# Synaptic Transmission Deficits in *Caenorhabditis elegans* Synaptobrevin Mutants

**Michael L. Nonet,[1] Owais Saifee,[1] Hongjuan Zhao,[1] James B. Rand,[2] and Liping Wei[1]**

[1]*Department of Anatomy and Neurobiology, Washington University School of Medicine, St. Louis, Missouri 63110, and* [2]*Program in Molecular and Cell Biology, Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma 73104*

Synaptobrevins are vesicle-associated proteins implicated in neurotransmitter release by both biochemical studies and perturbation experiments that use botulinum toxins. To test these models *in vivo*, we have isolated and characterized the first synaptobrevin mutants in metazoans and show that neurotransmission is severely disrupted in mutant animals. Mutants lacking *snb-1* die just after completing embryogenesis. The dying animals retain some capability for movement, although they are extremely uncoordinated and incapable of feeding. We also have isolated and characterized several hypomorphic *snb-1* mutants. Although fully viable, these mutants exhibit a variety of behavioral abnormalities that are consistent with a general defect in the efficacy of synaptic transmission. The viable mutants are resistant to the acetylcholinesterase inhibitor aldicarb, indicating that cholinergic transmission is impaired. Extracellular recordings from pharyngeal muscle also demonstrate severe defects in synaptic transmission in the mutants. The molecular lesions in the hypomorphic alleles reside on the hydrophobic face of a proposed amphipathic–helical region implicated biochemically in interacting with the t-SNAREs syntaxin and SNAP-25. Finally, we demonstrate that double mutants lacking both the v-SNAREs synaptotagmin and *snb-1* are phenotypically similar to *snb-1* mutants and less severe than syntaxin mutants. Our work demonstrates that synaptobrevin is essential for viability and is required for functional synaptic transmission. However, our analysis also suggests that transmitter release is not completely eliminated by removal of either one or both v-SNAREs.

*Key words: synaptobrevin; VAMP; exocytosis; synaptic vesicle protein; mutants;* Caenorhabditis elegans*; v-SNARE*

Synaptic transmission is a calcium-regulated secretory process that shares many similarities with other secretory mechanisms in eukaryotic cells. A growing number of molecular components of the synaptic machinery have been identified via both biochemical and genetic approaches (for review, see Bennett and Scheller, 1994; Sudhof, 1995). One class of molecules that participate in the fusion event is the SNAP receptors (SNAREs; Sollner et al., 1993a). Distinct, but homologous, members of the v- and t-SNARE families are thought to mediate trafficking events in different cellular compartments (for review, see Ferro-Novick and Jahn, 1994). In neurons the v-SNAREs synaptobrevin (also called VAMP) and synaptotagmin are found specifically associated with synaptic vesicles (Trimble et al., 1988; Perin et al., 1990), and the t-SNAREs syntaxin and SNAP-25 are localized primarily on the plasma membrane (Bennett et al., 1992; Garcia et al., 1995). Stable synaptobrevin/syntaxin/SNAP-25 biochemical complexes can be assembled and disassembled *in vitro* (Sollner et al., 1993b), and these biochemical reactions are proposed to mediate vesicular fusion.

Synaptobrevin was first identified as a protein associated with synaptic vesicles isolated from electric organs of the ray (Trimble et al., 1988). Subsequently, homologs have been isolated from a variety of organisms, including yeast, *Drosophila*, and human (Sudhof et al., 1989; Archer et al., 1990; Gerst et al., 1992; Protopopov et al., 1993). A variety of evidence suggests that synaptobrevin family members play an essential role in regulating fusion events. *Saccharomyces cerevisiae snc1 snc2* double mutants lacking the yeast homologs of synaptobrevin have severe secretory defects (Gerst et al., 1992; Protopopov et al., 1993). Studies using several botulinum neurotoxins directly implicate synaptobrevin in the regulation of synaptic transmission (for review, see Tonello et al., 1996). These potent inhibitors of synaptic transmission are metalloproteases that cleave SNAREs. Tetanus toxin (TeTx) and several clostridial toxins cleave synaptobrevin at unique sites (Schiavo et al., 1992, 1993, 1994). In fact, expression of these toxins in neurons blocks synaptic transmission *in vivo* (Sweeney et al., 1995). Furthermore, in $\beta$-islet cell lines, the TeTx-block in $Ca^{2+}$-induced release of insulin can be overcome by introducing TeTx-resistant forms of synaptobrevin (Regazzi et al., 1997). However, these toxins also cleave other cellular homologs of synaptobrevin, such as cellubrevin, which are expressed in all cells (McMahon et al., 1993). Indeed, tetanus toxin can inhibit cell membrane repair in fibroblasts and *Xenopus* oocytes, suggesting that synaptobrevin-like molecules regulate other fusion events (Steinhardt et al., 1994). Thus, although TeTx toxin action suggests a requirement for synaptobrevin in synaptic release, the possibility remains that protease cleavage of other targets contributes to the transmission abnormalities in toxin-treated cells.

To examine the role of synaptobrevin in regulating synaptic transmission, we isolated and characterized *Caenorhabditis elegans* synaptobrevin mutants. SNB-1 was expressed in neurons

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Correspondence should be addressed to Dr. Michael L. Nonet, Department of Anatomy and Neurobiology, Campus Box 8108, Washington University School of Medicine, 660 South Euclid Avenue, St. Louis, MO 63110.

and colocalized with other synaptic vesicle proteins. Mutants lacking synaptobrevin function died shortly after completing embryogenesis. Viable hypomorphic mutants exhibited a variety of behavioral, pharmacological, and physiological defects, indicating that functional synaptobrevin is required for normal synaptic transmission. Characterization of the molecular lesions in the hypomorphic mutants implicates a proposed amphipathic–helical region of SNB-1 in synaptobrevin function.

## MATERIALS AND METHODS

*Growth and culture of* C. elegans. *C. elegans* was grown at 22.5°C on solid medium, as described by Sulston and Hodgkin (1988). All mapping, complementation, and deficiency testing was performed by standard genetic methods (Herman and Horvitz, 1980). Aldicarb, 2-methyl-2-[methylthio]proprionaldehyde *O*-[methylcarbamoyl]oxime, was obtained from Chem Services (West Chester, PA) and was prepared as a 100 mM stock solution in 70% ethanol. Aldicarb was added to the agar growth medium after autoclaving.

*DNA and RNA manipulations. C. elegans* genomic DNA was isolated as described by (Sulston and Hodgkin, 1988). cDNA was made by reverse-transcribing RNA, using random hexanucleotide primers as described by Sambrook et al. (1989). Poly($A^+$)-selected RNA was isolated from a mixed-stage culture of the wild-type strain N2 as previously described (Nonet and Meyer, 1991). Manipulations of DNA and RNA, including electrophoresis, blotting, and probing of blots, were performed with standard procedures except where noted (Sambrook et al., 1989).

*Cloning of* C. elegans *synaptobrevin gene.* Degenerate oligonucleotides SB-1 (5′ TNCARCARACNCARGC 3′) and SB-2 (5′ CCNARNATDATCATCAT 3′), corresponding to regions conserved among the bovine, *Torpedo*, and *Drosophila* synaptobrevin proteins (Trimble et al., 1988; Sudhof et al., 1989), were used to amplify the *C. elegans* gene specifically. PCR reactions were performed as described by Innis et al. (1990). SB-1/SB-2 products were gel-purified, cloned into pBluescript KS($^-$), and sequenced. Insert DNA was used to screen a λZAP cDNA library (Lichtsteiner and Tjian, 1993). The inserts of two cDNA clones isolated were excised from λZAP *in vivo* to create pSB100 and pSB101. pSB101 cDNA represents the full-length transcript because it contains a partial SL1 trans-spliced leader sequence and a 3′ poly($A^+$) sequence and is of comparable size to the *snb-1* message observed on Northern blots (see Fig. 3). Analysis of the DNA sequence and the deduced amino acid coding sequence of the gene were performed on a SPARC station with the Genetics Computer Group program package (Devereux et al., 1984). The *snb-1* cDNA sequences was deposited into GenBank (accession number AF003281).

