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

Tim Fergestad,¹ Mark N. Wu,² Karen L. Schulze,³ Thomas E. Lloyd,² Hugo J. Bellen,^{2,3,4} and Kendal Broadie¹

¹Department of Biology, University of Utah, Salt Lake City, Utah 84112-0840, and Departments of ²Molecular and Cellular Biology and ³Molecular and Human Genetics, ⁴Howard Hughes Medical Institute, Baylor College of Medicine, Houston, Texas 77030

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; *syx*⁴ mutant) to examine their significance in synaptic transmission *in vivo*. The *syx*⁴ 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 1* mutants. However, the *syx*⁴ mutants show no alterations in synaptic protein levels *in vivo* or syntaxin partner binding interactions *in vitro*. Rather, *syx*⁴ 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 *Syx*⁴ 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

Syntaxin is a t-SNARE expressed ubiquitously in the plasma membrane, but it acts as the central member of the core complex mediating synaptic vesicle (SV) fusion only at presynaptic active zones (Jahn and Hanson, 1998; Weber et al., 1998; Chen et al., 1999). Targeted vesicle fusion is regulated by a large number of syntaxin-binding interactions that control its functional conformation (Bajjalieh and Scheller, 1995; Dulubova et al., 1999; Seagar et al., 1999; Brunger, 2000; Yang et al., 2000). Immunoprecipitation studies using anti-p35 (syntaxin) antibody suggested an interaction between syntaxin and N-type calcium channels (Bennett et al., 1992), an interaction widely postulated to tether synaptic vesicles at the sites of Ca²⁺ influx (Rettig et al., 1997). Nevertheless, the significance of this proposed tethering interaction is unclear because synaptic vesicle docking occurs normally in *Drosophila* syntaxin null mutants (Broadie et al., 1995).

More recently, coexpression of syntaxin and calcium channels in *Xenopus* oocytes has suggested a functional role for this interaction, because syntaxin appears to attenuate Ca²⁺ influx and

slow the kinetics of channel inactivation (Bezprozvanny et al., 1995). Syntaxin cleavage by botulinum toxin C1 results in increased Ca²⁺ influx in purified synaptosomes, further suggesting that syntaxin inhibits calcium channel function (Bergsman and Tsien, 2000). It has been proposed that ubiquitous syntaxin inhibits Ca²⁺ influx in extrasynaptic membrane, permitting Ca²⁺ influx only in the presence of a synaptic vesicle (SNARE complex formation) at the active zone. Thus, the formation and stability of the SNARE complex might be linked directly to the interaction of syntaxin and the calcium channel.

Syntaxin, SNAP-25, and synaptotagmin all show biochemical and functional interactions with calcium channels (Leveque et al., 1992; Abe et al., 1993; Sheng et al., 1997; Wiser et al., 1999; Wu et al., 1999; Zhong et al., 1999). These proteins interact with each other to couple SNARE complex and calcium channel function (Wiser et al., 1996, 1997; Sheng et al., 1998; Tobi et al., 1998; Seagar et al., 1999). Formation of a quarternary complex of syntaxin, SNAP-25, synaptotagmin, and the calcium channel, termed the “excitosome” (Wiser et al., 1999), has been proposed to be required for efficient, synchronous neurotransmitter release at active zones (Kim and Catterall, 1997). Importantly, syntaxin inhibits N-type calcium channels through a site distinct from the synprint cytosolic loop of the calcium channel. This regulation is specifically disrupted by two point mutations (A240V and V244A) in the syntaxin H3 helical domain (Bezprozvanny et al., 2000). It has been proposed that this specific interaction provides a mechanism for coupling excitosome function with Ca²⁺ influx at active zones.

Our goal in this study was to introduce the comparable double point mutations (A243V and V247A; *syx*⁴ mutant) into *Drosophila* syntaxin1A to assay the impact on neurotransmitter release

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T.F. and M.N.W. contributed equally to this work.

Correspondence should be addressed to Dr. Kendal Broadie, Department of Biology, University of Utah, 257 South 1400 East, Salt Lake City, UT 84112. E-mail: broadie@biology.utah.edu.

