

# Temperature-Sensitive Paralytic Mutations Demonstrate that Synaptic Exocytosis Requires SNARE Complex Assembly and Disassembly

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## Summary

The neuronal SNARE complex is formed via the interaction of synaptobrevin with syntaxin and SNAP-25. Purified SNARE proteins assemble spontaneously, while disassembly requires the ATPase NSF. Cycles of assembly and disassembly have been proposed to drive lipid bilayer fusion. However, this hypothesis remains to be tested *in vivo*. We have isolated a *Drosophila* temperature-sensitive paralytic mutation in *syntaxin* that rapidly blocks synaptic transmission at nonpermissive temperatures. This paralytic mutation specifically and selectively decreases binding to synaptobrevin and abolishes assembly of the 7S SNARE complex. Temperature-sensitive paralytic mutations in *NSF* (*comatose*) also block synaptic transmission, but over a much slower time course and with the accumulation of syntaxin and SNARE complexes on synaptic vesicles. These results provide *in vivo* evidence that cycles of assembly and disassembly of SNARE complexes drive membrane trafficking at synapses.

## Introduction

Membrane trafficking in eukaryotic cells involves vesicular intermediates that are shuttled between cellular organelles. A highly conserved vesicle fusion apparatus containing *n*-ethylmaleimide-sensitive fusion factor (NSF) has been postulated to function in multiple vesicular transport pathways (i.e., ER to Golgi, intra-Golgi, Golgi to lysosome, Golgi to plasma membrane), including the exocytosis of synaptic vesicles (Ferro-Novick and Jahn, 1994; Söllner and Rothman, 1994). NSF is a homomultimeric ATPase that binds via soluble NSF attachment proteins (SNAPs) to specific SNAP receptors anchored on vesicle and target membranes (known as v- and t-SNAREs, respectively) (Söllner et al., 1993b). SNAREs are members of multigene families with compartment-specific proteins present in all eukaryotic cells (Weimbs et al., 1997; Advani et al., 1998). One of the more highly regulated trafficking pathways occurs at the synapse, where neurotransmitter-filled synaptic vesicles undergo local exocytotic and endocytotic cycles to mediate neuronal communication (Scheller, 1995; Südhof, 1995; Hanson et al., 1997a). The interaction of the synaptic

vesicle v-SNARE synaptobrevin with the t-SNAREs syntaxin and SNAP-25 results in the formation of an SDS-resistant complex that migrates at 7S in density gradients (Söllner et al., 1993b; Hayashi et al., 1994). The 7S SNARE complex serves as the membrane receptor for SNAPs and NSF. Hydrolysis of ATP by NSF is coupled to disassembly of the 7S complex. The sequential interactions of these proteins led to a model known as the SNARE hypothesis for vesicle trafficking. In early models, disassembly was proposed to drive bilayer fusion (Söllner et al., 1993a).

Recently, the function of the SNARE complex has been addressed with studies as diverse as the analysis of homotypic vacuolar fusion in yeast to investigations of tetanus and botulinum toxin-poisoned synapses (Schiavo et al., 1992; Blasi et al., 1993a, 1993b; Mayer et al., 1996; Nichols et al., 1997). These studies led to refinements in the SNARE hypothesis that include a proposed predocking role for NSF in dissociating SNARE complexes that reside in the same membrane, thereby activating t-SNAREs for future SNARE assembly (Ungermann et al., 1998). These studies also suggest that the SNAREs themselves are likely to mediate a postdocking function in the vesicle cycle (Hunt et al., 1994; Broadie et al., 1995; Sweeney et al., 1995). The SNARE hypothesis provides an attractive model for membrane fusion as assembly of SNAREs via parallel coiled-coil interactions would bring the vesicle and presynaptic membranes into close apposition (Hanson et al., 1997b; Lin and Scheller, 1997). The assembly of SNAREs into an extremely stable complex might provide sufficient energy for fusion. Recently, reconstitution experiments have demonstrated that SNAREs (syntaxin, SNAP-25, and synaptobrevin) may represent the minimal machinery capable of mediating vesicular fusion *in vitro* (Weber et al., 1998).

Although the SNARE hypothesis provides an attractive model for compartment-specific membrane fusion, it has yet to be tested *in vivo*. Specifically, it is not known whether assembly of the 7S complex is required for neuronal exocytosis, although individual SNARE proteins are clearly required at some point in the release pathway (Broadie et al., 1995; Schulze et al., 1995; Deitcher et al., 1998). Addressing this issue requires loss-of-function mutations in one or more SNARE proteins that specifically affect assembly or disassembly of the SNARE complex.

We have used an unbiased genetic approach to identify mutations that disrupt synaptic transmission by screening for temperature-sensitive paralytic mutants in *Drosophila*. Temperature-sensitive paralytic mutations have been an important tool in the genetic dissection of membrane excitability, including studies of ion channels (*paralytic* [*para*], *no action potential* [*nap*], *temperature-induced paralysis E* [*tipE*], *slowpoke* [*slo*], *seizure* [*sei*]), endocytosis (*shibire* [*shi*], *stoned*), and exocytosis (*comatose* [*com*], *cysteine string protein* [*csp*]). This approach allows mutations in proteins important in synaptic transmission to be identified on the basis of behavioral paralysis, without any prior knowledge of the

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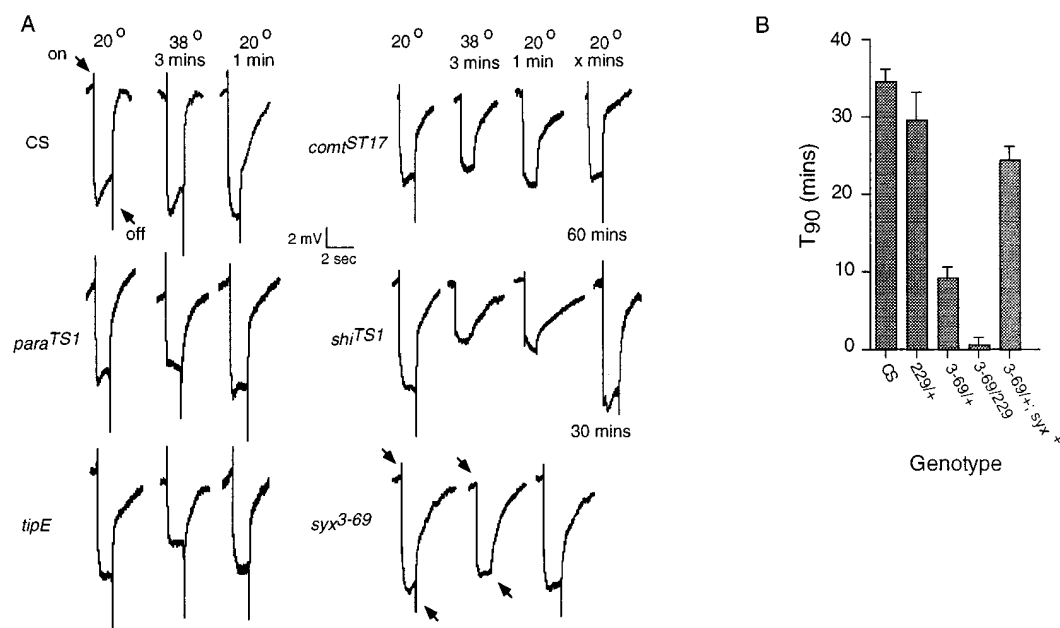


Figure 1. Analysis of Electretinogram (ERG) and Paralytic Behavior of a Temperature-Sensitive *syntaxin* Mutant

(A) ERGs recorded from wild-type and various temperature-sensitive paralytic mutants. Flies were rapidly heated from 20°C to 38°C and maintained at 38°C for 3 min. Flies were then rapidly cooled to 20°C and allowed to recover in darkness, with test light pulses given at regular intervals. Note the loss of on- and off-transients (arrows) with a normal photoreceptor depolarization in *syx<sup>3-69</sup>* flies. The on-/off-transients recover rapidly when shifted back to the nonpermissive temperature. In contrast, *comt<sup>ST17</sup>* and *shi<sup>TS1</sup>* mutants take substantially longer to recover. Identical defects as shown for *comt<sup>ST17</sup>* were observed for *comt<sup>ST53</sup>*, *comt<sup>TP7</sup>*, and allelic combinations thereof. The loss of on-/off-transients is specific to synaptic transmission mutants, as *para* and *tipE*, which affect axonal conduction, do not alter the ERG. Three to ten individuals of each genotype aged 1–5 days were tested with similar results to those shown.

