Genetic Screens for Synaptic Function Mutants in the Nematode *C. elegans*

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The synapse is the site of a remarkable conversion of electrical signals into chemical signals. This conversion takes place when a depolarizing impulse enters the axon terminal and opens voltage-sensitive calcium channels; calcium then flows into the cell and initiates the fusion of synaptic vesicles with the plasma membrane. The precise mechanisms of synaptic vesicle dynamics remains a mystery. We would like to understand the specialized molecular machinery that is required to dock and prepare vesicles for release, fuse the vesicular and plasma membranes, and finally to recycle vesicular components to regenerate mature synaptic vesicles.

We are identifying proteins involved in neurotransmission using a genetic strategy to analyze the nematode *C. elegans*. There are aspects of this nematode that make it well-suited for studies of the nervous system. First, the nervous system in *C. elegans* is largely nonessential under laboratory conditions. Although the two sexes can mate to produce hybrid progeny, the hermaphrodite can fertilize her eggs internally in the absence of males (Wood, 1988). For this reason coordinated locomotion is not required for mating. Second, since the nematode is maintained on a lawn of bacteria, it does not need to search for its food. Ingestion of bacteria is mediated by the pharynx. Because contractions of the pharyngeal muscle can be driven by myogenic action potentials, food intake can occur even with a severely defective nervous system (Avery & Horvitz, 1989). For these reasons, mutants with severe defects in neurotransmission can be propagated and studied. Nevertheless, some nervous function is required for survival since the absence of acetylcholine or the death of certain neurons will cause the animal to die (Avery & Horvitz, 1989; Rand, 1989).

A second advantage to using *C. elegans* for these studies arises from our detailed knowledge of the nervous system. The number of neurons is invariant among individuals: there are 302 neurons with fixed positions in the adult animal (Sulston & Horvitz, 1977). In addition, the connectivity of the nervous system has been reconstructed from serial electron micrographs (White, Southgate, Thomson & Brenner, 1986). Because *C. elegans* is transparent, identified neurons can be killed with a laser microbeam and changes in behavior analyzed in a living animal (Chalfie, Sulston, White, Southgate, Thomson & Brenner, 1985; Avery & Horvitz, 1989). These behavioral abnormalities in conjunction with the known connectivity of the nervous system can be used to deduce the function of a particular neuron in the context of a simple circuit. Finally, the spectrum of neurotransmitters used by *C. elegans* is essentially the same as in other animals, and many of the pharmaceuticals that have been characterized in vertebrates act similarly in nematodes (Lewis, Wu, Levine & Berg, 1980; Horvitz, Chalfie, Trent, Sulston & Evans, 1982; Avery & Horvitz, 1990; McIntire, Jorgensen, Kaplam & Horvitz, 1993b). These drugs can then be used to characterize existing mutants or to iso-

late new mutants in genetic screens.

We have pursued two classes of genes required for neurotransmission: genes required for the functioning of a single neurotransmitter, specifically, the neurotransmitter GABA, and genes required for the functioning of all neurotransmitters. The critical step in obtaining mutations in these processes is the design of an appropriate genetic screen. We discuss three screens designed to obtain mutants in these processes. We then describe the phenotype of animals with mutations in the synaptic vesicle protein synaptotagmin and propose that this protein functions in the regeneration of synaptic vesicles.

GENES REQUIRED FOR GABA FUNCTION

Any given neuromuscular junction will require two kinds of proteins: those that mediate synaptic functions common to all synapses, such as the proteins required for synaptic vesicle exo- and endocytosis, and the proteins required for the specific neurotransmitter used by that synapse. These neurotransmitter-specific proteins would include proteins required for the selection of a proper synaptic partner, the biosynthetic enzymes for the neurotransmitter, the transporters that pump the neurotransmitter through the plasma and vesicular membranes, and the receptors and receptor-assembly proteins in the post synaptic cell.

