Targeted Mutations in the Syntaxin H3 Domain Specifically Disrupt SNARE Complex Function in Synaptic Transmission

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The cytoplasmic H3 helical domain of syntaxin is implicated in numerous protein–protein interactions required for the assembly and stability of the SNARE complex mediating vesicular fusion at the synapse. Two specific hydrophobic residues (Ala-240, Val-244) in H3 layers 4 and 5 of mammalian syntaxin1A have been suggested to be involved in SNARE complex stability and required for the inhibitory effects of syntaxin on N-type calcium channels. We have generated the equivalent double point mutations in Drosophila syntaxin1A (A243V, V247A; syx4 mutant) to examine their significance in synaptic transmission in vivo. The syx4 mutant animals are embryonic lethal and display severely impaired neuronal secretion, although non-neuronal secretion appears normal. Synaptic transmission is nearly abolished, with residual transmission delayed, highly variable, and nonsynchronous, strongly reminiscent of transmission in null synaptotagmin I mutants. However, the syx4 mutants show no alterations in synaptic protein levels in vivo or syntaxin partner binding interactions in vitro. Rather, syx4 mutant animals have severely impaired hypertonic saline response in vivo, an assay indicating loss of fusion-competent synaptic vesicles, and in vitro SNARE complexes containing Syx4 protein have significantly compromised stability. These data suggest that the same residues required for syntaxin-mediated calcium channel inhibition are required for the generation of fusion-competent vesicles in a neuronal-specific mechanism acting at synapses.

Key words: Drosophila; SNARE complex; core complex; syntaxin; synaptotagmin; calcium channel
at the synapse. Binding assays with Syx\(^4\) show normal biochemical interactions with syntaxin binding partners, Syx\(^4\) displays in vitro SNARE complex formation, and these interactions are consistent with the normal non-neuronal secretion observed in mutants. However, syx\(^4\) displays severely compromised neurotransmission, including a high rate of failures. Residual responses display decreased amplitude and increased variability and are temporally uncoupled from the stimulus. The syx\(^4\) mutations also compromise the stability of the SNARE complex in vitro and severely reduce the response to hyperosmotic saline application in vivo. Our results indicate that the same H3 residues that mediate Ca\(^{2+}\) channel inhibition also govern SNARE complexes through increased complex stability/assembly. We propose that these coupled processes ensure rapid SNARE complex formation and excitation-secretion coupling at the active zone.

**MATERIALS AND METHODS**

Generation of the syx\(^4\) mutant. Site directed mutagenesis in vivo was performed as described (Wu et al., 1999). Briefly, mutations in the syntaxin open reading frame (ORF) (A243V, V247A) were generated using the Quikchange kit (Stratagene, La Jolla, CA). After sequencing, the mutated ORF was subcloned (XhoI-KpnI) into a 13.5 kb genomic rescue fragment in pCaSpeR3 (Pirrotta, 1998). Independent transgenic lines bearing this construct were generated as described (Rubin and Spradling, 1982) and crossed into the null syx\(^{229}\) background. Flies were balanced over TM6B, Tb Hu (Lindsley and Zimm, 1992) or TM3, Kr-GFP (a gift of D. Casso and T. Kornberg, University of California San Francisco). Mutant embryos were identified by the absence of the Green Fluorescent Protein (GFP) balancer or by using outcrossed strains for the mutant ORF was subcloned (Lindsley and Zimm, 1992) or cross between control (Maier et al., 1990) was used at 1:1000. Cuticles were prepared as described (Harrison et al., 1994), except that blots were performed as described (Wu et al., 1999). Briefly, fly heads were crushed in a mortar and pestle in buffer B (5 mm HEPES, pH 7.4, 100 mM NaCl, 2 ml/ml heads). After homogenization with a Dounce homogenizer, cuticular debris was pelleted at 5000 x g. Membranes were solubilized with 1% Triton X-100 at 4°C for 1 hr, and insoluble material was removed by spinning at 50,000 rpm for 20 min in a TL-100.2 rotor. GST-syntaxin protein (0.25 μg) was incubated O/N at 4°C with 500 μg of head extract. Beads were washed as above. Proteins on beads were released by boiling in 20 μl sample buffer, and bands were detected by Western blotting and ECL. Antibodies were used as described (Schulze et al., 1995). Synprint was detected using anti-Xpress primary antibody 1:5000 (Invitrogen, Carlsbad, CA). For dose–response binding curves, GST, GST-syntaxin, or GST-Syx\(^4\) bound to glutathione-Sepharose beads was incubated with SNAP-25 (0.5 μM) and n-synaptobrevin (0.01, 0.02, 0.05, 0.1, 0.2 μM) or synaptotagmin (0.05, 0.02, 0.5, 1, 1.5 μM) or CSP (0.02, 0.2, 0.5, 1, 1.5 μM) in 200 μl. Known amounts of n-synaptobrevin or synaptotagmin, or CSP, were added on the same gel as standards. Band intensities were quantified using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA). Binding curves with values that fell within the linear range were used.

