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A retrograde signal is involved in activity-dependent remodeling at a *C. elegans* neuromuscular junction

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SUMMARY

We have characterized how perturbations of normal synaptic activity influence the morphology of cholinergic SAB motor neurons that innervate head muscle in *C. elegans*. Mutations disrupting components of the presynaptic release apparatus, acetylcholine (ACh) synthesis or ACh loading into synaptic vesicles each induced sprouting of SAB axonal processes. These sprouts usually arose in the middle of the normal innervation zone and terminated with a single presynaptic varicosity. Sprouting SAB neurons with a similar morphology were also observed upon reducing activity in muscle, either by using mutants lacking a functional nicotinic ACh receptor subunit or through muscle-specific expression of a gain-of-function potassium channel. Analysis of temperature-sensitive mutants in the choline acetyltransferase gene revealed that the sprouting response to inactivity was developmentally regulated; reduction of synaptic activity in early larval stages, but not in late larval stages, induced both sprouting and addition of varicosities. Our results indicate that activity levels regulate the structure of certain synaptic connections between nerve and muscle in *C. elegans*. One component of this regulatory machinery is a retrograde signal from the postsynaptic cell that mediates the formation of synaptic connections.

Key words: Synaptogenesis, Activity, Synaptic plasticity, Neuromuscular junction, Retrograde signal, *C. elegans*

INTRODUCTION

Patterns of neural activity play essential roles in establishing and maintaining precise patterns of neuronal connectivity in many systems (Katz and Shatz, 1996). For example, in the vertebrate brain, activity regulates the segregation of visual inputs into alternating columns in the vertebrate visual cortex (Hubel and Wiesel, 1970; Stryker and Harris, 1986) and is required for the refinement of retinotectal connections after the initial formation of a coarse topographic map (Cline and Constantine-Paton, 1990). At vertebrate neuromuscular junctions, synaptic activity is required for synaptic elimination during the transition from polyinnervated muscle fibers in the neonate to singly innervated muscle fibers in the adult (Nguyen and Lichtman, 1996). Activity-dependent processes are also observed in invertebrate systems. In *Drosophila melanogaster*, motor ending sprouting can be induced by denervation of muscle similar to that observed in vertebrates (Keshishian et al., 1993). Furthermore, when neural activity is reduced either genetically or pharmacologically during late embryogenesis and early larval development, motor neurons form ectopic synaptic connections, suggesting neural activity functions to maintain precise connectivity during normal development (Jarecki and Keshishian, 1995). In addition, increases in axonal branches and synaptic varicosities are seen in mutant combinations with increased nerve excitability (Budnik et al., 1990).

Although the underlying mechanisms of these activity-dependent remodeling events are largely unknown, genetic analysis is beginning to provide some insights into the process. The cAMP cascade may mediate the activity-dependent regulation of synaptic connectivity in *Drosophila*, since synaptic mutants affecting cAMP-specific phosphodiesterase and calmodulin (CaM)-dependent adenylyl cyclase display similar abnormal connections seen in mutants with increased nerve excitability (Zhong et al., 1992). In addition, a downregulation of Fasciculin II (Fas II) in these mutants indicates an involvement of Fas II in the process (Schuster et al., 1996a). It is supported by the observation that in Fas II mutants, sprouting increases or decreases depending on the level of Fas II (Schuster et al., 1996b). In mammals, a number of different signaling molecules, which are upregulated by denervation, are possible candidates for activity-dependent regulation of structural plasticity. For example, insulin-like growth factors 1 and 2 are capable of inducing collateral sprouting both in vitro and in vivo (Caroni, 1993). Overexpression of growth-associated protein GAP-43 in neurons induces nerve sprouting both at neuromuscular junctions and in the termini of hippocampal mossy fibers (Aigner et al., 1995). Other molecules including CGRP and NCAM may also be involved (Covault and Sanes, 1985; Sala et al., 1995).

Retrograde signals from the postsynaptic cell appear to participate in regulating changes in presynaptic structure and

function (Davis et al., 1998; Harish and Poo, 1992; Holland and Brown, 1980; Nguyen and Lichtman, 1996). In mouse, blocking the postsynaptic receptor activity in muscle by injecting irreversible acetylcholine receptor antagonist can invoke terminal sprouting of motor neurons (Holland and Brown, 1980). Retrograde signals are also implicated in local withdrawal of nerve terminals during the synaptic elimination process (Balice-Gordon and Lichtman, 1994). At the *Drosophila* neuromuscular junction, a decrease in postsynaptic receptors leads to an increase in presynaptic transmitter release, probably through a phosphorylation process mediated by protein kinase A (Davis et al., 1998; Petersen et al., 1997).

Although retrograde signals appear to regulate both presynaptic structural and functional plasticity, the molecular and cellular nature of such signals has remained elusive. Characterizing such signals in a genetically tractable system would permit the use of molecular genetic approaches. *C. elegans* is one such system, which allows easy combination of genetic, molecular and cellular techniques. Along with *Drosophila*, studies in the nematode have provided insights into a variety of processes underlying neuronal development and function, including axonal outgrowth, chemo- and mechanosensation, and synaptic transmission (Antebi et al., 1997; Bargmann and Mori, 1997; Davis and Goodman, 1998; Driscoll and Kaplan, 1997; Keshishian et al., 1996; Rand and Nonet, 1997b). The nematode in particular, possesses a remarkably simple nervous system consisting of only 302 neurons that form approximately 7000 synapses. The connectivity of the vast majority of these neurons has been determined from the analysis of serial electron micrographs (White et al., 1986). The synaptic outputs of neurons in *C. elegans* are very stereotypic. For example, both the number and type of synaptic connections formed by an individual neuron are very similar to contacts made by its bilateral partner within an individual, and very similar to the contacts formed in other individuals (Hall and Russell, 1991; White et al., 1986). Hence, the pattern of synaptic contacts formed during both embryogenesis and early larval development seems to be inscribed in the developmental program of the nematode. Indeed, even some rewiring is developmentally programmed. For example, during the first stage of larval development, DD motor neurons re-specify their synaptic connections (White et al., 1978). These changes are regulated by the developmental timing gene *lin-14*, but do not appear to be regulated by activity (Hallam and Jin, 1998; Jin et al., 1999).

Recent studies of *C. elegans* suggest that neural activity plays an important role in the maintenance of sensory axon morphology after an initial pattern of innervation is established. Sensory inputs are required for maintaining normal axon morphology (Peckol et al., 1999). Furthermore, mutations that alter the excitability of sensory neurons lead to axonal branching or the failure of axons to terminate at normal positions (Coburn et al., 1998; Peckol et al., 1999). This requirement for normal sensory inputs and sensory transduction continues even in the adult (Coburn et al., 1998; Peckol et al., 1999). In addition, communication between motor neurons and muscles has been suggested in several cases in *C. elegans*. For example, muscles can target to ectopic motor axons, suggesting that attractive signal(s) are released by motor terminals (Hall and Hedgecock, 1991; Hedgecock et al., 1990). Moreover, retrograde signaling also appears to exist in *C. elegans*. When the target muscle cells are laser-ablated, the motor neurons sprout and form presynaptic varicosities on ectopic muscle cells, indicating that the *C. elegans* muscle possesses the potential to induce sprouting and synaptogenesis in associated neurons (Plunkett et al., 1996). Despite these indications, it is not clear whether activity-dependent remodeling exists at the synaptic level in *C. elegans* and whether retrograde signaling is involved in the process. Here, we characterize conditions that induce morphological changes in a class of motor neurons innervating head muscle of *C. elegans*. Our experiments reveal a case of neuronal plasticity in *C. elegans* mediated by synaptic activity and further indicate that communication between motor neurons and muscle cells underlies the decision to alter these synaptic connections.

MATERIALS AND METHODS

Nematode strains and culture

Bristol strain N2 and mutants were grown at 22.5°C on solid medium as described by Sulston and Hodgkin (1988), except in the temperature-shift experiments.

