficking. Syt 4 mRNA and protein levels are bi-directionally regulated by neuronal activity, with seizure induction increasing Syt 4 levels and decreased activity reducing Syt 4 expression. Bi-directional manipulations of postsynaptic Syt 4 levels *in vivo* demonstrate that it regulates synaptic growth at NMJs, with *Syt 4* mutants showing reduced varicosity number. Postsynaptic over-expression of Syt 4 enhances synaptic growth and requires Ca^{2+} binding by both the C2A and C2B domains, as well as serine 284, an evolutionarily conserved substitution for a key C2A Ca^{2+}-binding aspartic acid found in other synaptotagmin isoforms. In addition, Syt 4 is required for activity-dependent structural plasticity at NMJs, including seizure-induced and temperature-dependent synaptic growth. These findings suggest retrograde vesicular trafficking mediated by Syt 4 contributes to activity-dependent growth of neuronal connections.

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Chapter 1: Activity Dependent Growth and Synaptic Transmission at *Drosophila* Synapses

Cynthia F. Barber and J. Troy Littleton

1 Introduction

Multicellular organisms depend upon cellular communication, allowing them to specialize yet, as a coordinated system, adapt and survive in their environment. A dramatic example of intercellular communication is in the nervous system, with its ability not only to mediate sensory perception in realtime, but also to form memories and even consciousness. In the 1940's, Donald Hebb postulated that selectivity of neuronal memory coding is achieved through the stabilization and strengthening of productive synaptic connections, and the elimination of unproductive ones. It is becoming increasingly clear that bidirectional communication at the synapse is key to this process. Retrograde signals released from the postsynaptic terminal are transmitted both in reponse to activity and constitutively, to modulate synaptic development and acute plasticity (Fitzsimonds and Poo, 1998; Kalinovsky and Scheiffele, 2004; Marques, 2005). In *Drosophila,* one member of the Synaptotagmin Ca^{2+} sensor family, Synaptotamin 4 (Syt 4), resides on a post-synaptic vesicle population, and may regulate the release of activity dependent retrograde signals (Adolfsen et al., 2004; Yoshihara et al., 2005). However, many questions remain about Syt 4 regulation, function, and the interacting signaling cascades. *Drosophila*, with its powerful genetic toolkit and testable behavioral repertoire, has already proven an important model system in the field of neurobiology. Using the *Drosophila* model we will examine the regulation of functional connectivity between neurons. First, I will describe *Drosophila* as a neurobiological model, its signaling cascades and known regulators of synaptic growth, then delve specifically into the area of synaptic transmission and how Ca^{2+} sensing by Synaptotagmin 4 (Syt 4) may function in the 'synaptic tagging' of productive synapses.

1.1 *Drosophila* as a Neurobiological System

Glutamatergic synapses, being the common excitatory connection in the mammalian central nervous system (CNS), are of obvious interest to neuroscientists. However, the difficulty of accessing individual synapses within the mammalian CNS, as well as the complex nature of the CNS, significantly complicates analysis. In contrast, *Drosophila* neuromuscular junctions (NMJs), formed on muscles along the larval bodywall, offer easily accessible glutamatergic synapses for both morphological and electrophysiological analysis. Like hippocampal synapses, the *Drosophila* NMJ is subject to experiential strengthening, with increased larval locomotion leading to increases in both the magnitude of evoked junctional potentials (EJPs), as well as the number of boutons (neuronal sites of active zones and synaptic connection) (Sigrist et al., 2003). During the larval stages (1st to 3rd instar), NMJs undergo significant synaptic elaboration, expanding >10 fold at muscle 6/7 NMJ, to compensate for rapid muscle growth (Fig 1.1). Forward and reverse genetic approaches available in *Drosophila* make the NMJ an exceptionally accessible model synapse for following both structural and functional changes. Not only can NMJ morphology be used as a target for genetic screens, the UAS/GAL4 system allows selective expression of transgenes on one or both sides of the synapse to define their pre- or post-synaptic function.

Beyond the synapse, *Drosophila* display a number of complex behaviors ranging from courtship and grooming to Pavlovian olfactory learning. Screens for mutants defective in such natural behaviors have identified molecular components important to learning, improving our understanding of how synaptic function translates to behavior. Meanwhile, additional screening approaches have identified temperature-sensitive (TS)

Figure 1.1

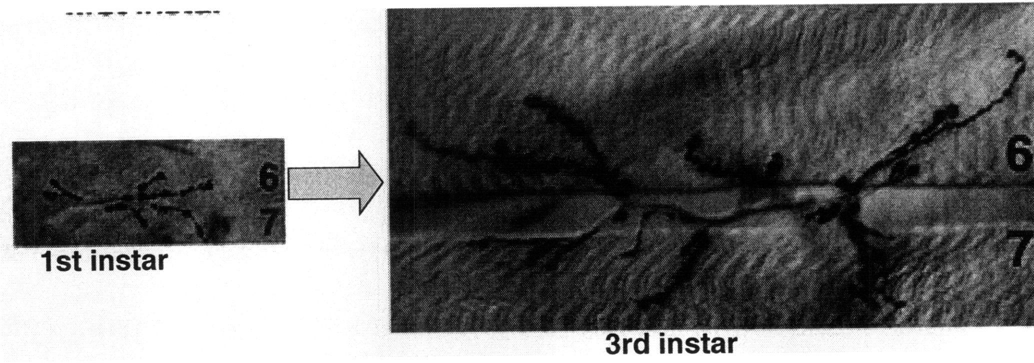


Figure 1.1: Between the first and third larval instar stages, the *Drosophila* NMJ undergoes a large expansion to accommodate the 10x increase in muscle surface area.

activity mutants that display seizures or paralysis due to changes in the excitability of neurons at high temperatures. These mutants provide tools to identify the mechanisms that link neuronal activity to synaptic modification by allowing conditional control of synaptic activity through simple manipulation of the animal's external temperature. The ability to move rapidly from mutational screens and molecular identification of mutated gene products, to the characterization of the underlying synaptic and behavioral defects, has made *Drosophila* a popular model for exploring gene function in learning and memory.

1.1.1 Learning and Memory in *Drosophila*

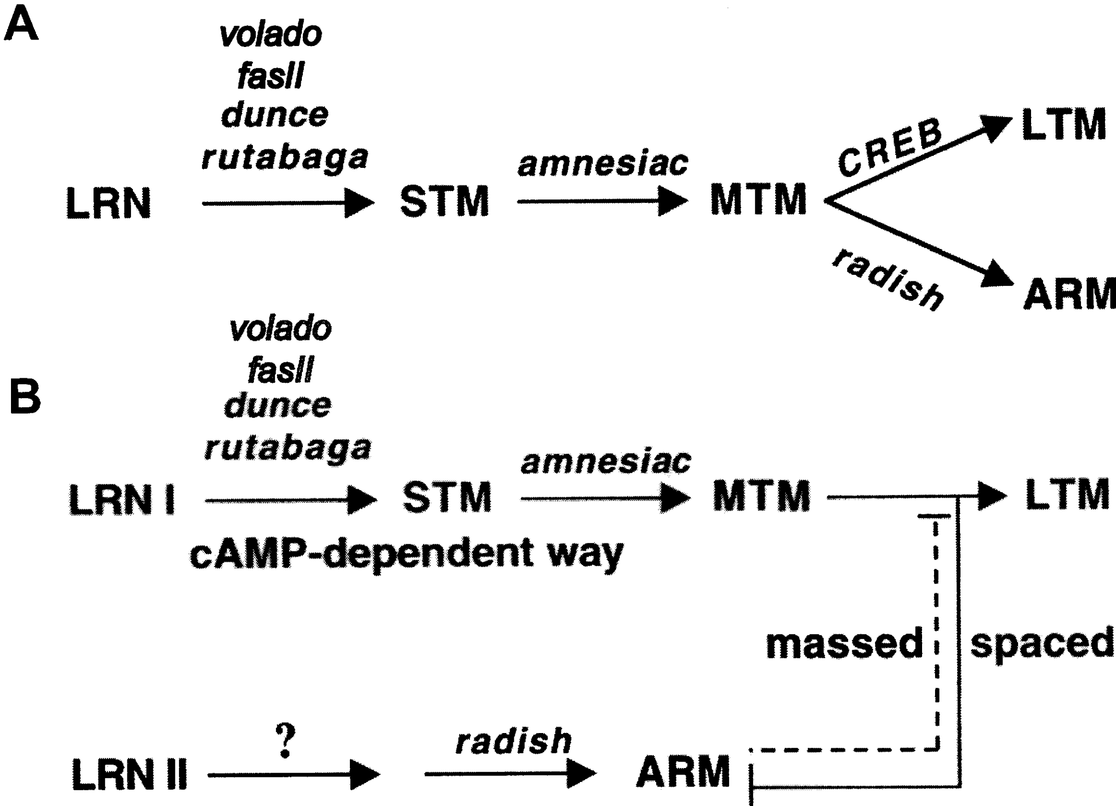
Efficient tests for *Drosophila* learning and memory behaviors were first developed in the 1980s, and styled after classical Pavlovian training paradigms with neutral stimuli (such as an odor) paired to negative reinforcement (electric shock) (Tully and Quinn, 1985). Olfactory-based learning is assessed by the fly's avoidance (or lack there of) of an odor previously paired with electric shock. In wildtype flies, avoidance behavior normally persists for up to 24 hours, and is taken as a measure of the flies' memory (Tully and Quinn, 1985). Such olfactory memories depend on mushroom body neurons in the CNS, which preferentially express a number of proteins important to learning. *Drosophila* can also learn to discriminate and remember visual landmarks in response to negative reinforcement. In contrast to olfactory learning, the anatomical sites important for memory formation and recall of visual-based behaviors have been mapped to a brain region in the central complex known as the fan-shaped body (Liu et al., 2006). Beside olfaction and visual-based forms of memory, the anatomical requirements for other forms

of memory have not yet been fully explored (Isabel et al., 2004). The general conclusion from anatomical mapping of learning and memory centers is that memory traces are not universally stored in one central location, but rather distributed to substructures of the fly brain that receive multimodal stimuli for the specific behavior in question. Whether different types of sensory-based memory traces require the same molecular pathway is unknown, and most efforts have focused on dissection of the molecular requirements for olfaction-based memory formation.

Mutant screens testing for defects in olfactory learning and memory have produced a series of mutants that define the pathways of memory formation, including the role of CREB-regulated transcription in long-term memory (LTM). While spaced learning trials with intermittent rest periods leads to the formation of LTM, massed learning trials without breaks leads to a separate type of memory termed anesthesia resistant memory (ARM). ARM is a form of memory likely found in all animals that can be disrupted immediately after training by concussion, anesthesia, or electroconvulsive shock, all of which disrupt the pattern of neural activity of the brain. Some disagreement still exists as to whether the molecular pathways of LTM and ARM interact with each other, although a recent study has suggested LTM formation leads to the extinction of ARM (Fig 1.2) (Isabel et al., 2004).

Learning in both memory pathways is mediated by cAMP signaling and disrupted in the *dunce (dnc)* cAMP phosphodiesterase mutant (which increases neuronal cAMP levels) and the *amnesiac (amn)* pituitary adenylyl cyclase activating peptide (PACAP) mutant (which decreases cAMP levels). In these learning deficient mutants, a rapid decay

Figure 1.2



Adapted from Isabel et al., 2004

Figure 1.2: Phases of memory storage **A,** Volado, FasII, Dunce and Rutabaga are essential to STM formation. Amnesiac is necessary for the transition to MTM and CREB for LTM formation. Along a separate pathway from MTM, Radish is involved in the transition to ARM. **B,** A new ARM, LTP pathway interaction model preposed by Isabel, Preat and colleagues in which ARM and LTP pathways suppress each other (Adapted from Isabel et al., 2004).

in conditioned olfactory avoidance occurs after only 30 minutes (Quinn et al., 1979; Feany and Quinn, 1995; Tully et al., 1985). While the chronic alterations in cAMP levels in either direction are sufficient to disrupt learning and memory, the molecular and morphological effects on synapses are distinct (Zhong and Wu, 2004). The high cAMP levels in *dnc* mutants cause more boutons to form at the NMJ, as well as greater quantal content during synaptic transmission (Davis et al., 1996). In contrast, *rutabaga (rut)* mutants, which disrupt the activity of an adenylyl cyclase, display low cAMP levels and have fewer NMJ boutons, each of which is larger than normal (Budnik et al., 1990). Comparing the effects of the mutations at the NMJ to those on behavior support the underlying assumption that specific patterns of activity may contribute to CNS memory formation through structural remodeling of synaptic connections, similar to what is observed at the NMJ.

To trigger LTM for olfactory-based behavioral changes in *Drosophila*, neuronal circuits must progress through the more intermediate, genetically separable phases of short-term memory (STM) and medium-term memory (MTM) (Fig 1.2). The intergrins Fasciclin II (FasII) and Volado (Vol) are essential for STM formation, and mutants in these proteins display memory defects within three minutes of training (Cheng et al., 2001; Grotewiel et al., 1998). The complex role of intergrins at the NMJ in forming and maintaining synaptic connections will be discussed in detail below. The transition from STM to MTM is abolished in the *rut* mutant, which eliminates a Ca^{2+} responsive adenylyl cyclase, as well as in *protein kinase A* (*PKA*) mutants (Livingstone et al., 1984; Li et al., 1996). The final step to LTM from MTM requires transcriptional induction of genes through CREB activity (Fig 1.2) (Yin et al., 1994; Yin et al., 1995). CREB-induced

expression of immediate-early genes (IEGs) is predicted to trigger permanent changes to neuronal structure and/or function.

CREB, a member of the bZIP transcription factor superfamily, is predicted to be the cAMP target largely responsible for triggering long-lasting transcriptional changes that alter synaptic structure and function in *Drosophila*. Two *CREB* genes have been identified in flies. *dCREB2-a* is a PKA-regulated cAMP-responsive transcriptional activator homologous to mammalian CREB, CREM, and ATF-1, and is the only cAMP-responsive *CREB* known to be encoded in the fly genome (Yin et al., 1995). A second member of the CREB family, *dCREB2-b*, functions as a CREB repressor and blocks CREB-dependent transcriptional upregulation and LTM formation in flies (Yin et al., 1994). Transgenic expression of the CREB suppressor, *dCREB2-b,* can also reduce the increased quantal content observed in *dnc* mutants, but does not reverse the structural changes at the synapse (Davis et al., 1996). Such observations suggest two independent pathways for plasticity, one regulating synaptic release and another effecting morphological expansion of the synapse. As such, cAMP increases caused by neuronal activity are proposed to trigger two parallel but interlinked pathways necessary for synaptic growth and increased neurotransmitter release.

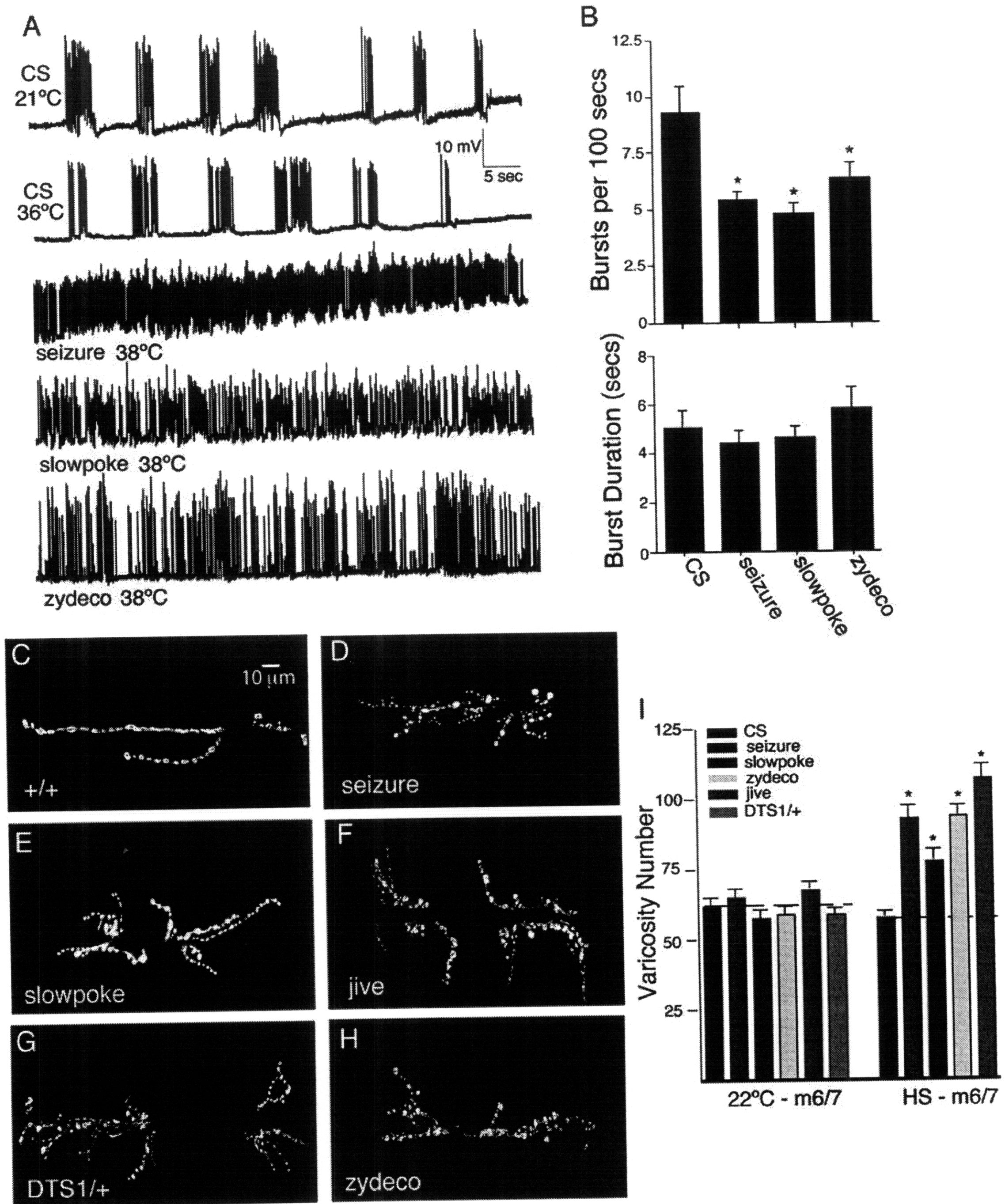
The role of regulated gene expression in the ARM pathway, which is specifically blocked in *Drosophila radish* (*rad)* mutants, is still unknown (Folkers et al., 1993). It has been reported that *rad* encodes a phopholipase-A2, which may trigger ARM through arachidonic acid induction of the atypical protein kinase C (aPKC) signaling pathway, previously implicated in regulation of synaptic bouton budding (Chiang et al., 2004; Ruiz-Canada et al., 2004). However, recent evidence indicates that the original gene

identification of *rad* as a phospholipase-A2 is incorrect (McGuire et al., 2005; Folkers et al., 2006). Folkers, Quinn and colleagues have mapped the *rad* mutation to a novel gene product encoded by the CG15720 locus. CG15720 encodes a mushroom-body enriched protein with multiple PKA phosphorylation sites and weak homology to RNA splicing factors. While the molecular function of the Rad protein is unclear, it could potentially regulate gene splicing. For in depth reviews of olfactory learning, LTM and ARM in *Drosophila*, see McGuire, Deshazer, and Davis (2005) and Margulies, Tully and Dubnau (2005).

1.1.2 Temperature-Sensitive Neuronal Excitability Mutants

While each *Drosophila* memory mutant disrupts a specific molecular pathway, conditional *Drosophila* mutants that alter neuronal firing patterns throughout the nervous system allow a broader evaluation of the effects of chronic and acute activity changes on gene expression. The hyperexcitability mutants *Shaker* (*Sh*), *ether-a-go-go* (*eag*), and *Hyperkinetic* (*Hk*) were originally isolated in screens for ether-induced leg shaking (Kaplan and Trout, 1969). TS activity mutants such as *paralytic* ($para^{TS1}$) and *seizure* (sei^{TS1}) were isolated in separate behavioral screens for adults displaying either paralysis or seizures at elevated temperatures (Ganetzky and Wu, 1986; Titus et al., 1997). The cloning of the neuronal activity mutations was originally important in defining the components of neuronal electrical responses (Na^+ channels in the case of *para*, and K^+ channels in the case of *Sh*). However, these mutants have also offered an opportunity to examine the molecular changes that occur at synapses under conditions of either reduced or enhanced activity. For example, the double mutant of *eag Sh*, which disrupts two

Figure 1.3



Adapted from Guan et al., 2005

Figure 1.3: Synaptic overgrowth following seizure induction. **A,** Enhanced firing of spontaneous central pattern generator assayed at muscle 6 of 3rd instar wandering larvae at non-permissive temperatures from *sei*^{TS1} (n=5), *slo*^{TS1} (n=10), and *zydeco* (n=7) compared to CS (n=10). **B,** Quantification of bursts occuring in 100s, (*sei*^{TS1} $p<0.0011$, *slo*^{TS1} $p<0.0005$, and *zydeco* $p<0.02$; compared to CS by student's T-test). **C-H,** Anti-Syt 1 staining of wandering 3rd instar larval NMJs at segment A3 following 37°C heat shock paradigm (75 min twice a day). **I,** Quantification of average bouton number (+/- SEM) at muscle 6/7 of segments A3-A5. * denotes $p<0.05$ by student T-test. (Adapted from Guan et al., 2005)

classes of potassium channels, results in repetitive neuronal firing and enhanced neurotransmitter release, leading to increases in cAMP levels and synaptic growth at the NMJ (Schuster et al., 1996 II). Examination of NMJ structure in a number of conditional mutants that increase synaptic activity has revealed a strong correlation between activity levels and synaptic bouton number, a correlation that is also observed with experiential strengthening of the synapse following increased locomotion (Budnik et al., 1990; Sigrist et al., 2003; Guan et al., 2005). With strengthening following enhanced locomotor activity in larvae, reversible increases in evoked synaptic responses occur after relatively short periods of increased locomotion (peaking after 2 hours). However, morphological changes occur on the order of several hours and include increases in the synaptic translational machinery, bouton number, and clustering of the glutamate receptor DGluR-IIA (Sigrist et al., 2003). Thus, rapid short-term changes in neuronal activity are thought to drive stable long-term plasticity, with separate molecular pathways regulating neurotransmitter release and synaptic structure.

1.2 Intracellular Signaling Pathways Involved in Synaptic Plasticity

cAMP and CREB play a well-characterized role in activity-dependent transcription-regulation in LTM formation. However, a number of other signaling pathways are also regulated by activity within the cell. Here we review some of the other pathways that respond and interact to balance synaptic growth and function.

1.2.1 PKA

A homolog of one of the well-known cAMP signaling targets, PKA, was identified in an enhancer trap screen for genes preferentially expressed in the mushroom bodies (Skoulakis et al., 1993). The *Drosophila* PKA homolog, DCO, is not only preferentially expressed in learning centers, it has also been demonstrated to play critical roles in learning and MTM formation (Skoulakis et al., 1993; Li et al., 1996). The requirement for PKA in MTM formation has recently been confirmed in honeybees (Friedrich et al., 2004), indicating that PKA is a critical downstream signaling module in cAMP-mediated induction of CREB transcriptional activity.

1.2.2 ERK/MAPK

It is well established that Ca^{2+} influx through voltage-sensitive Ca^{2+} channels trigger vesicle fusion in response to neuronal activity. Additional cellular signaling cascades are also linked to intracellular Ca^{2+} signaling, coupling transcriptional regulation to neuronal activity. In mammals, MAPK/ERK kinase (MEK) interacts directly with neuronal Ca^{2+} channels. Following Ca^{2+} influx, MEK activates ERK (Ras/ extracellular signal-regulated kinase) through phosphorylation (Dolmetsch et al., 2001). Conservation of Ca^{2+} -dependent MEK induction of ERK in *Drosophila* was demonstrated using the TS mutant *comt*^{tp7}; Ca-*P60A*^{Kum170}, which display long-lasting seizure activity following heat pulses. After a 4-minute pulse at 40°C, these animals showed a robust MEK-dependent phosphorlyation of ERK to Dp-ERK (Hoeffer et al., 2003). Additional studies at the larval NMJ showed that both pre- and post-synaptic activation of ERK requires MEK. Postsynaptic phosphorylation of ERK drives

translocation of the protein to muscle nuclei, where it induces CREB-dependent transcription of IEGs such as Fos (*kayak*) and c/EBP (*slbo*) (Hoeffer et al., 2003). Nuclear ERK signaling also influences the function of Jun, one component of the transcription factor AP-1.

AP-1 is the conserved heterodimer of the basic leucine zipper transcriptional activators Fos and Jun. In *Drosophila*, AP-1 regulates synaptic growth at the larval NMJ and increases quantal content of evoked synaptic potentials (Sanyal et al., 2002). Expression of either the D-Fos repressor (Fbz-) or the D-Jun repressor (Jbz-) results in reduced synapse size and higher synaptic FasII expression (Sanyal et al., 2002). AP-1's effect on neurotransmitter release is blocked by *dCREB2-b*, while its effect of synaptic varicosity number is not, suggesting that AP-1 can act upstream of CREB, possibly through direct binding of the CREB regulatory sequence (Sanyal et al., 2002). AP-1-dependent synaptic growth can also be induced through positive feedback from CREB-dependent transcriptional changes (Sanyal et al., 2002). In addition to ERK, AP-1-dependent synaptic growth can be induced by the MAPK, JNK (*basket*) (Peverali et al., 1996; Sanyal et al., 2002). Thus, Ca^{2+} signals accompanying neuronal activity are able to alter the function of at least two transcription factors through MAPK signaling cascades.

1.2.3 CaMKII

Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is another Ca^{2+}-dependent signaling protein enriched in neuronal tissues, and localizes to pre- and postsynaptic compartments. At low Ca^{2+} concentrations in resting neurons, CaMKII is autoinhibited and the kinase activity of the protein is Ca^{2+}/calmodulin-dependent. However, when

neurons are active and Ca^{2+} levels increase, autophosphorylation occurs at the T287 residue (T286 in mammals) and the autoinhibitory domain is released. Release of the inhibitory domain allows the catalytic domain to become Ca^{2+}-independent (for a review of CaMKII biochemistry, see Hanson and Schulman, 1992). While the protein is known to translocate to the nucleus, the mechanism by which this occurs is currently unknown (for a review, see Griffith et al., 2003).

CaMKII activity in *Drosophila* has been linked to behavioral memory and NMJ plasticity (for a review of CaMKII function in memory, see Lisman et al., 2002). Flies expressing a peptide inhibitor of CaMKII activity (Ala) perform poorly in a number of learning tasks, whereas expression of constitutively active CaMKII can enhance learning (Griffith et al., 1993; Mehren and Griffith, 2004). However, the effects of CaMKII expression differ depending on the neuronal population, suggesting some neurons may have a CaMKII saturation point beyond which additional expression has no effect (Mehren et al., 2004). At the NMJ, over-expression of constitutively active CaMKII results in enlarged individual boutons with abnormal spacing, while inhibiting CaMKII produces the opposite affect (Koh et al., 1999). The ability of CAMKII to modulate morphological synaptic changes appears to rely upon CaMKII-dependent regulation of intergrins, which are discussed in greater detail below.

During induction of LTM, the RNA interference (RISC) pathway has been implicated in regulation of synaptic components such as CaMKII (Ashraf et al., 2006). The 3' UTR of *Drosophila CaMKII* is both necessary and sufficient for mRNA localization to dendrites (as in mammals) and *CaMKII* mRNA is found in the antennal lobe of flies during olfactory training (Ashraf et al., 2006). The genetic interactions

between CaMKII and components of the RISC pathway suggest that the expression and synaptic localization of CaMKII is under control of RISC components (Ashraf et al., 2006).

1.3 Intercellular Retrograde Signaling Pathways in Plasticity

Traditionally, synaptic signaling through release of small molecules and neuropeptides was thought to occur only at axons. This view was propagated in part due to the clear geometric polarity of neurons, and the lack of assays for presynaptic response to acute retrograde signal release. However, dendritic release of a number of neuromodulators such as dopamine, ATP, GABA, and neuropeptides has now been documented in many areas of the brain, as well as bidirectional synaptic signaling through transmembrane complexes (Geffen et al., 1976; Heeringa and Abercrombie, 1995; Cheramy et al., 1981; Araneda and Bustos, 1989; Israel and Meunier, 1978; Isaacson, 2001; Pow and Morris, 1989; Knoll and Drescher, 2002; Ludwig and Pittman, 2003; Li et al., 2005). A common theme in retrograde signaling research has been the role of such signals in synaptic plasticity and development.

The cellular machinery that regulates release of retrograde signals is not well understood. However, Ca^{2+}-dependent exocytosis of postsynaptic vesicles in mammalian neurons has been documented (Maletic-Savatic and Malinow, 1998; Li et al., 2005). Amperometric recording of somatodendritic dopamine release reveals kinetics similar to presynaptic quantal events, with release occurring in less than a millisecond (Li et al., 2005). Other evidence suggests that SNARE proteins, whose role in presynaptic release is discussed below, are also necessary for dendritic release of dopamine (Fortin et al.,

2006) as well as Ca^{2+}-dependent exocytosis of hippocampal dendritic vesicles (Maletic-Savatic and Malinow, 1998). The parallels between presynaptic and postsynaptic release, and in particular the role of Synaptotagmins in Ca^{2+} sensing of release, will be discussed in the more detail below. First, I will discuss what is known about retrograde signaling in *Drosophila* and the targets of activity dependent regulation.

1.3.1 Transmembrane Signaling

Transmembrane signaling complexes, such as the ephrin/ephrin receptor, have already been demonstrated to function in bi-directional signaling (Knoll and Drescher, 2002). Other transmembrane complexes likely to be involved in bi-directional signaling include cadherins, the neurexin-neuroligin complex, and SynCAMs. However, differentiating the role of cellular adhesion molecules in synaptic organization versus acute signaling is a challenging task. In *Drosophila*, the best characterized example is the neuronal cell adhesion molecule (NCAM), FasII.

NCAMs and intergrins are two important regulators of cellular movement and growth. One of the molecular changes observed during experiential strengthening of the NMJ is downregulation of the NCAM Fasciclin II (FasII) (Sigrist et al., 2003). As mentioned above, an inverse relationship between synaptic activity and FasII expression is also observed in a number of *Drosophila* epilepsy and learning mutants, including *eag Sh* and *dnc*. Both of these mutants exhibit enhanced neurotransmitter release and synaptic growth, increased cAMP concentrations, and a decrease in FasII levels (Schuster et al., 1996 II; Davis et al., 1996; Sanyal et al., 2002). Reduction in the levels of FasII expression by ~50% is sufficient to increase the number of boutons at larval NMJs

(Schuster et al., 1996 I). Over-Expression of FasII in *eag Sh*, *dnc*, or AP-1 mutant backgrounds can prevent the increased bouton growth observed in these mutants, but does not rescue the enhanced neurotransmitter release phenotype (Schuster et al., 1996 II). These findings suggest that alteration in FasII levels is a key regulatory pathway that controls synaptic structure. CREB-dependent transcriptional changes are thought to occur in parallel to FasII modulation, allowing additional regulation of neurotransmitter release.

The *fasII* gene produces three isoforms differentially expressed in *Drosophila*; one is attached to the plasma membrane via GPI linkage, while two isoforms contain transmembrane domains with a short carboxy termini (one of which contains a PEST protein degradation sequence) (Grenningloh et al., 1991; Lin and Goodman, 1994). During axon outgrowth, transmembrane-containing FasII isoforms are expressed throughout motor neurons. By the 2nd instar larval stage, expression is limited to synaptic terminals (Schuster et al., 1996 I). Early synapse formation in *fasII* null mutants appears normal. However, a few hours after hatching, mutant larvae become sluggish and eventually die as synapses retract (Schuster et al., 1996 I). These findings suggest a model whereby a threshold level of FasII is necessary for synapse stabilization, with further asymmetric increases in FasII levels at the synapse serving to inhibit additional synaptic expansion. Elevated levels of FasII may curb NMJ growth by 'over-stabilizing' synapses, as over-expression of FasII leads to the persistence of ectopic motor neuron synapses at mismatched nerve-muscle connections in larval animals (Davis et al., 1997).

FasII plays a similar stabilizing role at synapses in the larval CNS and on adult flight muscles (Baines et al., 2002; Hebbar and Fernandes, 2005). The effects of FasII

levels on adult CNS function, and specifically in learning and memory, were assessed using *Drosophila fasII* hypomorphs lacking expression in the mushroom bodies. While mushroom body neurons appear to form normally in *fasII* hypomorphs, the mutants are deficient in olfactory memory formation (Cheng et al., 2001). Such data support the idea that FasII may play a similar role in the *Drosophila* CNS as observed at the larval NMJ, and that acute regulation of synaptic growth is important in olfactory-cued learning. Rescue of the *fasII* null phenotype at the NMJ requires expression of FasII on both sides of the synapse, consistent with data obtained in cell culture that indicates FasII forms transmembrane homophilic complexes that span the synaptic cleft (Schuster et al., 1996 I).