We are identifying the proteins required for the functioning of the neurotransmitter gamma aminobutyric acid (GABA). GABA is the main inhibitory neurotransmitter in both vertebrates and invertebrates. We have undertaken a cellular, genetic, and molecular analysis of GABA neurotransmission in *C. elegans*. We have two goals: first, we would like to know how the GABAergic neurons function in the context of a known circuit; and second we would like to use the knowledge gained about the circuit to identify the molecules that are required specifically for GABA transmission. Our strategy consists of the following steps. First, we identified the GABAergic neurons in the nematode using immunohistochemistry. Second, we killed these cells to characterize their roles in the control of *C. elegans* behavior. Third, we are identifying genes that function in GABA transmission by looking for mutants that behave similarly to the operated animals.

Staining worms with an anti-GABA antibody demonstrated that of the 302 neurons in *C. elegans*, 26 express the neurotransmitter GABA (Fig. 1, McIntire, Jorgensen & Horvitz, 1993a). We killed these cells using a laser microbeam and identified functions for 25 of these 26 neurons (McIntire *et al.* 1993a). GABA functions can be classified as either inhibitory or excitatory based on the effect of GABA on muscle contractions.

Inhibitory GABA Function: The DD and VD GABAergic motor neurons provide contralateral inhibition to antagonistic muscle groups, that is, they insure that if one side of the body is contracted, the other side is relaxed so that the animal can bend in a coordinated fashion while swimming. Lack of these cells causes animals to hypercontract or "shrink" when they are touched (Fig. 2). The RME motor neurons synapse to the anterior body muscles and are required to dampen the oscillations of the head and nose as the animal forages.

Excitatory GABA Function: The AVL and DVB neurons innervate the enteric muscles that compress the intestine and open the anus. Animals lacking the AVL and DVB neurons lack contractions of the enteric muscles. GABA is usually an inhibitory neurotransmitter that acts by opening anion channels. However, GABA appears to drive contractions in these muscles by an alternative mechanism indicating a novel excitatory action of GABA. This conclusion is supported by the connectivity of these neurons and by our pharmacological and genetic data. First, the AVL and DVB neurons directly innervate the enteric muscles and appear to be the only motor neurons forming neuromuscular junctions with these muscles. Second, GABA agonists can induce contractions of the enteric muscles in the absence of innervation from AVL and DVB, but the GABA drugs that

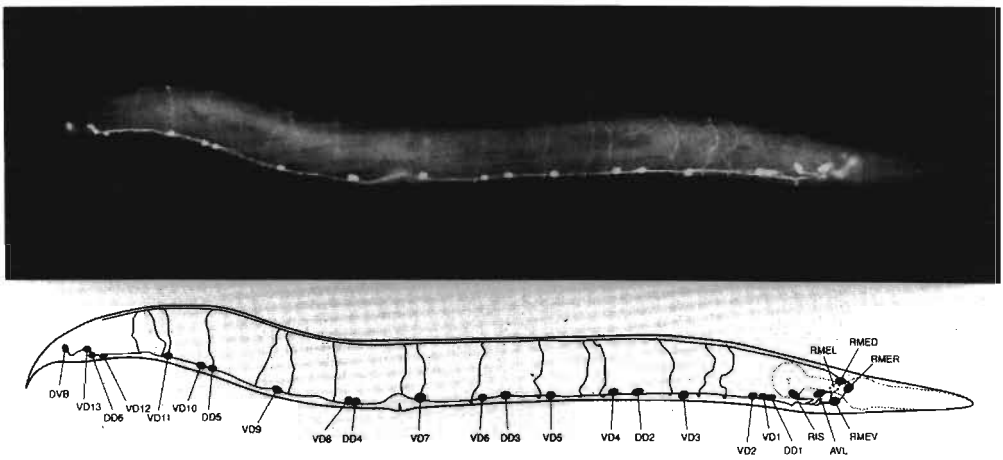

Fig. 1. GABA immunoreactivity in *C. elegans*
Fluorescent photomicrograph and a tracing of a wild-type adult hermaphrodite stained with an antiserum raised against GABA. The 26 neurons stained with the anti-GABA antiserum are indicated (McIntire *et al.* 1993). Anterior is to the right, and dorsal is up. The pharynx is indicated by the dotted line.

affect muscle contraction are different from the ones causing the body muscles to relax. Third, gene products required for muscle relaxation (*unc*-49) and muscle contraction (*exp*-1) are different (see below). These gene products are required postsynaptically and presumably encode two different GABA receptors.