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at the synapse. Binding assays with Syx⁴ show normal biochemical interactions with syntaxin binding partners, Syx⁴ 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 Ca²⁺ 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 mutant ORF was subcloned (*Xba*I–*Kpn*I) into a 13.5 kb genomic rescue fragment in pCaSpeR3 (Pirrotta, 1988). 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 which all non-hatchers were mutant embryos.

Phenotypic characterization of embryos. Mutant embryonic Western blots were performed as described (Harrison et al., 1994), except that four embryos were used per lane rather than one. Proteins were detected by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech, Piscataway, NJ). I378 (anti-rat syntaxin) (Hata et al., 1993) was used at 1:5000, 4F8 [anti-ras-opposite promoter (ROP)] (Harrison et al., 1994) was used at 1:1000, Dsyt2 (anti-synaptotagmin) (Littleton et al., 1993a) was used at 1:2000, and 49/92 [anti-cysteine string protein (CSP)] (Zinsmaier et al., 1990) was used at 1:1000. Cuticles were prepared as described (Ashburner, 1990) from control (*y w*), *y w*; *syx*²²⁹, and *y w*; P{*syx*⁴}; *syx*²²⁹ embryos. *y w*; P{*syx*^{wt}}; *syx*²²⁹ embryos resembled *y w* controls (data not shown).

SDS-resistant complex formation. Core complexes were formed during overnight incubation of glutathione S-transferase (GST)-syntaxin or GST-Syx⁴ (1.2 μM, immobilized on glutathione-Sepharose beads) with SNAP-25 (4 μM) and n-synaptobrevin (4 μM) at 4°C in buffer A (50 mM HEPES, pH 7.4, 150 mM potassium acetate, 0.05% Tween 20). Samples were washed once with buffer A + 1 mg/ml gelatin and twice with buffer A + 5% glycerol. Samples were divided into eight tubes and resuspended in 1× sample buffer (50 mM Tris, pH 6.8, 2.5% β-mercaptoethanol, 2% SDS, 5% glycerol, and 0.025% bromophenol blue). Seven samples were incubated for 5 min at 25, 37, 42, 48, 52, 60, or 66°C using a Robocycler (Stratagene). The eighth aliquot was boiled for 5 min. The proteins were then resolved by SDS-PAGE, transferred to nitrocellulose membranes, and detected with a polyclonal n-syb antibody (R29) at 1:2000 using ECL.

In vitro binding assays. For GST-syntaxin fusion protein constructs, the cytoplasmic domains (aa 1–272) of syntaxin and Syx⁴ were PCR amplified using *Pfu* polymerase (Stratagene) and cloned into pGEX-4T-1 (Amersham Pharmacia Biotech). The open reading frame of each construct was sequenced entirely. Constructs expressing target proteins have been described previously (Wu et al., 1999). GST-fusion and His-tagged proteins were produced according to manufacturer's protocols (Amersham Pharmacia Biotech, and Novagen, Milwaukee, WI, respectively). To exchange buffers for His-tagged proteins, proteins were concentrated using Centrprep columns (Amicon/Millipore, Bedford, MA) and washed twice with 10 ml of PBS (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3). SDS-PAGE and Coomassie blue staining were used to estimate protein concentrations, using bovine 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 (Syt), 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/1 ml heads). After homogenization with a Dounce homogenizer, cuticular debris was pelleted at 5000 × 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 μM) 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 antibody 1:5000 (Invitrogen, Carlsbad, CA). For dose–response binding curves, GST, GST-syntaxin, or GST-Syx⁴ 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, synaptotagmin, or CSP were run on the same gel as standards. Bands 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, Syx⁴, and SNAP-25 (1 μM) were performed overnight at 4°C in binding buffer (0.02 M HEPES, pH 7.6, 0.15 M potassium acetate, 0.5% Triton X-100, and 2.5% 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-Dsyt2 (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 mM EGTA or 1 mM CaCl₂. After 2 hr incubation at room temperature, the beads were washed three times with 1 ml wash buffer (0.02 M HEPES, pH 7.6, 0.15 M potassium acetate, 0.5% Triton X-100). Beads were resuspended with 1× sample buffer and boiled before electrophoresis, except those samples being tested for SDS-denaturation. The primary antibody used in Western detection was affinity purified anti-n-synaptobrevin (rat R29) at a 1:1000 dilution (Wu et al., 1999). Quantification of binding was performed by using an ¹²⁵I-labeled secondary antibody (anti-rat Ig, whole antibody; Amersham Pharmacia Biotech).

