

Distinct Requirements for Evoked and Spontaneous Release of Neurotransmitter Are Revealed by Mutations in the *Drosophila* Gene *neuronal-synaptobrevin*

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Two modes of vesicular release of transmitter occur at a synapse: spontaneous release in the absence of a stimulus and evoked release that is triggered by Ca²⁺ influx. These modes often have been presumed to represent the same exocytotic apparatus functioning at different rates in different Ca²⁺ concentrations. To investigate the mechanism of transmitter release, we have examined the role of synaptobrevin/VAMP, a protein involved in vesicular docking and/or fusion. We generated a series of mutations, including null mutations, in *neuronal-synaptobrevin* (*n-syb*), the neuronally expressed synaptobrevin gene in *Drosophila*. Mutant embryos completely lacking *n-syb* form morphologically normal neuromuscular

junctions. Electrophysiological recordings from the neuromuscular junction of these mutants reveal that the excitatory synaptic current evoked by stimulation of the motor neuron is abolished entirely. However, spontaneous release of quanta from these terminals persists, although its rate is reduced by 75%. Thus, at least a portion of the spontaneous “minis” that are seen at the synapse can be generated by a protein complex that is distinct from that required for an evoked synaptic response.

Key words: exocytosis; synaptobrevin; VAMP; *Drosophila*; synapse; neuromuscular junction; synaptic vesicle; spontaneous release; mini; regulated release

VAMP or synaptobrevin, syntaxin, and SNAP-25 bind to each other *in vitro* in a complex that is thought to be at the heart of vesicle docking and fusion (for review, see Jahn and Südhof, 1994). Support for their central role in exocytosis comes from the finding that homologs of these proteins are essential in many vesicular transport events within all eukaryotic cells (for review, see Calakos and Scheller, 1996).

In vertebrate synapses the importance of syntaxin, synaptobrevin, and SNAP-25 has been substantiated by studies with clostridial neurotoxins that block synaptic transmission by cleaving these proteins (for review, see Schiavo et al., 1994b). Synaptobrevin, in particular, is the target of tetanus toxin and botulinum toxins B, D, F, and G (Schiavo et al., 1992, 1994a; Yamasaki et al., 1994).

It has been hypothesized that the specificity of vesicle targeting to appropriate receptor membranes is dependent on a vesicle protein (v-SNARE) interacting specifically with the target membrane proteins (t-SNARE). Thus, the targeting of the synaptic vesicles to the active zone would be accomplished by VAMP/

synaptobrevin binding to syntaxin and SNAP-25 (Söllner et al., 1993; Calakos et al., 1994). However, the application of clostridial toxins that proteolyze these components has not been observed to alter the docking of vesicles at active zones in several electron microscopic studies (Hunt et al., 1994; Broadie et al., 1995).

Although the clostridial neurotoxin experiments have provided useful information, some caution is appropriate. The toxins may not cleave 100% of their target proteins, especially if the protein is complexed tightly with other proteins. Furthermore, clostridial toxins cleave their protein targets near their C termini, and it is unclear if the truncated proteins have residual function. The clostridial toxins also may have additional undefined proteolytic targets or have additional enzymatic activities (Ashton et al., 1995; Foran et al., 1996). A genetic approach sidesteps these problems.

Two synaptobrevin homologs have been described in *Drosophila*. One such homolog, *synaptobrevin* or *syb* (Südhof et al., 1989), is expressed most strongly in the gut (Chin et al., 1993), although it may be present in all tissues at a low level. Another homolog, *neuronal-synaptobrevin* (*n-syb*), is highly expressed in the nervous system (DiAntonio et al., 1993a). *n-syb* is expressed in the embryonic CNS and PNS from ~12 hr after egg laying until adulthood; thus *n-syb* is an excellent candidate for a synaptic v-SNARE. Here we describe the generation of mutations in *n-syb*. Analysis of a null mutation has provided strong evidence that spontaneous fusions and evoked release differ in their requirements for this central component of the exocytotic complex.

Received June 30, 1997; revised Dec. 2, 1997; accepted Dec. 23, 1997.

This work was supported by a Silvio Conti Center for the Neurosciences award from the National Institute of Mental Health (T.L.S.); by a grant-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (Y.K.); and by fellowships from the Muscular Dystrophy Association (D.L.D.), American Heart Association (S.B.), Human Frontiers Program (B.A.S.), and the National Science Foundation and National Institutes of Health (R.W.B.). We thank Irene Inman for invaluable technical assistance and Huai Yu Mi for help in peptide coupling. We also thank Kendal Broadie for advice on dissections, Corey Goodman for the gift of monoclonal antibody 1D4 (anti-FasII), and Stephen DiNardo for the gift of *Drosophila* line 34.

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MATERIALS AND METHODS

Cosmid screening, identification of P1 clones, and preparation of probes. A cosmid library prepared from the isogenic strain *Iso-1* (kindly provided by John Tamkun, University of California, Santa Cruz, CA) was plated

on nylon membranes (Amersham, Arlington Heights, IL); the filters were processed by the standard Grunstein/Hogness method and hybridized with a cDNA probe, including the entire open reading frame (ORF) of *n-syb* in genomic hybridization buffer (Church and Gilbert, 1984). Seven positive clones were isolated, and one clone, 4D, was used for preparing probes for the P-element screen. Cosmid 4D was digested with a battery of enzymes, blotted, and hybridized with a cDNA probe as above. *Bam*HI (New England Biolabs, Beverly, MA) digestion produced two hybridizing bands of 8 and 10 kb. These fragments were subcloned into pBluescript SK⁺ (Stratagene, La Jolla, CA). The resulting plasmids were *Bam*HI-digested; the fragments were gel-purified twice and used as probes to identify pools from the P-element mutagenesis that contained a P-element insertion near *n-syb*. P1 clones from the area around 62A and 62B were obtained from the *Drosophila* Genome Center at Stanford (Stanford, CA; kindly provided by Matthew Scott). These clones were digested with *Bgl*II and *Xho*I, separated by agarose gel electrophoresis, blotted, and probed with an 8 kb *Eco*RI genomic fragment from *n-syb* and the flanking DNA around the line 34 starter P-element (see *Drosophila* stocks below) to assess how far it was from the *n-syb* gene.

Drosophila stocks. Flies were grown at 22°C on standard cornmeal/agar media. S. Dinardo (Rockefeller University, New York, NY) kindly provided line 34, which contains a single insertion of the plasmid-rescueable P-element, *PlacW*, at polytene band 62A/B. The third chromosome of line 34 was made isozygotic after recombining away an unrelated third chromosome-lethal mutation with the third chromosome from a *yw* stock. The transposase line containing the $\Delta 2-3$ source of transposase on the *TMS* balancer chromosome (marked with *Stubble*, *Sb*) was provided by the Bloomington *Drosophila* Stock Center (Bloomington, IN).

P-element mutagenesis. Line 34 females were crossed to *w*; *Dr/TMS*, $\Delta 2-3$, *Sb* males and F1 *w*+, *Sb* males and females were selected. 750 F1 *w*+, *Sb* females and 700 F1 *w*+, *Sb* males (both with mottled eyes) were mated individually to *yw* males and females, respectively. The F2 progeny were examined, and a single darker-eyed F2 male (that was not marked with *Sb*) was selected from each vial. Approximately 900 darker-eyed F2 males were, in turn, mated individually to *yw* females. After 5 d, the males were retrieved from the vials and were pooled into groups of 30 for plasmid rescue.

