

The Products of the *Drosophila stoned* Locus Interact with Synaptic Vesicles via Synaptotagmin

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The *stoned* locus of *Drosophila melanogaster* encodes two novel proteins, stonedA (STNA) and stonedB (STNB), both of which are expressed in the nervous system. Flies with defects at the *stoned* locus have abnormal behavior and altered synaptic transmission. Genetic interactions, in particular with the *shibire* (dynamin) mutation, indicated a presynaptic function for *stoned* and suggested an involvement in vesicle cycling. Immunological studies revealed colocalization of the stoned proteins at the neuromuscular junction with the integral synaptic vesicle protein synaptotagmin (SYT). We show here that *stoned* interacts genetically with *synaptotagmin* to produce a lethal phenotype. The STNB protein is found by co-immunoprecipitation to be associated with synaptic vesicles, and glutathione S-transferase pull-downs demonstrate

an *in vitro* interaction between the μ 2-homology domain of STNB and the C2B domain of the SYTI isoform. The STNA protein is also found in association with vesicles, and it too exhibits an *in vitro* association with SYTI. However, we find that the bulk of STNA is in a nonmembranous fraction. By using the *shibire* mutant to block endocytosis, STNB is shown to be present on some synaptic vesicles before exocytosis. However, STNB is not associated with all synaptic vesicles. We hypothesize that STNB specifies a subset of synaptic vesicles with a role in the synaptic vesicle cycle that is yet to be determined.

Key words: *Drosophila melanogaster*; protein interactions; *stoned*; *synaptotagmin*; synaptic vesicles

The proteins encoded by the *stoned* locus of *Drosophila*, stonedA (STNA) and stonedB (STNB), are critical for normal synaptic function. Viable mutant alleles of *stoned*, *stoned-temperature-sensitive* (*stn^{ts}*) (Grigliatti et al., 1973) and *stoned^C* (*stn^C*) (Homyk and Sheppard, 1977), are behaviorally abnormal, whereas lethal alleles (Miklos et al., 1987) demonstrate that *stoned* products are essential. Mosaic and Northern analysis suggested neural-specific expression (Petrovich et al., 1993; Andrews et al., 1996), and both STNA (Stimson et al., 1998) and STNB proteins (Fergestad et al., 1999) have been localized to presynaptic terminals at larval neuromuscular junctions.

The functions of the stoned proteins are unknown, but a combination of viable mutations at both the *stoned* and *shibire* loci is synthetically lethal (Petrovich et al., 1993). The *shibire* gene encodes dynamin (Chen et al., 1991; van der Blik and Meyerowitz, 1991), a GTPase with a pivotal role in endocytosis and vesicle recycling (Masur et al., 1990; Takei et al., 1995; Vallee and Okamoto, 1995). STNB has amino acid sequence homology to μ -adaptin proteins (Andrews et al., 1996). The μ 2-adaptin (Thurieu et al., 1988) is a subunit of AP2, a clathrin adaptor-protein-complex (for review, see Robinson, 1994; Kirchhausen, 1999). STNA contains signature sequences, DPF, (Stimson et al., 1998) that can bind the appendage domain of α -adaptin (Owen et al., 1999), also a subunit of AP2 and required for synaptic vesicle endocytosis (Gonzalez-Gaiten and Jäckle, 1997). In addition, the lethal alleles of *stoned* show alterations in the size and morphology of synaptic vesicles (Fergestad et al., 1999), a phenotype similar to

that observed in *Drosophila* with defects in the clathrin adaptor-protein AP180 (Zhang et al., 1998). All of these observations imply that *stoned* proteins function in endocytosis.

In contrast to the above, electrophysiological studies of *stoned* mutants, involving either the adult visual system (Kelly, 1983; Homyk and Pye, 1989) or the embryonic and larval neuromuscular junctions (Stimson et al., 1998; Fergestad et al., 1999), all show defects in synaptic transmission. Furthermore, both *stn^C* and *stn^{ts}* mutations increase the frequency of miniature-endplate potentials (Stimson et al., 1998). These alterations are consistent with changes in exocytosis and neurotransmitter release.

Hypomorphic mutations at the *Drosophila melanogaster* *sytI* locus result in behavioral, electrophysiological, and morphological phenotypes similar to those seen in *stn* mutants (DiAntonio et al., 1993; Littleton et al., 1993a, 1994; Reist et al., 1998). Synaptotagmin I (SYTI) is a synaptic vesicle protein with a proposed role in both exocytotic and endocytotic functions in both mammals (for review, see Zhang et al., 1994; Südhof and Rizo, 1996) and invertebrates (DiAntonio et al., 1993; Littleton et al., 1993a, 1994; Nonet et al., 1993; Jorgensen et al., 1995).

In this paper we report a synthetically lethal interaction in flies with mutations at both the *sytI* and *stn* loci, that STNB interacts with synaptic vesicles *in vivo* via SYTI, and that this interaction may be maintained throughout the vesicle cycle. In addition, *in vitro*, studies show that domains of both STNA and STNB bind the C2B domain of the SYTI protein.

MATERIALS AND METHODS

Drosophila strains and crosses. The *Drosophila* strains that we used—[*white stoned^{ts1}* (*wstn^{ts1}*), *white stoned^{ts2}* (*wstn^{ts2}*), *shibire^{ts1}* (*shi^{ts1}*), *shibire^{ts3}* (*shi^{ts3}*), *Suppressor of stoned* (*Su(stn)*), and *Oregon-R*]—were as described previously (Lindsley and Zimm, 1992; Petrovich et al., 1993). The synaptotagmin mutants *w/w; sytAD4/CyO*; *+/+* and *w/w; sytD27/Gla, Bc*; *P[elav-syt I]/+*, both strains homozygous/hemizygous for the *w* mutation, were supplied by T. L. Schwarz (Stanford University Medical Center, Stanford, CA) and were as published (DiAntonio et al., 1993; DiAntonio and Schwarz, 1994).

Crosses to produce flies hemizygous for each of the viable *stoned* alleles, *stn^{ts}* and *stn^C*, doubly heterozygous for the *syt* null mutations, *sytD27* and *sytAD4*, and heterozygous for the *syt⁺* minigenes, were as outlined in Figure 1. Control flies were genetically identical for the *syt* alleles but wild-type at the *stoned* locus.