By screening for mutants that display the shrinking defect (Fig. 2; McIntire *et al.* 1993b) or the expulsion defect (Jorgensen & Horvitz, unpublished), we identified six genes required specifically for either the inhibitory or the excitatory functions of GABA as well as genes required for all GABA functions (Table 1).

One of these genes, *unc-25*, encodes the biosynthetic enzyme for GABA, glutamic acid decarboxylase (Y. Jin & H.R. Horvitz, unpublished data). *unc-30* encodes a homeodomain protein that is required for normal connectivity of and GABA expression in the DD and VD GABAergic neurons (Jin, Hoskins & Horvitz, 1994). *unc-49* is required postsynaptically and encodes a $GABA_A$-like receptor (B. Bamber & E. Jorgensen, unpublished data). *unc-46* and *unc-47* are required for all GABA functions and appear to encode presynaptic proteins. *exp-1* is required only for the postsynaptic excitatory GABA function (Jorgensen & Horvitz, unpublished results) and may therefore encode a novel receptor.

Table 1. Genes required for GABA function

gene	inhib. fx	excit. fx
unc-25	+	+
unc-30	+	−
unc-46	+	+
unc-47	+	+
unc-49	+	−
exp-1	−	+

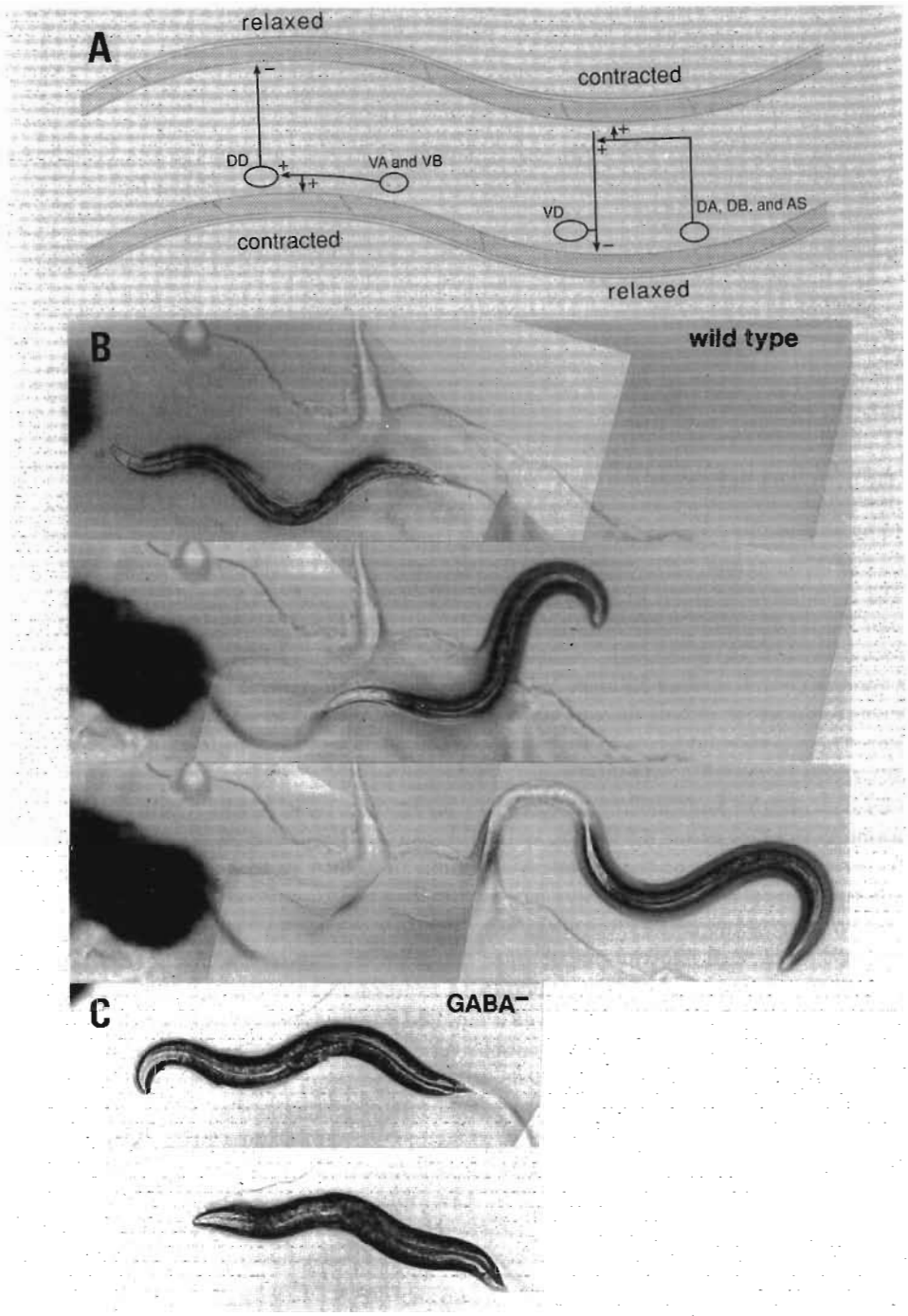

Fig. 2. Loss of GABAergic function causes hypercontraction of the body muscles
A, A model for DD and VD neuronal function (McIntire *et al.* 1993). The excitatory motor neurons (VA, VB, DA, and AS) excite the body muscles (shaded) and activate the GABAergic DD or VD neurons, which cause relaxation of the dorsal and ventral muscles, respectively. Locomotory behavior (B) in a wild-type animal and (C) in an *unc-25* (*e156*) mutant, which lacks GABA expres-

GENES REQUIRED FOR THE FUNCTIONING OF ALL SYNAPSES

All neuromuscular junctions must possess the machinery that converts an electrical signal into the secretion of neurotransmitters contained in synaptic vesicles. The components required for synaptic vesicle dynamics might be shared by multiple transmitters include cytoskeletal proteins that maintain the reserve pool of vesicles, voltage-sensitive calcium channels, vesicle docking and fusion proteins, and finally proteins required for the recycling and regeneration of vesicles. We used a genetic strategy to identify