Plasmid rescue. Thirty pools of 30 males were homogenized, and the genomic DNA from each pool was purified (Kaiser and Goodwin, 1990) and resuspended in 100 μ l of 10 mM Tris-Cl, pH 8.0, and 1 mM EDTA (TE) supplemented with 100 μ g/ml RNase A (Sigma, St. Louis, MO). Ten microliters (three fly equivalents) were digested with 100 U of *Eco*RI (New England Biolabs) for 2 hr at 37°C, phenol/ CHCl_3 -extracted, CHCl_3 -extracted, ethanol-precipitated, rinsed with 70% ethanol, and resuspended in 50 μ l of TE, pH 8.0. Each sample was ligated overnight at 18°C in a volume of 400 μ l (to promote intramolecular ligations) containing (in mM) 70 Tris-Cl, pH 7.5, 10 MgCl_2 , 1 spermidine-HCl, 1 ATP, and 10 DTT plus 90 μ g/ml nuclease-free BSA (New England Biolabs) and 1200 U of T4 DNA ligase (New England Biolabs). Ligation reactions were phenol/ CHCl_3 -extracted, CHCl_3 -extracted, ethanol-precipitated, rinsed with 70% ethanol three times, and resuspended in 10 μ l of H_2O . The ligated DNA was added to 50 μ l of Electromax *DH10B Escherichia coli* (Life Technologies, Gaithersburg, MD) and electroporated with a Bio-Rad Genepulser (Bio-Rad, Hercules, CA). The bacteria were plated on 150 mm LB-agar plates supplemented with 100 μ g/ml ampicillin and grown overnight. The 300–1000 colonies resulting from each pool were scraped off the plates; their DNA was purified, digested with *Eco*RI, electrophoresed on 0.8% agarose (Life Technologies) gels, transferred to nylon membranes (Amersham), and UV cross-linked (Stratagene). The blots were probed with two randomly primed ³²P-labeled genomic *Bam*HI fragments (8 and 10 kb) and hybridized as described for the P1 blots. Blots were exposed to XAR film (Kodak, Rochester, NY) for several hours with an intensifying screen, and the two pools showing significant hybridization were identified. The two positive pools were subdivided and screened as described above until two different individual lines were identified, F33 and F82. Balanced stocks were established for lines F33 and F82 over *TM3*, *Sb* or *TM6*, *Ubx*, *y*+ or *TM6B*, *Hu*, *Tb*.

F33, a homozygous-lethal mutation, contained a P-element insertion in exon 1 of *n-syb* 150 bp 5' of the initiation ATG (in addition to the original starter P-element of line 34), as determined by sequencing the rescued plasmid and by Southern blot analysis. The original line 34 insertion in the F33 line was recombined away from the *n-syb* insertion; that stock was used in subsequent experiments and will be referred to as *n-syb*^{F33-R}. The

additional P-element insertion in F82 was ~4 kb downstream of the *n-syb* gene and was homozygous-viable; it apparently did not affect the transcription of the *n-syb* gene.

P-element excisions. *n-syb*^{F33-R} and *n-syb*^{F82} females were mated to *w*; *Dr/TMS*, $\Delta 2-3$, *Sb* males. *w*⁺, *Sb* F1 males were mated to *w*; *CXD/TM3*, *Sb* virgin females. Approximately 700 *w* F2 males were selected and tested for lethality in combination with *n-syb*^{F33}. Lines were established from lethal excisions *n-syb* ^{Δ F33B}, *n-syb* ^{Δ F33OO}, *n-syb* ^{Δ F82C}, and *n-syb* ^{Δ F33-8} over the balancers *TM3*, *Sb*, *TM6*, *Ubx*, *y*+ and *TM6B*, *Hu*, *Tb*. Seven excision lines that were viable in combination with *n-syb*^{F33} and were homozygous-viable for the excision chromosome also were established. Genomic DNA from the viable lines was digested with *Eco*RI, blotted, probed with the 2 kb *Eco*RI *n-syb* genomic fragment, and exposed to film as described above.

F2 lethal screen. An F2 lethal screen was performed to generate EMS alleles of *n-syb*. Briefly, isozygotic *red ebony* (*red e*) males were treated with EMS (ethane methyl sulfonate, Sigma) and mated to *w*; *CXD/TM3*, *Sb* virgin females. The resulting F1 *red e**/*TM3*, *Sb* males were crossed to *n-syb*^{F33}/*TM3*, *Sb* virgins; vials were scored after 14 d. Vials containing only *Sb* flies were selected. Two lines, I4 and I18, that initially showed lethality in the F2 lethal screen were identified. Subsequently, recombining these lines to *n-syb*^{F33}/*TM3* resulted in viable *red e**/*n-syb*^{F33} adult flies that were severely uncoordinated. Stocks were generated of the hypomorphic alleles of *n-syb*^{I4} and *n-syb*^{I18}.

PCR and Southern blot analysis of excisions. DNA was prepared from each of the 65 *n-syb*^{F33-R} excisions that failed to complement the *n-syb*^{F33-R} P-element insertion, and these DNAs were used in a PCR assay with a primer to the 31 bp repeat in the P-element (5'-CGACGGGACCACCTTATGTTATTTTCATCATG-3') and a downstream primer in exon 1 of *n-syb* (5'-GCACGATGCACCTTGGCCTCTTTC-3') with the polymerase *Tfl* (Epicentre Technologies, Madison, WI). The amplification conditions included a denaturation temperature of 95°C, an annealing temperature of 50°C, and an extension temperature of 72°C (each for 1 min) for 35 cycles, followed by a 10 min extension at 72°C. Reaction products were electrophoresed on 3% agarose gels (2% NuSieve GTG, FMC, and 1% agarose; Life Technologies). The six *n-syb*^{F33} excision lines that did not produce a PCR product (indicating a deletion that either removed the 31 bp repeat of the P-element or the sequence 3' of the P-element insertion) were analyzed by Southern blotting, along with the F82 excisions and some 40 additional *n-syb*^{F33} excision lines. DNA was prepared as above and digested with *Eco*RI, *Pst*I, and *Xho*I (New England Biolabs) and in every double-digest combination and blotted as described. Southern blots were probed with a series of genomic probes that spanned the entire *n-syb* ORF and extended both 5' and 3' of the gene (see Fig. 2). Several excision lines were identified that deleted portions of the *n-syb* gene, including *n-syb* ^{Δ F33B}, *n-syb* ^{Δ F33OO}, *n-syb* ^{Δ F82C}, and *n-syb* ^{Δ F33-8}.

Antibody preparation. Peptide NKLGLIGGEQPPQYQYPPQYM was synthesized at the Beckman Center Peptide and Nucleic Acid Facility (Stanford, CA). The peptide was coupled to thyroglobulin, using glutaraldehyde as described (Harlow and Lane, 1988), and used as an immunogen. Antisera were prepared, affinity-purified, and stored as described (Mi et al., 1995).

Western blots. Heads or bodies from wild-type and *n-syb* mutant lines were dissected and homogenized in 50 μ l of 5% SDS, 0.2 M Tris base, 10% glycerol, and 0.1% bromophenol blue and heated at 95°C for 4 min. The samples were run on a 15% acrylamide gel and blotted onto Immobilon P (Millipore, Bedford, MA) membranes. Membranes were blocked in PBT (PBS plus 0.05% Tween 20) supplemented with 5% nonfat dry milk. Blots were incubated with either a 1:500 (see Fig. 3B) or a 1:2000 (Fig. 3A) dilution of affinity-purified anti-*n-syb* antisera in PBT plus 1% BSA for 1 hr at room temperature, washed extensively for 30 min in PBT, and then incubated with a 1:20,000–1:50,000 dilution of anti-rabbit-HRP conjugate (Amersham) in PBT plus 1% BSA and washed as above. The blots were incubated with chemiluminescent substrate according to the ECL kit directions (Amersham) and exposed to Biomax ML film (Kodak). Exposures ranged from 1 min to 1 hr, depending on the signal intensity.

Immunofluorescence. *yw*; *n-syb* ^{Δ F33B}/*n-syb* ^{Δ F33B} mutant embryos were collected from the stock *yw*; *n-syb* ^{Δ F33B}/*TM6* *Ubx*, *y*+ and *yw*; *n-syb* ^{Δ F33B}/*TM6* *Ubx*, *y*+ siblings served as controls.

Embryos were collected on grape juice plates at 25°C. The animals were hand-dechorionated and devitelinated. Late stage 17 embryos were affixed to SYLGARD-coated (Dow Corning, Midland, MI) slides with Nexaband (Veterinary Products Laboratories) glue. The glue was ap-

plied in small drops from the end of a glass micropipette. The head and tail of the animal were glued down first before the dorsal midline of the animal was perforated with a sharp glass micropipette. Then an incision was made along the perforation, the animal was laid out along this incision, and the flaps of cuticle were glued to the slide. The gut, fat bodies, and connective tissue then were removed to expose the CNS and musculature. Dissections were performed in HL3 physiological solution, as described by Stewart et al. (1994).

