

Postsynaptic regulation of synaptic plasticity by synaptotagmin 4 requires both C2 domains

Cynthia F. Barber,^{1,2} Ramon A. Jorquera,^{1,2} Jan E. Melom,^{1,2} and J. Troy Littleton^{1,2}

¹Department of Biology and ²Department of Brain and Cognitive Sciences, The Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA 02139

Ca²⁺ influx into synaptic compartments during activity is a key mediator of neuronal plasticity. Although the role of presynaptic Ca²⁺ in triggering vesicle fusion through the Ca²⁺ sensor synaptotagmin 1 (Syt 1) is established, molecular mechanisms that underlie responses to postsynaptic Ca²⁺ influx remain unclear. In this study, we demonstrate that fusion-competent Syt 4 vesicles localize postsynaptically at both neuromuscular junctions (NMJs) and central nervous system synapses in *Drosophila melanogaster*. Syt 4 messenger RNA and protein expression are strongly regulated by neuronal activity, whereas altered levels of postsynaptic Syt 4 modify

synaptic growth and presynaptic release properties. Syt 4 is required for known forms of activity-dependent structural plasticity at NMJs. Synaptic proliferation and retrograde signaling mediated by Syt 4 requires functional C2A and C2B Ca²⁺-binding sites, as well as serine 284, an evolutionarily conserved substitution for a key Ca²⁺-binding aspartic acid found in other synaptotagmins. These data suggest that Syt 4 regulates activity-dependent release of postsynaptic retrograde signals that promote synaptic plasticity, similar to the role of Syt 1 as a Ca²⁺ sensor for presynaptic vesicle fusion.

Introduction

Information transfer at neuronal synapses requires release of small, diffusible neurotransmitters after presynaptic vesicle fusion. 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). Retrograde signals can be released constitutively or in an activity-regulated fashion to modulate synaptic development and acute plasticity (Fitzsimonds and Poo, 1998). Ca²⁺-regulated vesicle trafficking also occurs in the postsynaptic compartment, suggesting that retrograde signaling may use vesicular trafficking mechanisms similar to those found presynaptically (Maletic-Savatic and Malinow, 1998; Adolfsen et al., 2004; Kennedy and Ehlers, 2006). One system in which retrograde signaling has been well characterized is at *Drosophila melanogaster* neuromuscular junctions (NMJs), where a strong correlation between neuronal activity levels and synaptic growth has been observed (Budnik et al., 1990; Sigrist et al., 2003; Guan et al., 2005). During the larval stages of development, an ~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 controls both synaptic growth and homeostatic plasticity during larval development (Paradis et al., 2001; Marqués et al., 2002; McCabe et al., 2003; Goold and Davis, 2007). Although retrograde signals are required for plasticity and synaptic growth at *Drosophila* NMJs, the underlying mechanisms mediating retrograde release remain largely unknown.

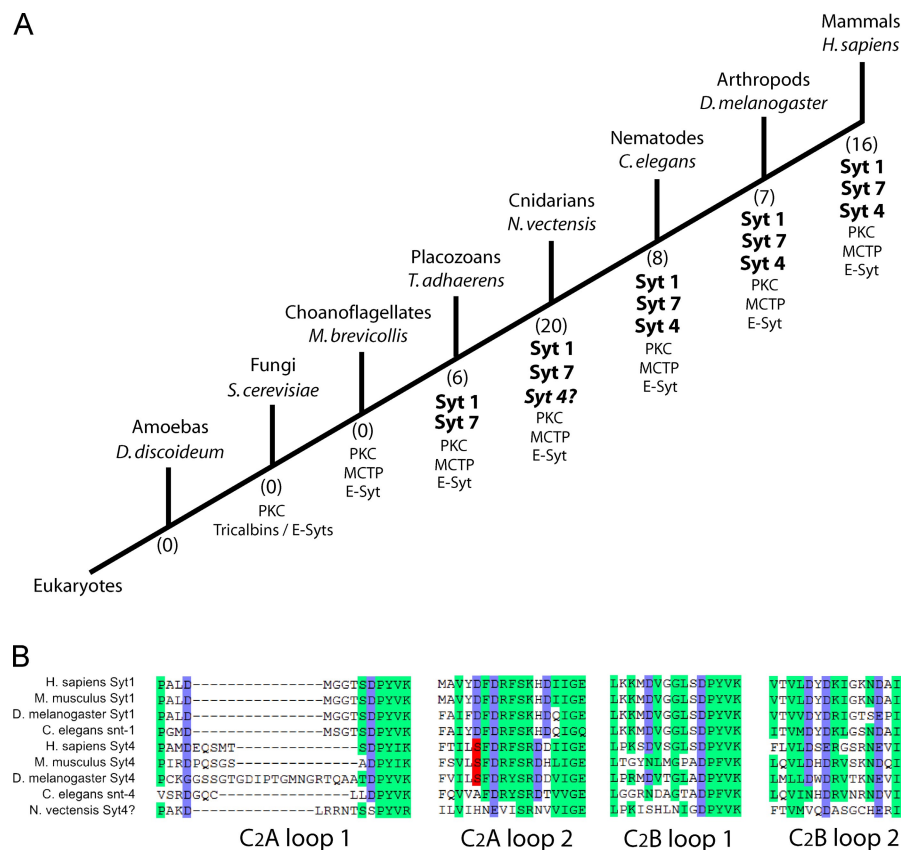
Synaptotagmins are a conserved family of vesicular Ca²⁺ sensors that share a common structure with a single transmembrane domain and two cytoplasmic Ca²⁺-binding C2 domains (Adolfsen and Littleton, 2001). Only two of the seven *Drosophila* synaptotagmins, synaptotagmin 1 (Syt 1) and Syt 4, are present at most if not all synapses (Adolfsen et al., 2004). Unlike the synaptic vesicle-associated Syt 1, Syt 4 is expressed postsynaptically 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). In this study, we show that Syt 4 fusion-competent vesicles localize postsynaptically in *Drosophila* central nervous system (CNS) neurons. Syt 4

Correspondence to J. Troy Littleton: troy@mit.edu

Abbreviations used in this paper: α -Cpx, α -complexin; ANOVA, analysis of variance; Brp, Bruchpilot; CNS, central nervous system; EPSC, excitatory postsynaptic current; mRFP, monomeric RFP; NMJ, neuromuscular junction; VNC, ventral nerve cord.

© 2009 Barber et al. This article is distributed under the terms of an Attribution-Noncommercial-Share Alike-No Mirror Sites license for the first six months after the publication date [see <http://www.jcb.org/misc/terms.shtml>]. After six months it is available under a Creative Commons License [Attribution-Noncommercial-Share Alike 3.0 Unported license, as described at <http://creativecommons.org/licenses/by-nc-sa/3.0/>].

Figure 1. Evolution of the synaptotagmin family. (A) Diagram of the emergence of C2 domain proteins in eukaryotes. The number of synaptotagmin family members in each organism is shown in parentheses. The emergence of the E-Syt and multiple C2 domain and transmembrane region proteins (MCTP) families is noted. (B) Sequence alignment of the C2A and C2B domains of Syt 1 and Syt 4 is shown. Critical aspartic acid residues involved in Ca^{2+} coordination are shown in blue, with similar amino acids shown in green. The substitution of D3 to S in C2A of Syt 4 is highlighted in red.



expression is bidirectionally regulated by neuronal activity and modulates synaptic growth and plasticity. Gain and loss of function experiments demonstrate that Ca^{2+} -binding sites in both C2 domains are required for Syt 4 activity. In addition, Syt 4 modulates activity-dependent structural plasticity at NMJs, including seizure-induced and temperature-dependent enhancements of synaptic growth. These findings suggest a role for Syt 4 in regulating retrograde vesicular trafficking that underlies activity-dependent synaptic growth at *Drosophila* NMJs.

Results

Evolutionary emergence of Syt 1 and Syt 4

Based on studies of the synaptic vesicle isoform Syt 1, synaptotagmins are postulated to mediate the Ca^{2+} dependency of regulated exocytosis (Yoshihara et al., 2003; Chapman, 2008). Given their expression pattern in *Drosophila*, 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, which is similar to the presynaptic role of Syt 1 in synaptic vesicle fusion. To analyze the emergence of the synaptotagmin family in relation to synapse evolution, we took advantage of recent genomic sequencing of some of the early branch points in metazoan evolution, beginning in single cell eukaryotes with the genomes of the amoeba *Dictyostelium discoideum* and the yeast *Saccharomyces cerevisiae* (Fig. 1 A). *D. discoideum* contains several single C2 domain proteins that do not show strong homology to known proteins in other species. In addition to PKC, the *S. cerevisiae*

genome encodes several members of the extended Syt (E-Syt; also termed tricalbins) family, which encode N-terminal transmembrane proteins followed by three or more C2 domains that are predicted to function in membrane trafficking (Creutz et al., 2004; Min et al., 2007). The choanoflagellate genome of *Monoisiga brevicollis*, thought to be the closest living single cell relative to animals (King et al., 2008), lacks synaptotagmins, though it does encode members of the PKC and E-Syt families. The multiple C2 domain and transmembrane region proteins, another evolutionarily conserved family with multiple C2 domain repeats (Shin et al., 2005), first appear in the choanoflagellates.

Given the lack of synaptotagmins at the single cell stage of evolution, we analyzed primitive metazoan branches before the evolutionary emergence of neurons and synapses. *Trichoplax adhaerens* is a placozoan consisting of a flat disc of epithelial-like cells surrounding a layer of multinucleate fiber cells with no nervous system. To our surprise, we identified synaptotagmin homologues in the *T. adhaerens* genome (Srivastava et al., 2008), including isoforms of Syt 1 and 7. Four additional tandem C2 domain proteins were also identified, although they showed weak homology to the bilateria synaptotagmins. Given the role of Syt 7 in plasma membrane repair via Ca^{2+} -dependent fusion of secretory lysosomes (Reddy et al., 2001), it is not surprising that this family member emerged early in evolution of multicellular eukaryotes when cell–cell tension issues arose. However, the finding of a Syt 1 homologue before the evolution of the synapse was surprising. Indeed, we identified homologues of many presynaptic and synaptic vesicle proteins in *T. adhaerens*, suggesting that a cell–cell communication pathway

ancestral to the synapse was present early in multicellular evolution. We did not identify a Syt 4 isoform in the *T. adhaerens* genome. The earliest evolution of a Syt 4-like protein coincides with the emergence of a primitive nervous system, as we identified a putative ancestral Syt 4 protein in the sea anemone *Nematostella vectensis* (Putnam et al., 2007). The characteristic D to S substitution in the Syt 4 C2A D3 residue does not appear until *Drosophila* (Fig. 1 B), suggesting continued evolution of Syt 4 function from its earliest appearance. We hypothesize that synaptotagmins evolved from diversification of function from an early PKC family member, as yeast PKC is implicated in the cell wall integrity signaling pathway required to remodel the cell surface during environmental stress and during growth and morphogenesis (Levin, 2005). Evolution of synaptotagmins from a Ca^{2+} -dependent cell membrane remodeling pathway is an attractive origin for a protein family underlying Ca^{2+} -dependent secretion. In summary, our genomic analysis indicates that Syt 1 and 7 are the founding members of the synaptotagmin family and arose before the origin of neurons, whereas Syt 4 emerged coincident with nervous system evolution.

Syt 4 localizes postsynaptically in the CNS

To determine whether Syt 4 localizes to the postsynaptic compartment in the CNS as well as muscles, we constructed transgenic UAS lines expressing YFP fused to the intravesicular N terminus of Syt 4 or monomeric RFP (mRFP) 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 third instar larvae showed punctate expression at the postsynaptic compartment of NMJ synapses (Fig. 2 A). To analyze Syt 4 targeting in the CNS, we took advantage of the defined polarity of CNS motor neurons in the ventral nerve cord (VNC) of *Drosophila* larvae and the motor neuron-specific GAL4 driver D42 (Fig. 2 B; Parkes et al., 1998). Motor neuron axons terminate at muscle NMJs, whereas their dendrites are restricted to the VNC where they receive synaptic input. Syt 4-YFP driven by D42-GAL4 prominently localized to motor neuron cell bodies and dendrites in the VNC (Fig. 2 A) where synaptic inputs are known to occur (Littleton et al., 1993). In contrast, Syt 4-YFP targeted poorly to presynaptic terminals at NMJs.