Electrophysiological analysis. The *syx*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 a glass suction electrode. 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 Ca²⁺. 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 EJC amplitudes at 0.1–0.4 mM Ca²⁺ 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.

Figure 1. Syntactin double point mutations lie in neighboring hydrophobic layers. *a*, Alignment of amino acids 229–267 of the H3 domains of *Drosophila*, *C. elegans*, rat, squid, and yeast syntactin-1A homologs. The central ionic layer is indicated by the zero, and the numbers indicate adjacent hydrophobic layers. These layer assignments are based on the crystal structure of the core complex (Sutton et al., 1998). The Syx⁴ protein is altered for the two boxed amino acids. *b*, A schematic showing the position of the Syx⁴ mutations in relation to the entire H3 domain of syntactin. The solid bars indicate the Syx⁴ mutations, and the hatched bars indicate the Ca²⁺ effector domain (Wu et al., 1999).

RESULTS

Targeted mutation of syntactin H3 residues A243 and V247

The syntactin 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 syntactin (Ala-240, Val-244) have been suggested to be required for SNARE complex stability and syntactin-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* syntactin1A 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 “Ca²⁺ effector domain” characterized previously (Fig. 1*a,b*) (Wu et al., 1999). Both the mutant form of syntactin (Syx⁴) and wild-type syntactin (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 Syx⁴ construct were crossed into a syntactin null deletion mutant (Syx²²⁹) 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 Syx⁴ constructs in the Syx²²⁹ null background express similar levels of syntactin protein. These data show that the Syx⁴ mutations do not significantly alter levels of syntactin protein *in vivo*, compared with Syx^{wt} controls. Likewise, different transgenic lines for both constructs display similar levels of syntactin expression (Fig. 2), showing that there are no significant position effects on transgene expression. To

Figure 2. Syx⁴ mutant animals exhibit normal levels of synaptic proteins. The levels of syntactin, ROP, synaptotagmin I, and CSP are unchanged in Syx⁴ mutants, compared with controls (Syx^{wt}). Westerns were performed on extract obtained from four embryos of the appropriate genotype, i.e., Syx^{wt-1}, Syx^{wt-2}, Syx⁴⁻¹, and Syx⁴⁻². Bands shown for a given protein were taken from the same exposure of a single gel. The smaller synaptotagmin I band represents a degradation product (Littleton et al., 1993b).

determine whether the Syx⁴ 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 Syx⁴ embryos and also between different transgenic lines of each construct. To examine the spatial and temporal localization of syntactin and synaptotagmin I, immunocytochemical staining of embryos was performed. Immunocytochemistry revealed an indistinguishable level and distribution of both proteins in multiple Syx^{wt} and Syx⁴ lines (data not shown). Hence, protein levels and distribution of all proteins tested in Syx⁴ 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*.

Syx⁴ mutants display defects in neuronal but not non-neuronal secretion

We first assessed the gross phenotypes of the Syx⁴ mutants. All Syx⁴ phenotypic analyses were performed in the Syx null (Syx²²⁹) background. We have shown previously that Syx²²⁹ embryos are late embryonic lethal (Schulze et al., 1995). The wild-type genomic construct (Syx^{wt}) can rescue null (Syx²²⁹) mutants to adulthood (Fig. 3*a*), demonstrating the normal function of the transgenic protein. In contrast, the genomic construct containing the Syx⁴ mutation is fully embryonic lethal in the null background (Fig. 3*a*). Hence, the Syx⁴ mutations must cause a severe loss of syntactin function.