Recent results indicate that the amyloid precursor protein (Appl), whose mammalian homolog (App) is implicated in Alzheimer's Disease, acts downstream of FasII in the regulation of NMJ structure. Although APPL is not necessary for synaptic stabilization (as observed in *FasII* mutants), *appl* mutants have smaller boutons than wildtype and are not capable of the synaptic overgrowth that *fasII* mutants produce (Ashley et al., 2005). Appl is postulated to provide a link between FasII and cytoskeleton regulation (Brouillet et al., 1999) that would be necessary for regulation of bouton budding that occurs during synaptic growth (Ruiz-Canada et al., 2004). One additional member of the pathway, dX11 (*mint*), has been identified as an interaction partner for both Appl and FasII via its PDZ domains, and may mediate transport of binding partners to FasII (Ashley et al., 2005). While most studies have explored the roles FasII plays as a homophilic cell adhesion molecule, FasII may also directly participate in cell signaling. This hypothesis is supported by data showing that the intercellular domain of FasII is

essential for promoting synaptic growth. FasII has also been shown to control proneural gene expression in sensory organ development (Garcia-Alonso et al., 1995). These findings argue that a FasII-APPL signaling pathway plays a critical role in the modulation of synaptic growth at the *Drosophila* NMJ.

As mentioned above, FasII appears to act downstream of AP-1-dependent transcription, though it also subject to non-transcriptional regulation. Clustering of FasII on both sides of the synapse, facilitated by the *Drosophila* PSD-95 homolog Dlg (which is also responsible for the clustering of Sh K^+ channels), is important to its function, and represents one opportunity for post-transcriptional regulation (Thomas et al., 1997). Clustering of FasII is disrupted when Dlg is phosphorylated by CaMKII, resulting in increased synaptic growth (Koh et al., 1999). In addition to Ca^{2+}, position specific intergrins, including αPS1 (*mew*), αPS2 (*if*), αPS3 (*vol*), and βPS (*mys*), can also interfere with CaMKII's ability to regulate FasII distribution within synapses (Beumer et al., 2002). In *mys* mutants, synaptic FasII levels are higher compared to control animals, and larva show synaptic growth defects. These synaptic growth defects can be rescued by changes in CaMKII expression (Beumer et al., 2002).

Analysis of AP-1 mutants also suggest that the MAPK encoded by the *jnk* locus contributes to regulation of FasII levels downstream of AP-1 induced transcription (Sanyal et al., 2002). ERK and the Ras1-MAPK pathway have also been implicated in regulation, as ERK activation correlates with reduced FasII activity and the induction of the IEGs Fos (*kayak*) and c/EBP (*slbo*) (Hoeffer et al., 2003). Both RasI and MAPK are present at synapses, and increases in RasI transgenes that activate MAPK promote synaptic growth (Koh et al., 2002). In MAPK loss-of-function mutants, the amount of

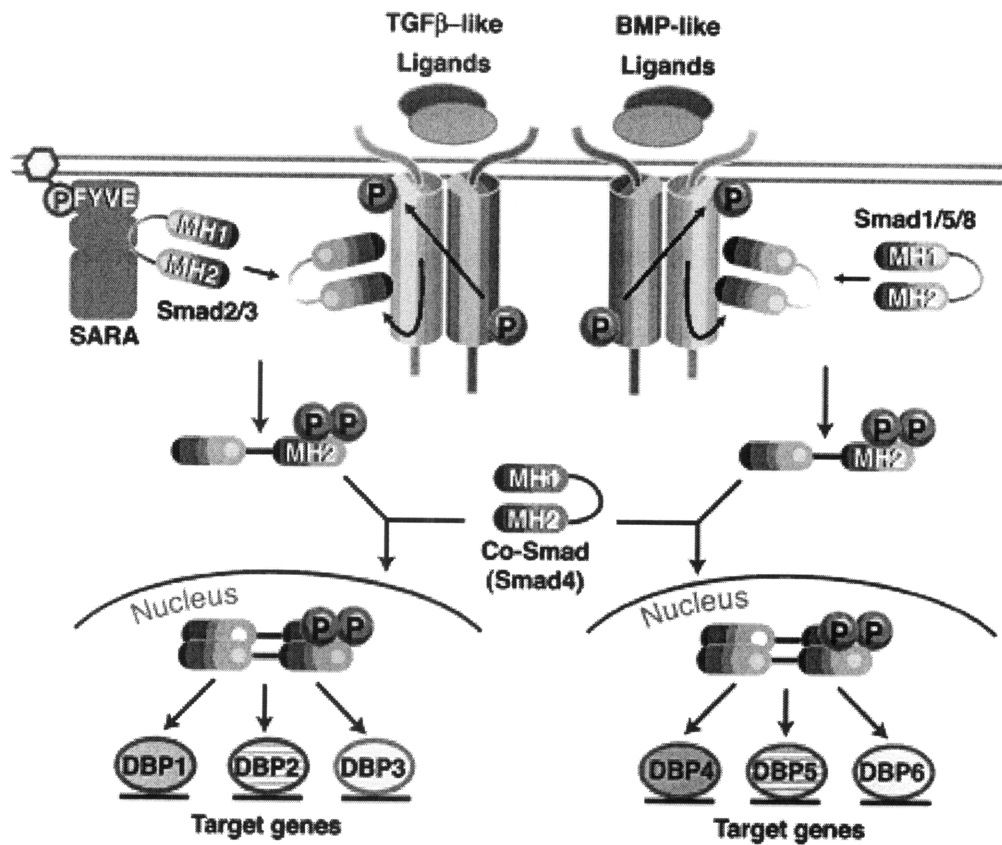
FasII co-immunoprecipitating with Dlg increases by 170% (Koh et al., 2002). Conversely, increasing the amount of MAPK leads to a reduction of FasII and corresponding increases in synaptic growth (Koh et al., 2002). Similar analysis of the Aplysia FasII homolog, apCAM, in long-term facilitation indicates that MAPK phosphorylates the apCAM PEST sequence, leading to ubiquitination and degradation of the protein (Michael et al., 1998; Bailey et al., 1997; Martin et al., 1997). While the full details of FasII regulation and signaling are unknown, FasII function, along with that of intergrins and other NCAMs, critically regulate synaptic structure and growth in response to activity.

1.3.2 TGF-β /BMP

A number of characterized synaptic retrograde signals, including BMP, are known to be released through postsynaptic secretion. Synaptic BMP signaling requires binding of a secreted ligand, triggering interactions between type I and type II BMP receptors in the presynaptic cell. Activation results in downstream signaling that drives synaptic growth at *Drosophila* NMJs (Wrana et al., 1994).

Both type I and II BMP receptors are single pass transmembrane serine/threonine kinases. Ligand binding by type II receptors results in phosphorylation of the GS box of type I receptors, triggering kinase activation and subsequent phosphorylation of receptor-mediated Smads (R-Smads) (Wrana et al., 1994). Phosphorylated R-Smads associate with co-Smads to form a complex that translocates into the nucleus and activates gene transcription (Wrana and Attisano, 2000). The *Drosophila* genome includes three type I

Figure 1.4



Adapted from Attisanto and Wrana, 2002

Figure 1.4: TGFβ signaling pathway. Ligands bind specific receptors, which in turn phosphorylate specific R-Smads. R-Smads bind Co-Smads, and the complex translocates to the nucleus. In the nucleus, the complexes associate with different DNA binding proteins to affect transcription. (Adapted from Attisanto and Wrana, 2002)

receptors (Tkv, Sax, Babo) and two type II receptors (Punt and Wit). Mutants for the R-Smad *mad*, the co-Smad *med*, and the type I receptors *tkv* and *sax,* all show reduced NMJ size and function, implicating BMP-dependent gene transcription in synaptic growth and function (McCabe et al., 2004). At the NMJ, BMP receptors are required in the presynaptic terminal, while BMP ligands are secreted from the muscle, indicating a retrograde signaling pathway regulates synaptic growth via changes to motor neuron gene expression (McCabe et al., 2004). A recent report suggests that the lack of rapid homeostatic compensation when exposed to PhTx in *gbb* mutants can be rescued by either pre- or post-synaptic expression of *gbb,* separating the homeostatic phenotype from those of reduced NMJ growth and baseline transmission (Goold and Davis, 2007). Release of BMPs from the muscle may provide a signaling mechanism that confirms productive synapse formation, triggering additional changes in neuronal gene expression that promote enhanced connectivity.

1.3.3 Trk/Neurotrophin

The neurotrophins, nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and neurotrophin 3 and 4/5 (NT3 and NT4/5), make up a family of small mammalian proteins involved in receptor tyrosine kinase (Trk receptor) signaling in regulation of neuronal survival and connectivity during development. In mammals, NGF binds TrkA, BDNF and NT4/5 bind TrkB, and NT3 binds TrkC (for neurotrophin reveiw see: Zweifel et al., 2005). Best characterized in developing neurons of the peripheral nervous system (PNS), target cell release of retrograde neurotrophic signals lead to axonal transport of the neurotrophin and downstream effectors to the cell body (Hendry,

1975). In cell culture systems, the transport of even small amounts of NGF from distal axons is sufficient to encourage neuronal survival (Campenot, 1977). The downstream effectors of the neurotrophins interact with CREB and other transcriptional regulation systems (Lonze and Ginty, 2002). Although there are no reported *Drosophila* BDNF or NGF homologs, NGF-like and Trk-like proteins have been reported in the earthworm invertebrate system, suggesting that the proteins are not evolutionarily limited to mammals (Davoli et al., 2002). A related family of receptor tyrosine kinases, the Ror-family, does contain homologs conserved from *Drosophila* to mammals (Wilson et al., 1993). Furthermore, the evolutionarily conserved *Drosophila* protein Numb plays a role in PC12 cells in regulating cellular response to NGF-induced differentiation (Pedersen et al., 2002). Together these results suggest that an, as of yet, unreported neurotrophic pathway may regulate plasticity in *Drosophila* neurons.

1.4 Targets of Activity-Dependent Regulation

The activation of IEGs and CREB-mediated transcription have been shown to be important in LTM in both vertebrates and invertebrates (Dash et al., 1990; Huang et al., 1994; Bito et al., 1996; Sanyal et al., 2002; Kelleher et al., 2004; Etter et al., 2005). We have already described above these and other signaling pathways that are activated by neuronal activity. The eventual targets of these pathways, including the Synaptotagmins, and their effects on the synaptic environment are explored below.

1.4.1 Futsch- Cytoskeleton

Cytoskeletal regulation is likely to be a critical target for modulation of synaptic growth. In particular, regulation of the microtubule-based cytoskeleton has been implicated in synapse proliferation in *Drosophila*. Mutations in *futsch*, which encodes a microtubule-binding protein of the MAP1B family, indicate that MAP1B positively regulates both axonal and dendritic growth (Hummel et al., 2000). The Futsch protein localizes to microtubule loops that occur in terminal boutons and at sites of NMJ branching (Hummel et al., 2000; Roos et al., 2000). Futsch staining is strongest at the proximal end of microtubule loops and may function to stabilize microtubules (Roos et al., 2000). Although the exact role of MAP1B in bouton formation is not clear, regulation of the formation of microtubule loops at terminal boutons appears to be critical for bouton division and synaptic branching (Roos et al., 2000). Loss-of-function *futsch* mutations disrupt microtubule loops, resulting in fewer, larger boutons (Roos et al., 2000). A partial rescue can be achieved by expressing the N-terminal of the protein, which contains the putative microtubule-binding domain, demonstrating that is the microtubule-related function that is responsible for synaptic growth (Roos et al., 2000).

Futsch-dependent morphological changes at the synapse appear to be regulated by the *Drosophila* fragile X related (dFxr) protein. Fragile X mental retardation is the most common cause of mental retardation in humans, suggesting a critical role for the fragile X protein in higher brain function. The *Drosophila* homolog, dFxr, is expressed in the cytoplasm of most neurons and suppresses Futsch translation through direct binding of *futsch* mRNA (Zhang et al., 2001). Over-Expression of dFxr causes phenotypes that mimic those observed in *futsch* null mutants, with reduced NMJ complexity and larger

synaptic boutons. *dfxr* null mutants have phenotypes affecting synaptic growth in the opposite direction, with larvae showing increased synaptic branching and bouton number (Zhang et al., 2001). The similarity of phenotypes indicates dFxr may regulate synaptic structure through direct regulation of *futsch* (Zhang et al., 2001). In addition to regulating *futsch*, dFxr may also control translation of factors modulating neurotransmitter release, as *dfxr* mutants have a significant reduction in evoked currents and a slight increase in mini amplitude (Zhang et al., 2001). In contrast, over-expression of dFxr can increase miniature junction current (mEJC) frequency and also slightly increases mEJC amplitude (Zhang et al., 2001). Similarly, loss-of-function and over-expression of dFxr in the central nervous system can disrupt synaptic transmission in the visual system (Zhang et al., 2001). These data indicate that synaptic signaling pathways can regulate both transcription and local mRNA abundance to modulate synaptic growth during larval development in *Drosophila*.

1.4.2 Highwire and Fat Facets- Ubiquitination

Regulation of the post-translational machinery at synapses provides additional control mechanisms that allow neurons to generate rapid changes in the molecular environment of the synapse. Ubiquitination is one of the prominent pathways through which protein half-life is regulated, and the activity of the conserved ubiquitin ligase Highwire (*hiw*) and the deubiquitin protease Fat Facets (*faf*) plays a critical role in controlling synaptic structure and function.

Hiw, a putative RING-H2 E3 ubiquitin-protein ligase, is localized to presynaptic terminals throughout development (Wu et al., 2005). Like *fasII* hypomorphs, *hiw* mutants have an increased number of type I boutons at NMJs, although each bouton is smaller and

has reduced neurotransmitter release (Wan et al., 2000). Similar to *hiw* mutants, Faf over-expression leads to proliferation of boutons, an increase in the length of synaptic innervation along the muscle, and a decrease in neurotransmitter release (DiAntonio et al., 2001). *faf* loss-of-function alleles can rescue the *hiw* loss-of-function phenotypes, while over-expression of Faf is lethal in the *hiw* null background (DiAntonio et al., 2001). Hiw/Faf regulation may interface with several signaling systems in the presynaptic terminal that independently regulate synaptic growth and function, as low levels of the *hiw* transgene can rescue neurotransmitter release defects, while high transgene expression is required to rescue structural overgrowth (Wu et al., 2005). Together, the manipulations of these two components of the ubiquitination pathway suggest an important role for regulated protein degradation at synapses in the control of signaling pathways that mediate synaptic growth (DiAntonio et al., 2001).

One target pathway known to be regulated by Hiw/Faf is the BMP signaling cascade. Mutants in the type II receptor *wit* have reduced quantal content, a smaller number of boutons, and misalignment of presynaptic active zones (Marques et al., 2002; Aberle et al., 2002). Mutants in the gene for the Wit ligand *glass bottom boat* (*gbb*) exhibit many of the same defects, including the decrease in bouton number, altered synaptic ultrastructure, and the lack of activated Mad in motor neurons (McCabe et al., 2003; Marques et al., 2003). In the background of either the BMP signaling mutant *wit* or *med,* the synaptic overgrowth observed in *hiw* loss-of-function mutants and Faf over-expression animals is reduced (McCabe et al., 2004), suggesting Hiw/Faf ubiquitination/ deubiquitination regulates synaptic structure in part through the BMP signaling cascade. Although over-expression of TGF-β signaling components at the synapse is not sufficient

to trigger synaptic overgrowth in wildtype terminals, over-expression in the *hiw* mutant background leads to increased synaptic proliferation (McCabe et al., 2004). These results suggest a model whereby Hiw ubiquitination provides a dampening system to modulate BMP-dependent signaling at the synapse.

Genetic interactions between *Hiw* and mutations in the gene for the MAPKKK *wallenda* (*wnd*), suggest that Hiw also regulates MAPK signaling cascades independent of *wit* (Collins et al., 2006). Indeed, Wnd expression is directly regulated by Hiw ubiquitination, and is essential for the synaptic overgrowth observed in both *hiw* loss-of-function mutants and *faf* gain of function strains (Collins et al., 2006). Meanwhile, Wnd over-expression is sufficient to phenocopy the *hiw* loss-of-function phenotype, suggesting it is an essential target of ubiquitination regulation (Collins et al., 2006). In contrast to *wnd's* suppression of the *hiw* synaptic overgrowth phenotype, the physiology defects in *hiw* mutants are not rescued, suggesting additional molecular pathways are likely modulated by Hiw function (Collins et al., 2006). Wnd is a member of a family of MAPKKKs involved in JNK and p38 signaling. Mutations in the *Drosophila* JNK homolog, *basket (bsk),* can rescue either *hiw* loss-of-function or *wnd* over-expression phenotypes (Collins et al., 2006). In contrast, mutations in the p38 signaling pathway are not sufficient for rescue in these genetic backgrounds (Collins et al., 2006). *Drosophila* Fos (but not Jun) is also necessary for *hiw* synaptic overgrowth phenotypes, suggesting that transcriptional changes secondary to altered ubiquitination may act through a non-AP-1 pathway (Collins et al., 2006). Hiw has been suggested to regulate a number of other proteins including the hamartin and tuberin complex (in *Drosophila dTsc1-dTsc2*), as well as components of the rapamycin/S6K/4E-binding protein signaling pathway

(Murthy et al., 2004). In summary, regulation of protein ubiquitination at NMJs can modulate multiple signaling pathways, providing an avenue for regulation of synaptic structure and function.

1.4.3 Intercellular Signaling

An additional class of genes subject to activity-regulation, and one that will be explored in further detail, are those that encode proteins that modulate membrane trafficking and secretion within neurons. In particular, mRNA levels for several members of the Synaptotagmin family of vesicle Ca^{2+} sensors have been shown to be activity regulated. Transcriptional upregulation of mRNA for Synaptotagmin 1 (Syt 1) has been identified in several *Drosophila* seizure mutants (Guan et al., 2005). Syt 1 localizes to presynaptic vesicles and acts as the Ca^{2+} sensor for synchronous neurotransmitter release in *Drosophila* and mammals (Yoshihara and Littleton, 2002; Nishiki and Augustine, 2004). Given that synaptic vesicle release has been shown to be modulated by the levels of Syt 1 (Yoshihara and Littleton, 2002), a direct regulation of its mRNA by activity provides an avenue for long-term control of synaptic vesicle fusion rates. Another member of the Synaptotagmin Family, Synaptotagmin 4 (Syt 4), has also been identified in rat brain and PC12 cells as an IEG induced by seizure activity, depolarization, or forskolin application (Vician et al., 1995; Ferguson et al., 1999). Syt 4 is a conserved member of the Synaptotagmin Ca^{2+} sensor family and localizes to postsynaptic vesicles, suggesting a role in coupling postsynaptic Ca^{2+} influx to release of retrograde signals (Adolfsen et al., 2004; Yoshihara et al., 2005). Mutations in Syt 4 disrupt the timing of normal NMJ synapse development, and abolish specific forms of synaptic plasticity at

embryonic NMJs (Yoshihara et al., 2005). Over-Expression of Syt 4 in the postsynaptic compartment causes an overproliferation of boutons at the NMJ, suggesting a role for Syt 4-regulated retrograde signaling in synaptic wiring (Yoshihara et al., 2005). *syt 4* null mutants in mice show deficiencies in hippocampal dependent learning and memory tasks, as well as problems with motor control (Ferguson et al., 2000; Ferguson et al., 2004a; Ferguson et al., 2004b). Together, these data suggest that activity-dependent regulation of transcription of members of the Synaptotagmin family may provide a long-term mechanism to modulate vesicle cycling in both pre- and post-synaptic compartments. Below I focus on the mechanics and regulation of vesicular fusion at the synapse.

1.5 SNARE Mediated Vesicular Fusion

All intracellular fusion, with the exception of mitochondia and peroxisomes, appears to rely on SNARE proteins and the generalized SNARE mechanism of fusion (Hermann et al., 1998; Sesaki and Jensen, 2001; Titorenko and Rachubinski, 2000). While unusual for its tight regulation by activity, Ca^{2+} dependent exocytosis in neurons is produced through the introduction of the Complexin (Cpx) fusion clamp/Syt Ca^{2+} sensor system to the more generalized SNARE fusion machinery (Giraduo et al., 2006; Schaub et al., 2006; Huntwork and Littleton, 2007; Yoshihara and Littleton, 2002). Below, I review SNARE function so as to understand the role of Synaptotagmins in its regulation.

1.5.1 SNARE Mechanism

The SNARE protein superfamily is defined by the presence of a 60-70 amino acid SNARE motif which includes coiled-coil like heptad repeats capable of close association with other SNARE domains (Weimbs et al., 1997; Fasshauer et al., 1998). The

superfamily includes 25 *Saccharomyces cereisiae*, 36 human, 54 *Arabidopsis thaliana* and 19 *Drosophila* members (Jahn and Scheller, 2006; Hong, 2005; Littleton, 2000). Most SNARE proteins are membrane bound by a C-terminal transmembrane domain, however, some are anchored to membranes through palmitoylation, or association with other membrane bound SNARE proteins (Fukasawa et al., 2004; Veit et al., 1996; Vogel et al., 2000). The SNARE theory proposed that SNARE complexes form between vesicle bound SNARE proteins (v-SNAREs) and target membrane bound SNARE proteins (t-SNAREs) that bring membranes close together, overcoming the energy barrier associated with membrane fusion (Sollner et al., 1993).

In mammalian neurons, the four helix SNARE-pin is formed by fusion of the tail-anchored v-SNARE Synaptobrevin2/VAMP (Syb2), the t-SNARE Syntaxin1A (Syx1A), which contributes a 'heavy chain,' and the t-SNARE SNAP-25, which contributes two 'light chains'(Fig 1.5) (Fukuda et al., 2000; Bennett et al., 1992; Oyler et al., 1989). The *Drosophila* homologs are respectively known as neuronal-Synaptobrevin (n-Syb), Syntaxin (Syx) and SNAP-25. The SNARE domains of the proteins zipper shut in an N- to C-terminal direction (toward the typically membrane anchored end) into the 4 helix structure (Sutton et al., 1998). SNARE mediated membrane fusion transitions through a hemifusion intermediate as demonstrated by fluorescence lipid mixing assay using the mammalian neuronal SNAREs (Lu et al., 2005). Post-fusion, NSF and α-SNAP cooperate in disassembly of the SNARE complex through NSF's ATPase activity (Hohl et al., 1998; Wimmer et al., 2001; Furst et al., 2003). Lipid mixing assays have demonstrated that the SNAREs represent the minimum machinery necessary for vesicular fusion, although the neuronal SNAREs are insufficient to reconstitute Ca^{2+}-dependent

Figure 1.5

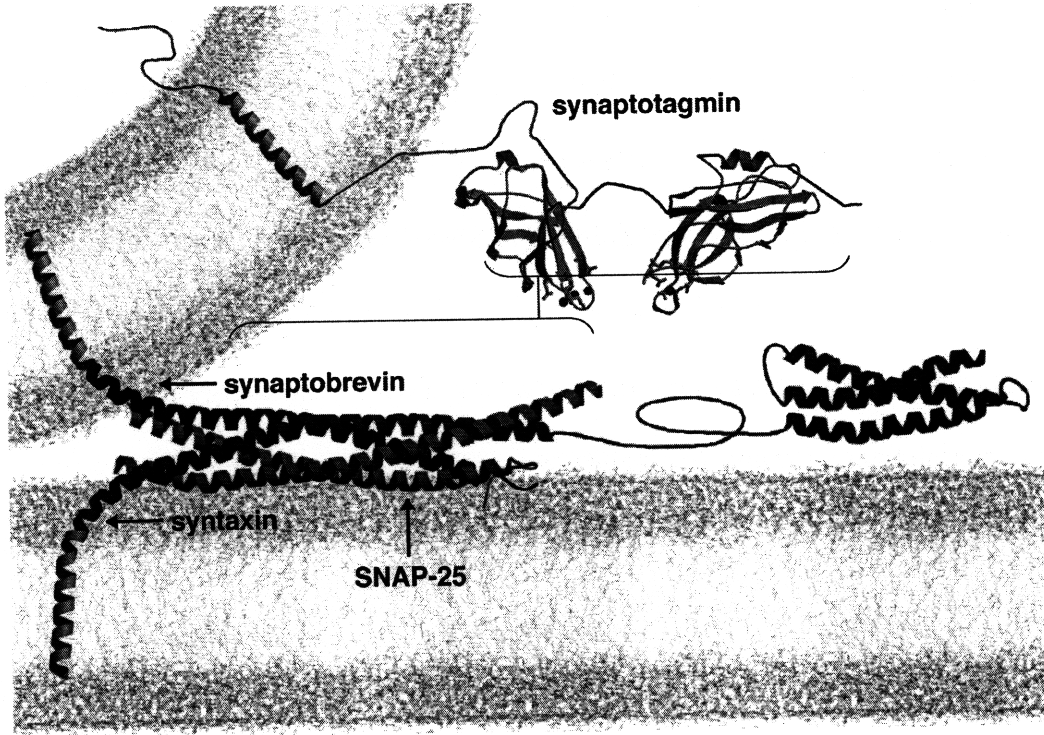


Figure 1.5: SNAREs in vesicular fusion. Vesicular fusion is mediated by the formation of the SNARE complex between vesicular Synaptobrevin, the target membrane bound Syntaxin, and soluble SNAP-25. Vesicular Synaptotagmin binds Ca^{2+} (shown in red), aids in formation of the SNARE complex, and bending of the target membrane (not shown).

fusion (Weber et al., 1998). The Syx family of t-SNAREs include 8 yeast, 11 *Drosophila*, and 9 *C. elegan* members (Littleton, 2000). The v-SNAREs have 10 yeast but only 5 *Drosophila* members including n-Syb, YKT6p, Sec22p, Gos28, and Vti1p (Littleton, 2000). Finally, the SNAP family of t-SNAREs have only three *Drosophila* and *C.elegan* members, suggesting they must be more promiscuous than the Syxs (Littleton, 2000). In *Drosophila* there are two homologs of NSF/Sec18 and three of α-SNAP/Sec17 (Littleton, 2000).

Although most often identified through the functional definitions v- or t-SNARE, structural evidence has led to a new SNARE protein designation system based upon the residue displayed on the binding surface of the SNARE motif. It is proposed that one member of each of the new designations, R, Qa, Qb, and Qc, is required to form a SNARE complex. The R-SNAREs include the VAMP/Syb family (VAMP1.2.3.4.5.7.8. Sec22b, and Ykt6), Qa the Syx subfamily, Qb the SNAP-25 N-terminal-like SNAREs, and Qc the SNAP-25 C-terminal-like SNAREs (Sutton et al., 1998; Fasshauer et al., 1998). Some Qa-Qb-Qc-R complexes occur that do not match the typical v-SNARE and t-SNARE designations, for instance some Qc SNAREs may function as v-SNAREs (Parlati et al., 2002).

1.5.2 SNAREs and the Vesicle Cycle

The SNARE hypothesis developed from co-evolving stories in yeast vesicular transport and mammalian neurobiology. In mammals, the SNARE story began when *N*-ethylmaleimide-sensitive fusion protein (NSF) was identified based on its ability to restore vesicular transport to a cell free Golgi system blocked by *N*-ethylmaleimide

(Wilson et al., 1989). NSF showed homology to yeast protein Sec18, suggesting that membrane fusion machinery may be conserved through evolution. Additional probing of the cell free system quickly lead to the identification of soluble-NSF attachment protein (αSNAP) (Clary et al., 1990). Using SNAP protein as bait in binding experiments with bovine brain extract, the SNAREs were identified as SNAP receptors (Sollner et al., 1993). Sollner, Rothman and colleagues proposed that SNAREs provide both a mechanism of membrane fusion, as well as specificity to fusion, based on the specificity of v-SNAREs for particular t-SNAREs (Sollner et al., 1993).

The real proof that SNARE proteins were responsible for neuronal vesicular fusion came from studies of botulism and tetanus toxins, which were known to disrupt vesicular fusion and synaptic transmission. The proof came by way of demonstration that specific tetanus (TTX) and botulism (BTX) toxins cleave Syb2 (Schiavo et al., 1992; Yamasaka et al., 1994), SNAP-25 (Schiavo et al., 1993; Binz et al., 1994), and Syx (Blasi et al., 1993) to arrest fusion. While the individual SNAREs can be cleaved by the toxins, the SDS-resistant SNARE complex, once formed is not susceptable to clevage (Hayashi et al., 1994).

Meanwhile, in yeast, Sec22 (a VAMP homolog) was identified as essential for vesicular transport (Newman et al., 1990). Snc1, another homolog of VAMP, was identified for it's ability to suppress loss of CAP function (part of the RAS-responsive S. cerevisiae adenylyl cyclase complex) (Gerst et al., 1992). Analysis of *snc1* and *snc2* mutants showed accumulation of post-golgi transport vesicles and that the proteins are involved in the post-Golgi secretory pathway (Protopopov et al., 1993). The Snc proteins were shown to tightly interact with Sec9 (SNAP-25 homolog), much like the SNARE

proteins in mammals (Couve and Gerst, 1994; Brennwald et al., 1994). Syx homologs Sso1p, Sso2p, Sed5 were also picked up in screens for vesicular transport mutants (Aalto et al., 1993; Hardwick and Pelham, 1992). Together Sso1, Sec9, and Snc1 were demonstrated to have SNARE-like interactions, with disassembly mediated by the α-SNAP homolog, Sec17 (Rossi et al., 1997).

In the proposed model of vesicle cycling, after SNARE-mediated vesicle fusion occurs, the SNARE complex is disassembled in the plasma membrane by NSF and α-SNAP. v-SNARE proteins are then endocytosed for use in another round of vesicular fusion. In *Drosophila, comatose (comt)* (NSF homolog), and *syx* TS mutants were used to reversibly block synaptic transmission, demonstrating the hypothesized SNARE-mediated synaptic vesicle cycle *in vivo* for the first time (Littleton et al., 1998). Yet, it remained to be shown conclusively where NSF disassembled the SNARE complex in the vesicle cycle. At the restrictive temperature, neurotransmission initially continued in *comt* mutants, but there was an accumulation of SDS-resistant SNARE complexes in the plasma membrane (Tolar and Pallanck, 1998). Simultaneously reducing fusion with a *para* TS mutation (discussed above) delayed the accumulation of SNARE complexes in *comt*, while simultaneously blocking endocytosis with a *shi* (dynamin) TS mutation lead to complete depletion of synaptic vesicles (Littleton et al., 2001). This demonstrated that NSF was indeed involved in post-fusion disassembly of the SNARE complex. For an in depth review of *Drosophila* SNARE mutants and their phenotypes, see Kidokoro 2003. SNARE proteins are also known to interact with vesicular coat proteins such as COPI, COPII, and epsinR, and may also play an active role in the endocytosis steps of the

vesicle cycle (Mossessova et al., 2003; Miller et al., 2003; Rein et al., 2002; Hirst et al., 2004; Deak et al., 2004).

In *Drosophila,* n-Syb and Syx are both essential for evoked release (Deitcher et al., 1998; Schulze et al., 1995). n-Syb is additionally necessary for 75% of spontaneous release (Deitcher et al., 1998). In mice, the same blockage of evoked and incomplete but severe blockage of spontaneous release, is seen in *syb2* mutants (Schoch et al., 2001). These two functions of Syb2 have been genetically separated by insertion of a short linker sequence between the transmembrane and SNARE domains. With a long linker insert, Syb2 cannot rescue either function, however with a short linker Syb2 can restore spontaneous but not evoked fusion (Deak et al., 2006).

Little is understood of the importance of spontaneous (sometimes called 'miniature' or 'quantal') release events, which involve the fusion of individual vesicles at the presynaptic terminal. Enhanced spontaneous release can trigger firing behavior in CA3 pyramidal cells, as well as alter firing behavior in interneurons (Sharma and Vijayaraghavan, 2003; Carter and Regehr, 2002). In the absence of evoked release (due to TTX treatment), rat neuronal culture experiments suggest that spontaneous release is sufficient to maintain dendritic structure (McKinney et al., 1999). A novel proposed activity of Syb is maintaining a separate spontaneous fusion vesicle population that does not participate in evoked release (Sara et al., 2005). Postsynaptic signaling pathways important to homeostasis, such as MAPK, may serve to integrate information from spontaneous firing (Murphy et al., 1994). Blocking spontaneous release in hippocampal neurons increases dendrite protein synthesis and, correspondingly, increasing spontaneous release reduces protein synthesis (Sutton et al., 2004). The suppression of

protein synthesis by spontaneous release events may fine tune the synaptic function of dendrites by stabilizing receptor populations (Sutton et al., 2006).

1.6 Synaptotagmins and Ca^{2+} Regulation of SNARE Fusion

Synaptotagmin proteins form a large and well conserved family of Ca^{2+} sensors found in organisms ranging from invertebrates to mammals (for review of evolutionary conservation across kingdoms, see Craxton 2004). Members of the family have an N-terminal intra-vesicular domain, a transmembrane domain, two C2 Ca^{2+} binding domains (C2A and C2B), and a short C-terminal tail. Syt 1 is the most thoroughly characterized member of the family. Below, I will discuss what is known about Syt 1 function, then discuss the variations found in other family members. Finally, I will focus in on Syt 4 and its postsynaptic role at the synapse.