*Production of antibodies and immunocytochemistry.* The *Bam*HI–*Bsa*BI fragment of pSB101 was inserted into pRSETB (Invitrogen, San Diego, CA) to create the plasmid pSB110. The plasmid encodes a fusion protein containing a six histidine tag and amino acids 13–88 of the *C. elegans* SNB-1 protein. The fusion protein was purified and used to immunize rabbits as described in Nonet et al. (1993). SNB-1 antiserum was immunoaffinity-purified by the method described in Pringle et al. (1991). Affinity-purified α-SNB-1 serum was used at 1:50 dilution. Immunocytochemistry was performed as previously described in Nonet et al. (1997).

*Chromosomal localization of the* snb-1 *gene.* pSB101 insert DNA was used to probe an ordered grid of yeast artificial chromosome (YAC) clones representing most of the *C. elegans* genome. The *snb-1* probe hybridized to three overlapping YAC clones: Y7E11, Y6G12, and Y44A7 (Coulson et al., 1988). The cosmid T10H9 was shown to contain *snb-1* by Southern analysis (Coulson et al., 1986). A 5.2 kb *Hin*dIII fragment from cosmid T10H9 containing the complete *snb-1* gene was cloned in both orientations into pBluescript KS($^-$) to create pSB102 and pSB103. Animals homozygous for *nDf18*, *nDf32*, and *sDf20* deficiencies were analyzed for the presence of *snb-1* and *xol-1* sequences (Rhind et al., 1995) by using single-worm PCR (Williams et al., 1992). Deficiency animals were isolated as dead eggs segregating from *Df/dpy-11(e224)* animals. *sDf20* and *nDf32* removed *snb-1*, but not the control locus (*xol-1*). The *snb-1::lacZ* fusion construct pSB111 was created by inserting a 3.2 kb *Hin*dIII–*Nae*I fragment of pSB102 into the *Msc*I--*Hin*dIII fragment of pPD21.28 (Fire et al., 1990). Transgenic animals expressing the fusion construct were examined by using X-gal as a substrate for *lacZ*. The genomic region was sequenced (GenBank accession number AF 003282).

*Isolation of* snb-1 *mutants. snb-1(md247)* was isolated in a genetic screen for aldicarb-resistant mutants (Miller et al., 1996). Additional alleles were isolated in three noncomplementation screens. Wild-type males were mutagenized with ethyl methanesulfonate and crossed to *dpy-11(e224) snb-1(md247) ric-4(md1088); xol-1(y9) flu-2(e1003)* in the first screen, to *dpy-11(e224) snb-1(md247); xol-1(y9) flu-2(e1003)* in the second screen, and to *dpy-11(e224) snb-1(js17); xol-1(y9) flu-2(e1003)* in the last screen. In each case, animals resistant to aldicarb were selected by placing the cross-progeny on plates containing either 0.5 or 0.75 mM aldicarb. *js17* was isolated from the first screen of ~30,000 cross-progeny, *js43* and *js44* from the second screen of 50,000 cross-progeny, and *js124* from the third screen of 32,000 cross-progeny. The entire coding region of the *snb-1* locus was sequenced from the mutants. *js124* is a C to T transition at position 1 of codon 50, *js17* and *js43* result from C to T transitions at position 1 of codon 62, *js44* results from a C to T transition at position 2 of codon 65, and *md247* results from the duplication of the sequence CGCTATCGTCGTCATTCTTAT (encoding amino acids 92–99) and its insertion after the first base of codon 100. The lesion is a 20 bp duplication separated by a TA sequence and may be the result of a Tc1 transposition event (Mori et al., 1988; Plasterk, 1991). Introduction of the pSB103 genomic construct by germline transformation (Mello et al., 1991) rescued the behavioral phenotypes of *md247* and *js124*. *snb-1* was mapped genetically between *dpy-11* and *unc-68*: from *dpy-11(e224) unc-68(r1158)/snb-1(md247)* animals, two of seven Dpy non-Unc recombinants carried the *snb-1(md247)* lesion. *snb-1(md247)* complements *nDf18* and *sDf36* but failed to complement *nDf32*, *sDf20*, and *sDf30*. All *snb-1* alleles failed to complement *nDf32*.

*Behavioral assays.* Locomotion was assayed by imaging L4-staged animals shortly after they were deposited onto fresh agar plates containing an *E. coli* lawn. CCD camera images were collected for 1 min with an LG3 frame grabber (Scion) at 1 sec intervals at a magnification between 1.6 and 2.5×. Distances traveled were calculated by summing movements in the position of the tail by using the program Scion Image (Scion). Defecation was observed under a dissecting microscope, and cycles were recorded with a simple computer program (Liu and Thomas, 1994). Pharyngeal pumping of mutant animals with slow pumping rates was assayed by counting pumping for 1 min intervals. Pharyngeal pumping of animals with fast rates (>120 pumps/min) was assayed by counting pumps from digital images captured at 15 frames/sec for 20 sec intervals.

*Resistance to aldicarb and levamisole.* Mutants were assayed for acute exposure to aldicarb and levamisole. Aldicarb resistance was examined by transferring individual animals to plates containing aldicarb and assaying them for paralysis 4 hr after exposure. Animals were considered paralyzed if they failed to move even if prodded with a platinum wire. Levamisole resistance was examined by placing 25 worms in S-basal medium (Sulston and Hodgkin, 1988) with 100 μM levamisole and scoring them for movement by examining video images of the animals at different time points.

*Electrophysiology.* Electropharyngeograms (EPGs) were recorded with an AC preamplifier (designed by David Brumley, University of Oregon) and LabView Acquisition software as previously described (Avery et al., 1995). Bath solution consisted of M9 with 2.5–5 mM serotonin to stimulate pumping. Only recordings from young adult hermaphrodites with at least 10 pharyngeal pumps were used in analysis. Analysis of the records was performed as described in Iwasaki et al. (1997) except that amplitudes were calculated as peak-to-peak.

## RESULTS

### Isolation of a synaptobrevin homolog in *C. elegans*

We isolated partial cDNA clones encoding a *C. elegans* synaptobrevin molecule with PCR. A complete cDNA representing the transcript from the gene was isolated from an embryonic cDNA library (see Materials and Methods). The deduced transcript is predicted to encoded a protein product with 68% identity to human synaptobrevin-2 (Fig. 1). The predicted protein is most similar to neuronal synaptobrevin molecules from metazoans (60–68% identity) but also is very similar to the ubiquitously expressed rat cellubrevin (66% identity) and a *Drosophila* synaptobrevin expressed in gut (59% identity). The gene was named *synaptobrevin (snb-1*) because it represents the first characterized member of this family in *C. elegans*.