We and others have shown previously that syntactin 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. 3*b*, control). In contrast, Syx null mutant embryos (Syx²²⁹/Syx²²⁹) fail to secrete detectable cuticle and show a complete absence of denticle belts and mouth hooks (Fig. 3*b*, Syx²²⁹). Surprisingly, the cuticular features of Syx⁴ mutants (Syx⁴/Syx⁴; Syx²²⁹/Syx²²⁹) are indistinguishable from wild-type

Figure 3. Gross phenotypes of *syx⁴* mutants suggest defects in neuronal but not non-neuronal secretion. *a*, Lethal phase and movement in the *syx* null (*syx²²⁹*) background. Spontaneous muscle contractions of embryos 22–24 hr after egg laying were observed for 5 min and quantified. Spontaneous peristaltic contractions of *syx⁴* embryos are significantly reduced, compared with *syx^{wt}* embryos. Evoked contractile responses, determined after a brisk tactile stimulation, were present in *syx⁴* although absent in the null allele. *b*, Cuticle secretion is not impaired in *syx⁴* mutants. Cuticles from control, *syx²²⁹*, and *syx⁴* (in *syx²²⁹* background) embryos were imaged using dark-field microscopy. Anterior is to the left. Cuticular structures, including denticle belts and mouth hooks, are absent in the null mutant but present in the control and *syx⁴* mutant.

controls and contain normal segmental denticles and mouth hooks developed from cuticle secretion (Fig. 3*b*, *syx⁴*). The *syx⁴* mutant embryos appear to have normally structured tissues in general, in sharp contrast to *syx²²⁹* nulls, 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/V247 are not required for vesicular fusion in 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 *syx²²⁹* mutants display a complete absence of both evoked and spontaneous coordinated movement attributable to a complete block of neurotransmission (Fig. 3*a*) (Schulze et al., 1995). As expected, the *syx^{wt}* genomic construct rescues both spontaneous and touch-evoked movement phenotypes (Fig. 3*a*). Likewise, the *syx⁴* mutant embryos, unlike *syx²²⁹* mutants, show spontaneous movement and touch-evoked muscle contraction. However, both behaviors are impaired, suggesting that neuromuscular transmission is reduced but not abolished (Fig. 3*a*). The *syx⁴* mutants display an approximately fourfold reduction in the

Figure 4. *syx⁴* mutants display a profound reduction in evoked neurotransmission. *a*, Representative excitatory junctional current (EJC) traces are shown for *syx^{wt}*, *syx²²⁹*, and *syx⁴*; *syx²²⁹* embryos. Recordings were performed at the embryonic muscle 6 NMJ (22–24 hr after egg laying). In addition to a severe reduction in the EJC amplitude in *syx⁴*, neurotransmitter release is clearly asynchronous. Four traces are superimposed; arrow indicates nerve stimulation artifact. *b*, Mean EJC amplitudes for *syx^{wt-1}* ($n = 5$), *syx^{wt-2}* ($n = 9$), *syx^{wt-3}* ($n = 5$), *syx⁴⁻¹* ($n = 3$), *syx⁴⁻²* ($n = 2$), and *syx⁴⁻³* ($n = 3$) embryos. *c*, Mean latency (time to peak) data are pooled for *syx^{wt}* ($n = 21$) and *syx⁴* ($n = 8$) embryos. *d*, Evoked neurotransmission is variable in *syx⁴* mutants, compared with *syx^{wt}*. Coefficient of variation (EJC amplitude SD/EJC amplitude) is plotted for *syx^{wt-1}*, *syx^{wt-2}*, *syx^{wt-3}*, *syx⁴⁻¹*, *syx⁴⁻²*, and *syx⁴⁻³* embryos. *e*, Percentage of failures of evoked response after nerve stimulation in 1.8 mM Ca^{2+} for *syx^{wt}* and *syx⁴* embryos. *syx^{wt}* transmission almost never fails, whereas *syx⁴* fails 50% of all stimuli. Error bars signify SEM. ** $p < 0.01$.

frequency of muscular contraction waves compared with wild-type controls (*syx^{wt}/syx^{wt}*; *syx²²⁹/syx²²⁹* = 1.7 ± 0.2 contractions per minute, $n = 20$; *syx⁴/syx⁴*; *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* mutants display severely impaired excitation–secretion coupling**