Microscopy

Live animals were anesthetized with 10 mM sodium azide, mounted on 2% agarose pads, and examined under epifluorescence using an Olympus BX60 or Zeiss Axioskop equipped for differential interference contrast microscopy. Time-averaged images were collected using a DAGE SIT68 camera or a Cohu CCD and captured using a CG-7 frame grabber (Scion Image). Confocal images were taken using a Biorad MRC1024 microscope.

Immunocytochemistry

Immunocytochemistry with RAB-3, SNT-1, UNC-64 and myosin antiserum was performed as described previously in Nonet et al. (1997).

Visualization of SAB and DA motor neurons using synaptobrevin-GFP

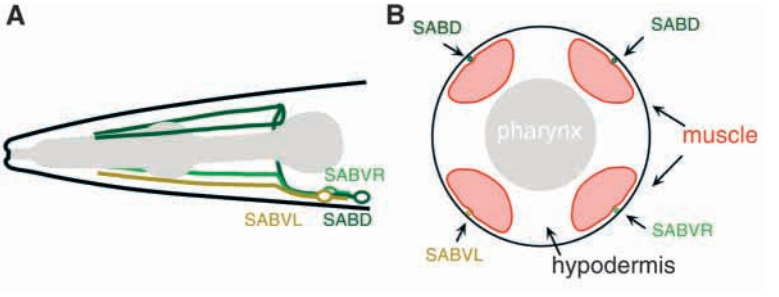
jsIs42 animals (Nonet, 1999) expressing synaptobrevin-GFP in SAB neurons and other A type motor neurons under the control of the *unc-4* promoter were used in our analysis (Miller and Niemeyer, 1995). Although expression of UNC-4 is transient in many of the VA and DA neurons (Miller and Niemeyer, 1995; M. L. N. and H. Z., unpublished data), expression of synaptobrevin-GFP under the control of the *unc-4* promoter in SAB neurons was maintained throughout development in *jsIs42* animals. Except where otherwise noted, SAB axons were examined in young adult hermaphrodites. The four SAB axons were scored independently in the same animal for the presence of altered morphology. A minimum of 120 axons (at least 30 animals) were scored for branching and looping phenotypes.

Plasmid constructions

$_{p}$*snb-1-cha-1*: oligonucleotides corresponding to the beginning and end of the *cha-1* coding region were used in a PCR to amplify the gene from wild-type genomic DNA. The product was inserted into pRM248, a clone containing the *snb-1* promoter.

$_{p}$*myo-3-unc-29*: oligonucleotides corresponding to the beginning and end of the *unc-29* coding region were used in a PCR to amplify the gene from wild-type genomic DNA. The product was into pPD96.52, a plasmid containing the *myo-3* promoter.

$_{p}$*myo-3-egl-36(E142K)* was a gift of Aguan Wei (Washington University School of Medicine). An *egl-36* cDNA carrying the Glu142Lys lesion (Johnstone et al., 1997) was inserted after the muscle-specific *myo-3* promoter in pPD96.52.



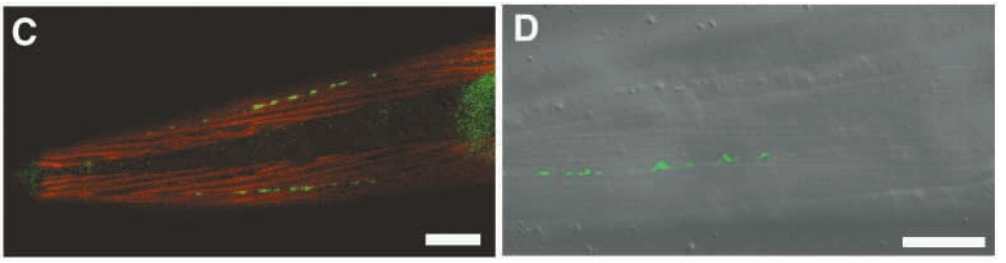


Fig. 1. Anatomy of SAB neurons. (A) Diagram of the head of *C. elegans* highlighting the processes of SAB neurons. SABVL, SABVR and SABD are each shown in a different shade of green. The pharynx is shown in gray. Muscle is not shown in this diagram. Anterior is left and dorsal is up in all images. (B) Diagram of a cross section through the head of a nematode showing SAB axons (green), muscle (red), the hypodermis (white) and the pharynx (gray). (C) Localization of the synaptic varicosities of SAB neurons on head muscle. A confocal image of lateral view of the head of a wild-type animal fixed and stained for myosin (red) to label head muscle and synaptotagmin (green) to label synaptic vesicles. SAB synaptic varicosities are in close apposition to head muscle. Mouse α-myosin and rabbit α-synaptotagmin antibodies were detected using rhodamine-conjugated and FITC-conjugated secondary antisera, respectively. (D) SAB processes extend on the surface of head muscle. Overlay of images of the same focal plane of the head of an adult *jsIs42* animal taken with Nomarski and epifluorescence optics. SAB varicosities visualized with a synaptobrevin-GFP tag (in green) run in the center of the muscle quadrant on the surface of the muscle cells. *jsIs42* animals express synaptobrevin-GFP in SAB neurons and other A type motor neurons under the control of the *unc-4* promoter (Miller and Niemeyer, 1995). Bars, 10 μm.

$_p$myo-3-egl-36(E142K G398E): oligonucleotides were used to engineer the Gly398Glu change in *$_p$myo-3-egl-36 (E142K)* using *Dpn*I and Pfu polymerase (Fisher and Pei, 1997).

$_p$cha-1-egl-36(E142K): the *egl-3*6(E142K) cDNA was inserted after the *cha-1* promoter in pPD49.26.

Germline transformation

Nematodes were transformed using the method described by Mello et al. (1991). Plasmids were injected at 10-30 ng/μl in conjunction with the dominant *rol-6(su1003)* transformation marker plasmid pRF4 at 140 ng/μl. Several independent lines were created for each construct examined.

Temperature-shift experiments

To synchronize animals, young adult *cha-1* hermaphrodites were transferred to plates, permitted to lay eggs at 15°C for 1 hour, and then removed. Plates were incubated at 15°C and these synchronized populations were then shifted at appropriate times to 22.5°C for 12 hours and returned to 15°C to assess the critical period of SAB sprouting. Synchronized L2 larvae were shifted for different time periods to assess the period of inactivity required for sprouting. Young adults from these plates were examined under fluorescence microscopy and the number of sprouting SAB axons were counted. Animals shifted at L3 and L4 stages were also examined as old adults 3 days later. Data were plotted using standard deviation (s.d.) as a measure of error.

RESULTS

Anatomy of the SAB neurons

Three cholinergic SAB neurons (SABVL, SABVR and SABD) are generated during embryogenesis in *C. elegans*. Their cell bodies are located in the retrovesicular ganglion, ventral to the posterior bulb of the pharynx and posterior to the nerve ring. The axonal projections of these neurons run anteriorly and innervate head muscle (Fig. 1). Head muscles are located in four quadrants in *C. elegans* (Fig. 1B). SABVL and SABVR each innervates a single ventral quadrant, while the SABD axon bifurcates, with each process innervating one dorsal muscle quadrant (Fig. 1A). In adult animals, we observed approximately a dozen synaptic varicosities along each SAB axonal segment in close apposition with muscle, a segment we refer to as the axon's 'innervation zone'. The varicosities stained strongly with antisera directed against synaptic vesicle-associated proteins including synaptotagmin, RAB-3 and synaptobrevin (Figs 1C, 2A; Nonet et al., 1998, 1997). Immunostaining with an α-myosin antibody to visualize muscle cells revealed that these varicosities extended over the most anterior 6-8 cells in each of the four muscle quadrants (Fig. 1C and data not shown). The varicosities could also be visualized on the surface of head muscle in living animals that express the synaptic vesicle marker synaptobrevin-GFP (Figs 1D, 2B; Nonet, 1999). SAB neurons thus differ from most other motor neurons in *C. elegans*, because most other motor neurons of *C. elegans* do not extend axons to contact muscle; rather, the muscles extend processes to contact the motor axons in the major process bundles of the nematode (White et al., 1986).