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Antibodies. Polyclonal antisera to an MBP/STNA (residues 20–349) fusion was raised in rabbits as described previously (Andrews et al., 1996) and used at a dilution of 1:20,000 on Western blots, or affinity-purified for immunoprecipitations. Rabbit anti-stonedB antisera were from two sources. Antiserum raised to an MBP/STNB (residues 1025–1261) fusion protein was used at a dilution of 1:1000 on Western blots or affinity-purified as described previously (Andrews et al., 1996). Rabbit anti-stonedB antisera raised against two combined peptides (residues 88–104 and 1244–1261) was affinity-purified against a glutathione S-transferase (GST)/STNB (residues 643–1261) fusion protein. The purified STNB antibodies were used in separate immunoprecipitation experiments. Experiments using both purified STNB antibodies gave identical results. Rabbit anti-synaptotagmin I (DSYT-2) antibody was a gift to M.R. from Troy Littleton and Hugo Bellen (Littleton et al., 1993b) (Baylor College of Medicine, Waco, TX). The DSYT1 antisera was used at a dilution of 1:2000, or when affinity-purified against an MBP/SYTI (cytoplasmic domain) fusion, at a dilution of 1:5000. Rabbit anti-synaptobrevin (SYB) raised against a peptide, CMADAAPAGDAPPNA, from the cytoplasmic domain was a gift from J. Roos and R. B. Kelly (University of California Medical School, San Francisco, CA). It was used at a dilution of 1:2000 on Western blots. Mouse anti-cysteine string protein (CSP) antibody (mAb49) was kindly provided by Konrad Zinsmaier (University of Pennsylvania School of Medicine, Philadelphia, PA) and was used at a dilution of 1:200. The mouse anti-syntaxin (SYX) antibody (mAb 8C3) was a gift to M.R. from Seymour Benzer (California Institute of Technology, Pasadena, CA) and was used at a dilution of 1:500.

Fractionation of head homogenates. For the differential centrifugation, heads (0.1 gm) from Oregon-R flies were homogenized, Teflon on glass (20 strokes at 400 rpm), in 1 ml of buffer A in the absence of Ca^{2+} [10 mM HEPES, pH 7.4, 1 mM EGTA, 0.1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF)] or in buffer A plus Ca^{2+} [10 mM HEPES, pH 7.4, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, 1 mM PMSF]. Homogenates were then centrifuged at $1000 \times g$ to produce the P1 pellet fraction. The supernatant (S1) was centrifuged at $25,000 \times g$ and $4^\circ C$ for 40 min to produce the P2 pellet fraction. The resulting S2 supernatant was then subjected to centrifugation at $4^\circ C$ at an average of $125,000 \times g$ for 1 hr in a TL-100.3 rotor (Beckman TL-100 Ultracentrifuge) to obtain the P3 pellet and the final supernatant, S3. For organelle immunoprecipitation the P3 pellet was then resuspended in 1 ml of the homogenization buffer.

To generate material for the glycerol gradients, heads were homogenized in buffer A at a tissue-to-buffer ratio of 1:2, and the resulting homogenate was centrifuged at $1000 \times g$ for 10 min. The S1 supernatant (150 μ l) was then loaded onto the surface of a 5–25% glycerol gradient (1.7 ml) over a 150 μ l 50% sucrose pad. The gradients were spun at 50,000 rpm using a TLS-55 rotor in a Beckman TL100 tabletop ultracentrifuge for 30 min. Fifteen 133 μ l fractions were collected from each gradient. SDS sample buffer, without reducing agents, was added to each fraction, and the samples were boiled immediately and stored at $-20^\circ C$. The absence of reducing agent allows the STNB protein to run at its expected molecular weight of 138 kDa (Andrews et al., 1996).

Co-immunoprecipitations. Anti-STNA and anti-STNB antibodies raised against MBP-STNA and MBP-STNB fusion proteins were affinity-purified (Andrews et al., 1996). Tosyl-activated magnetic beads (Dyna, Dynabeads M-500 Subcellular) were prepared per manufacturer's instructions using Protein-A as the linker protein (120 μ g/6 $\times 10^7$ beads). The Protein A-linked beads were incubated overnight at $4^\circ C$ with either affinity-purified anti-STNB antibodies, affinity-purified anti-STNA antibodies, nonspecific rabbit IgG (preimmune serum), or no antibodies. The IgGs were covalently linked to the Protein A beads using dimethylpimelidate as described (Harlow and Lane, 1988). The beads were mixed with the P3 (vesicle enriched) fraction overnight at $4^\circ C$, and then washed with 0.1% bovine serum albumin in PBS. Bound material was eluted by boiling in reducing SDS sample buffer, electrophoresed on SDS PAGE, and Western-blotted. The blots were prepared and probed as previously described (Andrews et al., 1996).

Scanning electron microscopy. Dynabeads (2 $\times 10^7$ beads per sample) prepared for the co-immunoprecipitation experiments were incubated with 25 μ l of P3 fraction (equivalent to proteins from 25 μ g wild-type fly heads). Beads (10^5) from each treatment were then washed in PBS and prepared for scanning electron microscopy by standard techniques. The beads were fixed in 2.5% glutaraldehyde, washed in PBS, attached to polyethyleneimine-coated coverslips, coated with OsO_4 , and subjected to critical point drying. Silver-coated, mounted samples were examined with a Phillips XL30 FEG Field Emission Scanning Electron Microscope (Phillips, Eindhoven, The Netherlands).

Fusion constructs. Fusion constructs were obtained by ligating restriction fragments from cDNA clones, and PCR fragments, in-frame, into pMalc2 (New England Biolabs) and pGEX-4T-1 (Pharmacia Biotech) expression vectors.

The in-frame restriction sites used at the 5' end of ORF 2 constructs were the *SpeI* site at 4529 bp and an introduced *EcoRI* site at 5249 bp. The *SmaI* site at 5399 bp, the *SallI* site at 5927 bp, and the *EcoRV* site at 6782 bp were used as 3' insert/vector ligation sites (the numbering of bases is from the corrected sequence GenBank accession no. U54982, with base 1 the A of the ATG initiating translation codon). A pGEX-4T-1 clone containing the *SpeI-EcoRV* fragment was produced by sequential subcloning of this fragment into Bluescript SK⁺ (Stratagene, La Jolla, CA), pMal,

then via Bluescript SK⁺, into the pGEX vector (see Fig. 5A, Construct 1). Other subclones were generated similarly to Construct 1.

DNA encoding the N-terminal 306 amino acids of STNA was obtained from both wild-type and *stm*^{ts2} genomic DNA by PCR using commercially obtained oligonucleotides and standard techniques. The sequence of the 5' oligonucleotide was 5'-CCGGATCCCTTAAGCTACCAAAAGGCC-3', incorporating an in-frame *BamHI* site at the initiating methionine codon. The sequence of the 3' oligonucleotide was 5'-GCTCGGCGTTC-GAAGTGG-3'. The PCR fragments were ligated into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced to identify any introduced errors. Clones with no errors were subcloned into pGEX4-T-1 using the introduced *BamHI* site and an *EcoRI* site present in the pGEM-T Easy vector polylinker. These clones represent constructs 1 and 3 of STNA in Figure 6. The 966 bp *XhoI* fragment (bases 79–1045) was subcloned in frame into the *XhoI* site of pGEX-4T-1 to produce GST/Xho-p33 (see Fig. 6, Construct 2).