mutants that lacked normal synaptic function. These mutants will be used to characterize the genes and gene products required for synaptic function. We used two screens to identify synaptic function mutants: a screen for mutants that behaved as if they lacked acetylcholine and GABA neurotransmission and a selection for mutants that failed to release neurotransmitter.

1. Behavioral screen

We used our knowledge about neurotransmitters in *C. elegans* to identify genes required for the functioning of all synapses. The *cha-1* gene encodes the biosynthetic enzyme choline acetyltransferase, and *cha-1* mutants lack acetylcholine (Rand & Russell, 1984). Such animals display a distinctive locomotory defect: they have jerky movements and often coil when moving backward. *unc-25* mutants, which lack GABA, as described above, shrink and lack contractions of the enteric muscles. Double mutants have an additive phenotype. Animals lacking normal synaptic transmission because of defects in neurotransmitter release would be expected to display all of these defective behaviors. By screening mutagenized animals for this combined mutant phenotype, we identified 14 mutations that defined 11 complementation groups (Table 2). To confirm that there was a decrease in released acetylcholine in these mutants, we demonstrated that they were resistant to an inhibitor of acetylcholinesterase (see below). Alleles of many of the genes identified in this screen had been

Table 2. Genes required for synaptic function identified in the behavioral screen

gene	alleles
snt-1	*n2665*
unc-41	*n2163, n2913*
unc-26	*n702, n1307*
ric-1	*n1337*
ric-7	*n2657*
unc-11	*n2954*
unc-13	*n2813*
unc-64	*n2917*
unc-1	*n2887* dm, *n2918* dm
unc-68	*n2226* sd
ric-6	*n2915* sd

snt, synaptotagmin; *unc*, uncoordinated; *ric*, resistant to inhibitors of cholinesterase.

sion (McIntire *et al.* 1993). The head of each animal was touched with a platinum wire. Touch to the head of the wild-type animal resulted in backward movement characterized by deep sinusoidal waves. Touch to the head of the *unc-25* mutant caused the ventral and dorsal muscles to contract simultaneously, resulting in no backward movement and a shrinkage of body length.