Synaptic transmission deficits cause axons of SAB neurons to sprout and form ectopic presynaptic varicosities

To determine the role of synaptic activity in the development of SAB neurons, their morphology was examined in mutants with deficits in synaptic transmission. Specifically, we considered animals with lesions in genes encoding components of the synaptic transmission machinery, including DAG/phorbol ester-binding protein *unc-13*, the syntaxin-binding protein *unc-18*, the calcium-binding protein

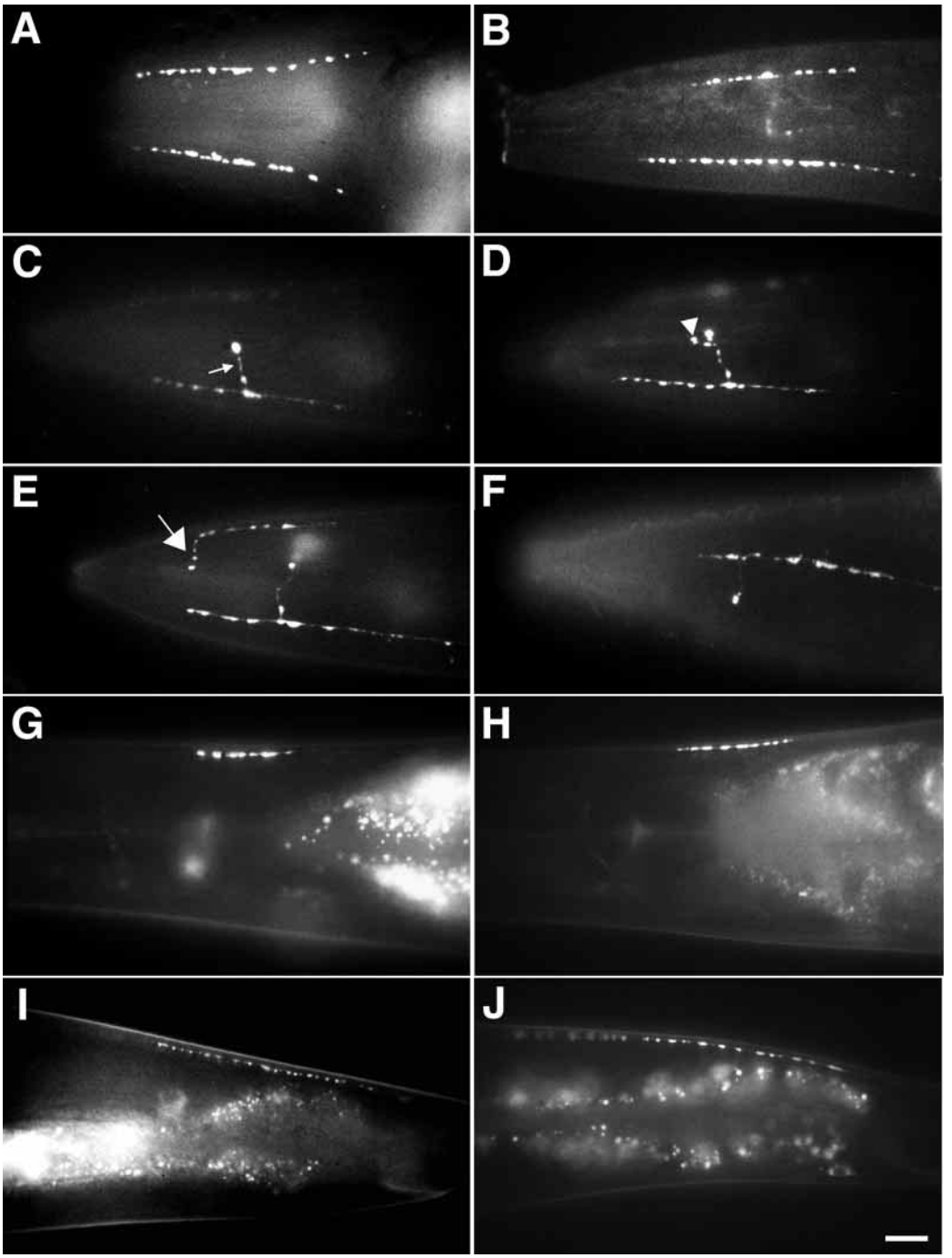


Fig. 2. Sprouting of SAB neurons in the synaptic transmission mutant *unc-18.* (A) A lateral view of SAB synaptic varicosities of a fixed adult wild-type animal visualized by incubation with primary antibody directed against the synaptic vesicle marker RAB-3 and FITC-conjugated secondary antibody. (B) Image of a lateral view of the head of an anesthetized wild-type adult animal expressing GFP-tagged synaptobrevin in SAB neurons (genotype: *jsIs42*). The fluorescence synaptic varicosities of two SAB axons are visible along the axons. (C-E) Images of a lateral view of the heads of the synaptic transmission mutant *unc-18*. SAB axons and sprouts are visualized using synaptobrevin-GFP (genotype: *unc-18(e81) jsIs42*). Most sprouting SAB axons extended a single branch (C) with a bright GFP-positive varicosity (small arrow). Secondary branching (D) was also observed in rare instances (large arrowhead). (E) 15% of SAB axons failed to terminate normally and extended a process after turning sharply towards lateral positions (top axon; large arrow). (F) Image of a lateral view of the head of a transgenic animal expressing a gain-of-function *egl-36* channel subunit under the control of the *cha-1* promoter (genotype: *jsIs42; jsEx479*). (G,H) Lateral view of wild-type and *unc-18* adult hermaphrodites showing the synaptic varicosities of the DA1 neuron. (I,J) Lateral view of wild-type and *unc-18* adult hermaphrodites showing the synaptic varicosities of the DA9 neuron (genotype of G and I: *jsIs42*; genotype of H and J: *unc-18(e81) jsIs42*). Bar, 10 μm.

synaptotagmin (*snt-1)*, and the GTP-binding protein *rab-3* (Gengyo-Ando et al., 1993; Maruyama and Brenner, 1991; Nonet et al., 1993, 1997). Our observations demonstrate that synaptic connectivity is not hard-wired in the developmental program of the nematode. Adult *unc-18* mutant animals showed at least two abnormalities (Fig. 2). First, SAB axons sprouted and formed ectopic varicosities. In most cases, sprouts arose from the middle of the innervation zone. Most of the sprouting axons contained a short single branch with several evenly distributed varicosities (Fig. 2C-E). The varicosity at the end of the branch often was more intense (Fig. 2C). Sprouts with secondary branches were occasionally seen (Fig. 2D). Similar abnormalities were observed in other synaptic transmission mutants (Table 1). The frequency of sprouting axons correlated with the severity of behavioral defects in the synaptic mutants. For example, *unc-18(e81)* mutants are virtually paralyzed; 88% of SAB neurons sprouted in these animals. By contrast, *unc-18/+* and *unc-13/+* heterozygotes and mutants with very mild behavioral and physiological abnormalities, such as *rab-3(y251)*, showed no significant differences in sprouting from the wild type (Table 1).