The cytoplasmic domain of synaptotagmin-I, 760–1780 bp in pET-15b, was used as a complete construct or cloned as separate C2A and C2B domains into the pMal vector. An *EcoRI* site was introduced at 408 bp into the coding sequence using the oligonucleotide 5'-cataGTGTGCGGGAATTCCTGAAGAAGCG-3', and an *XhoI* site was generated immediately 3' to the translation stop codon using the oligonucleotide 5'-gcagctcgactCATGTTCCTCAG-3' (lower case bases are homologous to the pET vector sequence of the cDNA clone). The 1020 bp *syfI* fragment produced was subcloned into pGEM-T Easy. The resulting *EcoRI-XhoI* fragment was then subcloned into pMalc2 to produce the MBP/SYT fusion protein. Separate constructs for each of the two C2 domains of the synaptotagmin-I cDNA, including some adjacent sequence, were obtained from this construct. DNA from the pGEM-T Easy clone was digested with *EcoRI* and *EcoRV* to obtain the fragment encoding the C2A domain. This fragment was subcloned into Bluescript and then into pMalc2 using *EcoRI* and *HindIII* restriction sites. The SYTC2A construct that was generated contains amino acid residues 137–327, (21,978 Da) of the SYT protein. The SYTC2B construct was produced by introducing an *EcoRI* site at 857 bp using the oligonucleotide 5'-CTGGTCAAGCGTTGAATTCGAGGGC-3', and a C-terminal *XhoI* site using the 3' oligonucleotide was used to produce the 1020 bp fragment. The 474 bp *syfI* fragment produced was subcloned into pGEM-T Easy and then into pMalc2 using the *EcoRI* and *SallI* sites in the pMal vector. The SYTC2B construct contains residues 317–473, (17,702 Da) of the SYT protein and so extends beyond the sequence encoding the C2B domain. The fusion proteins were bound to amylose resin, and the SYT domains were cleaved from the MBP in 50 μ l digests using FactorXa (New England Biolabs) according to the manufacturer's instructions. After digestion, EGTA was added to the samples to inactivate Ca^{2+} in the digestion buffer, and the isolated SYTI domains were identified on SDS-PAGE gels and by Western blotting. Equivalent amounts of the SYT C2A and SYT C2B fragment did not give equivalent signals on Western blots, and so the amounts of the two fragments used in binding to STN fusion proteins were adjusted to give approximately equivalent signals.

The C2B domain of SYTIV was generated by PCR from *Drosophila* genomic DNA using a 5' oligonucleotide that introduced an *EcoRI* site at 1114 bp (Accession No. AF181098), 5'-GCCACGGGAATTCAGATTC-GAGCC-3', and a 3' oligonucleotide that introduced a *HindIII* site at 1581bp 5'-GCGCCAGAAGCTTCCTGCC-3'. The PCR product was subcloned into pGEMT and sequenced to confirm that there were no *Taq*-induced errors. After the 467 bp fragment was subcloned into the pMal vector using the introduced restriction sites, SYTIV C2B/MBP was prepared and digested with FactorXa as described above for the SYTI constructs.

SYT binding experiments. GST fusion proteins were incubated with glutathione-Sepharose4B resin at $4^\circ C$ overnight on a rocking platform. The resin was then washed with 10,000-fold volumes of PBS, or when performing studies involving Ca^{2+} , it was washed with Tris-buffered saline (TBS). Bound protein was eluted from 20 μ l of resin and assayed by the Bradford method (Bio-Rad) to determine protein eluted per microliters of resin. The protein-bound resin was incubated with a crude cell extract of the SYT fusion and washed with 10,000-fold volumes of buffer (or buffer containing Ca^{2+}). For Ca^{2+} studies, the resin was washed with an additional 2000-fold volumes of TBS to remove Ca^{2+} , before elution. Protein eluted from resin was separated by SDS-PAGE, Western-blotted, and probed with affinity-purified SYTI polyclonal antibodies.

Sequencing. Sequencing was performed by the Sanger method (Sanger et al., 1977). Fragments generated by PCR using commercially obtained oligonucleotides (Bresatec) were subcloned into the pGEM-T and pGEM-T Easy vectors (Promega). Wild-type cDNA and genomic clones, and genomic DNA and cDNA derived from total RNA, were used as controls to identify strain polymorphisms and when sequence obtained from the mutant differed from the published sequence. Some errors in the published sequence were identified and have been corrected in the data base (GenBank accession no. U54982).

RESULTS

Genetic interactions of stoned and synaptotagmin

The aim of these experiments was to determine whether either of the viable *stm* alleles, when in combination with mutations at the *synaptotagmin I* locus, enhanced or suppressed the *syf I* phenotype.

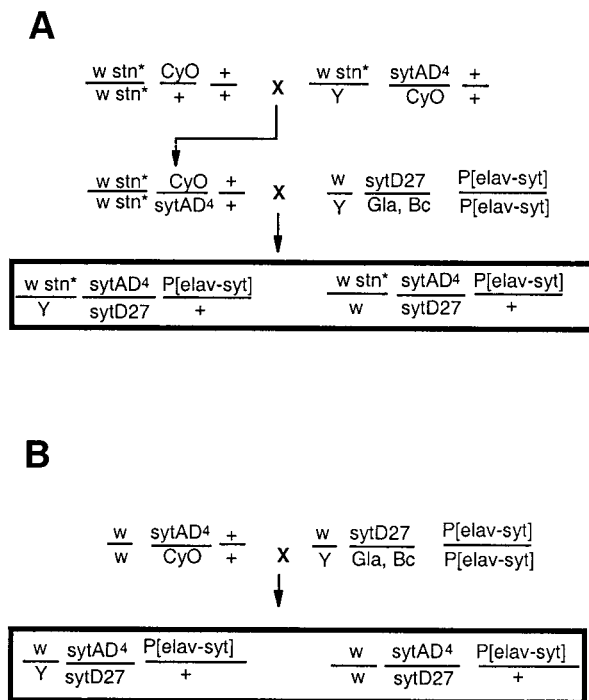


Figure 1. Generation of the *stoned*–*synaptotagmin* double mutants. The diagram shows the crossing strategy used to generate flies hemizygous for *stn* and with the *sytI* hypomorphic combination, that is, doubly heterozygous for *sytI* null mutations *sytAD4* and *sytD27* but heterozygous for the *sytI*⁺ transgene on chromosome 3. (For simplicity, we have not shown the early crosses introducing the *stn* alleles into the strain carrying the *sytAD4* mutation, and the genotypes shown are only those of offspring used in later crosses and of the double mutants from the crosses.) **A**, The experimental crosses. Three separate crosses were established. The crosses differed only in the *stn* mutation present on chromosome 1, that is, *stn*^{*} indicates *stn*^{ts1}, *stn*^{ts2}, or *stn*^C. **B**, The control cross. This generated flies identical in genotype to the experimental crosses except for being wild type at the *stn* locus.

The *stn*^C and *stn*^{ts} alleles are homozygous viable as adult flies and were isolated in the same genetic background, and both possess the same insertional polymorphism as the original Oregon-R strain (Andrews et al., 1996). Flies heterozygous for two *syt I* null mutations, *sytAD4* and *sytD27*, were supplied by the Schwarz laboratory. The P-element-mediated transposition of a *syt I* minigene to the third chromosome provides 10% of wild-type SYTI protein levels and allows flies with lethal null mutations on both chromosomes at the *sytI* locus to survive to produce fertile adults (DiAntonio et al., 1993; DiAntonio and Schwarz, 1994). Double mutant combinations were constructed (Fig. 1), and the viability and behavior of the resulting flies were investigated.