1.6.1 Synaptotagmin 1

Syt 1 was originally identified by Perin, Sudhof and colleague. Named p65 for its molecular weight, the synaptic vesicle protein contained C2 domains similar to those in PKC (Perin et al., 1990). Unlike PKC, the C2 domains of Syt 1 not only bound acidic phospholipids but also calmodulin, suggesting Syt 1 might mediate Ca^{2+}-dependent phospholipid binding and vesicular fusion (Perin et al., 1990). SNAP-25, Syx, Syb and Syt 1 are together sufficient to reconstitute Ca^{2+}-dependent membrane fusion (Tucker et al., 2004). Furthermore, recent evidence has shown that Syt 1 promotes membrane fusion by bending the target membrane while facilitating SNARE zippering (Martens et

al., 2007). Syt 1's role in the synaptic vesicle cycle is a complex one, with each of the C2 domains taking part in different functions.

1.6.1.1 Syt 1: Ca^{2+} Sensor

Syt 1 is neuronal in mammals (by RNA blots and Westerns), *Drosophila* (by mRNA *in situ* and immunohistochemistry), and *C.elegans* (by immunohistochemistry) (Adolfsen et al., 2004; Li et al., 1995; Littleton et al., 1993; Nonet et al., 1993). It is also found in non-neuronal secretory cells of *C.elegans* that exhibit regulated secretion (Nonet et al., 1993). *syt 1* null mutations in *Drosophila, C.elegans* (*snt-1*), and mice eliminate fast synchronous neurotransmitter release in response to action potentials, although evoked asynchronous release remains (Chapman and Jahn, 1994; DiAntonio and Schwarz, 1994; Geppert et al., 1994; Littleton et al., 1993; Maximov and Sudhof, 2005; Nonet et al., 1993). Thus, Syt 1 acts not only to promote fast synchronous synaptic vesicle fusion, but also to suppress asynchronous vesicle fusion. Vesicle docking is also impaired in *Drosophila syt 1* null mutants, with fewer, larger vesicles located at some distance from the membrane (Loewen et al., 2006; Reist et al., 1998). *syt 1* null larvae are still capable of impaired movement, although mutants become non-viable upon reaching adulthood, suggesting that asynchronous release is sufficient for some level of synaptic communication (Adolfsen et al., 2004). There is disagreement in the literature over whether an increase in spontaneous release is also characteristic of *syt 1* null mutants (Broadie et al., 1994; Pang et al., 2006; Yoshihara and Littleton, 2002; Okamoto et al., 2005; Geppert et al., 1994).

In wild-type *Drosophila,* synchronous release of neurotransmitter in response to neuronal action potentials occurs with a Ca^{2+} cooperativity of 3.5 (in nonsaturating Ca^{2+} ~0.2-0.5 mM) (Yoshihara and Littleton, 2002; Littleton et al., 1994). This is consistent with data from mammalian systems suggesting that binding of approximately four Ca^{2+} ions is necessary to account for the rate of fusion (Heidelberger et al., 1994; Dodge and Rahamimoff, 1967). Syt 1 Ca^{2+} sensing and phospholipid binding is tuned to levels appropriate to synaptic transmission, which will be talked about in greater detail below (Brose et al., 1992; Davis et al., 1999). When Ca^{2+} triggered penetration of membranes and binding of SNAREs is followed by engineered Trp fluorescence, the speed also matches that necessary for synaptic transmission (Davis et al., 1999). Causation has also been demonstrated through *syt 1* Ca^{2+}-binding gain-of-function mutations, tryptophan additions to the C2A or C2B domains, which lead to a predicted increase in neurotransmitter release (Rhee et al., 2005).

In addition to Ca^{2+} and phospholipids, Syt 1 also binds Syb, the C-terminal of SNAP-25, the C-terminal of Syx, and the assembled SNARE complex (Chapman et al., 1995; Ernst and Brunger, 2003; Gerona et al., 2000; Li et al., 1995; Rickman and Davletov, 2003; Schiavo et al., 1997). Syt 1 Ca^{2+}-dependent binding of the C-terminal of SNAP-25 is necessary for Ca^{2+}-dependent binding of both SNAP-25*Syx and SNAP-25*Syx*VAMP complexes (Gerona et al., 2000). Indeed, Ca^{2+} induces simultaneous binding of Syt 1 to phospholipids and the SNARE complex (Dai et al., 2007). The only Ca^{2+}-independent binding action is full length Syt 1 binding of the SNARE complex (Rickman and Davletov, 2003). Through these interactions, Syt 1 promotes formation of the mature SNARE complex and vesicular membrane fusion

(Littleton et al., 2001). The SNARE proteins themselves may also contribute to the Ca^{2+} cooperativity of fusion, as hypomorphic *syx* and *syb* mutants in *Drosophila* alter not only the amount of release but also the cooperativity of release (Stewart et al., 2000). Syt 1 phosphorylation by casein kinase II, CaMKII and PKC is reported to alter Syt 1 interaction with SNARE proteins, suggesting cellular mechanisms for regulation of Syt 1 function (Bennett et al., 1993; Davletov et al., 1993; Hilfiker et al., 1999; Verona et al., 2000).

Syt 1's intimate association with Ca^{2+} ion channels may also contribute to rapid vesicle fusion. Syt 1's C2A and C2B domains both bind isoforms of P/Q-type Ca^{2+} channels, while C2B binds the N-type Ca^{2+} channels that mediates Ca^{2+} influx for fast exocytosis (Chapman et al., 1998; Kim and Catterall, 1997; Sheng et al., 1997). N-type Ca^{2+} channels have also been shown to bind Syt 3 and 4 in a Ca^{2+} independent manner (Chapman et al., 1998).

1.6.1.2 Syt 1: C2A Domain

The first C2 domain (C2A) of Syt 1 is composed of an eight stranded beta-sandwich attached to a 'C2 key,' where Ca^{2+} binding occurs (Sutton et al., 1995). Early reports suggested that the isolated bovine Syt 1 C2A domain had the same phospholipid binding properties as full length Syt 1, such that C2A may be responsible for Syt 1's phosphobinding affinity (Li et al., 1995). The C2A domain of Syt 1 can bind Ca^{2+} and phospholipids as observed through both classical *in vitro* phosphobinding assays and NMR (Davletov and Sudhof, 1993; Chae et al., 1998). One report suggested Ca^{2+} binding triggers a conformational shift in the C2A domain to allow phospholipid binding

(Chapman and Davis, 1998). However, the crystal structures of C2A with and without Ca^{2+} showed an electrostatic change, rather than conformational, upon Ca^{2+} binding (Shao et al., 1998). Ca^{2+} binding of C2A also appears to act as an electrostatic switch allowing binding of Syx by Syt 1 (Shao et al., 1997).

The crystal structure of Syt 1's C2A domain defined five Asp residues (D1-D5) and one Ser residue responsible for coordinating binding of three Ca^{2+} ions (Shao et al., 1998). *In vitro* phosphobinding assays and NMR confirmed the importance of the Asp and Ser residues in Ca^{2+} and Ca^{2+}-dependent phospholipid binding (Zhang et al., 1998). However, when Syt 1 was expressed in a *Drosophila* null background with mutations to the key Asps of the C2A domain, it did not appear to alter the Ca^{2+} dependence of neurotransmission (Robinson et al., 2002). This suggested that *in vivo* the C2A domain alone does not account for Syt 1 Ca^{2+}-dependent triggering of release. On the other hand, a different set of Syt 1 C2A point mutants (R233Q and K236Q) reduced Ca^{2+} binding two-fold, and when introduced into mice by homologous recombination decreased sensitivity to evoked release by two-fold, leaving spontaneous release unaltered (Fernandez-Chacon et al., 2001).

1.6.1.3 Syt 1:C2B Domain

Like the C2A domain, the Syt 1 C2B domain binds phospholipids in a Ca^{2+} dependent manner (Schiavo et al., 1996; Fernandez et al., 2001). Syt 1 C2B crystal structures also show binding of 2-3 Ca^{2+} ions by the 5 conserved Asp residues (D1-D5) (Cheng et al., 2004). The crystal structure of Syt 3 C2B showed some differences from C2A that may account for the observed functional differences in the two domains. The

major structural difference being a seven-residue alpha-helix inserted between the beta-strands of C2B, something not seen before in the structure any C2A domains, but present in the C2B domain of rabphilin 3A (Sutton et al., 1999; Ubach et al., 1999). Complexin (Cpx) is hypothesized to act as a fusion clamp in the presynaptic terminal, preventing vesicular fusion until Ca^{2+}-triggered release (Giraduo et al., 2006; Schaub et al., 2006; Huntwork and Littleton, 2007). Syt 1 C2B and Cpx interactions with the SNARE complex have been seen in crystal structures and confirmed by single-molecule FRET, suggesting that this is the key domain in release of Cpx inhibition (Bowen et al., 2005).

The Syt 1 *Drosophila* mutant *syt* 1^{AD1} eliminates the C2B domain and results in a loss of the fourth order kinetics of fusion *in vivo* (DiAntonio and Schwarz, 1994; Littleton et al., 1994). The *syt* 1^{AD1} mutant is deficient in both exo- and endocytosis, demonstrating Syt 1's role in both steps of vesicle cycling (Littleton et al., 2001). Syt 1 C2B also has a demonstrated role in endocytosis of *C.elegans* and squid (Jorgensen et al., 1995; Llinas et al., 2004). Both in mammals and *Drosophila,* C2B binding of AP-2, a protein involved in clathrin coated vesicle endocytosis, is necessary for proper synaptic endocytosis (Zhang et al., 1994; Littleton et al., 2001). While *syt* 1^{AD1} cannot facilitate endocytosis, the C2A domain retains some independent phospholipid binding activity, which in embryos is sufficient to trigger limited synchronous release (Davis et al., 1999; Littleton et al., 2001; Yoshihara and Littleton, 2002). *syt* 1^{AD4}, a *syt 1* mutant with an early stop codon before the transmembrane domain (eliminating both C2 domains), lacks even this limited release (DiAntonio and Schwarz, 1994; Yoshihara and Littleton, 2002). Thus, the C2B domain is essential to both Syt 1's exocytosis and endocytosis functions.

Milder *syt 1* C2B domain mutants, C2B(D416,418N) (corresponding to D3,4 of the Ca^{2+}-binding domain), still have a >95% reduction in neurotransmission due to the altered Ca^{2+}-binding motif (Mackler et al., 2002; Loewen et al., 2006). This mutation has also been shown to alter the rate of endocytosis in a manner rescued by higher extracellular calcium (Poskanzer et al., 2006). The vesicle distribution within the neuron looks like wild-type, suggesting that this phenotype is not due to impaired vesicle docking but to impaired vesicle fusion (Loewen et al., 2006). While *syt 1* C2B(D3,4N) reduces the quantal content and Ca^{2+} dependency of fusion, C2B(D1,2N) actually inhibits high K^+ release (Tamura et al., 2007). When Syt 1 proteins mutant in the C2B domain are injected into *syt 1* null mouse hippocampal neurons, mutating D2 or D3 prevents rescue of synchronous release, while suppressing asynchronous release (Nishiki and Augustine, 2004). Mutating D1 or D4 alone allows partial rescue of both functions, and mutating D5 does not inhibit rescue (Nishiki and Augustine, 2004). A final *Drosophila* C2B mutant, *syt* 1^{AD3}, contains a Y to N substitution in the Ca^{2+}-binding domain that shows similar results (DiAntonio and Schwarz, 1994). In the mutant there is a defect in exocytosis that high Ca^{2+} rescues, but no defect in endocytosis (Littleton et al., 2001). The coopertivity of release is unaltered in *syt* 1^{AD3} mutants, although the quantal content of release is significantly reduced (Yoshihara and Littleton, 2002). Yet, *syt* 1^{AD3} remains capable of suppressing asynchronous release, where as *syt* 1^{AD1} cannot (Yoshihara and Littleton, 2002). These results show the importance of Ca^{2+} sensing within the Syt 1 C2B domain not only for coordination of fast synchronous release, but also suppression of asynchronous release.

A separate function of the C2B domain that is poorly understood is mediation of Syt oligomerization. Several early studies reported that the C2B domain drives heteroligomerization in a Ca^{2+}-dependent manner between various combinations of Syt 1, 2, 3, and 4, even suggesting that Syt 1 oligomerization may be essential to release (Chapman et al., 1998; Desai et al., 2000; Sugita et al., 1996). Later studies suggested that although C2B binds Ca^{2+} it does not cause oligomerization in solution (Ubach et al., 2001; Garcia et al., 2000). However, EM studies have revealed that oligomerization does occur on membranes (Wu et al., 2003). The biological significance of Syt oligomerization has not be established.

1.6.1.4 Syt 1:C2A/B

Fluorescence studies have now shown that during fusion both C2 domains penetrate the membrane simultaneously (Bai et al., 2002; Hui et al., 2006). Crystal structures of human Syt 1 C2A/B reveal a network of interactions between the C2A and C2B domains that may alter function, and allow regulation of C2A Ca^{2+} binding by C2B's C-terminal alpha-helix (Fuson et al., 2007). In biochemical studies, Ca^{2+} appears to trigger an intramolecular interaction between the C2 domains (Garcia et al., 2000). Furthermore, tethering of a Syt 1 C2A Ca^{2+}-binding mutant to C2B revealed that C2A affects the membrane-binding properties of C2B (Hui et al., 2006). Besides phospholipid binding, both C2A and C2B are necessary for SNAP-25 and SNARE Ca^{2+}-dependent binding, including the same residues responsible for phospholipid binding (Earles et al., 2001; Lynch et al., 2007). While SNAP-25, Syx and Syb along with Syt 1 are sufficient to reconstitute Ca^{2+}-regulated membrane fusion, the Syt 1 C2 domains had to be tethered

to each other (Tucker et al., 2004). Together these experiments emphasize the importance of taking the whole protein in context, and explain some early conflicting results about the function of individual C2 domains.

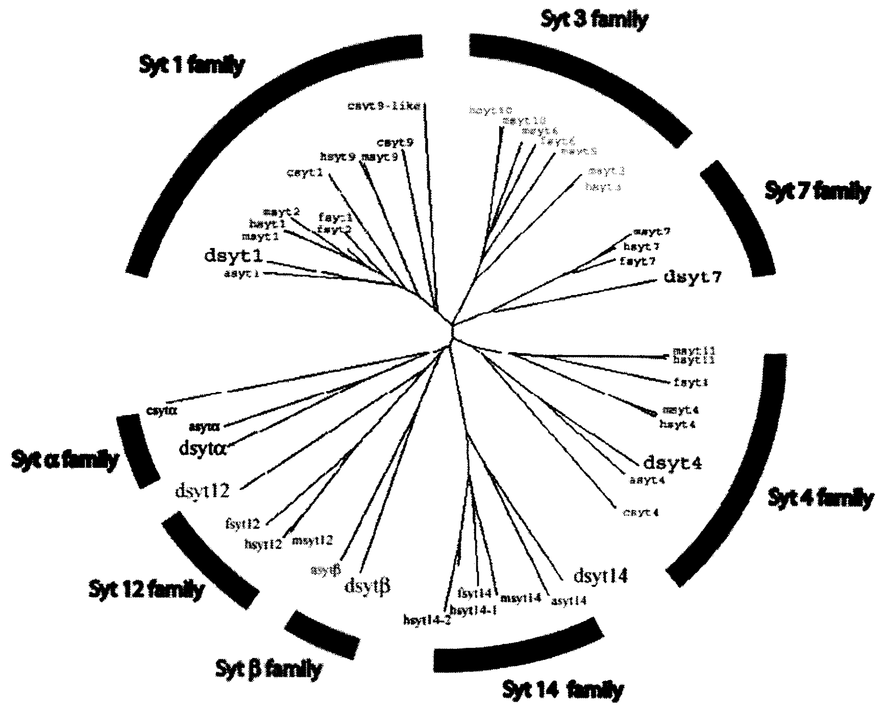
1.6.2 Diversity in the Synaptotagmin Family

Synaptotagmins constitute a large protein family with ~14 mammalian isoforms, 7 *Drosophila* isoforms, and 8 *C.elegan* isoforms (Fig 1.6). Several studies have grouped the members of the Syt family into functional categories based on Ca^{2+} sensing, phospholipid binding, localization, or formation of oligomers. It has been hypothesized that different Syt members work in combinatorial fashion to fine-tune regulation of vesicle fusion (Saegusa et al., 2002; Tucker et al., 2003), or to regulate release via hetero-oligomer interactions (Desai et al., 2000; Wang et al., 2001). However, Syt family members exhibit distinct localizations, Ca^{2+} affinities, and binding affinites for phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) that suggest many, if not all, members regulate separate cellular functions.

1.6.2.1 Syt 2

Syt 2 is a mammalian neuronal Syt, with unique spacotemporal expression from Syt 1 and other mammalian neuronal Syts (Li et al., 1995; Xu et al., 2007; Fox and Sanes, 2007). Three mammalian neuronal Syts (Syt 1, 2, and 5(also known as 9)) all appear to trigger fast synchronous release (Xu et al., 2007; Fox and Sanes, 2007). Mice carrying a mutation in *syt 2* have a decrease in evoked release at the calyx of held synapses, and an

Figure 1.6



Adapted from Adolfsen et al., 2004

Figure 1.6: Dendrogram of Synaptotagmin family members from *Drosophila*, *C. elegans*, *A. Gambiae*, *F. rubripes*, *M. musculus*, and *H. sapiens*. Subfamilies are named according to mammalian nomenclature. Subfamilies without vertebrate members are designated with greek letters. (Adapted from Adolfsen et al., 2004)

increase in spontaneous release, causing these mutant synapses to look similar to *syt 1* null synapses (Pang et al., 2006).

The similarities between Syt 1 and Syt 2 are also seen in biochemical assays of their function. Both have similar Ca^{2+}-dependent binding of PS (EC_{50} 3-6uM) and Syx (Li et al., 1995). Of the Syt family members that tightly bind phospholipids as a function of Ca^{2+} (in 25%PS, 74% PC assays) Syt 1 and 2 are among those with the fastest kinetics of dissociation (50msec) (Hui et al., 2005). They also both showed enhanced PS binding with Ca^{2+} in a reconstituted fusion system monitored by FRET (v-SNARE membrane 30%PE, 52%PC, 15%PS: t-SNARE membrane 30%PE, 55%PC, 15%PS) and including mammalian Syb2, Syx1A, and SNAP-25 (Bhalla et al., 2008). However, unlike Syt 1, Syt 2 does not bind AP2 independent of Ca^{2+} (Li et al., 1995). Syt 2 is also unique in that its Ca^{2+} binding activity is regulated in a Ca^{2+}-dependent manner by WNK1 phosphorylation within the C2 domains (Lee et al., 2004).

The similarities between Syt 1 and Syt 2 function extend to the biochemical studies of individual domains. Like Syt 1, the C2A domain of mouse Syt 2 binds the acidic phospholipids PS and PI in a Ca^{2+}-dependent manner, but does not interact with PC or PE (Fukuda et al., 1996). Correspondingly it binds any 1:1 w/w pairing of phospholipids excepting PE/PC (Fukuda et al., 1996). Like the full length proteins, Syt 1 and Syt 2 C2A domains alone share similar Ca^{2+}/phospholipid binding properties, with EC_{50}~10-20μM in a 25%PS 75% PC assay (Sugita et al., 2002). Both C2B domains also bind Syt 1 *in vitro* in a Ca^{2+} dependent manner (Sugita et al., 1996). One report suggests that rat brain vesicles contain Syt 1 and 2 in the same vesicle population, and that Ca^{2+} drives formation of hetero-oligomers (Osborne et al., 1999). However, reports conflict

over the whether Syt 2 oligomerization is moderately Ca^{2+} independent or only occurs under very high Ca^{2+} (5mM) conditions (Fukuda and Mikoshiba, 2000; Garcia and Godwin, 2004). Oligomerization of the proteins on a single vesicle population seems unlikely, as the similarities in biochemical function and previous reports of distinct spacotemporal expression suggest the two Syts play similar roles in different mammalian neuron populations.

1.6.2.2 Syt 3 and 7

It has been reported that Syt 3 and 7 form a unique class of Syts localized to plasma membranes, as opposed to a vesicle population, in mouse neurons and PC12 cells (Butz et al., 1999; Sugita et al., 2001; Saegusa et al., 2002). Researchers have suggested that these plasma membrane Syts may facilitate vesicle fusion through a non-SNARE mechanism, or through hetero-oligomeriztion with vesicle bound Syts (Shin et al., 2002). However, many reports have placed both isoforms within various vesicle populations.

Syt 3 is a reported mammalian neuronal Syt isoform, while Syt 7 is seen ubiquitously in both neuronal and non-neuronal tissues of rat and *Drosophila* (Li et al., 1995; Adolfsen et al., 2004). Syt 3 and 7 have both been identified on insulin secretory granules, rather than the plasma membrane, of pancreatic beta cells, (Brown et al., 2000; Gao et al., 2000; Gustavsson et al., 2008; Mizuta et al., 1997). Syt 7 does not colocalize with Syx1A, n-Syb, or ROP in sucrose gradients of *Drosophila* heads, and cannot rescue Syt 1 function (Adolfsen et al., 2004). However, Syt 7 is also not observed on the plasma membrane but in distinct cellular compartments present in a number of cell types (Adolfsen et al., 2004). Reports from mammals have placed Syt 7 on lysosomes/late

endosomes whose exocytosis plays a role in regulating bone remodeling, glucose release and neurite outgrowth in different cell types (Zhao et al., 2008; Gustavsson et al., 2008; Arantes and Andrews, 2006). Syt 7 is also involved in slow exocytotic release from adrenal chromaffin cells (Schonn et al., 2008). Taken together, these reports suggests that contrary to the plasma membrane hypothesis, Syt 3 and 7 likely participate in standard SNARE mediated vesicle-fusion.

Studies generally agree that Syt 3 Ca^{2+}-dependent phospholipid binding is similar to that of Syt 1 and 2. Just as in Syt 1, fluorescence shows that the C2A and C2B domains of Syt 3 bind phospholipids in tandem (Bai et al., 2002). Also like Syt 1, the Syt 3 C2A domain binds PS phospholipids with an EC50 of 1-6uM and has fast kinetics of dissociation (50msec) (Li et al., 1995; Sugita et al., 2002; Hui et al., 2005). The Syt 1 and Syt 3 C2A phospholipid binding preferences are also similar, Syt 3 C2A binds the acidic phospholipids PS and PI in a Ca^{2+}-dependent manner and does not bind pure PC or PE (Fukuda et al., 1996). In contrast to Syt 1 and 2, and potentially due to differences at the amino terminus, Syt 3 does bind PS/PE and PI/PE lipids in a Ca^{2+}-independent manner (Fukuda et al., 1996). The biological importance of this difference is not clear, but might reflect differences in the lipid environments that each Syt facilitates fusion. Syt 3 has been reported to have strong Ca^{2+}-independent oligomerization, while the the Syt 3 C2B domain has also been reported to mediate Ca^{2+}-dependent hetero-oligomerization between Syt 1, 3 and 4 (Fukuda and Mikoshiba, 2000; Chapman et al., 1998). The striking similarities between Syt 3 and Syt 1 biochemical functions, further support a vesicle fusion role for Syt 3 *in vivo*.

There have been mixed reports about Syt 7's Ca^{2+}-dependent functions. One study shows that it binds Ca^{2+} with an EC_{50}~1-2uM (Sugita et al., 2002). Another that while it binds phospholipids (25%PS, 74% PC, 1% tracer), it does so in a Ca^{2+}-independent manner, and with slow dissociation (500 msec) (Hui et al., 2005). Finally, in the FRET reconstituted fusion system (v-SNARE membrane: 30%PE, 52% PC, 15%PS; t-SNARE membrane: 30%PE, 55%PC, 15%PS with Syb2, Syx1A, SNAP-25) Syt 7 actually showed Ca^{2+}-inhibition of binding (Bhalla et al., 2008). To further confuse matters, the isolated C2B domain in a standard lipid binding assay, showed Ca^{2+}-dependent binding 10x that of Syt 1 (Sugita et al., 2002). The C2B domain is also reported to exhibit Ca^{2+}-dependent oligomerization (Fukuda and Mikoshiba, 2000; Fukuda and Mikoshiba, 2001). While many questions remain as to the binding properties of Syt 7, the *in vivo* results discussed above have already begun to expose the essential role Syt 7 plays in several exocytosis systems.

1.6.2.3 Syt 5/9

Syt 5, also known as Syt 9, is another mammalian neuronal Syt that localizes to separate regions of the brain where it triggers fast synchronous release (Li et al., 1995; Xu et al., 2007). By GFP labeling, Syt 5 is found to localize to dense-core vesicles in PC12 cells, where it has been suggested it aids Syt 1 in regulation of Ca^{2+}-dependent secretion (Saegusa et al., 2002; Fukuda et al., 2002). Like the other neuronal Syts, Syt 5 shows Ca^{2+} enhanced PS binding in the reconstituted FRET fusion system mentioned above (Bhalla et al., 2008). The Syt 5 C2A domain also binds PS phospholipids with a Ca^{2+} EC_{50} similar to Syt 1 (3-6μM), but shows a medium time to dissociation (250 msec)

(Hui et al., 2005; Li et al., 1995; Sugita et al., 2002). The C2A domain of mouse Syt 5 tested in a purified lipid system binds the acidic phospholipids PS and PI in a Ca^{2+}-dependent manner, does not bind PC, and weakly binds PE (Fukuda et al., 1996). Correspondingly, the only pairwise combination it cannot bind is PE/PC (Fukuda et al., 1996). Syt 5 also binds Syx with Ca^{+2}-dependence and AP2 in Ca^{2+}-independent manner (Li et al., 1995). These binding interactions, so similar to the other neuronal Syts suggest that Syt 5 plays a similar role in its separate neuronal population.

1.6.2.4 Other Syt Family Members

The Syt family members discussed above appear to all play roles in Ca^{2+} regulated vesicle fusion in neuronal and secretory cells. Other than Syt 4, which will be discussed in greater detail below, the remaining Syt family members are less well understood, expressed in different tissues, some species specific, and with a variety of reported Ca^{2+} binding affinities and activities. To give a sense of what is known about the remaining Syt family members, they are briefly reviewed here.

A number of Syt isoform subfamilies are only expressed in highly restricted cell types. *Drosophila* embryonic mRNA *in situ* shows insect specific Syt α and β in highly restricted neuronal populations, whose current function is unknown (Adolfsen et al., 2004). Restricted expression is also seen in the Syt 14 subfamily. This Ca^{2+}-independent isoform is mainly expressed in the heart and testis of mice, and only at low levels in *Drosophila* embryos (Fukuda, 2003; Adolfsen et al., 2004).

Other isoforms, such as Syt 6 and 8, are widely expressed in mammalian non-neuronal tissues (Li et al., 1995). These two isoforms in particular are known to be

essential for the mouse sperm acrosome reaction (Hutt et al., 2005; Li et al., 1995). The other roles of Syt 6 are not so clear. In PC12 cells, Syt 6-GFP is known to localize to ER like structures, and *in vitro* Syt 6 exhibits Ca^{2+}-dependent PS binding (Bhalla et al., 2008; Saegusa et al., 2002). However, this does little to illuminate the biological role Syt 6 must play in a broad number of tissues.

The expression pattern of some isoforms suggests a specific developmental role. Syt 12 is expressed in mouse brain increasingly over the postnatal period. Over-expression of Syt 12 increases spontaneous release, and phosphorylation by PKA in the the C2 linker region is apparently necessary for this function (Maximov et al., 2007). Similar to prenatal mice, Syt 12 levels are below detection in *Drosophila* embryos (Adolfsen et al., 2004). Syt 12 reportedly does not bind phospholipids in a Ca^{2+}-dependent manner (25%PS, 74% PC, 1% tracer), and in the reconstituted fusion system showed Ca^{2+}-inhibition of binding (Bhalla et al., 2008; Hui et al., 2005). How this relates to Syt 12's *in vivo* functions is still not clear, and like many of the Syt isoforms, further studies will be required before the *in vivo* function in neurons is identified.

1.7 Synaptotagmin 4 and 11

Next to Syt 1, the most conserved isoform of the Syt family is Syt 4. Syt 4 is neuronal in both mammals and *Drosophila,* with a conserved Asp to Ser substitution in D3 of the C2A Ca^{2+} binding pocket (Li et al., 1995; Littleton et al., 1999). In mammals, the subfamily contains two members, Syt 4 and 11 (von Poser et al., 1997). The subfamily's capacity for Ca^{2+}-dependent phospholipid binding, and their role as either a positive or negative regulator of vesicular fusion, have been the subject of on going

debate in the literature. Here I will review *in vitro* and *in vivo* observations that suggest Syt 4 functions in the nervous system.

1.7.1 Biochemical Properties of Syt 4 Subfamily

As mentioned above, members of the Syt 4 subfamily contain a conserved Asp to Ser substitution in D3 of the C2A Ca^{2+} binding pocket (Littleton et al., 1999). This substitution has been theorized to inactivate C2A Ca^{2+}-binding by removing a negative charge necessary for coordination of binding, thus rendering Syt 4 a Ca^{2+}-independent isoform. Indeed, the isolated C2A domains of mammalian Syt 4 and 11 do not exhibit Ca^{2+}-dependent phospholipid binding *in vivo* (von Poser et al., 1997). In addition, replacement of the Ser with Asp endows the domain with Ca^{2+}-dependent phopholipid binding activity, and stabilizes the structure by circular dichroism (von Poser et al., 1997). Several other studies have also reported that mammalian Syt 4, as well as Syt 1(C2A)/Syt 4(C2B) and Syt 4(C2A)/Syt 1(C2B) chimeras, do not bind 25%PS, 75%PC phospholipids in a Ca^{2+}-dependent manner (Hui et al., 2005; Bai et al., 2002; Dai et al., 2004). Assayed by FRET (as described above), mammalian Syt 4 recently even showed Ca^{2+}-inhibition of PS binding (Bhalla et al., 2008). The only study reporting *in vitro* Syt 4 Ca^{2+}-dependent binding suggested that *Drosophila* Syt 4 binds PS/PC lipids while rat Syt 4 and 11 do not (Dai et al., 2004). Yet, apparent Ca^{2+}-dependent fusion of Syt 4 vesicles has been observed in *Drosophila* and PC12 cells (Yoshihara et al., 2005; Fukuda and Yamamoto, 2004). It is possible that the difference in *in vivo* and *in vitro* results is due to Syt 4 C2A phosphorylation-dependent regulation at the conserved Ser, much like the phosphorylation-dependent regulation seen in Syt 2 or 3. Phosphorylation of the Ser

would theoretically provide the negative charge necessary to allow it to participate in Ca^{2+} coordination, mimicking the Asp residues present at the site in other Syts.

Another possible explanation for the conflicting results may be found in the phospholipid composition of membranes used for the binding assays. Tested individually, mammalian Syt 4 C2A was found to bind the acidic phospholipids PS and PI in a Ca^{2+}-dependent manner, but did not interact with PC or PE (Fukuda et al., 1996) Tested in pairwise combinations (1:1, w/w) Syt 4 C2A only bound PS/PI and did not bind any combination containing PC or PE (Fukuda et al., 1996). Placing a Syt 4-like Ser substitution in D3 of Syt 2's C2A Ca^{2+} binding domain (D231S or D231N) reportedly yields the Syt 4 phospholipid binding profile (Fukuda et al., 1996). Meanwhile, changing the Syt 4 Ser to an Asp allowed PS/PC binding (Fukuda et al., 1996). Adding to the complexity of the binding data, full length Syt 4 bound liposomes containing PS or PS +PC (clusters in non-uniform distribution) but not PS/PC (uniform distribution) (Fukuda et al., 1996). It is easy to imagine a specialized region of the membrane dedicated to exocytosis could contain a favorable lipid composition differing from that used in *in vitro* lipid binding assays.

Few of Syt 4's other binding activities have been characterized. Syt 4, like Syt 1 and the majority of other Syts, binds AP2 in a Ca^{2+}-independent manner, suggesting that it plays a role in endocytosis of vesicles following fusion (Li et al., 1995). Unlike other Syts, the Syt 4 C2A domain is reported to bind Syx in a Ca^{2+}-independent manner (Li et al., 1995). However, the potential biological implications of this are unclear, especially given the observation of Ca^{2+}-dependent fusion of Syt 4 vesicles *in vivo*.

Debate also remains as to the oligomerization properties of Syt 4. Reports suggest that the C2B domain of Syt 4 does not allow homo-oligomer formation but can mediate Ca^{2+}-dependent formation of hetero-oligomers with Syt 1 and 3 (Fukuda and Mikoshiba, 2000; Chapman et al., 1998). One report suggests that the C2B domain mediates oligomerization with Syt 1 in PC12 cells, although others suggests that they do not co-localize, which would impede oligomerization (Thomas et al., 1999; Fukuda et al., 2002). In mammals, a group reported vesicles from rat brain contained both Syt 1 and 4, but did not find any oligomers (Syt 1/2 oligomers were found) (Osborne et al., 1999). Thus, it remains unclear both if Syt 4 can participate in oligomerization, and what the biological relevance would be of such an interaction. Taken together, the controversies of Syt 4's biochemical properties make it clear that further work needs to be done in characterizing this protein.

1.7.2 Syt 4 *in vivo*

Syt 4 was originally identified in a cDNA library screen of PC12 cells for immediate early gene products up-regulated by K^+ depolarization, Ca^{2+}-ionophore, ATP, and forskolin application; it was also found to be up-regulated by Kainic acid-induced seizures in rats (Vician et al., 1995). Additionally, Syt 4 is reported to be up regulated by cocaine, methamphetamine and MDMA (ecstasy) in mammals (Simantov, 2004; Isao and Akiyama, 2004; Yuferov et al., 2003). It is also specifically induced in the song circuit of zebra finch during singing, although not by metrazole induced seizure activity (Poopatanapong et al., 2006). Together these results give a clear picture of Syt 4 up-regulation in response to cellular activity in mammalian and zebra finch neurons.