To further characterize Syt 4 localization, we generated transgenic animals coexpressing Syt 4-mRFP and Syt 1-GFP with the D42-GAL4 driver, allowing us to compare targeting of a synaptic vesicle protein with Syt 4 in the same neurons. As shown in Fig. 2 B, Syt 1-GFP was not found in dendrites, but robustly targeted to presynaptic boutons, as expected for a synaptic vesicle protein. In contrast, Syt 4-mRFP showed low expression in presynaptic terminals but strong expression in the CNS dendritic neuropil. To determine whether Syt 4 vesicles were 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) whose fluorescence increases when exposed to the extracellular space after fusion (Miesenböck et al., 1998). 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 C), indicating that Syt 4 vesicles only fuse within the dendritic compartment of motor neurons. We did not observe activity-dependent increases in Syt 4-pHluorin signals using either application of high K^+ or electrical stimulation, which is likely the result of the small number of postsynaptic vesicles undergoing fusion. We conclude that fusion-competent Syt 4 vesicles target to postsynaptic compartments in CNS dendrites and muscle NMJs.

Regulation of Syt 4 expression by neuronal activity

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 up-regulated by seizure induction and neuronal depolarization (Vician et al., 1995). To determine whether activity-dependent regulation of *syt 4* is evolutionarily conserved, we tested whether the *Drosophila* homologue is also regulated by neuronal activity. Our first indication that activity levels modulate Syt 4 expression was the observation that Syt 4 immunoreactivity is reduced in *syt 1*^{-/-} larvae (Fig. 3, A and B), which have reduced synaptic activity (Yoshihara and Littleton, 2002). We next analyzed Syt 4 levels in *Drosophila* temperature-sensitive (TS) mutants that enhance or reduce neuronal activity (Ganetzky and Wu, 1986; Littleton et al., 1998). Syt 4 staining at larval NMJs of wild-type, *para*^{ts1}, and *sei*^{ts1} strains demonstrated that Syt 4 protein expression correlates with the level of neuronal activity (Fig. 3, C–H). This regulation was not restricted to NMJs, as we observed similar results in the CNS by Western blot analysis of Syt 4 levels from adult *Drosophila* head extracts (Fig. 3, I and J). Quantification of Syt 4 protein demonstrated a statistically significant fivefold reduction in *para*^{ts1} and *syx*³⁻⁶⁹ mutants and a statistically significant 46% increase in *sei*^{ts1} mutants (Fig. 3 I).

Syt 4 expression could be regulated by a direct change in protein levels through altered protein stability or degradation or via activity-dependent transcriptional control of *syt 4* RNA. 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 a 38°C heat shock for 20 min followed by a 30-min recovery (Fig. 3 K). Relative to wild-type, *sei*^{ts1} showed a twofold increase in Syt 4 mRNA levels at the permissive 25°C, likely reflecting the excess chronic activity differences in the mutant. In addition, *sei*^{ts1} flies given a 20-min heat shock to induce acute seizures displayed a 2.9-fold increase in Syt 4 mRNA expression compared with *sei*^{ts1} 25°C controls and a 12-fold increase compared with heat-shocked wild-type controls. *para*^{ts1} animals displayed a 70% reduction in *syt 4* mRNA levels, which is consistent with results from Western analysis. These findings indicate that Syt 4 mRNA and protein levels are bidirectionally modified by changes in neuronal activity, suggesting that Syt 4-dependent postsynaptic function may be regulated by the prior activity history of neurons.

Syt 4-dependent structural plasticity requires C2A and C2B Ca^{2+} -binding sites

Our previous data suggested that Syt 4 overexpression in postsynaptic muscles stimulates synaptic varicosity formation and

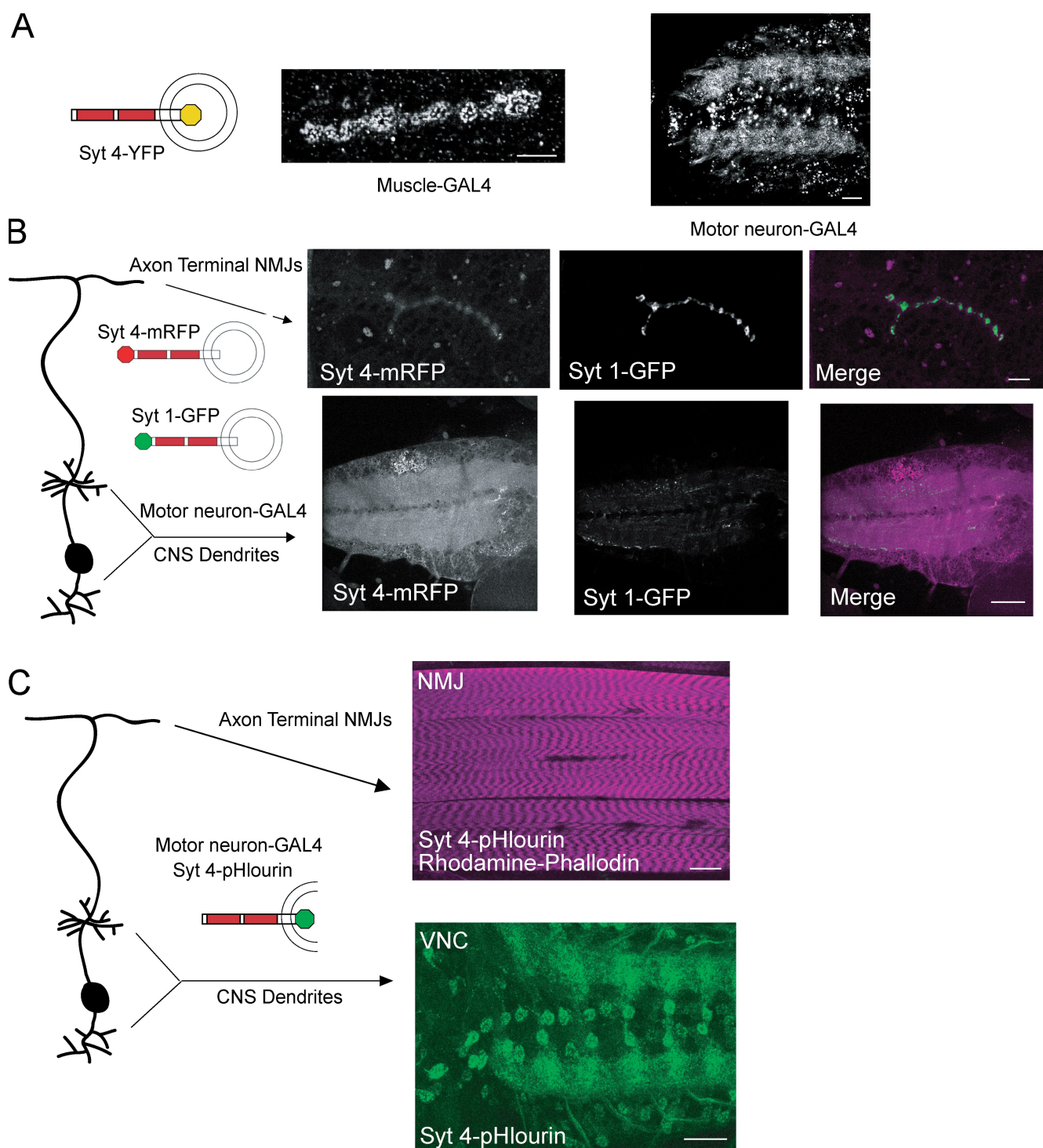
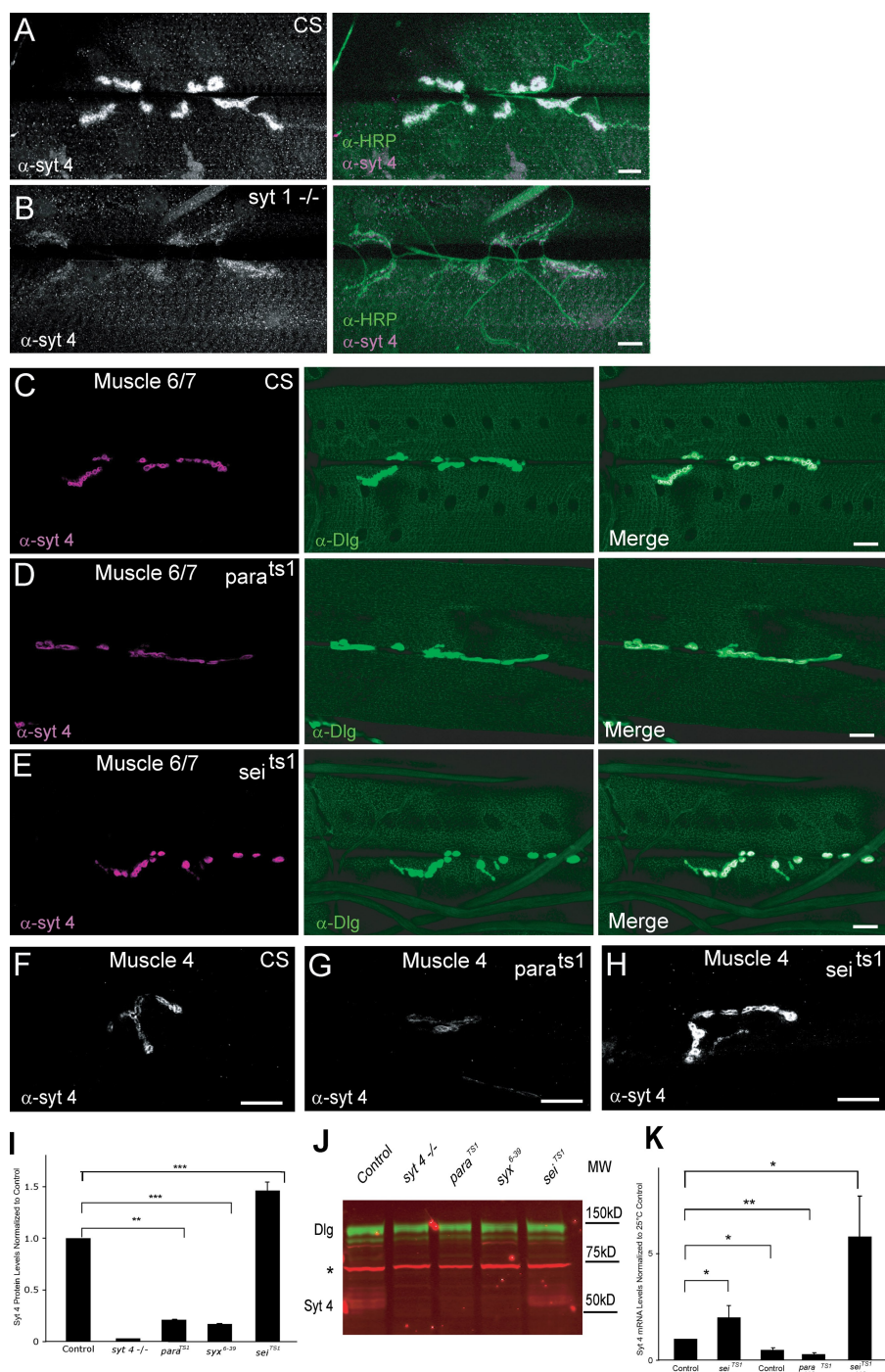


Figure 2. Syt 4 localization to postsynaptic compartments. (A) Diagram of N-terminal YFP-tagged Syt 4 (left). Expression in muscle with the *Mhc-GAL4* driver (middle) and motor neurons with *D42-GAL4* (right) is shown. Bars: (middle) 5 μ m; (right) 20 μ m. (B) Model of Syt 1-GFP and Syt 4-mRFP transgenic proteins expressed in motor neurons (left). Syt 1-GFP shows characteristic synaptic vesicle localization to presynaptic boutons at NMJs, whereas Syt 4-mRFP localizes to postsynaptic dendrites within the CNS. Merged image is displayed in the right panels. Bars: (top) 20 μ m; (bottom) 50 μ m. (C) Model of Syt 4-pHlourin transgenic protein expressed in motor neurons. No Syt 4 vesicle fusion is detected in presynaptic boutons at NMJs (top; counterstained with rhodamine-phalloidin to highlight muscle 6/7). In contrast, Syt 4-pHlourin is readily detected in cell bodies and dendritic membranes in the CNS. Bars: (top) 50 μ m; (bottom) 20 μ m.