Targeted mutations in *Drosophila* syntaxin 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 *syx^{wt}* and *syx⁴* transgenic embryos. As shown in Figure 4, *a* and *b*, evoked EJC amplitude is severely reduced in *syx⁴* mutants, to ~10% of the levels of *syx^{wt}* transgenic controls

(1.1 ± 0.1 nA for *syx^{wt}*, $n = 19$; 0.12 ± 0.02 nA for *syx^d*, $n = 8$; $p < 0.0001$). An identical phenotype was observed in three independent transgenic lines (Fig. 4*b*). Transmission in *syx^d* 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 *syx^d* and *syx^{wt}* controls (1.59 for *syx^{wt}*; 1.47 for *syx^d*). 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 *syx^d* mutants.

Neurotransmission in *syx^d* 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 excytosome 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 *syx^d* mutants is strikingly asynchronous, demonstrates low fidelity to identical stimuli, and exhibits a high failure rate. In control *syx^{wt}* NMJ synapses, stimulation-evoked transmission occurs consistently within ~ 5 msec after nerve stimulation, whereas in *syx^d* mutants, evoked release occurs at delayed (twofold) latencies (Fig. 4*c*) (5.8 ± 0.2 msec for *syx^{wt}*, $n = 21$; 10.4 ± 0.5 msec for *syx^d*, $n = 8$; $p < 0.0001$). This increased latency suggests reduced kinetics of excitation–secretion coupling. As shown in Figure 4*d*, *syx^d* 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 *syx^{wt}*, 1.2 ± 0.08 for *syx^d*; $p < 0.0001$). Finally, although *syx^{wt}* controls always release neurotransmitter in response to nerve stimulation in 1.8 mM extracellular Ca^{2+} (no failures), *syx^d* mutants fail to respond $>50\%$ of the time (Fig. 4*e*). 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 *syx^d* 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 *syx^d* mutants (0.19 ± 0.02 nA for *syx^{wt}*, $n = 10$; 0.26 ± 0.01 nA for *syx^d*, $n = 12$; $p < 0.01$), but no changes in the kinetics of transmitter release were observed. The underlying reason for the increase in quantal size is unclear, but the increase is specific for the *syx^d* 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 *syx^d* mutants, this result demonstrates that the postsynaptic receptor field is present and the severe decrease in *syx^d* neurotransmission is attributable to a presynaptic defect. In line with evoked defects, *syx^d* mutants reveal a significant decrease in mEJC frequency (0.042 ± 0.010 Hz for *syx^{wt}*, $n = 11$; 0.023 ± 0.006 Hz for *syx^d*, $n = 13$; $p < 0.05$). This result suggests that core complexes containing Syx⁴ protein show a decreased ability to mediate vesicular fusion. In conclusion, *syx^d* 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 *syx^d* 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

Figure 5. The frequency of spontaneous mEJCs is reduced in *syx^d* mutants. *a*, Representative mEJC traces are shown for *syx^{wt}*, *syx²²⁹*, and *syx^d*; *syx²²⁹* animals. Recordings were performed in 0.5 mM Ca^{2+} + TTX as described in Materials and Methods. *b*, Mean mEJC frequency in *syx^{wt}* ($n = 11$) and *syx^d* ($n = 13$) animals. Data from individual transgenic lines were not statistically different and thus were pooled. *syx^d* mutants show a 50% reduction in mEJC frequency relative to controls. *c*, Mean mEJC amplitude in *syx^{wt}* and *syx^d* animals. *syx^d* mutants display a slight, but significant, increase in quantal amplitude. Error bars represent SEM. * $p < 0.05$; ** $p < 0.01$.