The axon outgrowth pattern in *unc-18* animals reflected a second deviation from the wild-type SAB pattern. Axons in

Table 1. Sprouting and looping axons in synaptic transmission mutants

Genotype	Sprouting axons	Looping axons	*n* [a]	Transmission defects[b]	Behavioral phenotypes
Wild type	2%	0%	120	Wild type	Wild type
unc-18(e81)[c]	88%[f]	15%[f]	120	General; severe	Paralyzed
unc-13(e51)[c]	50%[f]	13%[f]	120	General; severe	Paralyzed
snt-1(md290)	25%[f]	7%[g]	120	General; moderate	Uncoordinated locomotion
unc-11(e47)	30%[f]	15%[f]	120	General; moderate	Uncoordinated locomotion
rab-3(y251)	3%	0%	400	General; mild	Slightly uncoordinated
cha-1(y226)[d]	53%[f]	5%[h]	120	Cholinergic; severe (ts)	Conditional (ts) lethal
unc-25(e156)	2%	4%[h]	120	GABAeric; severe	Poor reversal of direction
eat-4(ky5)[e]	1%	0%	120	Glutamergic; moderate	Feeding deficits
cat-1(e1111)[e]	3%	0%	120	Monoamine; severe	Altered foraging
unc-54(e190)	3%	0%	120	none	Muscle paralyzed
unc-29(e1072)	23%[f]	5%[h]	120	Cholinergic; moderate	Uncoordinated locomotion

[a]Number of axons examined. Only animals in which all four SAB axons were scored are represented.
[b]Transmission deficits are considered general if behavioral, pharmacological and/or physiological evidence suggests that multiple different transmitter systems are effected.
[c]Heterozygous animals that have no difference in behavior from wild-type animals were examined: 3% and 2% of axons sprouted in *unc-18(e81) jsIs42/+* and *unc-13(e51)/+; jsIs42/+* animals, respectively.
[d]Animals reared for 12 hours as L1 larvae at non-permissive temperature, then returned to permissive temperature.
[e]Sprouting and looping were assessed using α-synaptotagmin antisera in these mutant backgrounds.
[f]Significantly greater than wild type ($P<0.001$).
[g]Significantly greater than wild type ($P<0.002$).
[h]Significantly greater than wild type ($P<0.01$).

these animals occasionally extended beyond their normal termination point and looped back toward the posterior of the animal. Most of the looping axons turned nearly 90° near the tip of the nose (Fig. 2E) while other axons turned twice, forming a safety pin-like structure. Looping and sprouting were sometimes observed in the same axon. However, looping occurred less frequently than sprouting and was less well correlated with the severity of the transmission mutants (Table 1).

The use of synaptic transmission mutants to disrupt activity differs from classical approaches, which block electrical activity. To mimic this classical approach, we expressed an activated *egl-36* potassium channel (Johnstone et al., 1997) in all cholinergic motor neurons under control of the *cha-1* promoter. The *egl-36* gain-of-function mutation shifts the voltage dependency such that the channel opens at more negative voltages (Johnstone et al., 1997). Thus, expression of the mutant channel likely reduces the excitability of cholinergic motor neurons. Transgenic animals expressing this construct were uncoordinated, and 25% of SAB axons (n=120) formed branches with ectopic synaptic varicosities (Fig. 2F). In summary, disruption in either synaptic activity or electrical activity altered the SAB axonal morphology and the distribution of synaptic varicosities.

Sprouting is specific to the SAB neurons

We also examined the morphology of synaptic contacts made by VA and DA motor neurons onto body wall muscle (Nonet, 1999). In our experiments, no sprouts or ectopic varicosities were formed by either DA1 or DA9 motor neurons that innervate dorsal muscle (Fig. 2G-J), or VA neurons that innervate ventral muscle (data not shown). Indeed, neither VA nor DA motor neurons were observed to sprout in any genetic background we examined. However, even if remodeling occurred at these neuromuscular junctions, the changes probably would not be detectable in the light microscope. Specifically, muscles send out processes, called muscle arms, to meet motor neurons whose axons reside in the major bundles of neuropil: the ventral nerve cord, the dorsal nerve cord, and the nerve ring (White et al., 1986). Hence, motor neurons do not leave the nerve fascicle to innervate their targets; rather the synapses are formed en passant. It is likely that we would not detect sprouting of motor neurons in the nerve cords since the muscle arms of multiple different body wall muscles are in very close proximity to the motor neuron. Thus, our analysis does not rule out the possibility that similar changes occur at other cholinergic neuromuscular junctions in the nematode.

Ectopic varicosities of SAB neurons remain in contact with muscle

Sprouts of SAB neurons extended in a stereotypic direction. Axons in the dorsal quadrant sprouted ventrally, while axons in the ventral quadrants sprouted dorsally. Further, axonal sprouts rarely crossed into neighboring muscle quadrants. Perhaps the hypodermis acts as a physical barrier, preventing the axons from extending beyond the edge of a muscle quadrant (see Fig. 1B). The stereotypic branching pattern suggests that these ectopic sprouts might be attracted to a specific target. We characterized those cells that are in close proximity to varicosities at the end of sprouts to determine whether muscle or neurons are opposed to these presynaptic specializations. Under Nomarski optics, it was evident that the varicosities in *unc-18(e81)* animals always remained in close proximity to muscle cells (Fig. 3A-B). Although this suggested that the new varicosities formed contacts with muscle, other neuronal processes running in close contact with head muscle could also be the postsynaptic targets of the ectopic presynaptic varicosities (White et al., 1986). To assess this possibility, we stained animals which show a sprouting phenotype with antibodies against the synaptic vesicle protein RAB-3 and the plasma membrane protein syntaxin. While RAB-3 antibodies label synaptic varicosities (Nonet et al., 1997), syntaxin antibodies label all neuronal membrane (Saifee et al., 1998). Although ectopic varicosities from SAB neurons were

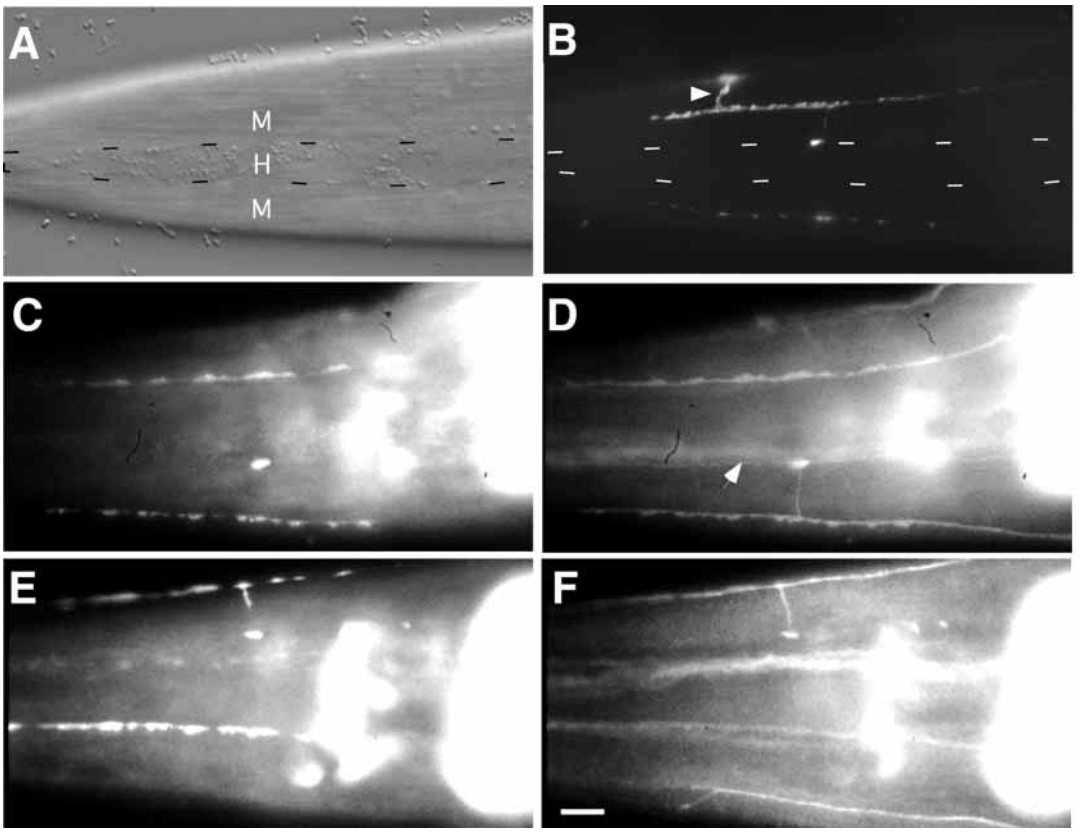


Fig. 3. Localization of the ectopic SAB varicosities. (A) Nomarski image of the lateral view of an adult *unc-18* mutant animal showing the surface of two quadrants of muscle (M) separated by hypodermis (H). The outline of the hypodermis is demarcated by a dashed line (genotype: *unc-18(e81) jsIs42)*. (B) Epifluorescent image in the same focal plane as A showing the branching pattern of an SAB neuron. The image illustrates a rare axon that extended both dorsal (arrowhead) and ventral branches. The varicosity of the ventral branch clearly remains in contact with muscle. The outline of the hypodermis is demarcated with a dashed line. (C-F) Lateral views of the head of adult *cha-1* animals fixed and double labeled with antibodies recognizing RAB-3 and syntaxin. (C,E) Presynaptic terminal of SAB neurons labeled with the α-RAB-3 antibody. (D,F) Axonal process of the same animals labeled with an α-syntaxin antibody. The synaptic varicosity of the SAB branch in C and D is juxtapositioned to an axon (D; small arrow), while the branch visible in E and F is not [genotype: *cha-1(y226ts)*]. Bar, 10 μm.