The total number of flies analyzed in each cross, the proportion of male to female offspring, and the proportion of doubly mutant offspring are shown in Table 1. Hemizygous mutant *stn* males eclosed later than females heterozygous for the same mutant *stn* allele, and the male/female ratios, ~60%, are as expected from previous viability data relating to *stn* mutants (Petrovich et al., 1993). Control males carrying only the *syt* hypomorphic combination (+/Y; *sytD27/sytAD4*; *P[elav-syt I]/+*), eclosed in approximately expected proportions (20.6%) as shown in Table 1. In contrast, the combination of the *syt* hypomorph with the *stn*^{ts} alleles significantly reduced the viability of these hemizygous *stn*^{ts} males. Only 9 doubly mutant males of the *stn*^{ts1}/Y; *sytD27/sytAD4*; *P[elav-syt I]/+* genotype (2.3% of total males) and 13 (5%) equivalent males carrying the *stn*^{ts2} allele eclosed. That the reduction in numbers of the double mutants carrying the *stn*^{ts} mutations was not caused by genetic background effects is demonstrated by the *stn*^C data. Double mutants with the *stn*^C allele (*stn*^C/Y; *sytD27/sytAD4*; *P[elav-syt I]/+*) eclosed in the expected numbers, as did female flies heterozygous for either *stn*^{ts} or *stn*^C (Table 1).

Table 1. Relative viabilities of *stn/syt* double mutants

Sex-chromosome genotype	<i>sytAD4/sytD27</i> <i>P[elav-syt⁺]</i> (as % of total)	<i>n</i>	Male/female ratio
<i>stn</i> ^{ts1} /Y	2.3*	399	
<i>stn</i> ^{ts1} /+	21.5	731	0.55
<i>stn</i> ^{ts2} /Y	5.8*	223	
<i>stn</i> ^{ts2} /+	23.8	416	0.54
<i>stn</i> ^C /Y	16.9	633	
<i>stn</i> ^C /+	24.9	884	0.72
+/Y	20.6	247	
+/+	18.2	248	0.99

+ indicates the presence of a wild-type *stoned* allele, and Y represents a Y chromosome. *n* = total number of flies of each sex resulting from the crosses (see Fig. 1). * denotes progeny values that are significantly different from expected values. (**p* < 0.001 using contingency χ^2 , actual numbers, not percentages, were used for calculation.)

Heterozygous females (*stn*^{ts1}/+; *sytAD4/sytD27*; *P[elav-syt I]/+*) showed abnormal behavior including a spread leg, “spider-like” appearance, and a slightly uncoordinated gait that was not seen in control flies. The few surviving *stn*^{ts}/Y; *sytAD4/sytD27*; *P[elav-syt I]/+* males had an extreme behavioral phenotype and were very sedentary and severely uncoordinated. These males all died within 48 hr of eclosion. It was not possible to discriminate between the surviving *stn*^{ts1} or *stn*^{ts2} combinations (see later data on the *stn*^{ts1} and *stn*^{ts2} mutations). Control males, +/Y; *sytAD4/sytD27*; *P[elav-syt I]/+*, although uncoordinated [see also DiAntonio et al. (1993)], appeared well coordinated by comparison with *stn*^{ts1}/Y; *sytAD4/sytD27*; *P[elav-syt I]/+* flies. A video showing *stn*^{ts}, the *syt I* hypomorph, and the doubly mutant males can be seen on <http://www.genetics.unimelb.edu.au/Kelly/Kelmov.html>.

The *stn*^C/Y; *sytAD4/sytD27*; *P[elav-syt I]/+* double mutants were behaviorally indistinguishable from *syt* hypomorphic controls (+/Y; *sytAD4/sytD27*; *P[elav-syt I]/+*).

These data clearly indicate a genetic interaction between the *stn*^{ts} mutations and *syt I*. Because synaptotagmins are an integral component of synaptic vesicle membranes, this data also suggests an interaction between the *stoned* protein(s) and synaptic vesicles.

Association of STNB with synaptic vesicle membranes

Hydrophobicity analysis of both the STNA and STNB proteins suggests that they should be soluble proteins. Wild-type fly head extracts, homogenized in both the presence and absence of calcium, were subjected to differential centrifugation to produce P1 (1000 × *g*), P2 (25,000 × *g*), and P3 (125,000 × *g*) pellets and a final supernatant fraction, S3 (see Materials and Methods). Western blots prepared from these fractions were probed with anti-STNA, anti-STNB, and anti-SYTI antibodies. The anti-SYTI antibodies were raised against the recombinant cytoplasmic region of the SYTI protein and recognize a number of isoforms of synaptotagmin (54–69 kDa), as described previously (Littleton et al., 1993b). Neither the STNA nor STNB proteins could be visualized in the supernatant fraction. The STNB protein co-sedimented with the synaptic vesicle protein marker SYT, primarily in the P2 and P3 fractions. STNA, on the other hand, preferentially partitioned into the P1 fraction, although some STNA was found in both the P2 and P3 fractions (Fig. 2A). This indicates that both *stoned* proteins preferentially partition into either membrane fractions or fractions containing large protein complexes. The association of STNA with the P1 fraction was investigated further. Solubilization of STNA from P1 was not achieved with Triton X-100, deoxycholate, or high NaCl concentrations; however, the chaotropic agent KI (1 M) effectively solubilized all of the STNA protein from the P1 fraction (data not shown). This indicates that STNA in the P1 fraction is not associated with heavy membranes but is more likely to be associated with a large protein complex.

As expected, the SYT isoforms were associated with both the P2

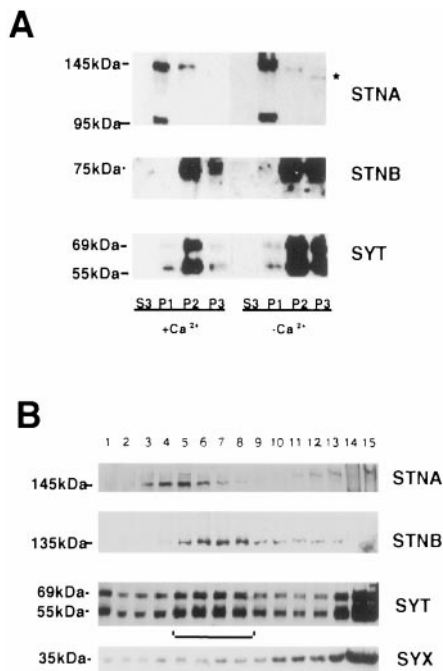


Figure 2. Distribution of STNA, STNB, and SYT proteins in head extracts. *A*, Heads were homogenized either in the presence or absence of Ca²⁺ and fractionated to produce a 1000 × g pellet (P1), a 25,000 × g pellet (P2), a 125,000 × g pellet (P3), and a final supernatant (S3). The protein content of each fraction was determined, and equal amounts of protein were electrophoresed. The resulting Western blots were probed with anti-STNA, anti-STNB, and anti-SYT antibodies. The figure shows that the bulk of the two *stoned* proteins partition into different fractions with STNA predominating in P1 fraction. The asterisk indicates that the STNA protein shows altered mobility in the P3 fraction. The distribution of the STNB protein parallels that of SYT. Under reducing conditions, the C-terminal fragment of STNB migrates with an apparent molecular weight of 75 kDa (Andrews et al., 1996). *B*, The numbered lanes are fractions from glycerol gradients of the S1 from wild-type flies. Fraction 1 represents the top of the gradient, and 15 represents the bottom of the gradient. Equal volumes from these fractions were then applied to nonreducing SDS-PAGE, Western-blotted, and probed with the antisera as indicated.