growth (Yoshihara et al., 2005). To characterize the effect of altered Syt 4 levels, we analyzed the requirement for C2A and C2B function in the promotion of synaptic growth. Studies of Syt 1 have demonstrated that mutations disrupting the C2B

Ca²⁺-binding domain have profound effects on release (Mackler et al., 2002), whereas C2A mutants do not (Robinson et al., 2002). Multiple negatively charged aspartic acid residues within loops emerging from the two C2 domains mediate Ca²⁺ binding



by forming a bridge between negatively charged lipids embedded in the plasma membrane (Chapman, 2008). We neutralized one or more of the five key aspartic acid residues (previously referred to as D1–D5; Mackler et al., 2002) in each C2 domain to asparagines to test their requirement for Syt 4 function (Fig. 4 A). Multiple transgenic lines expressing each mutant protein under control of the UAS–GAL4 system were generated, and strains overexpressing similar levels of Syt 4 were selected for analysis (Fig. S1). Third 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. We observed a 60% increase in bouton number when wild-type

Syt 4 was overexpressed postsynaptically (Fig. 4 B). Mutating aspartic acids in both C2A (D292N [D5]) and C2B (D427N [D3] and D429N [D4]) Ca^{2+} -binding domains eliminated the ability of Syt 4 to facilitate synaptic proliferation (Fig. 4, B and C). To determine whether the C2B domain plays a critical role in Syt 4 function, we generated transgenic lines that overexpress Syt 4 with D427N and D429N substitutions in C2B that are required for in vitro Ca^{2+} binding (Mackler et al., 2002). Postsynaptic overexpression of this transgene failed to promote synaptic growth compared with wild-type Syt 4, indicating that the C2B Ca^{2+} -binding pocket is required for Syt 4 function, which is similar to its role in Syt 1.

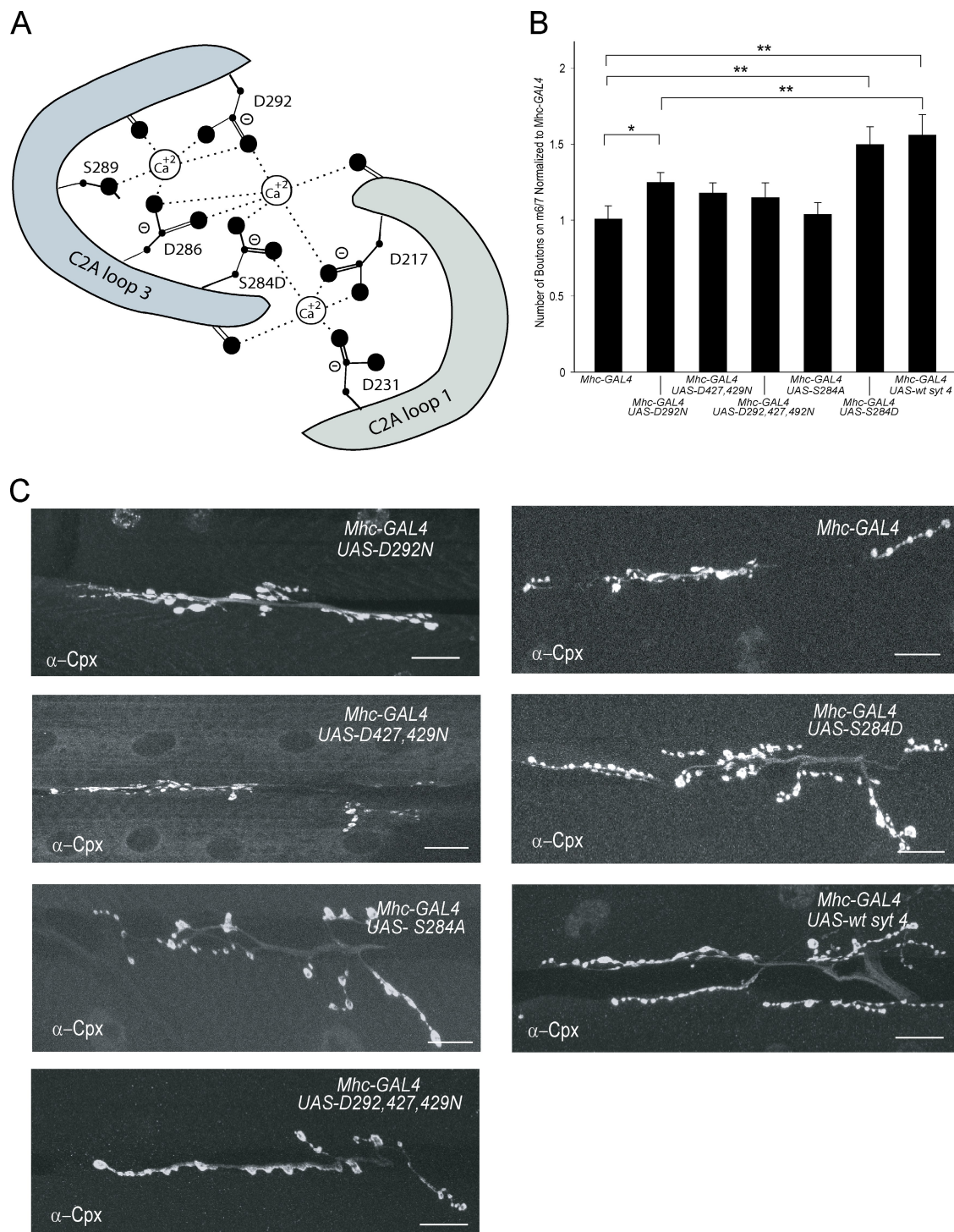


Figure 4. Structure-function analysis of Syt 4 C2 domains. (A) Model depicting key residues involved in coordination of Ca^{2+} binding in the Syt 4 C2A domain. S284D indicates the location of the key Ca^{2+} -binding residue that is an aspartic acid in other Syt family members. (B) Quantification of Syt 4-stimulated synaptic growth at muscle 6/7 of segment A3 (ANOVA, $P < 0.0006$). Third instar wandering larvae of the indicated genotypes reared at 25°C were immunostained with α -Cpx antisera. The data were analyzed by single-factor ANOVA ($P < 0.0006$), with Student's t test used as a secondary test. Quantified data normalized to Mhc-GAL4: Mhc-GAL4, 1.0 ± 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 by Student's t tests: D292N and Mhc-GAL4 control, $P < 0.05$; D292N and wild-type Syt 4, $P < 0.0025$; S284D and Mhc-GAL4 control, $P < 0.0025$; wild-type Syt 4 and Mhc-GAL4 control, $P < 0.0025$. (C) Representative immunocytochemical staining of third instar larval muscle 6/7 NMJs of the indicated genotypes with α -Cpx. *, $P < 0.05$; **, $P < 0.01$. Error bars indicate SEM. Bars, 20 μm .

In contrast to C2B, the C2A domain of Syt 4 contains a conserved substitution (D284S) in a key aspartic acid residue (D3) required for coordination of Ca^{2+} binding of Syt 4 C2A in vitro (Fernández-Chacón et al., 2002). Combined with data suggesting that C2B appears to be the most critical Ca^{2+} -binding domain in Syt 1, 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 (D5 of C2A). Surprisingly, overexpression of D292N was less efficient than wild type at promoting synaptic growth (Fig. 4, B and C), although the transgene did retain some residual function compared with C2B mutants. These data suggest that C2A Ca^{2+} binding is likely important for Syt 4 activity in vivo and that serine 284 (S284), which is conserved in Syt 4 isoforms from *Drosophila* to humans (Fig. 1 B), might not simply be an inactivating mutation for Ca^{2+} binding. Given that no other amino acid substitution has been allowed at S284, it would be unusual if the sole purpose were to inactivate Ca^{2+} binding. One 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. 4 A). To test whether 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 to determine whether it was capable of promoting synaptic growth. If the function of S284 is to eliminate Ca^{2+} binding, an S284A change should not alter Syt 4's ability to enhance synaptic growth. In contrast to this prediction, the C2A S284A mutant completely eliminated Syt 4 function, suggesting that this residue is essential in vivo (Fig. 4, B and C). To determine whether phosphorylation of S284 might contribute to Ca^{2+} binding by C2A, we constructed a phosphomimetic serine to aspartic acid construct (S284D), which has been previously shown to restore Ca^{2+} binding of the Syt 4 C2A domain in vitro (von Poser et al., 1997). When this construct was overexpressed postsynaptically in the muscle, it promoted NMJ growth similar to wild-type Syt 4, which is in sharp contrast to the nonfunctional serine to alanine mutation (Fig. 4, B and C). 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. Phosphorylation of S284 in Syt 4 in an activity-dependent fashion would be expected to enhance Ca^{2+} binding, potentially requiring less synaptic input to trigger future retrograde signaling. We conclude that Ca^{2+} binding by both C2A and C2B of Syt 4 is essential for its in vivo function and that the highly conserved serine at the D3 position of C2A is critical for the in vivo properties of the Syt 4 subfamily.

Syt 4 regulation of synaptic growth and neurotransmitter release

To further examine how Syt 4 regulates synaptic function, we compared synaptic growth, neurotransmitter release, and synaptic plasticity in response to high frequency stimulation in third instar larvae of *syt 4* mutants and *Mhc*-GAL4, UAS-syt 4

overexpression lines. Immunocytochemistry with α -Cpx antisera revealed that *syt 4*-null mutants reduce synaptic growth at the NMJ with a 24% decrease in varicosity number compared with controls (Fig. 5 A). *syt 4* mutants in trans to *Df(3R)rn¹⁶*, which removes the *syt 4* locus, showed a 28% reduction in synaptic bouton number (Fig. 5 A). Expression of Syt 4 using the muscle-specific *Mhc*-GAL4 driver rescued the synaptic growth defect (Fig. 5 A). To determine whether C2A and C2B function were required for Syt 4-dependent synaptic growth, we expressed Ca^{2+} -binding domain mutants in C2A (S284A) or C2B (D427N and D429N) in the null background and tested their ability to rescue the synaptic growth defect. Neither mutant transgene was able to restore synaptic growth to the levels observed with wild-type transgene expression, with C2A rescue mutants having a more severe defect than their C2B counterparts (Fig. 5 B). To analyze corresponding changes in the number of presynaptic release sites (active zones), we calculated the active zone to bouton ratio at NMJs of controls, *syt 4*-null mutants, and Syt 4 overexpression lines by costaining with α -Cpx to identify the number of varicosities and with α -Bruchpilot (α -Brp; nc82) to label active zones. Quantification revealed that active zone number changed parallel to bouton number, resulting in a similar active zone to bouton ratio in *syt 4* mutants and *Mhc*-GAL4; UAS-syt 4 overexpression lines compared with controls (Fig. 5, C–G).

We next examined physiological properties of synapses in *syt 4* mutants and postsynaptic overexpression lines under voltage clamp conditions at *Drosophila* third instar larval NMJs. *syt 4* mutants reduced spontaneous neurotransmitter release in the absence of nerve stimulation, whereas postsynaptic overexpression of Syt 4 enhanced spontaneous release (Fig. 6 A), which is consistent with the changes in varicosity number. Evoked responses to nerve stimulation in moderate extracellular Ca^{2+} (0.5 mM) showed a similar pattern, with the loss of Syt 4 reducing evoked neurotransmission, whereas postsynaptic overexpression of Syt 4 enhanced neurotransmitter release (Fig. 6 B). These changes in evoked responses were not observed in 2.0 mM extracellular Ca^{2+} (Fig. 6 C), suggesting that alterations in release probability or vesicle pool size may compensate for the altered number of release sites. To examine the basis for the response in higher Ca^{2+} , we tested short-term depression to 10-Hz stimulation. Fast and slow components of synaptic depression have been described at *Drosophila* NMJs (Delgado et al., 2000; Wu et al., 2005). The fast component represents the immediately releasable pool, whereas the slow component is correlated with delivery of synaptic vesicles from the readily releasable pool. The fast component of release during the initial stimulation was unchanged in *syt 4* mutants or overexpression lines, but distinct depletion profiles for the slow component of release were observed (Fig. 6 D). *syt 4* mutants displayed a lower depletion of synaptic vesicles and maintained a higher baseline-evoked response during steady-state stimulation. In contrast, Syt 4 overexpression larvae showed more depression and a smaller steady-state response during the train. Previous experiments have shown that retrograde signaling mechanisms can trigger homeostatic plasticity at the NMJ to compensate for altered synaptic structure (Davis et al., 1996).