Binding of core complex proteins to GST-synaptotagmin I was performed as described (Gerona et al., 2000). Incubations of n-synaptobrevin, syntaxin, Syx\(^{4}\), and SNAP-25 (1 μM) were performed overnight at 4°C in binding buffer (0.02 μM HEPES, pH 7.6, 0.15 μM potassium acetate, 0.5% Triton X-100, and 0.5% bovine serum albumin) to generate binary and ternary complexes. After the preincubation, complexes were diluted to 0.2 μM with binding buffer and incubated with 2 μg GST-Dyst2 (aa 134–474 of synaptotagmin I, provided by J. Troy Littleton, Massachusetts Institute of Technology, Cambridge, MA) bound to glutathione-Sepharose beads. The reactions were supplemented with 2 μM EGTA or 1 μM CaCl\(_2\). After 2 hr incubation at room temperature, the beads were washed three times with 1 ml wash buffer (0.02 μM HEPES, pH 7.6, 0.15 μM potassium acetate, 0.5% Triton X-100, and 1% bovine serum albumin) to remove unbound proteins, and 10 μl were washed twice with 10 μl of PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na\(_2\)HPO\(_4\), 1.8 mM KH\(_2\)PO\(_4\), pH 7.3). SDS-PAGE and Coomassie blue staining were used to visualize core complex proteins, proteolytic cleavage, and glycosylation of binary serum albumin as a standard. Typical binding incubations used 0.15–0.30 μM GST-syntaxin bound to glutathione-Sepharose beads and 2 μM n-synaptobrevin, 1 μM SNAP-25, 0.3–0.6 μM synaptotagmin I (Syx\(^{4}\), 2 μM synprint, or 1 μM CSP in a total volume of 200 μl with buffer A. Binding was generally performed for 1–2 hr at 4°C, except for SNAP-25 and ternary core complex formation [overnight (O/N)]. Beads were washed two times with buffer A + 1 mg/ml gelatin and three times with buffer A + 5% glycerol. Because no N-type synprint has been clearly identified in *Drosophila*, we used the mammalian N-type synprint as a surrogate, assuming conservation of structural homology between species. In our assay, because synaptotagmin and CSP showed nonspecific binding to beads, 20–100 μg of bacterial extract was added to those binding assays as a nonspecific competitor (Assubel, 1996). Because we were unable to produce soluble recombinant ROP, we detected the syntaxin–ROP interaction by performing pull-down experiments from head extracts. Briefly, fly heads were crushed in a mortar and pestle in buffer B (5 mM HEPES, pH 7.4, 100 mM NaCl, 2 ml/ml heads). After homogenization with a Dounce homogenizer, cuticular debris was pelleted at 5000 x g. Membranes were solubilized with 1% Triton X-100 at 4°C for 1 hr, and insoluble material was removed by spinning at 50,000 rpm for 20 min in a TL-100.2 rotor. GST-syntaxin protein (0.25 μg) was incubated O/N at 4°C with 500 μg of head extract. Beads were washed as above. Proteins on beads were released by boiling in 20 μl sample buffer, and bands were detected by Western blotting and ECL. Antibodies were used as described (Schulze et al., 1995). Synprint was detected using anti-Xpress primary antibody 1:5000 (Invitrogen, Carlsbad, CA). For dose–response binding curves, GST, GST-syntaxin, or GST-Syx\(^4\) bound to glutathione-Sepharose beads was incubated with SNAP-25 (0.5 μM) and n-synaptobrevin (0.01, 0.02, 0.05, 0.1, 0.2 μM) or synaptotagmin (0.05, 0.02, 0.5, 1, 1.5 μM) or CSP (0.02, 0.2, 0.5, 1, 1.5 μM) in 200 μl. Known amounts of n-synaptobrevin or synaptotagmin, or CSP, were added on the same gel as standards. Band intensities were quantified using a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA). Binding curves with values that fell within the linear range were used.

