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The *Caenorhabditis elegans unc-64* Locus Encodes a Syntaxin That Interacts Genetically with Synaptobrevin

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We describe the molecular cloning and characterization of the *unc-64* locus of *Caenorhabditis elegans. unc-64* expresses three transcripts, each encoding a molecule with 63–64% identity to human syntaxin 1A, a membrane- anchored protein involved in synaptic vesicle fusion. Interestingly, the alternative forms of syntaxin differ only in their C-terminal hydrophobic membrane anchors. The forms are differentially expressed in neuronal and secretory tissues; genetic evidence suggests that these forms are not functionally equivalent. A complete loss-of-function mutation in *unc-64* results in a worm that completes embryogenesis, but arrests development shortly thereafter as a paralyzed L1 larva, presumably as a consequence of neuronal dysfunction. The severity of the neuronal phenotypes of *C. elegans* syntaxin mutants appears comparable to those of *Drosophila syntaxin* mutants. However, nematode syntaxin appears not to be required for embryonic development, for secretion of cuticle from the hypodermis, or for the function of muscle, in contrast to *Drosophila* syntaxin, which appears to be required in all cells. Less severe viable *unc-64* mutants exhibit a variety of behavioral defects and show strong resistance to the acetylcholinesterase inhibitor aldicarb. Extracellular physiological recordings from pharyngeal muscle of hypomorphic mutants show alterations in the kinetics of transmitter release. The lesions in the hypomorphic alleles map to the hydrophobic face of the H3 coiled-coil domain of syntaxin, a domain that in vitro mediates physical interactions with similar coiled-coil domains in SNAP-25 and synaptobrevin. Furthermore, the *unc-64* syntaxin mutants exhibit allele-specific genetic interactions with mutants carrying lesions in the coiled-coil domain of synaptobrevin, providing in vivo evidence for the significance of these domains in regulating synaptic vesicle fusion.

INTRODUCTION

Communication between neurons at synaptic contacts occurs through the regulated release of chemical neurotransmitters. This release process is mediated by fusion of transmitter-filled vesicles with the plasma membrane. Through both biochemical and genetic studies, several dozen proteins have been implicated in this fusion process in neurons (Bennett and Scheller, 1994; Sollner and Rothman, 1994; Sudhof, 1995). A central player is the integral membrane protein syntaxin (Bennett *et al.*, 1992). In neurons, syntaxin is found in abundance on the plasma membrane and also in low levels in synaptic vesicles (Bennett *et al.*, 1992; Galli *et al.*, 1995; Walch-Solimena *et al.*, 1995). A vast array of experiments all indicate that syntaxin plays a critical role in regulating neurotransmitter release in neurons. For example, serotype C neurotoxin from *Clostridium botulinum*, which is a potent inhibitor of synaptic transmission, is a metalloprotease with high specificity for cleaving syntaxin (Blasi *et al.*, 1993; Schiavo *et al.*, 1995) Moreover, physiological recordings from *Drosophila syntaxin* mutants reveal the absence of both induced and spontaneous fusion events,

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suggesting an absolute requirement for syntaxin function (Broadie *et al.*, 1995). Despite these and many other studies, the precise role of syntaxin in vesicle fusion remains mysterious. Specifically, is syntaxin an integral component of the fusion machinery (i.e., capable of fusing two lipid bilayers) or does it regulate that machinery?

A reason to suspect that syntaxin is a critical component of the release apparatus per se is that in vitro syntaxin associates with numerous synaptic proteins. First, syntaxin forms an extremely stable ternary complex with the synaptic vesicle-associated membrane protein synaptobrevin (also called VAMP) and SNAP-25, a protein associated with the plasma membrane (Sollner *et al.*, 1993; Hayashi *et al.*, 1994). This and other related complexes between vesicle-associated and plasma membrane-associated proteins have been hypothesized to represent intermediates in the fusion process. However, this hypothesis remains effectively untested as biochemical studies of the ternary complex have been performed in the absence of membrane. Furthermore, a multitude of other proteins show strong interactions with syntaxin. These include Munc-13, Munc-18, calcium channels, synaptotagmin, and several other proteins (Bennett *et al.*, 1992; Hata *et al.*, 1993; Sollner *et al.*, 1993; Garcia *et al.*, 1994; Pevsner *et al.*, 1994; Sheng *et al.*, 1994; Betz *et al.*, 1997; Edwardson *et al.*, 1997). Although genetic and perturbation experiments suggest many of these proteins also play pivotal roles in regulating the release process, it is unclear whether the interactions of these proteins with syntaxin are even functionally significant in vivo. Elucidating the roles of each of these proteins in regulating vesicle fusion remains a complex task.

Caenorhabditis elegans is a powerful model system with which to study mechanisms of neurotransmission (Rand and Nonet, 1997). Genetic selection for mutants with transmission defects is feasible in this organism using the acetylcholinesterase inhibitor aldicarb (Rand and Russell, 1985; Miller *et al.*, 1996). This drug kills wild-type animals, presumably through hyperstimulation of muscle, but spares mutants with reduced cholinergic transmission. Distinct approaches aimed at identifying synaptic regulators, including biochemistry in vertebrates and genetics in *C. elegans*, have identified a largely overlapping set of genes (Sudhof, 1995; Rand and Nonet, 1997). At least five genes identified by mutation in *C. elegans*, namely *snb-1* (synaptobrevin), *snt-1* (synaptotagmin), *unc-2* (calcium channel α-subunit), *unc-13,* and *unc-18*, encode homologs of proteins that interact in vitro with vertebrate syntaxin (Maruyama and Brenner, 1991; Gengyo-Ando *et al.*, 1993; Nonet *et al.*, 1993; Schafer and Kenyon, 1995; Nonet *et al.*, 1998). Loss-of-function mutations in each of these *C. elegans* genes result in severe behavioral and synaptic function defects (Brenner, 1974; Nonet *et al.*, 1993, 1998). Although, the *C. elegans* proteins have not yet been reported as interacting with syntaxin as occurs in vertebrates, the extensive conservation of these molecules makes it likely that such interactions exist. Disrupting, through mutation, specific interactions between syntaxin and its many interacting partners will disclose in vivo functions of the proteins that require syntaxin interaction.

In this study, we describe the isolation and characterization of mutants defective in syntaxin function. We demonstrate that *C. elegans* syntaxin is encoded by the *unc-64* gene. Three distinct gene products derive from the locus; all share an identical cytoplasmic domain but contain different C-terminal membrane anchors and are expressed in distinct but overlapping secretory tissues. *unc-64* syntaxin appears to be essential for neuronal function, as mutants lacking the gene product arrest as paralyzed larvae. However, the gene is not required for embryogenesis or secretion of the cuticle, suggesting that *C. elegans* syntaxin is not required for secretion in all cells. Viable hypomorphic mutants carrying lesions in the coiled-coil domain show milder dysfunction of synaptic transmission. These mutants exhibit a variety of behavioral deficits and alterations in the kinetics of transmission. Furthermore, these mutants show specific genetic interactions with mutants carrying lesions in the coiled-coil domain of synaptobrevin (Nonet *et al.*, 1998). Our studies provide in vivo evidence for a critical role for the coiled-coil domains of both syntaxin and synaptobrevin in regulated secretion.

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 were performed using standard genetic methods (Herman and Horvitz, 1980). Aldicarb, 2-methyl-2-[methylthio]propionaldehyde *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 or added directly to plates.

DNA and RNA Manipulations

C. elegans genomic DNA was isolated as described by Sulston and Hodgkin, (1988). cDNA was synthesized 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 was performed using standard procedures except where noted (Sambrook *et al.*, 1989). Analyses of DNA sequences and amino acid sequences were performed on a SPARC station using the GCG program package (Devereux *et al.*, 1984).

Isolation of the C. elegans Syntaxin Gene

Oligonucleotide primers TX1 (5′-TTY TTY GAR CAR GTN GAR GAR AT) and TX5 (5′-CAT NGC CAT RTC NTN RAA CAT) corresponding to regions of syntaxin conserved between rat syntaxin

1A and 1B (Bennett *et al.*, 1992) were used to amplify a specific sequence from cDNA made as described by Nonet *et al.*, (1993). The fragment was cloned, sequenced, and used as a probe to isolate three cDNA clones from an embryonic cDNA library (Lichtsteiner and Tjian, 1993). The largest cDNA (*Not*I fragment) was subcloned into pBluescript KS(−) to create pTX4. Sequencing of the cDNA revealed it began with the trans-spliced leader sequence found on many *C. elegans* messages and ended with a poly A sequence (accession number AF047885). The full-length clone was used as a probe on a Northern blot of mixed-staged poly $(A)^+$ selected RNA and identified three transcripts. Sequencing of a genomic clone of the locus revealed the presence of potential alternative terminal exons. Two additional transcripts were amplified from cDNA using PCR and confirmed that the exons are utilized in vivo (accession numbers AF047886 and AF047887). We have not confirmed the structure of the noncoding portion of the larger messages identified on Northern blots because the region between the exons encoding transmembrane regions contains numerous high-copy repetitive elements (rep A, C, H, and W). The *unc-64* probe hybridized to YAC clones Y39E4, Y43F4, Y106H1, and Y109C2 on chromosome III of the physical map of *C. elegans* (Coulson *et al.*, 1988). As cosmid clones were not available for this genomic region, clone λTXg.1 was obtained by probing a genomic lambda library (Barstead and Waterston, 1989), and clone H20G23 was obtained by probing a fosmid library with assistance from the Genome Sequencing Center, Washington University School of Medicine, St. Louis, MO. pTX20 contains a 16.2-kilobase (kb) *Pst*I fragment from H20G23 inserted into pBluescript KS(−). pTX6 was created by inserting an 8.2-kb *Nco*I–*Nhe*I fragment of λTXg.1 into pRSETC. An *unc-64::lacZ* fusion plasmid, pTX9, was created by inserting the 3.1-kb *Xba*I–SpeI lacZ fragment from pPD95.03 (kindly provided by A. Fire, J. Ahnn, G. Seydoux, and S. Xu) into the *Nhe*I site of pTX6. pTX12, containing a functional syntaxin minigene, was created by inserting the 5.2-kb *Xho*I-*Nhe*I fragment of pTX6 into pTX4. Three GFP^{S65T}-tagged transmembrane domain constructs were created by amplifying sequences from pTX20 with an oligonucleotide in exon 5 (TX-17 5′-GCGCAAGACGAACCCAGAG) and oligonucleotides (TX-52 5′-ATCGGCCGACCGGTTTGAAAAAATGTATAATACTTTC, TX-53 5′-ATCGGCCGACCGGTATGCCAGGAATATACTGAATGAG) that replaced the stop codon in the exon with a linker sequence containing both an *Age*I and *Eag*I restriction site. The amplified fragments were introduced into pTX12 after cleavage at the *Nhe*I and *Eag*I restriction sites, replacing the cDNA sequences of pTX12. Green fluorescent protein (GFP) from pPD93.65 (kindly provided by A. Fire, J. Ahnn, G. Seydoux, and S. Xu) was added subsequently using the *Age*I and *Eag*I sites to create pTX34 and pTX37 (Figure 1). Using a similar amplification scheme as above (TX-55 5′-TTACCGGTCGAACACGAAAACGTTGAGAAGAC), pTX38 was created in a single step by replacing the *Nhe*I–*Age*I fragment of pTX34 with the smaller fragment tagging syntaxin C.