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SNB-1 worm          MDAQGDAGAQ-G-----GSQGGPRP---SNKRLQQTQAQVDEVVGIMKVNVEKVLERDQKLSQLDDRADALQEGASQFEKS   72
SYB1 human        MSAPAQPP•E•TE•TAP•------•GPP••P•NMT••R•••••••••E•••D•IR•••D••••••••••E•••••••••A••••••S•   82
SYB2 human        MSATAATAPPAAPAGE--------•GPPA•P•NLT••R•••••••••••••D••R•••D••••••••••E•••••••••A••••••T•   80
n-syb fly      MADAAPAGDAPPNA•APAGEG•DGEIV•GPHN•QQ-IAAQ•••••••••••••••D••RT•••••••••T•••E•••••••••Q••••••QQ   89
cellubrevin                  MST-----•VPS•SSAATG••R••••••N••••••D••R•••D••••••••••E•••••••••A••••••T•   67
SNC1 yeast         MSSSTPFDPY•LSEHD-------EER•QN-VQ•KS•TAEL••EI•DT••••RD•IN••A••GER•TSIE•K••N•AVS•QG•KRG   77

SNB-1 worm      AATLKRKYWWKNIKMMIIMCAIVVILIIIIVLWAGGK                                                    109
SYB1 human      ••K•••••••••C••••MLG••CA•IVVV••IYFFT                                                    118
SYB2 human      ••K••••••••••••••LGV•CA•IL•••IVYFSS                                                     116
n-syb fly       •GK•RK•F•LQ•••••••GV•GLVVVG••ANKL•LIGGEQPPQYQYPPQYMQPPPPPPQQPAGGQSSLVDGAGAGDGGAGGSAGAGHGGV   180
cellubrevin     ••K••••••••••••WA•GISVL••IV•••IV•CVS                                                    103
SNC1 yeast      •NRVRKAM•Y•D•••KMCLALVII••LVV•IVPIAVHFSR                                                117
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*Figure 1.* Similarity among synaptobrevin molecules. Alignment of synaptobrevin family proteins from *C. elegans* (*SNB-1 worm*), *Drosophila* (*n-syb fly*; DiAntonio et al., 1993), rat (*cellubrevin*; McMahon et al., 1993) human (*SYB1* and *SYB2*; Archer et al., 1990), and *S. cerevisiae* (*SNC1 yeast*; Gerst et al., 1992). The standard single letter amino acid code is used. *Dots* represent identity with the *C. elegans* sequence. Amino acid numbering appears at the *right*.

### *C. elegans* synaptobrevin is expressed in the nervous system

To ascertain the expression pattern of the gene in the nematode, we raised antisera directed against a bacterially expressed SNB-1 fusion protein. An affinity-purified synaptobrevin antiserum was incubated with fixed whole adult *C. elegans* animals, and the antibodies were detected by FITC-conjugated secondary antisera. SNB-1 was detected in the nervous system. The majority of synaptobrevin immunoreactivity was restricted to the major process bundles of *C. elegans*. The nerve ring, ventral cord, and dorsal cord were highly immunoreactive (Fig. 2*A–C*). Faint fluorescence also was detected in some neuronal cell bodies. By contrast, synaptobrevin was not detected in most commissural and dendritic processes. Previously characterized *C. elegans* synaptic vesicle proteins RAB-3 (Fig. 2*D*) and synaptotagmin (data not shown) colocalized with synaptobrevin (Nonet et al., 1993, 1997). In addition, synaptobrevin immunoreactivity was mislocalized in cell bodies in *unc-104*, as would be expected of a synaptic vesicle-associated protein (Hall and Hedgecock, 1991; Nonet et al., 1993, 1997). *unc-104* encodes a kinesin required for the transport of synaptic vesicles from the cell body to the synapse (Otsuka et al., 1991). Thus, *C. elegans* SNB-1 shows the expected distribution of a synaptic vesicle-associated protein.

Because it was not feasible to determine whether all neurons expressed SNB-1 from the examination of immunohistochemical-stained samples, the expression pattern of the gene was examined by using a translational *lacZ* fusion (see Materials and Methods). Consistent with our immunohistochemistry data, SNB-1 was expressed in the vast majority of all neurons in the nerve ring ganglia (Fig. 2*G*) and the ventral cord (Fig. 2*H*). Moreover, the expression of *snb-1* assayed by *lacZ* was restricted to neuronal tissue, although expression also has been observed in the spermatheca with green fluorescent protein (GFP) fusions (data not shown). We conclude that *snb-1* encodes a neuronal synaptobrevin.

### Isolation of synaptobrevin mutants

As a first step toward isolating mutations in *snb-1*, the gene was positioned on the physical map of *C. elegans* (Fig. 3; see Materials and Methods). The gene mapped to YAC clones containing DNA from the center of chromosome V. The genetic position was refined further by determining whether *snb-1* was removed by a variety of deficiencies of the region (Fig. 3*A*). The mapping data positioned the gene in the 0.3 map unit interval between the *dpy-11* and *unc-68* loci. A genomic clone containing the entire coding region was isolated from a cosmid clone (Fig. 3*B*; Coulson et al., 1986), and the single intron of the gene was identified by sequencing of the genomic region (Fig. 3*C*).

A variety of mutants encoding *C. elegans* synaptic components share a common attribute; they are resistant to the acetylcholinesterase inhibitor aldicarb (Nonet et al., 1993, 1997; Nguyen et al., 1995; Miller et al., 1996; Iwasaki et al., 1997). The mutation *ric(md247)* was isolated in a selection for aldicarb-resistant mutants (Miller et al., 1996), and genetic mapping placed *md247* close to the physical position of *snb-1*. *md247* was demonstrated to be a lesion in the *snb-1* gene by two criteria. First, *md247* mutants transformed with a genomic clone of the synaptobrevin gene are rescued to wild type, as assayed by both behavioral and aldicarb resistance assays. Second, sequencing of the *snb-1* coding region identified a mutation in *md247* that could account for the gene defect.

Additional *snb-1* mutations were isolated in noncomplementation screens. Wild-type males were mutagenized and crossed to marked *snb-1* strains (see Materials and Methods). Cross-progeny were screened for behavioral defects, or aldicarb resistance was used to identify new *snb-1* mutations. Four independent mutations, *js17*, *js43*, *js44*, and *js124*, were isolated in the three genetic screens. *js17, js43*, and *js44* are all similar to *snb-1(md247)* in that homozygous mutant animals are viable and also viable *in trans* to a deficiency of the region. By contrast, the *js124* mutation is homozygous lethal. These animals arrest development just after completing embryogenesis and hatching, and they tend to adopt a coiled position similar to that observed in lethal *cha-1* choline acetyltransferase mutants (Rand, 1989). Animals heterozygous for *js124* were not resistant to aldicarb and showed no behavioral abnormalities, indicating that the lesion had no dominant or gain-of-function characteristics. In addition, *js124 in trans* to a deficiency of the region phenotypically resembled the *js124* homozygotes. Finally, the lethal phenotype of *js124* was rescued to wild type by the introduction of a wild-type *snb-1* genomic clone, thus confirming that this phenotype is solely the result of the lesion in the *snb-1* locus.