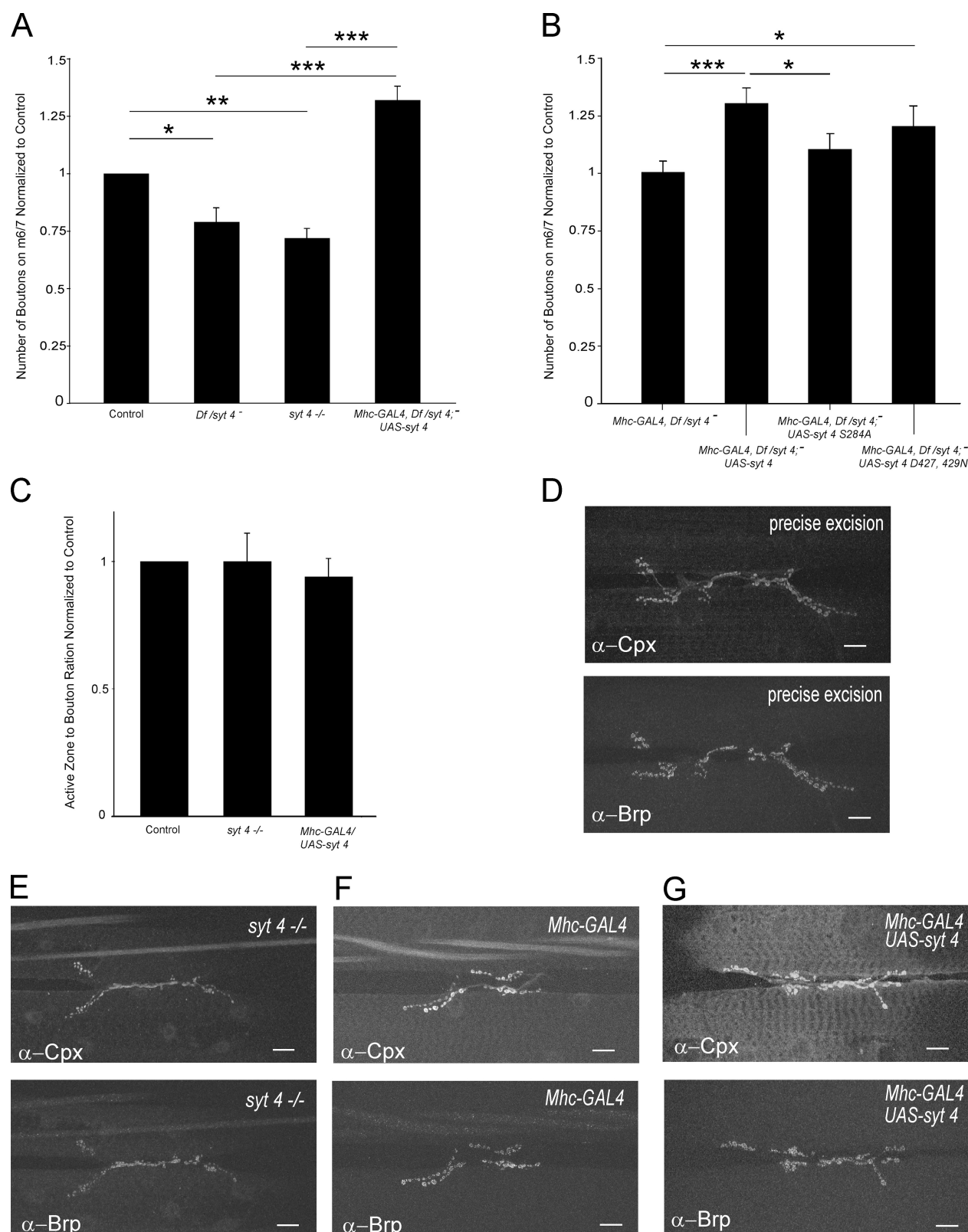


Figure 5. Syt 4 regulates synaptic growth at the NMJ. (A) Quantification of synaptic bouton number at muscle 6/7 in segment A3 of wandering third instar larvae by α -Cpx immunocytochemistry. Homozygous *syt 4*-null mutants in trans to a deficiency show a significant reduction in bouton number compared with controls, which is rescued by expression of wild-type Syt 4 postsynaptically (ANOVA, $P < 0.001$). The data were analyzed by single-factor ANOVA ($P < 0.001$). Data normalized to precise excision: control, 1 ± 0.04 ($n = 38$); *syt 4^{-/-}*, 0.76 ± 0.03 ($n = 34$). Data normalized to *Df/precise excision*: control, 1 ± 0.06 , ($n = 13$); *Df/syt 4^{-/-}*, 0.72 ± 0.04 ($n = 7$). Data normalized to *Mhc-GAL4, Df/precise excision*: control, 1 ± 0.05 ($n = 16$); *Mhc-GAL4, Df/syt 4^{-/-}*, 1.3 ± 0.06 ($n = 15$). Student's *t* test revealed significant differences between control and *syt 4^{-/-}* ($P < 0.001$), control and *Df/syt 4^{-/-}* ($P < 0.01$), *Df/syt 4^{-/-}* and *Mhc-GAL4, Df/syt 4^{-/-}; UAS-syt 4* ($P = 0.0008$), and *syt 4^{-/-}* and *Mhc-GAL4, Df/syt 4^{-/-}; UAS-syt 4* ($P = 0.0007$). (B) Quantification of synaptic bouton number in rescued animals (ANOVA, $P < 0.017$). The data were analyzed by single-factor ANOVA ($P < 0.017$). *Mhc-GAL4, Df/syt 4^{-/-}*:

Given the changes in synaptic vesicle pool size we observed, Syt 4 may not be essential for retrograde signaling that mediates homeostatic compensation. Alternatively, Syt 4-dependent retrograde signals may act to repress development of the readily releasable vesicle pool.

Previous studies at embryonic NMJs of *syt 4* mutants demonstrated that loss of Syt 4 disrupts retrograde signaling required for enhanced spontaneous presynaptic release after high frequency stimulation (Yoshihara et al., 2005). Whether this pathway is unique to developing synapses or also functions at mature synapses was unknown. Likewise, it is unclear whether the enhanced synaptic growth caused by postsynaptic overexpression of Syt 4 is linked to alterations in short-term synaptic plasticity. To examine synaptic plasticity at mature NMJs of third instar animals, we tested the effects of high frequency stimulation (four 1-s 100-Hz stimuli spaced 2 s apart) under voltage clamp conditions. High frequency stimulation triggered a long-lasting enhancement of spontaneous release at mature third instar NMJs (Fig. S2), although the fold increase in mini frequency is smaller than observed at embryonic NMJs. There is no change in quantal size after stimulation (Fig. S2 C) but rather in the appearance of multiquantal events, indicating increased presynaptic release probability as opposed to postsynaptic effects. Spontaneous release after high frequency stimulation remained elevated for ~5 min, indicating a long-lived effect on presynaptic release properties. In contrast to controls, *syt 4*-null mutants failed to show increased spontaneous release after stimulation (Fig. 7, A–D), suggesting that stimulation-dependent enhancement of presynaptic release requires Syt 4 function. Overexpression of Syt 4 in postsynaptic muscles triggered an elevation in the frequency of spontaneous fusion after high frequency stimulation compared with wild-type synapses (Fig. 7, A–D), with spontaneous release rates often >15 Hz. To determine whether C2A and C2B function were required, we expressed Ca²⁺-binding mutants in C2A (S284A) or C2B (D427N and D429N) in the null background and tested their ability to support stimulation-dependent enhancements in spontaneous release. Mutations in C2A or C2B eliminated the ability of Syt 4 to induce presynaptic release (Fig. 7 E), which is similar to their effects on synaptic growth (Fig. 4 B). We next tested whether the phosphomimetic serine to aspartic acid construct (S284D) could rescue release in *syt 4* mutants. When expressed postsynaptically, Syt 4 S284D promoted enhanced spontaneous presynaptic release to an even greater extent than wild-type Syt 4 (Fig. 7 E). In summary, our findings indicate that the loss of Syt 4 disrupts short-term enhancement of neurotransmitter release and synaptic growth, whereas overexpression of Syt 4 in the postsynaptic compartment enhances presynaptic plasticity and subsequent synapse proliferation.

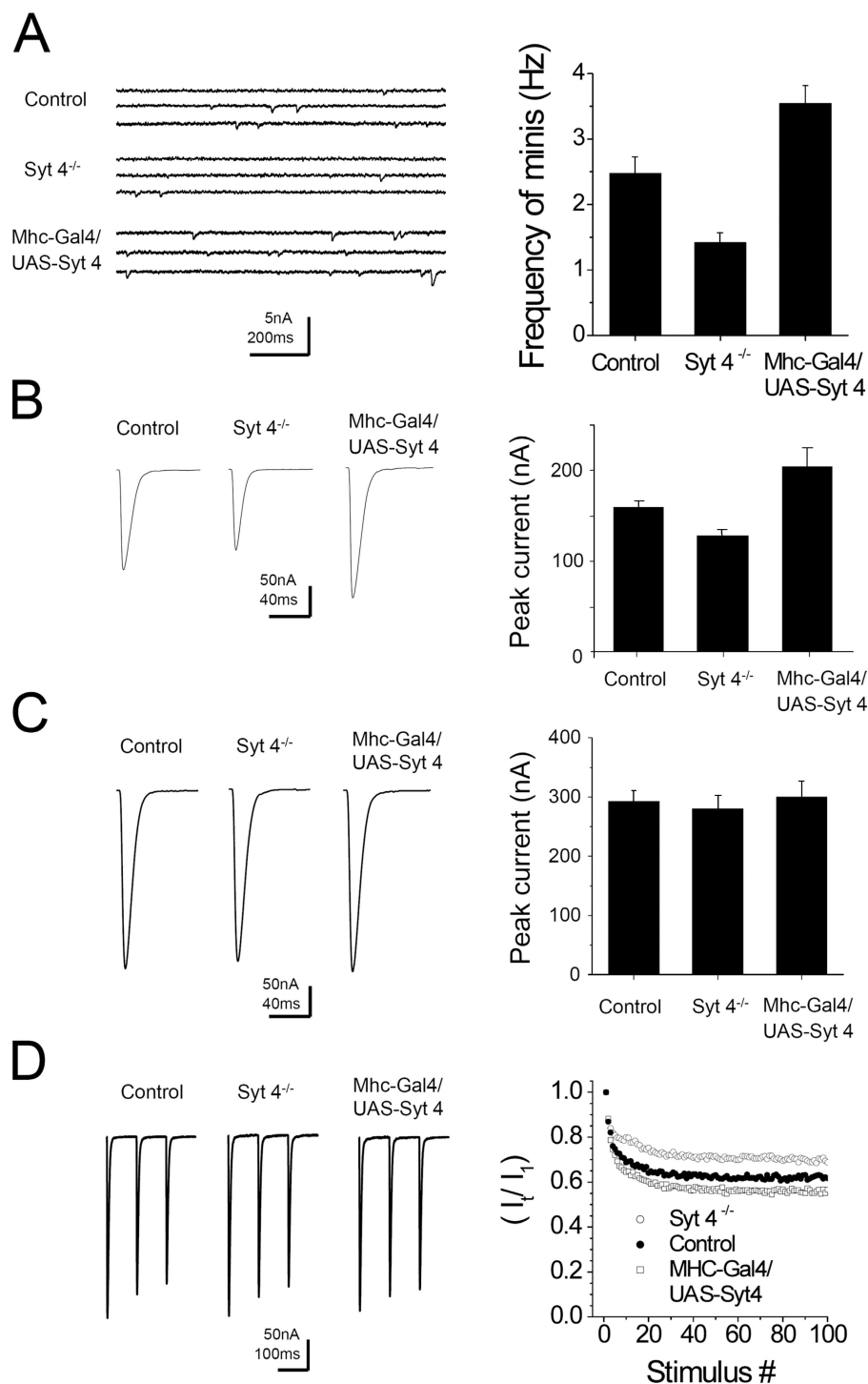
Syt 4 is required for activity-dependent structural plasticity at NMJs