identified previously by Sydney Brenner (1974), who isolated many mutants that could not move normally and named these genes after this uncoordinated phenotype, Unc. Two of the genes identified in our screen, *ric-6* and *ric-7*, represent previously unidentified loci, demonstrating that screens for such loci are not yet saturated. Of the genes we identified, only *unc-13* and *snt-1* have been characterized at a molecular level. *unc-13* encodes a large protein with a phorbol ester-binding domain (C1) and a calcium-binding domain (C2) (Maruyama & Brenner, 1991). Although the domain structure of the UNC-13 protein is provocative, the precise role of this protein in synaptic function is still unknown. *snt-1* encodes the synaptic vesicle protein synaptotagmin (Nonet, Grundahl, Meyer & Rand, 1993). We have used our synaptotagmin mutant to characterize the function of this protein in the synapse (see below).

2. A screen for lethal mutations that disrupt synaptic transmission

The behavioral screen for transmission mutants described above has two disadvantages. First, it is laborious: behavioral screens require that each mutagenized animal be observed before it can be selected as a candidate for further characterization. Second, the screen will miss genes that are mutated to lethality, since the screen requires homozygous mutants to survive until adulthood.

Mutations in genes that are required for neurotransmission may be lethal for two reasons: (1) Mutants lacking all nervous system function are lethal. Mutants lacking acetylcholine (*cha-1* mutants synthesize no acetylcholine) die as coiled L1 larvae (Rand & Russell, 1984). Also, animals in which the M4 pharyngeal motor neuron or the CAN neurons have been killed also die (Avery & Horvitz, 1989; Manser & Wood, 1990). (2) Some of the components of the synaptic vesicle fusion complex may also be components of the exocytotic machinery of all cells (Bennett & Scheller, 1993); eliminating such gene products would generate an inviable animal because of defective secretion in non-neuronal cells.

We have devised a selection that circumvents these drawbacks. First, by using a pesticide that kills wild-type animals we performed a *selection* for neurotransmission mutants instead of a *screen* for behavioral defects. Acetylcholine transmission is terminated when the neurotransmitter is cleaved by the enzyme acetylcholinesterase. Inhibitors of acetylcholinesterase, such as the pesticide aldicarb, cause toxic levels of acetylcholine to build up in the synaptic cleft of nematodes (Rand & Russell, 1985). In the presence of the drug, the body muscles hypercontract, the pharynx is paralyzed and the animal eventually dies. Mutations in some genes encoding synaptic components reduce the release of neurotransmitter into the synaptic cleft and thereby confer resistance to these drugs (Brenner, 1974; Alfonso, Grundahl, Duerr, Han & Rand, 1993; Gengyo-Ando, Kamiya, Yamakawa, Kodaira, Nishiwaki, Miwa, Hori & Hosono, 1993; Nonet *et al.* 1993). For example, mutants that fail to release acetylcholine because they fail to synthesize the neurotransmitter (*cha-1*) or fail to package the neurotransmitter into vesicles (*unc-17*) are resistant to aldicarb (Alfonso *et al.* 1993; Alfonso, Grundahl, McMaus & Rand, 1994).

Second, by performing the selection in a sensitized background we can isolate homozygous lethal mutations in heterozygous viable animals. Specifically, when the mutation *unc-13(n2813)* is homozygous (genotype: *unc-13/unc-13*) the strain is strongly resistant to aldicarb (Fig. 3). When this mutation is heterozygous (genotype: *unc-13/+*) the strain is sensitive to the drug. This genotype represents our sensitized background in which heterozygous mutations at a second locus can be isolated. For example, mutations in a single copy of the gene encoding synaptotagmin (genotype: *snt-1/+*; *unc-13/+*) make our sensitized strain aldicarb-resistant. This synthetic drug resistance was observed in double mutants with several genes known to be involved in neurotransmission (*snt-1*, *unc-41*, and *unc-32*). Thus, in the screen we simply looked for new aldicarb resistant mutants in this sensitized background (Fig. 3). Based on the results of the reconstruction experiments described above, we should be able to isolate more alleles of homozygous viable mutations like *snt-1* and *unc-41*. In addition, because these newly-induced mutations can be complement-