Sequencing of the alleles revealed that the *md247* lesion duplicates 20 bp and results in a shift in the reading frame at amino acid 100, midway in the transmembrane domain of SNB-1 (Fig. 4). *js17* and *js43* are independently isolated missense mutations resulting in an L62F substitution, whereas *js44* consists of a missense change resulting in an A66G substitution (Fig. 4). None of the mutants,

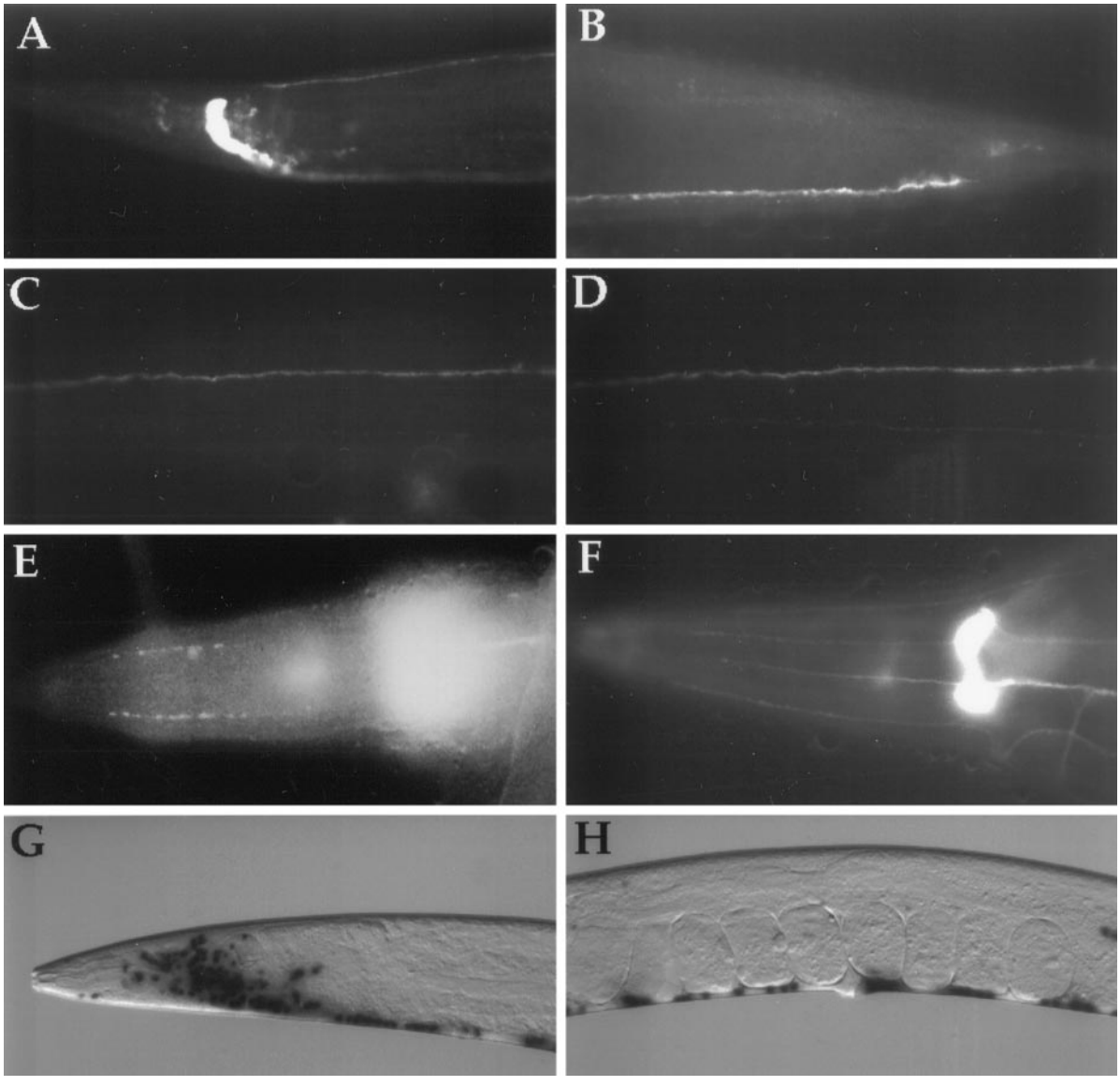


*Figure 2.* Expression of *snb-1* in the *C. elegans* nervous system. *A–F*, Whole worms fixed and stained with α-SNB-1, α-RAB-3, and α-UNC-64 (syntaxin) primary antibodies and visualized with FITC- or Cy3-conjugated antibodies. *A*, Lateral view of the head region of a wild-type adult hermaphrodite showing SNB-1 immunoreactivity in the nerve ring, pharyngeal nervous system, and ventral and dorsal nerve cords. *B*, Ventral view of the tail region of a wild-type adult hermaphrodite showing SNB-1 immunoreactivity in the ventral nerve cord. *C, D*, Ventral view of the midsection of a wild-type adult hermaphrodite showing colocalization of SNB-1 (*C*) and RAB-3 (*D*) immunoreactivity in the ventral nerve cord. *E*, Ventral view of the head of SNB-1 immunoreactivity in an adult *snb-1(md247)* mutant. The SNB-1 mutant protein remains limited to the synaptic regions of the SAB neurons. *F*, Similar view of UNC-64 syntaxin immunoreactivity in a wild-type animal reveals all processes in the head. *G, H*, Transgenic *jsEx96* animals expressing the $_{P}$*snb-1*::*lacZ* reporter construct pSB111 stained for β-galactosidase activity, using X-gal. *lacZ* contains an SV40 nuclear localization signal. *G*, Lateral view of the head region of an adult hermaphrodite showing staining neurons in the head ganglia and pharynx. *H*, Lateral view of the vulval region of an adult hermaphrodite showing staining in the motor neurons in the ventral cord and two neurons in the deirid sensillum.

*js17*, *js43*, *js44*, nor *md247*, shows immunohistochemical abnormalities (Fig. 2*E*) (data not shown). *js124* results in a Q50>STOP nonsense lesion (Fig. 4); hence the *js124* phenotype likely represents the *snb-1* null phenotype.

### Phenotypes of synaptobrevin mutants

The *snb-1* mutants all exhibit a variety of behavioral abnormalities. The viable mutants all remain capable of coordinated movement. However, their movements are easily differentiated from the wild type (Table 1). The animals are lethargic and have a tendency to jerk, especially during backward motion. Synaptobrevin null *js124* animals arrested as L1 larvae and often adopted a coiled position. These animals also remained capable of making some movements. To characterize the movements, we examined the animals via video microscopy. Time-lapse imaging (Fig. 5) demonstrated that these animals were capable of making semi-coordinated movements (Fig. 5). Additionally, pharyngeal pumping, which mediates feeding behavior, was depressed in all of the mutants (Table 1). Furthermore, the defecation cycle time was longer, and enteric muscle contraction step of the defecation cycle (Avery and Thomas, 1997) failed more frequently than in the wild type (Table 1). These behavioral defects presumably all represent manifestations of the underlying impairment of synaptic trans-