and P3 fractions, plasma membrane, and vesicle-enriched fractions, respectively. Also observed was a coincidental shift of SYT and STNB from the P3 to the P2 fraction when homogenization was performed in the presence of Ca²⁺. The supernatant fraction from the P1 centrifugation (S1) was applied to a glycerol gradient and centrifuged to separate membrane components. A peak of SYT (Fig. 2*B*, fractions 5–8), corresponding to the synaptic vesicle fraction, was observed as previously described (van de Goor et al., 1995). STNB protein co-sedimented with the SYT peak, whereas STNA, although entering the gradient, peaked in fractions 3–6 (Fig. 2*B*). These two results, the coincident redistribution of STNB and SYT in the presence of Ca²⁺ and their co-sedimentation in glycerol gradients, are consistent with an association of the STNB protein with synaptic vesicles. The plasma membrane marker syntaxin was also present in the gradients, probably indicating fragmentation of plasma membrane during homogenization, although its distribution did not mirror that of synaptotagmin/STNB or STNA.

To determine whether the STNB present in the P3 fraction is associated with synaptic vesicles, anti-STNB antibodies were attached to Protein A-coated magnetic beads, and incubated with a P3 fraction prepared from *Drosophila* head homogenates in the absence of calcium. These beads were then analyzed for the presence of STNB and the synaptic vesicle protein markers SYT, CSP, and SYB as well as the plasma membrane marker SYX. The results indicate that a major proportion of the STNB protein in the P3 fraction is immunoprecipitated (Fig. 3*A*). The STNB antibodies coprecipitate all three synaptic vesicle markers (SYT, CSP, and SYB), but not SYX (Fig. 3*A*). Although multiple species of SYT can be identified in P3 fractions, as was previously described for

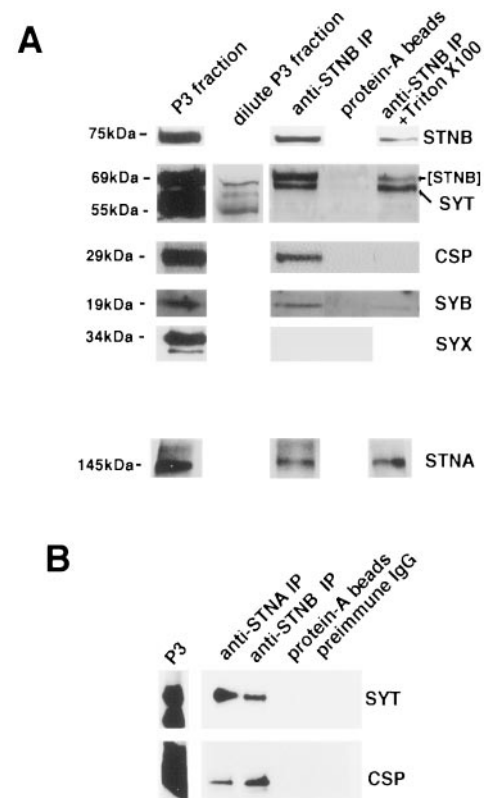


Figure 3. Immunoprecipitation studies using anti-STN antibodies. *A*, Western blots of protein eluates from immunoprecipitation experiments using the beads as shown in *A*. Blots were probed with anti-STNB, anti-SYT, anti-CSP, anti-SYB, anti-SYX, and anti-STNA antibodies. The lanes show the P3 fraction, the bead-associated fraction, the fraction associated with Protein A-only beads, and the bead-associated fraction remaining after washing with Triton X-100. The material shown in all lanes was derived from the same P3 fraction. The additional blot shown in the STNB + SYT panel contains a reduced amount of the original P3 fraction probed with anti-SYT antibodies showing the relative positions of the various SYT isoforms. The SYT panel is the blot from the top panel (STNB alone) reprobed with anti-SYT antibodies showing the presence of both the 69 kDa SYTI and the signal remaining from STNB. *B*, Immunoprecipitation of vesicles by anti-STNA antibodies. Western blot of protein eluates from beads coated with Protein A bound to anti-STNA, anti-STNB, Protein A alone, or nonspecific antibodies (preimmune IgG) as well as a P3 fraction. These blots were probed with anti-SYT and anti-CSP. All gels used in these assays were run under reducing conditions.

head homogenates (Littleton et al., 1993b), only the 69 kDa SYTI isoform is present in these precipitates.

On the basis of its deduced amino acid sequence, STNB does not contain any putative transmembrane segments and is unlikely to be an integral membrane protein. What then is the molecular nature of the STNB/vesicle association? To address this question, fractions of the STNB immunoprecipitations were washed extensively with 1% Triton X-100. The presence of the detergent entirely removed CSP from the precipitates and considerably reduced the amount of SYB present. However, Triton X-100 had no effect on the amount of SYTI bound to the beads (Fig. 3*A*). This result suggests that STNB is not associating with the lipid components of the vesicle membrane and that the interaction may be via SYTI.

There was relatively little STNA seen in the P3 fraction on the Western blots (Fig. 2*A*). However, we did observe STNA protein on the glycerol gradients (Fig. 2*B*), and although there was no coincidence of the peak fractions, there was overlap between STNA and the synaptic vesicle peak. The immunoprecipitations were therefore further probed for the presence of STNA. The STNA protein was found to be associated with the immunoprecipitates and to be insensitive to the Triton X-100 washes (Fig. 3*A*). When immunoprecipitations were performed using anti-STNA antibodies attached to beads, again SYTI and CSP were coprecipi-

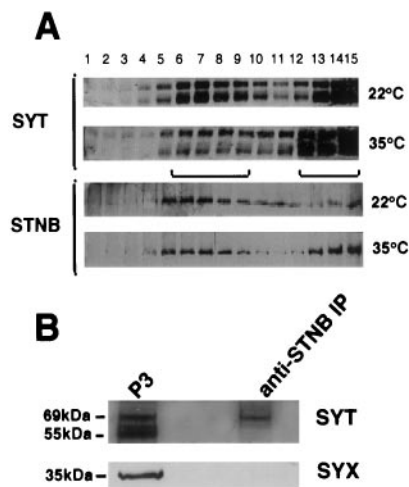


Figure 4. The effects of heat-treating *shi*^{ts1} flies on the distribution of STNA and STNB. *A*, Glycerol gradients were loaded with equal quantities of S1 material from heat-treated and untreated *shi*^{ts1} flies, and fractions 1–15 were collected as for Figure 2*B*. Western blots were probed with anti-SYT, anti-STNA, and anti-STNB antibodies. A peak of free synaptic vesicles was observed in fractions 6–9, most of which shifts to the bottom of the gradient in extracts from heat-treated flies. A similar shift is observed with both STNB and SYT. *B*, A P3 fraction from heat-treated *shi*^{ts1} flies (left-hand lane) was applied to Protein A/anti-STNB-coated beads, and the eluants were Western-blotted and probed with anti-SYT and anti-SYX antibodies (right-hand lane), showing that anti-STNB continues to immunoprecipitate synaptic vesicles but not plasma membrane.

tated (Fig. 3*B*). Therefore, both STNB and STNA can be found associated with synaptic vesicles in the P3 fraction.