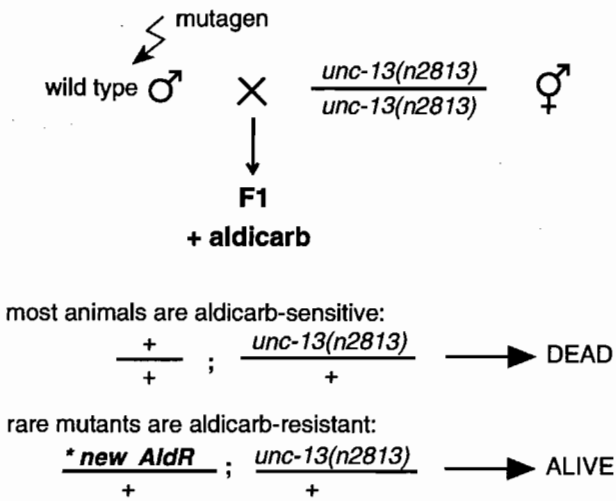

Fig. 3. Selection for mutations that enhance aldicarb resistance in an *unc-13*/+ sensitized background (see text)

ed by the wild-type chromosome, we hoped to recover lethal mutations that enhance drug resistance.

We screened 2,300 F1 animals (2,300 genome equivalents) and isolated 123 synthetic aldicarb-resistant strains. Given that known uncoordinated mutants exhibit drug resistance, we expected to isolate viable uncoordinated strains. As expected we isolated many such strains (about half of our strains, Table 3). Most of these strains were either coilers or inactive animals with a weak jerky backing phenotype—precisely the behavioral phenotypes observed in existing aldicarb-resistant strains. Although the genetic characterization of these mutants has only just begun, we recovered mutations in *unc-26* and in *unc-2*, genes known to mutate to aldicarb-resistance (E. Jorgensen, unpublished data). These results demonstrated that the screen successfully isolated new aldicarb-resistant mutations.

Some resistant mutations after separation from the *unc-13* mutation led to a behaviorally wild-type phenotype. It is known that mutants in at least one protein associated with synaptic vesicles, rab3a, are aldicarb-resistant but have no known behavioral abnormality (M. Nonet, personal communication). Therefore, such mutations were also expected.

Table 3. Mutations isolated in synthetic aldicarb-resistance selection

phenotype	#strains	
Total lethal mutations	62	
embryonic lethal		18
L1 arrest		9
larval arrest		16
sterile uncoordinated adults		19
Total viable (*unc-13*-indep. ald. resis.)	61	
uncoordinated		48
wild-type		13
TOTAL	123	

Of our aldicarb-resistant mutants, 62 segregated either inviable or sterile adults. Although these strains have not been outcrossed, the lethal phenotype segregates with that of the aldicarb-resistance. Thus, we expect that the lethal mutation confers aldicarb-resistance to these strains and that it is not a second unrelated mutation in the background of the strain. These lethal mutations could not have been isolated in screens for homozygous mutants and are precisely the kind of mutations we had hoped to obtain. Future characterization of these strains may demonstrate that these gene products are components of the secretion machinery in neuronal and non-neuronal cells.

SYNAPTOTAGMIN

One of the eleven genes identified in our behavioral screen, *snt-1*, was shown by others to encode synaptotagmin (Nonet *et al.* 1993). Synaptotagmin is an integral membrane protein of the synaptic vesicle (Matthew, Tsavaler & Reichardt, 1981; Perin, Fried, Mignery, Jahn & Südhof, 1990) and interacts with proteins of the plasma membrane (Bennett, Calakos, Kreiner & Scheller, 1992; Leveque, Hoshino, David, Shoji-Kasai, Leys & Omori, 1992; Hata, Davletov, Petrenko, Jahn & Südhof, 1993). Because it binds calcium (Brose, Petrenko, Südhof & Jahn, 1992), synaptotagmin has been proposed to be the calcium sensor that regulates exocytosis at the synaptic terminal. Two divergent models have been suggested regarding synaptotagmin function: synaptotagmin could either facilitate or inhibit synaptic vesicle release (Popov & Poo, 1993). Both of these models implicate a role for synaptotagmin in exocytosis. By contrast, our results suggest that synaptotagmin is required in the recycling of vesicles. We propose that synaptotagmin is a multifunctional protein that acts in both exocytosis and the regeneration of synaptic vesicles.