Electrophysiological analysis. The syntx\(^4\) mutants are late embryonic lethal, and therefore electrophysiological recordings were performed at the embryonic neuromuscular junction (NMJ) as reported previously (Broadie and Bate, 1993; Wu et al., 1999). All recordings were made at 18°C using standard whole-cell patch-clamp (~60 mV) techniques from muscle 6 in anterior abdominal segments A2–A3 at 22–24 hr after fertilization (incubated at 25°C). Excitatory junctional currents (EJCs) were evoked by brief stimulation of the motor nerve (1 msec) with positive current using 25–30 μA of stimulus current. Mean EJC amplitudes were determined from 25 consecutive EJCs evoked at each frequency, including response failures. Data were acquired and analyzed using PCLAMP 6.0 software (Axon Instruments, Foster City, CA). All miniature EJC (mEJC) recordings were done in 0.1 μM tetrodotoxin (TTX; Sigma, St. Louis, MO) at 0.5 mM external Ca\(^{2+}\). mEJC amplitude and frequency were analyzed using Mini Analysis software 3.0 (Jaejin Software, Leonia, NJ). Calcium dependence of evoked transmission was characterized by the power relationship of basal mEJC amplitudes at 0, 0.1, 0.4, and 1.0 μM Ca\(^{2+}\) concentrations (Broadie et al., 1994). Hyperosmotic saline, consisting of bath saline with 850 mM sucrose added, was pressure ejected onto the neuromuscular junction for 3 sec using an unpolished patch pipette (Aravamudan et al., 1999). Statistical analyses were done with Instat (Graphpad software, San Diego, CA). All significance values were calculated using Mann–Whitney U tests.
RESULTS

Targeted mutation of syntaxin H3 residues A243 and V247

The syntaxin H3 cytosolic domain, which mediates coiled-coil interactions with other members of the SNARE complex, is highly conserved across species and absolutely required for vesicular fusion (Schulze et al., 1995; Wu et al., 1999). Specific H3 residues support different protein-binding interactions, which both repress and enhance the efficiency of excitation-secretion coupling at Drosophila synapses (Wu et al., 1999). Two highly conserved H3 residues in mammalian syntaxin (Ala-240, Val-244) have been suggested to be required for SNARe complex stability and syntaxin-mediated inhibition of N-type calcium channels in vitro (Kee et al., 1995; Bezprozvanny et al., 2000). We generated double point mutations in the equivalent residues in Drosophila syntaxin1A using methods identical to our earlier mutational analyses of the H3 domain (Wu et al., 1999). The two point mutations (A243V, V247A) disrupt residues that lie at the end of the H3 coiled-coil domain in hydrophobic layers 4 and 5 within the core complex-forming bundle (Kee et al., 1995) and just outside the “Ca\(^{2+}\) effector domain” characterized previously (Fig. 1ab) (Wu et al., 1999). Both the mutant form of syntaxin (syx\(^4\)) and wild-type syntaxin (syx\(^{wt}\)) were introduced into the Drosophila genome using transgenic constructs (see Materials and Methods).