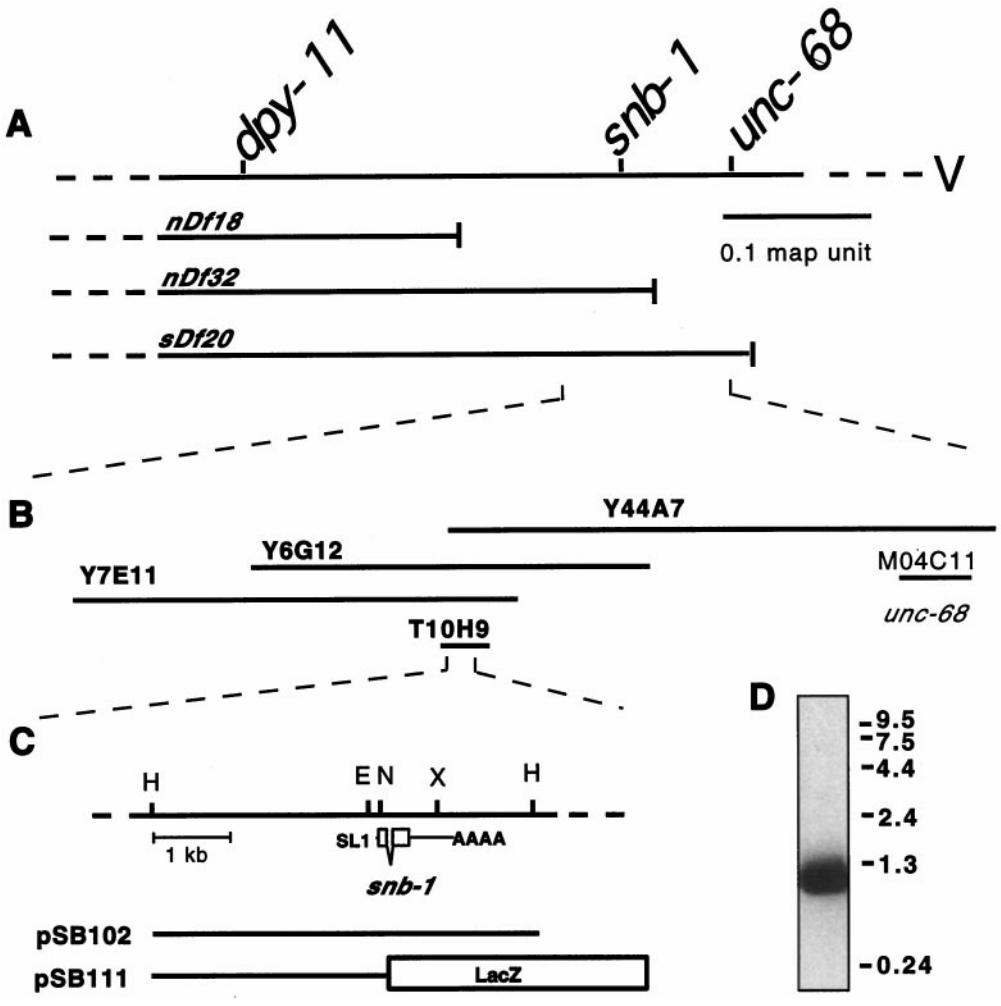


*Figure 3.* The *snb-1* locus. *A*, Genetic map of the *snb-1* region of chromosome V showing three deficiencies used to position the locus. *B*, Physical map of the *snb-1* region showing cosmid and yeast artificial chromosome clones. The position of the *unc-68* gene is shown also. *C*, Restriction map of the genomic *snb-1* gene. *Below* the restriction map a schematic diagram shows the intron–exon structure of the *snb-1* gene and the sequences contained in plasmid constructs used in this study. The pSB103 insert is identical to the pSB102 insert shown. *E*, *Eco*RI; *X*, *Xba*I; *N*, *NaeI*; *H*, *Hin*dIII restriction endonuclease sites. *D*, Autoradiograph of a Northern blot probed with a *snb-1* cDNA fragment. Poly(A$^+$) RNA (10 $\mu$g) isolated from a culture of mixed-stage hermaphrodites was loaded on the gel. Size standards, labeled in kilobases, are on the *right*.

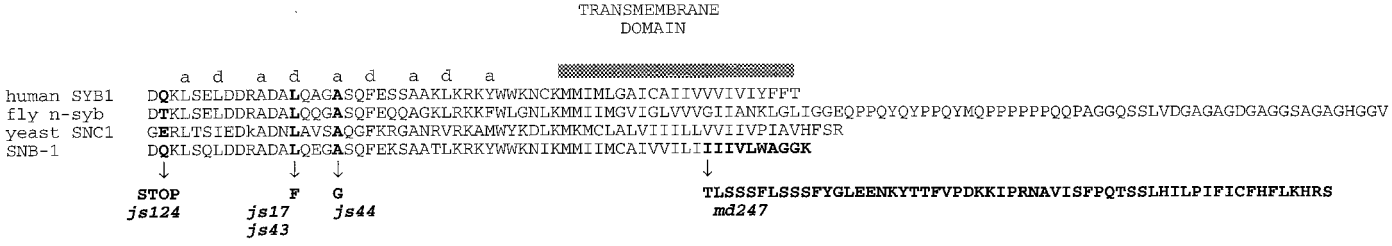


*Figure 4.* Molecular lesions in *snb-1* mutants. The carboxyl sequences of four synaptobrevin sequences are shown aligned. The standard single letter amino acid code is used. The hydrophobic transmembrane region of the proteins is indicated. Sequences forming an $\alpha$-helical structure form a repeating seven-amino-acid unit. The amino acids of the helix are labeled *a* through *g*. *a* and *d above* the sequence alignment represent the amino acids on the hydrophobic face of a predicted amphipathic–helix structure that could be formed by the proteins. The lesions found in the four *snb-1* mutations are labeled in *bold*. The molecular lesions are detailed in Materials and Methods.

mission. However, the movements of *snb-1* null mutants, although much less vigorous than those of the wild type, are evidence that some neuromuscular transmission persists in the absence of synaptobrevin.

### Synaptic transmission defects in synaptobrevin mutants

To assess neurotransmission in *snb-1* mutants, we examined their response to pharmacological agents. We quantified the resistance of *snb-1* mutants to the acetylcholinesterase inhibitor aldicarb (Fig. 6). All three viable alleles were resistant to aldicarb, as was the acetylcholine receptor mutant *unc-29* (Fleming et al., 1997) The aldicarb resistance suggests that cholinergic transmission is impaired in the mutants. We also examined the response of *snb-1* mutants to the acetylcholine receptor agonist levamisole (Lewis et al., 1980a,b). *snb-1* mutants and wild-type animals showed comparable sensitivity to the drug (Fig. 6). By contrast, the acetylcholine receptor mutant *unc-29* was extremely resistant to the drug. Thus, the assay indicates that postsynaptic responses are fundamentally intact, suggesting that cholinergic transmission is impaired presynaptically in *snb-1* mutants.

We also examined electrical activity in the pharynx, using an extracellular recording method devised by Raizen and Avery

**Table 1. Behavioral defects of synaptobrevin mutants**

| | Locomotion[a] | | | Defecation[d] | |
|---|---|---|---|---|---|
| Strain | Speed ($\mu$m/sec) | Efficiency[b] | Pharyngeal pumps/min[c] | Cycle time (sec) | EMCs (%)[e] |
| N2 | 160 ± 36 | 0.63 | 250 ± 30 | 42.0 ± 3.3 | 98 |
| *md247* | 89 ± 20 | 0.43 | 64 ± 20 | 66.1 ± 11.1 | 85 |
| *js17* | 44 ± 11 | 0.26 | 206 ± 24 | 47.2 ± 3.9 | 62 |
| *js44* | 73 ± 33 | 0.32 | N.D. | 51.5 ± 5.5 | 83 |

All errors are listed as SD from the mean.

[a] Data shown represent the mean of at least 19 animals. This methods underestimates the distance traveled in fast-moving animals because of the sinusoidal locomotion pattern of worms.

[b] Efficiency is defined as displacement/total distance traveled. *snb-1* mutants have a tendency to alternate short periods of forward and backward motion.

[c] Data Shown represent the mean of at least 15 young animals for each genotype.

[d] Data shown represent observations of the defecation cycles of at least 10 animals (over 100 defecation cycles in total.)

[e] The percentage of defecation cycles with an enteric muscle contraction (see Avery and Thomas, 1997).

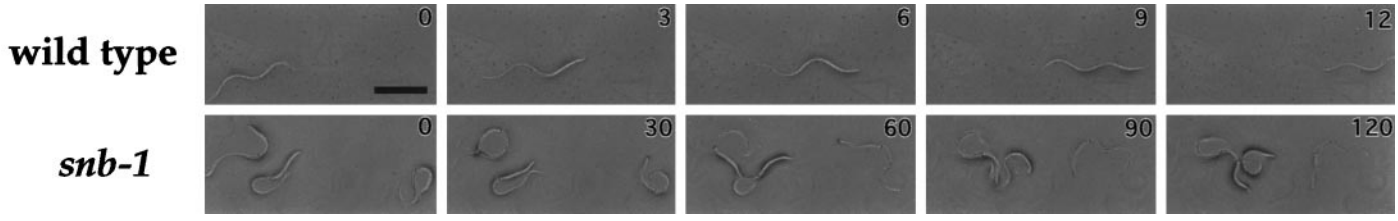


*Figure 5.* Locomotion of *snb-1* mutants. Shown are bright-field images of wild-type and *snb-1(js124)* L1 larvae on an *E. coli* lawn at indicated time intervals (*top right*, in seconds). Scale bar, 100 $\mu$m.