The dissected preparations were fixed in Bouin's fixative (15:5:1 mixture of saturated picric acid, 37% formaldehyde, and glacial acetic acid) for 15–30 min, washed in PBT (PBS plus 0.1% Triton X-100) for 30–60 min, blocked in 5% normal goat serum in PBT for 30 min, and then incubated in affinity-purified rabbit anti-*n-syb* antiserum (1:250) and mouse anti-Fasciclin II monoclonal (1:50) overnight at 4°C. The preparations were washed in PBT for 30–60 min, blocked in 5% normal goat serum in PBT for 30 min, incubated in FITC-conjugated goat anti-rabbit (1:250) and Texas Red-conjugated goat anti-mouse (1:250) (Jackson ImmunoResearch Labs, West Grove, PA) for 2 hr at room temperature, and then washed in PBT for 30–60 min. Mutant and control samples were dissected on the same slide.

The preparations were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and viewed on a Molecular Dynamics (Sunnyvale, CA) confocal microscope. All images comparing mutant and wild-type animals were acquired at the same gain.

Electrophysiology. Embryos from the stock *yw; n-syb*^{ΔF33B/TM6}, *Ubx*, *y+* and *yw; n-syb*^{F33-R/TM6}, *Ubx*, *y+* and *yw; line 34/TM6, *Ubx*, *y+* were collected, and *y*⁻ *n-syb* homozygotes were identified. Dissecting and recording procedures of synaptic currents were described elsewhere (Kidokoro and Nishikawa, 1994; Nishikawa and Kidokoro, 1995). The dissecting procedures were performed in Ca²⁺-free, Mg²⁺ saline (see below). The ventral ganglion was kept intact. The preparation was treated for 3 min with 1 mg/ml collagenase (Type IV; Sigma) in 0.1 mM Ca²⁺ saline.*

Recordings were mainly from longitudinal muscles 4, 6, and 7. The miniature synaptic current frequency was counted visually for 5 min on a CRT screen with simultaneous recording on a paper recorder (Nihon-Kohden, Japan). There were spontaneous synaptic currents with a slow time course mixed with fast ones, attributable to electrical coupling of muscle cells with neighbors (Gho, 1994; Kidokoro and Nishikawa, 1994; Ueda and Kidokoro, 1996). In this study only synaptic currents with a fast time course were counted. For nerve stimulation, a microelectrode filled with 4 M K-acetate was inserted in the middle of the ventral ganglion, and positive pulses of ~2 μA in intensity and 2 msec in duration were delivered.

Solutions. The ionic composition of the solutions used in the experiments are as follows (in mM). In normal external saline: 140 NaCl, 2 KCl, 5.5 MgCl₂, 0.5 CaCl₂, and 5 HEPES-NaOH, pH 7.1. In Ca²⁺-free external solution: 140 NaCl, 20 KCl, 6 MgCl₂, and 5 HEPES-NaOH, pH 7.1. The ionic composition of the internal solution was (in mM): 158 CsCl, 1 Mg-ATP, 5 EGTA, and 10 HEPES-NaOH, pH 7.1. Tetrodotoxin (TTX) was purchased from Sigma.

RESULTS

Generation of *n-syb* mutations

The *Drosophila neuronal-synaptobrevin* gene, *n-syb*, is located on the left arm of the third chromosome at the border between polytene bands 62A and 62B (DiAntonio et al., 1993a). Because no preexisting mutations or chromosomal aberrations affected *n-syb* (data not shown), we decided to mutate the gene by mobilizing a P-element transposon. Because these elements, when mobilized by the transposase, are predisposed to insert themselves in the vicinity of their site of origin (Tower et al., 1993), we sought a P-element-containing line that carried an insertion within 100 kb of *n-syb*. We obtained a line of flies (line 34) containing a PlacW-type P-element insertion at the border between polytene bands 62A and 62B (Gonczy et al., 1992). PlacW P-elements contain the bacterial origin of replication and the ampicillin resistance gene, permitting the isolation of the DNA sequences flanking the site of the P-element insertion (Bier et al., 1989). The DNA sequence flanking the line 34 P-element insertion was isolated by digesting line 34 genomic DNA with *EcoRI*,

circularizing it with T4 DNA ligase, and using the resulting plasmid to transform bacteria. To determine how close the line 34 P-element insertion was to the *n-syb* gene, we inquired whether it fell within the same P1 clone (genomic clones containing 70–95 kb of genomic DNA) as the *n-syb* gene. Five P1 clones spanning the region from early 62A to late 62B were obtained from *Drosophila* Genome Center, digested, electrophoresed, blotted, and hybridized with probes derived from the *n-syb* gene and from the sequence surrounding the line 34 P-element insertion (Fig. 1A). Of these, one P1 clone (17–42; lane 1) was recognized by both probes, whereas others contained either the *n-syb* region (29–89 and 39–43; lanes 2 and 3) or the P-element site of insertion (55–41; lane 5), but not both. Because clone 17–42 hybridized with both probes, the distance between the P-element insertion and the *n-syb* gene could not exceed 100 kb, the maximum size of the insert in a P1 clone.

The P-element was mobilized by crossing it to a line expressing a constant source of transposase (Fig. 1B). Flies containing both the P-element and the source of transposase (700 males and 750 females) then were crossed individually to *yw* flies. The F2 progeny from each vial were examined, and a single male containing a likely additional P-element insertion (based on a darker eye phenotype) was selected from each vial. 900 novel insertions were selected, and these flies then were screened to see if any of the new P-element insertions landed in or near the *n-syb* gene via a modification of the plasmid rescue procedure (Zinsmaier et al., 1994). Two insertions were identified near the *n-syb* gene. One, called F82, has a P-element insertion 3–4 kb 3' of the *n-syb* gene (in addition to the starter P-element from line 34) and apparently did not disrupt the *n-syb* transcript because it was homozygous-viable. A second insertion landed 150 bp upstream of the initiation ATG and was homozygous-lethal. We will designate this mutation *n-syb*^{F33}. The position of the F33 insertion was determined by sequencing the rescued P-element plasmid, by PCR with primers to the 31 bp repeat of the P-element and to exon 1 sequences of *n-syb*, and by Southern blotting, as shown in Figure 1C. The Southern blot revealed that the P-element had inserted in a 2 kb *EcoRI* fragment just 5' to the *n-syb* ORF.

Several lines of evidence indicate that the lethality of *n-syb*^{F33} is attributable to the insertion near *n-syb*. When the F33 insertion was recombined away from the starter line 34 P-element, the lethality remained with the F33 insertion. Furthermore, when the P-element in F33 was excised precisely (by reintroducing the transposase), homozygous-viable lines were generated, indicating that the lethality was attributable to the insertion. Southern blot analysis of the viable excision lines revealed that the P-element precisely or nearly precisely excised in four of seven lines (Fig. 1D, lanes 1, 2, 6, and 7), and in the remaining three lines (Fig. 1D, lanes 3, 4, and 5) only several hundred base pairs of the P-element remained behind. Lines that retained larger fragments of the P-element remained lethal.

n-syb^{F33} is a severe allele of *n-syb* (it is an embryonic-lethal), but because the insertion does not interrupt the ORF of *n-syb*, this allele might produce some *n-syb* protein; thus by analyzing its phenotype, we might underestimate the role of *n-syb*. We therefore generated an unambiguous null mutation by imprecise P-element excision. From ~700 excisions, 105 lines were identified that were lethal in combination with *n-syb*^{F33}. Additional deletions were generated by excising the F82 P-element. Many of the excisions were internal deletions of the F33 P-element (identified by using PCR primers to the 31 bp repeat of the P-element and to exon 1 of *n-syb*; see Fig. 2A, bottom) and were unlikely to