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 by genetic mutations that increase neuronal excitability (Budnik et al., 1990). We hypothesized that increased neuronal activity could trigger Syt 4-dependent retrograde signaling secondary to Ca²⁺ influx post-synaptically during elevated motor neuron firing. To test this model, we quantified growth after enhanced neuronal activity in *syt 4*-null mutants and precise excision lines. We first examined structural plasticity at larval NMJs that occurs after increased larval locomotion stimulated by rearing at elevated temperatures (Sigrist et al., 2003). Although control larvae showed a 33% increase in bouton number when reared at 31°C compared with 25°C, animals lacking Syt 4 displayed no statistically significant increase in bouton number at 31°C (Fig. 8, A and B). Next, we examined synaptic overgrowth that occurs secondary to mutations that enhance neuronal activity. When *sei^{ts1}* mutants lacking the *Drosophila* ERG potassium channel are subject to developmental heat shocks, increased NMJ activity causes expansion of the larval NMJ (Guan et al., 2005). To test the requirement for Syt 4, the *sei^{ts1}* allele was moved into the *syt 4*^{−/−}, and precise excision background and larvae were reared at 25 and at 31°C (Fig. 8 C). *sei^{ts1}* mutants in the precise excision background displayed a 60% increase in bouton number at 31°C compared with 25°C. In contrast, *sei^{ts1}* in the *syt 4*^{−/−}-null background showed no significant change in bouton number between 25 and 31°C.

To determine whether neuronal activity is also required for the enhanced synaptic growth observed when Syt 4 is overexpressed postsynaptically, we examined the effects of reducing synaptic activity in Syt 4 overexpression strains. Previous studies have shown that mutations that reduce sodium channel function (*para^{ts1}*) suppress synaptic overgrowth induced by hyperexcitability mutants (Budnik et al., 1990) or elevated temperature rearing (Sigrist et al., 2003). We compared synaptic growth properties in Syt 4 overexpression strains in the *para^{ts1}* genetic background at permissive and nonpermissive temperatures (Fig. 8 D). Similar to previous observations (Sigrist et al., 2003), *para^{ts1}* mutants in the *Mhc-GAL4* genetic background suppressed temperature-induced synaptic overgrowth. In addition, *para^{ts1}* was able to dramatically reduce Syt 4-induced synaptic overgrowth when animals were reared at 31°C compared with 25°C. In summary, these findings indicate that synaptic proliferation triggered by postsynaptic Syt 4 overexpression at *Drosophila* NMJs occurs in an activity-dependent fashion.

1 ± 0.05 (n = 16); *Mhc-GAL4*, *Df/syt 4*^{−/−}; UAS-*syt 4*: 1.3 ± 0.06 (n = 15); *Mhc-GAL4*, *Df/syt 4*^{−/−}; UAS-*syt 4* S284A: 1.1 ± 0.07 (n = 18); *Mhc-GAL4*, *Df/syt 4*^{−/−}; UAS-*syt 4* D427N D429N: 1.2 ± 0.09 (n = 16). Student's *t* tests revealed significant differences between control and UAS-*syt 4* rescue (P = 0.0003), UAS-*syt 4* and UAS-*syt 4* S284A rescue (P < 0.03), and control and UAS-*syt 4* D427N D429N rescue (P = 0.02). (C) Quantification of active zone to bouton number at muscle 6/7 of segment A3 in wandering third instar larvae by α-Brp and α-Cpx immunocytochemistry. No significant difference was seen in mean active zone number per bouton in the null or overexpression lines. Data normalized to precise excision: precise excision, 1 ± 0.09 (n = 6 NMJs); *syt 4*^{−/−}, 1.0 ± 0.11 (n = 5 NMJs). Overexpression lines normalized to *Mhc-GAL4*: *Mhc-GAL4*, 1 ± 0.11 (n = 7 NMJs); *Mhc-GAL4*/UAS-*syt 4*, 0.94 ± 0.07 (n = 10 NMJs). (D–G) Representative staining of third instar NMJs of the indicated genotypes with α-Cpx (left) and α-Brp (right). *, P < 0.05; **, P < 0.01; ***, P < 0.001. Error bars indicate SEM. Bars, 20 μm.

Figure 6. Syt 4 regulates neurotransmitter release. (A) Representative traces of spontaneous postsynaptic currents (minis) in control, *syt 4*-null mutants, and Syt 4 overexpression lines in 0.5 mM external Ca^{2+} . Mean mini frequency in the strains: control, 2.47 ± 0.25 Hz; *syt 4*^{-/-}, 1.42 ± 0.15 Hz; *Mhc-GAL4/UAS-syt 4*, 3.54 ± 0.27 Hz. The data are the mean of at least four different animals. Student's *t* tests revealed significant differences between control and *syt 4*^{-/-} ($P < 0.01$) and between control and *Mhc-GAL4/UAS-syt 4* ($P < 0.01$). (B) Evoked Excitatory postsynaptic currents (EPSCs) recorded in 0.5 mM external Ca^{2+} in control, *syt 4*-null mutants, and Syt 4 overexpression lines. Mean peak amplitude in the strains: control, 153 ± 6 nA; *syt 4*^{-/-}, 123 ± 7 nA; *Mhc-GAL4/UAS-syt 4*, 196 ± 20 nA. Student's *t* tests revealed significant differences between control and *syt 4*^{-/-} ($P < 0.01$) and between control and *Mhc-GAL4/UAS-syt 4* ($P < 0.01$). (C) Evoked EPSCs recorded in 2 mM external Ca^{2+} in control, *syt 4* mutants, and Syt 4 overexpression lines. Mean peak amplitude in the strains: control, 292 ± 19 nA; *syt 4*^{-/-}, 280 ± 24 nA; *Mhc-GAL4/UAS-syt 4*, 300 ± 28 nA. Student's *t* tests revealed no significant differences between control and *syt 4*^{-/-} or *Mhc-GAL4/UAS-syt 4*. (D) Evoked EPSCs recorded in 2 mM external Ca^{2+} during tetanic stimulation of the nerve at 10 Hz. The initial stimuli are shown on the left, and the mean evoked amplitude during 10 s of stimulation is displayed on the right. The depression parameter is expressed as $1 - \text{EPSC}_{ss}/\text{EPSC}_1$. The extent of depression estimated from steady state at the end of the stimulation episode was as follows: control, 0.38 ± 0.03 ; *syt 4*^{-/-}, 0.30 ± 0.02 ; *Mhc-GAL4/UAS-syt 4*, 0.44 ± 0.04 ; and was significantly different between control and *syt 4*^{-/-} ($P < 0.01$) and between control and *Mhc-GAL4/UAS-syt 4* ($P < 0.01$). Error bars indicate SEM.



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 (Knöll and Drescher, 2002), membrane-permeable factors (Hawkins et al., 1994), and conventional neurotransmit-

ters (Ludwig and Pittman, 2003; Regehr et al., 2009). Recent experiments have also established the presence of membrane trafficking components in the postsynaptic compartment, including ER, Golgi, and postsynaptic vesicles (Kennedy and Ehlers, 2006). In contrast to the well-described mechanisms for presynaptic vesicle trafficking, how retrograde signals are released from the postsynaptic compartment is poorly understood. In this study, we show that the Syt 1 homologue, Syt 4, plays a postsynaptic role in regulation of synaptic growth and plasticity in an activity-dependent manner at *Drosophila* synapses.