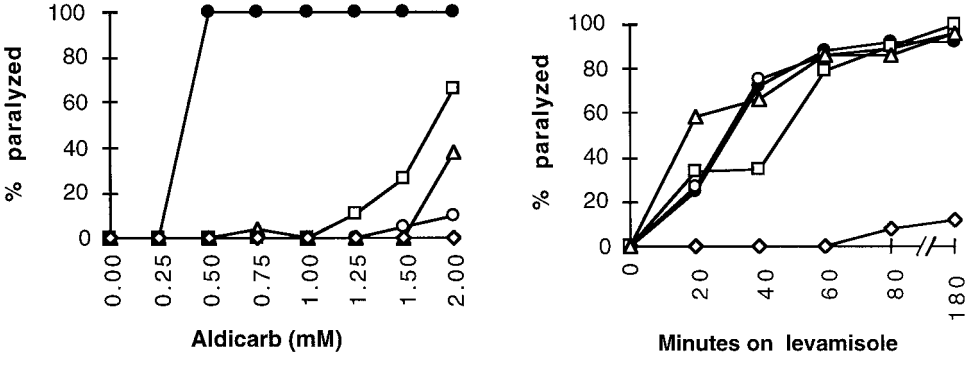


*Figure 6.* Pharmacological properties of *snb-1* mutants. *A*, Aldicarb sensitivities of *snb-1* mutants and the wild type. Shown is the percentage of animals paralyzed after a 4 hr exposure to various concentrations of aldicarb on plates seeded with *E. coli*: wild type (●), *js17* (□), *js44* (△), *md247* (○), and *unc-29* (◇). *B*, Levamisole sensitivities of *snb-1* mutants and the wild type. Shown is the percentage of animals paralyzed after exposure of adult animals to 100 $\mu$M levamisole for various times: wild type (●), *js17* (□), *js44* (△), *md247* (○), and *unc-29* (◇).

(1994). EPGs allow for the examination of currents intrinsic to pharyngeal muscle as well as for those resulting from synaptic input. We examined inhibitory currents elicited by the glutamatergic motor neuron M3, which shortens the length of the contraction of pharyngeal muscle (Avery, 1993). Wild-type animals exhibited three to five distinct M3-induced transients during each pharyngeal pump (Fig. 7*A*, Table 2). By contrast, M3 transients were usually completely absent or extremely small in recordings from *snb-1(md247)* mutants (Fig. 7*B*, Table 2). M3 transients of reduced amplitude also were observed in records of both *js17* and *js44* animals (Fig. 7*C,D*, Table 2). Consistent with a reduction in M3 activity, pharyngeal pump duration was increased in all of the mutants (Table 2). The activity of MC neurons, a pair of motor neurons that stimulate pharyngeal contractions (Raizen et al., 1995), also was altered in *snb-1* mutants. Specifically, we noted an increase in MC transients among pumps that failed to elicit pharyngeal contractions (Fig. 7*B–D*). These subthreshold MC failures were observed rarely in wild-type animals (data not shown) (Iwasaki et al., 1997). In summary, our pharmacological and physiological assays demonstrate that both cholinergic and glutamatergic transmission are impaired in *snb-1* hypomorphic mutants.

### v-SNARE double mutants remain capable of movement

Biochemical analysis that led to the SNARE hypothesis identified both synaptobrevin (Sollner et al., 1993a,b) and synaptotagmin (Schiavo et al., 1995) as v-SNARES of synaptic vesicles. To assess whether the residual behaviorally assessed neurotransmission was a consequence of synaptotagmin function, we constructed *snt-1(md290)*; *snb-1 (js124)/dpy-11(e224)* animals and examined *snt-1*; *snb-1* double mutants segregating from the parents. The double mutants were phenotypically very similar to *snb-1(js124)* animals. They were capable of some movement (Fig. 8) and arrested development in the first larval stage. By contrast, mutants lacking the t-SNARE *unc-64* syntaxin were virtually completely paralyzed (Fig. 8). Behaviorally, the *snb-1* and *snt-1*; *snb-1* double mutants were distinguishable because pharyngeal pumping was reduced in the double mutant (*snb-1*, 11.7 ± 7.2 pumps/min; *snt-1*; *snb-1*, 0.5 ± 0.8 pumps/min; *unc-64* 0.15 ± 0.3 pumps/min). However, in contrast to *unc-64* syntaxin mu-