Where does STNB act in the vesicle cycle?

The structure of STNB, containing as it does a μ 2 homology domain (Andrews et al., 1996), along with the interaction of *stn*^{ts} with *shibire* (Petrovich et al., 1993) and the alteration in the size of vesicles in *stoned* lethal mutants (Fergestad et al., 1999), all suggest that STNB may play a role in the synaptic vesicle endocytotic pathway (Zhang et al., 1998). Because STNB can be found associated with free synaptic vesicles in the P3 fraction, it might be presumed that these vesicles have recently been endocytosed. We investigated this possibility by looking at the distribution of STNB in extracts from heat-treated *shi*^{ts1} flies. If STNB becomes associated with synaptic vesicles only as they are budding from the plasma membrane, then in heat-treated *shibire* flies, where endocytosis is blocked at the budding stage, STNB should no longer colocalize with free synaptic vesicles. If recruitment of STNB to budding vesicles occurs after dynamin activity, then STNB should be in the supernatant fraction (the top of the gradient). If recruitment of STNB occurs before dynamin action, then in heat-treated *shibire* flies, STNB should be associated with the plasma membrane fraction (bottom of the gradient). To block endocytosis but not deplete all synapses of synaptic vesicles, the *shibire* flies were heat-treated in the dark. Figure 4*A* shows glycerol gradients prepared using S1 extracts from *shi*^{ts1} flies kept at 22°C (control) compared with similar flies treated, in the dark, for 15 min at 35°C. Contrary to expectation, the behavior of STNB mimics that of SYTI. As reported previously (van de Goor et al., 1995), there is a heat treatment-dependent shift in the SYT isoforms from the synaptic vesicle fraction (fractions 5–8) to the bottom of the gradient (fractions 12–15), although less than in previous studies, as the flies were dark-adapted. There is also a partial shift of STNB to the bottom of the gradient, but some STNB, like SYTI, remained associated with the free synaptic vesicle fraction (Fig. 4*A*). There is no indication of a shift of STNB to the soluble fractions (top of the gradient). The distribution of STNA on the gradients is unaffected by the heat treatment (data not shown).

To check that STNB in the vesicle fractions of heat-treated *shi*^{ts1} extracts remains bound to synaptic vesicles, P3 fractions from

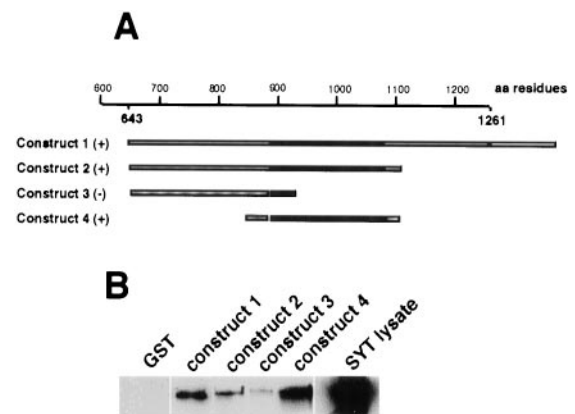


Figure 5. Molecular analysis of SYT binding to domains of STNB. *A*, A diagram indicating the STNB μ 2-like domain regions that were subcloned into the pGEX expression vector. The black regions represent the (discontinuous) μ 2-like domain (Andrews et al., 1996), the hatched region represents coding region, and the unfilled region represents the 3' untranslated sequence. *B*, Western blots probed with affinity-purified anti-SYT antibody. Lane 1 is a negative control with GST bound to the resin, showing that GST does not bind SYT. The other lanes are as labeled, with the final lane being a sample of the *Escherichia coli* lysate expressing the ~39 kDa 6x-His SYT.

control and heat-treated *shibire* flies were immunoprecipitated with anti-STNB antibodies. Figure 4*B* shows that anti-STNB antibodies co-immunoprecipitate SYTI from P3 fractions of heat-treated *shi*^{ts1} flies. These immunoprecipitates still do not have syntaxin associated with them and so are unlikely to have arisen from fragmented plasma membrane. These results suggest that STNB-associated vesicles do not represent only those recently endocytosed.

Molecular interactions between STNB and SYTI

The continued association of SYTI with immunoprecipitated STNB even after Triton X-100 treatment suggested a direct interaction between STNB and SYTI. It appeared likely that this interaction would be via the μ 2-like domain of STNB. To investigate this, a 621 residue protein containing the μ 2-like region, residues 883–1089, and adjacent sequence up to residue 1261 (Fig. 5*A*, Construct 1), was expressed in the pGEX expression vector to produce a GST fusion. The resin-bound GST/ μ 2-like fusion protein was incubated overnight with a crude extract of bacterial cells expressing the cytoplasmic portion of *Drosophila* SYTI as a 6x-His (pET) fusion protein of 39 kDa. When proteins eluted from the resin were Western-blotted and the blot was exposed to anti-SYTI antibody, the expected 39 kDa SYTI fusion protein was identified (Fig. 5*B*). The GST protein itself was unable to bind SYTI in this assay (Fig. 5*B*).

A series of fusion constructs with different regions of the μ 2-like domain of STNB (Fig. 5*A*) were similarly assayed. In repeated experiments, fusion proteins terminating at amino acid residue 930 (Fig. 5*A*, Construct 3) were unable to bind SYTI. However, SYTI bound to all fusion proteins containing amino acid residues 847–1108 of the STNB protein. Because residues 883–1138 of STNB constitute the μ 2-like domain (Andrews et al., 1996), this domain is sufficient for SYTI binding *in vitro*. It is possible, however, that residues outside the μ 2-like domain influence the strength of this interaction *in vivo*.

The effect of Ca²⁺ on binding of SYTI to the GST/STNB (Fig. 5*A*, Construct 1) was assayed by performing the overnight incubations and washes in the presence or absence of added Ca²⁺ up to 250 μ M. SYTI bound to the fusion in both the presence and absence of Ca²⁺, indicating that the interaction of the STNB μ 2-like domain with SYTI, like that of AP2 (Zhang et al., 1994), is independent of Ca²⁺ (data not shown).