(B) Ten flies of the indicated genotypes were tested in blind trials at 38°C, and the time required for 90% of the flies to remain on the bottom is plotted on the ordinate. Error bars represent SEM. Between three and 20 separate trials were performed for each genotype. Wild-type flies, as well as flies with 50% of wild-type levels of syntaxin (*syx<sup>229/+</sup>*), remain active for greater than 30 min. In contrast, flies carrying a *syx<sup>3-69</sup>* allele and a *syntaxin* null allele (*syx<sup>229</sup>*) become paralyzed within seconds. *syx<sup>3-69/+</sup>* flies remain on the bottom of the vial after approximately 8–10 min of exposure to 38°C. Addition of an extra copy of *syntaxin<sup>+</sup>* to *syx<sup>3-69/+</sup>* flies restores behavior at 38°C to near normal.

products involved. We now report the characterization of temperature-sensitive paralytic mutations in syntaxin and NSF that define an *in vivo* requirement for SNARE complex assembly and disassembly in neuronal exocytosis.

## Results

### Identification of Temperature-Sensitive Paralytic Mutations Disrupting Neurotransmitter Release

To begin to address the *in vivo* requirements for vesicular trafficking at the synapse, we have screened for conditional mutations in *Drosophila* that block synaptic transmission. Temperature-sensitive paralytic mutations were generated by chemical (EMS) or P-element mutagenesis and tested for paralysis in preheated glass vials in a 38°C water bath in F1 and F2 screens. Flies demonstrating abnormal motor behavior and paralysis were maintained as stocks. Sixty-six temperature-sensitive paralytic mutations were identified and screened for electrophysiological defects in synaptic transmission in the visual system at permissive (20°C) and nonpermissive temperatures (38°C). The electroretinogram (ERG) is an extracellular recording from the *Drosophila* eye

that measures light-induced depolarization of photoreceptors and synaptic activation of second order neurons in the visual pathway (Hotta and Benzer, 1969; Pak et al., 1969). These synaptic events occur at the onset and termination of a light pulse and are represented by the on- and off-transients of the ERG. Several mutations known to block synaptic transmission, including *synaptotagmin* (DiAntonio and Schwarz, 1994), *rop* (Harrison et al., 1994), and *csp* (Zinsmaier et al., 1994), decrease or abolish the on-/off-transients. Twenty-six of the paralytic mutants altered some aspect of the ERG (J. T. L. and B. G., unpublished data), but only four of the mutants specifically lost the on-/off-transients at 38°C (Figure 1). These include additional alleles of the endocytotic mutation *shi* and the previously identified *comt* mutations (Siddiqi and Benzer, 1976). The loss of on-/off-transients in *comt* confirms that NSF is essential for synaptic transmission *in vivo* and provides an electrophysiological correlate of the behavioral paralysis (Pallanck et al., 1995). Two other mutations also lost on-/off-transients at 38°C, including *ts<sup>3-69</sup>* and an additional mutation that is currently being characterized.

To identify the gene altered by *ts<sup>3-69</sup>*, the mutation was mapped by recombination to the third chromosome at position 3-81. Deficiency mapping within this interval

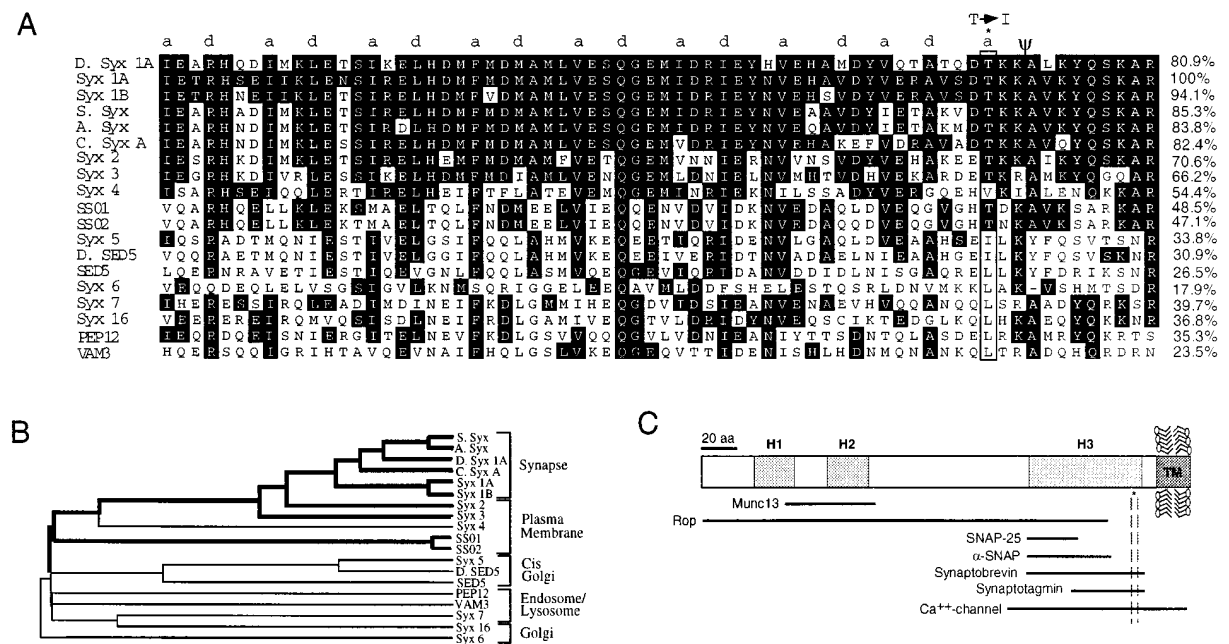
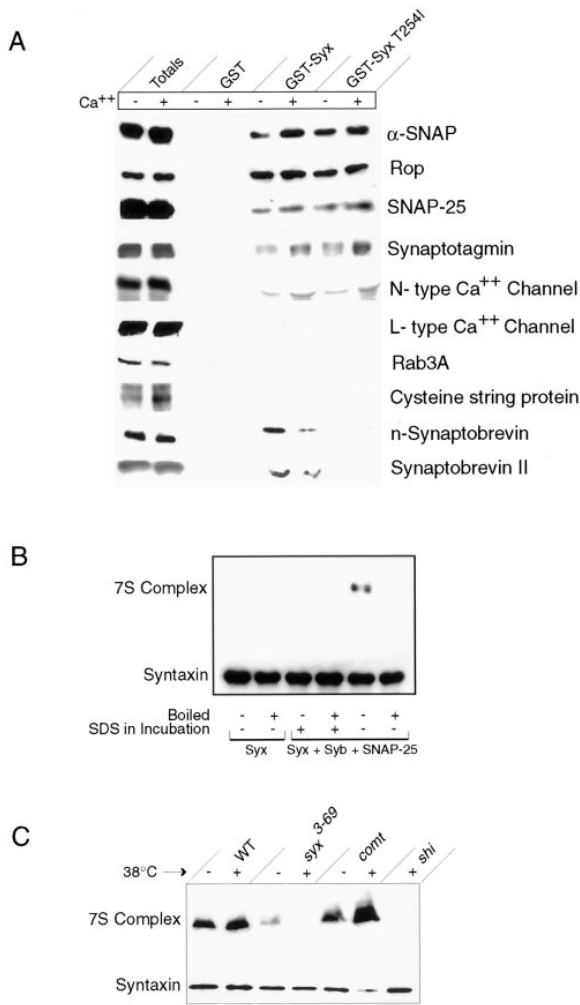


Figure 2. Sequence Analysis of *syx<sup>3-69</sup>*. (A) The H3 domain of the syntaxin family is shown with homologies to rat syntaxin 1A highlighted and identities indicated at the right. The "a" and "d" sites on the hydrophobic face of the heptad repeat of the H3 domain are shown above the sequence. The botulinum C1 cleavage site is indicated by the  $\psi$ . The *syx<sup>3-69</sup>* paralytic phenotype results from a single amino acid change (threonine to isoleucine) at position 254 (boxed in the sequence comparisons) of the *Drosophila* syntaxin 1A sequence. Abbreviations: S., squid; A., *Aplysia*; C., *C. elegans*; D., *Drosophila*. (B) Dendrogram of the syntaxin t-SNARE family based on homology within the H3 domain is shown. All syntaxin members present at the synapse or plasma membrane (with the exception of syntaxin 4) have a threonine at the site of the mutated residue in *syx<sup>3-69</sup>* and are highlighted with bold lines. The subcellular locations of the syntaxin homologs are indicated. (C) Schematic diagram of syntaxin indicating the locations of the three coiled-coil motifs. The corresponding regions of syntaxin that are required for binding to its synaptic partners are shown below the diagram. The T254I mutation (\*) falls within the domain previously shown to be required for binding synaptobrevin, synaptotagmin, and N-, P/Q-type  $Ca^{2+}$  channels.