occasionally in proximity to an axon (probably ALM; Fig. 3C-D), more often, the ectopic varicosities were not adjacent to any axon (Fig. 3E-F). Furthermore, when ALM was absent due to a mutation in the *mec-3* gene (Way and Chalfie, 1989), the frequency of SAB sprouting was unaffected (data not shown). Taken together, these data suggest that the SAB axons sprout over and form novel varicosities that contact muscle cells in the quadrant the axon initially innervates.

Disruption of cholinergic transmission induces motor neuron sprouting

Acetylcholine (ACh) is the major excitatory neurotransmitter at most *C. elegans* neuromuscular junctions (for a review, see Jorgensen and Nonet, 1995). To determine whether reduction of ACh-mediated synaptic activity is sufficient to induce sprouting in SAB neurons, mutants with a deficiency in ACh synthesis (Alfonso et al., 1994) or ACh vesicular transport (Alfonso et al., 1993) were examined for sprouting and ectopic varicosities. Since the synaptic inputs onto SAB motor neurons are not cholinergic, mutants that disrupt cholinergic function likely disrupt synaptic, but not electrical, activity in SAB motor neurons. In the viable hypomorphic choline acetyltransferase mutants *cha-1(b401)* and *cha-1(p1152)*, 33% and 35% of SAB axons sprouted, respectively. Sprouting was also observed in two temperature-sensitive *cha-1* mutants (see below). When vesicular loading of ACh into synaptic vesicles was impaired by lesions in the ACh vesicular transporter *unc-17*, SAB axons also sprouted: 13% in *unc-17(e113)* animals and 20% in *unc-17(e245*) animals. Locomotion deficits in the viable *cha-1* and *unc-17* mutants are milder than those of *unc-13* and *unc-18* mutants (Rand, 1989), suggesting differences in the level of residual cholinergic activity in these mutants may account for the variability in sprouting frequency. We were not able to examine animals carrying complete loss-of-function *cha-1* or *unc-17* mutations because both of these animals arrest development as L1 larvae (Alfonso et al., 1993, 1994).

We confirmed that the sprouting defect seen in *cha-1* mutants is specific to the absence of CHA-1 protein in neurons. We transformed temperature-sensitive *cha-1(y226ts)* mutant animals with a $_{p}$*snb-1*-CHA-1 construct, which directs expression of wild-type CHA-1 in all neurons and a few secretory cells under the control of the synaptobrevin promoter. Transformed animals at the non-permissive temperature behaved like the wild type, while those lacking this construct arrested as L1 larvae. The frequency of sprouting in SAB neurons was reduced to 15% and 9% in two independent transgenic lines compared to 53% in untransformed *cha-1(y226ts)* animals. The failure to completely abolish sprouting likely results from the mosaic nature of transgenic expression (Mello and Fire, 1995). Thus, expression of *cha-1* in all neurons rescued both the behavioral and the sprouting defects, indicating that reduction of presynaptic cholinergic activity is responsible for the induction of axonal sprouting of SAB neurons.

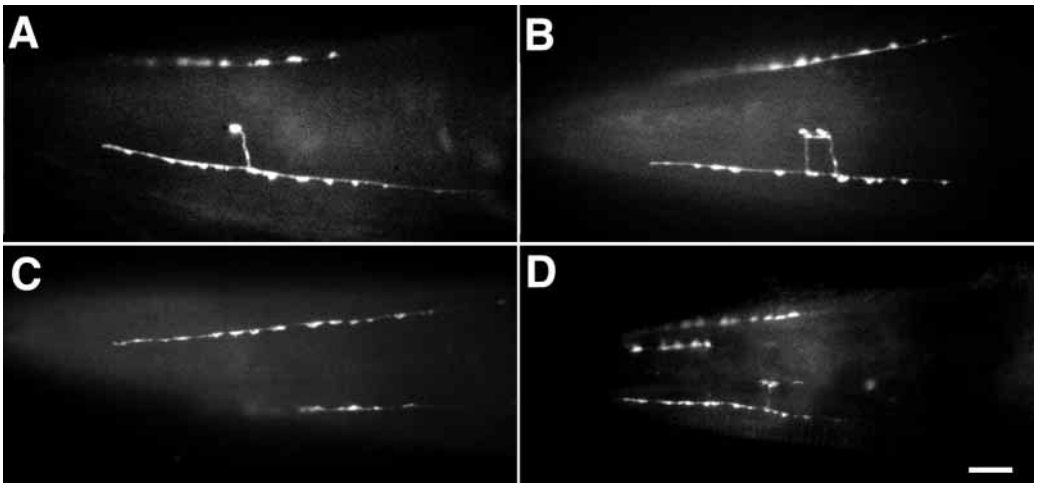


Fig. 4. Decreasing head muscle excitability induces axonal sprouting. Images of a lateral view of the head of anesthetized adult hermaphrodites expressing GFP-tagged synaptobrevin. (A,B) Examples of SAB axonal sprouting in *unc-29* animals lacking a non-α nicotinic AChR subunit (genotype: *unc-29(e1072); jsIs42*). (C) Image of the head of a transgenic animal expressing the inactive *egl-36(E142K G398E)* potassium channel that exhibits normal SAB axonal structure (genotype: *jsIs42; jsEx486*). (D) SAB axonal sprouting in animals expressing a gain-of-function *egl-36* potassium channel in muscle under the control of the *myo-3* promoter (genotype: *jsIs42; jsEx352*). Bar, 10 μm.

We next determined whether the sprouting defect is specifically associated with dysfunction in cholinergic transmission. First, disruption of other specific neurotransmitter systems in *C. elegans* did not induce sprouting. GABA is known to be an inhibitory neurotransmitter acting on head and body wall muscle of *C. elegans* (McIntire et al., 1993; White et al., 1986). Disruption of GABAergic transmission in the nematode did not lead to a sprouting defect (Table 1): less than 2% of SAB axons sprouted in *unc-25(e156)* mutants, which lack glutamic acid decarboxylase and thus are defective in GABA synthesis (Jin et al., 1999). Similarly *eat-4* mutants (Lee et al., 1999), which disrupt glutamatergic transmission, and *cat-1* mutants (Duerr et al., 1999), which disrupt monoamine transmission, also exhibited normal SAB morphology (Table 1). Second, we eliminated the possibility that the sprouting defect in the synaptic transmission mutants is simply a consequence of developmental defects associated with the poor health of the severe synaptic transmission mutants. Specifically, we examined *unc-54* mutants that lack the major muscle myosin heavy chain. These mutants are as paralyzed as, and slower growing than, *unc-18(e81)* mutants and produce smaller broods. Nevertheless, sprouting was not observed in *unc-54* animals (Table 1). Taken together, our observations suggest that reduction of cholinergic activity can cause axonal sprouting and the formation of ectopic presynaptic varicosities at neuromuscular junctions in *C. elegans*.