The cellular effects of Syt 4 have been studied in more detail in PC12 cells. Forskolin application to PC12 cells not only increases Syt 4 protein level, but also triggers transport of Syt 4 from golgi, where it is found in undifferentiated PC12 cells, to mature and immature dense core vesicles (Fukuda and Yamamoto, 2004). Application of NGF to undifferentiated PC12 cells also triggers movement of Syt 4 from golgi to fusion competent mature dense core vesicles (Fukuda et al., 2002). Syt 4's vesicle population appears distinct from other described populations, and Syt 4 does not colocalize with Syt 1, Syt 5/9, Rab3A, Rab27A, or Synaptophysin (Fukuda et al., 2002). As far as its function, Syt 4 has been implicated as taking part in the maturation of the PC12 secretory granules (Ahras et al., 2006). It has also been directly implicated in regulation of various neurotransmitter release events. For instance, over-expression of Syt 4 was reported to reduce release of human growth hormone, but not when the C2A Ca^{2+} binding Ser was mutated to Asp (Machado et al., 2004). After measuring fusion pore dynamics in mutant PC12 cells through detection of NE release, it was hypothesized that Syt 1 functions to stabilize the fusion pore, while Syt 4 destabilizes it (Wang et al., 2001). Thus, it was preposed that Syt 4 regulates the dynamics of fusion, making the decision between kiss-and-run and complete fusion.

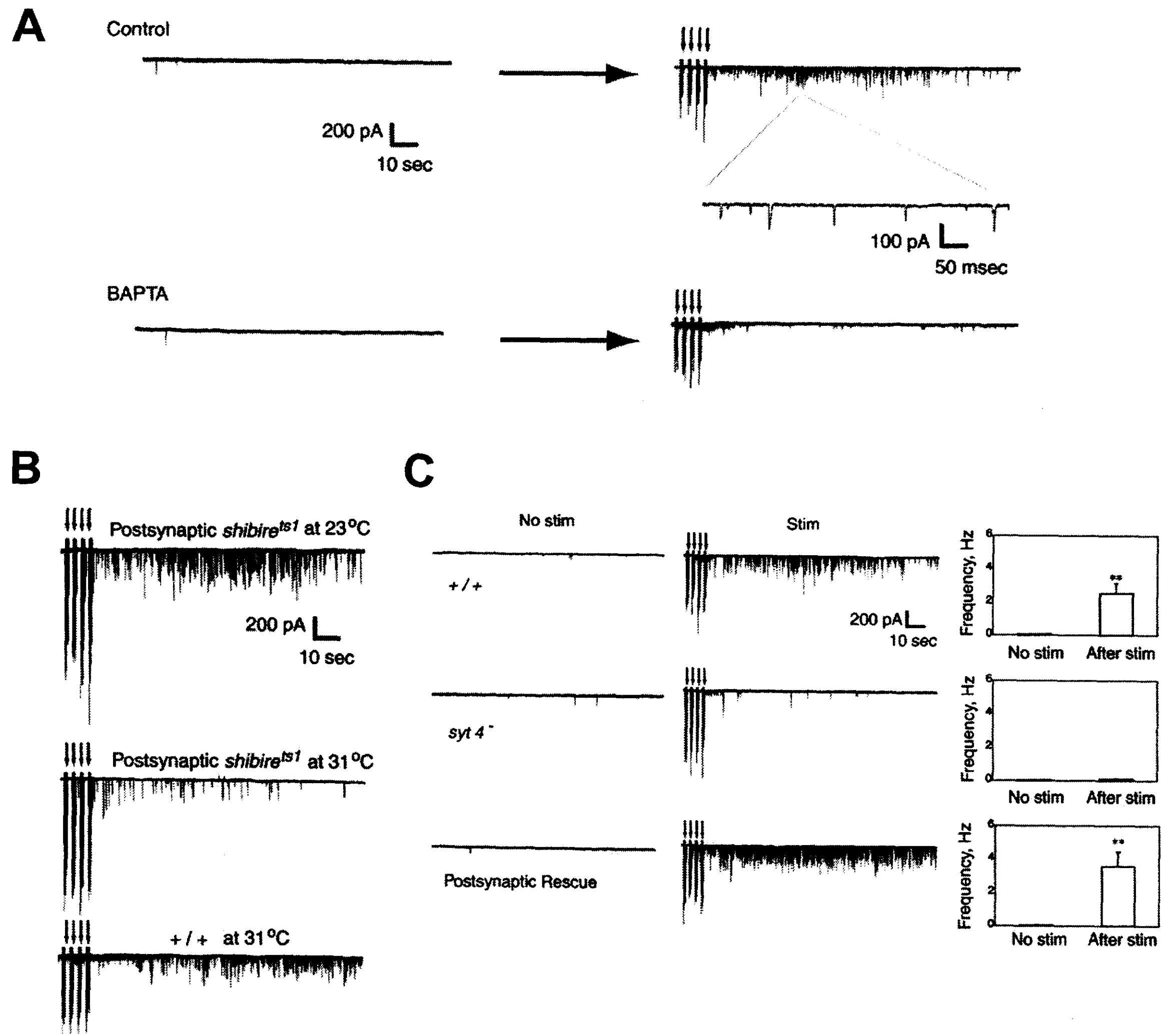
Several lines of evidence make it unlikely that Syt 1 and 4 acts as positive and negative regulators of the fusion pore. Over-expression of Syt 4 in cultured mouse hippocampal neurons does not alter release probability, Ca^{2+}-dependence of release, short-term plasticity, or fusion pore kinetics (mutant Syt 1 did alter the kinetics) (Ting et al., 2006). In *Drosophila,* Syt 1 is presynaptic, while Syt 4 is postsynaptic (Adolfsen et al., 2002) making it impossible for the two to cooperate in regulation of the fusion pore.

Furthermore, the two Syts do not appear to co-localization even in PC12 cells (Fukuda et al., 2002; Pawlu et al., 2004; Wang et al., 2003). Together the data makes Syt 4 activity as a negative regulator of release seem unlikely.

To further explore Syt 4 activity in nervous systems, *syt 4* null mutants have been generated in both mice and *Drosophila*. *syt 4* null mutant mice have problems with fine motor coordination, lower anxiety levels, reduced depression-like behavior, as well as defects in hippocampal dependent tasks such as contextual fear conditioning and associative passive avoidance memory, but not novel object recognition, suggesting a role for Syt 4 in the hippocampus (Ferguson et al., 2000; Ferguson et al., 2001; Ferguson et al., 2004). *syt 4* null mouse neurons also have enhanced short-term plasticity, paired-pulse facilitation and post-tetanic potentiation in the CA1 area (Ferguson et al., 2004). Despite the changes to behavior and electrophysology, the hippocampus of *syt 4* null mice looks normal by immunohistochemistry (Ferguson et al., 2004). Syt 4 was not found in the presynaptic terminals of the hippocampus, but was expressed in the astroglia in situ (Zhang et al., 2004). Results from RNAi and injection of Syt 4 C2B suggest that Syt 4 may regulate release of glial glutamate and this might provide an explanation for the observed *syt 4* null hippocampal phenotypes (Zhang et al., 2004).

Drosophila Syt 4 is postsynaptic and on sucrose gradients does not co-localize with Syx1A, n-Syb, or ROP, but does colocalize with the postsynaptic density protein, DPAK by immunohistochemistry (Adolfsen et al., 2004; Yoshihara et al., 2005). It also cannot rescue Syt 1 function (Adolfsen et al., 2004). Syt 4 function was investigated using a new paradigm for testing plasticity in the embryonic NMJ (~30 active zones). In wildtype embryos, after 100-Hz stimulation there is 100x increase in spontaneous release

Figure 1.7



Adapted from Yoshihara et al., 2005

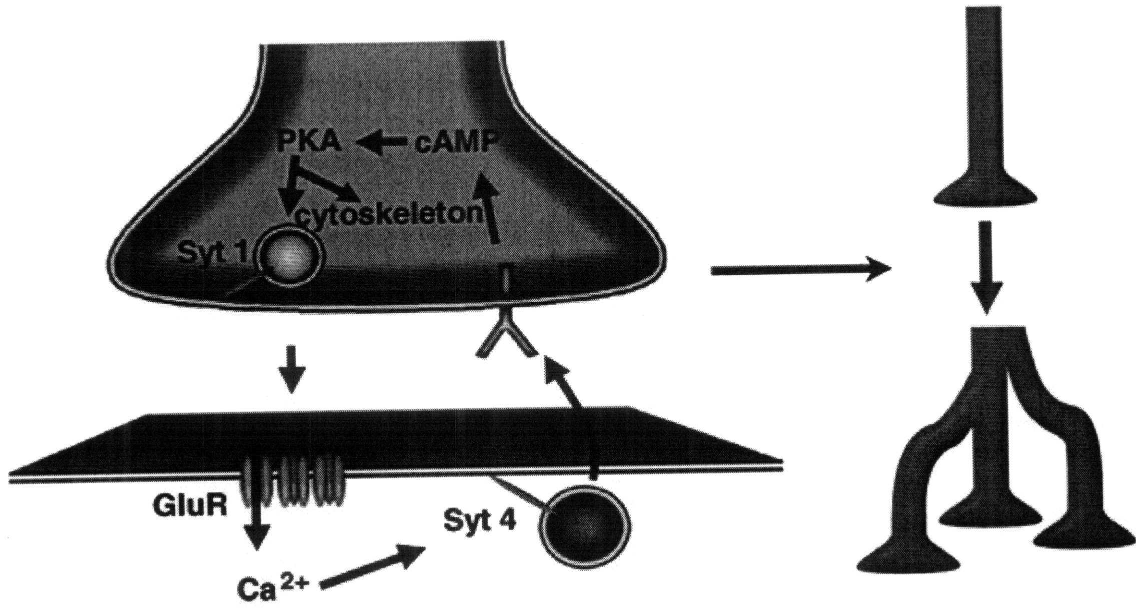
Figure 1.7: High-Frequency-stimulation-induced Miniature Release (HFMR) in *Drosophila* embryos **A,** In wild-type embryos, after 100-Hz stimulation there is 100x increase in spontaneous release (0.03 to 5 Hz) starting within 1 minute and lasting up to 20 minutes. HFMR is absent when the Ca^{2+} chelator, BAPTA, is present in the postsynaptic recording electrode. **B,** At the restrictive temperature, postsynaptic expression of shi^{TS1} significantly reduces HFMR. **C,** HFMR is absent in *syt 4* null mutants. This phenotype can be rescued by postsynaptic expression of Syt 4. (Adapted from Yoshihara et al., 2005)

(0.03 to 5 Hz) starting within 1 minute and lasting up to 20 minutes (Fig 1.7). This plasticity is termed High-Frequency-stimulation-induced Miniature Release (HFMR) (Yoshihara et al., 2005).

HFMR requires postsynaptic Ca^{2+} and vesicular fusion, as demonstrated through the abolishment of HFMR by postsynaptic Ca^{2+} chelators EGTA and BAPTA (Fig 1.7), as well as reversible blockage of post-synaptic endocytosis with *UAS-shi*^{ts1} under control of the post-synaptic *myosin heavy chain*-Gal 4 driver (Fig 1.7) (Yoshihara et al., 2005). Like HFMR, fusion of postsynaptic Syt 4 vesicles requires postsynaptic Ca^{2+}, suggesting that postsynaptic fusion of Syt 4 vesicles may be important to HFMR plasticity (Yoshihara et al., 2005). The postsynaptic glutamate receptors DGluRIIA and DGluRIIB are responsible for postsynaptic currents induced by nerve stimulation, and NMJ growth of both glutamate receptor and *syt 4* mutants embryos appear similarly stunted, again placing Syt 4 vesicle fusion in an important Ca^{2+}-dependent postsynaptic function (DiAntonio et al., 1999; Yoshihara et al., 2005).

syt 4 null larvae are HFMR deficient (Fig 1.7), rescuable by expression of *UAS-syt 4* in the muscle but not the neuron (Yoshihara et al., 2005). HFMR also failed to be rescued by expression of the *syt 4* Ca^{2+} binding mutant *syt 4 [C2A(D4N)C2B(D3,4N)]* (Yoshihara et al., 2005). This further supports that the high frequency stimulation may cause fusion of postsynaptic Syt 4 vesicles (in a Ca^{2+}-dependent manner), which results in up-regulation of spontaneous release from the neuron. Given how little is known about the role of spontaneous release in neuronal plasticity, it is difficult to make an immediate guess as to how up-regulation of spontaneous release would effect synaptic plasticity. However, postsynaptic over-expression of Syt 4 at the *Drosophila* NMJ causes

Figure 1.8



Adapted from Yoshihara et al., 2005

Figure 1.8: The proposed Syt 4-mediated retrograde signaling model describing synaptic plasticity and growth at Drosophila NMJs. (Adapted from Yoshihara et al., 2005)

neuronal overgrowth in a synapse specific manner, while over-expression of *syt 4 [C2A(D4N)C2B(D3,4N)]* does not (Yoshihara et al., 2005). *cpx* mutants that exhibit a dramatic up-regulation in spontaneous release, also show neuronal overgrowth (Huntwork and Littleton, 2007). Together this suggested that the fusion of Syt 4 vesicles might reinforce a productive synapse through increasing growth (Fig 1.8).

What remains is to fit these observations into the known signaling pathways involved in neuronal plasticity. The electrophysiology phenotype of HFMR looks similar to forskolin induction of PKA, and the PKA null mutant DCO^{B3} shows no HFMR or forskolin response (Yoshihara et al., 1999; Yoshihara et al., 2005). *syt 4* null mutants still show a forskolin response, suggesting that forskolin can by-pass Syt 4 in induction of spontaneous release (Yoshihara et al., 2005). Similarly, constitutively active presynaptic PKA can caused overgrowth similar to postsynaptic over-expression of Syt 4, and the PKA transgene can rescue morphology in both the PKA mutant and Syt 4 mutant embryos (Yoshihara et al., 2005). This provides the first link in the pathway between neuronal activity, Syt 4, and enhanced synaptic function.

1.8 Conclusion

In response to learning and changes in neuronal activity, several classes of gene products are subject to regulation of their expression level. It is clear that both transcriptional and post-translational mechanisms are activated by enhanced neuronal activity and contribute to long-term alterations in neuronal connectivity and function. Retrograde signals, transmitted both through transmembrane signaling complexes and secreted neurotransmitters, play a unique role in the regulation of this neuronal plasticity.

Analysis of the signaling pathways that mediate synaptic growth at the *Drosophila* NMJ has provided a foundation for understanding the mechanisms by which changes in cell adhesion and transsynaptic signaling mediate structural changes in synaptic strength. One compelling player in these mechanisms is Syt 4.

Syt 4 is an evolutionarily conserved neuronal isoform of the Synaptotagmin family of vesicular Ca^{2+} sensors. Recent evidence has suggested that Syt 4, localized to postsynaptic vesicles in *Drosophila* NMJs, may play a role in Ca^{2+}-dependent release of retrograde signals in response to neuronal activity, thus 'tagging' productive synapses for increased growth and connectivity. However, much work remains to substantiate Syt 4's role at the synapse, tie it into the known signaling pathways, and demonstrate whether it indeed takes part in Hebb's postulated mechanism of neuronal memory encoding.

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Chapter 2: Activity-Dependent Regulation of Synaptotagmin 4

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Bill Adolfsen, Kathy Galle and Robin Stevens contributed to generation and localization of transgenic fluorescently labeled proteins.

2.1 Introduction

Information transfer at neuronal synapses requires release of small diffusible neurotransmitters following presynaptic vesicle fusion (Schweizer and Ryan, 2006; Sudhof, 2004). Synaptic signaling can also occur in the reverse direction, with postsynaptic targets transmitting retrograde signals that alter the function or structure of presynaptic terminals (Tao and Poo, 2001). Such retrograde signals can be released in an activity-regulated fashion, or transmitted constitutively, to modulate synaptic development and acute plasticity (Fitzsimonds and Poo, 1998; Kalinovsky and Scheiffele, 2004; Marques, 2005). Ca^{2+}-regulated vesicle fusion also occurs in the postsynaptic compartment (Adolfsen et al., 2004; Maletic-Savatic and Malinow, 1998; Park et al., 2006; Yoshihara et al., 2005; Hanus and Ehlers, 2008), suggesting retrograde signaling may use conserved vesicular trafficking mechanisms.

Although the presence of retrograde signaling in neurons is established, the biological function is largely unknown. One system where retrograde signaling has been well characterized is the *Drosophila* NMJ, where a strong correlation between activity levels and synaptic growth has been observed (Budnik et al., 1990; Guan et al., 2005; Sigrist et al., 2003). During the larval stages of development, a ~100-fold increase in muscle surface area is accompanied by enhanced synaptic strength and growth of the innervating motor neuron. Retrograde release of molecules from the postsynaptic muscle, including the TGF-β ligand *glass-bottom-boat*, is required for this synaptic growth (Aberle et al., 2002; Marques et al., 2002; Marques et al., 2003; McCabe et al., 2003). In addition, retrograde signals control homeostatic plasticity at the NMJ that maintains synaptic transmission within appropriate levels (Daniels et al., 2004; Frank et

al., 2006; Goold and Davis, 2007; Haghighi et al., 2003; Paradis et al., 2001). Although retrograde signaling is required for plasticity and synaptic growth at *Drosophila* NMJs, the underlying mechanisms remain largely unknown.

Synaptotagmins are a conserved family of Ca^{2+} sensors that localize to intracellular vesicular compartments in neurons and endocrine cells and share a common structure with a single transmembrane domain and two cytoplasmic C2 domains. Besides the synaptic vesicle Ca^{2+} sensor, Syt 1, the most evolutionarily conserved isoform of the family is Syt 4. Like Syt 1, Syt 4 expression is abundant in the nervous system and localizes specifically to *Drosophila* synapses (Adolfsen et al., 2004; Littleton et al., 1999). However, Syt 4 is expressed on a population of postsynaptic vesicles at the NMJ (Adolfsen et al., 2004), and mutants lacking Syt 4 show abnormal development and function of the embryonic NMJ (Yoshihara et al., 2005). Here we report that Syt 4 localizes postsynaptically at both NMJ and CNS synapses. Syt 4 mRNA and protein expression are strongly regulated by neuronal activity, while altered levels of Syt 4 modify synaptic growth at the NMJ. In addition, Syt 4 is necessary for enhanced synaptic growth following increased neuronal activity. Together, these results suggest that Syt 4 modulates activity-dependent release of postsynaptic retrograde signals that promote synaptic proliferation at *Drosophila* NMJs.

2.2 Results

2.2.1 Syt 4 Localizes Postsynaptically in the CNS

Synaptotagmins form a family of vesicular trafficking proteins found in vertebrates and invertebrates, though they are absent in yeast and plant genomes (Adolfsen and Littleton,

2001; Sudhof, 2004). Based on studies of the synaptic vesicle isoform, SytI, synaptotagmins are postulated to mediate the Ca^{2+} dependency of regulated exocytosis (Chapman, 2008; Yoshihara et al., 2003). Only two of the seven *Drosophila* synaptotagmins are present at most synapses – Syt 1 and Syt 4 (Adolfsen et al., 2004). In contrast to Syt 1, Syt 4 localizes postsynaptically at *Drosophila* NMJs (Adolfsen et al., 2004), suggesting it may mediate Ca^{2+}-dependent retrograde signaling. Based on their expression pattern, we hypothesize that Syt 1 and 4 form the core evolutionary conserved synaptotagmin family present at synapses, with Syt 4 mediating Ca^{2+}-dependent trafficking of postsynaptic cargos, similar to the presynaptic role of Syt 1 in synaptic vesicle fusion.

To begin to test this model, we determined whether Syt 4 was also expressed in postsynaptic compartments of *Drosophila* CNS neurons. Although Syt 4 localizes postsynaptically at the NMJ and is expressed in the CNS neuropil (Adolfsen et al., 2004), its subcellular localization within individual CNS neurons was unknown. To determine Syt 4 localization, we constructed transgenic UAS lines expressing yellow fluorescent protein (YFP) fused to the intravesicular N-terminus of Syt 4, or cyan fluorescent protein (CFP) fused to the cytoplasmic C-terminus of Syt 4, allowing cell-type specific expression with defined GAL4 driver strains. Imaging of Syt 4-YFP expressed under control of the muscle specific driver *Myosin heavy chain (Mhc)*-GAL4 in wandering 3rd instar larvae revealed expression at punctate sites at the postsynaptic compartment of NMJ synapses (Fig. 1A), similar to the expression of a Syt 4 N-terminal pHlourin construct we previously generated (Yoshihara et al., 2005). Like Syt 4-pHlourin, Syt 4-YFP showed a more prominent synaptic plasma membrane expression at the NMJ than

observed by antibody staining, where the protein localizes to vesicular structures in the postsynaptic compartment. We infer that the YFP-tagged protein is less efficiently endocytosed following fusion of Syt 4 vesicles, a commonly observed phenomenon with GFP-tagged vesicle proteins. However, the prominent accumulation of Syt 4-YFP at postsynaptic sites of exocytosis provides a powerful tool for labeling this compartment at the NMJ.

To analyze Syt 4 targeting in the CNS, we took advantage of the defined polarity of CNS motorneurons in the ventral nerve cord (VNC) of *Drosophila* larvae (Fig. 2.1B). Motorneuron axons terminate at muscle NMJs, while their dendrites are restricted to the VNC, where they receive synaptic input. This defined polarity allowed us to analyze targeting of fluorescently labeled proteins to dendritic versus axonal terminals by expressing them under the control of the motor neuron GAL4 driver, D42 (Parkes et al., 1998). Syt 4-YFP driven by D42-GAL4 prominently localized to motor neuron cell bodies and dendrites in the VNC (Fig. 1A), where synaptic inputs are known to occur (Littleton et al., 1993). In contrast, small amounts of Syt 4-YFP targeted to presynaptic terminals at NMJs, suggesting Syt 4 localizes predominately to postsynaptic sites in CNS dendrites. To more precisely analyze the specificity of targeting of Syt 4 to the postsynaptic compartment, we generated Syt 4-CFP and n-Syb-YFP expressing transgenic animals, allowing us to compare the targeting of a known synaptic vesicle protein (the synaptic vesicle v-SNARE, n-Synaptobrevin) with Syt 4 in neurons of the same animal Expression of Syt 4-CFP and n-Syb-YFP by D42-GAL4 resulted in differential trafficking within motor neurons. Both proteins were visualized in the cell body and axon initial segment from which VNC dendrites sprout (Fig. 2.1B).

Figure 2.1

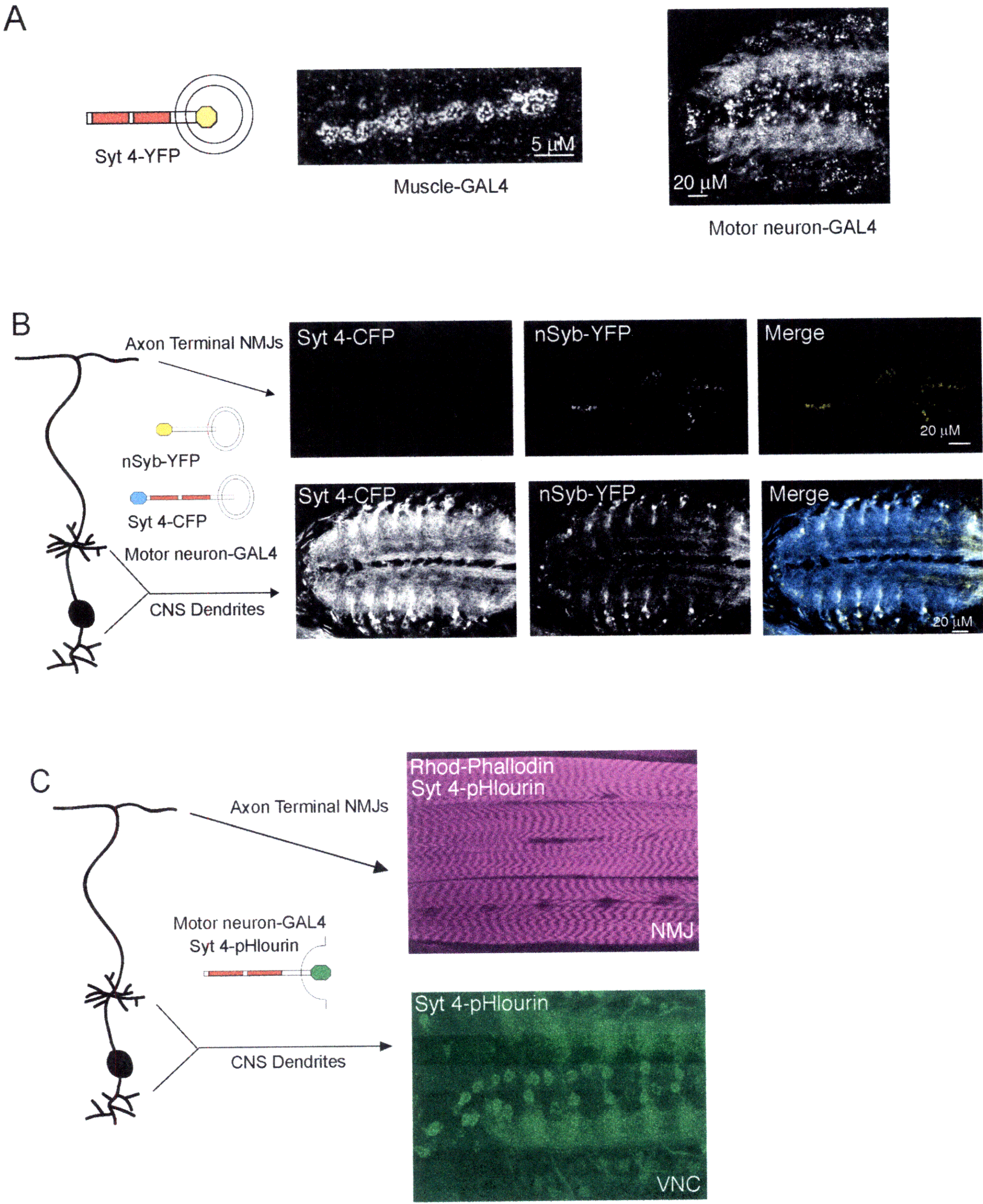

Figure 2.1: Syt 4 Localization to Postsynaptic Compartments. **A**, Diagram of N-terminal YFP tagged Syt 4 used to generate transgenic animals (left panel). Expression in muscle with the *Mhc-GAL4* driver reveals localization to postsynaptic sites (middle panel). Expression in motor neurons with D42-GAL4 results in punctate expression in the dendritic neuropil of the CNS ventral nerve cord (right panel). **B,** Model of n-Syb-YFP and Syt 4-CFP transgenic proteins expressed in motor neurons (diagramed on the left) by D42-GAL4. nSyb-YFP shows characteristic synaptic vesicle localization to presynaptic boutons at NMJs, while Syt 4-CFP localizes to postsynaptic dendrites within the CNS. The merged image is displayed in the right panels. **C,** Model of Syt 4- pHlourin transgenic protein expressed in motor neurons by D42-GAL4. No Syt 4 vesicle fusion is detected in presynaptic boutons at NMJs (top panel – counterstained with rhodamine-phallodin to highlight muscle 6/7). In contrast, Syt 4-pHlourin is readily detected in cell bodies (possibly reflecting ER/Golgi trafficking of the protein) and in puncta on dendritic membranes in the CNS.

However, they showed differential targeting to peripheral synapses. Using identical confocal settings, we imaged presynaptic terminals at NMJs and dendritic synapses in the VNC for each transgenic protein to determine their respective targeting properties. As shown in Fig. 2.1B, n-Syb-YFP was found at lower levels in CNS dendrites, but robustly present at presynaptic boutons, as expected for a synaptic vesicle protein. In contrast, Syt 4-CFP showed low expression in presynaptic terminals, but robust targeting and expression in the CNS dendritic neuropil, indicating Syt 4 preferentially targets to postsynaptic sites in CNS neurons.

2.2.2 Postsynaptic CNS Syt 4 Vesicles are Fusion Competent

To determine if Syt 4 vesicles are fusion competent in dendritic compartments of CNS neurons, we analyzed transgenic animals expressing a Syt 4 transgene tagged with a pH-sensitive GFP variant (ecliptic pHluorin) (Miesenbock et al., 1998). The fluorescence of pHluorin dramatically increases when exposed to the extracellular space from the acidic lumen of intracellular vesicles during fusion. Expression of Syt 4-pHluorin in motor neurons resulted in intense fluorescence on dendritic membranes in the VNC, with no fluorescence observed at presynaptic NMJs (Fig. 2.1C), indicating Syt 4 vesicles only fuse within the dendritic compartment of motor neurons. We conclude that fusion-competent Syt 4 vesicles target to postsynaptic compartments in CNS dendrites and muscle NMJs, suggesting Syt 4 may be a general regulator of Ca^{2+}-dependent trafficking events within the postsynaptic compartment, similar to the role of Syt 1 in presynaptic terminals.

2.2.3 Syt 4 Protein Levels are Altered in Activity Mutants

Given the potential role of Syt 4 in regulating Ca^{2+}-dependent retrograde signaling, modulation of Syt 4 function or expression could alter the capacity for postsynaptic targets to transmit retrograde signals. Mammalian *syt 4* was originally identified as an immediate-early gene (IEG) upregulated by seizure induction and neuronal depolarization (Ferguson et al., 1999; Vician et al., 1995). To determine if activity-dependent regulation of *syt 4* is evolutionarily conserved, we tested whether the *Drosophila* homolog is also regulated by neuronal activity. Our first indication that activity levels strongly modulate Syt 4 expression in *Drosophila* was the observation that Syt 4 levels are altered in *syt 1* -/- larvae. Mutants in *syt 1* exhibit a dramatic reduction in synaptic activity due to loss of the synchronous Ca^{2+} sensor for synaptic vesicle fusion (Yoshihara and Littleton, 2002). Immunohistochemistry with α-Syt 4 antisera at control and *syt 1* -/- larval NMJs revealed a dramatic decrease in postsynaptic Syt 4 levels compared to wildtype (Fig. 2.2 A, B).