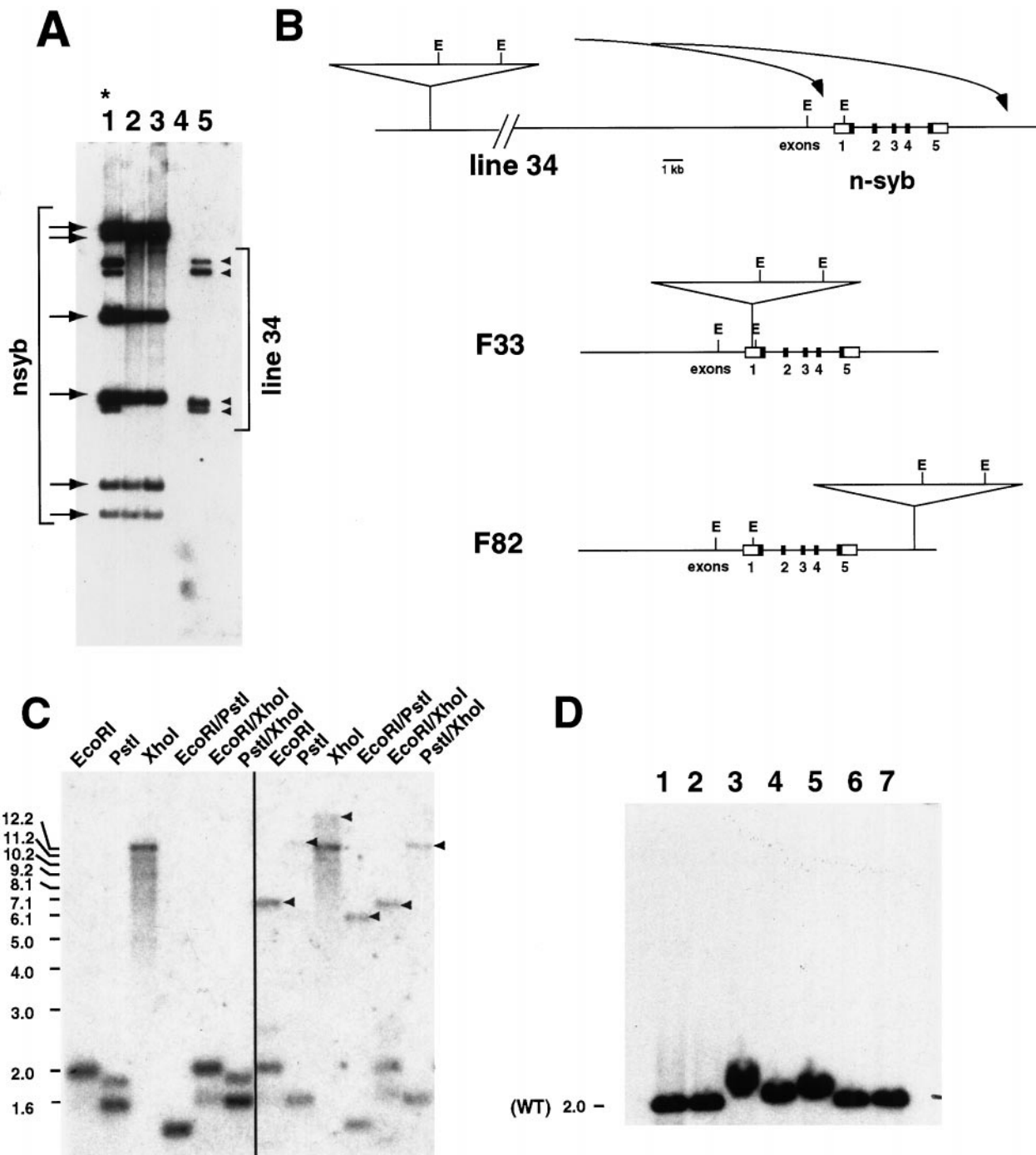


Figure 1. Mapping of P-element insertions near the *n-syb* locus. **A**, Lanes 1–5 contain, in order, P1 genomic clones 17–42, 29–89, 39–43, 40–41, and 55–41 from polytene bands 62A and 62B. Their DNA was digested with *Bgl*III and *Xho*I and simultaneously probed with *n-syb* probe and the flanking sequence from the starter P-element. *n-syb*-specific bands are indicated with arrows, and the P-element flanking sequence is indicated with arrowheads. 17–42 hybridizes with both probes (asterisk). **B**, Map of P-elements near *n-syb*. The P-element upstream of *n-syb* in line 34 was mobilized (arrows) to give rise to the insertions in F33 and F82. Untranslated exon sequences are indicated by open boxes, translated exons by shaded boxes, and *Eco*RI sites by the letter E. Top, Although the orientation of the P-element and the *n-syb* gene and the distance between the two have not been determined, they must fall within ~100 kb of each other to be contained on P1 17–42. Middle, The F33 P-element inserted in exon 1 of the *n-syb* gene 150 bp from the initiation ATG. Bottom, The F82 P-element inserted 3–4 kb from the 3' end of *n-syb*. **C**, Southern blot of genomic DNA from wild-type (left) and F33/*TM3, Sb* heterozygote (right). Lanes are digested with the indicated restriction enzymes, and the molecular weights of the bands are indicated in kilobase pairs. Both blots are probed with a 2 kb *Eco*RI fragment of *n-syb* from the 5' untranslated region. Arrowheads indicate new bands that result from the F33 insertion. **D**, Southern blot of viable, revertant excision lines. Lanes 1–7 are genomic DNA from seven different F33 excisions digested with *Eco*RI and probed with a 2 kb *Eco*RI *n-syb* genomic fragment. The wild-type band of 2 kb is indicated, and all of the excisions are within 200–300 bp of the wild-type size.

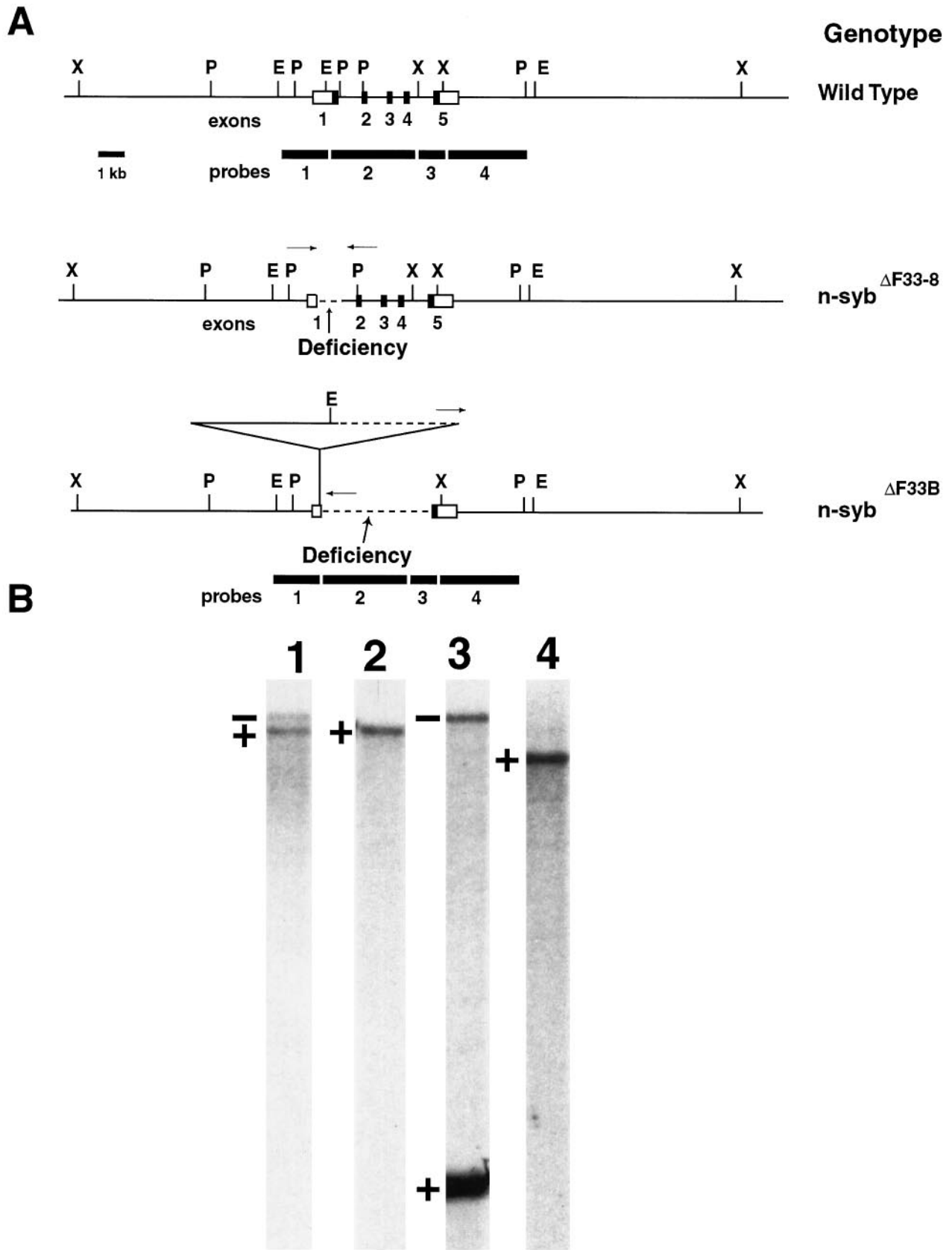


Figure 2. Restriction mapping of excision alleles. *A*, Restriction map of the *n-syb* locus (top), the excision allele *n-syb*^{ΔF33-8} (middle), and the excision allele *n-syb*^{ΔF33B} (bottom). The restriction site abbreviations are the following: *Eco*RI, *E*; *Xho*I, *X*; *Pst*I, *P*. Deficiencies are indicated by a dashed line, and exons are shown as boxes (shaded boxes are coding regions, and unshaded boxes are noncoding regions). Probes used in *B* are diagrammed below the wild-type *n-syb* and the *n-syb*^{ΔF33B} loci. *B*, Genomic Southern blots of DNA (Figure legend continues)