The *stn*^{ts} mutation

Because the *stn*^{ts} alleles are known to interact with other mutant neurological loci, including *sytl*, the nature of these *stn*^{ts} mutations

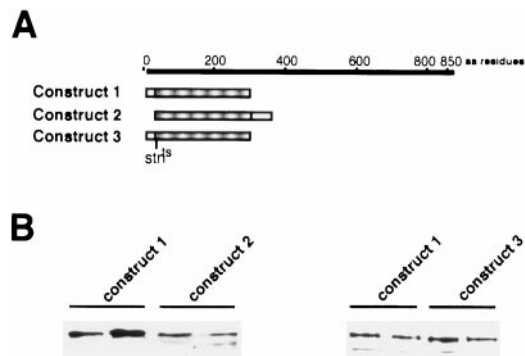


Figure 6. The N-terminal domain of STNA binds synaptotagmin. *A*, A diagram showing the STNA regions that were subcloned into the pGEX expression vector. The line represents the complete STNA protein. The hatched box represents the domain common to the fusion constructs that bound SYT. The position of the *stn*^{ts} mutation is also shown. *B*, Western blots of protein eluates probed with SYT antibodies. The first blot shows the SYT bound to approximately equal quantities of fusion proteins from *Constructs 1* and *2*. The second blot is a separate but equivalent experiment comparing the ability of the wild-type (*Construct 1*) and the *stn*^{ts} mutant fusion protein (*Construct 3*) to bind SYT.

was important. Genomic DNA and cDNAs obtained from *stn*^{ts2} mutant flies were sequenced. The only sequence difference found between wild type and *stn*^{ts2} was an A to T transversion present 104 bp into the coding sequence of ORF 1, resulting in a change from lysine (K) to methionine (M) at residue 35. The A to T substitution was found to be present in *stn*^{ts1}, *stn*^{ts2}, and *Su(stn);stn*^{ts2} flies but not in *stn*^C mutants, *shi*^{ts} mutants, or Oregon-R wild-type controls. Because both *stn*^{ts} alleles and *stn*^C and *shi*^{ts3} were isolated from the same Oregon-R strain, the absence of the mutation in *stn*^C and *shi*^{ts3} shows that this is not a polymorphism. These data also imply that there is only a single *stn*^{ts} mutation, *stn*^{ts1} being identical to *stn*^{ts2}.

Molecular interactions between STNA and SYTI

We have shown (Fig. 3) that STNA can be found associated with synaptic vesicles immunoabsorbed by the STNB antibodies. To determine whether the STNA protein might be associated directly with synaptotagmin, SYTI binding to STNA fusion proteins was analyzed as described previously for the STNB constructs. Three constructs were used. The first was a fusion protein that included the first 290 residues of STNA (GST/5'STNA), the second included residues 26–350 (GST/STNA Xho-p33), which removes most of the N-terminal region that would be missing if the methionine in the *stn*^{ts} mutant acted as a novel translation initiation site, and the third was identical to GST/5'STNA but contained the sequence encoding the K to M substitution found in the *stn*^{ts} flies (Fig. 6*A*). The results (Fig. 6*B*) indicate that residues 26–290 of the amino terminal region of STNA can bind SYTI. This region includes the sequence altered in the *stn*^{ts} mutation. Binding of SYTI to the GST/5'STNA construct containing the *stn*^{ts} mutation showed that this mutant protein also bound SYTI (Fig. 6*B*). The affinity of STNA for SYTI appears less than that seen with the STNB constructs; however, the binding of STNA to SYTI *in vitro* was observed consistently.

Domain specificity of stoned–synaptotagmin interactions

There are two C2 domains, C2A and C2B, in the SYTI monomer, and the two domains have been found to interact with different intracellular components (Davletov and Südhof, 1993; Fukuda et al., 1995; Li et al., 1995; Sugita et al., 1996). Two SYTI protein constructs were produced, each containing one of the C2 domains, and the ability of these protein constructs to bind to both the STNA/GST and STNB/GST fusions was investigated. The protein containing the cytoplasmic sequences including the C2A domain (residues 137–327) but excluding the C2B domain failed to bind to either STNA or STNB. However, the protein containing only the

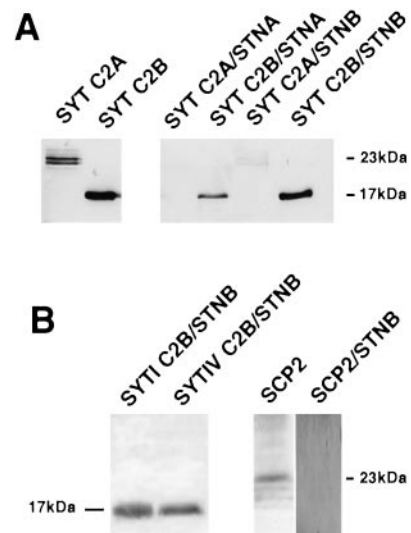


Figure 7. Stoned proteins bind to the C2B domain of synaptotagmin. *A*, The SYTC2A domain, or SYTC2B domain, were cleaved from maltose-binding-protein fusions (see Materials and Methods) and are shown in lanes 1 and 2. The SYTC2B binds to the STNA and STNB fusions (lanes 4 and 6) but SYTC2A does not (lanes 3 and 5). *B*, The SYTC2B domains of both SYTI and SYTIV are capable of binding to the μ 2-homology domain of STNB. The cleaved SYTI and SYTIV pMal fusion proteins both produce 17 kDa fragments. As a control for the specificity of this interaction, the *Drosophila* SCP2 (Kelly et al., 1997) protein fused in pMAL and cleaved from the fusion protein to produce a 23 kDa protein was used in the binding assay. No SCP2 protein was found associated with the beads.

C2B domain (residues 317–473) was found to bind to both STNA and STNB (Fig. 7*A*).

The immunoprecipitation studies indicated that only the 69 kDa SYTI isoform was coprecipitated with STNB. However, Littleton et al. (1999) identified genes for other synaptotagmin species in *Drosophila*, including a SYTIV homolog of 55 kDa that was shown to be associated with synaptic vesicles. The C2B domain (residues 325–374) of SYTIV was expressed as a pMAL fusion protein and shown to cross-react with the polyclonal anti-SYTI antibodies. We asked, therefore, whether STNB could interact with SYTIV *in vitro*. The SYTIV fusions were then assayed for their ability to bind to STNB as described above for the SYTI C2 domains. The SYTIV C2B domain was shown to interact with the μ 2-homology region of STNB (Fig. 7*B*).