To determine if reduced Syt 4 expression is specific to loss of Syt 1, or more generally reflects alterations in neuronal activity, we analyzed Syt 4 levels in *Drosophila* temperature-sensitive (TS) mutants that enhance or reduce neuronal activity (Ganetzky and Wu, 1986; Littleton et al., 1998). At non-permissive temperatures, TS mutants show loss of locomotor function as a result of reduced membrane excitability (as in the case of the sodium channel mutant, $para^{ts1}$, and the t-SNARE mutant, $syx^{3\text{-}69}$) or uncontrolled seizures (as in the potassium channel mutant, sei^{ts1}). Studies have also demonstrated that these TS mutants show chronic alterations in excitability, even at permissive temperatures

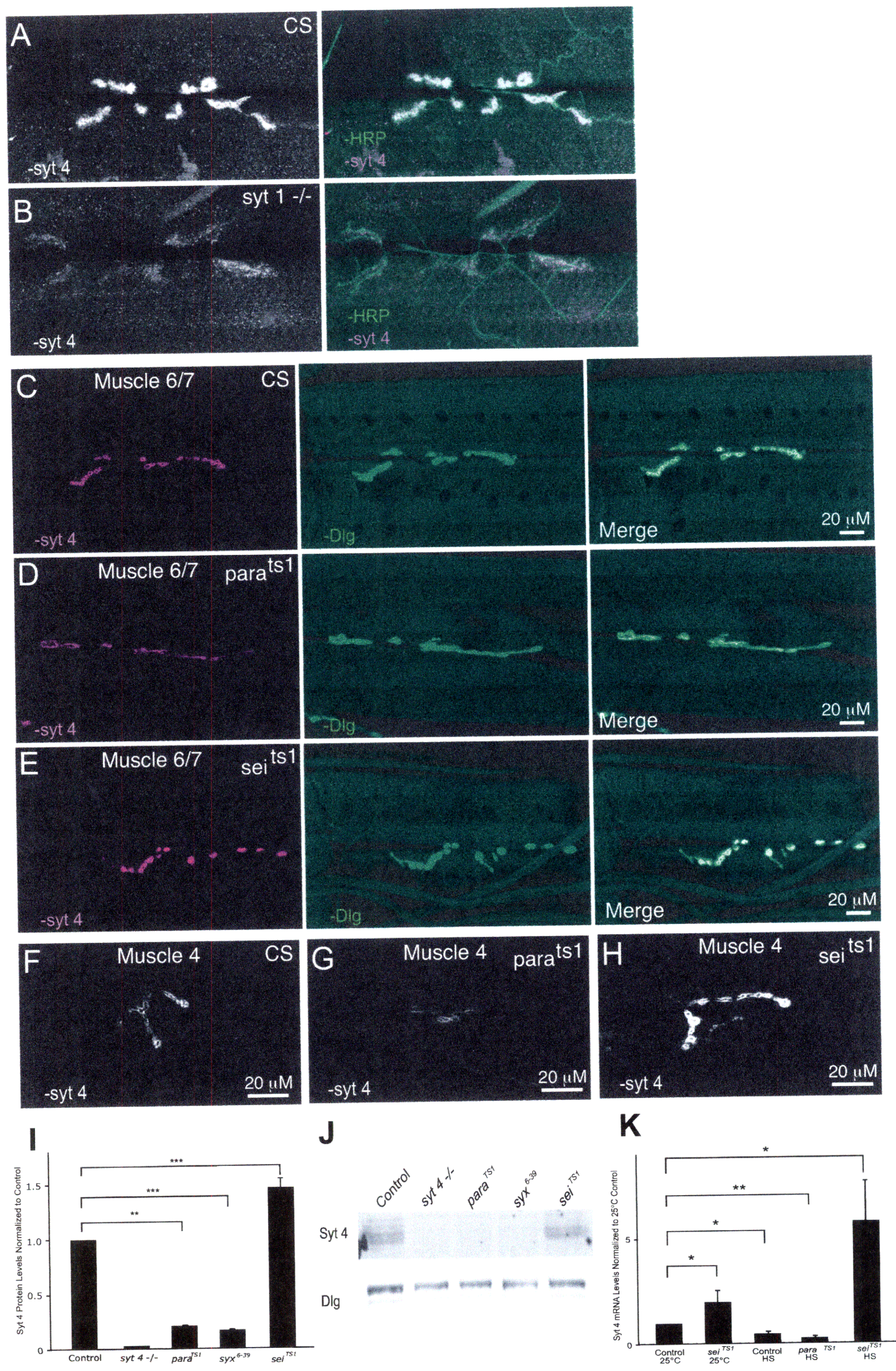
A
CS
-syt 4
-HRP
-syt 4
B
syt 1 -/-
-syt 4
-HRP
-syt 4
C
Muscle 6/7
CS
-syt 4
-Dlg
Merge
20 µM
D
Muscle 6/7
para ts1
-syt 4
-Dlg
Merge
20 µM
E
Muscle 6/7
sei ts1
-syt 4
-Dlg
Merge
20 µM
F
Muscle 4
CS
-syt 4
20 µM
G
Muscle 4
para ts1
-syt 4
20 µM
H
Muscle 4
sei ts1
-syt 4
20 µM
I
J
K
Control
syt 4 -/-
para TS1
syx 6-39
sei TS1
Syt 4
Dlg

Figure 2.2: Activity-Dependent Regulation of Syt 4 Expression. **A-B,** Immunostaining with α-Syt 4 (left panel) and α-HRP (merged image on right) of wandering 3rd instar CS (**A**) or *Syt 1 -/-* (**B**) larvae at muscle fiber 6/7. **C-E,** Immunostaining of CS, $para^{ts1}$, and sei^{ts1} with α-Syt 4 and α-Dlg at muscle fiber 6/7 in 3rd instar larvae reared at 25°C. Syt 4 is down-regulated in $para^{ts1}$ and upregulated in sei^{ts1} mutants. Identical confocal settings were used for all images. **F-H,** Immunostaining of CS, $para^{ts1}$, and sei^{ts1} with α-Syt 4 at muscle fiber 4 in 3rd instar larvae reared at 25°C. Identical confocal settings were used for all images. **I,** Quantification of Syt 4 protein levels in head extracts of CS, *syt* 4^{BA1} nulls, $para^{ts1}$, $syx^{3\text{-}69}$ and sei^{ts1} at 25°C. Quantification normalized to control (CS): $para^{ts1}$: 0.21 +/- 0.004, n=4, student's T-test $p<0.01$; $syx^{3\text{-}69}$: 0.17 +/- 0.003, n=8, student's T-test $p<0.0005$; sei^{ts1}: 1.46 +/- 0.08, n=7, student's T-test $p<0.0005$. ANOVA analysis F=38.82, $p<0.0001$. **J,** Western Blot analysis of head extracts of the indicated genotypes probed with α-Syt 4 and α-Dlg (loading control). **K,** Quantification of Syt 4 mRNA expression by RT-PCR in the indicated genotypes reared at 25°C, with or without a 20 min 38°C heat shock (HS). GAPDH1 mRNA was used as control. Quantification normalized to CS at 25°C: CS HS: 0.47 +/- 0.12, n=4, student's T-test $p<0.025$; sei^{ts1} 25°C: 1.68 +/- 0.51, n=4, student's T-test $p<0.05$; sei^{ts1} HS: 3.00 +/- 0.84, n=4, student's T-test $p<0.025$; $para^{ts1}$ HS: 0.32 +/- 0.06, n=4, student's T-test $p<0.01$. ANOVA analysis F=6.09, p=0.004.

(Guan et al., 2005), which are exacerbated at 38°C. Syt 4 staining at larval NMJs of wildtype, *para^{ts1}*, and *sei^{ts1}* strains demonstrated that Syt 4 protein expression does indeed correlate with the level of neuronal activity (Fig. 2.2 C-H). Syt 4 levels are reduced in *para^{ts1}* mutants, which have reduced neuronal excitability due to a reduction in sodium channel number, and elevated in *sei^{ts1}* mutants, which have enhanced neuronal activity due to loss of an ERG potassium channel. The effect of activity on Syt 4 levels was not restricted to the NMJ, as we observed similar results in the CNS by western blot analysis of Syt 4 levels from adult *Drosophila* head extracts taken from the mutants (Fig. 2.2 I-J). Quantification of Syt 4 levels by Li-Cor Odyssey infrared imaging demonstrated a statistically significant 5-fold reduction in *para^{ts1}* (student's T-test $p<0.01$) and *syx$^{3\text{-}69}$* (student's T-test $p<0.005$) mutants, and a statistically significant 46% increase (student's T-test $p<0.005$) in *sei^{ts1}* mutants (Fig. 2I).

2.2.4 Syt 4 mRNA Levels are Altered in Activity Mutants

The regulation of Syt 4 protein expression could represent a direct change in protein levels through altered protein stability or degradation, or might reflect activity-dependent transcriptional control of *syt 4* RNA expression. To examine transcriptional regulation of the locus, we used RT-PCR to compare *syt 4* mRNA levels in head extracts from TS excitability mutants raised at 25°C with those subjected to an acute 20 minute 38°C heat shock followed by a 30 minute recovery (Fig. 2.2 K). Relative to wildtype, *sei^{ts1}* showed a 2-fold higher level of Syt 4 mRNA at the permissive 25°C (student's T-test $p<0.05$), likely reflecting the excess chronic activity differences in the mutant. In addition, *sei^{TS1}* flies given a 20 minute heat shock to induce seizure activity in the mutant

displayed a 1.8 fold increase compared to *sei*^{TS1} 25°C controls, and a 6.4 fold increase in Syt 4 mRNA expression levels compared to heat shocked wildtype (student's T-test $p<0.025$). Heat-shocked *para*^{ts1} animals displayed a 70% reduction in *syt 4* mRNA levels (student's T-test $p<0.01$), consistent with the results from western analysis. We conclude that Syt 4 mRNA and protein levels are bi-directionally modified by neuronal activity changes, suggesting retrograde signaling mediated by Syt 4 may be regulated by the prior history of activity in neurons.

2.2.5 Syt 4 Levels Regulate Bouton Number

Retrograde signaling at *Drosophila* NMJs has been shown to regulate acute synaptic plasticity (Yoshihara et al., 2005), synaptic growth (McCabe et al., 2003; Yoshihara et al., 2005) and homeostatic plasticity (Goold and Davis, 2007). Syt 4 could mediate release of retrograde signals that regulate each of these pathways, or play a more specific role in only specific aspects of bi-directional signaling at the NMJ. Our previous analysis suggested that Syt 4 can stimulate synaptic varicosity formation and growth (Yoshihara et al., 2005). To further analyze the effect of Syt 4 levels on NMJ growth, we compared synaptic growth properties in *syt 4 -/-* larvae versus Syt 4 over-expression strains. Wandering 3rd instar larvae reared at 25°C were dissected and bouton number was quantified at muscle 6/7 of segment A3 by α-complexin (α-cpx) immunocytochemistry. The NMJs of *syt 4* null mutants exhibited a 24% decrease in bouton number (+/+ *n*=38; -/- *n*=34; student's T-test $p<0.001$) compared to controls (Fig. 3A). In contrast, there was a 44% increase in bouton number when Syt 4 was

Figure 2.3

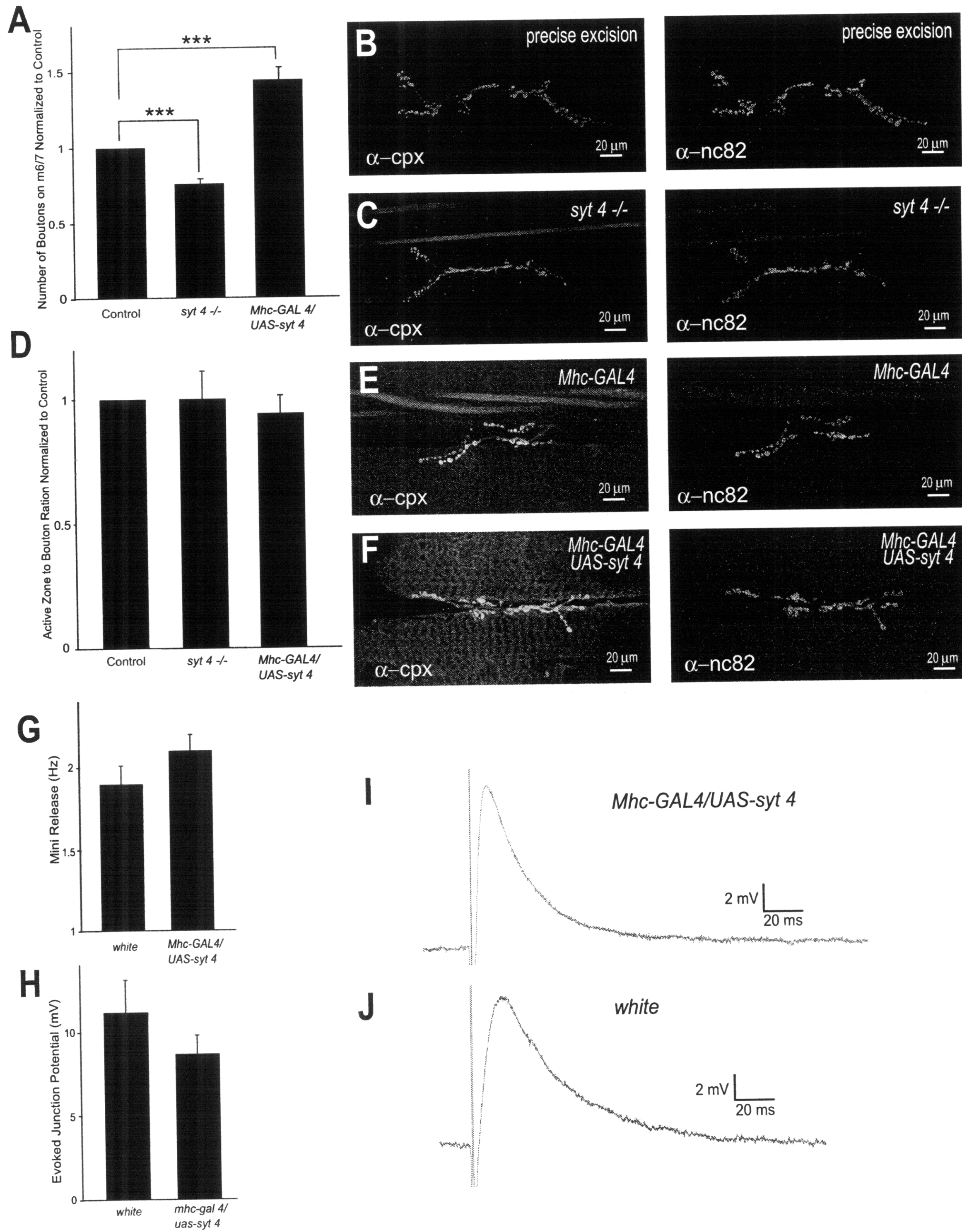

Figure 2.3: Syt 4 Regulates Synaptic Growth at the NMJ. **A,** Quantification of synaptic bouton number at muscle 6/7 in segment A3 of wandering 3rd instar larvae by α-cpx immunocytochemistry. *Syt 4* null mutants display 24% fewer boutons than precise excision controls (normalized to precise excision: control: 1 +/- 0.04, *n*=38; *syt 4 -/-*: 0.76 +/- 0.03, *n*=34; student's T-test p<0.001), while over-expression of Syt 4 by *Mhc-GAL4* results in a 44% increase in bouton number (normalized to *Mhc-GAL4: Mhc-GAL4:* 1 +/- 0.07, *n*=11; *Mhc-GAL4/UAS-syt 4:* 1.56 +/- 0.09, *n*=13; student's T-test p<0.001). **B,** Quantification of active zone to bouton number at muscle 6/7 of segment A3 in wandering 3rd instar larvae by α-nc82 and α-cpx immunocytochemistry. No significant difference in ratio in the null (normalized to precise excision: precise excision: 1 +/- 0.09, *n* = 6 NMJs; *syt 4 -/-*: 1.0 +/- 0.11, *n* = 5 NMJs) or over-expression lines (normalize to *Mhc-GAL4*: *Mhc-GAL4:* 1 +/- 0.11, *n*= 7 NMJs; *Mhc-GAL4/UAS-syt 4*: 0.94 +/- 0.07, *n*=10 NMJs) was observed. **C-F**, Representative immunocytochemical staining of 3rd instar larval NMJs of the indicated genotypes with α-cpx (left panels) and α-nc82 (right panels). **G,** Quantification of mEJP frequency in control and Syt 4 over-expression 3rd instar larvae at 0.2 mM Ca^{2+} (*white*: 1.9 Hz +/- 0.19, *n* = 10 NMJs; *Mhc-GAL4/UAS-syt 4*: 2.1 Hz +/- 0.14, *n* = 9 NMJs). **H,** Quantification of EJP amplitude in control and Syt 4 over-expression 3rd instar larvae at 0.2 mM Ca^{2+} (*white*: 11.2 mV +/- 1.9, *n* = 9 NMJs; *Mhc-GAL4/UAS-syt 4*: 8.7 mV +/- 1.1, *n* = 9 NMJs). **I-J**, Representative EJP traces from Syt 4 over-expression (**I**) and control (**J**) larval NMJs.

over-expressed postsynaptically (*Mhc-GAL4 n*=11; *Mhc-GAL4/UAS-syt 4 n*=13; student's T-test $p<0.001$) (Fig. 2.3A). We conclude that alterations in the levels of postsynaptic Syt 4 modulate synaptic growth at *Drosophila* NMJs.

2.2.6 Syt 4 Expression does not Alter Active Zone Ratio

To determine if alterations in Syt 4 expression alters the number of presynaptic release sites, in addition to modulating bouton number, we calculated the active zone to bouton ratio at larval NMJs of controls, *syt 4 -/-* and Syt 4 over-expression lines. NMJs were co-stained with α-cpx to label synapses and with α-bruchpilot (nc82) to label active zones. Quantification at 3rd instar larval m6/7 NMJs at segment A3 revealed no significant difference in the active zone to bouton ratio in *syt 4 -/-* mutants (normalized to control: control: 1+/- 0.09, *n* = 6; *syt 4 -/-*: 1.0 +/- 0.11, *n* = 5) or *Mhc-GAL4; UAS-syt 4* over-expression lines (normalized to control *Mhc-GAL4*: 1 +/- 0.11, *n*= 7; *Mhc-GAL4/ UAS-syt 4*: 0.94 +/- 0.07, *n*=10) (Fig. 3B-F). These findings indicate that Syt 4-dependent retrograde trafficking coordinately regulates both active zone and bouton number.

2.2.7 Synaptic Function

Modulation of synaptic growth and neurotransmitter release requires distinct signaling pathways at the NMJ, as they have been genetically separated in multiple synaptic growth mutants (Davis et al., 1996; Goold & Davis, 2007). In addition, previous studies have shown that retrograde signaling mechanisms can trigger homeostatic plasticity at the NMJ (Davis et al., 1996), with mutants displaying enhanced synaptic release properties without altered synaptic growth. Despite the decrease in bouton

number at the 3rd instar NMJ, evoked release is not altered in *syt 4 -/-* mutants (Saraswati et al., 2007). These findings suggest Syt 4 is not essential for retrograde signaling that mediates homeostatic compensation. To determine if the increased bouton number driven by postsynaptic over-expression of Syt 4 enhances neurotransmitter release, we examined synaptic properties, including EJP amplitude and mini EJP frequency, at 0.2 mM extracellular Ca^{2+} in over-expression lines (Fig. 2.3 G-J). Recordings from muscle 6/7 in segment A3 of 3rd instar larvae revealed no significant difference in either mini frequency (control: 1.9 Hz +/- 0.19, n = 10; *Mhc-GAL4/UAS-syt 4*: 2.1 Hz +/- 0.14, n = 9) or EJP amplitude (control: 11.2 mV +/- 1.9, n = 9; *Mhc-GAL4/UAS-syt 4*: 8.7 mV +/- 1.1, n = 9). These findings suggest that Syt 4-dependent retrograde signaling does not trigger increased evoked neurotransmitter release, despite enhanced synaptic growth. Alternatively, homeostatic compensation mechanisms triggered by postsynaptic activity are sufficient to maintain normal synaptic transmission levels despite a ~50% increase in bouton and active zone number in over-expression strains. In summary, our findings indicate that the loss of Syt 4 disrupts retrograde signaling mechanisms required for synaptic growth, while over-expression of Syt 4 in the postsynaptic compartment enhances retrograde signaling and subsequent synapse proliferation.

2.2.8 Syt 4 is Necessary for Temperature-Dependent Plasticity

Enhanced synaptic growth in response to increased neuronal activity has been well characterized at *Drosophila* NMJs (Barber and Littleton, 2007), and can be triggered by seizure episodes (Guan et al., 2005), enhanced larval locomotion at elevated rearing temperatures (Sigrist et al., 2003) or genetic mutations that increase neuronal excitability

Figure 2.4

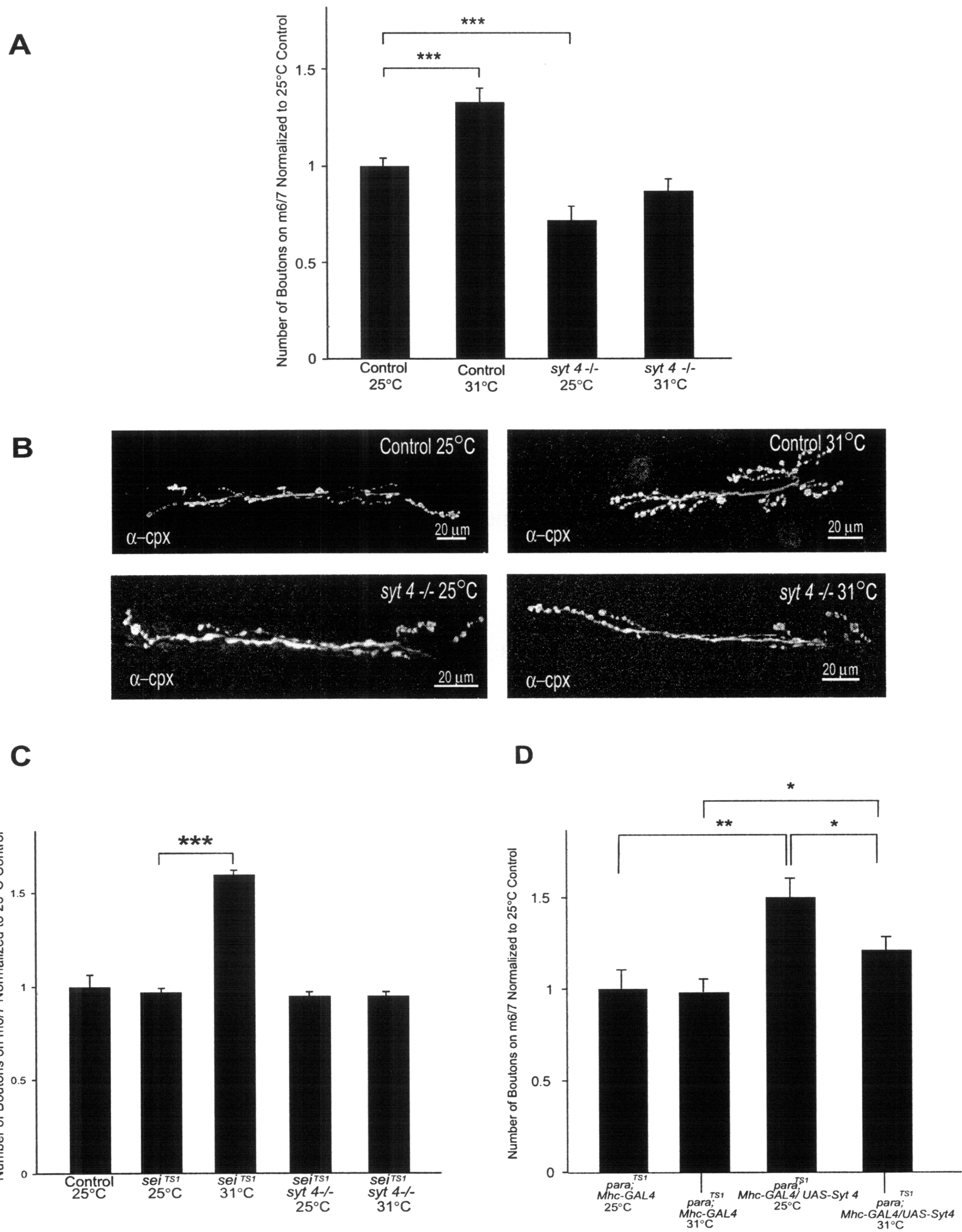

Figure 2.4: Syt 4 Regulates Activity-dependent Synaptic Growth at the NMJ. **A,** Quantification of bouton number at muscle 6/7 of segment A3 in 3rd instar wandering larvae of the indicated genotypes reared at 25°C or 31°C. Temperature dependent synaptic growth is suppressed in *syt 4 -/-* mutants. Quantification normalized to 25°C precise excision control larvae: control 25°C: 1 +/- 0.06, *n*=14; control 31°C: 1.33 +/- 0.06, *n*=12; *syt 4 -/-* 25°C: 0.72 +/- 0.04, *n*=11; *syt 4 -/-* 31°C: 0.87 +/- 0.07, *n*=13. Significant differences were detected in bouton number: control at 25°C and 31°C, student's T-test $p<0.001$; control and *syt 4 -/-* at 25°C, student's T-test $p<0.001$. ANOVA analysis, $F=16.37$, $p<0.0001$. **B,** Representative immunocytochemical staining of 3rd instar larval muscle 6/7 NMJs with α-cpx of the indicated genotypes reared at 25°C or 31°C. **C,** Quantification of bouton number at muscle 6/7 of segment A3 in 3rd instar wandering larvae of the indicated genotypes reared at 25°C or 31°C. *sei*^{ts1} mutants reared at 31°C show an increase in bouton number compared to 25°C (normalized to *sei*^{ts1}: *sei*^{ts1} 25°C: 1 +/- 0.07, *n*=14; *sei*^{ts1} 31°C: 1.35 +/- 0.06; student's T-test $p<0.0005$). This synaptic overgrowth is abolished in *sei*^{ts1} *syt 4 -/-* double mutants (normalized to *sei*^{ts1}: *sei*^{ts1}*; syt 4 -/-* 25°C: 0.95 +/- 0.02, *n* = 15; *sei*^{ts1}*; syt 4 -/-* 31°C: 0.95 +/- 0.02, *n* = 10). ANOVA analysis $F=19.9$, $p<0.0005$. **D,** Quantification of bouton number at muscle 6/7 of segment A3 in 3rd instar wandering larvae of the indicated genotypes reared at 25°C or 31°C. Over-Expression of Syt 4 in a *para*^{ts1} background caused bouton overgrowth at the permissive temperature (normalized to *para*^{ts1}; *Mhc-GAL4*: *para*^{ts1}; *Mhc-GAL4* 25°C: 1 +/- 0.1, *n*=10; *para*^{ts1}; *Mhc-GAL4/UAS-Syt 4* 25°C: 1.5 +/- 0.1, *n*=11; student's T-test $p<0.003$). At the non-permissive temperature, the enhanced growth is eliminated in controls (*para*^{ts1}; *Mhc-GAL4* 31°C: 0.98 +/- 0.07, *n*=15) and impaired in Syt 4 over-

expression larvae (*para*^{ts1}; *Mhc-GAL4/UAS-Syt 4* 31°C: 1.21 +/- 0.07, n=11, student's T-test $p<0.05$). ANOVA analysis $F=8.09$, $p=0.0002$.

(Budnik et al., 1990). These forms of structural plasticity elicited by enhanced neuronal activity are strikingly similar to the synaptic overgrowth we observe when Syt 4 is over-expressed postsynaptically at NMJs (Fig. 2.3, 2.4). We hypothesized that increased neuronal activity could trigger Syt 4-dependent retrograde signaling secondary to Ca^{2+} influx postsynaptically during elevated motor neuron firing. To test if Syt 4 is necessary for activity-dependent synaptic growth in *Drosophila*, we examined synaptic growth following enhanced neuronal activity in *syt 4* null mutants and in control precise excision lines from the genetic background in which the *syt 4* mutant was generated. We first examined structural plasticity at larval NMJs that occurs following increased larval locomotion stimulated by rearing the animals at elevated temperatures (Sigrist et al., 2003). We compared synaptic growth in *syt 4 -/-* larvae and control strains raised at 25°C and 31°C (Fig. 2.4 A, B). As described earlier, *syt 4* null mutants displayed decreased synaptic growth at 25°C compared to controls. While control larvae showed a 33% increase in bouton number when reared at 31°C compared to 25°C (student's T-test $p<0.001$), animals lacking Syt 4 displayed no statistically significant increase in bouton number at 31°C (normalized to control; 25°C: 0.72 +/- 0.04, n = 11; 31°C: 0.87 +/- 0.07, n = 13) (Fig. 5A, B). We conclude that enhanced synaptic growth induced by temperature-dependent increases in larval locomotion requires Syt 4-dependent retrograde signaling.

2.2.9 Syt 4 is Necessary for Plasticity in Neuronal Excitability Mutants

To extend this finding, we examined synaptic overgrowth that occurs secondary to mutations that enhance neuronal excitability. If Syt 4 is required to transmit essential

synaptic growth promoting signals during enhanced neuronal firing, we would anticipate synaptic overgrowth would be compromised when Syt 4-dependent vesicular fusion is abolished. When *sei^{ts1}* mutants lacking the *Drosophila* ERG potassium channel (Titus et al., 1997) are subject to developmental heat shocks, increased NMJ activity causes expansion of the larval NMJ (Guan et al., 2005). To test if Syt 4 activity is necessary for this form of structural plasticity, the *sei^{ts1}* allele was moved into the *syt 4 -/-* null background, and as a control into the *syt 4* precise excision background. Larvae of each genotype were reared at 25°C and at 31°C (Fig. 2.4C). *sei^{ts1}* mutants in the precise excision background displayed a 60% increase in bouton number at 31°C compared to 25°C (student's T-test $p<0.0005$). In contrast, *sei^{ts1}* in the *syt 4 -/-* null background showed no significant change in bouton number between 25°C and 31°C (normalized to control; 25°C: 0.95 +/- 0.02, n = 15; 31°C: 0.95 +/- 0.02, n = 10), indicating Syt 4 is required for seizure-induced synaptic growth.

2.2.10 Activity is Necessary for Syt 4 Facilitation of Growth

To test if neuronal activity is also required for the enhanced synaptic growth observed when Syt 4 is over-expressed postsynaptically, we examined the effects of reducing synaptic activity in Syt 4 over-expression strains. Previous studies have shown that mutations that reduce sodium channel function (*para^{ts1}*) can profoundly suppress synaptic overgrowth induced by hyperexcitability mutants (Budnik et al., 1990) or elevated temperature rearing (Sigrist et al., 2003). To analyze the effect of reduced activity on Syt 4-induced NMJ growth, we compared synaptic growth properties in Syt 4 over-expression strains in the *para^{ts1}* genetic background at permissive and non-

permissive temperatures (Fig. 2.4 D). Similar to previous observations (Sigrist et al, 2003), *para*^{ts1} mutants in the *Mhc-GAL-4* genetic background suppressed temperature-induced synaptic overgrowth (*para*^{ts1}; *Mhc-GAL-4* 25°C, 1 +/- 0.1, *n* = 10; 31°C: 0.98 +/- 0.07 normalized to 25°C, *n* = 15). In addition, *para*^{ts1} was able to dramatically reduce Syt 4-induced synaptic overgrowth when animals were reared at 31°C compared to 25°C (student's T-test p<0.05, normalized to *para*^{ts1}; *Mhc-GAL-4* at 25°C: *para*^{ts1}; *Mhc-GAL4/UAS-syt 4* at 25°C: 1.5+/-0.1, *n*=12; *Mhc-GAL4/UAS-syt 4* at 31°C: 1.21+/-0.07, *n*=11). In summary, these findings indicate that synaptic proliferation triggered by postsynaptic Syt 4 over-expression at *Drosophila* NMJs occurs in an activity-dependent fashion.

2.3 Discussion

Over the last decade, there has been a reversal in our appreciation of retrograde signaling at synapses, as experimental evidence has challenged the well-established view that synaptic information flows unidirectionally from the presynaptic terminal to the postsynaptic side. Retrograde signaling can be mediated by cell-cell contact through surface attached adhesion complexes, such as the ephrin/ephrin receptor (Knoll and Drescher, 2002). Membrane-permeable factors also mediate retrograde signaling, including the gases nitric oxide (NO) and carbon monoxide (CO), and bioactive lipid mediators arachidonic acid (AA), platelet-activating factor (PAF) and endocannabinoids (Hawkins et al., 1994). Retrograde communication can also be mediated through vesicular release of soluble factors via constitutive or regulated fusion pathways. Dendritic release of dopamine, ATP, GABA, and neuropeptides has been documented in many areas of the brain (Geffen et al., 1976; Cheramy et al., 1981; Israel and Meunier,

1978; Isaacson, 2001; Pow and Morris, 1989; Ludwig and Pittman, 2003; Li et al., 2005). Recent studies have also established the presence of membrane trafficking components in the postsynaptic compartment, including ER, Golgi and postsynaptic vesicles (Horton and Ehlers, 2004; Kennedy and Ehlers, 2006; Hanus and Ehlers, 2008). Fusion of cargos stored in post-Golgi vesicles can be triggered by secretagogues, including rises in intracellular Ca^{2+} levels. Ca^{2+}-regulated secretion has been best characterized for synaptic vesicle fusion, where Syt 1 functions as the presynaptic Ca^{2+} sensor. In contrast to the well-described mechanisms for neurotransmitter release, how retrograde signals are released from the postsynaptic compartment is poorly understood. Here we show that the Syt 1 homolog, Syt 4, plays an essential role in regulation of synaptic growth in an activity-dependent at *Drosophila* NMJ synapses, likely through regulation of retrograde signaling.

Members of the Synaptotagmin family are attractive candidates for postsynaptic Ca^{2+}-sensors capable of regulating retrograde signaling. Seven family members are encoded in the *Drosophila* genome, five of which are conserved in mammals, and two that are insect specific (Adolfsen and Littleton, 2001; Adolfsen et al, 2004). Structure-function studies have divided the synaptotagmin superfamily into Ca^{2+}-dependent and Ca^{2+}-independent isoforms based on conservation or evolutionarily divergence of the aspartic acid residues within the Ca^{2+} binding sites. Of those with predicted Ca^{2+}-binding properties, SytI, 4 and 7 are conserved and expressed in the CNS (Adolfsen et al., 2004). Syt 7 does not localize to *Drosophila* synapses (Adolfsen et al., 2004; Saraswati et al., 2007), and mutants disrupt dense core vesicle release from adrenal chromaffin cells (Schonn et al., 2008) and Ca^{2+}-dependent lysosomal exocytosis in non-neuronal cells

(Chakrabarti et al., 2003). Syt 1 and Syt 4 are the only isoforms that are expressed at most, if not all, *Drosophila* synapses. At the NMJ, where resolution of presynaptic versus postsynaptic compartments is easily determined, Syt 1 is presynaptic and Syt 4 postsynaptic. By imaging the localization of YFP-, CFP-, and pHlourin-tagged Syt 4 in CNS motorneurons, we demonstrate that Syt 4-fusion competent vesicles are also found in CNS dendrites, suggesting the protein may play a general role in Ca^{2+} regulation of postsynaptic membrane traffic.

Previous characterization of NGF and target-derived neurotrophic factors has defined their role in regulation of neuronal survival and connectivity during development (Zweifel et al., 2005). Recent studies have also suggested an important role for other target-derived secreted growth factors as retrograde signals in synaptic growth, including fibroblast growth factor (FGF), Wingless/Wnt and transforming growth factor (TGF-β) peptides (Umemori et al, 2004; Salinas, 2005; Hall et al., 2000). Although the cargo contained in Syt 4 vesicles is currently unknown, manipulations of Syt 4 levels postsynaptically suggest the retrograde signal(s) modulate synaptic growth. We observe a modest decrease in the number of boutons at NMJs lacking Syt 4. Over-Expression of postsynaptic Syt 4 has the opposite effect, inducing bouton proliferation. In neither case is there a change in neurotransmitter release or the number of active zones per bouton. These findings indicate Syt 4 regulates the release of signals involved in NMJ structural plasticity, but may not be essential for homeostatic mechanisms that control synaptic transmission. Previous studies have demonstrated that structural.growth at the NMJ is a prerequisite for the enhancement of synaptic output, and is regulated in part by the expression of cell adhesion factors such as FasII (Schuster et al., 1996a; Schuster et al.,

1996b). Secondary signals in the presynaptic terminal would then be required to facilitate synaptic transmission.