Production of Syntaxin Antibodies

Sequences from pTX4 coding for amino acids 1–266 were amplified by PCR using oligonucleotides TX-7 (5′-GCTCTAGAATGACTAAGGACAGATTG) and TX-8 (5′-CAAGATCTACTTCTTCCTTCGCGCCTTC) and cloned into pRSETB (Invitrogen, San Diego, CA) to create the plasmid pTX10. The plasmid expresses a 32-kDa fusion protein containing a six-histidine tag on the amino terminus of a 30.5- kDa cytoplasmic domain of the UNC-64 protein. The protein was purified and used to immunize rabbits as described by Nonet *et al.* (1993). UNC-64 antiserum was affinity purified using the a method described previously (Smith and Fisher, 1984). Affinity-purified antibodies were used at 1:50 dilution, and sera were used at 1:1000 dilution. Immunocytochemistry was performed as previously described using Bouin's fixative (Nonet *et al.*, 1993).

Genetic Analysis of unc-64

Wild-type males were mutagenized with 50 mM ethyl methanesulfonate for 4 h and crossed to *dpy-18(e364) unc-64(e246); xol-1(y9) flu-2(e1003)*, and the cross-progeny was screened for lethargic non-Dpy animals. Two additional alleles of *unc-64* were isolated in a screen of 5200 genomes. Each mutant was outcrossed to wild-type and subsequently balanced with *bli-5(e518)*. The lethal *unc-64* alleles are balanced effectively by the *bli-5* mutant as no recombinants between *bli-5(e518)* and *unc-64(e246)* were isolated in more than 1500 animals examined. The entire coding region and all intron–exon junctions of *e246, js21, js115, js116, md130,* and *md1259* were sequenced to characterize the molecular lesions. The identified lesions are C to T in the second base of codon 248 in *e246*, C to T in the second base of codon in 241 in *js21*, C to T in the first base of codon 71 in *js115*, G to A in the second base of codon 283 (transmembrane exon A) in *js116*, a G to A in the base after exon 6 in *md130*, and a G to A in the first base after exon 3 in *md1259.*

To examine the phenotype of animals harboring *js21* and *e246* mutations *in trans* to a deficiency, *unc-64/+* males were crossed to *eDf2; eDp6* hermaphrodites and *eDf2/unc-64* animals were examined. In the case of *js115*, which confers a lethal phenotype, single *js115/+* males were mated to single *tra-2(q122)/+;eDf2/+* female animals isolated from a cross of *tra-2(q122)* males with *e246/eDf2. js115/eDf2* animals segregating from *js115/eDf2; eDp6* were also analyzed. The phenotypes of *e246, js21* and *js115 in trans* to the deficiency *eDf2* in each case were very similar to that of animals homozygous for the respective mutation. In addition, *js115/e246* animals were phenotypically indistinguishable to *eDf2/e246* animals. In concert, these results indicate that *js115* behaves like a null mutant. *unc-64; snb-1* mutants were created by crossing *dpy-11(e224)/snb-1* males to *unc-64; dpy-11(e224)* yielding *unc-64/+; dpy-11/snb-1* animals. After selecting animals homozygous for *unc-64, unc-64; snb-1* animals were identified as animals that failed to segregate Dpy animals. The *unc-64* genotype in the double mutants was confirmed by PCR analysis.

Transformation and RNA Injections of C. elegans

Germline transformation was accomplished by coinjecting the plasmid of interest with either *rol-6* (pRF4 at 150 μg/ml) as a dominant marker or *lin-15* (pJM23 at 50 μg/ml) injected into *lin-15(n765)* as a recessive transformation marker (Mello *et al.*, 1991; Huang *et al.*, 1994). The dominant marker *rol-6* was used to rescue *e246* and *js115* mutants by the introduction of pTX20 (20 μg/ml). *js115* and j*s116* were also rescued by the minigene construct pTX12 (20 μg/ml). LacZ (pTX9) and GFP (pTX34, 37, 38) expression constructs were introduced at 10 μg/ml into *lin-15(n765)* using *lin-15* as marker. For RNA interference experiments, full-length antisense RNA was synthesized in vitro using T7 RNA polymerase from a PCR-amplified pTX4 insert DNA template and injected into the germline of wild-type animals at 0.8 mg/ml as described by Rocheleau *et al.* (1997).

Mosaic Analysis

To examine animals with no germline contribution, *js115; jsEx136* animals were created. The *jsEx136* extrachromosomal array was created by coinjection of the *unc-64 (+)* plasmid pTX20 (20 μg/ml), the dominant *rol-6* marker pRF4 (150 μg/ml), and pMR1 (10 μg/ml) into *js115/bli-5(e518)*. pMR1, an *unc-54* promoter::GFP^{S65T} construct that expresses high levels of GFP^{S65T} in body wall muscle and sex muscles, was constructed by inserting a *Kpn*I--*Apa*I GFP^{S65T} fragment from pPD93.51 into pPD30.38 (both plasmids kindly provided by A. Fire, J. Ahnn, G. Seydoux, and S. Xu). More than 140 rollers were examined and none that segregated no progeny were identified. Among 354 nonroller mosaic animals, 10 that segregated no viable progeny were isolated. One produced no progeny, and the other 9 produced from 12 to greater than 100 progeny (a total of >740). Ten to 30 of the L1-arrested animals from each mother were cloned and examined for the presence of GFP in muscle and the

general organization of structures including the cuticle, nerve ring ganglia, intestine, and pharynx. The animals appeared similar to *js115* homozygotes from a *unc-64(js115)/bli-5(e518)* heterozygote. The plates also were examined for arrested unhatched eggs. A few progeny of 4 of the 9 animals failed to hatch. Of the 10 eggs found, only 3 were arrested before threefold stage. Finally, of approximately 2500 progeny screened under a fluorescent dissecting microscope for progeny lacking fluorescence in muscle, we identified none lacking fluorescence in all muscle and analyzed the locomotion of 14 lacking GFP in large sections of muscle.

Behavioral Assays

Locomotion was assayed by imaging L4-staged animals shortly after they were deposited onto fresh agar plates containing an *Escherichia coli* lawn. Charge-coupled device camera images were collected for 1 min with an LG3 frame grabber (Scion) at 1-s intervals at a magnification between 1.6× and 2.5×. Distance traveled was calculated by summing displacements in the position of the tail. Defecation was observed under a dissecting microscope and cycles were recorded using a simple computer program (Liu and Thomas, 1994). Pharyngeal pumping of mutant animals with slow pumping rates was assayed by counting the number of pumps in a 1-min interval. Pharyngeal pumping of animals with faster rates (>120 pumps/min) was assayed by counting pumps from digital images captured at 15 frames/s for 20-s 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 for paralysis 4 h 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 for movement by examining video images of the animals at different time points.

Electrophysiology

Electropharyngeograms (EPGs) were recorded using a AC preamplifier (designed by David Brumley, University of Oregon, Eugene, OR) and LabView Acquisition software (National Instruments, Austin, TX) as previously described (Avery *et al.,* 1995). Bath solution consisted of M9 or Dent's saline (Avery *et al.,* 1995) with 2.5 mM serotonin to stimulate pumping. Only recordings from young adult hermaphrodites with at least 10 pharyngeal pumps were analyzed. Analysis of the records was performed as described by Iwasaki *et al.* (1997) except that amplitudes were calculated as peak-to-peak, and the threshold was set at 5% (rather than 10%) of the mean amplitude of the R-phase transients to identify M3 transients in a record.

RESULTS

unc-64 Encodes a Syntaxin Homolog

unc-64 was first described by Brenner (1974) as a mutant with locomotory defects. *unc-64* was suspected to encode a component regulating synaptic transmission because it shares a common phenotype with many other synaptic mutants in *C. elegans*: resistance to the acetylcholinesterase inhibitor aldicarb (Alfonso *et al.*, 1993, 1994; Nonet *et al.*, 1993, 1997, 1998; Nguyen *et al.*, 1995; Miller *et al.*, 1996; Iwasaki *et al.*, 1997). We isolated a *C. elegans* gene with strong similarity to rat syntaxin 1 that mapped to a region of chromosome III on the physical map, roughly corresponding to the genetic map position of the *unc-64* gene (see Figure 1 and MATERIALS AND METHODS for details). We tested whether the syntaxin-like gene was encoded by *unc-64* using germline transformation (Mello *et al.*, 1991). *unc-64(e246)* mutants harboring a genomic clone of the *C. elegans* syntaxin gene were phenotypically rescued to wild-type, as determined by both behavioral and aldicarb resistance assays. Additionally, sequencing of the coding region of the candidate syntaxin gene from *unc-64* mutant alleles in each case revealed a molecular lesion that could account for the genetic defect (Figure 1A). Thus, we conclude that *unc-64* encodes a *C. elegans* syntaxin homolog.