DISCUSSION

The results presented here clearly show that there is both an *in vivo* and an *in vitro* interaction between the products of the *stoned* gene and the integral synaptic vesicle protein synaptotagmin I. We hypothesized that a genetic interaction would be observed between the viable *stn* alleles and the *syt* hypomorphic combination of genes, and our data show a synergistic lethality between the *stn*^{ts} mutation and *sytI*. Individually, both the *stn*^C mutation and the *syt* hypomorphic mutations remove the transient responses of the ERG (Homyk and Pye, 1989; DiAntonio and Schwarz, 1994), increase the frequency of miniature end plate potentials, and decrease the amplitude of the excitatory junction current (EJC) at the larval neuromuscular junction (Littleton et al., 1993a; DiAntonio and Schwarz, 1994; Stimson et al., 1998). Given the phenotypic similarities between these mutants, we anticipated a phenotypic enhancement in the *stn*^C/*Y*; *sytD27/sytAD4*; *P[elav-syt]*/+ doubly mutant fly. However, we found no genetic interaction between these mutations. In contrast, the *stn*^{ts} mutant, which shows an increase in the off-transient amplitude (Kelly 1983) and no effect on the larval neuromuscular EJC (Stimson et al., 1998), shows a lethal interaction with *sytI* as was previously observed for *shibire* and *dunce*. The *shibire* product, dynamin, is essential for vesicle endocytosis and links *stoned* to the endocytotic branch of the vesicle cycle. The

dunce locus encodes a cAMP phosphodiesterase, and mutation at this locus results in a chronic increase in cAMP levels (Byers et al., 1981). The calcium dependence of neurotransmitter release is altered in *dunce* mutants (Zhong and Wu, 1991), suggesting an indirect effect of cAMP on exocytosis. A retrograde effect of cAMP on quantal output from larval presynaptic terminals has also been observed (Davis et al., 1998). Therefore the interaction between *stoned* and *dunce* suggests a possible effect of *stoned* on exocytosis. Because synaptotagmin has been implicated in both exocytosis and endocytosis, the *stm/sytl* genetic interaction does not discriminate between the endocytotic or exocytotic branches of vesicle cycling; however, the data strengthens the argument for a role for *stoned* in these processes.

Investigation of possible protein interactions have led to the conclusion that STNB and SYTI interact directly *in vivo*. Both SYTI and STNB are localized to nerve terminals (Fergestad et al., 1999), although the co-sedimentation properties and the colocalization on glycerol gradients of STNB and SYT reported here are the first indications that the proteins might interact. Western blot analysis of the proteins attached to the anti-STNB antibody-coated beads indicates the presence of several integral synaptic vesicle proteins and clearly identifies the vesicles as free synaptic vesicles. The absence of SYX from these immunoprecipitates suggests low or no contamination with fragmented plasma membrane. That the detergent solubilization of vesicle membranes removed CSP and SYB but not SYT from these precipitates strongly implied a direct binding of STNB to SYT *in vivo*. The ability of STNB to directly bind to SYT was confirmed by the STNB-GST pull-downs showing that the μ 2-homology domain of STNB can bind to the C2B domain of SYTI independently of Ca^{2+} .

We observe that anti-STNB antibodies precipitate synaptic vesicles, but only those containing the 69 kDa isoform of synaptotagmin. More than one band is recognized by affinity-purified anti-SYTI antibodies on the gradients and in the P3 fraction. Although we cannot exclude the possibility that proteolysis of SYTI causes at least some of the bands that are seen (Littleton et al., 1993b), we would expect that SYTIV would be identified by the anti-SYTI antibodies as a band at 55 kDa in P3 fractions. SYTIV is present in *Drosophila* head protein homogenates and is an integral synaptic vesicle protein (Littleton et al., 1999). Our results show that SYTIV is recognized by the antibody and can bind to STNB *in vitro* (Fig. 7B). It is possible that STNB and SYTIV are never present at the same synapses, although a previous report states that SYTI and SYTIV are present together, in at least some synapses (Littleton et al., 1999). It seems likely that STNB binds specifically to SYTI and not to SYTIV *in vivo*, but a physiological interaction between STNB and SYTIV cannot be excluded.

The behavior of STNB and SYT in heat-treated *shi^{ts1}* flies indicates that STNB is not restricted to vesicles that have been recently endocytosed but may be constitutively associated with vesicles. There is movement of a high proportion of the 55 kDa band recognized by the SYTI antibodies to the plasma membrane fractions in heat-treated *shi^{ts1}* flies [van de Goor et al. (1995), and Fig. 4, this report]. The 55 kDa band may be proteolyzed SYTI, SYTIV, or a combination of both proteins, but the *shibire* data suggest that vesicles containing this moiety are capable of exocytosis and, according to van de Goor et al. (1995), endocytosis. Our immunoprecipitation data show no association between STNB and vesicles containing anti-SYTI cross-reacting proteins with molecular weights lower than 69 kDa (Fig. 3). We conclude that the presence of STNB on synaptic vesicles is not essential for either exocytosis or endocytosis of all vesicles. The constitutive association of STNB, therefore, marks a specific pool of vesicles and suggests that they are somehow differentiated from the remainder of the vesicle pool. A number of structurally and physiologically differentiated pools have been proposed (Koenig and Ikeda, 1996; Kuromi and Kidokoro, 1998) that are involved in both clathrin-dependent and clathrin-independent recycling (for review, see Palfrey and Artalejo, 1998). Because both AP2 and STNB bind SYTI, it is possible that STNB competes with and inhibits AP2 and

prevents clathrin-mediated cycling, perhaps resulting in fast vesicle cycling. The finding that α -adaptin mutant embryos lack vesicles in presynaptic terminals (Gonzalez-Gaitan and Jäckle, 1997) is evidence against this hypothesis. However, currently there is insufficient data to determine how a chronic loss of α -adaptin may affect vesicle biogenesis and recycling. An understanding of the number of vesicle pools, their physiological role, and the contribution to these pools made by STNB remains to be determined.

The distribution of the STNA protein differs markedly from that of STNB. The presence of STNA protein in the P1 fraction, along with its solubilization only in the presence of the highly chaotropic agent KI, suggests that most of this protein is involved in a dense multiprotein complex. It is tempting to speculate that STNA is associated with cytoskeletal elements, but this has yet to be shown. The presence of small amounts of STNA protein in the P3 fraction and the association of this STNA protein with STNB-containing vesicles implies an interaction between STNA and STNB. The observation that the P3 STNA has an altered mobility by comparison with the bulk of the protein may indicate that post-translational modification of STNA affects its interaction with the P1 protein complex or with synaptic vesicles, or both.

We have shown that the *stm^{ts}* mutation is located in the STNA protein. The amino terminal domain of STNA (residues 26–290) binds to the C2B domain of synaptotagmin *in vitro*. The ORF containing STNA is highly unusual in that it contains no sequences encoding internal methionine residues (Andrews et al., 1996). The *stm^{ts}* mutation, on the other hand, introduces such an internal methionine codon early in the STNA ORF. Because STNA and STNB are products of a dicistronic mRNA (Andrews et al., 1996), which in eukaryotes is rare, we currently have no understanding of the effects of this novel methionine on translation initiation or the read-through mechanism translating the second ORF. However, preliminary data indicate no major quantitative alteration in the levels of either STNA or STNB in *stm^{ts}* flies. We suggest that STNA has a specific role to play in the regulation of synaptic output, but it is unclear at present how and where this protein acts in the vesicle cycle.

The function of both the STNA and STNB proteins remains unknown; however, in this report we have shown that both of these proteins can associate with synaptic vesicles via SYTI and hypothesize that the association of STNB with SYTI defines a physiologically distinct pool of synaptic vesicles.

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