The enhanced synaptic growth observed with Syt 4 over-expression at the NMJ is strikingly similar to that seen in response to increased neuronal activity. Indeed, we find Syt 4-dependent synaptic growth requires neuronal activity, as the effect can be blocked by decreased action potential firing in *para*^{ts1} mutants. In addition, Syt 4 is required for an endogenous form of activity-induced synaptic growth triggered by rearing animals at higher temperatures and increased larval locomotion. Bouton overproliferation in the seizure-inducing *sei*^{ts1} mutant is also impaired in *syt 4* nulls, suggesting Syt 4-dependent retrograde signaling is an important modulator of activity-dependent synaptic growth and plasticity.

Two orthologs of *Drosophila* Syt 4 are found in mammalian genomes, encoded by the Syt 4 and Syt 11 genes. Although the localization of mammalian Syt 4 is controversial, both EM and immunocytochemical studies have found the protein associated with the Golgi and in vesicles that are present in postsynaptic dendrites (Ibata et al., 2002; Berton et al., 2000). The subcellular localization of Syt 11 has not been reported, but *syt 11* mRNA is abundantly expressed in the brain (von Poser et al, 1997). In addition, Ca^{2+}-dependent exocytosis of postsynaptic vesicles in mammalian neurons has been documented (Maletic-Savatic and Malinow, 1998; Li et al., 2005). Ca^{2+}-dependent fusion of somatodendritic vesicles containing the VMAT2 monoamine transporter has also been demonstrated by both immunocytochemistry and amperometric recording of dopamine release (Li e al., 2005). Surprisingly, the kinetics of somatodendritic dopamine release is similar to quantal events recorded at presynaptic sites, with release occurring in

less than a millisecond. Although future studies will be required to determine the location and function of Syt 4 and Syt 11 at mammalian synapses, they represent exciting candidates for regulating Ca^{2+}-dependent trafficking of postsynaptic cargos in mammalian neurons. Our observation that Syt 4 mRNA and protein levels are modulated by neuronal activity, similar to findings in mammals (Vician et al., 1995) and birds (Poopatanapong et al., 2006), suggests that not only has Syt 4's primary sequence been conserved, but also the transcriptional modulation by neuronal activity. Given the profound effects we observe on synaptic growth when postsynaptic Syt 4 levels are altered, it is exciting to speculate that activity-dependent modulation of Syt 4 may be a general mechanism for regulating synaptic growth and plasticity at activated synapses.

2.4 Materials and Methods

2.4.1 Generation of *Drosophila* Stocks

Drosophila were cultured on standard media at the indicated temperatures. Generation of the *syt 4* null mutant, *syt* 4^{BA1}, and precise excision control was previously described (Adolfsen et al., 2004). *Mhc-GAL4* and *C155-GAL4* lines were provided by Corey Goodman. *UAS-syt 4* tagged lines were obtained by injection of the construct into w^{1118} flies at the Duke Model Systems Transgenic Facility. YFP-*syt 4* cDNA fusions were generated by joining cDNA encoding YFP to the 5'-beginning of the *syt 4* coding sequence in pUAST and generating transgenic animals as described above. Syt-4-CFP lines were generated in a similar fashion by fusing the CFP cDNA to the 3'-end of the coding region of *syt 4* and deleting the stop codon. n-Synaptobrevin-YFP strains were

generated by subcloning of the YFP cDNA to the 5'-end of *n-Syb's* coding cDNA. All constructs were verified by DNA sequencing prior to injection.

2.4.2 Immunostaining

Immunostaining of wandering 3rd instar larvae reared at 25°C or 31°C was performed as previously described (Yoshihara and Littleton, 2002; Rieckhof et al., 2003). Primary antibody dilutions used were: α-Cpx (Huntwork and Littleton, 2007) for bouton counting 1:1000, nc82 α-Bruchpilot (Developmental Studies Hybridoma Bank) for active zone counting 1:50, α-syt 4 (Adolfsen et al., 2004) 1:250. Cy2-conjugated goat α-rabbit and Cy5-conjugated goat α-mouse (Jackson ImmunoResearch Laboratories) were used at 1:250. Samples were mounted in 70% glycerol. Images were taken using confocal microscopy on an Axoplan 2 microscope (Carl Zeiss MicroImaging, Inc.) using PASCAL software (Carl Zeiss MicroImaging, Inc.). Bouton and active zone number were quantified at muscle 6/7 of segment A3. All error measurements are standard error of the mean (SEM). Statistical significance was calculated using student's T tests, with * denoting $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

2.4.3 Western Blot / Quantification of Protein expression

α-Syt 4 antibody (Adolfsen et al., 2004) was used at 1:750; α-Dlg antibody 4F3 (Developmental Studies Hybridoma Bank) was used at 1:2000. Secondary antibodies were used at 1:10,000, including goat α-rabbit IgG Alexa 680 (Invitrogen) and goat α-mouse IR dye 800 (Rockland). Western Blots were visualized on the Li-Cor Odyssey

infrared imaging system and protein expression quantified with the accompanying software.

2.4.4 RT-PCR

RT-PCR was performed on male *Drosophila* head RNA preparations after a single acute 20 minute 38°C heat shock followed by 30 minute recovery, as previously described in Guan et al., (2005). Briefly, RNA was isolated from heads of males aged 3-4 days post eclosion. All flies were sacrificed between 2 and 4 PM to reduce any circadian-dependent transcriptional changes. Following seizure induction and recovery, *Drosophila* were frozen in liquid nitrogen and vortexed to separate heads from bodies. From purified total RNA from heads, mRNA was isolated using the Qiagen Oligotex mRNA extraction kit. cDNA was created with T7-$(dT)_{24}$ primer (Geneset Corp) using the GibcoBRL/ Invitrogen cDNA kit and purified by Phase Lock Gel extraction (Eppendorf). Primers used for RT-PCR quantification include:

GAPDH1 primers: 5' end: aatcaaggctaaggtcgaggag: 3' end: taaccgaactcgttgtcgtacc;

syt 4 primers: 5' end: cattgattcggagagg: 3' end: cttattgcgttcggtttgag.

2.4.5 Electrophysiology

Electrophysiological analysis of wandering 3rd instar larvae was performed in *Drosophila* HL3.1 saline (70 mM NaCl, 5 mM KCl, 4 mM $MgCl_2$, 10 mM $NaHCO_3$, 5 mM Trehalose, 115 mM sucrose, and 5 mM Hepes-NaOH, pH 7.2) with 0.2 mM $CaCl_2$ at muscle 6/7 at segment A3. Recordings were taken using an Axoclamp 2B amplifier (Axon Instruments, Inc.) at 22°C as described previously (Rieckhof et al., 2003).

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Chapter 3: Synaptotagmin 4 Ca^{2+} Binding

Cynthia F. Barber and J. Troy Littleton

Dina Volfson contributed to phosphorylation assays of the Syt 4 C2A domain.

3.1 Introduction

The Synaptotagmins (Syts) form a family of vesicular trafficking proteins implicated in regulation of Ca^{2+}-dependent vesicular fusion (Chapman, 2008). The most conserved member of the family, Syt 1, is believed to function as the Ca^{2+} sensor in fast spontaneous evoked release (Chapman, 2008; Yoshihara et al., 2003). Syt 1 is thought to facilitate membrane fusion, and thus neurotransmitter release, by bending the target membrane and simultaneously facilitating SNARE zippering (Martens et al., 2007). As the most extensively studied Synaptotagmin isoform, most of what we know about Synaptotagmin function is based on biochemical and genetic studies of Syt 1.

Synaptotagmin family members contain a short N-terminal intra-vesicular domain, a transmembrane domain, and a cytosolic segment composed of two C2 Ca^{2+}-binding domains. The C2 domains (C2A and C2B) share homology with the C2 domains of PKC, although are unique in their ability to bind both phospholipids and Ca^{2+} ions (Perin et al., 1990). The crystal structure of the isolated Syt 1 C2A domain reveals an eight stranded beta-sandwich attached to a 'C2 key,' where Ca^{2+} binding occurs (Sutton et al., 1995). Crystal structures and NMR on the Syt 1 C2A domain define five Asp residues (D1-D5) and one Ser involved in coordinating and binding of three Ca^{2+} ions (Shao et al., 1998; Zhang et al., 1998). Binding of the Ca^{2+} ions causes an electrostatic shift in the protein that is thought to assist with the binding of acidic phospholipids and SNARE proteins (Shao et al., 1997; Shao et al., 1998). The overall structure of the C2B domain is very similar to that of the C2A domain, with the addition of a seven-residue alpha-helix inserted between the beta-strands (Sutton et al., 1999). Much like C2A, C2B crystal

structures show binding of 2-3 Ca^{2+} ions by the 5 conserved Asp residues (D1-D5) (Cheng et al., 2004).

Although often studied in isolation, the two C2 domains are known to work together in facilitation of vesicular fusion, with both C2 domains penetrating the membrane simultaneously (Bai et al., 2002, Hui et al., 2006). Crystal structures of the human Syt 1 C2A/B reveal a network of interactions between the C2A and C2B domains that may alter function, and allow regulation of C2A Ca^{2+} binding by C2B's C-terminal alpha-helix (Fuson et al., 2007). The positive charge of Ca^{2+} ions mediate the formation of a bridge between the negatively-charged aspartic acids of both domains and the acidic phospholipids of the plasma membrane (Chapman, 2008).

There are seven Synaptotagmin isoforms in *Drosophila,* only two of which are present at most synapses – Syt 1 and Syt 4 (Adolfsen et al., 2004). In contrast to Syt 1, Syt 4 localizes postsynaptically at *Drosophila* NMJs (Adolfsen et al., 2004; Yoshihara et al., 2005), suggesting it may mediate Ca^{2+}-dependent retrograde signaling. However, the Ca^{2+} binding properties of Syt 4 have been a matter of intense debate. Many *in vitro* studies have reported that Syt 4, as well as its isolated C2A domain, fail to bind phospholipids in a Ca^{2+}-dependent manner (Bai et al., 2002, Bhalla et al., 2008, Dai et al., 2004, Hui et al., 2005, von Poser et al., 1997). A conserved D3 to serine substitution in the C2A Ca^{2+}-binding domain of Syt 4 has been preposed to evolutionarily 'inactivate' Syt 4 Ca^{2+} binding (von Poser et al., 1997). However, *in vivo* observations from *Drosophila* and PC12 cells suggest that Syt 4 containing vesicles fuse in Ca^{2+}-dependent manner (Yoshihara et al., 2005, Fukuda and Yamamoto 2004). Here we report that synaptic growth induced by Syt 4-dependent retrograde signaling requires functional

Ca^{2+}-binding sites in both the C2A and C2B domains. Furthermore, the conserved Ser substitution, S284, appears to play an essential role in Syt 4 function.

3.2 Results

In *Drosophila,* Syt 4 is localized to a fusion competent vesicle population in the postsynaptic compartment of the NMJ (Adolfsen et al., 2004, Yoshihara et al., 2005). One established *in vivo* assay for Syt 4 function in *Drosophila* is over-expression of a *UAS-syt 4* transgene under control of the postsynaptic *myosin heavy chain*-Gal 4 (*Mhc-GAL4*) driver (Yoshihara et al., 2005). When wild-type Syt 4 is over-expressed in the muscle, synaptic growth is stimulated, and as assayed in wandering 3rd instar larvae *UAS-syt 4;Mhc-GAL4* lines demonstrate a ~50% increase in bouton number (Yoshihara et al., 2005). To assess whether Ca^{2+} binding is required for Syt 4 function, we examined the role of C2A and C2B in Syt 4-dependent synaptic growth by neutralizing one or more of the five key aspartic acid residues (referred to as D1-D5 in previous literature) in each C2 domain to asparagines (Fig. 3.1). Multiple transgenic lines expressing each mutant protein under control of the UAS/GAL4 system were generated and strains over-expressing similar levels of Syt 4 were selected for analysis (Fig. 3.1).

3.2.1 C2 Ca^{2+} Binding Sites are Required for Syt 4 Function

Only two known Ca^{2+} binding sites exist in the Syt 4 protein, the 'C2 keys' of the C2A and C2B domains. To determine if the Ca^{2+} binding sites were important to Syt 4 function we simultaneously mutated key aspartic acids in both the C2A (D292N (D5)) and C2B (D427N (D3), D429N (D4)) Ca^{2+}-binding domains. Mutations to the analogous

Figure 3.1

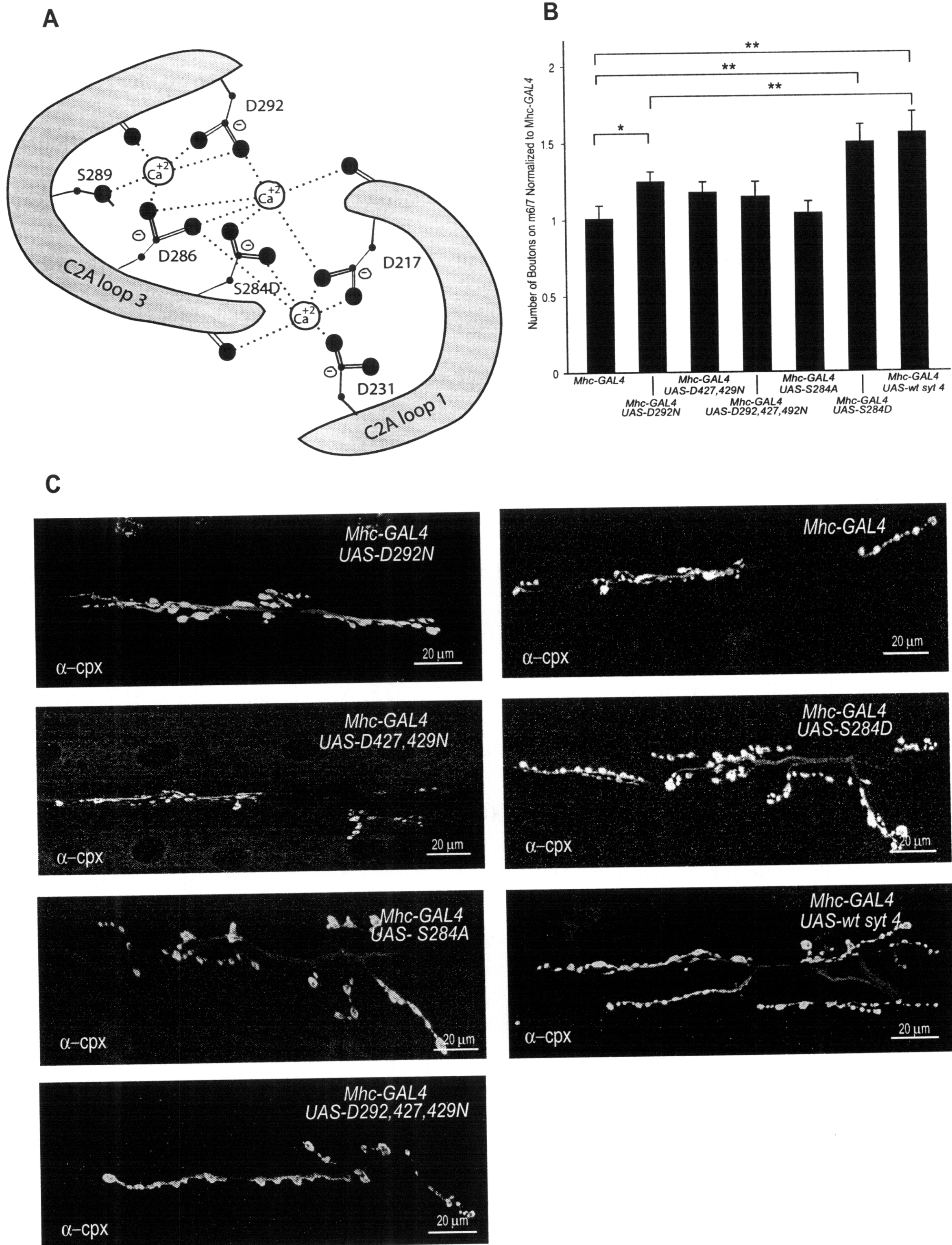


Figure 3.1: Structure-Function Analysis of Syt 4 C2 Domains. **A,** Model depicting key residues involved in coordination of Ca^{2+} binding in the Syt 4 C2A domain. S284D indicates location of the key Ca^{2+} binding residue that is an aspartic acid in other synaptotagmin family members, and a serine in the Syt 4/11 family. **B,** Quantification of Syt 4-stimulated synaptic growth at muscle 6/7 of segment A3. 3rd instar wandering larvae of the indicated genotypes reared at 25°C were immunostained with α-cpx antisera. Quantified data normalized to *Mhc-GAL4: Mhc-GAL4*: 1 +/- 0.09, *n*=11; *Mhc-GAL4/UAS-syt 4*: 1.6 +/- 0.1, *n* = 9; *Mhc-GAL4/+*; *UAS-syt 4 D292N*: 1.23 +/- 0.07, *n* = 10; *Mhc-GAL4/UAS-syt 4 D427N D429N*: 1.17 +/- 0.07, *n* = 13; *Mhc-GAL4/UAS-syt 4 D292N D427N D429N*: 1.1 +/- 0.1, *n* = 9; *Mhc-GAL4/+*; *UAS-syt 4 S284A*: 1.0 +/- 0.08, *n* = 11; *Mhc-GAL4/+*; *UAS-syt 4 S284D*: 1.5 +/- 0.1, *n* = 11. Significant differences were detected in bouton number: *D292N* and *Mhc-GAL4* control, student's T-test $p<0.05$; *D292N* and wildtype Syt 4, student's T-test $p<0.0025$; *S284D* and *Mhc-GAL4* control, student's T-test $p<0.0025$; wildtype Syt 4 and *Mhc-GAL4* control, student's T-test $p<0.0025$. ANOVA analysis $F=4.58$, $p=0.0006$. **C,** Representative immunocytochemical staining of 3rd instar larval muscle 6/7 NMJs of the indicated genotypes with α-cpx.

residues in Syt 1 proteins had previously established their ability to eliminate Ca^{2+} binding activity (Mackler et al., 2002; Robinson et al., 2002; Zhang et al., 1998). When over-expressed in the muscle, the C2A/C2B mutant *syt 4* transgene failed to facilitate synaptic overgrowth (normalized to *Mhc-GAL4* control: 1 +/- 0.09, *n*=11; *Mhc-GAL4/UAS-syt 4 D292N D427N D429N*: 1.1 +/- 0.1, *n* = 9; *Mhc-GAL4/UAS-syt 4:* 1.56 +/- 0.09, *n*=13) (Fig. 3.1). This confirms earlier results by Yoshihara, Littleton and colleagues that Syt 4 functions in a Ca^{2+}-dependent manner, and may indeed regulate Ca^{2+}-dependent retrograde signaling leading to enhanced synaptic growth. More importantly, it confirms that despite the *in vitro* evidence, *in vivo* Syt 4 appears to function as a Ca^{2+} sensor.

3.2.2 The C2B Ca^{2+}-Binding Domain is Required for Syt 4 Function

In Syt 1, the C2B domain plays a critical role in regulation of Ca^{2+}-dependent phospholipid binding and facilitation of vesicular fusion (Littleton et al., 2001; Mackler et al., 2002). Syt 1 proteins with mutations to the D3,4 residues in the C2B Ca^{2+} binding site fail to bind Ca^{2+} *in vitro* or rescue the *syt 1* null phenotype *in vivo* (Mackler et al., 2002). To determine if the C2B domain plays a similar role in Syt 4 function, we generated *UAS-syt 4 D427, 429N* transgenic lines that mutate D3,4 of the C2B Ca^{2+} binding domain.

Postsynaptic over-expression of this transgene, driven by *MHC-GAL4*, failed to promote synaptic growth compared to over-expression of wildtype Syt 4 (normalized to control; *UAS-syt 4 D427N D429N*: 1.17 +/- 0.07, *n* = 13). This indicates that the C2B Ca^{2+} binding pocket of Syt 4 is required for protein function, similar to its role in Syt 1.

This finding has important implications as it confirms a central assumption that there is both structural and significant functional homology between the Synaptotagmin family members.

3.2.3 The C2A Ca^{2+}-Binding Domain is Required for Syt 4 Function

In contrast to C2B, the C2A domain of Syt 4 contains a conserved substitution (D284S) in a key aspartic acid residue (D3) that has been hypothesized as an evolutionary inactivation of C2A Ca^{2+}-binding (von Poser et al., 1997). Indeed, the presence of an aspartic acid at the site is required for coordination of Syt 4 C2A Ca^{2+} binding *in vitro* (Fernández-Chacón et al., 2002). Because of this substitution, and data suggesting that C2B appears to be the most critical Ca^{2+}-binding domain in Syt 1 (Mackler et al., 2002), we hypothesized that Ca^{2+} binding by C2A would not be required for Syt 4 function *in vivo*.

To directly test the relevance of the C2A Ca^{2+}-binding site, we generated transgenic lines expressing an aspartic acid to asparagine mutation at D292. Surprisingly, over-expression of *UAS-syt 4 D292N* in the muscle was less efficient at promoting synaptic growth than the wildtype transgene (normalized to control; *Mhc-GAL4/+; UAS-syt 4 D292N/+*: 1.23 +/- 0.07, n = 10; *Mhc-GAL4/UAS-syt 4*: 1.6 +/- 0.1, n = 9) (Fig. 3.1). Although the C2A mutant transgene did retain some residual function compared to C2B mutants, this data suggests that C2A Ca^{2+} binding is necessary for Syt 4 activity *in vivo*. Furthermore, this suggests that Serine 284, which is conserved in all Syt 4 isoforms from *C. elegans* to humans, might not simply be a mutation for inactivation of Ca^{2+} binding.

3.2.4 S284 is Required for Syt 4 Function

Given the conservation of S284, and that no other amino acid substitution has been allowed during evolution, it would be unusual if the sole purpose of the serine was to inactivate Ca^{2+} binding. It has previously been proposed, based on *in vitro* phospholipid binding assays, that the serine is involved in determination of Syt 4 phospholipid binding affinity (Fukuda et al., 1996). Another explanation for the conserved serine is that it represents a site of phosphorylation, potentially regenerating a negative charge in the C2A Ca^{2+}-binding pocket normally formed by an aspartic acid in other synaptotagmin isoforms (Fig. 3.1). Given the requirement of the C2A Ca^{2+}-binding site for Syt 4 function, the phosphorylation theory predicts, in contrast to the inactivation theory, that S284 is also required for Syt 4 function.

To test if S284 is required for Syt 4 function *in vivo*, we generated transgenic animals expressing *UAS-syt 4 S284A* and expressed the construct postsynaptically under the control of *Mhc-GAL4*. If indeed S284 serves to eliminate Ca^{2+} binding, a S284A change would not alter Syt 4's ability to enhance retrograde signaling and synaptic growth. If, however, the serine is subject to phosphorylation that completes the Ca^{2+} binding site and is required for Syt 4 function, S284A would impair Syt 4 function. In fact, the C2A S284A mutant completely eliminated Syt 4 function in the neuronal overgrowth assay (normalized to control; control: 1 +/- 0.09, n = 11; *Mhc-GAL4/+; UAS-syt 4 S284A/+*: 1.0 +/- 0.08, n = 11), suggesting this residue is essential to Syt 4 *in vivo* activity (Fig. 3.1).

3.2.5 Phosphomimetic S284D is Sufficient for Syt 4 Function

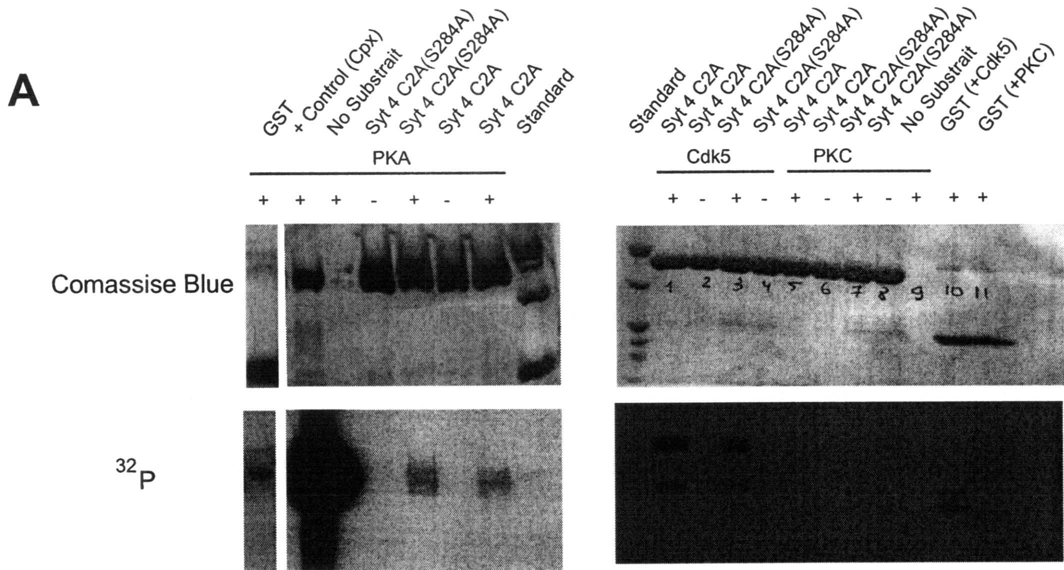
To determine if phosphorylation of S284 might contribute to Ca^{2+} binding by C2A, we constructed a phosphomimetic serine to aspartic acid construct (S284D). The aspartic acid mimics the size and charge of a phosphorylated serine, and in Syt 4 such a substitution was previously shown to restore Ca^{2+} binding of the Syt 4 C2A domain *in vitro* (von Poser et al., 1997). Thus, we hypothesized that Syt 4 containing the substitution would function at a level similar to or greater than wildtype.

When the *UAS-syt 4 S284D* construct was over-expressed postsynaptically in the muscle, it promoted a ~50% increase in bouton number (Fig 3.1). Thus, the *UAS-syt 4 S284D* construct was able to promote NMJ growth at a level similar to wildtype, and in sharp contrast to the non-functional serine to alanine mutation (control: 1 +/- 0.09, n=11; *Mhc-GAL4/+; UAS- syt 4 S284D/+*: 1.5 +/- 0.1, n = 11; *Mhc-GAL4/UAS-syt 4*: 1.6 +/- 0.1, n = 9) (Fig. 3.1). Phosphorylation of S284 is an attractive mechanism for setting thresholds for retrograde plasticity, as less active synapses might have Syt 4 in an unphosphorylated state that is less efficient at promoting retrograde signaling. We conclude that Ca^{2+} binding by the C2A and C2B domains of Syt 4 is essential for its *in vivo* function, and that the highly conserved serine at the D3 position of C2A in Syt 4 is critical for the *in vivo* properties of the Syt 4 subfamily.

3.2.6 S248 is Not Phosphorylated by PKA, PKC, Cdk5 or CaMKII

Given the role of Syt 4 in mediating translation of neuronal activity to synaptic growth, phosphorylation of S284 in an activity-dependent manner provides an attractive mechanism for regulation of Syt 4 function. Phosphorylation of S284 in Syt 4 in an

Figure 3.2



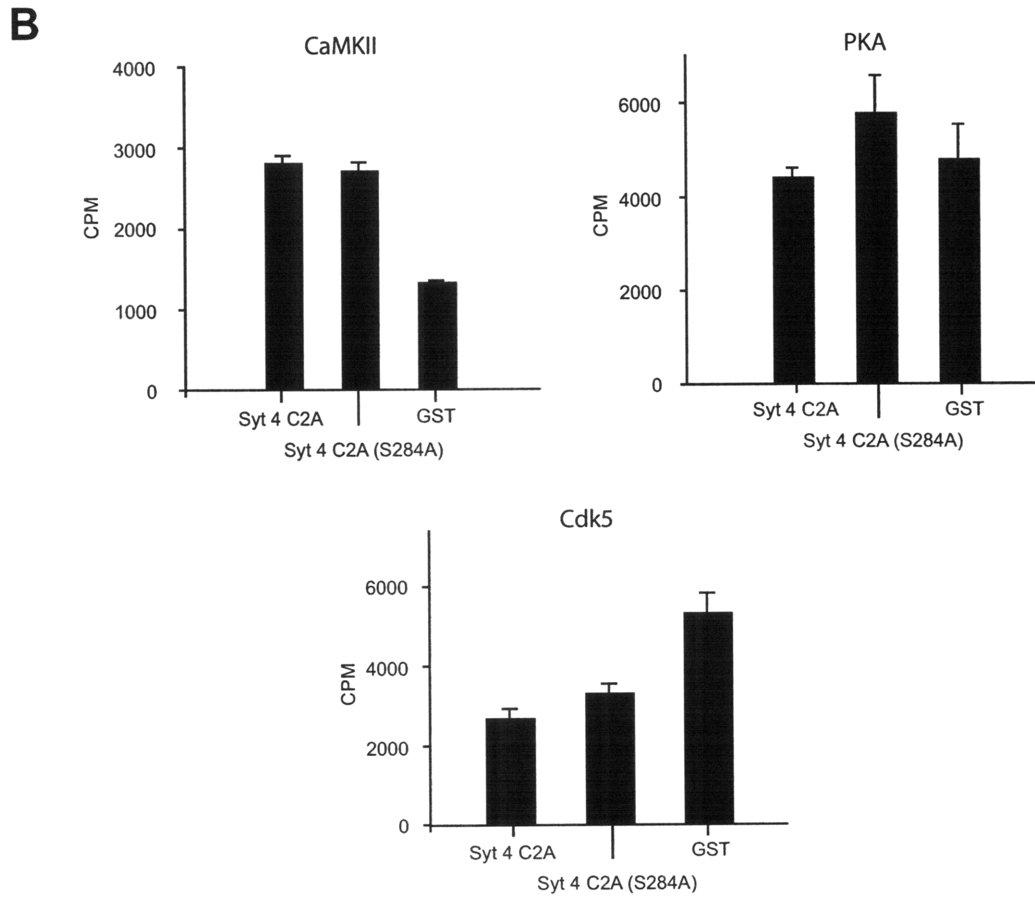


Figure 3.2: Phosphorylation of Syt 4 C2A and Syt 4 C2A (S284A) constructs fused to GST. **A,** Crude protein is shown by Coomassie Blue staining of SDS-PAGE gels, corresponding ^{32}P films are shown below. Film did not reveal ^{32}P labeling of either construct after PKC treatment and up to 3 days exposure. ^{32}P labeling of both constructs was detected after PKA and Cdk5 treatment. However, there was no difference in labeling between the two constructs. In both cases, significant levels of labeling were also seen on GST alone. This suggests that any phosphorylation that does occur on C2A is not linked to S284. Films are pictured after 24 hour exposure. **B,** Quantification of ^{32}P labeling by scintillation counter. PKA and Cdk5 labeled GST alone at levels equal to or greater than fusion protein labeling, suggesting no specific phosphorylation of the constructs by either kinase. CaMKII specifically labeled the constructs, but there was no difference in labeling between the native and mutant C2A domains, suggesting S284 is not the site of CaMKII phosphorylation.

activity-dependent fashion would be expected to enhance Ca^{2+} binding, potentially requiring less synaptic input to trigger future retrograde signaling. Analysis of the Syt 4 protein sequence does not reveal a known kinase consensus sequence surrounding S284. However, a number of kinases are known to participate in activity-dependent phosphorylation of neuronal targets, and could potentially play a role in Syt 4 phosphorylation. These include PKA, PKC, Cdk5, and CaMKII.

PKA, whose activity is regulated by fluctuations in cyclic AMP levels, is known to be involved in the transition from medium-term to long-term memory storage in *Drosophila*, as well as high frequency-stimulated miniature release (HFMR) plasticity (Yin et al., 1995; Yoshihara et al., 2005). Classical PKC phosphorylation requires Ca^{2+}, and PKC activity is known to be required for new bouton formation (Ruiz-Canada et al., 2004). Cdk5 is an neuronal Cdk whose transcription is regulated by Fos, and upregulated after seizure or administration of dopamine or cocaine (Dhariwala and Rajadhyaksha, 2007). Finally, the kinase CaMKII, which also requires Ca^{2+} to function, is known to be involved in *Drosophila* behavioral memory and NMJ plasticity (Lisman et al., 2002; Koh et al., 1999).

To test the role of PKA, PKC, Cdk5 and CaMKII in phosphorylation of Syt 4 S284, transgenic Syt 4 C2A domains were purified with either the wildtype sequence or an S284A mutation. The ability of each kinase to phosphorylate the C2A domain with and without the S284A mutation was assessed using radioactive P^{32} labeling (Fig. 3.2). If a kinase were responsible for phosphorylation of S284, a lower level of P^{32} labeling would be expected in the S284A sample than the wildtype C2A domain sample. None of the three kinases tested showed such a pattern. While this does not rule out

phosphorylation of S284, it suggests that a kinase other than PKA, PKC, Cdk5 or CaMKII would be responsible. Future studies will employ IP of endogenous Syt 4 and subsequent mass spectrometry to determine if S284 is phosphorylated *in vivo*.