Three Distinct Forms of Syntaxin Are Encoded by unc-64

We isolated cDNA clones derived from the *unc-64* syntaxin locus. Several complete cDNA clones representing one transcript from the *C. elegans* syntaxin locus were isolated from an embryonic cDNA library, and partial cDNAs representing two additional transcripts were isolated from first-strand cDNA using the PCR. Each transcript is predicted to encode a product with 63% or 64% identity to human syntaxin 1A. The three transcripts differ only in the last of eight coding exons and thus encode products with identical cytoplasmic domains but different transmembrane anchor domains (Figure 1, A and B). We refer to these three products as the syntaxin A, B, and C products of the *unc-64* locus. At least three distinct transcripts of approximately 1.1 kb, 2.6 kb, and 3.8 kb are present on Northern blots of mixed-staged RNA (Figure 1C). The size of the full-length syntaxin B cDNA isolated from the cDNA library is of the appropriate size to correspond with the smaller transcript. The larger transcripts are of an appropriate length to represent the syntaxin A and C cDNAs if they utilize the same polyadenylation site. The splicing of the transmembrane domain of syntaxin in *C. elegans* is reminiscent of the splicing pattern observed for the rat syntaxin 2 and the mouse syntaxin 3 genes (Bennett *et al.*, 1993; Ibaraki *et al.*, 1995).

unc-64 Syntaxin Is Expressed in the Nervous System and Secretory Tissues

The *C. elegans* syntaxin sequences are most similar to the vertebrate neuronal-specific syntaxin 1 molecules and hence are likely to be expressed in the nematode nervous system. To examine the expression pattern, antisera were raised against bacterially expressed *C. elegans* syntaxin fusion protein. An affinity-purified anti-syntaxin antiserum was incubated with fixed whole adult animals, and the antibodies were detected using FITC-conjugated secondary antisera. As expected, syntaxin was detected in the nervous system (Figure 2). Syntaxin immunoreactivity was very

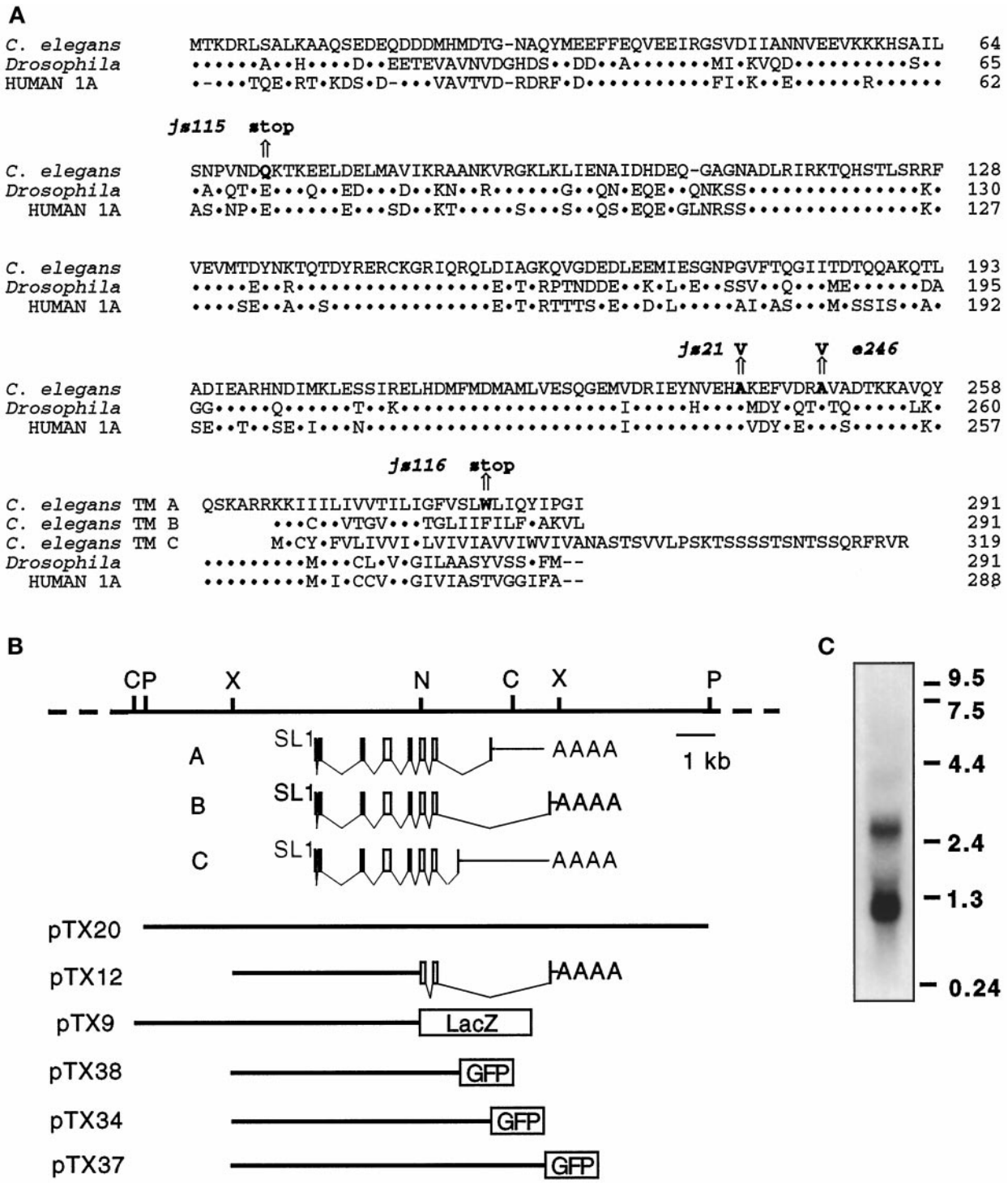


Figure 1. The *unc-64* syntaxin locus. (A) Similarity among syntaxin molecules. An alignment of syntaxin family proteins from *C. elegans*, *Drosophila* (Schulze *et al.*, 1995), and human (Osborne *et al.*, 1997). The position of the molecular lesions identified in selected mutations in the *unc-64* syntaxin gene (bold) and the resulting change in amino acid sequence are indicated above an arrow. The standard single-letter amino acid code is used. Dots represent identity with the *C. elegans* sequence. Amino acid number appears at the right. (B) Restriction map of the genomic *unc-64* gene. Below the restriction map is a schematic diagram showing the intron–exon structure of three *unc-64* products and the sequences contained in plasmid constructs used in this study. Gray represents predicted message structures. C, *Nco*I; X, *Xho*I; N, *Nhe*I; P, *Pst*I restriction endonuclease sites. (C) Autoradiograph of a Northern blot probed with a syntaxin cDNA fragment; 10 μg of mixed-staged poly A(+) selected RNA isolated from wild-type hermaphrodites was loaded on the gel. RNA size standards (kb) are on the right.

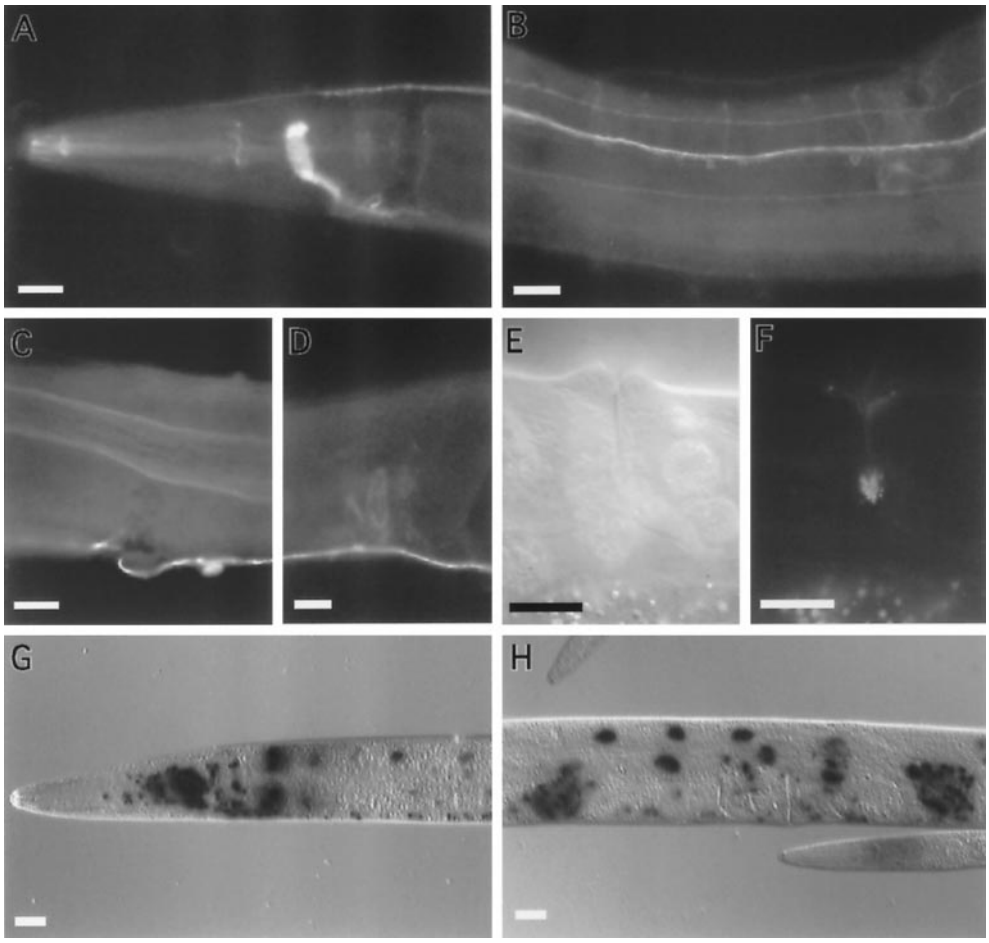


Figure 2. Expression of *unc-64* syntaxin. (A–D) Whole adult wild-type hermaphrodite worms fixed and stained with α-UNC-64 primary antibodies and visualized with FITC-conjugated antibodies. (A) Lateral view of the head region showing immunoreactivity in the nerve ring, dorsal cord, and pharyngeal nervous system. (B) Ventral view of the midbody region showing expression in ventral cord axons, neuronal cell bodies, and in commissural and sublateral processes. (C) A lateral view of the midbody showing UNC-64 immunoreactivity on the basolateral surface of the intestine. (D) A lateral view of the midbody showing immunoreactivity in the hermaphrodite spermatheca. The male vas deferens also stains. (E) A lateral view of the vulva viewed using differential interference contrast microscopy. (F) Same plane of focus as panel E showing fluorescence from the UNC-64 A::GFP product in the uv1 cells of the vulva. (G–H) Whole adult wild-type animals fixed and stained for *LacZ* gene activity expressed under the control of the *unc-64* promoter. *LacZ* activity assayed using X-gal as a substrate. The *lacZ* gene contains a nuclear localization signal. (G) Lateral view of the head region showing expression of *unc-64 ::lacZ* in neuronal and intestinal nuclei. (H) Lateral view of the midbody of an adult hermaphrodite showing *unc-64::lacZ* expression in intestinal nuclei, neuronal nuclei, and the spermatheca. Scale bar, in all panels, 20 μm.