Transgenic animals bearing either the genomic rescue syx\(^{wt}\) construct or the genomic syx\(^4\) construct were crossed into a syntaxin null deletion mutant (syx\(^{229}\)) background (Schulze et al., 1995). Multiple insertion lines of each construct were compared with Western blots for protein expression levels. Figure 2 shows that two different lines of both syx\(^{wt}\) and syx\(^4\) constructs in the syx\(^{229}\) null background express similar levels of syntaxin protein. These data show that the syx\(^4\) mutations do not significantly alter levels of syntaxin protein in vivo, compared with syx\(^{wt}\) controls. Likewise, different transgenic lines for both constructs display similar levels of syntaxin expression (Fig. 2), showing that there are no significant position effects on transgene expression. To determine whether the syx\(^4\) mutations alter the expression of other proteins implicated in synaptic transmission, Western blots were probed for ROP (Munc-18 homolog), synaptotagmin I, and CSP. As shown in Figure 2, the levels of these proteins are similar between syx\(^{wt}\) and syx\(^4\) embryos and also between different transgenic lines of each construct. To examine the spatial and temporal localization of syntaxin and synaptotagmin I, immunocytochemical staining of embryos was performed. Immunocytochemistry revealed an indistinguishable level and distribution of both proteins in multiple syx\(^{wt}\) and syx\(^4\) lines (data not shown). Hence, protein levels and distribution of all proteins tested in syx\(^4\) mutants were indistinguishable from wild-type controls. We used these transgenic animals to assay the function of the disrupted residues in vesicle fusion in vivo.

syx\(^4\) mutants display defects in neuronal but not non-neuronal secretion

We first assessed the gross phenotypes of the syx\(^4\) mutants. All syx\(^4\) phenotypic analyses were performed in the syx null (syx\(^{229}\)) background. We have shown previously that syx\(^{229}\) embryos are late embryonic lethal (Schulze et al., 1995). The wild-type genomic construct (syx\(^{wt}\)) can rescue null (syx\(^{229}\)) mutants to adulthood (Fig. 3a), demonstrating the normal function of the transgenic protein. In contrast, the genomic construct containing the syx\(^4\) mutation is fully embryonic lethal in the null background (Fig. 3a). Hence, the syx\(^4\) mutations must cause a severe loss of syntaxin function.

We and others have shown previously that syntaxin is absolutely required for both neuronal and non-neuronal secretory events in Drosophila (Schulze et al., 1995; Schulze and Bellen, 1996; Burgess et al., 1997). For example, epidermal cells secrete cuticular proteins from their apical surface; hence, this process represents a polarized form of vesicle transport similar to neurotransmission. Mature wild-type embryos display numerous cuticular structures, most obviously including segmental denticle belts and anterior mouth hooks (Fig. 3b, control). In contrast, syx null mutant embryos (syx\(^{229}\)/syx\(^{229}\)) fail to secrete detectable cuticle and show a complete absence of denticle belts and mouth hooks (Fig. 3b, syx\(^{229}\)). Surprisingly, the cuticular features of syx\(^4\) mutants (syx\(^4\)/syx\(^{wt}\); syx\(^{229}\)/syx\(^{229}\)) are indistinguishable from wild-type conditions in vivo.
controls and contain normal segmental denticles and mouth hooks developed from cuticle secretion (Fig. 3b, syx4). The syx4 mutant embryos appear to have normally structured tissues in general, in sharp contrast to syx4null, which display grossly abnormal gut and nerve cord development (data not shown) (Schulze et al., 1995). These data show that the syx4 mutations do not detectably impair non-neuronal secretion, suggesting that H3 residues A243 and V247 play an important role in a process constitutive secretory processes.

Mature wild-type embryos display robust, neurally driven peristaltic muscle contractions before hatching. Spontaneous contractions strongly resemble postembryonic locomotory movement, and tactile stimulation increases the strength and frequency of this movement. Null syx4null mutants display a complete absence of both evoked and spontaneous coordinated movement attributable to a complete block of neurotransmission (Fig. 3a) (Schulze et al., 1995). As expected, the syx4null genomic construct rescues both spontaneous and touch-evoked movement phenotypes (Fig. 3a). Likewise, the syx4 mutant embryos, unlike syx4null mutants, show spontaneous movement and touch-evoked muscle contraction. However, both behaviors are impaired, suggesting that neumuscular transmission is reduced but not abolished (Fig. 3a). The syx4 mutants display an approximately fourfold reduction in the frequency of muscular contraction waves compared with wild-type controls (syx4null/syx4null; syx4null/syx229; syx229/syx229 = 1.7 ± 0.2 contractions per minute, n = 20; syx4null/syx4null; syx229/syx229 = 0.4 ± 0.1 contractions per minute, n = 11; p < 0.01). These results suggest that the H3 residues A243 and V247 play an important role in a process specific for neuronal secretion at the synapse.