Post-synaptic disruption of cholinergic transmission induces sprouting

Mutants lacking functional nicotinic acetylcholine receptor (nAChR) subunits were examined for the presence of sprouts to assess whether reduction of postsynaptic activity is sufficient to induce sprouting of SAB neurons. In the *unc-29(e1072)* mutant, which lacks a non-α nicotinic AChR subunit (Fleming et al., 1997), 23% of SAB axons contained sprouts (Fig. 4A,B). The varicosities at the termination of sprouts were strongly fluorescent and were similar in appearance to those seen in mutants with reduced presynaptic activity. Nevertheless, *unc-29*-mediated disruption of postsynaptic activity caused a lower incidence of sprouting than that seen when presynaptic activity was blocked. Perhaps this is explained by the presence of other cholinergic receptors in muscle (Richmond and Jorgensen, 1999).

The UNC-29 acetylcholine receptor is expressed not only in body wall muscle, but also in certain classes of neurons (Fleming et al., 1997). To demonstrate that the sprouting effect was a result of impaired postsynaptic receptor function, we directed the expression of the wild-type *unc-29* gene in muscle by fusing to the *myo-3* promoter (Okkema et al., 1993). The locomotion defect was alleviated by expression of the wild-type UNC-29 receptor subunit in muscle; the animals behaved indistinguishably from wild-type animals expressing only the dominant transformation marker *rol-6(su1003)*. Moreover, muscle-specific expression suppressed the sprouting phenotype. Only 5% of SAB axons (n=120) sprouted among transformed *unc-29* animals we examined. The fact that disruption of acetylcholine receptor in muscle generates sprouting of the neuron indicates that the decision to sprout is not made independently by the neuron, but rather involves communication between the neuron and muscle.

Removal of the UNC-29 acetylcholine receptor likely generates sprouting by reducing the excitation of head muscle. To mimic this reduction in excitability, we expressed the activated form of the *egl-36* potassium channel subunit (Johnstone et al., 1997) in muscle, under control of the *myo-3* promoter. Transgenic animals expressing this construct were slow growing, unhealthy and severely uncoordinated. Further, 57% of SAB axons (n=120) formed branches with ectopic synaptic varicosities (Fig. 4D). To confirm that this effect is due to the hyperpolarizing activity of *egl-36* channel, we introduced a Gly398Glu substitution into the core region of the activated form of the channel subunit. This mutation has been shown to genetically suppress the dominant activity of *egl-36* (Johnstone et al., 1997). Animals expressing the channel subunit carrying both the activating and Gly398Glu lesions showed a 3% sprouting frequency, comparable to the baseline observed in wild-type animals (Fig. 4C). Hence, dysfunction in muscle excitation, either by disruption of an acetylcholine receptor or by hyperpolarization of muscle,

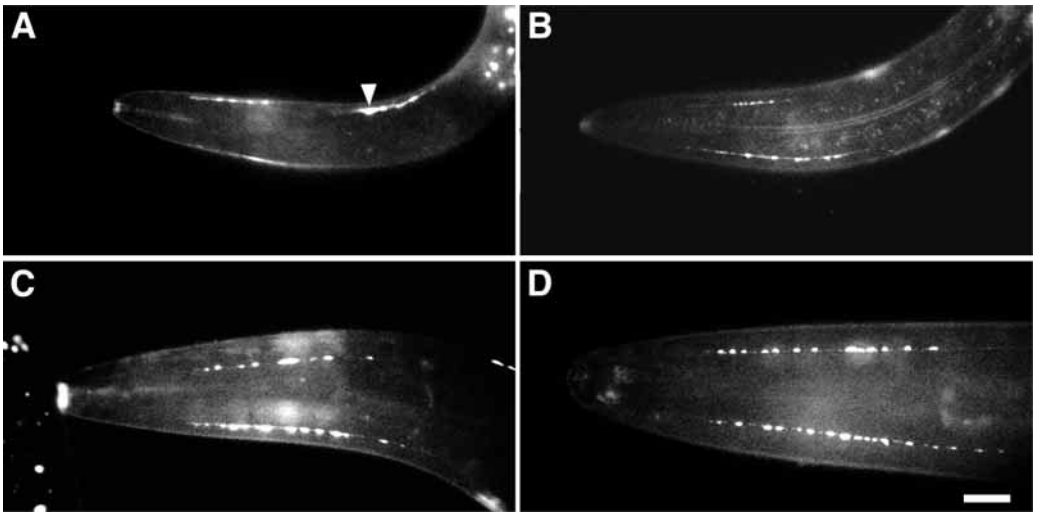


Fig. 5. Development of SAB synaptic varicosities. Images of a lateral view of the head of anesthetized hermaphrodites expressing GFP-tagged synaptobrevin. The SAB varicosities are visible during each developmental stage: (A) L1 larva, (B) L2 larva, (C) L3 larva, (D) L4 larva. The synaptic varicosities of the DA1 neurons are also visible in the L1 larva shown in A (arrowhead). The number of varicosities increases during development: L1 larvae (4.8±2.2), L2 larvae (5.8±1.4), L3 larvae (7.2±1.6), L4 larvae (9.4±1.4) and adults (12.1±2.1). Values are means ± s.d.; n=50 for all stages. Genotype of all animals is *jsIs42*. Bar, 10 μm.

altered SAB axon morphology, suggesting muscle inactivity is sensed by the axon to yield modulation of synaptic contacts.

SAB neurons add synaptic varicosities throughout development

We examined the presynaptic specialization of SAB neurons in wild-type animals expressing synaptobrevin-GFP at various time points throughout development. SAB neurons extend axonal projections and innervate head muscle during embryogenesis. Subsequent to hatching, L1 larvae had established an average of 4.8±2.2 (n=50) varicosities per axon (Fig. 5A). The number of varicosities and the length of the innervation zone increased slowly during the subsequent larval stages (Fig. 5B-D). For example, the number of varicosities had nearly tripled to 12.1±2.1 (n=50) by the adult stage. In summary, SAB neurons are born and establish differentiated synaptic terminals embryonically, then continue to add synaptic varicosities throughout the larval development stages.

Reduction of activity in early larval stages is required for induction of sprouting

We took advantage of two temperature-sensitive alleles, *cha-1(y226ts)*, and *cha-1(md39ts)*, to assess the critical period in which SABs are capable of modifying their synaptic connections. Both mutants develop normally at 15°C, but exhibit severe behavioral defects and arrest development in the L1 larval stage when raised continuously at 22.5°C (M. Nonet and J. Rand, unpublished data). The behavioral defects associated with a shift of the *cha-1(y226ts)* animals to a non-permissive temperature become apparent within 15 seconds of the shift and are quickly reversible upon shifting back to a permissive temperature (data not shown). Hence, these mutants allow manipulation of cholinergic activity during development. To assess the time period when reduction of cholinergic activity could affect sprouting, we shifted animals at different stages to 22.5°C for 12 hours, then returned the animals to 15°C, and allowed them to develop to adults. *cha-1(y226ts)* and *cha-1(md39ts)* animals raised continuously at 15°C exhibited normal SAB morphology (Fig. 6A and data not shown). However, a high frequency of SAB axons sprouted when L1 and L2 larvae were temperature shifted (Fig. 7). For example, among animals shifted to non-permissive temperature during the first larval stage, 53% of *cha-1(y226ts)* and 56% of *cha-1(md39ts)* SAB axons sprouted. The majority of the sprouts were single short branches arising from the middle of the axons, and ended with a large varicosity (Fig. 6B). In some animals, the sprouts bifurcated and developed two or more evenly-distributed varicosities (Fig. 6C,D). The sprouting frequency decreased dramatically when animals were shifted during the L3 stage and approached baseline in animals shifted at L4 or adult stage (Fig. 7A). Similar results were obtained using *cha-1(md39ts)* (data not shown). We also examined a fraction of animals shifted during the L3 and L4 stage as older adults. The frequency of sprouting is similar among the two populations (Fig. 7A), indicating that the absence of sprouting was not simply a consequence of the shorter period of time these animals remained at the permissive temperature after the shift. Taken together, our results suggest that SAB neuronal morphology is developmentally regulated, and that reduction of activity before the L3 stage is most efficient to induce sprouting.