strong and distributed uniformly along the major process bundles: the nerve ring (Figure 2A), ventral cord (Figure 2B), and dorsal cord (Figure 2A). Syntaxin immunoreactivity also was detected in the vast majority of neuronal cell bodies as well as in commissural and dendritic processes (Figure 2B). By contrast, the vesicle-associated markers synaptobrevin, synaptotagmin, and RAB-3 are restricted to the synaptic-rich regions of the nervous system where they show a punctate, rather than uniform, appearance (Nonet *et al*., 1993, 1997, 1998). Syntaxin was also detected in the pharyngeal nervous system (Figure 2A). Furthermore, syntaxin was detected outside the nervous system. Immunoreactivity was strong in the uv1 cells of the vulva, in the intestine (Figure 2C), and in the spermatheca (Figure 2D). Detectable levels of syntaxin protein were not observed in other tissues. Our protein expression data suggest that *unc-64* encodes the homolog of the vertebrate syntaxin 1 gene (Bennett *et al*., 1992, 1993).

As it was not feasible using immunohistochemistry to determine which neurons expressed syntaxin, the expression pattern of the SNARE gene was examined using a translational *lacZ* fusion. A genomic syntaxin fragment (Figure 1C) was inserted into a plasmid containing a nuclear-localized *lacZ* reporter gene (Fire *et*

al., 1990). As expected, syntaxin was expressed in the vast majority of all neurons (Figure 2, G and H). In addition, syntaxin expression was high in intestinal nuclei, in the spermatheca, and in the uv1 cells of the vulva (Figure 2H). In summary, *C. elegans* syntaxin is expressed in both secretory and neuronal tissues. In neurons, the protein is not specifically associated or concentrated at synaptic release sites; rather, it is ubiquitously distributed.

The three identified products deriving from the *C. elegans* syntaxin locus differ only in those sequences that are predicted to lie in the lipid bilayer and act as transmembrane anchors. Because production of antibodies capable of distinguishing these products did not seem feasible, we engineered constructs that tagged each product by appending the GFP-coding region to the last codon of each predicted transmembrane domain. The constructs were introduced into wild-type animals by germline transformation, and the expression pattern of the GFP-tagged syntaxins was examined. Syntaxin A and C were expressed in neurons and in five nonneuronal tissues. By contrast, expression of syntaxin B was limited to the five nonneuronal cell types. In neurons, syntaxin A and C were found ubiquitously on neuronal cell bodies, axons, and dendrites. Also stained were the uv1 secretory cells of the vulva, the excretory gland cells, the distal tip cells, the spermatheca, and the intestinal muscle (Figure 2, E and F). In the uv1 cells, GFP fluorescence was concentrated subcellularly (Figure 2, E and F). The expression pattern from the GFP, *lacZ*, and antibody studies were consistent, except that GFP reporter expression was observed in the intestine only with syntaxin C fusions. This expression was limited to the posterior half of the intestine. It is possible that sequences that direct gut expression are absent from the GFP clones since sequences further upstream of the initiation codon are present in the lacZ constructs. In summary, the alternative UNC-64 syntaxin products are expressed in distinct but overlapping neuronal and nonneuronal secretory cells.

Isolation of Additional unc-64 Syntaxin Mutants

Additional alleles of the *unc-64* gene were isolated in three independent screens. *md130* and *md1259* were isolated in a screen for aldicarb- resistant mutants occurring spontaneously in a strain with high levels of Tc1 transposition (Miller *et al.*, 1996). *js21* was isolated in a general screen for aldicarb-resistant mutants using EMS as a mutagen (M.L.N., unpublished data). Finally, two lethal alleles, *js115* and *js116*, were isolated in a noncomplementation screen (see MATERIALS AND METHODS). Molecular characterization revealed that the *e246* and *js21* lesions are missense mutations that result in alanine-to-valine substitutions at codons 241 and 248, respectively. These codons are in the H3 domain of syntaxin, a region thought to assume an amphipathic helical structure capable of forming coiled-coil interactions with other proteins (Hardwick and Pelham, 1992; Calakos *et al.*, 1994; Fasshauer *et al.*, 1997). The point mutations in *md130* and *md1259* disrupt splicing sites (see MATERIALS AND METHODS for details). The *js116* mutation results in premature termination in the transmembrane domain of the syntaxin A product, while the *js115* lesion results in premature termination at codon 71. Immunohistochemical examination of whole fixed animals revealed that syntaxin product remained in the nervous system of all viable mutants, although the level of syntaxin was reproducibly lower in the *md1259* mutant. UNC-64 staining was not detected in *js115*. All available evidence suggests that *js115* represents the null phenotype (see MATERIALS AND METHODS).

Behavioral Defects of unc-64 Syntaxin Mutants

All six *unc-64* mutants exhibit locomotory abnormalities. The lethal mutants exhibit the most severe motor defects. *js115* animals are virtually completely paralyzed and rarely maintain a sinusoidal posture, although they occasionally will make slow head movements (Figure 3). *js116* animals exhibit very slow motor movements (Figure 3) and tend to adopt a coiled position. Both *js115* and *js116* animals arrest development just after hatching and eventually die as L1 larvae. Behaviorally, the viable *js21* and *e246* mutants are very lethargic, although they retain the ability to move briefly when prodded. Locomotory abnormalities were quantified by examining the rate of movements of animals imaged using a charge-coupled device camera (Table 1). Despite exhibiting severe locomotory defects, other behaviors of the hypomorphic mutants were only weakly affected (Table 1). The rate of pharyngeal pumping was relatively normal in the hypomorphic mutants *e246* and *js21*, although pumping was completely abolished in the *js115* null mutant. The motor defecation program was only slightly abnormal in hypomorphs, but completely abolished in *js115*. It is interesting to note that, compared with other synaptic mutants with similarly severe locomotory defects (e.g., *unc-18* and *unc-13*), the *unc-64(e246)* mutant has much milder deficiencies in other behaviors. *unc-64(e246)* mutants also exhibit a tendency to form enduring dauer larvae (Iwasaki *et al.*, 1997), a developmental decision regulated by sensory input (Bargmann and Horvitz, 1991). Two other mutants, *md130* and *md1259*, also exhibit milder behavioral defects, but minimal analysis was performed on these alleles due to the difficulty in determining, with certainty, the resulting gene products. Finally, at the morphological level, muscle, the intestine, the

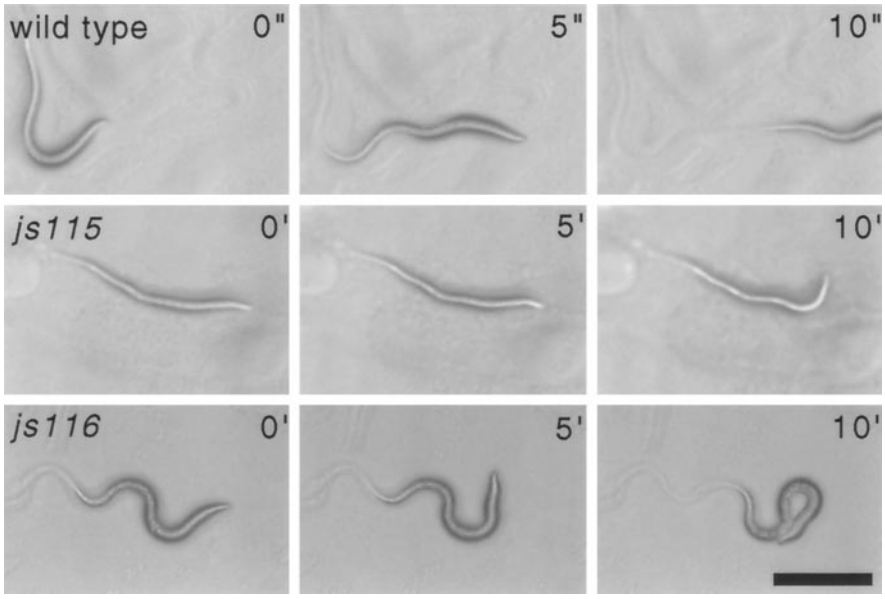


Figure 3. Phenotype of lethal *unc-64* syntaxin mutants. Bright field images of first larval stage animals of wild-type, *unc-64(js115),* and *unc-64(js116)* animals on an *E. coli* bacterial lawn at the indicated time intervals (wild-type in seconds and mutants in minutes). Scale bar, 100 μm.

pharynx, the cuticle, and the organization of the nervous system appear normal in both the hypomorphs and the null mutant. In summary, a variety of genetic lesions in the syntaxin gene result in behavioral defects of varying severity. Presumably, all these phenotypes represent manifestations of an underlying impairment of synaptic transmission.