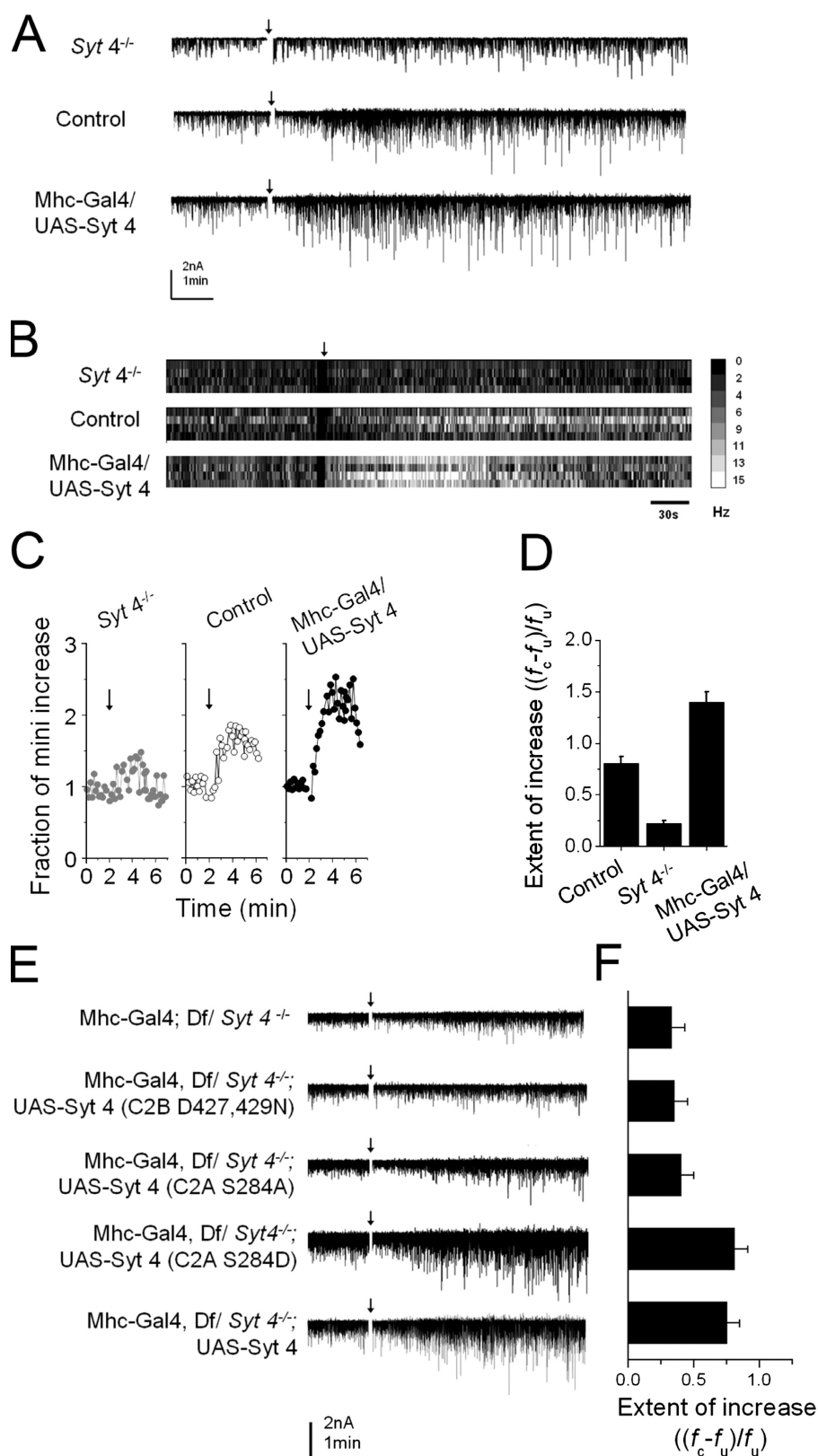


Figure 7. Posttetanic enhancement of spontaneous miniature release is regulated by Syt 4. (A) Current recordings of spontaneous miniature activity 2 min before and 9 min after the conditioning nerve stimulation (arrows) in *syt 4*-null mutants, control, and *Mhc-GAL4/UAS-syt 4* overexpression lines in 0.5 mM Ca^{2+} . (B) Grayscale representation of spontaneous miniature frequency before and after conditioning nerve stimulation (arrow) in four representative experiments. Each bit represents the frequency measured in 1 s. (C) Mean spontaneous miniature release normalized to the unconditioned spontaneous miniature frequency (f_u). (D) Mean extent of spontaneous miniature frequency increase after 2 min of conditioned stimulus. The data are presented as the mean of at least five different larvae and expressed as the fraction of increase $[(f_c - f_u)/f_u]$, where f_u and f_c are the unconditioned and conditioned spontaneous miniature frequency, respectively. The extent of increased spontaneous release for each genotype: control, 0.8 ± 0.07 ($n = 5$); *syt 4^{-/-}*, 0.22 ± 0.02 ($n = 5$); *Mhc-GAL4/UAS-syt 4* 1.4 ± 0.1 ($n = 5$). Student's *t* tests revealed significant differences between control and *syt 4^{-/-}* ($P < 0.01$) and between control and *Mhc-GAL4/UAS-syt 4* ($P < 0.01$). (E) Representative traces (left) before and after tetanic stimulation to induce posttetanic mini frequency increases in the indicated genotypes. The extent of increase in mini frequency (right) was estimated as in D. Mean enhancements are *Mhc-GAL4, Df/syt 4^{-/-}*: 0.33 ± 0.03 ; *Mhc-GAL4, Df/syt 4^{-/-}; UAS-syt 4 [D427 and 429N]/+*: 0.35 ± 0.04 ; *Mhc-GAL4, Df/syt 4^{-/-}; UAS-syt 4 [S284A]/+*: 0.4 ± 0.04 ; *Mhc-GAL4, Df/syt 4^{-/-}; UAS-syt 4 [S284D]/+*: 0.81 ± 0.07 ; *Mhc-GAL4, Df/syt 4^{-/-}; UAS-syt 4/+*: 0.75 ± 0.08 . Student's *t* tests revealed significant differences between *Mhc-GAL4, Df/syt 4^{-/-}* and *Df/syt 4^{-/-}; UAS-syt 4 [S284D]/+* ($P < 0.01$) and between *Mhc-GAL4, Df/syt 4^{-/-}* and *Mhc-GAL4, Df/syt 4^{-/-}; UAS-syt 4* ($P < 0.01$). No significant differences were found between the *syt 4*-null mutant and the *UAS-syt 4 D427, 429N*, and *UAS-syt 4 S284A* rescue lines. Arrows indicate onset of stimulation. (F) Quantification of posttetanic mini frequency increase in the indicated genotypes. Error bars indicate SEM.

Our phenotypic analysis of *syt 4*-null mutants reveals a decrease in the number of NMJ synaptic varicosities, a reduction in neurotransmission, and defective-enhanced spontaneous release after high frequency stimulation. Our results at mature third instar synapses are qualitatively similar to those we observe

at newly formed synapses (Yoshihara et al., 2005). However, although high frequency stimulation can induce an approximately two- to threefold increase in mini frequency at mature NMJs, similar stimulation of embryonic synapses results in an ~ 100 -fold change, indicating that newly formed connections are

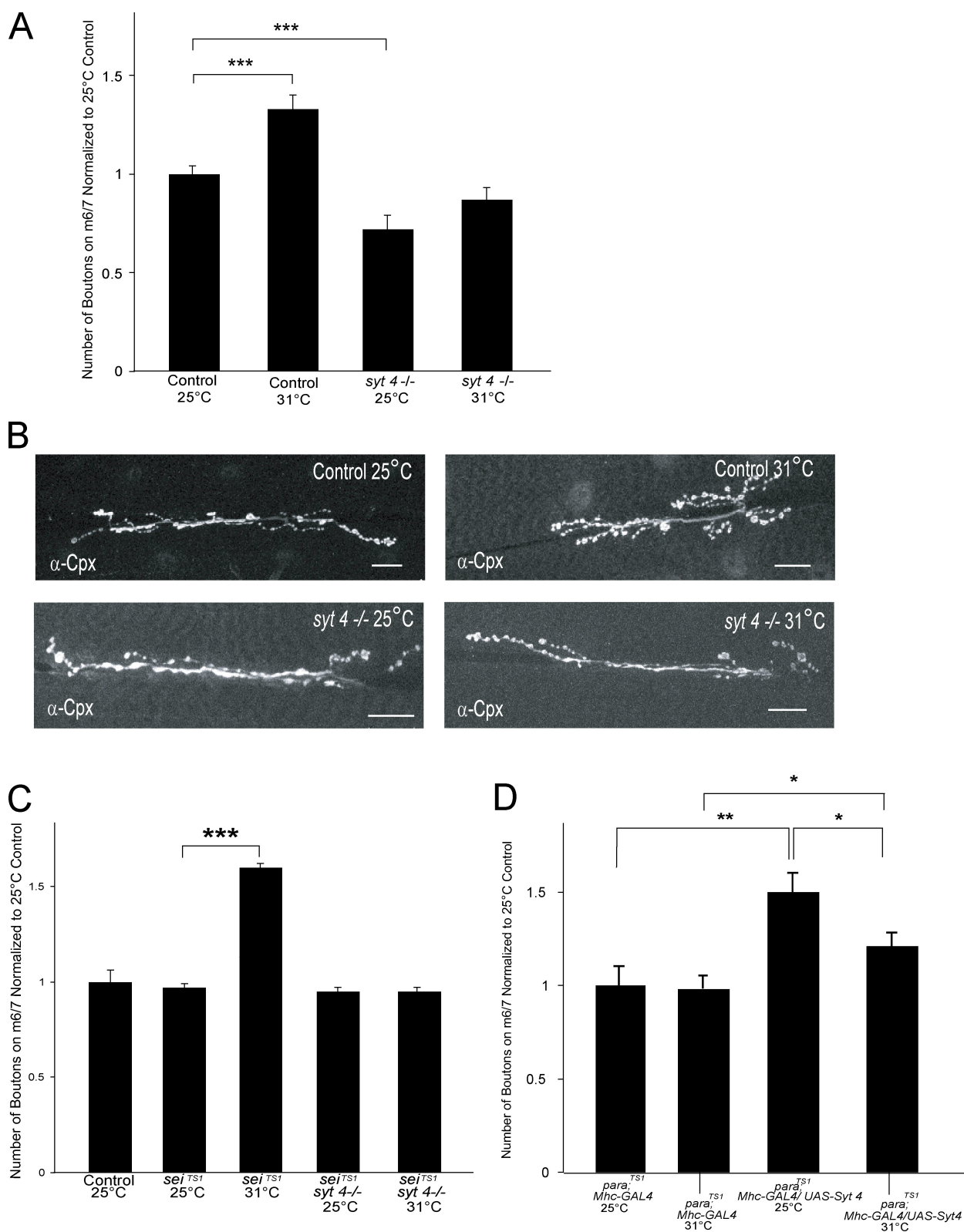


Figure 8. Syt 4 regulates activity-dependent synaptic growth at the NMJ. (A) Quantification of bouton number at muscle 6/7 of segment A3 in third instar wandering larvae of the indicated genotypes reared at 25 or 31°C (ANOVA, $P < 0.00001$). Quantification normalized to 25°C precise excision control 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 ($n = 13$). The data were analyzed by single-factor ANOVA ($P < 0.00001$) with Student's *t* tests used for secondary analysis: control at 25 and 31°C, $P < 0.001$; control and *syt 4^{-/-}* at 25°C, $P < 0.001$. (B) Representative immunocytochemical staining of third instar larval muscle 6/7 NMJs with α -Cpx of the indicated genotypes reared at 25 or 31°C. Bars, 20 μ m. (C) Quantification of bouton number at muscle 6/7 of segment A3 in third instar wandering larvae of the indicated genotypes reared at 25 or 31°C (ANOVA, $P < 0.00001$). Data normalized to 25°C control: *sei^{TS1}* 25°C, 0.96 ± 0.06 ($n = 14$); *sei^{TS1}* 31°C, 1.6 ± 0.09 ($P < 0.0005$); *sei^{TS1}; syt 4^{-/-}* 25°C, 0.95 ± 0.07 , ($n = 15$); *sei^{TS1}; syt 4^{-/-}* 31°C, 0.95 ± 0.07 ($n = 10$). The data were analyzed by single-factor

exquisitely sensitive to Syt 4-dependent retrograde signaling. In contrast to the loss of Syt 4 function, overexpression of Syt 4 in the postsynaptic compartment has the opposite effect, inducing bouton proliferation and enhancing spontaneous release after high frequency stimulation. Whether Syt 4 overexpression increases the likelihood of individual postsynaptic vesicle fusion events by altering the Ca^{2+} -dependent release machinery or results in an increased number of postsynaptic vesicles is currently unknown. Similar increases in spontaneous release frequency have been observed after repetitive pulses of high K^+ saline at the NMJ (Ataman et al., 2008). Although the link between increased mini frequency after strong stimulation and synaptic growth triggered by Syt 4 postsynaptic overexpression is unclear, it is tempting to speculate that increased Syt 4 levels enhance stimulation-dependent release of retrograde signals that act on presynaptic release and the synaptic growth machinery. We hypothesize that enhanced spontaneous release may function to prolong postsynaptic Ca^{2+} transients that normally occur after high frequency neuronal firing, enhancing generation of second messengers that promote synaptic proliferation. 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 contributes to activity-induced synaptic growth triggered by rearing animals at higher temperatures or by seizure-inducing mutations. By imaging tagged versions of Syt 4 in CNS motor neurons, we find that Syt 4 fusion-competent vesicles are also found in CNS dendrites, suggesting that the protein may play a general role in Ca^{2+} regulation of postsynaptic membrane traffic.

What is the role of Syt 4 in the postsynaptic compartment? We hypothesize that Syt 4 functions as a postsynaptic Ca^{2+} sensor for regulating retrograde signaling by activating postsynaptic vesicle fusion. This would result in release of a diffusible ligand from the postsynaptic compartment or insertion of a transmembrane protein that would trigger bidirectional signaling. Given the role of Syt 1 in presynaptic neurotransmitter release and the acute effects on synaptic plasticity we observed with manipulation of Syt 4 levels, we favor release of a diffusible signal. Alternatively, Syt 4 might be required for endocytosis of a transsynaptic signaling complex that would regulate short-term changes in bidirectional signaling. A role for Syt 4 solely in endocytosis is unlikely, as the protein is found on a vesicle compartment and not associated with the plasma membrane at rest (Adolfson et al., 2004), where it presumably would be required for endocytotic reactions. In addition, interactions between Syt 1 and the clathrin adapter AP-2 do not require Ca^{2+} binding by Syt 1. We have demonstrated that Syt 4's ability to drive synaptic growth and enhance presynaptic release requires C2 domain Ca^{2+} -binding residues. Finally, Syt 4 could have a unique postsynaptic function

that is unrelated to vesicle trafficking. Given the established role of synaptotagmins in regulating membrane trafficking, we view this as a less likely option. The specific cargo contained in Syt 4 vesicles is currently unknown. Similar to the diverse set of cell type-specific neurotransmitters found in Syt 1-containing synaptic vesicles, we expect a similar diversity of Syt 4 postsynaptic vesicle cargos. Several target-derived retrograde factors function in synaptic growth, including FGF, Wingless/Wnt, TGF- β peptides, and the NGF family of neurotrophic factors (Hall et al., 2000; Umemori et al., 2004; Salinas, 2005; Zweifel et al., 2005). Recent work indicates that mammalian Syt 4 is localized to postsynaptic vesicles in hippocampal neurons that contain brain-derived neurotrophic factor where it regulates their release and subsequent effects on long-term potentiation (Dean et al., 2009), supporting an evolutionarily conserved role of Syt 4 as a regulator of postsynaptic fusion events.