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. 6*a*, GST), GST-syntaxin (Fig. 6*a*, Syx^{wt}), and GST-Syx⁴ (Fig. 6*a*, Syx^d). 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. 6*a*). 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. 6*b–d*). Similar dose–response binding curves for Syx^{wt} and Syx^d were obtained for each of these proteins: n-synaptobrevin, synaptotagmin I, and CSP. Together, these data suggest that the *syx^d* 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-

Figure 6. Mutant *Syx*⁴ protein interacts normally with syntaxin binding partners. *a*, GST pull-down assays were performed by incubating GST, GST-*Syx*^{wt}, or GST-*Syx*⁴ with recombinant target proteins or *Drosophila* head extract, and bands were detected by immunoblotting as described in Materials and Methods. Bands shown for a given target protein were taken from a single exposure of a single gel. *b*, The *syx*⁴ mutation does not significantly alter ternary complex formation. Ternary complex formation was assessed by incubating immobilized GST-*Syx*^{wt} or GST-*Syx*⁴ with SNAP-25 and increasing amounts of n-synaptobrevin. Bound n-synaptobrevin was determined by immunoblotting and ECL, followed by densitometry with known standards. *c*, Dose-response binding for GST-*Syx*^{wt} and GST-*Syx*⁴ to synaptotagmin I. Increasing amounts of synaptotagmin I were incubated with either GST-*Syx*^{wt} or GST-*Syx*⁴, and bound synaptotagmin I was determined by immunoblotting and ECL. *d*, *Syx*⁴ and *Syx*^{wt} show similar dose-response binding to CSP.

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 accelerate core complex formation *in vitro* (Littleton et al., 2001). Therefore, to further test whether the *syx*⁴ mutation affects the ability of the core complex to bind synaptotagmin, GST fused with the cytoplasmic domain of synaptotagmin was immobilized on glutathione-Sepharose beads and exposed to n-synaptobrevin alone, preformed binary complexes (n-synaptobrevin-SNAP-25; n-synaptobrevin-syntaxin, or n-synaptobrevin-*Syx*⁴), or preformed ternary complexes (n-synaptobrevin-SNAP-25-syntaxin or *Syx*⁴). GST-synaptotagmin did not bind monomeric n-synaptobrevin or binary complexes of n-synaptobrevin-SNAP-25, n-synaptobrevin-syntaxin, or n-synaptobrevin-*Syx*⁴ in the presence or absence of Ca^{2+} (Fig. 7*a*, lanes 1–6). Ternary complexes composed of syntaxin and *Syx*⁴ were both bound by GST-synaptotagmin (Fig. 7*a*, lanes 7–14). Quantification of this binding using ¹²⁵I-labeled secondary antibody indicates that complexes composed of syntaxin and *Syx*⁴ bound GST-synaptotagmin with equal intensity in the absence of Ca^{2+} . Both forms of syntaxin demonstrated slightly increased binding in the presence of Ca^{2+} (Fig. 7*b*). We point out that synaptotagmin binding was enormously more variable in complexes containing *Syx*⁴ than in those containing *Syx*^{wt} (Fig. 7*b*); however, the mean binding was indistinguishable, and the significance of the increased variability in *Syx*⁴ is presently unclear. We conclude,

Figure 7. Ternary complexes containing *Syx*⁴ mutant protein interact normally with synaptotagmin I. Binary and ternary complexes of n-synaptobrevin, syntaxin, *Syx*⁴, and SNAP-25 were compared for their ability to bind GST-synaptotagmin in the presence and absence of Ca^{2+} . *a*, Complexes were detected by Western blotting with anti-synaptobrevin antibody. Lanes 1–6 are control proteins and binary combinations that do not bind GST-synaptotagmin. Lanes 7–14 show that core complexes formed with mutant *Syx*⁴ protein bind GST-synaptotagmin as readily as wild type (lanes 7–10 are boiled and lanes 11–14 are unboiled). *b*, The graph shows the percentage binding to GST-Syt as quantified using ¹²⁵I-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 $\pm$ 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 8*a*, 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.,

Figure 8. Core complex stability *in vitro* is impaired by the *syx*⁴ mutation. The heat lability of SDS-resistant core complexes containing Syx⁴ is increased, compared with core complexes containing Syx^{wt}. Complexes were formed by incubating His-tagged SNAP-25 and n-synaptobrevin overnight with GST-Syx^{wt} or GST-Syx⁴ immobilized on glutathione-Sepharose beads. *a*, Syx⁴-containing complexes are resistant to 2% SDS at 25°C, similar to control complexes (Syx^{wt}). *b*, 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 Syx⁴ relative to Syx^{wt} protein.