Late Arrest of unc-64 Syntaxin Mutants Is Not the Result of Maternal Contribution

In *D. melanogaster,* mosaic clones that lack syntaxin cannot be isolated, suggesting the molecule is required in all cells (Schulze and Bellen, 1996). Furthermore, syntaxin is also required early in development during cellularization of the *Drosophila* embryo (Burgess *et al.*, 1997). As our most severe mutant arrests development after completing embryogenesis, we examined whether *unc-64* syntaxin mutants completed embryogenesis using maternally derived products. We constructed a strain homozygous for *js115*, which also harbored an extrachromosomal array bearing a visible marker (*rol-6*), a muscle-specific *unc-54::GFP* construct, and the wild-type *unc-64* syntaxin gene. These animals segregate both wild-type and mutant progeny as the array is not transmitted to all progeny. We examined the progeny of nine mosaic animals lacking syntaxin product in the germline. These animals were identified on the basis of their failure to produce viable progeny. Nearly 99% (n = 740) of the progeny of these mosaic animals completed embryogenesis and arrested as first larval stage animals with a phenotype indistinguishable from the *js115* progeny of heterozygous mothers. The secreted cuticle of these animals appeared normal, containing well organized alae (Figure 4). Most of the remaining 1% of progeny arrested late in embryonic development as threefold embryos. None of the progeny of the mosaic animals we examined expressed GFP, confirming that the *unc-64(+)* array was absent from the germline. We also examined viable mosaic animals that lacked *unc-64* expression in muscle. Three mosaics that lacked GFP in muscle anterior of the vulva and 11 mosaics that lacked GFP in both dorsal body wall muscle quadrants all moved normally, suggesting that syntaxin is not required in muscle. Additionally, most of the severely uncoordinated mosaic animals we examined retained GFP expression in muscle, consistent with a neuronal site of action for syntaxin.

In separate experiments, we injected antisense RNA into the germline of wild-type animals in an attempt to inactivate the *unc-64* gene by RNA-mediated interference (Guo and Kemphues, 1995; Rocheleau *et al.*, 1997). This technique phenocopies the null phenotype of most genes that provide maternal contributions to the embryo, but is much less efficacious in phenocopying late-acting zygotic genes. Our injections failed to confer the *js115* phenotype or an aldicarb-resistant phenotype. In summary, we find no evidence for either a maternal contribution of *unc-64* or for a requirement for zygotic syntaxin in the early embryo.

Table 1. Behavioral defects *unc-64* mutants

Strain	Locomotion		Pharyngeal pumps/min	Defecation	
	Speed (μm/sec)	Efficiency[a]		Cycle time (s)	EMC[b]
N2[c]	161 ± 36	0.63	250 ± 30	42.0 ± 3.3	98%
e246	13 ± 8	0.18	209 ± 20	39.3 ± 3.3	87%
js21	51 ± 22	0.43	234 ± 10	48.8 ± 9.2	80%
js115[d]	0	NA	0.2 ± 0.6	None	NA
js116[d]	0	NA	3 ± 2	None	NA

Data shown as mean ± SD. A minimum of 18 animals were analyzed. NA, Not applicable.

[a] Efficiency is defined as displacement/total distance traveled. *unc-64* mutants have a tendency to alternate between forward and backward motion.

[b] Percentage of defecation cycles with an enteric muscle contraction (EMC).

[c] Data from Nonet *et al*. (1998).

[d] L1 larvae scored less than 4 h after hatching.

Synaptic Transmission Defects in unc-64 Mutants

To assess neurotransmission in *unc-64* mutants, we began by examining the effect of cholinergic pharmacological agents. We quantified the sensitivity of *unc-64* mutants to aldicarb, a potentiator of released acetylcholine, and to levamisole, an agonist of nicotinic acetylcholine receptors in the nematode (Lewis *et al.*, 1980a,b). All of the viable *unc-64* mutants examined were resistant to high levels of aldicarb (Figure 5A), as was the acetylcholine receptor mutant *unc-29* (Fleming *et al.*, 1997). This strongly suggests that cholinergic transmission is reduced in these mutants. However, unlike *unc-29*, the *unc-64* syntaxin mutants were sensitive to levamisole (Figure 5B), suggesting an intact postsynaptic apparatus and thus a presynaptic defect. Finally, examination of the lethal mutations revealed that aldicarb enhanced *js116* movement but failed to stimulate movement of *js115* animals, providing evidence that cholinergic synaptic transmission may be completely abolished in the null mutant.

To provide direct evidence of synaptic transmission defects in *C. elegans* syntaxin mutants, we used an extracellular recording technique developed by Raizen and Avery (1994). This method permits the detection of postsynaptic potentials in pharyngeal muscle resulting from the action of both excitatory and inhibitory motor neurons. MC is an excitatory motor neuron thought to stimulate pharyngeal muscle contraction (Raizen *et al.*, 1995). Its activation is visualized as a single depolarizing transient preceding many pumps. In *unc-64* syntaxin mutants, MC neuronal function appeared defective. Specifically, we observed a series of multiple excitatory postsynaptic potentials before initiation of pharyngeal pumps (arrows, Figure 6), which may reflect nonsynchronous transmitter release from MC. A similar phenotype has previously been observed in *aex-3*, *rab-3*, and *snb-1* mutants (Iwasaki *et al.*, 1997; Nonet *et al.*, 1997, 1998).

Our analysis concentrated on the examination of inhibitory postsynaptic potentials (IPSPs) produced by the motor neuron M3, a glutaminergic neuron that regulates the duration of pharyngeal muscle contractions (Avery, 1993; Dent *et al.*, 1997). In wild-type animals, M3 transients were regularly spaced and initiated 28 ± 5 ms after depolarization of the pharynx. In *unc-64 (e246)* animals, the first detectable IPSP often was greatly delayed (102 ± 43 ms; Figure 6). This is most easily illustrated by examining the distribution of IPSPs as a function of time after the initiation of the pharyngeal pump (Figure 7, A and B). While IPSPs occurred relatively synchronously in wild-type, they

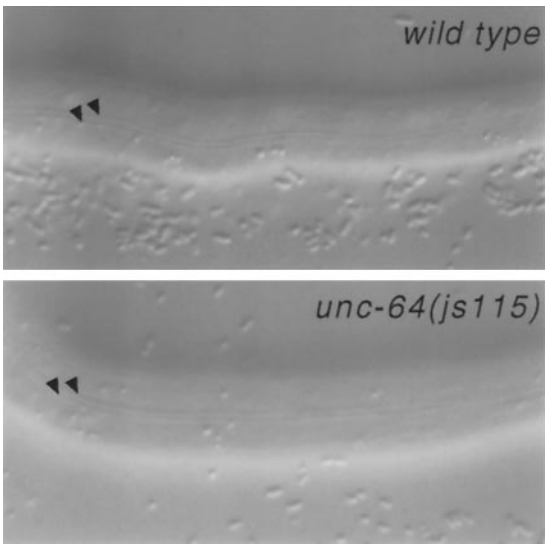


Figure 4. Cuticle of L1 *js115* syntaxin mutant animals. Lateral view of the cuticle structures (alae) of the L1 larvae of wild-type and homozygous *unc-64(js115)* animals segregating from a mosaic animal lacking *unc-64(+)* in the germline. Arrowheads point to the alae (cuticular specializations) observed in L1 larvae. Scale bar, 20 μm.

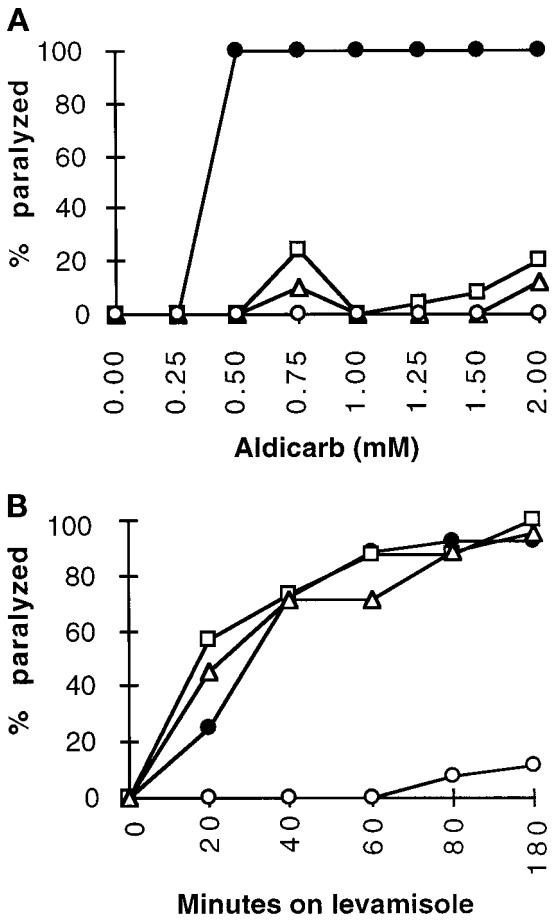


Figure 5. Pharmacological properties of *unc-64* mutants. (A) Aldicarb sensitivity of *unc-64* mutants and the wild type. Shown is the percentage of adult animals paralyzed after a 4-h exposure to various concentrations of aldicarb on plates seeded with *E. coli* wild-type (●), *js21*(□), *e246* (Δ), and *unc-29* (○). (B) Levamisole sensitivity of *unc-64* mutants and the wild type. Shown is the percentage of adult animals paralyzed after exposure to 100 μM levamisole at various times. Wild-type (●), *js21*(□), *e246* (Δ), and *unc-29* (○).

were broadly distributed in *e246* mutants. However, normalizing the time of the IPSP relative to the first IPSP within the pump reveals that, although the initial IPSP was variably delayed in the mutant, subsequent IPSPs were temporally synchronized (Figure 7, C and D). A second difference between wild-type and mutant was that the amplitude of M3 IPSPs was relatively constant in wild-type but increased substantially in *e246*. While in wild-type, the initial IPSP was slightly larger than the final IPSP, in *e246* there was a threefold increase in IPSP amplitude by the end of the pump (Figure 7, C and D). *js21* exhibited a similar but less severe defect (our unpublished data). These particular defects in M3 transmission are specific to the syntaxin helical mutants. The splicing mutants *md130* and *md1259* do not exhibit these alterations but instead resemble *aex-3(y255)*, *rab-3(y250)*, and *snb-1(js17)* in that their IPSPs were consistently decreased in amplitude yet initiated without delay after contraction of the pharynx (Figure 6). In summary, lesions in the H3 domain of syntaxin affect both the kinetics of transmitter release and the amplitude of postsynaptic potentials.