Table 1. Summary of *n-syb* alleles

Allele	Type of mutation	Severity	Phenotype
F33	P insertion	Nearly null	Embryonic lethal
Δ F33B	Excision	Null	Embryonic lethal
Δ F3300	Excision	Likely null	Embryonic lethal
Δ F33-8	Excision	Likely null	Embryonic lethal
Δ F82C	Excision	Likely null	Embryonic lethal
I4	EMS	Hypomorph	Viable, sluggish
I18	EMS	Hypomorph	Viable, sluggish

Summary of *n-syb* alleles and their phenotypes. The phenotype described is for the given allele in the homozygous state, with the exception of I4 and I18. The phenotype described for I4 and I18 is over the null allele *n-syb* ^{Δ F33B}.

produce a mutation more severe than the original F33 insertion. Several lines, however, deleted portions of the *n-syb* gene: Δ F33B, Δ F3300, Δ F82C, Δ F33-8, and Δ F82C. In Δ F33B and Δ F33-8 the extent of the deletions was determined (Fig. 2A). In Δ F33-8 the *Eco*RI site just 3' to the F33 insertion and the 3' end of exon 1, including the initiation ATG, was deleted, as judged by Southern blotting (data not shown). The deletion extended into intron 1 but did not extend into exon 2, nor did the deletion extend 5' from the F33 insertion site, as determined by PCR, between a primer 5' of the insertion and an exon 2 primer (see Fig. 2A, middle). The deletion of the initiation ATG in Δ F33-8 caused an abnormal protein to be made (see below).

A greater portion of the *n-syb* gene was deleted in Δ F33B. Southern blots indicated that much of exon 1 (including the start codon) and all of exons 2, 3, and 4 were removed (Fig. 2B). The deletion produced by the Δ F33B excision removed the first 122 of the 181 amino acids of *n-syb*. The remaining portion consisted of seven amino acids from the membrane-spanning domain and 52 from the intravesicular tail; thus, in the unlikely event that it was translated, it still would lack all of the functionally important and conserved domains, including those responsible for binding to syntaxin and SNAP-25. Thus *n-syb* ^{Δ F33B} constitutes a null mutation. Further *n-syb* alleles were generated by performing an F2 lethal screen. Flies were treated with the chemical mutagen ethyl methane sulfonate (EMS), which often induces point mutations. These flies were crossed to a third chromosome balancer stock, and the resulting F1 males were crossed to the original *n-syb*^{F33} allele so that flies that failed to complement *n-syb*^{F33} could be selected. Two alleles, *n-syb*^{I4} and *n-syb*^{I18}, were isolated and found to be hypomorphic alleles of *n-syb*; in combination with *n-syb*^{F33} or *n-syb* ^{Δ F33B}, the EMS alleles can survive until adulthood, but they are very sluggish and often remain motionless for minutes at a time.

A summary of the mutations is listed in Table 1. Seven alleles were isolated; they range from weak hypomorphic alleles that clearly retain *n-syb* function to nulls. The severe alleles were embryonic-lethals, whereas the weaker alleles were viable even as adults. To establish the function of *n-syb*, we concentrated on characterizing the phenotype of the null allele *n-syb* ^{Δ F33B}.

n-syb protein is enriched in synapses

To examine the distribution of *n-syb* protein, we raised an anti-serum against the intravesicular tail of *n-syb*, a region that shares no homology to other synaptobrevins, including the synaptobrevin ubiquitously expressed in *Drosophila*, *synaptobrevin* (*syb*) (see Materials and Methods). This affinity-purified antiserum recognizes a band of ~22 kDa, which is enriched in *Drosophila* heads relative to the rest of the body (Fig. 3A, wt heads vs wt bodies). Heads from mutant heterozygotes were analyzed with this antiserum (Fig. 3A) to characterize the mutations further. The 22 kDa band representing wild-type protein was seen, as expected, in all of the heterozygotes and in the parental line (line 34). In the mutants, however, the signal was decreased, as predicted by the loss of one of the two copies of the *n-syb* gene. Longer exposures of Western blots of protein extracts from the *n-syb* mutant heterozygotes revealed that the *n-syb*^{F33-8} mutant produced a faint band at a slightly higher molecular weight than the wild-type *n-syb* protein (data not shown). To examine this band more closely, we subjected a more concentrated protein extract from *n-syb* ^{Δ F33-8} heterozygotes to SDS-PAGE, blotted the extract, and probed it with the anti-*n-syb* antiserum. The results of this Western blot are shown in Figure 3B. A band running just above the wild-type *n-syb* band is evident. In that mutant a deletion removes the normal initiation ATG (see above), but the presence of a higher molecular weight protein suggests that an upstream, in-frame ATG from intron 1 was used to make a larger protein. In *n-syb* ^{Δ F33B}, no lower molecular weight band arises; thus, in this deletion, the fragment of the intravesicular tail that theoretically might be produced is not present at appreciable levels.

To localize the *n-syb* product in the embryo, we double-stained dissected preparations with this affinity-purified anti-*n-syb* antiserum and an anti-Fasciclin II antibody. In Figure 4A, the *n-syb* staining is strong and uniform in the ventral nerve cord (VNC), and faint staining is seen in axon commissures and segmental and intersegmental nerves that could represent vesicles en route to synapses. In the absence of the primary antibody, no staining was observed (data not shown).

The presence of *n-syb* protein at the neuromuscular junctions (NMJs) on muscles 6 and 7 of a wild-type embryo was demonstrated by labeling with anti-Fasciclin II antibody to identify the nerve (Fig. 4B) and with the affinity-purified anti-*n-syb* antiserum (Fig. 4C). The *n-syb* staining is concentrated in the synaptic zones at the NMJ. Thus, the subcellular localization of *n-syb* is consistent with an important role in synaptic function, and its presence at this synapse enables us to characterize the physiological consequences of the mutations at this well characterized synapse.

n-syb is not required for formation of the NMJ, but null mutants are paralyzed

The extension of growth cones during neural development is thought to involve the addition of membrane. Because membrane addition at the mature synapse involves SNARE proteins and because inhibition of SNAP-25 expression was shown to inhibit

← from *n-syb* ^{Δ F33B} heterozygote. Lanes 1–4 are all digested with *Xho*I and are hybridized with probes 1–4, respectively. Mutant (–) and wild-type (+) bands are indicated. In lane 1, probe 1 hybridized to both the wild-type and mutant *Xho*I restriction fragments. The larger size of the mutant band results from upstream *n-syb* sequences and the remainder of the P-element that failed to excise fully. In lane 2, probe 2 recognized the same wild-type band as in lane 1. The higher molecular weight mutant band failed to hybridize as the 3' half of exon 1, and all of exons 2, 3, and 4 are deleted in the mutant. In lane 3, probe 3 hybridized to a wild-type band of ~1 kb and the high molecular weight mutant band from lane 1. Probe 3 hybridized to the mutant band because the *Xho*I restriction site between exons 4 and 5 was deleted, but the deletion did not extend to exon 5. In lane 4, probe 4 hybridized to a single unaltered band because the probe is outside the deleted region.