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. 8*b*). In contrast, core complexes made with Syx⁴ denature at much lower temperatures. The Syx⁴ complexes remain stable up to 25°C (Fig. 8*a*) but are degraded at 37°C and undetectable above ~48°C (Fig. 8*b*). 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, 1996; Aravamudan et al., 1999). A 3 sec focal burst of hypertonic saline was applied to *syx*⁴ and control (*syx*^{wt}) embryonic synapses (Aravamudan et al., 1999). Synaptic responses from control animals consisted of many repetitive, high-frequency secretion events (Fig. 9*a*). In contrast, the hyperosmotic response of *syx*⁴ mutant animals is extremely reduced (Fig. 9*a*). The total charge elicited for *syx*^{wt}, *syx*⁴, and *syx*²²⁹ (null) is shown in Figure 9*b* (222 nA · msec for *syx*^{wt}; 47 nA · msec for *syx*⁴; 20.1 nA · msec for *syx*²²⁹). *syx*⁴ dramatically reduces hyperosmotic saline response, a phenotype comparable to, but slightly less severe than, complete deletion of syntaxin. Note also that there is an increased latency in the hyperosmotic response in *syx*⁴ (Fig. 9*a*), similar to the delay in stimulation-evoked neurotransmission (Fig. 4*c*).

Because *syx*⁴ mutant transmission is phenotypically very similar to *synaptotagmin I* null mutants (*syx*^{AD4}) (Broadie et al., 1994), the hyperosmotic response in *syx*^{AD4} was assayed in parallel. Hyperosmotic saline application to *syx*^{AD4} yielded reduced secretion

Figure 9. Core complex function is impaired in *syx*⁴ mutant synapses. *a*, Representative current traces are shown for *syx*^{wt}, *syx*²²⁹, *syx*⁴, *syx*²²⁹, *syx*²²⁹, and *syx*^{AD4} animals in response to 3 sec application of hyperosmotic saline (1175 mOsm; diagonally striped bar). The *syx*^{wt} synapse responds with robust, high-frequency secretory events, whereas the *syx* null (*syx*²²⁹) displays no detectable response. Note the obvious response reduction with increased latencies in both *syx*⁴ and *syx*^{AD4}. *b*, Total charge induced by transmitter release was measured from the area of the current trace responses (nanoamperes times millisecond). Average responses are shown for *syx*^{wt} (*n* = 6), *syx*⁴ (*n* = 7), *syx*²²⁹ (*n* = 5), and *syx*^{AD4} (*n* = 6) animals. Data from individual transgenic lines were not statistically different and thus were pooled. Error bars represent SEM. ***p* < 0.01; ****p* < 0.001.

events that were qualitatively (Fig. 9*a*) and quantitatively (Fig. 9*b*) very similar to *syx*⁴. The defects in both *syx*⁴ and *syx*^{AD4} animals shown here are reminiscent of *unc13* null mutants in *Drosophila*, *Caenorhabditis elegans*, and mouse (Aravamudan et al., 1999; Augustin et al., 1999; Richmond et al., 1999), where formation of the SNARE complex is severely reduced. Thus, these data suggest that the syntaxin H3 domain residues A243 and V247 are required for normal levels of fusion-competent SVs, and this explains both the severely compromised neurotransmission and the embryonic lethality in *syx*⁴ mutants.

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 (*syx*^Δ mutant) to assay their significance during *in vivo* secretory events.

In *Drosophila*, syntaxin1A 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, *syx*^Δ 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 *syx*^Δ 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, *syx*^Δ 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 *syx*^Δ 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 *syx*^{H3-C} mutant, which deletes the Ca²⁺ effector domain to severely reduce binding to synaptotagmin I (Wu et al., 1999). The *syx*^Δ 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 *syx*^Δ mutations impair this regulation.

We tested this hypothesis by assaying the protein binding properties of *syx*^Δ 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 *Syx*^Δ 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 *Syx*^Δ core complex may possibly indicate that rapid core complex formation in *syx*^Δ 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 (*syx*^{ΔD4}) and *syx*^Δ mutant synapses (Fig. 9). Although *Syx*^Δ 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 *syx*^Δ 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, synaptobrevin (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 *syx*^Δ 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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