demonstrated that *ts<sup>3-69</sup>* is uncovered by *Df(3R)crb<sup>87-4</sup>*, a deletion that uncovers the previously identified t-SNARE syntaxin 1A (Schulze et al., 1995). Indeed, flies heterozygous for *ts<sup>3-69</sup>* and a *syntaxin* null allele (*syx<sup>229</sup>*) become paralyzed in seconds at 38°C (Figure 1B). *ts<sup>3-69</sup>* shows partial dominance, since *ts<sup>3-69</sup>/+* heterozygotes also become immobile at 38°C, although with substantially slower kinetics than for homozygotes. This dominant phenotype is rescued by an extra copy of the wild-type *syntaxin* gene in transgenic flies (Figure 1B). These results confirm that *ts<sup>3-69</sup>* (referred to hereafter as *syx<sup>3-69</sup>*) is an allele of *syntaxin*. The paralysis caused by a single copy of *syx<sup>3-69</sup>* is likely due to impaired activity of a syntaxin multimeric complex rather than to loss of function, as a 50% reduction in the amount of syntaxin did not cause paralysis (Figure 1B), and the total amount of syntaxin present in *syx<sup>3-69</sup>* flies is unaltered on Western blots (Figure 3C). *syx<sup>3-69</sup>* flies have some behavioral defects at room temperature, including a decreased ability to fly, suggesting a defect in basal synaptic function as well. To further determine how *syx<sup>3-69</sup>* blocks synaptic transmission, the molecular defect in *syx<sup>3-69</sup>* was identified and its biochemical consequences were characterized.

Sequence comparison of *syntaxin* in *syx<sup>3-69</sup>* and the

parental strain revealed a single nucleotide change (ACC to ATC) that results in an amino acid substitution (threonine to isoleucine) at position 254. The syntaxin family of proteins has a similar domain structure, including a C-terminal transmembrane anchor and three cytosolic domains that are predicted to form coiled coils. The T254I mutation lies in the "a" position on the hydrophobic face of a heptad repeat at the end of the third coiled-coil motif of syntaxin (the H3 domain), three amino acids upstream of the botulinum C cleavage site (Schiavo et al., 1995). The H3 domain of syntaxin binds to synaptotagmin, SNAP-25, synaptobrevin,  $\alpha$ -SNAP, and N-, P/Q-type presynaptic calcium channels (Figure 2C) (Chapman et al., 1994, 1995; Hayashi et al., 1994; Sheng et al., 1994; Kee et al., 1995). Comparative sequence analysis reveals that threonine 254 is not a conserved residue among the syntaxin family (Figure 2A). However, all syntaxin members associated with the plasma membrane or the presynaptic membrane contain a threonine at this site (with the exception of syntaxin 4, which contains a conservative substitution), including mammalian syntaxins 1A, 1B, 2, and 3 and the yeast homologs SS01p and SS02p. The remaining syntaxins contain an isoleucine or leucine at this position and are located in different cellular compartments such as the *cis*-Golgi (SED5p,



**Figure 3.** Comparison of the Interactions of Wild-Type and T254I Syntaxins with Synaptic Proteins

(A) Wild-type GST-syntaxin and GST-syntaxin T254I showed  $Ca^{2+}$ -dependent binding to synaptotagmin and the synprint peptide of N-type  $Ca^{2+}$  channels and  $Ca^{2+}$ -independent binding to ROP,  $\alpha$ -SNAP, and SNAP-25. Neither fusion protein showed significant binding to Rab3A, CSP, or the synprint peptide of L-type  $Ca^{2+}$  channels. In contrast, wild-type syntaxin bound native *Drosophila* n-synaptobrevin and recombinant rat synaptobrevin II, while syntaxin T254I showed decreases in binding to both. The total lanes represent starting material treated in identical fashion but without exposure to GST fusion proteins.

(B) Recombinant rat SNAP-25, syntaxin 1A, and synaptobrevin-2 were incubated either in the absence or presence of SDS for 30 min at room temperature and then placed in SDS sample buffer and run on a 9%/15% discontinuous SDS-PAGE gel, transferred to nitrocellulose, and probed with the anti-syntaxin monoclonal antibody HPC-1. Similar results were seen when blots were probed with anti-synaptobrevin or anti-SNAP-25 antisera (data not shown).

(C) 7S complexes from 10 wild-type, *syx<sup>3-69</sup>*, *comt<sup>ST17</sup>*, and *shi<sup>751</sup>* flies that were kept at room temperature or given a 20 min 38°C heat shock were isolated. Syntaxin was present in a 35 kDa monomeric form and in a 73 kDa complex with SNAP-25 and synaptobrevin in wild-type flies at room temperature and 38°C. A reduction in the 7S complex was found in *syx<sup>3-69</sup>* flies at room temperature, and after exposure to 38°C no SNARE complex could be detected. This is in marked contrast with *comt*, which causes a dramatic accumulation of the 7S complex after exposure to 38°C. In *shi* flies at 38°C, where nerve terminals are completely depleted of synaptic vesicles, no 7S

*Drosophila* Sed5, syntaxin 5), trans-Golgi (syntaxin 6), or lysosome/endosome (PEP12p, syntaxin 7, VAM3p) (Weimbs et al., 1997). These sequence comparisons suggest that the amino acid substitution caused by *syx<sup>3-69</sup>* does not disrupt the overall conformation of the H3 domain but is more likely to affect a compartment-specific interaction of syntaxin. Because NSF and  $\alpha$ -SNAP represent conserved machinery required in multiple trafficking pathways, we hypothesized that the interaction with the compartment-specific v-SNARE (n-synaptobrevin) might be altered. Indeed, binding specificity between different syntaxin and synaptobrevin isoforms has been demonstrated (Calakos et al., 1994; Pevsner et al., 1994), consistent with the evolutionary conservation of compartment-specific v-/t-SNARE interactions.

### *syx<sup>3-69</sup>* Disrupts v-/t-SNARE Pairing and Blocks Formation of the 7S Complex

To test this hypothesis, we generated full-length GST fusion proteins containing either wild-type *Drosophila* syntaxin or the mutant version with the T254I substitution. Membrane and cytosolic fractions from wild-type *Drosophila* heads were incubated with GST alone, GST-syntaxin, or GST-syntaxin T254I immobilized on glutathione-Sepharose beads at 4°C for 2 hr in 1 mM  $Ca^{2+}$  or 2 mM EGTA. As there has been no previous biochemical analysis of interactions between synaptic proteins in *Drosophila*, we examined the evolutionary conservation of the interactions that have been described for mammalian syntaxin. *Drosophila* syntaxin showed  $Ca^{2+}$ -dependent binding to synaptotagmin and  $Ca^{2+}$ -independent binding to ROP,  $\alpha$ -SNAP, and SNAP-25 (Figure 3A). In addition, syntaxin bound the neuronal v-SNARE synaptobrevin (n-syb) with a slight decrease in binding in the presence of  $Ca^{2+}$  (observed in five separate binding assays). Significant binding of *Drosophila* syntaxin to Rab3A or CSP was not observed. Mammalian syntaxin also binds to N- and P/Q-type  $Ca^{2+}$  channel synprint peptides via a region encompassing the H3 domain of syntaxin (Sheng et al., 1994). As no synprint-containing  $Ca^{2+}$  channels have yet been identified in *Drosophila*, and given the 81% identity between rat and *Drosophila* H3 domains, we used His-tagged rat synprint peptides in binding assays. The synprint peptides from N-type  $Ca^{2+}$  channels bound *Drosophila* syntaxin, with stronger binding in the presence of  $Ca^{2+}$ , while the corresponding region of the L-type  $Ca^{2+}$  channel did not bind. Syntaxin T254I displayed normal binding to  $\alpha$ -SNAP, ROP, SNAP-25, synaptotagmin, and N-type  $Ca^{2+}$  channels but showed a marked decrease in binding to synaptobrevin in five separate binding assays (Figure 3A). Synaptobrevin binding was not completely abolished, as some reduced binding to syntaxin T254I could be detected upon longer enhanced chemiluminescence (ECL) exposures of the immunoblots. To confirm a direct defect in binding of v-/t-SNAREs, we tested the ability of recombinant His-tagged rat synaptobrevin-2, the major mammalian synaptic v-SNARE, to bind wild-type and T254I syntaxins.

complex can be detected. The slight variability in the amount of syntaxin in each lane is secondary to inherent differences in head sizes, but the trends shown were consistently observed.

As shown in Figure 3A, synaptobrevin-2 bound with much lower affinity to the T254I mutant, confirming a direct defect in the interaction between synaptobrevin and syntaxin T254I.