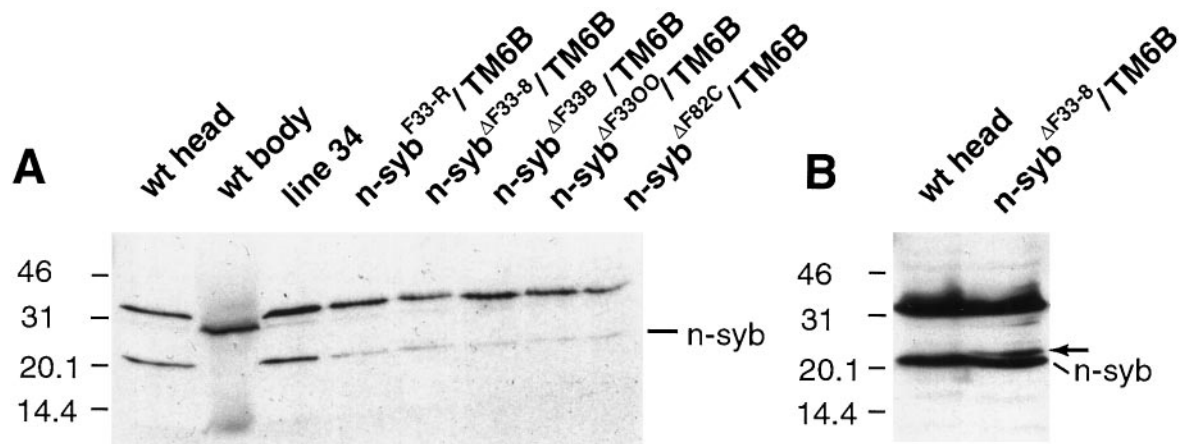


Figure 3. Western blots of protein extracts from wild-type and *n-syb* mutant heterozygotes probed with anti-*n-syb* antiserum. The molecular weight size markers are in kilodaltons, and the band corresponding to size of the *n-syb* protein (~22 kDa) is indicated. Proteins extracts from either 10 heads or 10 bodies (*A*) or 20 heads (*B*) from the indicated lines were prepared as described in Material and Methods. *A*, *n-syb* protein is enriched in wild-type (*wt*) heads as compared with the rest of the body (*wt body*); the starter P-element line, *line 34*, has wild-type levels of *n-syb*. All of the *n-syb* mutant heterozygotes have reduced levels of *n-syb* protein. *B*, *n-syb*^{ΔF33-8} produces a slightly higher molecular weight form of *n-syb* (indicated with an arrow) than wild type. This is most likely attributable to the use of an alternative initiation ATG in intron 1. The higher molecular weight band present in all of the lanes, running at ~35 kDa, appears to be a protein that cross-reacts with the *n-syb* antiserum because it does not decrease in intensity in the *n-syb* mutants.

axonal growth both *in vivo* and *in vitro* (Osen-Sand et al., 1993), we therefore examined whether *n-syb* is necessary for axonal outgrowth. *n-syb*^{ΔF33B} null mutants were stained with anti-Fasciilin II antibody (Fig. 4*D,F*). The longitudinal tracts of the VNC, the motor nerves, and the terminals of the motor neurons all appeared normal despite the absence of *n-syb*. When null mutants were stained with anti-*n-syb* antisera, no staining was observed in VNC or motor nerves or the motor neuron terminals (Fig. 4*E,G*). Despite the normal appearance of the VNC and motor neurons, late stage *n-syb*^{ΔF33B} homozygous embryos failed to move unless probed and never hatched from their egg cases.

Evoked neurotransmitter release is blocked in *n-syb* null mutant

To assess the role of *n-syb* in evoked neurotransmitter release, we recorded from stimulated embryonic NMJs, using whole-cell patch-clamp methods. Late embryos, homozygous for the *n-syb*^{ΔF33B} deletion or the *n-syb*^{F33-R} insertion, were dissected to expose the CNS and longitudinal muscles. Line 34 embryos were used as controls. A patch electrode recorded synaptic currents from muscle 4, 6, or 7 while the ventral ganglion was stimulated at 0.3 Hz. In *n-syb*^{ΔF33B} and *n-syb*^{F33-R} mutant embryos, stimulation of the nerve failed to elicit any evoked currents in the muscle (Fig. 5). In the parental controls, stimulation produced currents in excess of 500 pA in 0.5 mM Ca²⁺. Increasing the external Ca²⁺ concentration to 6 mM did not restore a detectable excitatory synaptic current (ESC) in the *n-syb* null. A potassium channel blocker, 4-aminopyridine (4-AP), is known to enhance synaptic transmission in *Drosophila* larvae, presumably by increasing Ca²⁺ influx (Jan and Jan, 1977). Therefore, 2 mM 4-AP was included in 2 mM Ca²⁺ external saline. Still no synaptic currents were evoked in *n-syb*^{ΔF33B} embryos (four cells were tested in three preparations). In contrast, prominent bursts of synaptic currents were observed in line 34 larvae with 1 mM 4-AP with 0.5 mM Ca²⁺ (data not shown). Thus, *n-syb* appears to be required for nerve-evoked release of neurotransmitter.

Spontaneous neurotransmitter release is reduced, but not abolished, in *n-syb* null mutant

Miniature excitatory synaptic currents (mESCs) at the NMJ are thought to arise from single synaptic vesicles fusing spontaneously with the presynaptic terminal membrane. To investigate the role of *n-syb* in this spontaneous release, we recorded mESCs at the NMJ of embryos homozygous for the *n-syb*^{ΔF33B} deletion or the *n-syb*^{F33-R} insertion. Again, the parental line 34 was used as a control. Recordings were performed in the presence of 3 μM TTX to eliminate nerve-evoked release, and the frequency of spontaneous events was recorded. The mESC frequency was reduced in both *n-syb* mutations by ~75%, as compared with line 34 controls (Fig. 6). These differences were statistically significant ($p < 0.05$). These results are in general agreement with previous reports in which the light chain of tetanus toxin was expressed in the *Drosophila* nervous system to reduce the level of *n-syb* (Broadie et al., 1995; Sweeney et al., 1995). Thus, *n-syb* appears to play a role in at least some of the spontaneous neurotransmitter release, but *n-syb* is not essential for spontaneous release.

To examine the mESCs more closely, we found that it was necessary to increase their frequency somewhat by depolarizing with high K⁺ in 0.5 mM Ca²⁺ in the presence of TTX to prevent action potentials. Representative traces of the mESCs from the different genotypes are shown in Figure 7*Aa–Ca*. From many such records the mean amplitude of mEPCs was calculated also. No significant difference in amplitude was observed between the control and *n-syb* mutant lines. The mean amplitudes were 155 pA ± 26 pA ($n = 5$) for *n-syb*^{ΔF33B}, 199 ± 16 pA ($n = 7$) for *n-syb*^{F33-R}, and 168 pA ± 24 pA ($n = 6$) for the control line 34, where n is the number of cells. The amplitudes of the mESCs were spread over a wide range of values (Fig. 7*Ab–Cb*) that may reflect some instances of the simultaneous release of more than one vesicle. Both the control line 34 and the *n-syb*^{ΔF33B} mutant show a similar amplitude distribution, with the largest number of events in the ~50 pA range and a pattern of smaller peaks at increasing current amplitudes. The mutant *n-syb*^{F33-R} has many