3.3 Discussion

An interesting question raised by earlier studies of Syt 4 is how similar the biochemical activities of Syt 1 and Syt 4 are *in vivo*. Syt 1 and Syt 4 cannot functionally substitute for each other at synapses (Adolfsen et al., 2004; Yoshihara et al., 2005), but this reflects the observation that the two proteins sort to distinct vesicle populations rather than implying dissimilar function. Syt 4-dependent retrograde signaling at NMJs shows Ca^{2+} dependence *in vivo* (Yoshihara et al., 2005; Fig. 3.1), although *in vitro* methods have produced conflicting evidence about its potential to bind Ca^{2+} (Dai et al., 2004; Robinson et al., 2002; Thomas et al., 1999; Fukuda et al., 1996).

Syt 1 preferentially requires C2B Ca^{2+} binding for activity (Mackler et al, 2002), with C2A playing a more minor role (Robinson et al., 2002). By examining the ability of mutant Syt 4 proteins to promote bouton growth, we demonstrate that Ca^{2+}-binding sites in both the C2A and C2B domain of Syt 4 are necessary for Syt 4 function. These results suggested not only that Syt 4 does function as a cellular Ca^{2+} sensor, but that the C2A Ca^{2+} site is required for its function *in vivo*. In particular, serine 284 of the C2A domain is required for Syt 4 activity. This was an unexpected finding, as the serine replaces an essential aspartic acid residue required for C2 domain Ca^{2+}-binding in other synaptotagmin isoforms. This aspartic acid to serine substitution is conserved from *C. elegans* to humans, suggesting an evolutionarily important role for the residue, previously

hypothesized to be inactivation of the C2A Ca^{2+}-binding site. However, our finding that S284 is required for Syt 4 function *in vivo* raises the possibility that the serine may be phosphorylated, reintroducing a negative charge into the Ca^{2+} binding pocket and allowing it to functionally substitute for the aspartic acid present in Syt 1's C2A Ca^{2+}-binding pocket. Indeed, substitution of an aspartic acid at S284 restores Ca^{2+} binding by Syt 4 *in vitro* (von Poser et al., 1997) and can promote synaptic growth when over-expressed *in vivo* (Fig. 3.1). A number of kinases may be responsible for S284 phosphorylation, three of which we tested in P^{32} phosphorylation assays. The assays revealed that neither PKA, PKC, Cdk5 or CaMKII appears to be responsible for phosphorylation of S284.

Phosphorylation of Syt 4 could potentially explain the conflicting *in vivo* and *in vitro* evidence concerning the protein's ability to sense Ca^{2+}. There is precedent for phosphorylation of synaptotagmins regulating Ca^{2+} binding, as Syt 2's C2 domains are phosphorylated by WNK1, in this case reducing its Ca^{2+}-dependent phospholipid binding affinity (Lee et al., 2004). Phosphorylation of Syt 4 such that it enhances activity in a retrograde signaling pathway, would provide a means for the cell to enhance plasticity - requiring less Ca^{2+} and thus less neuronal activity to trigger the retrograde signaling pathway. We conclude from our findings that Syt 4 likely acts as a Ca^{2+} sensor *in vivo* and that phosphorylation of S284 may provide a means of activity dependent regulation of Syt 4 function.

3.4 Materials and Methods

3.4.1 Generation of *Drosophila* Stocks

Drosophila were cultured on standard media at the indicated temperatures. *Mhc-GAL4* and *C155-GAL4* were provided by Corey Goodman. *UAS-syt 4* mutant lines were constructed in the *syt 4* cDNA by site-directed mutagenesis of *pUAST-syt 4*, and injected into w^{1118} flies at the Duke Model Systems Transgenic Facility. All constructs were verified by DNA sequencing prior to injection.

3.4.2 Immunostaining

Immunostaining of wandering 3rd instar larvae reared at 25°C was performed as previously described (Yoshihara and Littleton, 2002; Rieckhof et al., 2003). Primary antibody dilutions used were: α-Cpx (Huntwork and Littleton, 2007) for bouton counting 1:1000, α-syt 4 (Adolfsen et al., 2004) 1:250. Cy2-conjugated goat α-rabbit and Cy5-conjugated goat α-mouse (Jackson ImmunoResearch Laboratories) were used at 1:250. Samples were mounted in 70% glycerol. Images were taken using confocal microscopy on an Axoplan 2 microscope (Carl Zeiss MicroImaging, Inc.) using PASCAL software (Carl Zeiss MicroImaging, Inc.). Bouton were quantified at muscle 6/7 of segment A3. All error measurements are standard error of the mean (SEM). Statistical significance was calculated using student's T tests, with * denoting $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3.4.3 Western Blot / Quantification of Protein expression

α-Syt 4 antibody (Adolfsen et al., 2004) was used at 1:750; α-Dlg antibody 4F3 (Developmental Studies Hybridoma Bank) was used at 1:2000. Secondary antibodies were used at 1:10,000, including goat α-rabbit IgG Alexa 680 (Invitrogen) and goat α-mouse IR dye 800 (Rockland). Western Blots were visualized on the Li-Cor Odyssey infrared imaging system and protein expression quantified with the accompanying software.

3.4.4 Protein Purification

Coding sequence for the Syt 4 C2A domain was cloned into pGEX-2T plasmid, and the S284A substitution made by PCR site directed mutagenesis. Constructs were expressed in E.coli cells (BL21) according to standard laboratory protocols. The fusion proteins were purified using glutathione-sepharose beads (Amersham Pharmacia).

3.4.5 Protein Phosphorylation Assay

Kinase assays were conducted in 25μl volume, with 10X kinase buffer (3.5μl), cold ATP (10mM; 0.07μl), hot γ-ATP (1 μl), kinase (1μl), ddH_2O (5.43 μl), with remaining volume protein in PBS. Solutions were incubated at room temperature for 30-40 minutes, run on SDS-PAGE gels, and stained overnight with Coomassie blue. The next day gels were destained and exposed to film. For quantification by scintillation counter, reaction mixtures were spotted onto phosphocellulose (P81) paper, air dried and washed 3x with 1% phosphoric acid. Samples were tested in scintillation counter with 3mL scintillation

cocktail (Cell Signaling Technology). All kinases and positive controls from Cell Signaling Technology.

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Chapter 4: Conclusions and Perspectives

Cynthia F. Barber and J. Troy Littleton

4.1 Summary

The Synaptotagmin (Syt) family of Ca^{2+} sensors have been shown to play an important role in the regulation of Ca^{2+}-dependent vesicular fusion. Studies of the best characterized member of the family, Syt 1, have provided the basis for our understanding and investigations into other family members. The Syt 1 protein is composed of an N-terminal tail, a transmembrane domain, and two cytosolic C2 domains. The C2 domains share homology with the C2 domain of PKC, and are capable of binding Ca^{2+} and acidic phospholipids in a cooperative manner (Chapman, 2008). Each C2 domain contains five aspartic acid residues (termed D1-D5) that are key to the Ca^{2+} and phospholipid binding activities of the protein (Cheng et al., 2004; Shao et al., 1998; Zhang et al., 1998). Together the aspartic acids of both domains contribute to SNARE complex zippering while binding Ca^{2+} and negatively charged phospholipids of the plasma membrane, inducing curvature into the membrane that facilitates fusion (Chapman, 2008; Martens et al., 2007). Syt 1 function is conserved across evolution, from *C.elegans* to *Drosophila* and mammals, where it functions in the presynaptic terminal to coordinate fast-synchronous fusion of vesicles in response to action potentials (Chapman, 2008; Yoshihara et al., 2003).

Previous work by Yoshihara, Adolfsen, Littleton and colleagues has suggested that the second most conserved member of the Synaptotagmin family, Syt 4, may play a postsynaptic role similar to Syt 1. At the *Drosophila* neuromuscular junction (NMJ), Syt 4 is postsynaptic, residing on a vesicle population that is fusion competent, and whose Ca^{2+}-dependent fusion is necessary for high frequency-stimulation-induced miniature release (HFMR) plasticity, possibly due to the release of a retrograde signal (Adolfsen et

al., 2004; Yoshihara et al., 2005). Regulation of vesicle fusion and neurotransmitter release on both sides of the synapse by the Syt 1 and Syt 4 pair may form a fundamental evolutionarily conserved mechanism of synaptic function. In such a model, postsynaptic activity triggers vesicle fusion by Syt 4, releasing a retrograde signal back to the presynaptic neuron, which triggers signaling pathways that lead to enhanced synaptic growth. Such a system fulfills Hebbs model of neuronal memory encoding which theorizes the enhancement of productive synapses versus unproductive synapses. It might also begin to explain the thus far poorly understood release mechanisms of known retrograde signals such as BMP, neurotrophin and others (McCabe et al., 2003; McCabe et al., 2004; Zweifel et al., 2005).

We have presented data here that lends significant support to the proposed function of Syt 4. First, using YFP and CFP tagged proteins we have demonstrated that the postsynaptic localization of Syt 4 is not confined to the *Drosophila* NMJ, but is also true of Syt 4 localization in the motor neurons of the central nervous system (Fig 2.1). Using the pH-sensitive-GFP tag, pHluorin, attached to the N-terminal of Syt 4 we have also demonstrated that the Syt 4 vesicles fuse in the postsynaptic connections of the motor neurons (Fig 2.1). This data shows the postsyaptic localization of fusion competent Syt 4 vesicles is conserved across different classifications of synapses and has the potential to be a universal system.

Activity-dependent up-regulation of Syt 4 levels has previously been described in mammals and zebra finch (Vician et al., 1995; Poopatanapong et al., 2006). This suggests that activity dependent regulation of Syt 4 levels may be another characteristic of a conserved Syt 4 system. To investigate the evolutionary conservation of Syt 4 activity-

dependent regulation in *Drosophila* we used RT-PCR, western blot and immunohistochemistry to characterize Syt 4 protein and mRNA levels (Fig 2.2). Our results report bi-directional regulation of Syt 4 protein levels in neuronal excitability mutants directly correlated to the level of neuronal activity. This confirms the conservation of Syt 4 regulation by activity in *Drosophila*.

We next sought to characterize the effect of changes in Syt 4 levels to the synapse. We have reported here that Syt 4 levels appear to regulate synaptic structure in a bidirectional manner, with *syt* 4^{BA1} null mutants exhibiting only 74% the number of boutons as wildtype, and postsynaptic *UAS-syt 4* over-expression stimulating a 50% increase in bouton number (Fig 2.3). Active zones were correlatively regulated, with no change to the active-zone to bouton number, and the electrophysology of the 3rd instar synapse was unchanged compared to wildtype (Fig 2.3). This suggest that Syt 4, and the putative retrograde signal released by it, are involved in regulation of the structural plasticity pathway. We went on to demonstrate that Syt 4 is necessary for the previously characterized structural plasticity seen in temperature-dependent plasticity and the neuronal excitability mutants (Fig 2.4). Furthermore, neuronal dampening by the *para*^{ts1} mutant, previously shown to disrupt known forms of activity dependent plasticity, also disrupts Syt 4 enhancement of synaptic growth (Fig 2.4).

Finally, we addressed the conflicting evidence about the Ca^{2+} sensing abilities of Syt 4. Using an *in vivo* postsynaptic Syt 4 over-expression assay, we tested the functionality of various Syt 4 Ca^{2+} sensing mutants. These experiments revealed that the Ca^{2+} sensing sites of both the C2A and C2B domains are necessary for full functionality of Syt 4 (Fig 3.1). This strongly argues that Syt 4 is in fact a functional Ca^{2+} sensor.

Furthermore, the assay showed that S284 of the C2A domain, previously thought to function only in inactivatation of C2A Ca^{2+} sensing, is essential for the protein's function (Fig 3.1). Syt 4 protein with a phosphomimetic aspartic acid substitution made at this site functions as well as wildtype Syt 4 (Fig 3.1). The exact function of S284 remains unclear. However, it raises the possibility that Syt 4 Ca^{2+} sensing is specifically regulated by phosphorylation of this serine. This would provide two layers of regulation to Syt 4: regulation of protein levels and activation of the protein through phosphorylation of S284.

4.2 Future directions

While the above studies have lent great support to the theorized role of Syt 4 in synaptic plasticity, a number of intriguing questions still remain about the function of the protein and how it fits into the pathways of activity-dependent synaptic plasticity.

4.2.1 Syt 4 Localization

The postsynaptic localization of Syt 4 has thus far only been demonstrated using relatively low resolution immunostaining and fluorescence imaging (Adolfsen et al., 2004; Yoshihara et al., 2005). Using a high quality antibody, immuno-EM imaging of Syt 4 vesicles at the postsynaptic density would provide the first direct evidence that Syt 4 resides on a docked vesicle population at the postsynaptic density. As with the immunostaining experiments (Yoshihara et al., 2005), *shi^ts1^* mutants could be used to block endocytosis and visualize a depletion of postsynaptic vesicles correlating to Syt 4 localization on the plasma membrane.

4.2.2 Identification of Vesicular Contents

One of the most intriguing questions raised by our studies and the model of Syt 4 as a regulator of a retrograde signaling system is the identity of the retrograde signal. Two approaches could be taken to identify the Syt 4 signal: an unbiased biochemical approach and a targeted genetic approach.

Biochemically, a highly specific Syt 4 antibody can be used to immunopurify Syt 4 vesicles from adult *Drosophila* nervous system. Once immunopurified, mass spectrometry analysis of purified components, similar to (Takamori et al., 2006), could allow not only identification of the vesicular contents (if they are proteins, as opposed to small neurotransmitters), but also other proteins resident on Syt 4 vesicles. This information would allow us to understand what signaling pathways Syt 4 triggers presynaptically, and what SNARE and other regulatory proteins are involved in postsynaptic vesicle fusion.

As mentioned above, a number of studies at the *Drosophila* NMJ have already identified proteins, and even retrograde signals, involved in regulation of neuronal plasticity. For instance, we already know that postsynaptic regulation of the intergrin FasII, and postsynaptic release of the BMP homolog, gbb, play a role in regulation of neuronal plasticity. This opens the door to testing for genetic interactions between these messages and Syt 4. FasII is unlikely to be the signal regulated by Syt 4 release, as experiential strengthening leads to an increase in Syt 4 levels (Fig. 2.2), but a reduction in FasII levels (Schuster et al., 1996). We have begun studies in the lab over-expressing Syt 4 in the genetic background of the presynaptic gbb receptor (*wit*) mutant to determine if

retrograde gbb signaling in necessary for Syt 4 enhancement of synaptic plasticity. Similar experiments may be planned as additional information about postsynaptic messages becomes available, such as identification of a *Drosophila* neurotrophin homolog. Overall, this approach is limited in that it requires analysis of known retrograde signals.

4.2.3 Further Analysis of the Plasticity Pathway

Currently, the only other known component of the Syt 4 pathway is PKA, which appears to function downstream of Syt 4 signaling, presumably on the presynaptic side of the synapse (Yoshihara et al., 2005). Given the richness of information available on NMJ signaling pathways regulating NMJ plasticity, the opportunity exists to identify pathways upstream and downstream of Syt 4-regulated signaling

Upstream of Syt 4, the question remains how Syt 4 expression is regulated by activity. 500bp of DNA separate the start of the Syt 4 cDNA to the next gene, and this sequence is likely to be the site of Syt 4 transcriptional regulation. When the 500bp is plugged into transcription factor prediction algorithms, there is no strong homology to known transcription factor binding sequences (data not shown). However, a clear degree of evolutionarily conserved activity-dependent regulation exists (Fig 2.2). As Syt 4 was originally identified as an immediate early gene in mammals (Vician et al., 1995), CREB is a possible regulator of Syt 4 transcription. The ability of CREB to bind the 500bp 5' region can be assayed through DNase foot printing.

Downstream of Syt 4 vesicle release, additional synaptic signaling pathways can be tested for genetic interaction with Syt 4. For instance, if gbb is the retrograde signal

released by the Syt 4 vesicles, then presynaptic pMad levels should be reduced in the presynaptic neurons of Syt 4 mutants (Ch 1.3.3). This can be assayed through immunohistochemistry using the published pMad antibody (McCabe et al., 2003; Marques et al., 2003).

There is also the question of whether Syt 4 synaptic vesicle release is involved in short-term homeostasis mechanisms at the synapse. One form of rapid synaptic homeostasis to be characterized at the *Drosophila* NMJ is response to Philanthotoxin (PhTx) (Frank et al., 2006; Goold and Davis, 2007). PhTx is a glutamate receptor antagonist, and application of sub-saturation concentrations of PhTx to the NMJ causes a decrease in both miniature-evoked postsynaptic potentials (mEPSP) and evoked postsynaptic potentials (EPSP) (Frank et al., 2006). Over the course of 10 minutes, EPSPs gradually increase in amplitude (Frank et al., 2006). It has been reported that Wit and Gbb are necessary for this form of synaptic plasticity, but in an intracellular rather than retrograde signaling capacity (Goold and Davis, 2007). A similar exploration of PhTx homeostasis in *syt 4 -/-* mutants could be undertaken. However, an important caveat to these studies is that PhTx has also been shown to alter cation movement across membranes, and any effect this may have on electrophysiology experiments has not been fully explored (Huang et al., 1999).

4.2.4 Behavioral Studies of *syt 4^{BA1}* Mutants

While our studies have focused largely on the NMJ of the fly, we have demonstrated that Syt 4 is also expressed postsynaptically in the CNS. Mutations to mammalian Syt 4 result in subtle, but testable, changes in hippocampal-related behavior

(Ferguson et al., 2000; Ferguson et al., 2004a; Ferguson et al., 2004b). One of the strengths of the *Drosophila* system, as mentioned in Chapter 1, is the ability to move between molecular, synaptic, and behavior studies. What we know to date about *syt 4-/-* behavioral defects in flies is limited. Saraswati, Littleton and colleagues have demonstrated that there is no crawling deficiency in the Syt 4 larvae, and the Syt 4 flies live to adulthood with no obvious behavior or motor deficiencies (Saraswati et al., 2007). Still open for analysis are detailed studies of olfactory and other memory functions, as well as complex behaviors such as mating and aggression.

4.2.5 Further Structure/Function Analysis

Above we have described a number of Ca^{2+} sensing mutants in the C2A and C2B domains of Syt 4. Thus far, these mutants have only been tested in the Syt 4 NMJ overgrowth assay. The other robust assay for Syt 4 function is the embryonic HMFR phenotype described in Yoshihara et al., 2005. If the mutant constructs fail to rescue the *syt 4 -/-* phenotype in the HMFR assay this would solidify the connection between previous results and findings reported here.

Finally, the functional studies of S284 described above have suggested that post-translational modifications may play an important role in Syt 4 function. Purification of Syt 4 from *Drosophila* heads by IP, followed by mass spectrometry analysis of the Syt 4 protein will allow us to determine what post-translational modifications Syt 4 undergoes. This information may be critical in explaining the discrepancy between *in vitro* biochemical studies conducted on transgenic purified protein and *in vivo* studies describing different Ca^{2+} sensing properties of Syt 4. Such mass spectrometry studies

may not only reveal phosphorylation, but also glycosylation and other modifications that are essential to Syt 4 function.

4.3 Conclusion

The results reported here have strengthened our understanding of Syt 4's function and its role in the regulation of synaptic plasticity. They have demonstrated the *in vivo* dependence of C2A and C2B Ca^{2+}-binding for Syt 4 function, as well as the surprisingly essential role of S284. They have also placed postsynaptic Syt 4 vesicle fusion in known synaptic structural plasticity pathways, suggesting a release mechanism for synaptic retrograde signals. However, a number of questions remain about post-translational modifications to Syt 4, the signal(s) released by Syt 4, and the up- and downstream components of the Syt 4 regulated plasticity pathways. These questions will form the basis of future research for years to come, and will ultimately lead to a better understanding of how bi-directional communication at the synapse contributes to neurobiology on a system-wide level.

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Appendix A: Background Effects on the *syt 4* ^{BA1} Phenotype

Cynthia F. Barber and J. Troy Littleton

A-1 The *syt* 4^{BA1} Null Phenotype

In the above sections we have discussed how Syt 4 levels affect the structural phenotype of the synapse. It is known in *Drosophila,* that due to the number of pathways regulating synaptic homeostasis, genetic background can have a large influence on NMJ structural phenotypes. This becomes especially important in characterization of modest, but statistically significant, phenotypes such as that of *syt* 4^{BA1} mutants.

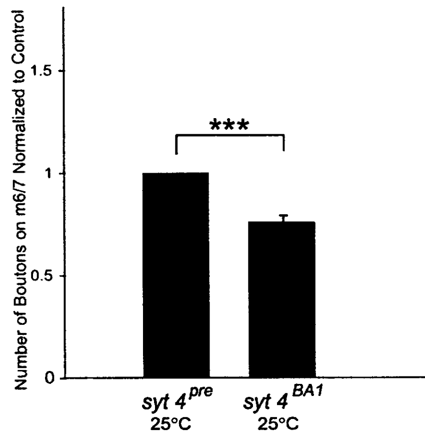
The *syt* 4^{BA1} putative null mutant was obtained through a p-element imprecise-excision screen (Adolfsen et al., 2002). The *syt* 4^{BA1} allele contains an approximately 1 Kb deletion that eliminates the first two exons of *syt 4,* including the ATG start site. As a byproduct of the screen, genetically identical control lines were produced in which the same p-element was precisely excised from the genome (*syt* 4^{pre} allele). The NMJ phenotype for *syt* 4^{BA1} mutant larvae was quantified by α-complexin (α-cpx) immunocytochemistry at muscle 6/7 of segment A3 in wandering 3rd instar larvae, and compared to the precise excision line. *syt* 4^{BA1} mutants exhibited a 24% decrease in bouton number (*syt* 4^{pre} n=38; *syt* 4^{BA1} n=34; student's T-test $p<0.001$) compared to the precise excision control (Fig. 5.1).

A-2 *syt* 4^{BA1} Phenotype in Different Genetic Backgrounds

In a series of experiments, the *syt* 4^{BA1} allele was moved into different genetic backgrounds that caused a change in the severity of the phenotype. In the first case, *syt* 4^{BA1} and *syt* 4^{pre} alleles were moved into the sei^{ts1} background (Fig 5.2). Raised at 25°C, there is no statistical difference in the absolute bouton count (Fig 5.1) (normalized to sei^{ts1};*syt* 4^{pre}: sei^{ts1};*syt* 4^{pre} 25°C: 1+/-0.07, n=14; sei^{ts1};*syt* 4^{BA1} 25°C: 0.95+/-0.02, n =

Figure A-1

A



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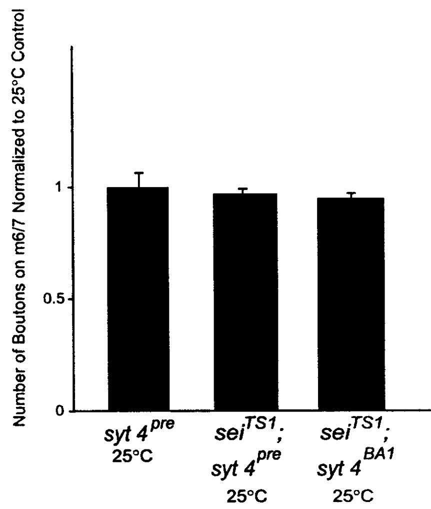
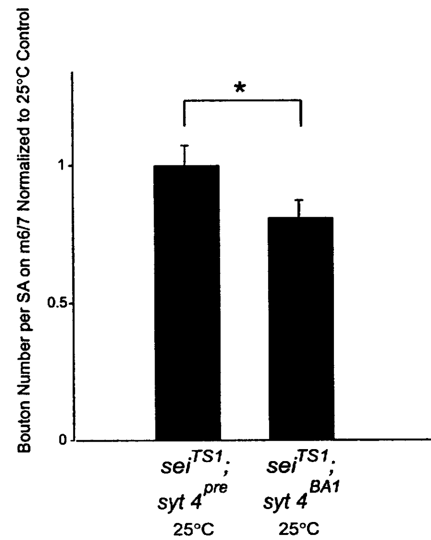




C

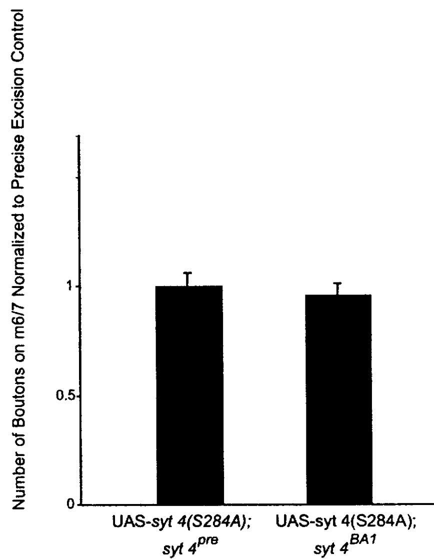


Figure A-1: *syt* 4^{BA1} phenotype in different genetic backgrounds. **A,** Quantification of synaptic bouton number at muscle 6/7 in segment A3 of wandering 3rd instar larvae by α-cpx immunocytochemistry. *syt* 4^{BA1} mutants display 24% fewer boutons than *syt* 4^{pre} controls (normalized to *syt* 4^{pre}: *syt* 4^{pre}: 1+/-0.04, *n*=38; *syt* 4^{BA1}: 0.76+/-0.03, *n*=34; student's T-test p<0.001). **B,** Quantification of bouton number at muscle 6/7 of segment A3 in 3rd instar wandering larvae of the indicated genotypes reared at 25°C. When bouton number at muscle 6/7 is counted, there is no statistically significant difference in bouton number at 25°C between sei^{ts1};*syt* 4^{pre} and sei^{ts1};*syt* 4^{BA1} (normalized to sei^{ts1};*syt* 4^{pre}: sei^{ts1};*syt* 4^{pre} 25°C: 1+/-0.07, *n*=14; sei^{ts1};*syt* 4^{BA1} 25°C: 0.95+/-0.02, *n*=15). However, when the bouton numbers were also normalized to the combined surface area of muscles 6/7 a statistically significant 18% reduction in bouton number is observed in sei^{ts1};*syt* 4^{BA1} compared to sei^{ts1};*syt* 4^{pre} (normalized to sei^{ts1};*syt* 4^{pre}: sei^{ts1};*syt* 4^{pre} 25°C: 1+/-0.07, *n*=14; sei^{ts1};*syt* 4^{BA1} 25°C: 0.82+/-0.06, *n*=15; student T-test p=0.04). **C,** Quantification of synaptic bouton number at muscle 6/7 in segment A3 of wandering 3rd instar larvae by α-cpx immunocytochemistry. When the genetic construct *UAS-syt 4 (S284A)* was moved into a *syt* 4^{BA1} and *syt* 4^{pre} background in preparation for rescue experiments, no significant difference was seen between the bouton number in the two lines (normalized to *UAS-syt 4 (S284A);syt* 4^{pre}: *UAS-syt 4 (S284A);syt* 4^{pre} 1+/-0.06, *n*=12; *UAS-syt 4 (S284A);syt* 4^{BA1} 0.95+/-0.05, n=13; Student T-test p=0.57).

Figure A-2

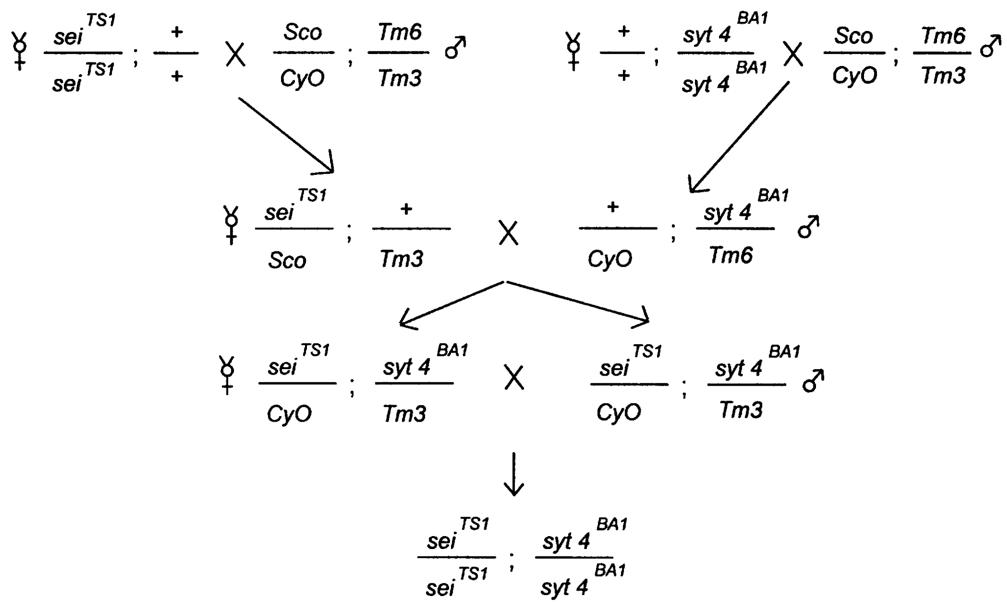


Figure A-2: Genetic crosses involved in constructing *sei*^{ts1}*;syt 4*^{BA1} homozygous lines. The same crosses were used to construct *sei*^{ts1}*;syt 4*^{pre} , with *syt 4*^{pre} in place of *syt 4*^{BA1}. The crosses were also used to obtain the *UAS-syt 4 (S284A);syt 4*^{BA1} and *UAS-syt 4 (S284A);syt 4*^{pre}, with *UAS-syt 4 (S284A)* in the place of *sei*^{ts1}.

15). However, when the number of boutons was normalized to the surface area of the muscle, a statistically significant 18% reduction in bouton number/surface area was seen in the *sei^{ts1};syt 4^{BA1}* lines compared to *sei^{ts1};syt 4^{pre}* (normalized to *sei^{ts1};syt 4^{pre}*: *sei^{ts1};syt 4^{pre}* 25°C: 1+/-0.07, *n*=14; *sei^{ts1};syt 4^{BA1}* 25°C: 0.82+/-0.06, *n* = 15; student T-test p=0.04). Bouton number is often normalized to muscle surface area when mutations cause a developmental phenotype that significantly changes muscle size and makes the absolute bouton numbers incomparable.

In the case of *sei^{ts1};syt 4^{pre}* and *sei^{ts1};syt 4^{BA1}*, the difference in ratio is due to a statistically significant difference in the surface area of the muscles in the two genotypes (*sei^{ts1};syt 4^{pre}* 25°C: 48,000+/-1,000 μm^2; *sei^{ts1};syt 4^{BA1}* 25°C: 59,000+/-2,000 μm^2; student T-test p=0.0002). However, there is no statistically significant difference in surface area observed in *sei^{ts1};syt 4^{pre}* 31°C (60,000+/-2,000 μm^2) and *sei^{ts1};syt 4^{BA1}* 31°C (57,000+/-2,000 μm^2). There is also no statistically significant difference seen in *syt 4^{BA1}* 18°C (77,000+/-2,000 μm^2), *syt 4^{BA1}* 31°C (72,000+/-3,000 μm^2), *syt 4^{pre}* 18°C (80,000+/-8,000 μm^2), and *syt 4^{pre}* 31°C (70,000+/-5,000 μm^2). The only significant difference seems to be an overall lower surface area in the *sei^{ts1}* background than the excision screen background. Thus, it appears that that the observed difference between *sei^{ts1};syt 4^{pre}* 25°C and *sei^{ts1};syt 4^{BA1}* 25°C bouton number to surface area ratio is likely an unreproducible anomaly. This leads back to the original data showing that in the *sei^{ts1}* background the *syt 4^{BA1}* phenotype observed in the parental lines is no longer statistically significant.

In a parallel experiment, the *UAS-syt 4 (S284A)* transgene was moved into the *syt 4^{BA1}* and *syt 4^{pre}* backgrounds in a variation of the same crosses used for *sei^{ts1}* (Fig 5.2).

This was done in preparation for a *syt 4 -/-* rescue experiment. However, once again, the series of crosses appeared to alter the genetic background sufficiently to eliminate the statistically significant difference in bouton number for the two pheonotypes (Fig 5.1) (normalized to *UAS-syt 4 (S284A);syt* 4^{pre}: *UAS-syt 4 (S284A);syt* 4^{pre} 1+/-0.06, *n*=12; *UAS-syt 4 (S284A);syt* 4^{BA1} 0.95+/-0.05, n=13; Student T-test p=0.57). In this case the muscle surface area was not measured, as there was no concern about a developmental phenotype.

A-3 Conclusions

In two examples involving three generations of crosses, the *syt* 4^{BA1} phenotype, although observable in the parental lines, could not be observed in the final offspring. Two possible theories can account for this outcome. Either, the *syt* 4^{BA1} phenotype is particularly sensitive to genetic background, or is a result of some unexpected difference between the *syt* 4^{BA1} and *syt* $4^{precise\ excision}$ lines that is unrelated to the *syt* 4^{BA1} allele. Experiments are currently underway to determine if the phenotype is present in *Drosophila* lines carrying *syt* 4^{BA1} over an appropriate deficiency. If so, it would suggest that the many homeostatic mechanisms present at the synapse are simply sufficient to mask the *syt* 4^{BA1} structural phenotype in some backgrounds.