Genetic Interactions between Syntaxin and Synaptobrevin Mutants

Syntaxin interacts tightly with synaptobrevin and SNAP-25 to form a stable ternary complex. This complex is proposed to represent an intermediate in the synaptic vesicle fusion cycle (Sollner *et al.*, 1993). In each of these proteins, the domains participating in the interaction are predicted to contain α-helices capable of assembling into coiled-coil structures (Hardwick and Pelham, 1992; Fasshauer *et al.*, 1997). Lesions that lie on the hydrophobic face of the presumed coiled helix region exist in both *C. elegans* syntaxin and synaptobrevin (Nonet *et al.*, 1998). We constructed double mutants between the synaptobrevin mutants *snb-1(js17)*, *snb-1(js44)*, and *snb-1(md247)* and the two missense helical domain syntaxin mutants *js21* and *e246* (Figure 8). When analyzing the phenotypes of all six double mutants, we observed strong synergistic effects with certain allelic combinations. The size of young adult animals (Figure 9) and the rates of pharyngeal pumping (Table 2) were drastically reduced in certain double-mutant combinations. For example, when the weakest synaptobrevin mutant, *js44*, was combined with the weakest syntaxin mutant, *js21*, the result was a more severe phenotype than when *js44* was paired with the stronger *e246* syntaxin lesion. Conversely, *snb-1(js17)* interacted strongly with *e246*, but not with *js21*. Neither syntaxin allele showed interactions with *snb-1(md247)*, whose lesion is outside the helical domain and alters the transmembrane domain of synaptobrevin (Nonet *et al.*, 1998). EPG analysis provided no additional insight, as even the healthier double mutants displayed more severe EPG phenotypes than the singles and failed to exhibit any significant M3 activity (Figure 6). This was not unexpected, as this assay examining M3 activity is very sensitive and is able to detect defects in mutants that exhibit only very mild behavioral changes (Nonet *et al.*, 1997).

DISCUSSION

We have cloned and characterized the *unc-64* locus of *C. elegans* and demonstrated that it encodes a gene with high similarity to the vertebrate neuronal synaptic protein syntaxin 1. The *C. elegans* syntaxin null mutant is paralyzed and arrests development after completing embryogenesis. Although this phenotype is consistent with a complete block in neurotransmit-

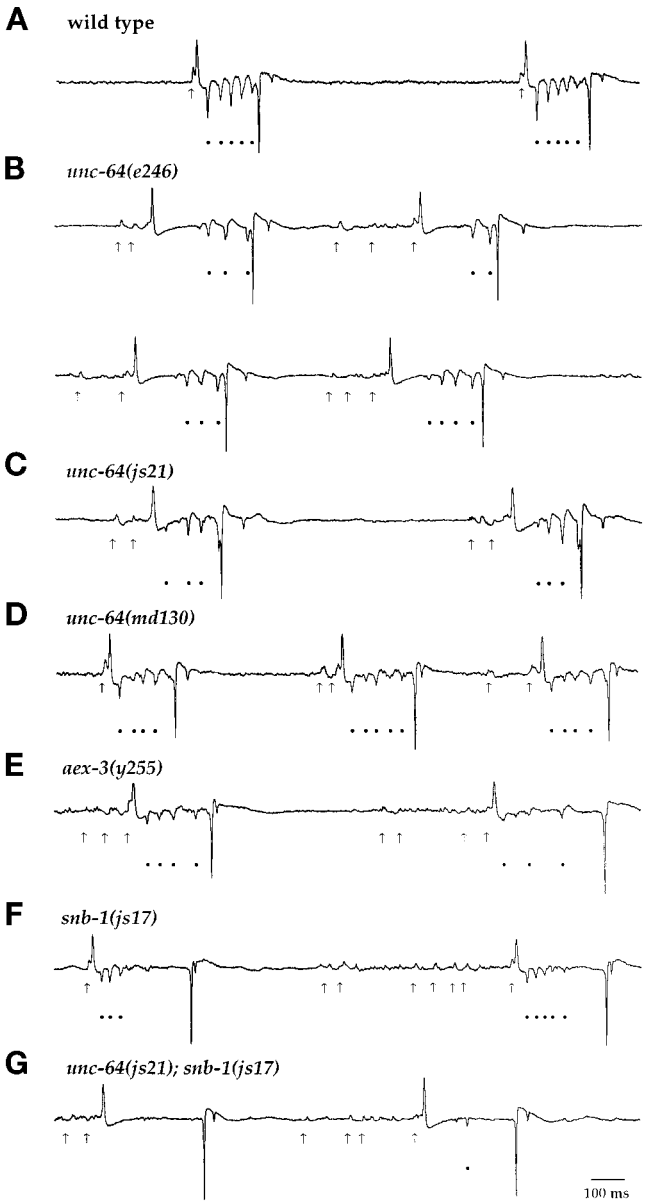


Figure 6. Pharyngeal recordings from wild-type and *unc-64* mutant animals. Shown are characteristic electrophysiological recordings from the wild-type strain N2 (A), *unc-64(e246)* (B), *unc-64(js21)* (C), *unc-64(md130)* (D), *aex-3(y255)* (E), *snb-1(js17)* (F), and *unc-64(js21); snb-1(js17)* (G). Arrows indicate MC-induced transients, and filled circles indicate M3-induced transients. All traces are millivolts versus time.

ter release, physiological techniques to document this directly are still lacking in *C. elegans*. The *unc-64* mutant phenotype is the most severe of all known synaptic mutants in *C. elegans*. Mutants in three genes encoding syntaxin-interacting proteins, namely *unc-13*, *unc-18*, and *snt-1* (synaptotagmin), all are viable although they exhibit severe behavioral defects (Maruyama and Brenner, 1991; Gengyo-Ando *et al.*, 1993; Nonet *et al.*, 1993). Although nematode synaptobrevin mutants also arrest developmentally as first larval stage animals, they remain capable of some movement (Nonet *et al.*, 1998). This is consistent with the phenotypes of syntaxin mutants in *Drosophila,* which also show the most severe synaptic transmission defect; both evoked and spontaneous transmitter release are blocked (Broadie *et al.*, 1995). *Drosophila* synaptotagmin and synaptobrevin mutants also show reduced evoked transmitter release, but do not eliminate spontaneous release (DiAntonio and Schwarz, 1994; Littleton *et al.*, 1994; Broadie *et al.*, 1995). Thus, among the molecular components of the release apparatus, absence of syntaxin is most disruptive to secretion.

unc-64 syntaxin expression is restricted to neurons and to several other tissues with secretory functions. Our expression data indicate that syntaxin is widely expressed in the nervous system, probably in all neurons, and is present in both axons as well as dendrites. In vertebrates, syntaxin is found predominantly on the plasma membrane, although its presence on synaptic vesicles has been reported recently (Bennett *et al.*, 1992; Galli *et al.*, 1995; Walch-Solimena *et al.*, 1995). *C. elegans* syntaxin does not appear to be concentrated at release sites, as detected by either antibodies or a GFP tag. This ubiquitous distribution of syntaxin on axons is inconsistent with the presence of large steady-state concentrations in *C. elegans* synaptic vesicles. Expression of syntaxin is also observed in several other tissues that have either a secretory or neuronal character, including the intestine, spermatheca, excretory gland cells, distal tip cells, and uv1 cells. The excretory gland cells are known to contain extensive populations of dense core vesicles and have been proposed to perform several functions, including osmoregulation and hormone secretion, in *C. elegans* (Nelson *et al.*, 1983; Nelson and Riddle, 1984). The uv1 cells exhibit characteristics of neurosecretory cells, as they contain neuropeptides of the FMRF-amide family (Schinkman and Li, 1992) and express other synaptic protein markers (Nonet *et al.*, 1993). The spermatheca is thought to be involved in secretion of the egg shell and expresses at least one other synaptic vesicle marker (Nonet *et al.*, 1993). Evidence also supports a secretory role for the distal tip cells. These cells extend processes around the end of the germline (Fitzgerald and Greenwald, 1995), express multiple other neuronal markers including synaptobrevin (Gao, Crittenden, and Kimble, personal communication), and are the source of signals that regulate germline function (reviewed by Schedl, 1997). Finally, yolk in *C. elegans* is synthesized in the intestine and secreted from the basolateral surface into the pseudocoelom (Kimble and Sharrock, 1983). Thus, with the lone exception of the intestinal muscle, a secretory role can be ascribed to all cells expressing syntaxin.