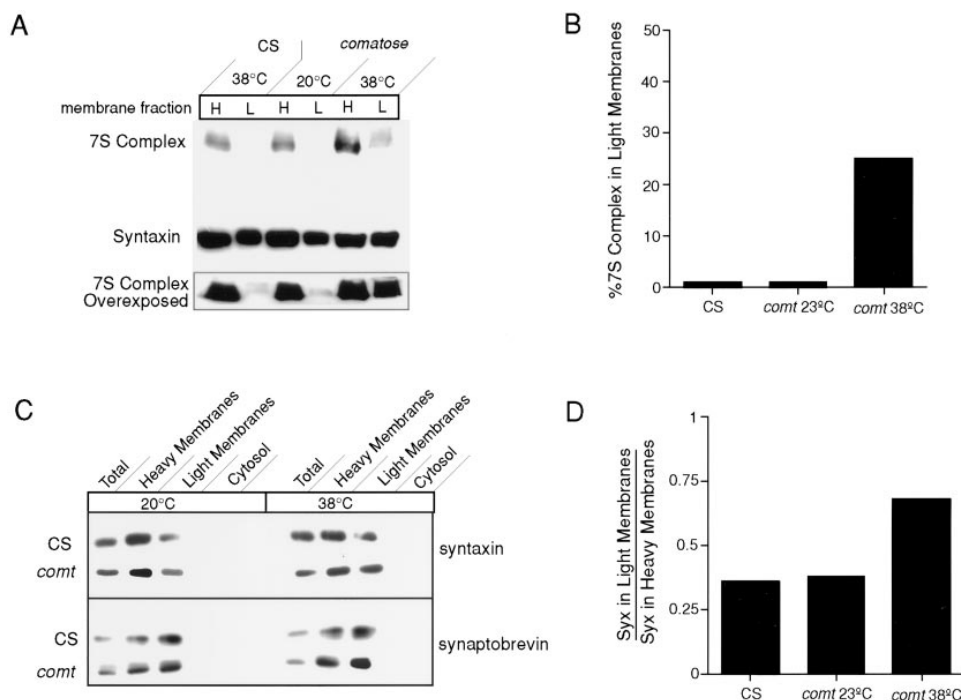
To determine whether the T254I substitution has similar consequences on the function of syntaxin *in vivo*, we examined the 7S complex in head extracts of *syx*<sup>3-69</sup> mutants. As shown in Figure 3B, syntaxin, synaptobrevin, and SNAP-25 form an SDS-resistant 7S SNARE complex that requires all three recombinant proteins. The SNARE complex does not form if SDS is included in the binding reaction, but once the complex is formed, it is resistant to dissociation by SDS unless the sample is boiled at 100°C for 3 min (Figure 3B). Native SNARE complexes can also be isolated from rat brain or *Drosophila* heads. Supernatants from homogenized wild-type fly heads were separated on SDS gels and probed with an anti-syntaxin antiserum. Monomeric 35 kDa syntaxin can be detected, as well as a 73 kDa SNARE complex containing syntaxin, synaptobrevin, and SNAP-25. Since 7S complex does not form in SDS (Figure 3B), the complex we detect corresponds to that already formed *in vivo*. The 7S complex is dissociated when the samples are boiled in SDS, and only monomeric syntaxin is detected on Western blots (data not shown). Head homogenates were similarly prepared from *syx*<sup>3-69</sup> flies that were kept at 23°C or given a 20 min exposure to 38°C. Whereas a 20 min heat pulse did not alter the amount of syntaxin in the 7S complex in wild-type flies, the complex was absent in *syx*<sup>3-69</sup> flies exposed to 38°C. All of the syntaxin in these flies migrates in the monomeric form (Figure 3C). Even at 23°C, *syx*<sup>3-69</sup> flies show a significant decrease in the amount of 7S complex, consistent with the defect in synaptobrevin binding. These data demonstrate that formation of the 7S complex is required for synaptic transmission *in vivo*.

#### ***comt* Mutants Accumulate Syntaxin and SNARE Complexes on Synaptic Vesicles**

In contrast with the reduction in 7S complex in *syx*<sup>3-69</sup> mutants, *comt*<sup>ST17</sup> flies given a 20 min heat pulse showed a dramatic accumulation of the 7S complex (Figure 3C). The *comt*<sup>ST17</sup> mutation is caused by a G274Q substitution in the D1 ATP-binding domain of NSF (Pallanck et al., 1995), a region essential for ATP hydrolysis and disassembly of the 7S complex *in vitro* (Nagiec et al., 1995). Thus, NSF is required *in vivo* to disassemble neuronal SNARE complexes. To localize the accumulation of SNARE complexes in *comt* mutants, differential centrifugation of *Drosophila* head homogenates was used to purify subcellular fractions containing heavy membranes enriched in presynaptic plasma membrane proteins and light membranes enriched in synaptic vesicle proteins. Quantitative immunoblots demonstrated that our purification protocol resulted in a 4.3-fold enrichment of synaptobrevin in light membrane fractions from total homogenates. In wild-type flies, the majority of the 7S complex was detected in the heavy membrane fraction, with only 1% of SNARE complexes found in light membranes (Figures 4A and 4B). In contrast, *comt*<sup>ST17</sup> mutants given a 20 min 38°C heat pulse showed a dramatic increase in the amount of 7S complex in light membranes

but only a modest increase in the 7S complex associated with the heavy membrane fraction (Figures 4A and 4B). These data provide evidence for the preferential accumulation of SNARE complexes on synaptic vesicles in *comt* mutants.

Two mechanisms can account for the increase in 7S complex on synaptic vesicles in *comt* at 38°C. One possibility is that NSF functions to disrupt 7S complexes that are assembled on synaptic vesicles (Otto et al., 1997) to allow productive *v-/t*-SNARE pairing between vesicle and target membranes at later stages in the cycle. An additional or alternative possibility is that there is a failure to dissociate SNARE complexes formed during the fusion process and these unresolved complexes are recycled on endocytosed vesicles. In this latter view, syntaxin content would go up on synaptic vesicles in *comt* mutants at 38°C. To address this question, we measured the amount of syntaxin in the light and heavy membrane fractions by quantitative immunoblot analysis using <sup>125</sup>I-conjugated secondary antibodies and phosphorimaging. In *comt*<sup>ST17</sup> mutants maintained at the permissive temperature, the ratio of syntaxin in light membranes to heavy membranes was 0.37. However, *comt*<sup>ST17</sup> flies given a 20 min heat pulse showed a 2-fold increase in syntaxin content in the light membranes, yielding a light-to-heavy membrane ratio of 0.68 (Figures 4C and 4D). Quantification of synaptotagmin as a control in wild-type and *comt*<sup>ST17</sup> flies given a 20 min 38°C heat pulse showed a ratio of 2.5 and 2.4, respectively, of synaptotagmin immunoreactivity in light membranes compared to heavy membranes, indicating that there is a selective enrichment of syntaxin on synaptic vesicles. These data demonstrate that the protein composition of synaptic vesicles is altered in *comt* at 38°C, with an increase in 7S complexes and a doubling of the normal amount of syntaxin. These findings suggest that NSF mediates disassembly of the 7S complex after fusion. The loss of NSF activity leads to increases in undissociated SNARE complexes on recycling synaptic vesicles that prevent productive *v-/t*-SNARE pairing at later stages of the exocytotic cycle. Consistent with this model, the disassembly of the SNARE complex by NSF is tightly coupled to exocytosis, as there is a complete absence of 7S complex in *shi* at 38°C, where all the SNAREs reside in the presynaptic plasma membrane (Figure 3C). Thus, NSF activity in *shi* mutants is capable of maintaining disassembled SNARE complexes, consistent with the ability of NSF to dissociate SNARE complexes that form in the same membrane (Otto et al., 1997). The absence of 7S complex in *shi* also suggests that NSF-mediated disassembly of the SNARE complex may be coupled to additional protein-protein interactions that prevent syntaxin from engaging *v*-SNAREs until later stages of the synaptic vesicle cycle. Together, these findings demonstrate that assembly and disassembly of the 7S SNARE complex is required for synaptic transmission in *Drosophila*, and that disruption of either process by *syx*<sup>3-69</sup> or *comt*<sup>ST17</sup> results in paralysis. To further characterize the defects associated with blocking either assembly or disassembly of the 7S complex, we compared the behavior and physiology of the *syx* and *comt* temperature-sensitive paralytic mutants. A comparison of the time course of the block in synaptic