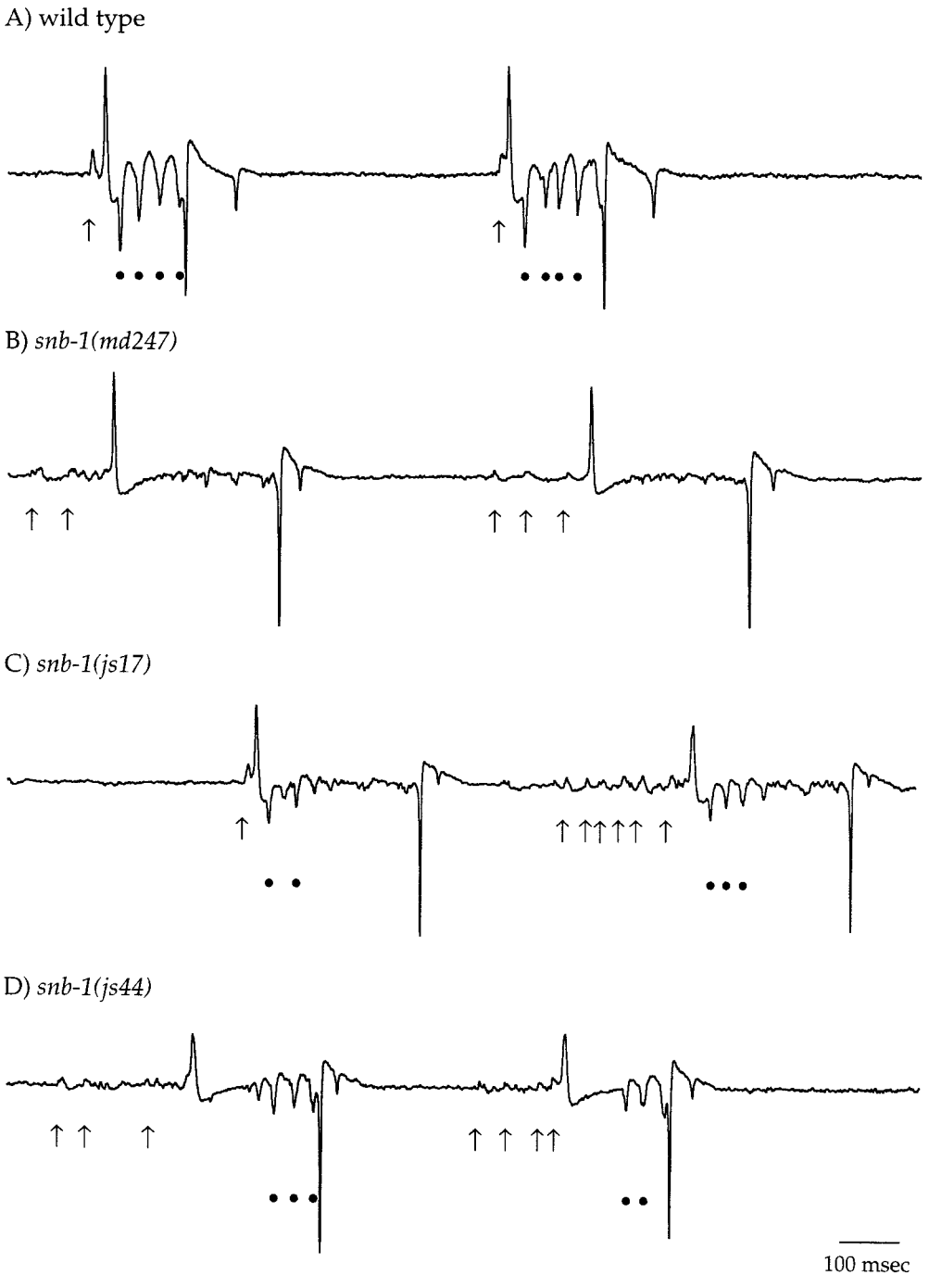


*Figure 7.* Pharyngeal recordings from wild-type and *snb-1* mutants. Shown are characteristic recordings of the wild-type strain N2 (*A*), *snb-1(md247)* (*B*), *snb-1(js17)* (*C*), and *snb-1(js44)* (*D*). *Arrows* indicate MC-induced transients, and the *filled circles* indicate M3-induced transients. All traces are millivolts versus time.

tants and *cha-1* choline acetyltransferase mutants (Avery and Horvitz, 1990), pumping of the double mutant could still be markedly stimulated by the addition of exogenous serotonin (*snt-1*; *snb-1* + 5HT, 12.1 ± 7.1; *unc-64* + 5HT, 0.05 ± 0.15). In summary, mutants lacking both *snb-1* and *snt-1*; *snb-1* closely resemble the *snb-1* mutant at a behavioral level; they remain capable of some neuromuscular signaling.

## DISCUSSION

We have isolated and characterized a gene encoding a *C. elegans* synaptobrevin that functions in the synaptic release pathway. SNB-1 is extremely likely the homolog of the synaptic vesicle-associated synaptobrevin-1 and -2 molecules expressed in the vertebrate nervous system. *snb-1* is expressed in the nervous system, and SNB-1 protein colocalizes with *C. elegans* RAB-3 and synaptotagmin, suggesting that the protein is synaptic vesicle-associated. Furthermore, *snb-1* mutants have synaptic transmission deficits, indicating that the molecule functions in neuronal exocytosis in *C. elegans*.

*snb-1* joins a long list of *C. elegans* mutants that are resistant to the drug aldicarb (Rand and Nonet, 1997). These mutants include genes encoding products involved in acetylcholine synthesis and vesicular loading (Alfonso et al., 1993, 1994), regulation of vesicle trafficking and fusion (Maruyama and Brenner, 1991; Gengyo-Ando et al., 1993; Nonet et al., 1993, 1997), and acetylcholine

**Table 2. Summary of physiological defects in *snb-1* mutants**

| Strain | Pump duration[a](msec) | M3s/pump[b] | M3 amplitude[c] |
|---|---|---|---|
| Wild type | 134 ± 2 | 4.04 ± 0.08 | 0.354 ± 0.005 |
| *md247* | 320 ± 5 | 0.65 ± 0.10 | 0.169 ± 0.004 |
| *js17* | 286 ± 6 | 2.20 ± 0.15 | 0.173 ± 0.003 |
| *js44* | 163 ± 4 | 2.47 ± 0.12 | 0.209 ± 0.004 |

Errors are displayed as SEM.

[a] Pump duration was defined as the time from the peak of the E-phase transient to the peak of the R-phase transient (Raizen and Avery, 1994).

[b] M3 transients were identified with an algorithm as described in Iwasaki et al.(1997).

[c] M3 amplitudes are normalized to the amplitude of the R-phase transient.

reception (these are not resistant to chronic aldicarb exposure; Lewis et al., 1980a; Fleming et al., 1997). Thus, the drug resistance of the hypomorphic mutants suggests that synaptic transmission is impaired in *snb-1* mutants. The pleiotropic behavioral abnormalities provide additional evidence for synaptic deficiencies. Finally, the electrophysiological abnormalities in M3 transmission in *snb-1* mutants also support a requirement for synaptobrevin for synaptic transmission. Unfortunately, our simple physiological assay cannot be applied to first-larval-stage animals. Thus, we have not confirmed that M3 activity is absent in our lethal *snb-1* mutant. However, M3 activity is often absent even in the viable *snb-1(md247)* mutants.

Our analysis of *snb-1* mutants clearly demonstrates that synaptobrevin is essential for viability of *C. elegans*. Animals lacking *snb-1* are able to complete embryogenesis but arrest development shortly after hatching and die several days later. This lethality is rescued by introduction of a transgene carrying wild-type *snb-1* sequences. The mutant animals are extremely uncoordinated and rarely move far from where they hatch. However, the animals are capable of some movements, including head foraging movements. This arrest phenotype is very similar to the phenotype of mutants lacking the *cha-1* choline acetyltransferase protein (Rand, 1989), the *unc-17* vesicular ACh transporter (Alfonso et al., 1993), and the *unc-104* synaptic vesicle kinesin motor protein (Hall and Hedgecock, 1991). However, the phenotype contrasts with that of mutants lacking syntaxin (*unc-64*), which are paralyzed on hatching (O. Saifee, L. Wei, and M. Nonet, unpublished data). Thus complete disruption of the v-SNARE synaptobrevin and corresponding t-SNARE syntaxin result in different defects in synaptic transmission.

Our analysis of the behavioral phenotypes of the *snb-1* null mutant suggests that transmitter release is not abolished completely in the absence of synaptobrevin. First, *snb-1* animals are still capable of some rudimentary movements. In fact, *snb-1(js124)* animals move better in the presence of aldicarb (data not shown), indicating that some ACh release is occurring at neuromuscular junctions in the absence of SNB-1. Additionally, pharyngeal pumping (10–20 pumps/min) is observed in *snb-1* null mutants. By contrast, pharyngeal pumping is essentially absent in both syntaxin null mutants (0.15 pumps/min) (O. Saifee, L. Wei, and M. Nonet, unpublished data) and *cha-1* choline acetyltransferase null mutants (<1 pump/min) (Avery and Horvitz, 1990). In *Drosophila*, expression of synaptobrevin-specific protease tetanus toxin in neurons blocks evoked release at the neuromuscular junction but does not eliminate spontaneous miniature release events (Broadie et al., 1995). It is possible that the movement and pumping in *C. elegans snb-1* mutants are mediated by spontaneous fusion events that do not require *snb-1*. A more likely possibility is that the residual release events use another synaptobrevin-like molecule expressed in the nervous system. For example, both cellubrevin and synaptobrevin-2 can contribute to exocytosis of insulin from pancreatic β-cells (Regazzi et al., 1997). A number of other synaptobrevin-related molecules apparently are expressed in *C. elegans*, because the Genome Sequencing Project has identified at least five genes with similarity to synaptobrevin. The most conserved of these sequences, F23H12.1, shares 47% identity with *snb-1*. However, this gene appears to be expressed primarily in the gut (L. Wei and M. Nonet, unpublished results). Despite this residual movement, which probably represents residual transmitter release, synaptobrevin is essential for the viability of *C. elegans*.