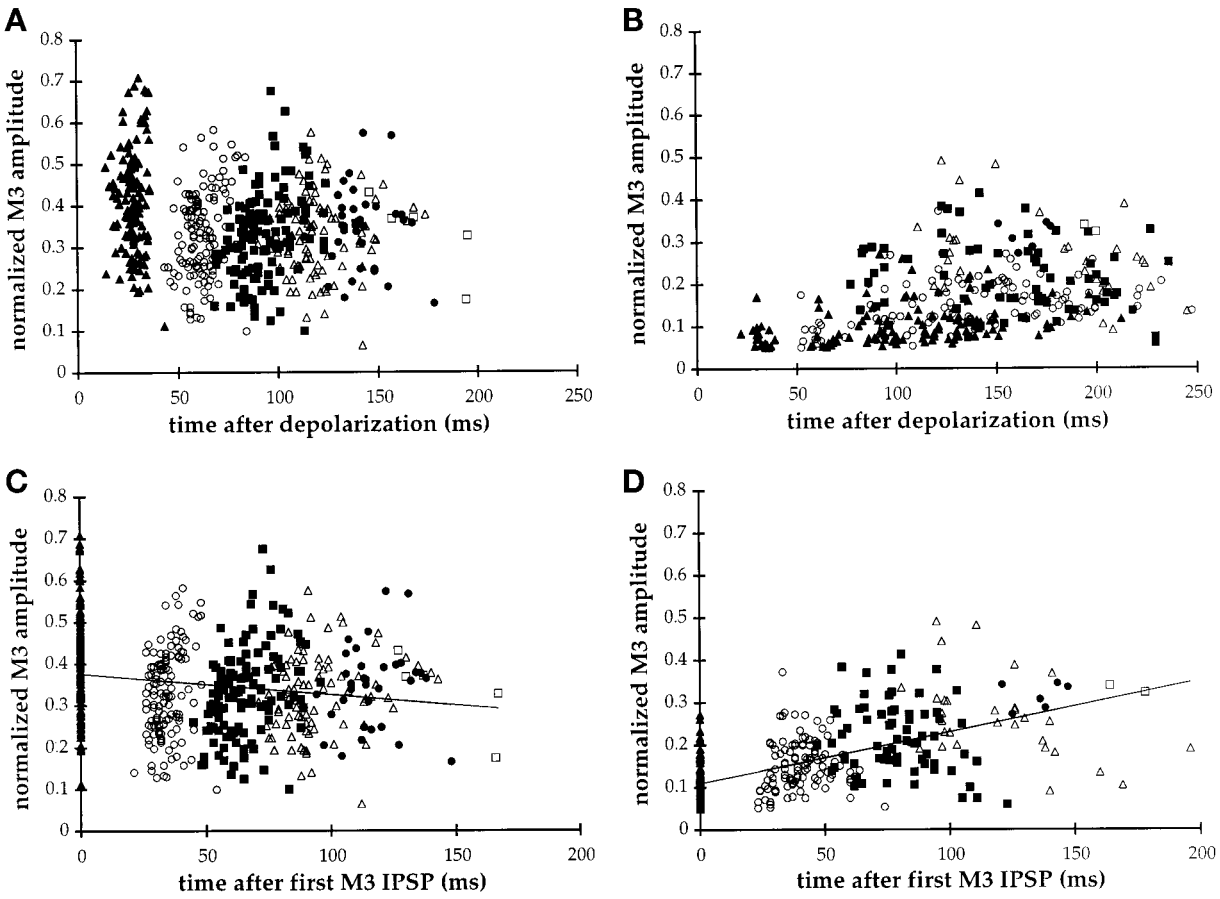
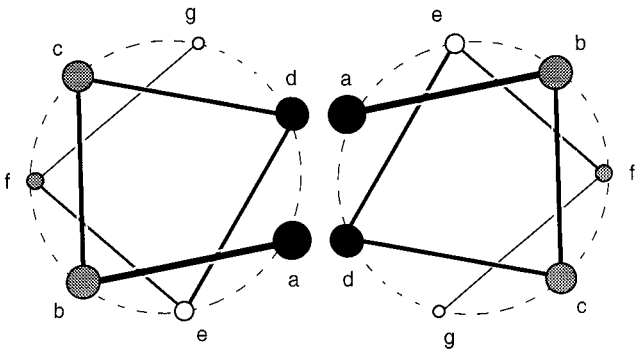


Figure 7. Analysis of M3 transmission in wild-type and *unc-64(e246)* animals. The amplitude of the first ([triof]), second (○), third (■), fourth (Δ), fifth (●), and six (□) M3-induced IPSPs within each pump (see Figure 6) is plotted as a function of time of occurrence. M3 amplitudes are normalized relative to the mean R-phase amplitude for each record. (A and B) Time of occurrence is plotted relative to initiation of pharyngeal muscle excitation. (C and D) Time of occurrence is normalized to the first M3 IPSP of the same pump. (A) Each group of M3 IPSPs cluster showing the high temporal synchrony of M3 transmitter release in wild-type (pump duration 148 ± 23 ms [mean ± S.D.], N = 130 pumps). (B) In *unc-64(e246)* temporal synchrony is lost as the timings of each group of M3 IPSPs are distributed over a larger time span. The first M3 IPSP occurs from 22 to 212 ms after muscle excitation (pump duration 209 ± 44 ms [mean ± S.D.], N = 130 pumps). (C) Aligning the IPSPs by timing of the first M3 IPSP again shows temporal clustering in wild-type. Mean IPSP spacings are [1st to 2nd] 34 ms, [2nd to 3rd] 31 ms, [3rd to 4th] 32 ms, [4th to 5th] 32 ms, and [5th to 6th] 36 ms. The downward sloping linear regression reveals that subsequent wild-type IPSP amplitudes tend to decrease slightly. (D) Similar alignment to the first M3 IPSP in *e246* reveals that subsequent IPSPs are, in fact, regularly spaced at [1st to 2nd] 41 ms, [2nd to 3rd] 42 ms, [3rd to 4th] 48 ms, [4th to 5th] 42 ms, and [5th to 6th] 43-ms mean intervals. The upward sloping regression line demonstrates that mutant IPSP amplitudes increase up to threefold with successive M3 firings.

Analysis of the defects in syntaxin mutants also suggests that *unc-64* syntaxin function is required only in neurons. For example, some cells have extensive secretory functions, yet do not require syntaxin. Most notably, secretion of first larval cuticle from the hypodermis is normal in the syntaxin null mutant. By contrast, *Drosphila* syntaxin mutants have severe cuticle defects (Schulze *et al.*, 1995). Furthermore, in *Drosophila*, syntaxin is also required during cellularization of the embryo (Burgess *et al.*, 1997). Indeed, *Drosophila* mosaic patches that lack syntaxin are not observed (Schulze and Bellen, 1996; Burgess *et al.*, 1997), suggesting that the protein is required for cell viability. In contrast, *C. elegans* animals lacking both maternal and zygotic contributions of syntaxin complete embryogenesis normally and arrest only when neuronal function is required for locomotion and feeding. Furthermore, mosaic animals lacking syntaxin in large patches of muscle show entirely normal locomotion. All of our results indicate that *C. elegans* syntaxin is required only in neuronal tissues. Why is *C. elegans* syntaxin not required in other tissues? In addition to the *unc-64* gene, at least three other divergent syntaxin homologs are found in *C. elegans*. Therefore, we propose that secretory function in different cell types has been segregated to different syntaxins. This is analogous to the situation in vertebrates in which at least 16 different syntaxins have been described with expres-



SYNTAXIN MUTANTS

```
          a  d  a  d  a  d  a  d  a  d  a  d  a  d  a  d  a  d  a  d
human 1A  IEtRHseIiKLEnSIrELHDMFMDMAMLVESQGEMiDRIEYNVEHAvdyVerAVsDTKKAVKYQSKARRKK
fly syt   IEARHqdImKLEtSIkELHDMFMDMAMLVESQGEMiDRIEYhVEHAmdyVqtAtqDTKKAlKYQSKARRKK
yeast SSO2 vqARHqellKLEktmaELtqlFnDMkeLVieQqEnvDvIdkNVEdAqqdVeqgVghTnKAVKsarKARknK
UNC-64    IEARHndImKLEsSIrELHDMFMDMAMLVESQGEMvDRIEYNVEHAkefVdrAVaDTKKAVqYQSKARRKK
                                                    ↓     ↓
                                                    V     V
                                                  js21   e246
```

TRANSMEMBRANE DOMAIN

SYNAPTOBREVIN MUTANTS

```
                                          a  d  a  d  a  d  a  d  a
human     NMTSNRRLQQTQAQVEEVVDIIRVNVDKVLERDQKLSeLDDRADALQaGASQFEssAakLkrKYWWKNcK
fly nsyb  -iaaqkRLQQTQAQVdEVVDImRtNVeKVLERDtKLSeLDDRADALQqGASQFEqqAgkLrkKfWlgNlK
yeast SNC1 -vqSksRtaelQAeiddtVgImRdNinKVaERgerLtsieDkADnLavsAqgFkrgAnrvrkamWyKdlK
SNB-1     ---SNkRLQQTQAQVdEVVgImKVNVeKVLERDQKLSqLDDRADALQeGASQFEksAatLkrKYWWKNiK
                                                ↓  ↓
                                                F  G
                                              js17 js44
```

TRANSMEMBRANE DOMAIN

Figure 8. Mutations in *snb-1* and *unc-64* on the hydrophobic faces of α-helices proposed to mediate interactions between synaptobrevin and syntaxin. Two model amphipathic α-helices composed of a repeating seven-amino acid pattern. The residues are labeled **a** through **g**. Residues in **a** and **d** positions are usually hydrophobic and are proposed to mediate interactions between binding partners. Below, a portion of the sequences of human, fly, yeast, and worm syntaxin and synaptobrevin (Archer *et al.*, 1990; DiAntonio *et al.*, 1993; Gerst *et al.*, 1992) are aligned. Hydrophobic residues in the **a** and **d** positions of the predicted α-helices are labeled. The site of lesions in *C. elegans* syntaxin and synaptobrevin (Nonet *et al.*, 1998) are also labeled. The two sequences are oriented assuming they will interact in a parallel manner (Hanson *et al.*, 1997; Lin and Scheller, 1997).

sion patterns often limited to selected tissues (Bennett *et al.*, 1993; Bock and Scheller, 1997). By contrast, *Drosophila* appears to utilize one syntaxin gene to regulate secretion in more varied cell types.

The *C. elegans* syntaxin gene expresses three distinct isoforms that differ only in sequences comprising the protein's transmembrane domain. Similar alternative isoforms of the vertebrate syntaxin 2 and syntaxin 3 genes have also been described (Bennett *et al.*, 1993; Ibaraki *et al.*, 1995). In fact, the position of four of eight splicing sites in the *unc-64* gene are precisely conserved within both the vertebrate syntaxin 1 and 3 genes (Ibaraki *et al.*, 1995; Osborne *et al.*, 1997). Paradoxically, the coding region of the more widely expressed *Drosophila* gene appears to be encoded within a single exon (although 6 different messages are transcribed from the locus [Schulze and Bellen, 1996]). Due to its assumed sole function as membrane anchor, more exotic roles of the transmembrane domain have been largely neglected. Furthermore, the vast majority of syntaxin biochemistry has been done in the absence of any transmembrane domain, because of the difficulty of working in vitro with protein containing a hydrophobic domain. However, evolutionary conservation of this diversity of membrane anchors from worm to man suggests that these different anchors are important for syntaxin function. Our data suggest that the transmembrane domains play distinct roles in the organism. First, the *js116* lesion in transmembrane domain A results in lethality despite the fact that the UNC-64C is expressed in the same cell types. Although we have not demonstrated that syntaxin A and C are coexpressed in all neurons, our data suggest that the functions of these proteins are not identical. Second, the *js116* lesion leaves 16 hydrophobic amino acids to act as a hydrophobic domain. This size domain should be sufficient to anchor a protein in the membrane (Whitley *et al.*, 1996), suggesting that syntaxin's hydrophobic domain may need to span both lipid layers of the membrane to function properly.