Brief disruption of synaptic activity is sufficient to induce sprouting

In order to define the time course of induction of sprouting of SAB neurons, L2 *cha-1(y226ts)* larvae were shifted from 15°C to 22.5°C and maintained at 22.5°C for varying periods ranging from 2-30 hours, then examined as adults. Sprouting was observed in animals exposed to the non-permissive temperature for as little as 2 hours. The frequency of sprouting was maximal after a 12 hour temperature shift (Fig. 7B). This suggests that the sprouting process is initiated very quickly upon a reduction in synaptic activity.

We also assessed if SAB sprouts that are generated by exposure to the non-permissive temperature retract after activity is restored to normal levels. L2 larvae kept at 22.5°C

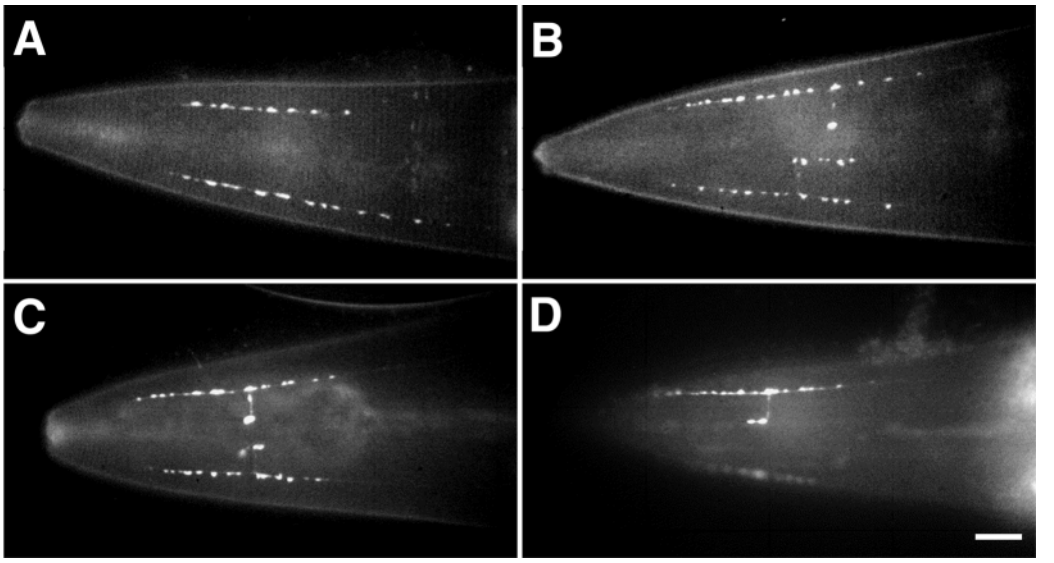


Fig. 6. Sprouting of SAB neurons caused by a brief reduction of cholinergic activity. (A-D) Images of a lateral view of the head of anesthetized adult animals expressing GFP-tagged synaptobrevin. (A) A temperature-sensitive *cha-1* animal grown at 15°C shows normal SAB morphology. (B-D) *cha-1* animals shifted at the L1 stage for 12 hours to 22.5°C and subsequently raised to adulthood at 15°C have sprouts and ectopic synaptic varicosities (genotype:*cha-1(y226ts);jsIs42*.) Bar, 10 μm.

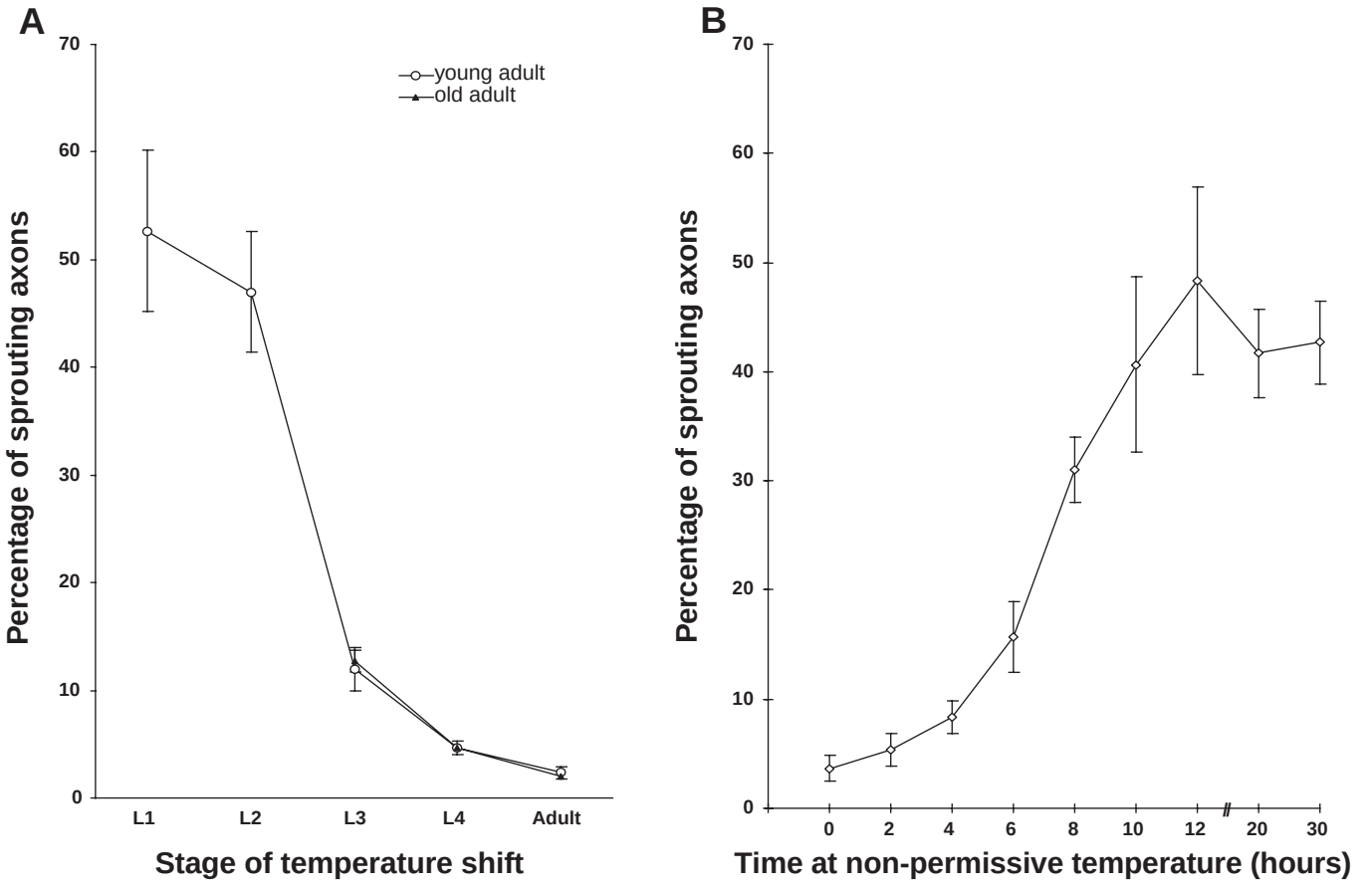


Fig. 7. Developmental regulation of SAB sprouting in response to reduction of cholinergic activity. (A) The frequency of SAB axons with sprouts is plotted as a function of developmental stage when *cha-1(y226ts)* animals were subjected to a 12 hour period at 22.5°C. Animals were scored for sprouting as young adults (open circle) or old adults (close triangle) after animals had finished development at 15°C. Values are means ± s.d. (B) The frequency of SAB axons with sprouts is plotted as a function of the length of time that *cha-1(y226ts)* animals were shifted to 22.5°C at the L2 stage. All animals were scored for sprouting as adults after the animals had finished development at 15°C. Values are means ± s.d.

for 12 hours were examined by fluorescence microscopy. Animals with sprouts were then raised at 15°C and re-examined as young adults. Among 54 axons with sprouts in the L2 larval stage, 15% (9 sprouts) had retracted. These results suggest that although SAB sprouts are initiated rapidly upon reduction of synaptic activity, a longer period of inactivity may

be required for the morphological changes to become stable and resistant to further alterations by changes in activity.