**Figure 4.** Accumulation of 7S SNARE Complexes and Syntaxin on Synaptic Vesicles in *comt<sup>ST17</sup>*  
 (A) 20  $\mu$ g of protein from the heavy (H) or light (L) membrane fractions were separated on a 9%/15% discontinuous gel and probed with the anti-syntaxin antiserum 8C3. The 7S complex showed a 2-fold accumulation in the heavy membrane fraction and a 33-fold accumulation in the light membrane fraction in *comt* flies given a 20 min 38°C heat pulse. Also, notice that the amount of monomeric syntaxin in the light membrane fraction from *comt* at 38°C has increased relative to controls. The inset at the bottom shows an overexposed portion of a blot containing the 7S complex to demonstrate the small amount of SNARE complex normally present on synaptic vesicles.  
 (B) Densitometric quantitation of the fraction of 7S in light membranes compared to heavy membranes.  
 (C) 20  $\mu$ g of protein from the indicated fractions were separated by SDS-PAGE on a 12% gel and probed for either synaptobrevin or syntaxin and developed with ECL. Whereas synaptobrevin is enriched in the light membrane fractions in each case, only in *comt* mutants given a 38°C heat pulse is syntaxin enriched in light membranes.  
 (D) Densitometric quantitation with <sup>125</sup>I-conjugated secondary antibodies of syntaxin content in the light versus heavy membrane fractions.

transmission caused by *syx<sup>3-69</sup>* and *comt* mutants is shown in Figure 5A. *syx<sup>3-69</sup>* showed a rapid loss and recovery of on-/off-transients when shifted between 20°C and 38°C. Thus, failure to assemble the 7S complex leads to a rapid block in synaptic transmission and paralysis, consistent with a late postdocking role for the SNARE complex. In contrast, *comt* mutants took 2–3 min at 38° before the on-/off-transients were lost and required substantial recovery times at 20°C for synaptic transmission to return to preblock levels. These data suggest that several rounds of vesicle cycling can occur in the absence of NSF activity (which is inactivated at 38°C), unlike the immediate block in *syx<sup>3-69</sup>* mutants. Alternatively, NSF may be slowly inactivated at 38°C, resulting in slower kinetics of synaptic failure. Several observations suggest that this latter explanation is unlikely. First, *comt* mutants given a 1 min heat pulse at 38°C and then returned to 20°C lost on-/off-transients several minutes after being shifted back to 20°C (Figure 5B). These results suggest that NSF activity is inactivated by a 1 min heat pulse, and, upon return to 20°C, the loss of NSF activity is phenotypically expressed as a delayed loss of on-/off-transients after the consumption of vesicles already acted upon by NSF prior to the

heat pulse. Second, three independent recessive *comt* mutations (*comt<sup>ST17</sup>*, *comt<sup>ST53</sup>*, and *comt<sup>1P</sup>*) that alter different amino acids in various locations of the NSF polypeptide showed similar behavior, suggesting that these defects are due to a general loss of NSF activity at 38°C rather than to a particular type of protein unfolding. Third, analysis of the recovery of on-/off-transients following a 38°C heat pulse confirmed that *syx<sup>3-69</sup>* and *comt* affect synaptic transmission differently (Figure 5C). Following the recovery of transients at 20°C after a heat pulse, repetitive light pulses consistently evoked on-/off-transients during the recovery period in *syx<sup>3-69</sup>*. In contrast, a single pulse of light was able to evoke on-transients in *comt*, but repetitive light pulses failed to do so. A longer period of recovery was required before an additional light pulse could trigger synaptic responses in *comt* mutants. The exhaustion of NSF activity during repetitive stimulation is consistent with a defect in the maintenance of fusion-ready vesicles and supports the idea of a requirement for NSF-mediated disassembly of SNARE complexes that accumulate in membranes of the presynaptic terminal and synaptic vesicles following previous rounds of exocytosis and endocytosis. Following the block of synaptic transmission at the

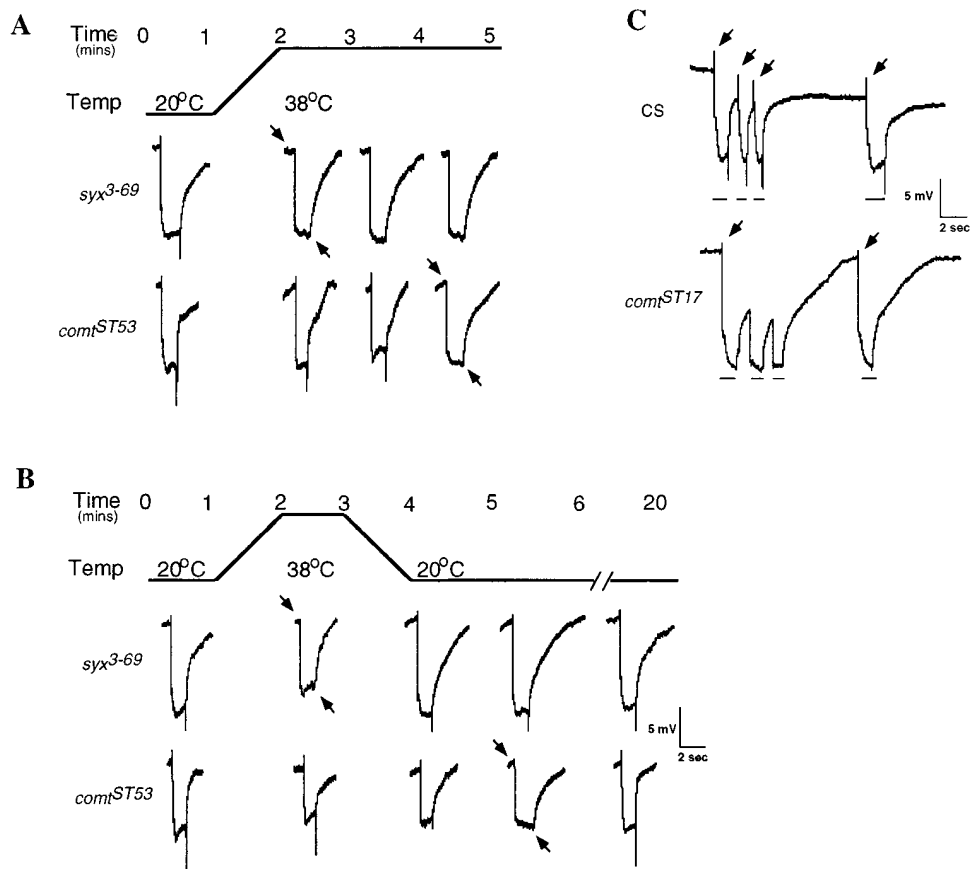


Figure 5. Time Course of the Loss and Recovery of On-/Off-Transients in *syx<sup>3-69</sup>* and *comt<sup>ST53</sup>* Mutants

(A) Flies were rapidly heated and maintained at 38°C. Test light pulses were given to monitor the loss of on-/off-transients. Whereas *syx<sup>3-69</sup>* mutants rapidly lose on-/off-transients, *comt* mutants took several minutes at 38°C before defects in synaptic transmission occurred.

(B) Flies were rapidly heated to 38°C and incubated for 1 min, followed by rapid cooling to 20°C. Test light pulses were given during the indicated time periods. The loss of on-/off-transients is indicated by the arrows. Whereas *syx<sup>3-69</sup>* mutants rapidly lose and recover on-/off-transients, *comt<sup>ST53</sup>* mutants did not lose on-/off-transients until several minutes later.

(C) Flies of the indicated genotype were rapidly brought to 38°C and incubated for 1 min. Flies were then returned to 20°C and the loss of on-/off-transients followed. The subsequent recovery of on-/off-transients during the next 30 min was monitored with test light pulses. The on-transients consistently return before the off-transients in all mutants tested, suggesting that the off-transient is more sensitive to the level of synaptic function. During this recovery period, both wild-type and *syx<sup>3-69</sup>* (data not shown, but identical to wild type) respond to rapid light pulses with on-/off-transients (arrows), whereas *comt* loses the on-transients during repetitive stimulation.

restrictive temperature in *comt*, NSF would have to disassemble a large accumulated pool of complexed SNAREs upon return to the permissive temperature for efficient synaptic transmission to resume. This would be a gradual process, given the slow enzymatic activity of NSF (Morgan et al., 1994). In contrast, the rapid loss and recovery of on-/off-transients in *syx<sup>3-69</sup>* suggests an instantaneous and rapidly reversible block in synaptic transmission.

The electrophysiological defects in the visual system correlated with the observed paralytic behavior of the flies at nonpermissive temperatures (Figure 6). *syx<sup>3-69</sup>* mutants become paralyzed within seconds at 38°C, whereas *comt* mutants take longer and become paralyzed within 2 min. Like mutants with defects in axonal conduction (*para*, *nap*, and *tipE*), *syx<sup>3-69</sup>* mutants recover rapidly when returned to the permissive temperature, regardless of the length of exposure to 38°C. In contrast,

*comt* mutants remain immobile for longer time periods that correlate with the length of exposure to 38°C.