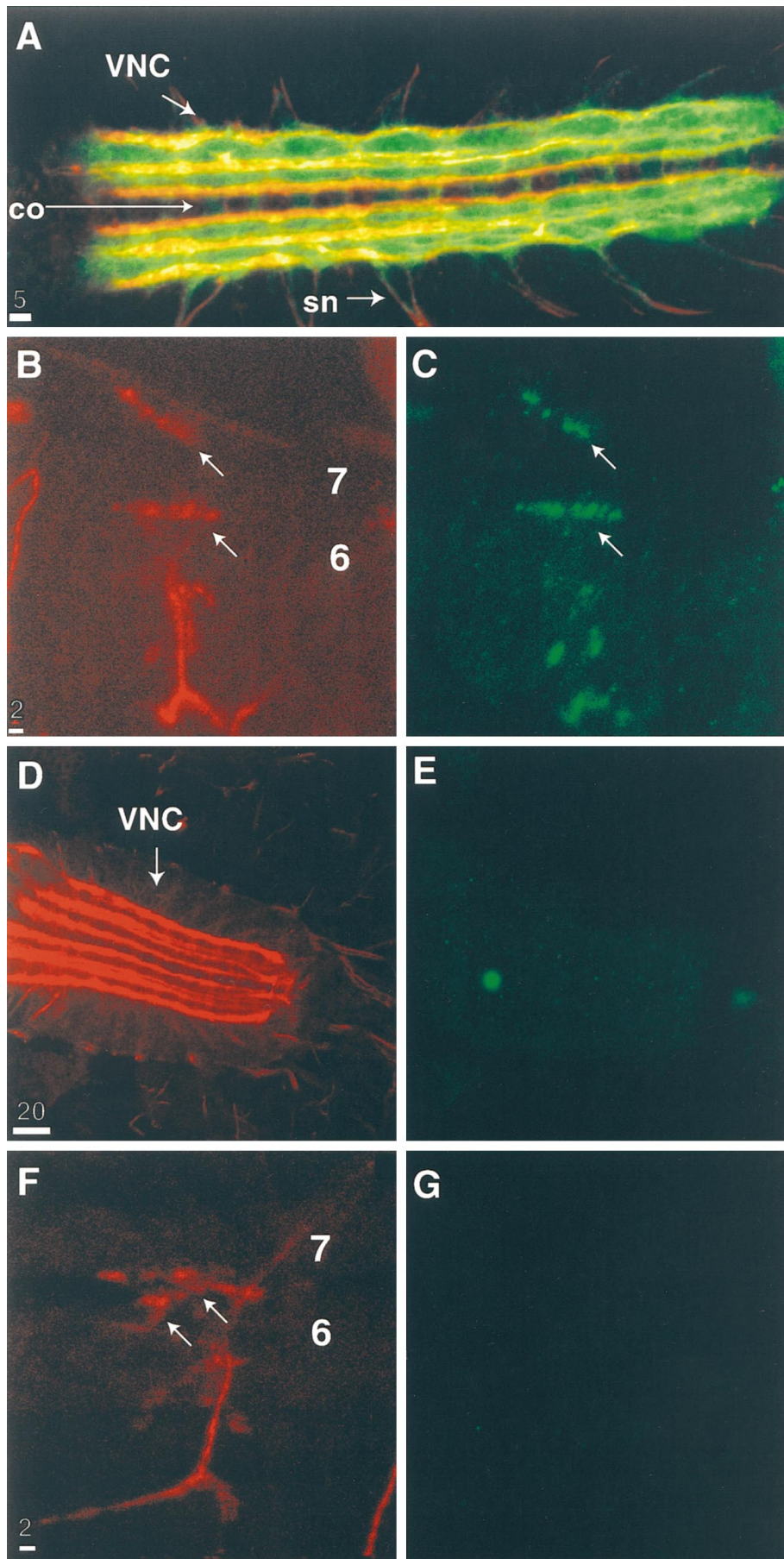


Figure 4. Immunocytochemistry of wild-type and *n-syb* mutant embryos. *A*, Wild-type embryo fillet double-stained with *n-syb* antiserum (green) and Fasciclin II antibody (red). *B*, *C*, Synapses (arrows) at a wild-type NMJ stained for FasII (*B*) and *n-syb* (*C*). *D–G*, *n-syb* null mutants (*n-syb*^{ΔF33B}) stained for FasII (*D*, *F*) and *n-syb* (*E*, *G*). Despite the absence of detectable *n-syb*, the morphology of the nerve cord and NMJ appears normal. Ventral nerve cord, *VNC*; axonal commissures, *co*; axons of the segmental nerves, *SN*; longitudinal muscles 6 and 7, 6, 7. Scale bar, 1 μm.



Figure 5. Nerve-evoked synaptic currents are absent from the neuromuscular junctions of *n-syb* mutants stimulated at 0.3 Hz. Evoked currents are lacking in *n-syb* null mutant *n-syb*^{ΔF33B} (*A*) and in the mutant *n-syb*^{F33-R} (*B*) but are present in the parental control, line 34 (*C*). The external solution contained 2 mM Ca²⁺ for lines *n-syb*^{ΔF33B} and *n-syb*^{F33-R} and 0.5 mM Ca²⁺ for line 34.

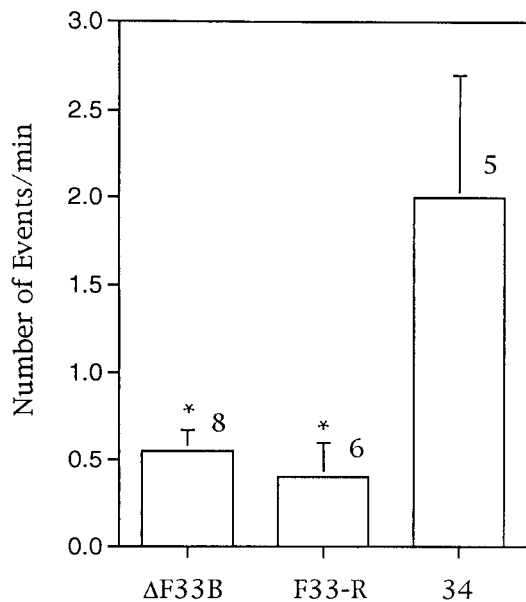


Figure 6. Frequency of miniature synaptic currents in *n-syb*^{ΔF33B}, *n-syb*^{F33-R}, and line 34. Error bars are SEM. Asterisks denote statistical differences from line 34 at $p < 0.05$ by the ANOVA test. Numbers indicate the number of cells examined. Miniature synaptic currents were recorded in the presence of 3 μ M TTX in 0.5 mM Ca²⁺ saline.

events in the 50 pA range, but it also has somewhat more events than the other lines in the 100 and 200 pA range. Interestingly, the lack of a major synaptic vesicle protein appears to have had little effect on the formation of synaptic vesicles or on the amount of neurotransmitter contained within the vesicles, as judged by the persistence of spontaneous events and their unaltered amplitude. Moreover, the responsiveness of the postsynaptic membrane must be roughly unchanged, and so the failure of nerve stimulation to evoke an ESC cannot be attributed to changes in postsynaptic sensitivity or transmitter packaging.

DISCUSSION

We have described the generation of mutations in the *n-syb* gene in *Drosophila melanogaster*. Among the alleles we generated is a deletion mutation, *n-syb*^{ΔF33B}, that removes most of the ORF and, by this molecular criterion as well as by protein analysis on immunoblots and immunocytochemistry, can be judged to be a null allele. *n-syb* protein and mRNA are not present in early embryos and therefore are not maternally deposited in the egg (DiAntonio et al., 1993a; D. Deitcher, unpublished data). Thus the homozygous null phenotype represents the complete absence of this gene product.

Embryos homozygous for this mutation are lethal and nearly paralyzed. From a morphological and electrophysiological analysis of these embryos, two major findings have emerged and are discussed below: (1) the outgrowth of axons and the formation of synapses is independent of the *n-syb* protein, and (2) spontaneous mESCs can occur without *n-syb*, whereas the action potential-evoked ESC cannot.

n-syb is required for synaptic function, but not synapse formation

Because the outgrowth of axons and the establishment of synapses require the addition of vesicles to the growth cone, we inquired whether or not this process involved *n-syb*. In the case of vertebrates, SNAP-25 and syntaxin, the same exocytotic proteins that function at the synapse, have been implicated in axon outgrowth as well (Osen-Sand et al., 1993; Igarashi et al., 1996). In *Drosophila*, two synaptic proteins, syntaxin and the n-sec homolog *rop*, have been shown to affect membrane trafficking in non-neuronal cells and are likely to be required for all membrane trafficking to the cell surface. *Syntaxin1* mutations have pleiotropic non-neuronal phenotypes (Schulze et al., 1995), have defects in the cellularization of the syncytial blastoderm (Burgess et al., 1997), and appear to be cell-lethal (Schulze and Bellen, 1996; Burgess et al., 1997). Although embryonic synapses and axons form in *syntaxin1* null mutations, the membrane addition for these processes is likely to be accomplished by *syntaxin1* protein and message that are deposited by the mother in the egg (Parfitt et al., 1995; Burgess et al., 1997). Mutations of the n-sec-1 ho-