syx4 nulls mutants display severely impaired excitation–secretion coupling

Targeted mutations in Drosophila syntaxain cause striking alterations in synaptic transmission, ranging from a complete loss of transmission in null mutants and a H3 deletion through marked elevated transmission in some H3 point mutations, revealing different regulatory functions of specific protein interactions (Broadie et al., 1995; Schulze et al., 1995; Wu et al., 1999). To address the in vivo role of H3 residues A243 and V247 in neurotransmission, whole-cell patch-clamp recordings were performed at the NMJ of syx4null and syx4 transgenic embryos. As shown in Figure 4, a and b, evoked EJC amplitude is severely reduced in syx4 nulls, to ~10% of the levels of syx4null transgenic controls.
(1.1 ± 0.1 nA for syxwt, n = 19; 0.12 ± 0.02 nA for syx4, n = 8; p < 0.0001). An identical phenotype was observed in three independent transgenic lines (Fig. 4b). Transmission in syx4 NMJs was similarly severely reduced in all external [Ca\(^{2+}\)] from 0.2 to 1.8 mM, but the Ca\(^{2+}\) cooperativity of transmission was similar for syx4 and syxwt controls (1.59 for syxwt; 1.47 for syx4). These data show that H3 residues A243 and V247 play a central, but nonessential, function in synaptic transmission and explain the embryonic lethality and severe loss of movement observed in the syx4 mutants.

Neurotransmission in syx4 mutants is characterized by several other obvious defects, which have been observed previously only in synaptotagmin null mutants in Drosophila (Broadie et al., 1994) and after disruption of the excitosome by synprint peptide injection (Mochida et al., 1996; Wiser et al., 1999). Specifically, as shown in Figure 4, a and c–e, neurotransmitter release in syx4 mutants is strikingly asynchronous, demonstrates low fidelity to identical stimuli, and exhibits a high failure rate. In control syxwt NMJ synapses, stimulation-evoked transmission occurs consistently within −5 msec after nerve stimulation, whereas in syx4 mutants, evoked release occurs at delayed (twofold) latencies (Fig. 4c) (5.8 ± 0.2 msec for syxwt, n = 21; 10.4 ± 0.5 msec for syx4, n = 8; p < 0.0001). This increased latency suggests reduced kinetics of excitation–secretion coupling. As shown in Figure 4d, syx4 mutants also show dramatically increased (fourfold) variability in the amount of neurotransmitter released per stimulus, compared with controls (coefficient of variation 0.31 ± 0.04 for syxwt, 1.2 ± 0.08 for syx4; p < 0.0001). Finally, although syx4 controls always release neurotransmitter in response to nerve stimulation in 1.8 mM extracellular Ca\(^{2+}\) (no failures), syx4 mutants fail to respond >50% of the time (Fig. 4e). Together, the strongly reduced, asynchronous, delayed, and variable release, combined with a high failure of evoked neurotransmission, indicate that excitation–secretion coupling of neurotransmitter release is severely impaired in syx4 mutants.

We next assayed spontaneous vesicle fusion in the absence of action potentials by recording mEJCs in the presence of TTX. As shown in Figure 5, mEJC amplitude is slightly increased in syx4 mutants (0.19 ± 0.02 nA for syxwt, n = 10; 0.26 ± 0.01 nA for syx4, n = 12; p < 0.01), but no changes in the kinetic of transmitter release were observed. The underlying reason for the increase in quantal size is unclear, but the increase is specific for the syx4 mutation, because other syntaxin point mutations that we have analyzed do not show an increase (Wu et al., 1999). Thus, because mEJC amplitude is increased in syx4 mutants, this result demonstrates that the postsynaptic receptor field is present and the severe decrease in syx4 neurotransmission is attributable to a presynaptic defect. In line with evoked defects, syx4 mutants reveal a significant decrease in mEJC frequency (0.042 ± 0.010 Hz for syx4, n = 11; 0.023 ± 0.006 Hz for syx4, n = 13; p < 0.05). This result suggests that core complexes containing Syx4 protein show a decreased ability to mediate vesicular fusion. In conclusion, syx4 mutants reveal a striking impairment, but not abolishment, of both evoked (Fig. 4) and spontaneous (Fig. 5) fusion events at the synapse.