#### Formation and Disassembly of the SNARE Complex Is Required after Vesicles Are Targeted to Active Zones

To examine the morphological consequences of the loss of v-/t-SNARE pairing and NSF function, and to determine where defects in assembly and disassembly of the SNARE complex block the synaptic vesicle cycle, we performed ultrastructural studies on wild-type, *syx<sup>3-69</sup>*, and *comt<sup>ST17</sup>* flies. The first optic neuropil (lamina ganglionaris), where the ERG defects in *syx<sup>3-69</sup>* and *comt<sup>ST17</sup>* originate, was chosen for investigation. The lamina contains over 800 stereotypic optic cartridges. Each cartridge is delimited by a glial sheath and contains six photoreceptor axons (R1–R6) clustered around several

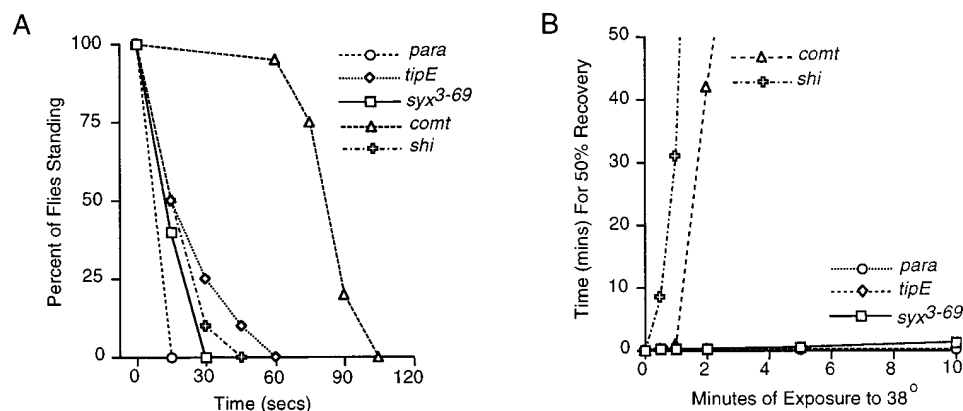


Figure 6. Kinetics of Paralysis of Temperature-Sensitive Paralytic Mutants

(A) Twenty male flies of each genotype were placed in a preheated vial at 38°C, and the percentage of flies climbing or standing was recorded in 15 s time intervals and plotted against the time of exposure to 38°C. *syx<sup>3-69</sup>* flies become paralyzed within 30 s at 38°C; *comt<sup>ST17</sup>* flies require a longer exposure to become paralyzed. In contrast, wild-type flies are still active after a 30 min exposure to 38°C. Five to ten trials with each genotype gave results similar to those shown. Flies were aged 1–4 days for all temperature shift experiments.

(B) For each time point on the abscissa, 20 flies of each genotype were heated at 38°C for the indicated time and allowed to recover at room temperature. The time for 50% of the flies to regain mobility was plotted on the ordinate. *syx<sup>3-69</sup>* flies rapidly recover from paralysis regardless of the length of exposure to 38°C (up to 10 min). In contrast, *comt<sup>ST17</sup>* and *shi<sup>f51</sup>* required progressively longer time periods to recover that correlated with the length of exposure to 38°C (the time for recovery continues to increase for flies held longer than 2 min at 38°C and is not shown). For *shi*, this recovery profile is likely to result from the depletion of synaptic vesicles and the time required to replenish these pools to preblock levels. For *comt*, the long recovery time is likely to represent slow time-dependent disassembly of accumulated SNARE complexes.

postsynaptic laminar neurons (L1 and L2). Synaptic contacts between photoreceptor axons and laminar neurons can be identified by the presence of presynaptic ribbons (T-bars) with their complement of docked synaptic vesicles and opposing postsynaptic specializations (Saint Marie and Carlson, 1982). We focused our analysis on the most common synapses in the cartridge, the R to L1/L2 histaminergic synapses that are responsible for the on-/off-transients of the ERG. Flies maintained in the dark or with minimal light stimulation have photoreceptor terminals filled with synaptic vesicles, a requirement for being able to respond rapidly to changing light stimuli. In *shi* mutants, the entire pool of vesicles at photoreceptor terminals can be depleted in several minutes with constant light stimulation at nonpermissive temperatures, while 20–30 min are required to completely refill the terminal with vesicles when the flies are returned to the permissive temperature (Koenig and Ikeda, 1996). We therefore selected a 10 min constant light stimulation protocol at 38°C that would drive vesicle cycling, allowing us to observe where in the pathway *comt* and *syx<sup>3-69</sup>* mutants are defective. This stimulation protocol results in relatively sparse numbers of vesicles in wild-type flies (Figure 7), as expected, because endocytosis is slower than exocytosis. A total of 131 micrographs were examined from wild-type, *syx<sup>3-69</sup>*, and *comt<sup>ST17</sup>* flies ( $n = 3–6$  flies for each genotype). The architecture of the optic cartridge is normal in mutant flies, with each photoreceptor axon containing synaptic vesicles 30–40 nm in diameter in all degrees of proximity to the T-bar. The most dramatic difference is an increase in the number of synaptic vesicles in photoreceptor terminals of *syx<sup>3-69</sup>* compared with wild type (Table 1), indicating that a syntaxin–synaptobrevin interaction is required for exocytosis. A similar but less striking increase

in vesicles was also found in *comt<sup>ST17</sup>* flies, confirming that the inability to disassemble SNARE complexes does not impair the endocytotic machinery. In addition, we observed clear increases in the number of vesicles that were clustered around T-bars in *syx<sup>3-69</sup>* and *comt<sup>ST17</sup>* mutants (Table 1 and Figure 7). Although *syx<sup>3-69</sup>* and *comt<sup>ST17</sup>* both had statistically significant increases in docked vesicles compared to wild type, there was no statistical difference in the number of docked vesicles between *syx<sup>3-69</sup>* and *comt<sup>ST17</sup>* ( $p < 0.45$ , unpaired Student's *t* test). These ultrastructural findings suggest that the defect in synaptic transmission in *syx<sup>3-69</sup>* and *comt<sup>ST17</sup>* mutants is secondary to a failure to exocytose vesicles at the synapse, resulting in increases in the number of morphologically docked synaptic vesicles. Thus, the 7S complex is required for vesicle fusion after synaptic vesicles are targeted to active zones. The buildup of vesicles around active zones in *comt* mutants is also consistent with this conclusion, as undissociated SNARE complexes on vesicles would result in a failure to form productive v-/t-SNARE pairings after vesicle docking.

## Discussion

Elucidation of the mechanisms underlying membrane trafficking constitutes a major focus in modern cell biology and is essential for understanding the basis for neuronal communication. We have demonstrated that cycles of assembly and disassembly of the SNARE complex are essential for neuronal exocytosis *in vivo*. The results of our studies extend from a behavioral phenotype of paralytic flies to an electrophysiological characterization of the underlying defect, an identification of

Table 1. Ultrastructural Defects in *syx*<sup>3-69</sup> and *comt*<sup>ST17</sup> Mutants

Genotype	Vesicles/Terminal	Vesicles/T-bar	Capitate Projections/Terminal
CS	17.2 ± 11.5 (43)	2.2 ± 1.8 (36)	2.6 ± 1.4 (50)
<i>syx</i> <sup>3-69</sup>	116.4 ± 37.5 (22) (p < 0.001)	6.0 ± 1.8 (21) (p < 0.001)	2.9 ± 1.5 (45) (p = 0.296)
<i>comt</i> <sup>ST17</sup>	60.4 ± 28.4 (23) (p < 0.001)	6.2 ± 1.7 (21) (p < 0.001)	2.6 ± 1.8 (19) (p = 0.896)

Data were obtained from three to six flies of each genotype maintained at 38°C for 10 min under constant light stimulation. Errors are standard deviation with the number of synapses examined in parentheses. Quantification of vesicles and capitate projections were made on cross-sectioned terminals of R1–R6 photoreceptors. Statistical significance was determined by unpaired Student's t test, with comparison to wild type shown in parentheses. Vesicles within 60 nM of a T-bar were included in the quantification of vesicles/T-bar.

the causative mutations, biochemical and in vivo molecular characterization of the consequences of point mutations in syntaxin and NSF, and finally an ultrastructural analysis of where these activities are required at the subcellular level. These data indicate that both syntaxin and NSF are required for efficient consumption of vesicles at the synapse and that mutations in either protein block synaptic transmission.