A-4 Materials and Methods

A-4.1 Generation of *Drosophila* Stocks

Drosophila were cultured on standard media at the indicated temperatures. *UAS-syt 4* mutant lines were constructed in the *syt 4* cDNA by site-directed mutagenesis of *pUAST-*

syt 4, and injected into w^{1118} flies at the Duke Model Systems Transgenic Facility. All constructs were verified by DNA sequencing prior to injection.

A-4.2 Immunostaining

Immunostaining of wandering 3rd instar larvae reared at 25°C, or the indicated temperature, was performed as previously described (Yoshihara and Littleton, 2002; Rieckhof et al., 2003). Primary antibody dilutions used were: α-Cpx (Huntwork and Littleton, 2007) for bouton counting 1:1000, α-syt 4 (Adolfsen et al., 2004) 1:250. Cy2-conjugated goat α-rabbit and Cy5-conjugated goat α-mouse (Jackson ImmunoResearch Laboratories) were used at 1:250. Samples were mounted in 70% glycerol. Images were taken using confocal microscopy on an Axoplan 2 microscope (Carl Zeiss MicroImaging, Inc.) using PASCAL software (Carl Zeiss MicroImaging, Inc.). Bouton were quantified at muscle 6/7 of segment A3. All error measurements are standard error of the mean (SEM). Statistical significance was calculated using student's T tests, with * denoting $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

A-5 References

Adolfsen B, Saraswati S, Yoshihara M, Littleton JT (2004) Synaptotagmins are trafficked to distinct subcellular domains including the postsynaptic compartment. J Cell Biol 166:249-260.

Appendix B: Genetic Interactions with the Syt 4 Pathway

Cynthia F. Barber and J. Troy Littleton

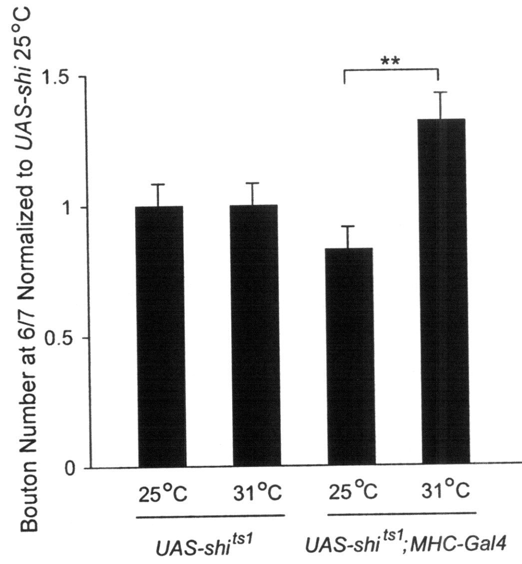
B-1 Shibire and SesB

Syt 4 resides on a postsynaptic vesicle population which cycles between the cytoplasm surrounding the postsynaptic density and the plasma membrane (Adolfsen et al., 2004; Yoshihara et al., 2005). Arrest of the Syt 4-vesicle cycle was previously visualized at the *Drosophila* NMJ through postsynaptic expression of the dominant negative dynamin allele, *UAS-shi^{ts1}* (Yoshihara et al., 2005). When *UAS-shi^{ts1}* postsynaptic expression is driven by *Mhc-GAL4* and the larvae are shifted to a non-permissive temperature (37°C), endocytosis is blocked at the postsynaptic membrane. This results in arrest of the vesicle cycle, and Syt 4 immunoreactivity translocating from postsynaptic puncta to the plasma membrane (Yoshihara et al., 2005).

Overexpression of Syt 4 in the postsynaptic muscle leads to an increase in bouton number (Fig 2.3; Yoshihara et al., 2005), while *syt 4^{BA1}* larvae show a modest decrease in bouton number (Fig 2.3). We decided to examine if chronic impairment of the postsynaptic vesicle cycle, through *UAS-shi^{ts1}* expression, would result in impairment of structural plasticity due to a reduction in Syt 4 vesicle fusion. A multiple insert *UAS-shi^{ts1}* line was crossed to *Mhc-GAL4*, and the larvae were raised at 31°C, not high enough to cause complete blockage of postsynaptic vesicle endocytosis but enough to theoretically reduce endocytosis efficiency. Wildtype larvae raised at 31°C typically exhibit a higher number of boutons than those raised at 25°C due to temperature dependent plasticity (Fig 2.4; Sigrist et al., 2003). Surprisingly, the *UAS-shi^{ts1}* control line without driver lacked this plasticity, and showed no difference in bouton number under the two conditions (Fig 6.1). Meanwhile, the line with postsynaptic overexpression

Figure B-1

A



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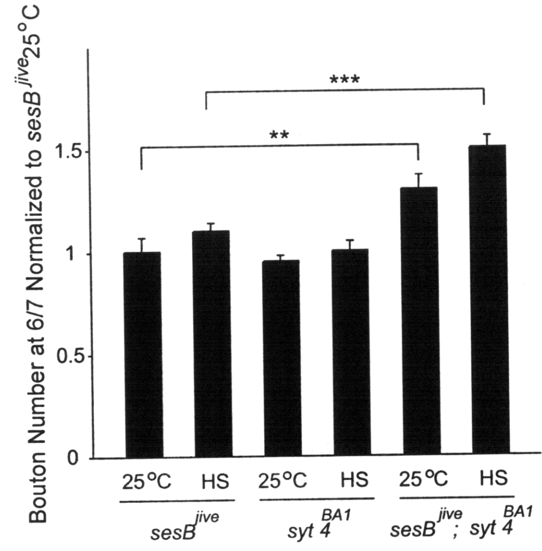

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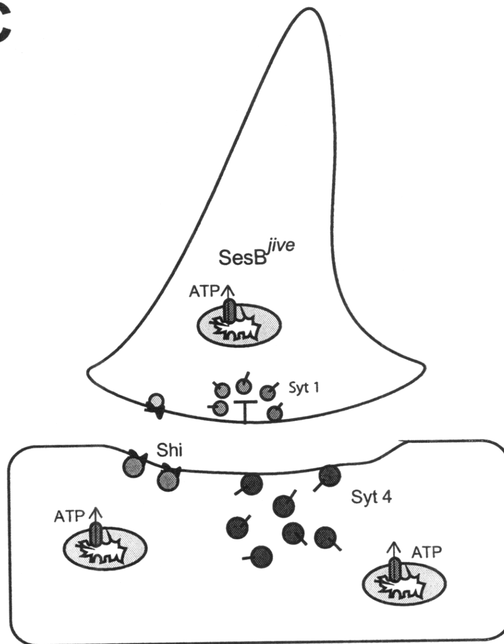


Figure B-1: Genetic interactions with the Syt 4 pathway. **A,** Quantification of synaptic bouton number at muscle 6/7 in segment A3 of wandering 3rd instar larvae by α-cpx immunocytochemistry. With no GAL4 driver, the *UAS-shi^{ts1}* line showed no change in bouton number when raised at 25°C and 31°C (normalized to *UAS-shi^{ts1}* 25°C: *UAS-shi^{ts1}* 25°C 1.0+/-0.08, *n*=14; *UAS-shi^{ts1}* 31°C 1.0+/-0.08, *n*=10). However, when the dominant negative allele was driven in the postsynaptic compartment by *Mhc-GAL4* a significant difference in bouton number at 25°C and 31°C was observed (normalized to *UAS-shi^{ts1}* 25°C: *UAS-shi^{ts1};Mhc-GAL4/+* 25°C 0.83+/-0.08, *n*=10; *UAS-shi^{ts1};Mhc-GAL4 /+* 31°C 1.32+/-0.1, *n*=8 student T-test $p<0.002$). **B,** Quantification of synaptic bouton number at muscle 6/7 in segment A3 of wandering 3rd instar larvae by α-cpx immunocytochemistry. There was no significant difference in bouton number between *sesB^{jive}* and *syt 4^{BA1}* lines raised solely at 25°C and those receiving a twice daily heat shock (HS) at 37°C (Normalized to *sesB^{jive}* 25°C: *sesB^{jive}* 25°C 1.0+/-0.08, *n*=13; *sesB^{jive}* HS 1.1+/-0.04, *n*=17; *syt 4^{BA1}* 25°C 0.95+/-0.03, *n*=80; *syt 4^{BA1}* HS 1.0+/-0.05, *n*=16). However, when the *sesB^{jive}* mutation was placed in the *syt 4^{BA1}* there was a significant difference between *sesB^{jive}* and *sesB^{jive};syt 4^{BA1}* under each set of conditions (Normalized to *sesB^{jive}* 25°C: *sesB^{jive}* 25°C 1.0+/-0.08, *n*=13; *sesB^{jive};syt 4^{BA1}* 25°C 1.3+/-0.07, *n*=20 Student T-test $p<0.006$; *sesB^{jive}* HS 1.1+/-0.04, *n*=17; *sesB^{jive};syt 4^{BA1}* HS 1.5+/-0.06, *n*=22 Student T-test $p<0.0001$). No significant difference was seen between *sesB^{jive};syt 4^{BA1}* under the two conditions. Results were similar when normalized to muscle 6/7 combined surface area. **D,** A schematic diagram depicting the sites of action for the mutant proteins. SesB^{jive} is a mutation of the mitochondrial ATP/ADP translocase, Shi^{ts1} is a temperature sensitive dynamin mutation known to impair endocytosis in a dominant negative manner.

of *UAS-shi*^{ts1} exhibited a large increase in the number of boutons under the 31°C condition.

The lack of temperature-dependent plasticity in the *UAS-shi*^{ts1} line may be due to leakiness in regulation of the multiple inserts, and an overall dampening of neuronal activity in the nervous system. Shi participates in endocytosis on both sides of the synapse (Fig 6.1), and pan-neuronal dampening of the vesicle cycle could easily disrupt normal plasticity. Similar impairment of temperature-dependent plasticity occurs in *para*^{ts1} and *syt 4*^{BA1} mutants (Fig 2.4, Sigrist et al., 2003). It is more difficult to understand the enhancement of synaptic plasticity that occurs with postsynaptic overexpression of *UAS-shi*^{ts1} at 31°C. Interestingly, similar results were obtained from studies involving SesB.

sesB^{jive} is a temperature sensitive allele of the mitochondrial ATP/ADP translocase, which causes seizures at non-permissive temperatures (Guan et al., 2005). To assess if Syt 4 was necessary for synaptic overgrowth observed in *sesB*^{jive} mutants subjected to twice daily heat shocks at a non-permisive temperature, *sesB*^{jive} was moved into a *syt 4*^{BA1} background. From the time of egg laying to dissection, larvae were raised at 25°C and subjected to twice daily 1-hour heat shocks at 37°C, control larvae were kept at 25°C. Unlike previous reports, no significant increase in bouton number was observed in *sesB*^{jive} mutants raised with the twice daily heat shocks. Adult *sesB*^{jive} mutants still experienced seizure at 37°C, suggesting the lack of plasticity in the heat shocked larva was probably due to an insufficient heat shock protocol. However, a significant increase in bouton number was observed in *sesB*^{jive};*syt 4*^{BA1} double mutants under both control and HS conditions (Normalized to *sesB*^{jive} 25°C: *sesB*^{jive} 25°C 1.0+/-0.08, n=13; *sesB*^{jive};*syt*

4^{BA1} 25°C 1.3+/-0.07, *n*=20 Student T-test p<0.006; *sesB^{jive}* HS 1.1+/-0.04, *n*=17; *sesB^{jive};syt 4^{BA1}* HS 1.5+/-0.06, *n*=22 Student T-test p<0.0001).

sesB^{jive} is the first allele of *sesB* shown to induce seizures. A previously characterized allele, *sesB^{org}*, was identified as an enhancer of *shi* paralysis phenotype (Rikhy et al., 2003). Shi requires GTPase activity, and reduced GDP is returned to GTP by an associated protein's ATPase activity. It is hypothesized that *sesB* mutants impair Shi function by reducing the local ATP concentration, there by reducing the efficiency of Shi. The mechanism of *sesB^{jive}* seizures remains unclear, however, given the nature of the SesB protein, the *sesB^{jive}* mutation is still likely to reduce local ATP concentration. Microarray data suggest that repeated HS of the *sesB^{jive}* mutants leads to activation of the ERK pathway, which can in turn alter transcription in the cell (Guan et al., 2005).

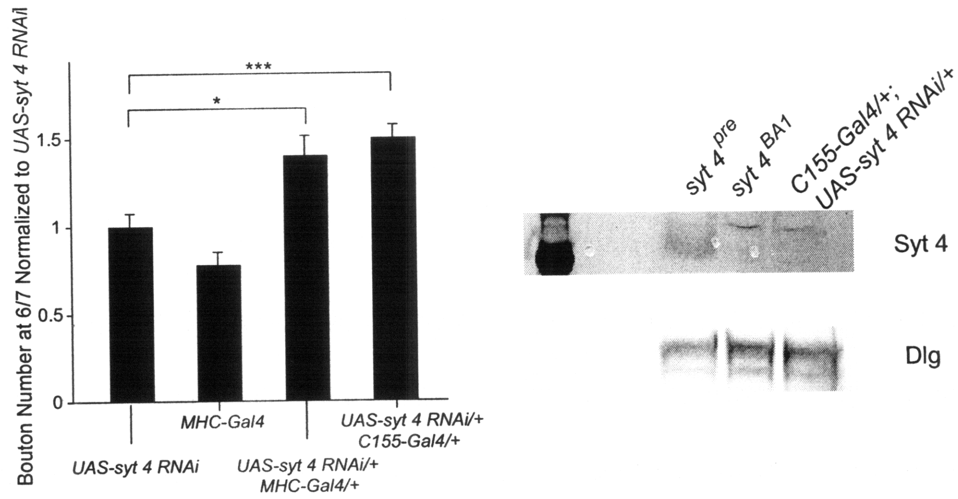
In the original *UAS-shi^{ts1}* and *sesB^{jive}* lines, neuronal function is likely modestly altered on both sides of the synapse. For *UAS-shi^{ts1}* this could manifest due to leakiness of regulation for multiple UAS inserts, and for *sesB^{jive}* due to the presence of mitochondria on both sides of the synapse. Under these conditions, there was no strong alteration to synaptic phenotype, except the loss of temperature dependent plasticity in *UAS-shi^{ts1}*. However, when inhibition was strongly induced postsynaptically, through the *Mhc-GAL4* driver and a temperature shift in the *UAS-shi^{ts1}* line, and the addition of the *syt 4^{BA1}* mutant to the *sesB^{jive}* line, bouton number increased. Given increased Syt 4 levels and postsynaptic vesicle release also lead to increased bouton proliferation, a unifying theory of the data is not immediately clear. It may be that in *syt 4^{BA1}* mutants, as in *syt 1 -/-*, an asynchronous component of release remains and that this component is sufficient to allow a reasonably normal level of growth. However, conditions that

severely limit postsynaptic release, such as those described here lead to bouton overgrowth. Thus, a substantial increase or decrease in postsynaptic release may both increase bouton number. This pattern would be consistent with regulation of a cellular adhesion molecule in which asymmetric levels on the two sides of the synapse lead to changes in growth. Alternatively, significant impairment of postsynaptic release may trigger complex homeostatic mechanisms that lead to bouton overgrowth. In *syt* 4^{BA1} mutants alone, the remaining asynchronous release might be sufficient to avoid activation of these mechanisms. Yet another possibility is that since dynamin (*shi*) function is required at multiple steps of vesicle trafficking, these results may reflect an additional requirement for endosomal/lysosomal trafficking of activated cell surface receptors that may trigger enhanced synaptic growth due to a lack of endocytosis and subsequent down-regulation.

B-2 Syt 4 RNAi

To further characterize the phenotype of larvae lacking Syt 4 activity, we drove postsynaptic expression of *UAS-syt 4 RNAi* with the *Mhc-GAL4* driver (Fig 6.2). Surprisingly, this lead to a statistically significant increase in bouton number (Normalized to *UAS-syt 4 RNAi*: *UAS-syt 4 RNAi* 1.0+/-0.07, *n*=8; *UAS-syt 4 RNAi; Mhc-GAL4/+* 1.4+/-0.11, *n*=12 student T-test $p<0.025$). A significant increase in bouton number was also seen in larvae with C155-Gal4 pan-neuronal driven expression of the construct (Normalized to *UAS-syt 4 RNAi*: *UAS-syt 4 RNAi* 1.0+/-0.07, *n*=8; *C155-Gal4/+; UAS-syt 4 RNAi/+* 1.5+/-0.07, *n*=12 student T-test $p<0.0001$). To confirm the ability of the RNAi line to downregulate Syt 4 expression, Syt 4 levels in the heads of adult

Figure B-2



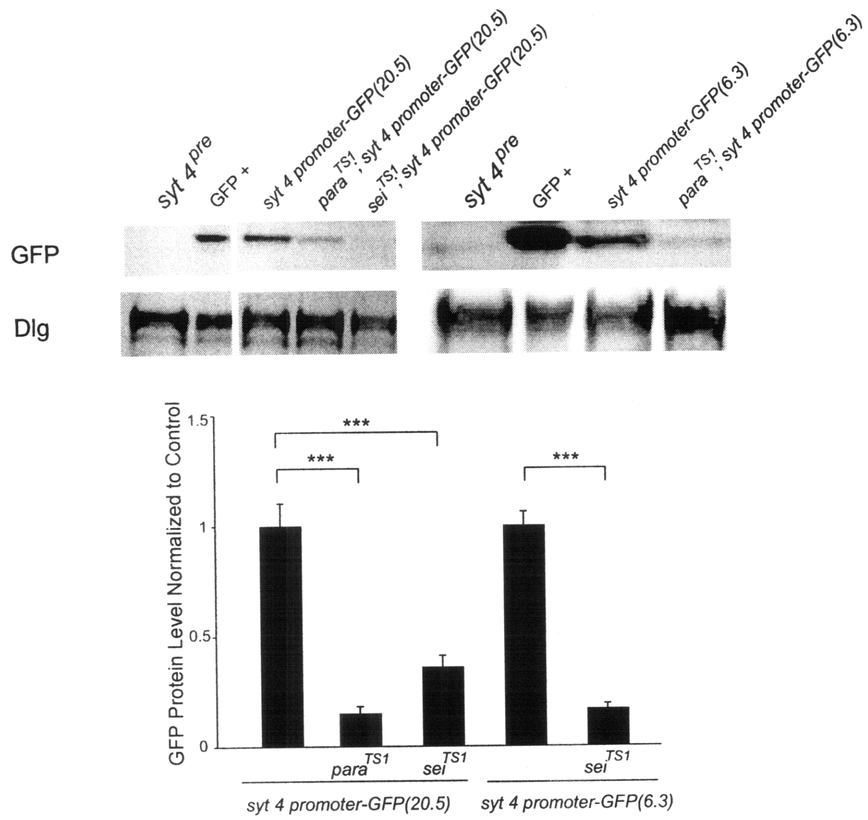


Figure B-2 **A,** Quantification of synaptic bouton number at muscle 6/7 in segment A3 of wandering 3rd instar larvae by α-cpx immunocytochemistry. A statistically significant increase in the bouton number was seen when the *UAS-syt 4 RNAi* construct was driven by either the *C155-Gal4* pan-neuronal (Student T-test $p<0.0001$) or the *Mhc-GAL4* muscle specific (Student T-test $p<0.025$) drivers. (Normalized to *UAS-syt 4 RNAi*: *UAS-syt 4 RNAi* 1.0+/-0.07, *n*=8; *MHC-GAL4* 0.78+/-0.07, *n*=11; *UAS-syt 4 RNAi/+;Mhc-GAL4/+* 1.4+/-0.11 *n*=12; *C155-Gal4/+;UAS-syt 4 RNAi/+* 1.5+/-0.07, *n*=12). Western blot confirms the kockdown of Syt 4 expression in *C155-Gal4/+;UAS-syt 4 RNAi/+* line, with Dlg as a loading control. **B,** Expression of GFP regulated by the putative promoter of *syt 4,* the 500bp upstream of the gene. GFP levels were visualized by western blot analysis with Dlg as a loading control. Two separate insertion lines were used to assay the regulation of GFP expression by activity. In the 20.5 line, GPF expression was significantly reduced when the construct was placed into either the $para^{TS1}$ or the sei^{TS1} backgrounds (normalized to 20.5 control: 20.5 control 1.0+/-0.1, *n*=6; 20.5;$para^{TS1}$ 0.15+/-0.05, *n*=5 student T-test $p=0.0002$; 20.5;sei^{TS1} 0.36+/-0.03, *n*=6 student T-test $p=0.0004$). The sei^{TS1} background results were confirmed with the 6.3 insertion line (normalized to 6.3 control: 6.3 control 1.0+/-0.06, *n*=4; 6.3; sei^{TS1} 0.17+/-0.02, *n*=4 student T-test $p<0.0001$).

C155-Gal4/+; *UAS-syt 4 RNAi/+* flies were assayed by western blot (Fig 6.2). Given the similarity between Syt family members, and the difference between the RNAi phenotype and that of *syt* 4^{BA1}, it seems likely that the RNAi phenotype is subject to off-target effects.

B-3 Transcriptional Regulation of Syt 4 Expression

Syt 4 is subject to conserved activity-dependent regulation, part of which likely occurs on the transcriptional level (Fig 2.3; Poopatanapong et al., 2006; Vician et al., 1995). In *Drosophila,* transcriptional regulation of the *syt 4* gene is likely to involve the 500bp between the upstream gene *gld* and the start of the *syt 4* gene. To analyze the role of this sequence in *syt 4* regulation, the region was cloned, linked to the *GFP* gene, and reinserted into the *Drosophila* genome. Thus, GFP expression was put under control of the 500bp region. The constructs were moved into $para^{ts1}$ and sei^{ts1} backgrounds, whose effects on Syt 4 expression have already been demonstrated (Fig 2.3).

GFP levels were significantly down-regulated in the $para^{ts1}$ flies to 15% of that in the wildtype background (student T-test p=0.0002), this is similar to the regulation of Syt 4 levels (Fig 6.2, Fig 2.3). However, GFP levels in two different insertion lines were also significantly downregulated in the sei^{ts1} background (student T-test p=0.0004 and p<0.0001). This is the opposite of what is seen with Syt 4 in the sei^{ts1} background (Fig 2.3). Together these results demonstrate that the 500bp are not sufficent to acount for Syt 4 activity-dependent regulation.

B-4 Conclusion

While the Shi and SesB data suggest that severe impairment of postsynaptic vesicle trafficking may induce bouton growth, it is likely that complex homeostatic mechanisms are triggered by the changes in postsynaptic function. Similarly, RNAi downregulation of Syt 4 presents the opposite phenotype of *syt* 4^{BA1}, likely due to off target effects altering pan-neuronal function. Finally, although Syt 4 mRNA and protein levels are coordinately regulated (Fig 2.3), the 500bp upstream of Syt 4 is not sufficient to recreate the bidirectional regulation. These data suggest some of the complexities that remain in understanding the Syt 4 pathway and its many interactions.

B-5 Materials and Methods

B-5.1 Generation of *Drosophila* Stocks

Drosophila were cultured on standard media at the indicated temperatures. Generation of the *syt 4* null mutant, *syt* 4^{BA1}, and precise excision control was previously described (Adolfsen et al., 2004). Identification of *sesB*^{jive} was previously described (Guan et al., 2005). *UAS-syt 4 RNAi* line provided by the Vienna *Drosophila* RNAi Center. *Mhc-GAL4* and *C155-GAL4* were provided by Corey Goodman. GFP fusion lines were constructed by cloning the 500bp region into a GFP plasmid. Constructs were injected into w^{1118} flies at the Duke Model Systems Transgenic Facility. All constructs were verified by DNA sequencing prior to injection.

B-5.2 Immunostaining

Immunostaining of wandering 3rd instar larvae reared at 25°C or 31°C was performed as previously described (Yoshihara and Littleton, 2002; Rieckhof et al., 2003). Primary antibody dilutions used were: α-Cpx (Huntwork and Littleton, 2007) for bouton counting 1:1000. Cy2-conjugated goat α-rabbit and Cy5-conjugated goat α-mouse (Jackson ImmunoResearch Laboratories) were used at 1:250. Samples were mounted in 70% glycerol. Images were taken using confocal microscopy on an Axoplan 2 microscope (Carl Zeiss MicroImaging, Inc.) using PASCAL software (Carl Zeiss MicroImaging, Inc.). Bouton and active zone number were quantified at muscle 6/7 of segment A3. All error measurements are standard error of the mean (SEM). Statistical significance was calculated using student's T tests, with * denoting $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

B-5.3 Western Blot / Quantification of Protein expression

α-Syt 4 antibody (Adolfsen et al., 2004) was used at 1:750; α-Dlg antibody 4F3 (Developmental Studies Hybridoma Bank) was used at 1:2000. Secondary antibodies were used at 1:10,000, including goat α-rabbit IgG Alexa 680 (Invitrogen) and goat α-mouse IR dye 800 (Rockland). Western Blots were visualized on the Li-Cor Odyssey infrared imaging system and protein expression quantified with the accompanying software.

B-6 References

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Appendix C: Expression of Mammalian Syt 4 and Syt 11

Cynthia F. Barber, Shaui Chen and J. Troy Littleton

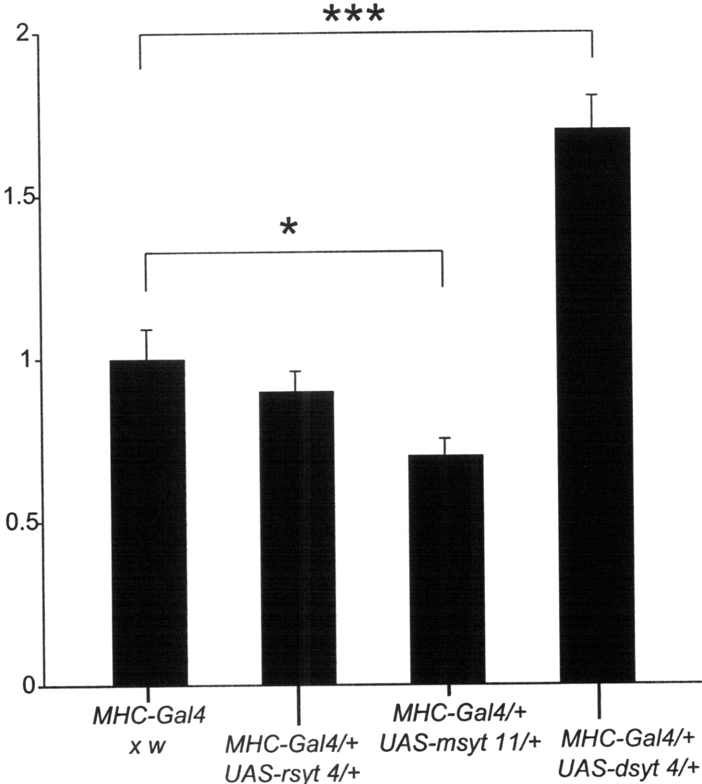
C-1 Expression of Mammalian Syt 4 and Syt 11

In mammals, the Syt 4 protein subfamily contains two members, Syt 4 and Syt 11 (von Poser et al., 1997). Syt 4 is neuronal in both mammals and *Drosophila,* and both contain the conserved Asp to Ser substitution in D3 of the C2A Ca^{2+} binding pocket (Li et al., 1995, Littleton et al., 1999). This along with similar activity-dependent regulation observed in both mammals (Vician et al., 1995) and *Drosophila* (Fig 2.3) suggest that the *Drosophila* and mammalian proteins may share a conserved evolutionary function.

To explore functional similarity between *Drosophila* Syt 4 (dSyt 4) and mammalian Syt 4 and Syt 11, the cDNAs of rat Syt 4 (rSyt 4) and mouse Syt 11 (mSyt 11) were placed under control of the UAS promoter. *UAS-rsyt 4* and *UAS-msyt 11* constructs were injected into white (*w*) eyed flies, with each of the constructs carrying a red eyed (w^+) marker. Thus, lines in which a construct sucsessfully intergrated into the DNA were identifiable by eye color. The degree of eye coloration (light orange, orange, dark orange, or red) also gave some indication of the construct's level of transcription. Several insertion lines were homozygozed for each construct.

A commercial α-mammalian Syt 4 antibody was used to identify a high expressing *UAS-rsyt 4* insertion line by western blot of adult fly heads where expression was under control of the pan-neuronal driver, C155-Gal4 (Fig 7.1). The high expressing line (line 7) also exhibited a dark eye color. Unfortunately, no mammalian Syt 11 antibody was available and lines had to be selected based on eye color alone. Several Syt 11 lines were tested, and a representative line is presented in Fig 7.1.

Figure C-1



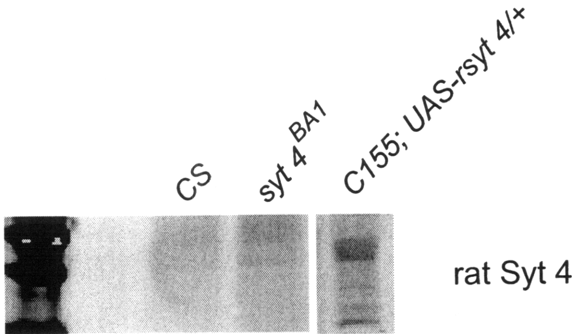


Figure C-1: Postsynaptic expression of rSyt 4 and mSyt 11. **A,** Quantification of synaptic bouton number at muscle 6/7 in segment A3 of wandering 3rd instar larvae by α-cpx immunocytochemistry. *UAS-dsyt 4* postsynaptic expression driven by *Mhc-GAL4* induces a statistically significant increase in bouton number (student T-test $p<0.0001$) compared to control (Normalized to control: *Mhc-GAL4* x *w* 1.0+/-0.09, *n*=19; *Mhc-GAL4/+;UAS-dsyt 4/+* 1.7+/-0.1, *n*=13). However, postsynaptic expression of neither *UAS-rsyt 4* (line 7) or *UAS-msyt 11* (line 19) driven by *Mhc-GAL4* induced bouton proliferation (Normalized to control: *Mhc-GAL4/+;UAS-rsyt 4/+* 0.9+/-0.06, *n*=11; *Mhc-GAL4/+;UAS-msyt 11/+* 0.7+/-0.05, *n*=14). Indeed, *Mhc-GAL4/+;UAS-msyt 11/+* showed a somewhat significant decrease in bouton number (student T-test $p=0.02$). **B,** Expression of rSyt 4 protein in *C155-Gal4*; *UAS-rsyt 4* (line 7) as demonstrated by western blot analysis of adult male fly heads.

When *UAS-rsyt 4* and *UAS-msyt 11* expression were driven in the muscle by *Mhc-GAL4*, none of the tested lines showed a significant increase in bouton number. This is in sharp contrast to the significant increase seen with postsynaptic overexpression of dSyt 4 (Fig 2.3; Fig 7.1). There are several potential explanations for the constructs' failure to promote bouton growth. One possible explanation is that the mammalian isoforms do not share an evolutionarily conserved function with dSyt4. This theory was previously suggested by the results of an *in vitro* lipid binding assay (Dai et al., 2004). However, it is likely that one of many potential problems exist with the constructs. With no antibody available to confirm mSyt 11 expression, and without careful calibration experiments to allow a comparison between *Mhc-GAL4* driven levels of rSyt 4 and dSyt 4, it is entirely possible that the comparable protein levels are simply not there. Other potential problems with moving the transcripts from one system to another include loss of an essential post-translational modification, or mislocalization of the proteins. Conflicts between *in vitro* experimental properties and *in vivo* observations have already demonstrated how important post-translational modifications can be to Syt protein function. Unfortunately, these experiments are, thus far, inconclusive as to the evolutionary conservation of Syt 4 function.

C-2 Materials and Methods

C-2.1 Generation of *Drosophila* Stocks

Drosophila were cultured on standard media at 25°C. *UAS-dsyt 4, UAS-rsyt 4* and *UAS-msyt 4* lines were obtained by cloning of the coding sequence into pUAST followed by injection of the construct into w^{1118} flies at the Duke Model Systems Transgenic Facility.

Mhc-GAL4 and *C155-GAL4* were provided by Corey Goodman. All constructs were verified by DNA sequencing prior to injection.

C-2.2 Immunostaining

Immunostaining of wandering 3rd instar larvae reared at 25°C was performed as previously described (Yoshihara and Littleton, 2002; Rieckhof et al., 2003). Primary antibody dilutions used were: α-Cpx (Huntwork and Littleton, 2007) for bouton counting 1:1000. Cy2-conjugated goat α-rabbit and Cy5-conjugated goat α-mouse (Jackson ImmunoResearch Laboratories) were used at 1:250. Samples were mounted in 70% glycerol. Images were taken using confocal microscopy on an Axoplan 2 microscope (Carl Zeiss MicroImaging, Inc.) using PASCAL software (Carl Zeiss MicroImaging, Inc.). Bouton and active zone number were quantified at muscle 6/7 of segment A3. All error measurements are standard error of the mean (SEM). Statistical significance was calculated using student's T tests, with * denoting $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

C-2.3 Western Blot / Quantification of Protein expression

α-Syt 4 antibody (Adolfsen et al., 2004) was used at 1:750; α-Dlg antibody 4F3 (Developmental Studies Hybridoma Bank) was used at 1:2000. Secondary antibodies were used at 1:10,000, including goat α-rabbit IgG Alexa 680 (Invitrogen) and goat α-mouse IR dye 800 (Rockland). Western Blots were visualized on the Li-Cor Odyssey infrared imaging system and protein expression quantified with the accompanying software.

C-3 References

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