**Syntaxin interactions are maintained with the syx4 mutations, but core complex stability is impaired**

What role do the syntaxin H3 residues A243 and V247 play that is so crucial to excitation–secretion coupling in the presynaptic terminal? Can we explain why these residues are central to secretion at presynaptic terminals but appear to play no role in non-neuronal secretion? We have shown previously that point mutations in the H3 domain of syntaxin can alter binding of specific syntaxin partners (Wu et al., 1999). Many of these proteins act as specific mediators of neuronal, but not non-neuronal, syntaxin function. One possibility is that the Syx4 mutations disrupt one or more of these known syntaxin interactions. Specifically, the physiological phenotype was very suggestive of an impairment of Syx4 interaction with synaptotagmin I, a putative Ca\(^{2+}\) sensor (Broadie et al., 1994).

To test whether the mutant Syx4 protein has altered interactions with known syntaxin binding partners, GST-pull-down assays were performed with GST alone (Fig. 6a, GST), GST-syntaxin (Fig. 6a, Syxwt), and GST-Syx4 (Fig. 6a, Syx4). GST alone did not bind any of the assayed proteins, including SNAP-25, n-synaptobrevin (within the ternary complex), ROP, synaptotagmin I, synprint, or CSP. However, GST-syntaxin and GST-Syx4 were both capable of interacting similarly with each of these binding partners (Fig. 6a). As shown previously by Kee et al. (1995), GST-Syx4 does show a reduction in this binary binding assay with n-synaptobrevin, compared with GST-syntaxin (data not shown). However, as described previously, this binary interaction is weak, easily disrupted, and unlikely to be physiologically significant (Kee et al., 1995; Wu et al., 1999).

More detailed binding assays were performed to specifically examine core complex formation as well as the possible interactions with synaptotagmin I and CSP (Fig. 6b–d). Similar dose–response binding curves for Syx4wt and Syx4 were obtained for each of these proteins: n-synaptobrevin, synaptotagmin I, and CSP. Together, these data suggest that the Syx4 mutations do not detectably alter binding of syntaxin to synaptotagmin I, CSP, SNAP-25, ROP, and synprint and do not alter core complex formation, as measured by a steady-state assay.

The C2A and C2B domains of synaptotagmin I bind the four-
helical bundle of the SNARE complex (Davis et al., 1999; Gerona et al., 2000) and may mediate or trigger Ca\(^{2+}\)-dependent exocytosis. Furthermore, the role of synaptotagmin in exocytosis may begin very early in SNARE complex formation (vesicle docking) (Reist et al., 1998) because it has been shown recently to also stabilize layers (Hayashi et al., 1994) of the H3 coiled-coil domain (Fig. 5). We assayed Drosophila SNARE complex stability containing either Syx\(^{wt}\) or Syx\(^{4}\). The graph shows the percentage binding to GST-Syt as quantified using \(^{125}\)I-labeled secondary antibody. Percentage binding was normalized, with the highest pixel value for each individual experiment being assigned 100%, from four independent experiments: syntaxin with EGTA, 59%; syntaxin with Ca\(^{2+}\), 99%; Syx\(^{4}\) with EGTA, 58%; Syx\(^{4}\) with Ca\(^{2+}\), 98%. Each bar represents the average of four independent experiments ± SEM. *p < 0.05.

Therefore, that the syx\(^{4}\) mutation does not appear to consistently alter the binding between the core complex and synaptotagmin I. Can the Syx\(^{4}\) mutations affect SNARE complex function directly? The core complex normally forms a highly stable four-helical bundle. The center of this SNARE bundle contains an ionic “layer” flanked by hydrophobic layers that mediate stabilizing interactions within the bundle of the core complex (Fasshauer et al., 1998; Sutton et al., 1998). The syx\(^{4}\) mutations lie in these stabilizing layers (+4 and +5) of the H3 coiled-coil domain (Fig. 1). Hayashi et al. (1994) have shown that the ternary core complex is resistant to SDS-denaturation up to 60°C. We assayed Drosophila SNARE complex stability containing either Syx\(^{wt}\) or Syx\(^{4}\).