An interesting question raised by our experiments is how similar the biochemical activities of Syt 1 and 4 are in vivo. Syt 1 and 4 cannot functionally substitute for each other at synapses (Adolfson et al., 2004; Yoshihara et al., 2005), but this reflects sorting to distinct vesicle populations. Syt 1 preferentially requires C2B Ca^{2+} binding for activity, with C2A playing a more minor role. Syt 4-dependent retrograde signaling at NMJs shows Ca^{2+} dependence in vivo (Fig. 4; Yoshihara et al., 2005), although in vitro methods have produced conflicting evidence about its potential to bind Ca^{2+} (Fukuda et al., 1996; Dai et al., 2004). By examining the ability of mutant Syt 4 proteins to promote synaptic growth and to rescue growth and plasticity defects in *syt 4* mutants, we demonstrate that Ca^{2+} -binding sites in both the C2A and C2B domains of Syt 4 are necessary. In particular, S284 of the C2A domain is required for Syt 4 activity in vivo. 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 *Drosophila* to humans, suggesting an evolutionarily important role for the residue, previously hypothesized to be inactivation of the C2A Ca^{2+} -binding site. 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 overexpressed in vivo (Fig. 4). Phosphorylation of Syt 4 could potentially explain the conflicting in vivo and in vitro data concerning the protein's ability to bind Ca^{2+} . There is precedent for phosphorylation of synaptotagmins regulating Ca^{2+} binding, as Syt 2 is phosphorylated by WNK1, reducing its Ca^{2+} -dependent, phospholipid-binding

ANOVA ($P < 0.00001$) with Student's *t* tests used for secondary analysis. (D) Quantification of bouton number at muscle 6/7 of segment A3 in third instar wandering larvae of the indicated genotypes reared at 25 or 31°C (ANOVA, $P < 0.0002$). Overexpression 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 [$n = 11$]; $P < 0.003$ by Student's *t* test). At the nonpermissive 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 overexpression larvae (*para^{ts1}*; *Mhc-GAL4/UAS-syt 4* 31°C, 1.21 ± 0.07 [$n = 11$]; $P < 0.05$ by Student's *t* test). The data were analyzed by single-factor ANOVA ($P < 0.0002$) with Student's *t* tests used for secondary analysis. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Error bars indicate SEM.

affinity (Lee et al., 2004). Current studies are under way to determine whether S284 of Syt 4 is phosphorylated in vivo and which kinases are involved.

Two orthologues of *Drosophila* Syt 4 are found in mammalian genomes encoded by the Syt 4 and Syt 11 genes. Although mammalian Syt 4 has been implicated in retrograde signaling (Dean et al., 2009), little is known about Syt 11 other than it is abundantly expressed in the brain (von Poser et al., 1997). However, 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 using immunocytochemistry and amperometric recording of dopamine release (Li et al., 2005). Surprisingly, the kinetics of somatodendritic dopamine release is similar to quantal events recorded at presynaptic sites, with release occurring in <1 ms. 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 its 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 and Syt 11 may be a general mechanism for regulating synaptic growth and plasticity at activated synapses.

Materials and methods

Generation of *Drosophila* stocks

Drosophila were cultured on standard media at the indicated temperatures. The syt 4-null mutant was generated through imprecise P-element excision of insert EY09259, 100-bp 5' of the syt 4 transcription start site (Adolfson et al., 2004). 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. 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 by injection into w^{1118} flies. Syt 4-mRFP lines were generated in a similar fashion by fusing the mRFP cDNA to the 3' end of the coding region of syt 4 and deleting the stop codon and were injected into w^{1118} flies by Genetic Services (Cambridge, MA). All constructs were verified by DNA sequencing before injection.

Sequence alignments and homology searches

The following proteins were used in the analysis shown in Fig. 1: *Homo sapiens* Syt 1 (NCBI Protein database accession no. NP_005630), *Mus musculus* Syt 1 (NCBI Protein database accession no. NP_033332), *Drosophila* Syt 1 (NCBI Protein database accession no. NP_523460), *Caenorhabditis elegans* snt-1 (NCBI Protein database accession no. NP_495394), *H. sapiens* Syt 4 (NCBI Protein database accession no. NP_065834), *M. musculus* Syt 4 (NCBI Protein database accession no. NP_033334), *Drosophila* Syt 4 (NCBI Protein database accession no. NP_477464), *C. elegans* snt-4 (NCBI Protein database accession no. NP_491853), and *N. vectensis* putative Syt 4 (NCBI Protein database accession no. XP_001633294). We used standard parameters in the field for identifying a synaptotagmin: a single transmembrane-containing protein lacking a signal peptide with tandem cytoplasmic C2 domains. For distinguishing Syt 1 and 4 family members, we relied on overall blast similarity to previously characterized family members and the presence of the aspartate to serine substitution at the C2A D3 residue found in known members of the Syt 4 family.

Immunostaining

Immunostaining was performed on wandering third instar larvae reared on standard media at 25 or 31°C as indicated in Figs. 1–8. Larvae were dissected in HL3.1 saline (70 mM NaCl, 5 mM KCl, 10 mM NaHCO_3 , 4 mM

MgCl_2 , 5 mM trehalose, 115 mM sucrose, and 5 mM Hepes, pH 7.2). Dissected larvae were fixed with 4% formaldehyde in HL3.1 for 45 min and washed three times for 5 min in PBS with 0.1% Triton X-100 (Huntwork and Littleton, 2007). Preparations were blocked for 1 h at room temperature with gentle agitation in 1% BSA and 0.1% Triton X-100 in PBS before primary antibodies were added. The following primary antibody dilutions were used: α -Cpx (Huntwork and Littleton, 2007) for bouton counting (1:1,000), nc82 α -Brp (Developmental Studies Hybridoma Bank) for active zone counting (1:50), and α -syt 4 (1:250; Adolfson et al., 2004). Larvae were incubated with primary antibodies overnight at 4°C and washed three times for 5 min in PBS with 0.1% Triton X-100. The secondary antibodies, Cy2-conjugated goat α -rabbit and Cy5-conjugated goat α -mouse (Jackson ImmunoResearch Laboratories), were used at 1:250 dilutions and incubated for 4 h at room temperature. Before mounting, the dissections were washed three times in PBS with 0.1% Triton X-100 and three times in PBS.

Morphology quantification

Samples were mounted in 70% glycerol and imaged with a 40 $\times$ NA 1.3 Plan Neofluar oil immersion lens (Carl Zeiss, Inc.) at room temperature. Images were captured with a laser-scanning confocal microscope (Pascal; Carl Zeiss, Inc.) using the accompanying software. Bouton and active zone number were quantified from confocal stacks taken of the entire NMJ at muscle 6/7 of segment A3 (Huntwork and Littleton, 2007). Published images were not subject to additional image processing. All error measurements indicate SEM. Statistical significance was calculated with single-factor analysis of variance (ANOVA), with Student's *t* tests used for secondary analysis. Statistical significance is noted as follows: *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001.

Western blotting and quantification of protein expression

α -Syt 4 antibody (Adolfson et al., 2004) was used at 1:750, and α -Dlg antibody 4F3 (Developmental Studies Hybridoma Bank) was used at 1:2,000. Secondary antibodies were used at 1:10,000, including goat α -rabbit IgG Alexa Fluor 680 (Invitrogen) and goat α -mouse IR dye 800 (Rockland). Western blots were visualized on an infrared imaging system (Odyssey; Li-COR Biosciences), and protein expression was quantified with the accompanying software.

RT-PCR

RT-PCR was performed on male *Drosophila* head RNA preparations after a single acute 20-min heat shock at 38°C followed by a 30-min recovery as described previously in Guan et al. (2005). In brief, RNA was isolated from heads of males aged 3–4 d after eclosion. All flies were sacrificed between 2 and 4 p.m. to reduce any circadian-dependent transcriptional changes. After 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 an mRNA extraction kit (Oligotex; QIAGEN). cDNA was created with T7-(dT)₂₄ primer (Genset Corp.) using a cDNA kit (Invitrogen) and purified by Phase Lock Gel extraction (Eppendorf). Primers used for RT-PCR quantification include: *GAPDH* primers (5' end) 5'-AATCAAGGCTAAGGTCGAGGAG-3' and (3' end) 5'-TAACCGAAGCTGTGCTGCTACC-3'; and syt 4 primers (5' end) 5'-CATTGATTCGAGAGG-3' and (3' end) 5'-CTATTGCGTTCGGTTTGAG-3'.

Electrophysiology

Evoked postsynaptic currents were recorded from ventral longitudinal muscle 6 at abdominal segment A3 in third instar larvae using two-microelectrode voltage clamp (OC725; Warner Instruments) at –80 mV holding potential (Acharya et al., 2006). All experiments were performed in modified HL3 solution consisting of 70 mM NaCl, 5 mM KCl, 4 mM MgCl_2 , 10 mM NaHCO_3 , 115 mM sucrose, and 5 mM Hepes-Na, pH 7.2. Extracellular Ca^{2+} concentration was adjusted to final values as indicated in Figs. 1–8. For stimulation, nerves were cut close to the ventral ganglion and sucked into a pipette filled with working solution. The nerve was stimulated at frequencies indicated in each experiment using a programmable stimulator (Master-8; AMPI). Data acquisition and analysis were performed using pClamp software (Axon Instruments).

Online supplemental material

Fig. S1 shows expression of Syt 4 in transgenic strains. Fig. S2 shows analysis of posttetanic induction of spontaneous release. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200903098/DC1>.

We thank Shuai Chen for help with data analysis and Bill Adolfson, Kathy Galle, and Robin Stevens for help with generation and localization of

transgenic fluorescently labeled proteins. The nc82 antibody against Brp developed by Erich Buchner and the 4F3 antibody against Dlg developed by Corey Goodman were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the National Institute of Child Health and Human Development and were maintained by the Department of Biological Sciences at the University of Iowa (Iowa City, IA).

This work was supported by the National Institutes of Health (grant NS40296) to J.T. Littleton.