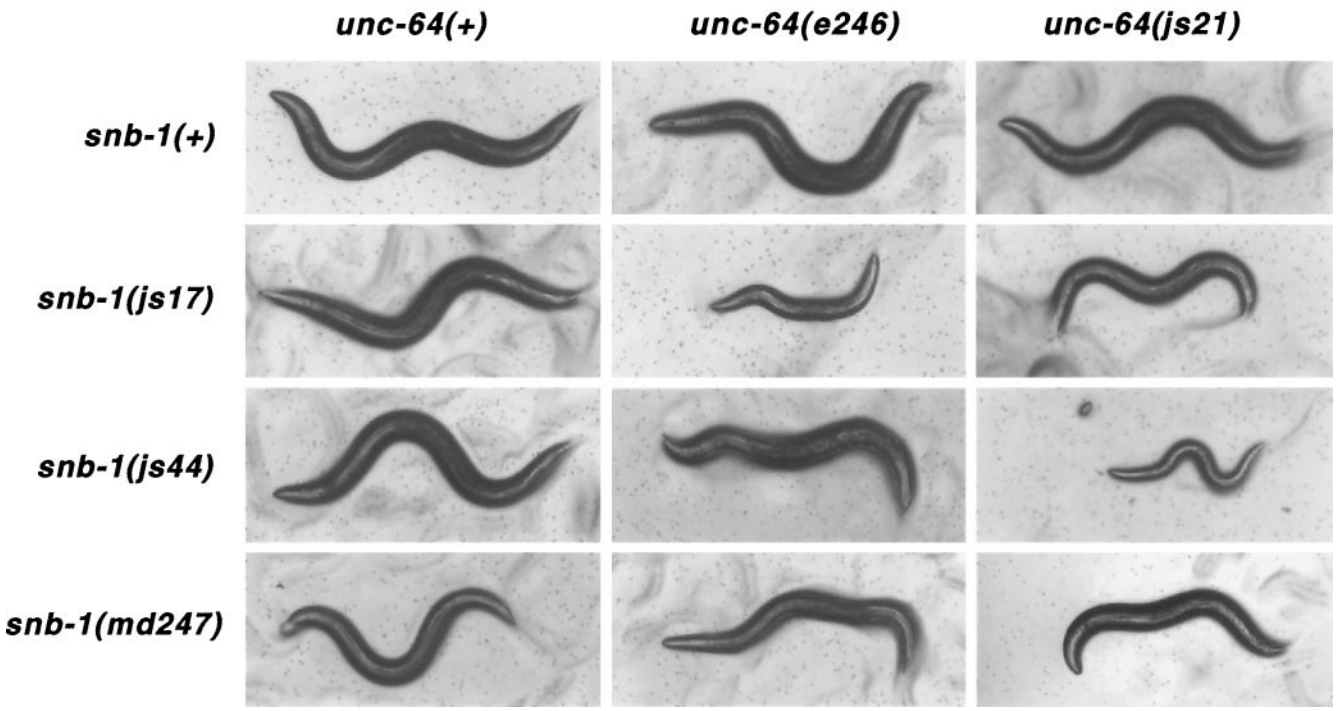


Figure 9. Phenotypes of *unc-64; snb-1* double- mutant animals. Bright field photographs of young adult animals singly and double mutant for *unc-64* syntaxin and *snb-1* synaptobrevin mutations. Animals were isolated as L4 larvae and then incubated 1 d before examination. Specific combinations of alleles cause severe behavioral, growth, and pharyngeal pumping defects.

Third, expression of syntaxin B (normally not expressed in neurons) under the control of the syntaxin promoter using a minigene construct is capable of rescuing the lethality of both *js115* (early stop codon) and *js116* mutants (our unpublished data). However, these rescued animals are not phenotypically wild-type, in contrast to animals rescued using a genomic construct (our unpublished data). This suggests that the functions of syntaxin A and B overlap significantly, but not completely. Our data indicate that the alternative forms of syntaxin do not play equivalent roles in the secretion process. Furthermore, our work suggests that the transmembrane domain is more than simply a membrane anchor for syntaxin. Isolation of lesions in the other transmembrane domains will probably be required to further dissect the role of the individual forms of syntaxin.

Table 2. Pharyngeal pumping in *unc-64 snb-1* double mutants

	unc-64(+)	*e246*	*js21*
snb-1(+)	250 ± 30	209 ± 20	234 ± 10
js17	206 ± 24	65 ± 24	127 ± 25
js44	215 ± 23	102 ± 21	27 ± 10
md247	64 ± 20	49 ± 10	73 ± 19

Pharyngeal pumping (pumps per min) of animals of genotype: *unc-64* (horizontal); *snb-1* (vertical).

Examination of the hypomorphic lesions in *C. elegans* syntaxin has provided the most direct evidence that syntaxin acts to regulate secretion of neurotransmitter in this organism. Our simple extracellular physiological assay demonstrates that M3 synaptic transmission to pharyngeal muscle is defective in syntaxin mutants. Specifically, the syntaxin mutants seem to alter the kinetics of release rather than the ability to release neurotransmitter per se. Although M3-induced transients in wild-type appear within 25–30 ms after depolarization of the pharyngeal muscle, the first transient in *e246* takes four times as long to appear. This defect cannot simply represent a slowing of the fusion process, because subsequent transients appear with a much more regular, although slightly increased, interspike interval. In wild-type animals, M3 transients are detected at a remarkably regular 30-ms cycle, whereas in *e246* this inter-M3 interval remains regular, but expands to 40 ms. The amplitudes of the M3 transients increase within a given pump. Since increasing amplitude on an EPG represents an increase in the rate of change of pharyngeal muscle membrane potential (Raizen and Avery, 1994; Avery *et al.*, 1995), this can be regarded as more efficacious signaling between M3 and the muscle. Therefore, after overcoming the initial

delay, efficacy of M3 transmission improves with each firing, but with a longer latency. This physiological defect is observed only in syntaxin mutants with lesions in the H3 domain, not in the *md130* or *md1259* splicing mutants (Figure 6 and our unpublished data). Furthermore, this defect is distinct from defects observed in *rab-3* and *aex-3* (RAB-3 nucleotide exchange protein) and certain synaptobrevin mutants. In these mutants the M3 transients appear uniformly smaller and less synchronous.

One model to explain the *unc-64(e246)* EPG defect would be that interactions between syntaxin (or more likely a complex containing syntaxin) and calcium channels is altered in the mutant. Syntaxin and N-type calcium channels are known to interact via the syntaxin H3 domain; moreover, this interaction is calcium dependent (Sheng *et al.*, 1994, 1996). Disruption of the syntaxin/calcium channel interaction alters the calcium dependence of transmitter release (Rettig *et al.*, 1997). Phosphorylation of the syntaxin- binding domain on calcium channels also inhibits the interaction with syntaxin, which could permit quick temporal regulation of release (Yokoyama *et al.*, 1997). In our mutants, transmitter release from M3 may be minimal during the first several attempted firings due to alterations in calcium sensitivity of the release machinery. Short-term modulation of the release apparatus may then account for the subsequent increase in release. While this highly speculative model is only one of numerous explanations for our recordings, it is consistent with our behavioral observations. Specifically, *unc-64(e246)* mutants are unusual in that they are differentially defective in distinct behaviors. Differences in the typical firing frequency of different synapses could account for this varied behavioral severity. Rapid firings, such as those required for normal pumping, would be less affected, while lower frequency excitations governing locomotion may be more severely impaired.

The most interesting lesions in *unc-64* are the point mutations that alter the hydrophobic face of the H3 coiled-coil domain of syntaxin. This domain is required in vitro for assembly of a ternary complex (Hayashi *et al.*, 1994; Kee *et al.*, 1995). Kee *et al*. (1995) also demonstrated that double-point mutations in hydrophobic residues (a and d; see Figure 8) of the coiled-coil domain of syntaxin led to reduced binding to synaptobrevin. One double mutant analyzed (A240V;V244A) includes the identical lesion found in the *js21* mutant. This double mutant retained the ability to bind to SNAP-25, α-SNAP, and n-sec1 (*unc-18* equivalent). However, all the point mutations in residues on the hydrophobic face that Kee *et al*. (1995) analyzed failed to block ternary complex formation. These observations are consistent with the fact that the *js21* mutant retains significant fusion activity. Furthermore, they suggest that complex formation may not serve as a sensitive assay for assessing functionality of syntaxin. In vivo, we have examined the interactions of the *js21* lesion with lesions in the proposed coiled-coil domain of synaptobrevin. The fact that combining *js21* with the weak *snb-1(js44)* mutant yields a much more severe behavioral phenotype than combining the mutant with the stronger *snb-1(js17)* mutant provides a genetic argument for functional interaction of these proteins. *unc-64(e246)* exhibits a reciprocal set of interactions with *snb-1* alleles and neither *unc-64* lesion significantly interacts with the *snb-1(md247)* lesion that maps outside the H3 domain. In a simplified coiled-coil model placing the two proteins in a parallel orientation (Hanson *et al.*, 1997; Lin and Scheller, 1997), the allelic combinations resulting in the most severe phenotypes are those in which lesions in each of the molecules are at positions in the coiled coil more distant from one another (Figure 8). These combinations of lesions may disrupt formation of interactions in a more extended region of the coiled-coil leading to synergy. It is hoped that an atomic level structure of the ternary complex will provide a more definitive molecular hypothesis regarding the perturbation of synaptic transmission in the coiled-coil lesions in both *snb-1* and *unc-64.* Regardless of the molecular defects, our mutants in both synaptobrevin and syntaxin provide strong in vivo evidence for the importance of the helical domains in synaptic transmission. Furthermore, the isolation of extragenic suppressors of these mutants may identify proteins that normally inhibit syntaxin function. Greater than 10 suppressors of *unc-64* lesions defining at least 4 different complementation groups have already been identified (Saifee and Nonet, unpublished results). These genetic approaches provide an alternative means of isolating novel molecules that regulate syntaxin function at the synapse.

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