We bound soluble His-synaptobrevin and His-SNAP-25 to immobilized GST-syntaxin and GST-Syx\(^{4}\) to examine the SDS resistance and heat lability of the complexes containing these variant proteins. As shown in Figure 8a, SDS-resistant core complexes migrate at \(~110\) kDa, and higher molecular weight bands are also present that likely represent a dimeric form (Hayashi et al., 1994; Hao et al., 1997), recently found to be increased by synaptotagmin I in the presence of Ca\(^{2+}\) (Littleton et al., 2001).
The wild-type core complex is stable through 54°C and partially denatured at 60°C in a sample buffer containing 2% SDS (Fig. 8b). In contrast, core complexes made with Syx^4 denature at much lower temperatures. The Syx^4 complexes remain stable up to 25°C (Fig. 8a) but are degraded at 37°C and undetectable above ~48°C (Fig. 8b). These observations show that the syx^4 mutation impairs the stability of the core complex and may provide a mechanistic explanation for the impaired excitation-secretion coupling in syx^4 synapses.

**syx^4** mutant synapses have significantly fewer SNARE complexes

To test the SV fusion competence in syx^4 mutants, the synaptic response to hyperosmotic saline application, which requires functional core complexes, was assayed (Rosenmund and Stevens, 2001). A 3 sec focal burst of hypertonic saline was applied to syx^4 mutants at 25°C, similar to control complexes (Syx^wt). Complexes were challenged in sample buffer for 5 min at the temperature shown. The lower molecular weight complexes (asterisk) correspond to the trimeric SNARE complex, whereas the higher molecular weight bands likely represent a dimeric form. Note the increased instability of complexes containing Syx^4 relative to Syx^wt protein.

**DISCUSSION**

Specific amino acids in the hydrophobic “layers” of the SNARE complex interact with a number of regulatory proteins to control the efficacy of neurotransmission (Littleton et al., 1998; Saifee et al., 1998; Wu et al., 1999). The two specific amino acids in the syntaxin H3 domain investigated here (A243, V247) have been proposed to mediate SNARE complex stability (Kee et al., 1995) and, more recently, to mediate calcium channel inhibition (Bez-
prozvanny et al., 2000). Our aim was to mutate these residues (syx4 mutant) to assay their significance during in vivo secretory events.

In Drosophila, syx1A is absolutely required for all vesicular fusion events throughout the animal (Schulze et al., 1995); null syntaxin mutants abolish both non-neuronal and neuronal secretion. In contrast, syx4 mutants display no detectable defects in non-neuronal secretion but rather specifically impaired synaptic transmission. These data show that constitutive vesicle fusion does not require residues A243 and V247 in the syntaxin H3 domain, implicating this site in mediating a process specifically involved in calcium-dependent synaptic vesicle fusion. Interaction with N-type Ca2+ channels is an obvious and attractive explanation for this synapse-specific function (Bezprozvanny et al., 2000). However, this interaction has been proposed to inhibit Ca2+ influx, which is not necessarily consistent with observed phenotypes. The syx4 mutants display a striking impairment of synaptic excitation–secretion coupling: action potential-evoked release reduced by ~90% and residual transmission, which is highly asynchronous, variable, and prone to failure. Thus, syx4 mutants are not capable of properly triggering robust, synchronized synaptic vesicle fusion in response to a Ca2+ influx. These defects are more consistent with an inability to rapidly generate functional SNARE complexes, as predicted (Kee et al., 1995).

The syx4 synaptic phenotypes are clearly distinct from those associated with other engineered point mutations in the H3 domain of syntaxin (Wu et al., 1999). However, the phenotypes are strikingly similar to those described previously for both the synaptotagmin I null mutant (Broadie et al., 1994) and the syx4P/C mutant, which deletes the Ca2+-effector domain to severely reduce binding to synaptotagmin I (Wu et al., 1999). The syx4 phenotypes also resemble the unreliable transmission observed in wild-type synapses at low (~0.4 mm) extracellular Ca2+ concentrations (Broadie et al., 1994; Wu et al., 1999). On the basis of these phenotypic similarities, it appears possible that core complex function in vivo is modulated at least in part by synaptotagmin I and that the syx4 mutations impair this regulation.