Submitted: 18 March 2009

Accepted: 15 September 2009

References

- Acharya, U., M.B. Edwards, R.A. Jorquera, H. Silva, K. Nagashima, P. Labarca, and J.K. Acharya. 2006. *Drosophila melanogaster* Scramblases modulate synaptic transmission. *J. Cell Biol.* 173:69–82. doi:10.1083/jcb.200506159.
- Adolfson, B., and J.T. Littleton. 2001. Genetic and molecular analysis of the synaptotagmin family. *Cell. Mol. Life Sci.* 58:393–402. doi:10.1007/PL00000865.
- Adolfson, B., S. Saraswati, M. Yoshihara, and J.T. Littleton. 2004. Synaptotagmins are trafficked to distinct subcellular domains including the postsynaptic compartment. *J. Cell Biol.* 166:249–260. doi:10.1083/jcb.200312054.
- Ataman, B., J. Ashley, M. Gorczyca, P. Ramachandran, W. Fouquet, S.J. Sigrist, and V. Budnik. 2008. Rapid activity-dependent modifications in synaptic structure and function require bidirectional Wnt signaling. *Neuron*. 57:705–718. doi:10.1016/j.neuron.2008.01.026.
- Barber, C.F., and J.T. Littleton. 2007. Synaptic growth and transcriptional regulation in *Drosophila*. In *Transcriptional Regulation by Neuronal Activity: To the Nucleus and Back*. S. Dudek, editor. Springer Science Publishing, New York. 253–275.
- Budnik, V., Y. Zhong, and C.F. Wu. 1990. Morphological plasticity of motor axons in *Drosophila* mutants with altered excitability. *J. Neurosci.* 10:3754–3768.
- Chapman, E.R. 2008. How does synaptotagmin trigger neurotransmitter release? *Annu. Rev. Biochem.* 77:615–641. doi:10.1146/annurev.biochem.77.062005.101135.
- Creutz, C.E., S.L. Snyder, and T.A. Schulz. 2004. Characterization of the yeast tricalbins: membrane-bound multi-C2-domain proteins that form complexes involved in membrane trafficking. *Cell. Mol. Life Sci.* 61:1208–1220. doi:10.1007/s00018-004-4029-8.
- Dai, H., O.H. Shin, M. Machius, D.R. Tomchick, T.C. Südhof, and J. Rizo. 2004. Structural basis for the evolutionary inactivation of Ca²⁺ binding to synaptotagmin 4. *Nat. Struct. Mol. Biol.* 11:844–849. doi:10.1038/nsmb817.
- Davis, G.W., C.M. Schuster, and C.S. Goodman. 1996. Genetic dissection of structural and functional components of synaptic plasticity. III. CREB is necessary for presynaptic functional plasticity. *Neuron*. 17:669–679. doi:10.1016/S0896-6273(00)80199-3.
- Dean, C., H. Liu, F.M. Dunning, P.Y. Chang, M.B. Jackson, and E.R. Chapman. 2009. Synaptotagmin-IV modulates synaptic function and long-term potentiation by regulating BDNF release. *Nat. Neurosci.* 12:767–776. doi:10.1038/nn.2315.
- Delgado, R., C. Maureira, C. Oliva, Y. Kidokoro, and P. Labarca. 2000. Size of vesicle pools, rates of mobilization, and recycling at neuromuscular synapses of a *Drosophila* mutant, *shibire*. *Neuron*. 28:941–953. doi:10.1016/S0896-6273(00)00165-3.
- Fernández-Chacón, R., O.H. Shin, A. Königstorfer, M.F. Matos, A.C. Meyer, J. Garcia, S.H. Gerber, J. Rizo, T.C. Südhof, and C. Rosenmund. 2002. Structure/function analysis of Ca²⁺ binding to the C2A domain of synaptotagmin 1. *J. Neurosci.* 22:8438–8446.
- Fitzsimonds, R.M., and M.M. Poo. 1998. Retrograde signaling in the development and modification of synapses. *Physiol. Rev.* 78:143–170.
- Fukuda, M., T. Kojima, and K. Mikoshiba. 1996. Phospholipid composition dependence of Ca²⁺-dependent phospholipid binding to the C2A domain of synaptotagmin IV. *J. Biol. Chem.* 271:8430–8434. doi:10.1074/jbc.271.14.8430.
- Ganetzky, B., and C.F. Wu. 1986. Neurogenetics of membrane excitability in *Drosophila*. *Annu. Rev. Genet.* 20:13–44. doi:10.1146/annurev.ge.20.120186.000305.
- Goold, C.P., and G.W. Davis. 2007. The BMP ligand Gbb gates the expression of synaptic homeostasis independent of synaptic growth control. *Neuron*. 56:109–123. doi:10.1016/j.neuron.2007.08.006.
- Guan, Z., S. Saraswati, B. Adolfson, and J.T. Littleton. 2005. Genome-wide transcriptional changes associated with enhanced activity in the *Drosophila* nervous system. *Neuron*. 48:91–107. doi:10.1016/j.neuron.2005.08.036.
- Hall, A.C., F.R. Lucas, and P.C. Salinas. 2000. Axonal remodeling and synaptic differentiation in the cerebellum is regulated by WNT-7a signaling. *Cell*. 100:525–535. doi:10.1016/S0092-8674(00)80689-3.
- Hawkins, R.D., M. Zhuo, and O. Arancio. 1994. Nitric oxide and carbon monoxide as possible retrograde messengers in hippocampal long-term potentiation. *J. Neurobiol.* 25:652–665. doi:10.1002/neu.480250607.
- Huntwork, S., and J.T. Littleton. 2007. A complexin fusion clamp regulates spontaneous neurotransmitter release and synaptic growth. *Nat. Neurosci.* 10:1235–1237. doi:10.1038/nn1980.
- Kennedy, M.J., and M.D. Ehlers. 2006. Organelles and trafficking machinery for postsynaptic plasticity. *Annu. Rev. Neurosci.* 29:325–362. doi:10.1146/annurev.neuro.29.051605.112808.
- King, N., M.J. Westbrook, S.L. Young, A. Kuo, M. Abedin, J. Chapman, S. Fairclough, U. Hellsten, Y. Isogai, I. Letunic, et al. 2008. The genome of the choanoflagellate *Monosiga brevicollis* and the origin of metazoans. *Nature*. 451:783–788. doi:10.1038/nature06617.
- Knöll, B., and U. Drescher. 2002. Ephrin-As as receptors in topographic projections. *Trends Neurosci.* 25:145–149. doi:10.1016/S0166-2236(00)02093-2.
- Lee, B.H., X. Min, C.J. Heise, B.E. Xu, S. Chen, H. Shu, K. Luby-Phelps, E.J. Goldsmith, and M.H. Cobb. 2004. WNK1 phosphorylates synaptotagmin 2 and modulates its membrane binding. *Mol. Cell.* 15:741–751. doi:10.1016/j.molcel.2004.07.018.
- Levin, D.E. 2005. Cell wall integrity signaling in *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* 69:262–291. doi:10.1128/MMBR.69.2.262-291.2005.
- Li, H., C.L. Waites, R.G. Staal, Y. Dobry, J. Park, D.L. Sulzer, and R.H. Edwards. 2005. Sorting of vesicular monoamine transporter 2 to the regulated secretory pathway confers the somatodendritic exocytosis of monoamines. *Neuron*. 48:619–633. doi:10.1016/j.neuron.2005.09.033.
- Littleton, J.T., H.J. Bellen, and M.S. Perin. 1993. Expression of synaptotagmin in *Drosophila* reveals transport and localization of synaptic vesicles to the synapse. *Development*. 118:1077–1088.
- Littleton, J.T., E.R. Chapman, R. Kreber, M.B. Garment, S.D. Carlson, and B. Ganetzky. 1998. Temperature-sensitive paralytic mutations demonstrate that synaptic exocytosis requires SNARE complex assembly and disassembly. *Neuron*. 21:401–413. doi:10.1016/S0896-6273(00)80549-8.
- Ludwig, M., and Q.J. Pittman. 2003. Talking back: dendritic neurotransmitter release. *Trends Neurosci.* 26:255–261. doi:10.1016/S0166-2236(03)00072-9.
- Mackler, J.M., J.A. Drummond, C.A. Loewen, I.M. Robinson, and N.E. Reist. 2002. The C(2)B Ca(2+)-binding motif of synaptotagmin is required for synaptic transmission in vivo. *Nature*. 418:340–344. doi:10.1038/nature00846.
- Maletic-Savatic, M., and R. Malinow. 1998. Calcium-evoked dendritic exocytosis in cultured hippocampal neurons. Part I: trans-Golgi network-derived organelles undergo regulated exocytosis. *J. Neurosci.* 18:6803–6813.
- Marqués, G., H. Bao, T.E. Haerry, M.J. Shimell, P. Duchek, B. Zhang, and M.B. O'Connor. 2002. The *Drosophila* BMP type II receptor Wishful Thinking regulates neuromuscular synapse morphology and function. *Neuron*. 33:529–543. doi:10.1016/S0896-6273(02)00595-0.
- McCabe, B.D., G. Marqués, A.P. Haghighi, R.D. Fetter, M.L. Crotty, T.E. Haerry, C.S. Goodman, and M.B. O'Connor. 2003. The BMP homolog Gbb provides a retrograde signal that regulates synaptic growth at the *Drosophila* neuromuscular junction. *Neuron*. 39:241–254. doi:10.1016/S0896-6273(03)00426-4.
- Miesenböck, G., D.A. De Angelis, and J.E. Rothman. 1998. Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. *Nature*. 394:192–195. doi:10.1038/28190.
- Min, S.W., W.P. Chang, and T.C. Südhof. 2007. E-Syts, a family of membranous Ca²⁺-sensor proteins with multiple C2 domains. *Proc. Natl. Acad. Sci. USA*. 104:3823–3828. doi:10.1073/pnas.0611725104.
- Paradis, S., S.T. Sweeney, and G.W. Davis. 2001. Homeostatic control of presynaptic release is triggered by postsynaptic membrane depolarization. *Neuron*. 30:737–749. doi:10.1016/S0896-6273(01)00326-9.
- Parkes, T.L., A.J. Elia, D. Dickinson, A.J. Hilliker, J.P. Phillips, and G.L. Boulianne. 1998. Extension of *Drosophila* lifespan by overexpression of human SOD1 in motoneurons. *Nat. Genet.* 19:171–174. doi:10.1038/534.
- Poopatanapong, A., I. Teramitsu, J.S. Byun, L.J. Vician, H.R. Herschman, and S.A. White. 2006. Singing, but not seizure, induces synaptotagmin IV in zebra finch song circuit nuclei. *J. Neurobiol.* 66:1613–1629. doi:10.1002/neu.20329.
- Putnam, N.H., M. Srivastava, U. Hellsten, B. Dirks, J. Chapman, A. Salamov, A. Terry, H. Shapiro, E. Lindquist, V.V. Kapitonov, et al. 2007. Sea anemone genome reveals ancestral eumetazoan gene repertoire and genomic organization. *Science*. 317:86–94. doi:10.1126/science.1139158.
- Reddy, A., E.V. Caler, and N.W. Andrews. 2001. Plasma membrane repair is mediated by Ca(2+)-regulated exocytosis of lysosomes. *Cell*. 106:157–169. doi:10.1016/S0092-8674(01)00421-4.
- Regehr, W.G., M.R. Carey, and A.R. Best. 2009. Activity-dependent regulation of synapses by retrograde messengers. *Neuron*. 63:154–170.

- Robinson, I.M., R. Ranjan, and T.L. Schwarz. 2002. Synaptotagmins I and IV promote transmitter release independently of Ca^{2+} binding in the C(2)A domain. *Nature*. 418:336–340. doi:10.1038/nature00915.
- Salinas, P.C. 2005. Retrograde signalling at the synapse: a role for Wnt proteins. *Biochem. Soc. Trans.* 33:1295–1298. doi:10.1042/BST20051295.
- Shin, O.H., W. Han, Y. Wang, and T.C. Südhof. 2005. Evolutionarily conserved multiple C2 domain proteins with two transmembrane regions (MCTPs) and unusual Ca^{2+} binding properties. *J. Biol. Chem.* 280:1641–1651. doi:10.1074/jbc.M407305200.
- Sigrist, S.J., D.F. Reiff, P.R. Thiel, J.R. Steinert, and C.M. Schuster. 2003. Experience-dependent strengthening of *Drosophila* neuromuscular junctions. *J. Neurosci.* 23:6546–6556.
- Srivastava, M., E. Begovic, J. Chapman, N.H. Putnam, U. Hellsten, T. Kawashima, A. Kuo, T. Mitros, A. Salamov, M.L. Carpenter, et al. 2008. The Trichoplax genome and the nature of placozoans. *Nature*. 454:955–960. doi:10.1038/nature07191.
- Tao, H.W., and M. Poo. 2001. Retrograde signaling at central synapses. *Proc. Natl. Acad. Sci. USA*. 98:11009–11015. doi:10.1073/pnas.191351698.
- Umemori, H., M.W. Linhoff, D.M. Ornitz, and J.R. Sanes. 2004. FGF22 and its close relatives are presynaptic organizing molecules in the mammalian brain. *Cell*. 118:257–270. doi:10.1016/j.cell.2004.06.025.
- Vician, L., I.K. Lim, G. Ferguson, G. Tocco, M. Baudry, and H.R. Herschman. 1995. Synaptotagmin IV is an immediate early gene induced by depolarization in PC12 cells and in brain. *Proc. Natl. Acad. Sci. USA*. 92:2164–2168. doi:10.1073/pnas.92.6.2164.
- von Poser, C., K. Ichtchenko, X. Shao, J. Rizo, and T.C. Südhof. 1997. The evolutionary pressure to inactivate. A subclass of synaptotagmins with an amino acid substitution that abolishes Ca^{2+} binding. *J. Biol. Chem.* 272:14314–14319. doi:10.1074/jbc.272.22.14314.
- Wu, Y., F. Kawasaki, and R.W. Ordway. 2005. Properties of short-term synaptic depression at larval neuromuscular synapses in wild-type and temperature-sensitive paralytic mutants of *Drosophila*. *J. Neurophysiol.* 93:2396–2405. doi:10.1152/jn.01108.2004.
- Yoshihara, M., and J.T. Littleton. 2002. Synaptotagmin I functions as a calcium sensor to synchronize neurotransmitter release. *Neuron*. 36:897–908. doi:10.1016/S0896-6273(02)01065-6.
- Yoshihara, M., B. Adolfsen, and J.T. Littleton. 2003. Is synaptotagmin the calcium sensor? *Curr. Opin. Neurobiol.* 13:315–323. doi:10.1016/S0959-4388(03)00063-1.
- Yoshihara, M., B. Adolfsen, K.T. Galle, and J.T. Littleton. 2005. Retrograde signaling by Syt 4 induces presynaptic release and synapse-specific growth. *Science*. 310:858–863. doi:10.1126/science.1117541.
- Zweifel, L.S., R. Kuruvilla, and D.D. Ginty. 2005. Functions and mechanisms of retrograde neurotrophin signalling. *Nat. Rev. Neurosci.* 6:615–625. doi:10.1038/nrn1727.