We inferred a role for synaptotagmin in the retrieval of vesicular components from an ultrastructural characterization of the mutant synapse. First, we examined electron micrographs of *snt-1* mutants and found that the density of synaptic vesicles at the neuromuscular junctions in these mutants is severely depleted (Fig. 4). Second, we demonstrated that the vesicles were generated normally at the cell body in the mutant and that vesicle components were transported to the neuromuscular junction. Specifically, we examined a double mutant containing defects in *snt-1* and *unc-104*, a gene encoding a neural-specific kinesin. In the double mutant, vesicles were observed in the cell body, since in the absence of kinesin the vesicles accumulated in the axon hillock. Furthermore, fluorescently-labelled synaptobrevin, a vesicular component, was transported to neuromuscular junctions in the mutant as in the wild type (K. Schuske & E. Jorgensen, unpublished data). Because a severe depletion of vesicles was observed by electron microscopy it is likely that the synaptic vesicle components have been displaced into the plasma membrane. Third, pharmacological experiments indicated that there is a decrease in neurotransmitter release. Specifically, synaptotagmin mutants were resistant to inhibitors of cholinesterase, suggesting that there is a decrease in released neurotransmitter. This drug resistance argues against a model in which the depletion of vesicles is caused solely by constitutive secretion, because unregulated release of neurotransmitter presumably would lead to a hypersensitivity to inhibitors of cholinesterase. Taken together, these results indicate that the defect seen in *snt-1* mutants is caused primarily by a defect in the endocytotic retrieval of vesicles. Recently, it has been shown that synaptotagmin is the clathrin-AP2 receptor in the plasma membrane of rat neurons (Zhang, Davletov, Südhof & Anderson, 1994), providing further evidence that synaptotagmin plays a role in endocytosis and vesicular recycling. This conclusion does not preclude synaptotagmin from playing a role in exocytosis—only that the primary defect seen in the *C. elegans snt-1* mutant is at the step of vesicular recycling. We suggest that synaptotagmin plays a role in the regulation of exocytosis, as demonstrated by others, and that this protein also acts to identify components of synaptic vesicles in the plasma or endosomal membrane and mark them for retrieval and for the regeneration of mature vesicles.

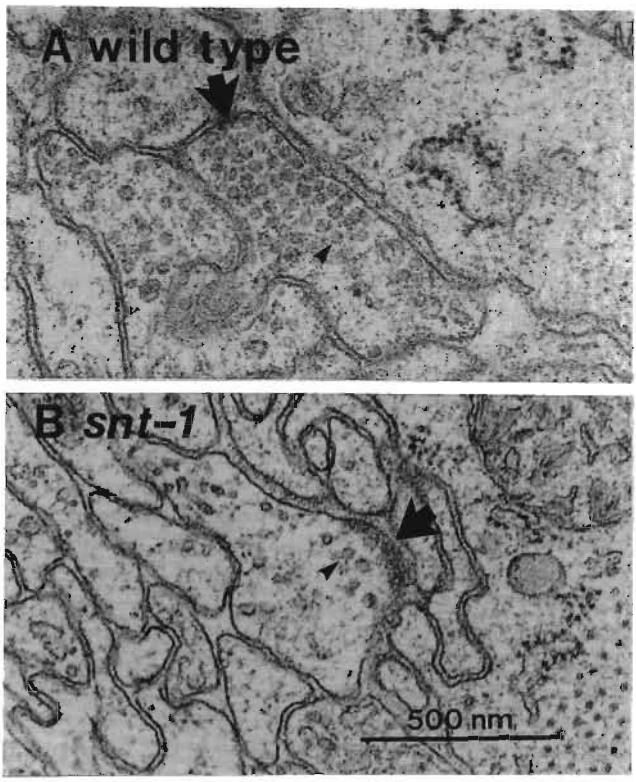

Fig. 4. Synaptic vesicles are depleted at neuromuscular junctions in *snt-1* mutants
A, Electron micrograph of a wild-type neuromuscular junction. Large arrow, dark thickening of an active zone. Arrowhead, one synaptic vesicle.
B, Electron micrograph of a *snt-1*(*md290*) neuromuscular junction.

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