We tested this hypothesis by assaying the protein binding properties of syx4 but were unable to identify impaired binding to synaptotagmin I, CSP, ROP/MUNC-18, the Ca2+ channel synprint site, or other members of the core complex. In particular, in numerous assays synaptotagmin I binding of the Syx4 core complex was not significantly different from controls, other than a dramatic increase in the variability of binding in the presence of Ca2+ (Fig. 7b). The increased variability of synaptotagmin I binding to the Syx4 core complex may possibly indicate that rapid core complex formation in syx4 mutants is impaired, because synaptotagmin I has recently been shown to accelerate core complex formation (Littleton et al., 2001). This is consistent with the evidence provided here showing a strong reduction of hyperosmotic saline-induced transmitter release in both synaptotagmin null (syx1D4) and syx4 mutant synapses (Fig. 9). Although Syx4 containing core complexes can be formed in vitro, on the basis of a steady-state assay, the resulting complexes display impaired stability manifested by increased heat lability. These observations suggest that the formation of the SNARE complex in vivo, which underlies neurotransmission, may be more rapid and substantially different from complex formation in vitro. These observations might reasonably explain why syx4 does not detectably perturb the slow, constitutive vesicle fusion in non-neuronal tissues, whereas it dramatically impairs the fast, Ca2+-dependent fusion at synapses.

Syntaxin, synaptotagmin, and SNAP-25 all dynamically interact with calcium channels and modify channel current properties (Wiser et al., 1996; Wiser et al., 1997; Catterall, 1998). Through these interactions, calcium channels have also been implicated in SNARE complex formation (Sheng et al., 1998; Seagar et al., 1999), possibly through an intermediate termed the excitosome where syntaxin, SNAP-25, and synaptotagmin all bind the channel in a complex awaiting the vesicle and its v-SNARE, synapto-binding (Wiser et al., 1999). Simplistically, the inhibition of Ca2+ influx by syntaxin (Bezprozvanny et al., 1995) predicts a negative role for the syntaxin–calcium channel interaction on neurotransmission. Therefore, removal of syntaxin-mediated inhibition of Ca2+ influx should result in increased presynaptic Ca2+ levels and increased vesicle fusion and transmission. However, we show that the double point mutations that remove syntaxin-mediated inhibition of calcium channels in vitro (Bezprozvanny et al., 2000) result in severely reduced transmission. We show here that these same residues of syntaxin are critical for normal response to hyperosmotic saline application. Therefore, these residues may play a coupled role in the regulation of Ca2+ channels and SNARE complexes, perhaps through the formation of an excitosome intermediate (Catterall, 1998; Wiser et al., 1999).

In Drosophila, we do not know which Ca2+ channels are present at presynaptic active zones and interact with the presynaptic SNARE complex. Therefore, we can provide no direct evidence for Drosophila syntaxin inhibiting calcium channels. However, the syntaxin interaction is maintained through different calcium channel types in vertebrates (Bezprozvanny et al., 1995; Wiser et al., 1999), and the specific residues mediating the interaction are highly conserved in Drosophila (Fig. 1). Thus, one focus of this study was to examine the significance of these calcium channel-inhibiting residues in vivo. Aberrant calcium channel openings, in the absence of syntaxin-mediated inhibition, might result in impaired excitation–secretion; however, because voltage activation of the channel is unaffected (Bezprozvanny et al., 2000) and mEJCs are less frequent in syx4 mutants (Fig. 4b), this is unlikely. Presently, the only functional link for the syntaxin–calcium channel interaction is through syntaxin residues 240 and 244 (243 and 247 in Drosophila). Therefore, alteration of these residues may impair the function of the SNARE complex by disruption of a calcium channel/excitosome intermediate.

If we have disrupted the only conserved syntaxin–Ca2+ channel interaction, as we believe, these data provide strong evidence for a positive role for this interaction. This model does not exclude an inhibitory role for syntaxin in calcium channel gating (Bezprozvanny et al., 2000) but suggests that these syntaxin residues, and the syntaxin–calcium channel interaction, are important for more than just inhibiting inappropriate Ca2+ influx. Examination of the interaction between syntaxin and Ca2+ channels may best be done by altering the Ca2+ channel instead of the multifunctional syntaxin, once the non-synprint site of interaction is identified.

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