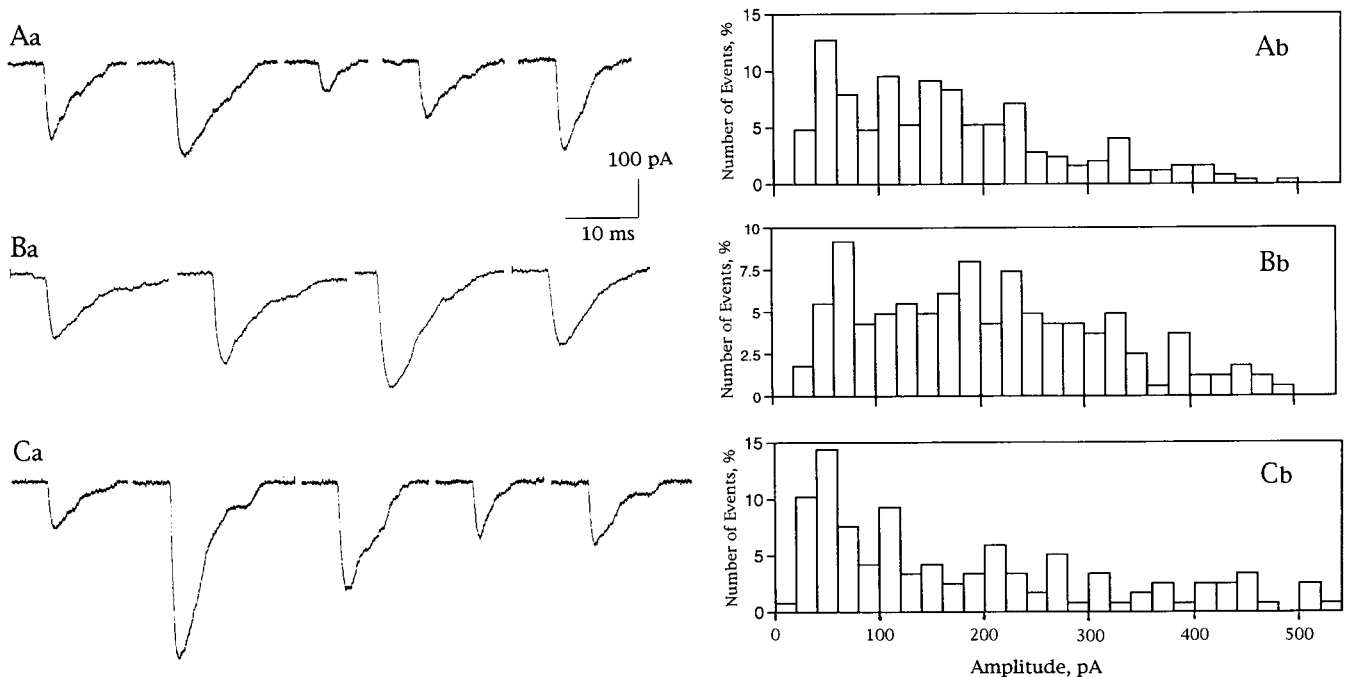


Figure 7. Representative miniature synaptic currents and amplitude histograms. Shown are miniature synaptic currents for *Aa*, *n-syb*^{ΔF33B}; *Ba*, *n-syb*^{F33-R}; and *Ca*, line 34. Amplitude histogram for *Ab* is *n-syb*^{ΔF33B}; for *Bb* is *n-syb*^{F33-R}; and for *Cb* is line 34. Miniature synaptic currents were recorded in high K⁺ saline (20 mM) to increase their frequency and in the presence of 3 μM TTX and 0.5 mM Ca²⁺. The mean amplitudes were *A*, 155 ± 26 pA (*n* = 5); *B*, 199 ± 16 pA (*n* = 7); and *C*, 168 ± 24 pA (*n* = 6), where *n* is the number of cells.

molog *rop* also have pleiotropic effects in non-neuronal cells that suggest an essential role in all membrane addition to the cell surface (Harrison et al., 1994). In contrast, synaptotagmin mutations in the fly and nematode do not interfere with neurite and synapse formation and are not implicated in any defects outside the functioning of the mature synapse (DiAntonio et al., 1993b; Nonet et al., 1993).

Thus studies of other synaptic proteins provide a precedent for a single protein acting in multiple cellular processes: general membrane addition, axonal outgrowth, and exocytosis from the mature synapse. Other proteins, however, appear to be specific for synaptic vesicle fusion. In the present case the transcript pattern for *n-syb* pointed to a neuron-specific function for this protein (DiAntonio et al., 1993a), and the presence of normal axon tracts and synapses in the *n-syb* null mutants (see Fig. 4) indicates that it is essential only to the functioning of the synapse and not to its development.

The discrimination of evoked responses from spontaneous miniature ESCs by mutations in *n-syb*

The dramatic effect on evoked release and the more moderate effect on spontaneous release of the *n-syb* mutant are noteworthy for their implication that the mechanism of vesicle fusion for these two types of synaptic events may differ. At the NMJ, a 0.3 Hz stimulus to the motor nerve did not produce an ESC in these mutants. These findings are consistent with an essential role for *n-syb* in evoked neurotransmitter. On the other hand, the *n-syb* protein is not essential for spontaneous neurotransmitter release but does reduce the frequency of spontaneous mESC significantly (by 75% in the *n-syb* null). Similar electrophysiological results were obtained by experiments in which the light chain of tetanus toxin was expressed in the *Drosophila* nervous system to reduce *n-syb* expression (Sweeney et al., 1995) and a smaller

reduction in mESC frequency (50%) was observed, although this difference was statistically insignificant. The key observation, however, that some mESCs persist in the absence of *n-syb* is confirmed by our study of a null allele.

Current models of VAMP/synaptobrevin function all invoke an action in concert with syntaxin and its cognate t-SNARE, and therefore it might be expected that the same phenotypes would arise if either member of the pair was disrupted. However, in *Drosophila syntaxin1* mutants, both evoked release and spontaneous mESCs are disrupted (Schulze et al., 1995). Although rare spontaneous events were seen occasionally, these are likely to be mediated by residual maternal *syntaxin1*. Thus, as mentioned above, *syntaxin1* appears to be required universally for fusion, whereas *n-syb* appears to be more specific.

The v-SNARE/t-SNARE model of VAMP/synaptobrevin function emphasizes a requirement for these proteins in targeting synaptic vesicles to active zones. However, EM data from tetanus toxin studies argue against this model. In both tetanus toxin-treated squid giant synapses (Hunt et al., 1994) and *Drosophila* NMJ (Broadie et al., 1995), synaptic vesicles are still “docked” at active zones. Although we have not yet studied the *n-syb* mutants by electron microscopy, the persistence of spontaneous vesicle fusions in our genetic study would indicate that many vesicles are, indeed, docked at the plasma membrane. Thus it appears unlikely that the morphologically docked vesicles observed in the earlier studies were attributable to uncleaved synaptobrevin or residual function in the proteolyzed products. Our study and those with the toxin all point to a disruption of the evoked response that lies downstream of morphological vesicle docking. There may be several biochemical stages that intervene between docking and fusion (Banerjee et al., 1996), and *n-syb* may be necessary for one of these or for promoting fusion itself.

The persistence of the spontaneous miniature EJC's in *n-syb* nulls raises two possibilities. The first is that the spontaneously fusing vesicles use an alternative isoform of synaptobrevin. Such an isoform, however, would not be redundant with *n-syb*; this homolog would be competent to mediate spontaneous fusions, but it would not be capable of responding to the Ca^{2+} signal that accompanies an action potential. One candidate is the other synaptobrevin isoform, *syb*, the widespread distribution of which in the organism suggests a role in constitutive trafficking. However, we have observed very low levels of this protein in the synaptic regions of the nerve cord in wild-type embryos or in *n-syb* mutants (S. Bhattacharya, personal communication), and only low levels of *syb* mRNA are found in the embryonic nervous system (Chin et al., 1993). *syb* is thus unlikely to be present on the majority of synaptic vesicles, although we cannot exclude that very low levels are present and suffice to produce the spontaneous events. In addition to *syb*, an as yet unidentified member of the synaptobrevin family also may be present.

Alternatively, the mESCs may occur in the absence of any VAMP/synaptobrevin. Synaptic vesicles have been shown to contain a substantial amount of syntaxin and SNAP-25, and it is possible that these are adequate vesicular components to accomplish fusion. A recent study of the requirements of yeast endosomes to fuse with one another indicated that fusion was most efficient with both "v-SNARES" and "t-SNARES" present on both of the fusing organelles. Surprisingly, however, t-SNARE/t-SNARE-mediated fusions (with a syntaxin homolog on both organelles, but no VAMP/synaptobrevin homolog on either) occurred at an appreciable rate that was approximately one-third as effective as having a v-SNARE on one side and a t-SNARE on the other (Nichols et al., 1997). With *syntaxin1* (and SNAP-25) present on both vesicles and plasma membrane, our *n-syb* mutants may provide an *in vivo* correlate to the *in vitro* experiment with yeast endosomes.

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