The finding of Broadie et al. (1995) that synaptic vesicles remained docked in *Drosophila* neurons expressing tetanus toxin and our finding that *C. elegans snb-1* null mutants remain capable of movement both suggest that synaptobrevin is not essential for the docking of synaptic vesicles before release. An alternative interpretation is that synaptobrevin and synaptotagmin both participate in docking and that docking requires only one of the two molecules. Schiavo et al. (1997) provided biochemical support of such a hypothesis by demonstrating a physical interaction between synaptotagmin and SNAP-25 that could account for docking in the absence of synaptobrevin. The difference in the phenotype of *C. elegans* mutants lacking *unc-64* syntaxin and double mutants lacking both synaptobrevin and synaptotagmin suggests that more release of transmitter occurs in the absence of both v-SNAREs than in the absence of the t-SNARE syntaxin. A likely explanation for this is that docking is still occurring in absence of the v-SNAREs. Because, in the absence of synaptobrevin in *Drosophila,* vesicle docking occurs at the ultrastructural level (Broadie et al., 1995), presumably one of the remaining vesicle proteins is involved in the docking of vesicles. *rab-3* would seem to be an ideal candidate because *C. elegans* mutants lacking *rab-3* result in a more diffuse distribution of vesicles around the synaptic release site, a phenotype that can be explained by a reduction in the efficiency of docking (Nonet et al., 1997). A combination of behavioral and morphological analyses of these and other double mutant and triple mutant combinations may help in the identification of these other required proteins.

The hypomorphic lesions we characterized provide some insight into the function of synaptobrevin. The *md247* mutation is the most unusual lesion we characterized. This lesion shifts the reading frame of SNB-1 half-way through the transmembrane domain, leaving only 13 amino acids of the native transmembrane domain to act as a membrane anchor (see Fig. 4). Because the mutant SNB-1 protein remains specifically localized to synaptic regions in *md247* animals (see Fig. 2), the mutant protein is probably still associated with vesicles. Consistent with this hypothesis, Whitley et al. (1996) have demonstrated that 12 hydrophobic residues are sufficient to anchor synaptobrevin into microsomal membranes. Synaptobrevin probably does not remain associated with the vesicle via interactions with other proteins, because deletion of the transmembrane domain of a GFP-tagged SNB-1 results in a ubiquitous distribution of this fusion protein in neurons (M. Nonet, unpublished results). Presently, we are characterizing the transmembrane sequences required for *snb-1* function by site-directed mutagenesis of this domain.

SNARE complex formation is proposed to be mediated by the assembly of a coiled-coil structure composed of hypothetical amphipathic–helical domains within the syntaxin, synaptobrevin,

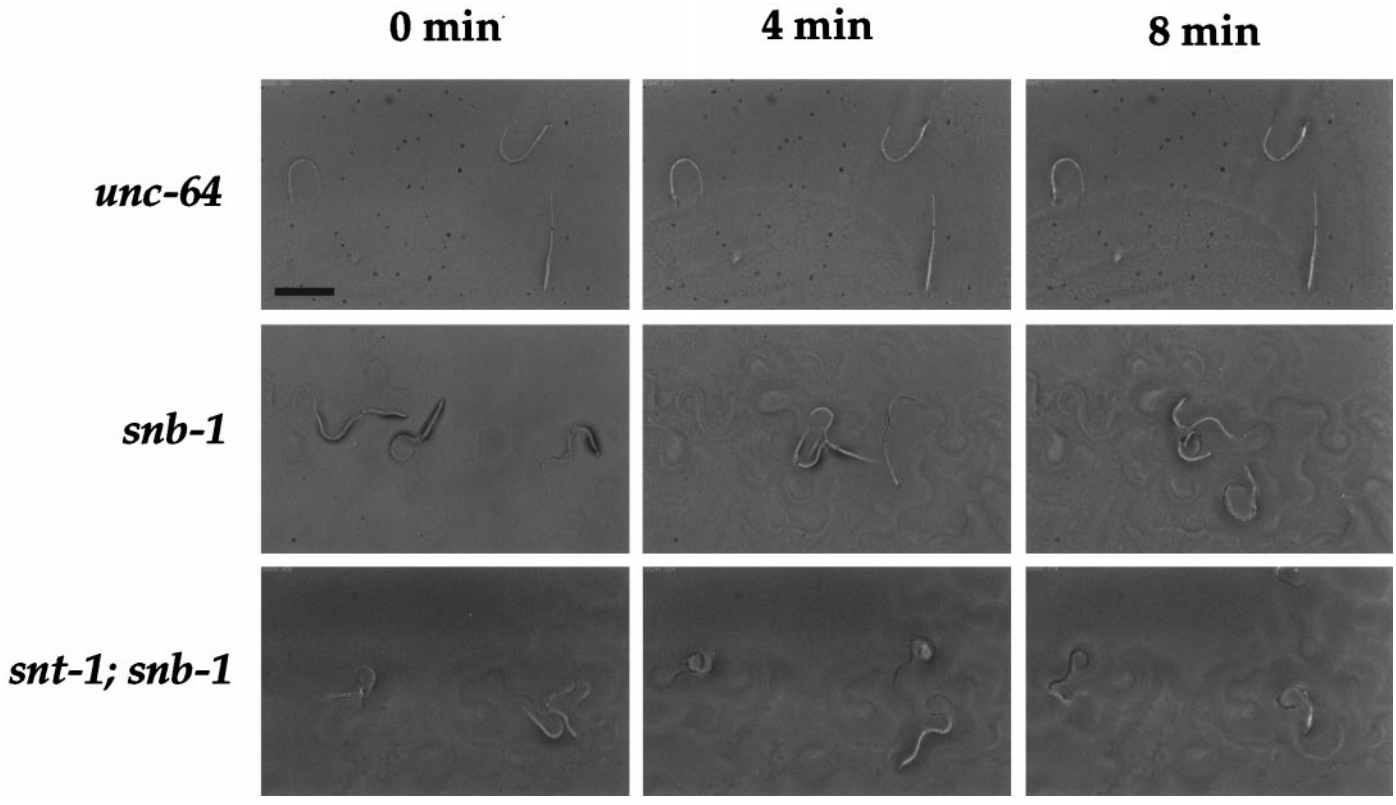


*Figure 8.* Locomotion of *snb-1 snt-1* double mutants. Shown are bright-field images of *snb-1(js124)*, *unc-64(js115)*, and *snt-1(md290); snb-1(js124)* L1 larvae on an *E. coli* lawn at indicated time intervals. Note the tracks in the *E. coli* lawn. Scale bar, 100 μm.

and SNAP-25 proteins (Chapman et al., 1994; Hayashi et al., 1994). The lesions in the hypomorphic *js17* and *js44* alleles lie on the hydrophobic faces of the proposed amphipathic–helix region of synaptobrevin that could mediate formation of a coiled-coil structure. Because protein levels are not altered dramatically in these mutants, the lesions probably do not affect *snb-1* function simply by altering the stability of the protein. Rather, they probably affect the interactions of synaptobrevin with the t-SNAREs or with other molecules. Further supporting this interpretation, these *snb-1* lesions exhibit allele-specific synergism with lesions in the amphipathic–helix domain of syntaxin (O. Saifee, L. Wei, and M. Nonet, unpublished data). Although synaptobrevin binds to syntaxin and SNAP-25, only a few specific amino acids of the protein have been implicated directly in SNARE complex formation. Hao et al. (1997) recently have examined the ability of point mutations and small deletions of synaptobrevin to form functional complexes with syntaxin and SNAP-25. Although deletions of most of the protein blocked interactions with syntaxin in an *in vitro* binding assay, a deletion of the region (corresponding to amino acids 63–73 in SNB-1) encompassing the *js44* lesion increased the affinity of synaptobrevin for syntaxin. However, in a yeast two-hybrid assay the same deletion failed to interact with syntaxin (Hao et al., 1997). The different behavior of lesions in this region *in vitro* and *in vivo* illustrates the need to correlate *in vivo* function and *in vitro* biochemistry to dissect the role of synaptobrevin in vesicle fusion efficiently. Our mutants provide the first *in vivo* evidence that the helical domain is important for synaptobrevin function.

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