Because our data show that vesicles are targeted to active zones even when formation of the 7S complex is abolished, other targeting mechanisms must be employed at the synapse that are likely to involve protein-protein interactions with components of the T-bar and cytoskeleton. Given the conservation of threonine 254 in syntaxin isoforms that localize to the plasma membrane, it is likely that specific v-/t-SNARE interactions have evolved not as targeting mechanisms for docking but rather as compartment-specific interaction pathways required for vesicle fusion. Analysis of tetanus or botulinum toxin-poisoned synapses, where synaptobrevin or syntaxin have been cleaved (Hunt et al., 1994; Broadie et al., 1995; Marsal et al., 1997; O'Connor et al., 1997), and of animals lacking syntaxin (Schulze et al., 1995) also indicate a postdocking role for SNARE proteins. The phenotypes observed in response to these manipulations are likely due to the complete removal of activity of either SNARE. In contrast, *syx*<sup>3-69</sup> mutants apparently retain all of syntaxin's interactions with other synaptic proteins, with the exception of synaptobrevin. Thus, this mutation has allowed us to demonstrate that a direct v-/t-SNARE interaction is required for exocytosis in vivo. The loss of the 7S SNARE complex in *syx*<sup>3-69</sup> confirms that this complex is an essential component of the fusion machinery. The rapid paralysis and block in synaptic transmission at 38°C and recovery at 20°C is consistent with the finding that the SNARE complex is capable of mediating vesicular fusion in vitro (Weber et al., 1998).

The exact role of NSF in vesicle trafficking is controversial. Studies have indicated a role in fusion (Orci et al., 1989; Söllner et al., 1993a) or in priming reactions before (Mayer et al., 1996; Mayer and Wickner, 1997; Ungermann et al., 1998) or after (Banerjee et al., 1996; Barlowe, 1997; Schweizer et al., 1998) docking. Our findings confirm that NSF is required in vivo to disassemble the 7S complex and that this reaction is required for neurotransmitter release. By comparing the kinetics of the paralysis and block in synaptic transmission in *syx*<sup>3-69</sup> and *comt*, we can begin to place temporally the requirements for assembly and disassembly of the SNARE

complex. Because both paralysis and blockage of synaptic transmission are slower in *comt* alleles than in *syx*<sup>3-69</sup> mutants, it is unlikely that NSF normally disassembles SNARE complexes at the time of fusion, as this should produce a rapid block in exocytosis when NSF activity is blocked in *comt* mutants. It is more likely that NSF disassembles SNARE complexes after fusion when the SNAREs reside in the presynaptic plasma membrane prior to synaptic vesicle recycling. In addition, the fact that there is no 7S complex in *shi* mutants at 38°C, when endocytosis is blocked, suggests that NSF can disassemble SNARE complexes before recycling when the SNAREs reside in the presynaptic membrane. The finding that *comt* mutants do not have any apparent defects in endocytosis indicates that the presence of unresolved SNARE complexes does not interfere with the endocytotic machinery. Under normal circumstances, NSF may also be required to disassemble any SNARE complexes still present on synaptic vesicles due to inefficient recycling of the complexes after fusion (Walch-Solimena et al., 1995). The increase in syntaxin content on synaptic vesicles in *comt* mutants due to the recycling of undissociated 7S SNARE complexes supports this idea. The accumulation of docked vesicles in *comt* suggests that undissociated SNARE complexes on vesicles and target membranes prevent formation of productive v-/t-SNARE pairings between vesicles and target membranes after several rounds of exocytosis. The accumulated unresolved complexes would have to be disassembled by NSF before additional cycles of fusion could resume. Such defects could explain the altered fusion kinetics observed in squid synapses injected with NSF peptides (Schweizer et al., 1998). The large number of stages in vesicle trafficking in which NSF has been found to act might reflect where, in each pathway, recycling and activation of SNARE complexes is necessary. At the synapse, where local recycling of vesicles occurs, an attractive model supported by our data is that SNARE disassembly following fusion is coupled with SNARE reactivation for further rounds of exocytosis. Recent structural studies indicate that v- and t-SNAREs form parallel coiled-coil structures to which a cylindrical hexameric NSF complex binds in a lock-and-key fashion (Hanson et al., 1997b; Lin and Scheller, 1997). The parallel SNARE complexes between the vesicle and presynaptic membrane should bring the membranes into close apposition. Consequently, the SNARE complex may be inaccessible to a large hexameric NSF complex at this stage of the exocytotic reaction.

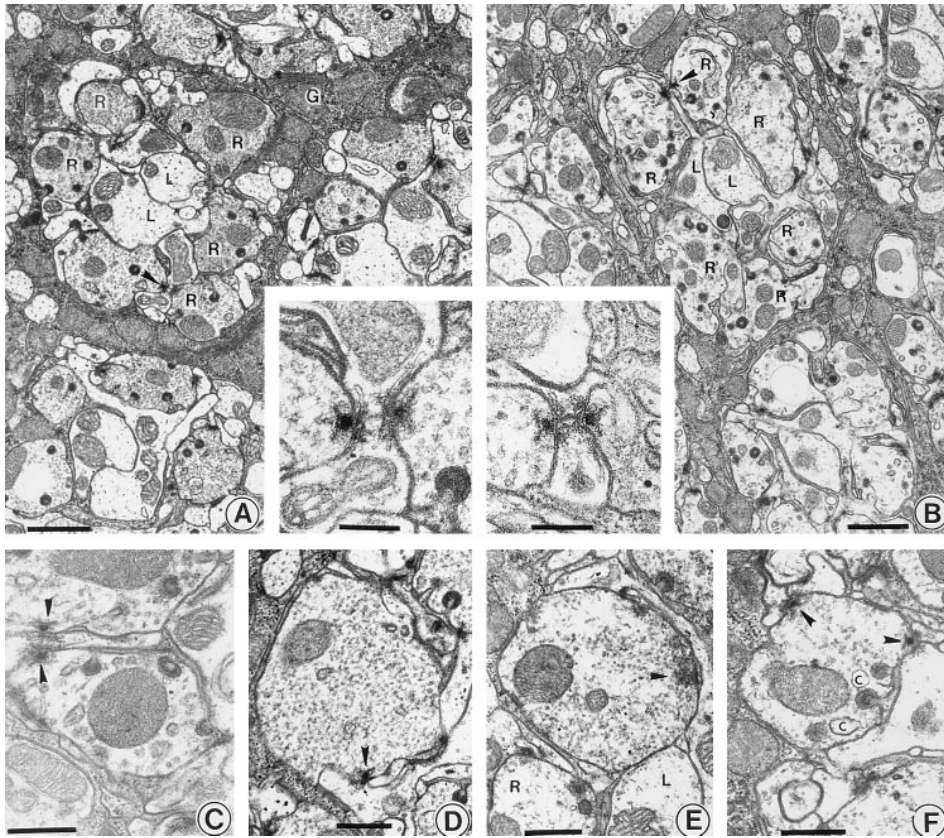


Figure 7. Ultrastructural Analysis of Wild-Type, *syx<sup>3-69</sup>*, and *comt<sup>ST17</sup>* Synapses Given a 10 min 38°C Heat Pulse under Constant Light Stimulation and Processed for EM

(A) A representative cross-sectioned optic cartridge in the lamina is shown with its glial sheath (G) in a *syx<sup>3-69</sup>* fly. Six reticular (R) axon terminals are clustered around several electron-lucent postsynaptic lamina neurons (L). The dark spheres are capitate projections invaginating into R axons from the adjacent epithelial glia and were abundant for all genotypes. T-bar active zones (arrowhead) at the R to L synapse are magnified in the inset. Abundant synaptic vesicles fill the terminals and can be found adjacent to and under T-bars. Scale bar, 1  $\mu\text{m}$ . Inset shows two opposing T-bar synapses from adjacent R axons onto a common L process. Scale bar, 0.25  $\mu\text{m}$ .

(B) A representative cross-sectioned optic cartridge from a wild-type fly shows relatively translucent R terminals due to the paucity of vesicles seen under these conditions. Scale bar, 1  $\mu\text{m}$ . Inset shows two R axons synapsing on a shared L cell process with relatively few vesicles (arrows) found near the active zone compared to *syx<sup>3-69</sup>*. Scale bar, 0.25  $\mu\text{m}$ .

(C) Cross-sectioned R axons of a wild-type fly demonstrate relatively few synaptic vesicles in the cytoplasm or at T-bars (arrowheads). Scale bar, 0.5  $\mu\text{m}$ .

(D) Cross-sectioned R axon of a *syx<sup>3-69</sup>* fly demonstrating a dramatic accumulation of vesicles filling the terminal. Scale bar, 0.5  $\mu\text{m}$ .

(E) Cross-sectioned R axon of a *comt<sup>ST17</sup>* fly. Again, the terminals are filled with synaptic vesicles that have not been consumed. Scale bar, 0.5  $\mu\text{m}$ .

(F) Cross-sectioned R axon of a *comt<sup>ST17</sup>* fly. Vesicles can be seen clustered around the two T-bars in this plane of sectioning (arrowheads). Two moderately sized cisterns (C) are also visible. Scale bar, 0.5  $\mu\text{m}$ .

One of the key questions in understanding the pathways of regulated and constitutive trafficking is determining what additional levels of control are needed to modulate membrane fusion. For regulated fusion pathways like synaptic transmission, additional components such as synaptotagmin are required to trigger fusion upon a calcium signal (Littleton et al., 1993b, 1994; Broadie et al., 1994; Geppert et al., 1994; Littleton and Bellen, 1995). Determining whether membrane fusion can be arrested after assembly of the 7S SNARE complex awaiting a calcium signal, or whether calcium entry itself promotes assembly of the 7S complex to drive fusion, have emerged as important points to address. The large number of synaptic mutations now available in *Drosophila* that block synaptic transmission

at specific points in the synaptic vesicle cycle should begin to allow us to explore these issues and shed light on the mechanism of regulated exocytosis.

#### Experimental Procedures

##### Fly Strains

Flies were cultured on standard medium at 23°C. *syx<sup>3-69</sup>* was generated in an F2 EMS screen for paralytic mutations on the third chromosome (marked with *st*) and mapped to 3-81 by recombination with *Gl Sb H* and *Pr Dr* chromosomes.

##### Binding Assays and Production of Recombinant Proteins

*Drosophila* full-length GST-syntaxin and GST-syntaxin T254I were generated in pGEX-2T, sequenced to verify the constructs, and subsequently expressed in JM109 cells. Recombinant proteins were

then prepared and immobilized on glutathione-Sepharose beads as previously described (Chapman et al., 1994). His-tagged synprint peptides from the  $\alpha_{1B}$  subunit of rat N-type  $Ca^{2+}$  channels (amino acids 718–963) and the  $\alpha_{1S}$  subunit of rat L-type  $Ca^{2+}$  (amino acids 670–800) channels (kindly provided by W. A. Catterall) and full-length His-tagged rat synaptobrevin II (Chapman et al., 1994) were expressed in JM109 cells, purified on  $Ni^{2+}$ -charged nitrilotriacetic acid agarose columns, and eluted with an 8–400 mM imidazole gradient. Eluates were run on SDS-PAGE and stained with Coomassie blue, and fractions containing the recombinant protein were pooled and dialyzed against TS buffer (20 mM Tris [pH 7.2], 150 mM NaCl) with 0.5% Triton X-100. The concentration of fusion proteins was determined by SDS-PAGE separation, Coomassie blue staining, and comparison with bovine serum albumin standards. Membrane and cytosolic preparations of wild-type *Drosophila* were made by freezing 10,000–20,000 flies in liquid nitrogen. Heads were obtained by sieving and were ground to a powder in liquid nitrogen. The powder was then homogenized on ice in TS buffer containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1  $\mu$ g/ml pepstatin) and subsequently solubilized with 1% Triton X-100 for 30 min at 4°C. Cell debris and cuticle were removed by centrifugation at 15,000  $\times$  g for 20 min, and the resulting supernatant was used in binding assays. Protein concentration of head supernatants was measured with Pierce BCA reagents. GST fusion proteins of *Drosophila* wild-type and T254I syntaxin (10  $\mu$ g) were immobilized on glutathione-Sepharose beads and incubated with *Drosophila* head fractions (1 mg of a 10 mg/ml preparation) or His-tagged fusion proteins (5  $\mu$ g of synaptobrevin II or N-type  $Ca^{2+}$  channel synprint and 10  $\mu$ g of L-type  $Ca^{2+}$  channel synprint) at 4°C for 2 hr in TS buffer containing either 2 mM EGTA or 1 mM  $Ca^{2+}$ . The beads were separated by centrifugation, and pellets were washed three times with TS containing either EGTA or  $Ca^{2+}$ . Equal fractions (1.25% of starting material for totals, 5% of starting material for GST lanes) were then subjected to SDS-PAGE, immunoblotting, and ECL. Monoclonal antiserum against syntaxin (8C3) was used at 1:2000, polyclonal anti-synaptobrevin at 1:2000 (van de Goor et al., 1995), monoclonal anti-cysteine string protein at 1:50 (Zinsmaier et al., 1990), polyclonal anti-Rab3a at 1:1000 (Schulze et al., 1995), polyclonal DSYT2 against synaptotagmin at 1:2000 (Littleton et al., 1993), monoclonal anti-ROP at 1:1000 (Harrison et al., 1994), monoclonal anti-SNAP-25 at 1:1000 (Schulze et al., 1995), monoclonal anti-synaptobrevin-2 (69.1) at 1:1000 (Edelmann et al., 1995), and anti-T7 for detection of synprint peptides at 1:1000 (Nøvgren).

For formation of recombinant 7S complexes, His-syntaxin 1A (amino acids 1–265) and His-synaptobrevin II (amino acids 1–94) were expressed in *E. coli* using pTrcHis vectors (Invitrogen) as described (Chapman et al., 1994). His-SNAP-25B (full length) was expressed using a modified version of pET11 called pOH2 (kindly provided by R. Jahn and H. Otto), resulting in a GNSHHHHH tag at the C terminus of the protein. All His fusion proteins were purified and quantified as described (Chapman et al., 1996). 7S “assembly” reactions were carried out by mixing His-syntaxin ( $7.25 \times 10^{-11}$  mol) with His-synaptobrevin and His-SNAP-25 ( $14 \times 10^{-11}$  moles of each) for 30 min at room temperature in 150  $\mu$ l of Tris buffered saline. As a control, His-syntaxin was incubated in the absence of synaptobrevin and SNAP-25. In parallel samples, SDS-PAGE sample buffer was added to syntaxin prior to the addition of synaptobrevin and SNAP-25. After the incubation, 75  $\mu$ l 2 $\times$  reducing SDS-PAGE sample buffer was added, and aliquots of each sample were heated to 100°C for 3 min. Boiled and unboiled samples were subjected to SDS-PAGE and analyzed by immunoblotting using the HPC-1 monoclonal antibody (Barnstable et al., 1985).

#### Preparation of 7S Complexes

Flies of the desired genotype were frozen in liquid nitrogen and vortexed, and equal numbers of heads (10) for each genotype were homogenized in 50  $\mu$ l of SDS sample buffer on ice. The samples were briefly centrifuged to pellet cuticle and 20  $\mu$ l of the supernatant was resuspended in 30  $\mu$ l of SDS sample buffer. Samples were loaded onto discontinuous 9%/15% SDS-PAGE gels without boiling and separated at 15 mA per gel to avoid the generation of excess heat (which breaks down the 7S complex). The gels were immunoblotted with anti-syntaxin monoclonal antibody 8C3 at 1:2000

dilution. Immunoreactive bands were visualized using ECL. Separation of subcellular fractions was carried out using 10,000–20,000 wild-type or *comt<sup>ST77</sup>* flies maintained at room temperature or given a 20 min 38°C heat pulse. Flies were frozen in liquid nitrogen, and heads were obtained by sieving and ground to a powder in liquid nitrogen. The powder was then homogenized on ice in 5 mM HEPES buffer (pH 7.4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 2  $\mu$ g/ml aprotinin, 1  $\mu$ g/ml leupeptin, 1 mM EDTA, and 1  $\mu$ g/ml pepstatin). Cell debris and cuticle were removed by centrifugation at 3,000  $\times$  g for 5 min. Heavy membranes were collected from the supernatant by centrifugation at 15,000  $\times$  g for 20 min. The resulting pellets were resuspended in 1% SDS, and light membranes were collected from the supernatant by centrifugation at 115,000  $\times$  g for 1 hr. The cytosol was collected and the pellet was resuspended in 1% SDS. Protein concentration of each fraction was measured with Pierce BCA reagents and was adjusted to 2 mg/ml. 7S complexes were run out on a 9%/15% gel as described above. Quantification of syntaxin content was done with monoclonal antiserum 8C3 on samples boiled for 5 min. Quantification of immunoblots was done on a Molecular Dynamics PhosphorImager or a Molecular Dynamics Personal Densitometer SI using ImageQuant software.

#### EM on Adult Heads

Heads of 1- to 3-day-old flies were fixed in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3) for 15 min at room temperature and then 2 hr on ice. After fixation, the specimens were rinsed in phosphate buffer and postfixed for 1 hr in 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.4) on ice. After a brief water rinse, the specimens were dehydrated and embedded in Spurr's resin. Sections were stained 10 min each in uranyl acetate and lead citrate and examined on a Philips EM 410 microscope.

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