DISCUSSION

In this study, we have shown that certain classes of motor neurons in *C. elegans* sprout and form new presynaptic specializations in response to perturbations in synaptic activity. Communication between nerve and muscle regulates these morphological alterations since either disruption of activity in muscle or a presynaptic blockade is sufficient to cause such neuronal changes. Further, the neuronal response to inactivity is developmentally regulated. In early larval stages, inactivity is potent in stimulating structural changes. By contrast, in late larval and adult stages, reduction of activity is insufficient to induce morphological alterations in the axon. Our studies demonstrate that at certain neuromuscular synapses in *C. elegans*, there exist mechanisms capable of sensing and retrogradely communicating the state of synaptic activity. Further, our findings suggest that these retrograde synaptic signaling mechanisms were likely incorporated into synaptic developmental processes early in evolution.

Motor neuron activity and sprouting

Our experiments suggest that a more complete blockade of neurotransmission correlates to higher frequency of sprouting in SAB neurons. The small size of this nematode precludes us from using physiological techniques to directly monitor the perturbations at the SAB neuromuscular junction. Nevertheless, for many mutants used in this study (e.g. *snt-1, snb-1, unc-13 and rab-3*), extracellular recordings of pharyngeal neuron function or intracellular recordings of body wall neuromuscular function have revealed defects in both cholinergic and glutamatergic synaptic transmission (Nonet et al., 1998, 1997; Raizen et al., 1995; Richmond et al., 1999), allowing us to extrapolate similar defects at the SAB synapses. Moreover, the synapses we have examined are neuromuscular; we have relied on behavioral assays (primarily locomotion) as indirect measures of the relative strength of the synaptic function defects. The phenotypes of synaptic mutants in *C. elegans* have correlated relatively closely to the phenotypes of mutants in other systems. For example, *rab-3* mutants in both *C. elegans* and mouse have mild behavioral and physiological defects (Geppert et al., 1994; Nonet et al., 1997). Syntaxin mutants of *C. elegans* show the strongest behavioral defects (lethality and complete paralysis) of nematode synaptic mutants and the analogous *Drosophila* mutants exhibit a complete block both of evoked and spontaneous transmission at the larval neuromuscular junction (Broadie et al., 1995; Saifee et al., 1998; Schulze et al., 1995). Furthermore, the viable *cha-1* mutations probably cause a dramatic decrease in cholinergic transmission since choline acetyltransferase activity levels in these animals are only at 1% or less of wild-type levels (Rand and Russell, 1984). Thus, although our assessments of synaptic function are indirect, it is very likely that the conditional lethal *cha-1* mutants and *unc-18* mutants exhibit a virtually complete block of transmission while other mutants, which retain significant capabilities for locomotion, only show a partial blockade of neuromuscular transmission.

We postulate that it is alterations in synaptic activity, not electrical activity, that generate the presynaptic sprouting defect. Motor output in *C. elegans* is regulated by the command neurons, which receive inputs from sensory neurons and interneurons and synapse onto SABs and other motor neurons (White et al., 1986). While SAB motor neurons are cholinergic, the command neurons and sensory neurons are not cholinergic (Rand and Nonet, 1997a; White et al., 1986). We have observed similar sprouting defects when synaptic transmission was blocked in all neurons or just in cholinergic neurons. The general synaptic transmission mutations likely disrupt both synaptic transmission between SAB neurons and muscle and between command and SAB neurons. Hence, synaptic activity and electrical activity is likely disrupted in SAB neurons in these mutants. By contrast, the perturbations using *cha-1* and *unc-17* mutations likely only disrupt cholinergic synaptic transmission between SABs and muscle, but not synaptic transmission between command neurons and the SABs. Hence, overall electrical activity in the SABs is unlikely to be disrupted either in *cha-1* or *unc-17* mutants. For these reasons, we conclude that level of synaptic activity, rather than general electrical activity, regulates the structural changes we have observed in SAB neurons. However, in the absence of a direct assessment of electrical signals in these neurons in the mutants, we cannot eliminate the possibility that changes in cholinergic activity lead indirectly to electrical silencing of SAB motor neurons.

Moreover, eliciting sprouting of SAB neurons requires silencing of all inputs to head muscle. Head muscle in *C. elegans* is unique because it has several different excitatory inputs, one from the SABs and others from the VA and VB motor neurons (White et al., 1986). In *cha-1* mutants, or in animals in which an activated potassium channel has been expressed under the control of the *cha-1* promoter, the function of all of these motor neurons is disrupted. However, expression of the activated potassium channel only in the SABs (under the control of the *unc-4* promoter) failed to elicit sprouting (data not shown). These findings suggest that SABs do not secrete a specific sprouting factor. Rather, the role of presynaptic cell in regulating morphology is likely simply to release acetylcholine to elicit electrical activity in muscle.

Morphological plasticity in *C. elegans*

Neuronal remodeling is a common theme in the development of circuits in vertebrates (Katz and Shatz, 1996) and also occurs in a variety of invertebrates (reviewed in Keshishian et al., 1996; Lnenicka and Murphey, 1989). In *C. elegans*, the most dramatic case of remodeling occurs in DD GABAergic motor neurons. These neurons innervate ventral muscle early in the first larval stage, but late in the first larval stage they retract these synaptic contacts and switch to innervate dorsal body wall muscle (White et al., 1978). Such reprogramming of DD synaptic connections has been shown to be regulated by the developmental timing gene *lin-14* (Hallam and Jin, 1998). In addition, studies by Plunkett et al. (1996) have demonstrated that GABAergic motor axons are capable of modifying their synaptic contacts by sprouting and extending axonal branches in response to ablation of their postsynaptic targets. However, no changes in these contacts were detected at either the light or ultrastructural level in a *unc-25* GAD mutant, which presumably eliminates all GABAergic transmission. Thus, it is likely that both of these phenomena occur independently of synaptic activity.

Activity-dependent structural plasticity has been observed in many invertebrates including crustaceans, insects, mollusks and nematodes (Bailey and Chen, 1983; Lnenicka et al., 1991; Lnenicka and Murphey, 1989). However, where it has been possible to disrupt electrical and synaptic activity independently, the structural changes in invertebrates seem to be regulated by electrical, but not by synaptic activity. For example, *C. elegans* sensory neurons form ectopic axons as a consequence of manipulation of the state of activity (Peckol et al., 1999). Reducing excitability, either by using mutants that structurally block sensory functions or through the expression of mammalian potassium channels in sensory neurons, induces the formation of ectopic axons. However, such ectopic axons were not observed even in severe synaptic transmission mutants such as *unc-18*. In *Drosophila*, activity regulates the formation and refinement of connections at neuromuscular junctions. First, a low level of electrical activity is required for expression and localization of GluRII receptors during synaptogenesis (Broadie and Bate, 1993). In addition, in mutants with altered potassium channels, such as *ether-à-go-go Shaker* double mutants, both the number of synaptic boutons and motor axonal branches are increased (Budnik et al., 1990). Motor neuron sprouting is observed in *Drosophila* when activity is reduced by disruption of sodium channel function or expression or by agents that block action potentials in neurons (Jarecki and Keshishian, 1995). However, the morphology of motor axons is not affected in *synaptotagmin* trans-heterozygotes where synaptic transmission is impaired (Jarecki and Keshishian, 1995), or when postsynaptic receptor antagonists are applied (Jarecki and Keshishian, 1995; Keshishian et al., 1993). Thus, changes in excitability caused by the electrical activity blockade in these cases may alter neuronal morphology in a cell-autonomous manner.

Our work implicates synaptic activity in the regulation of neuromuscular junction morphology at certain synapses in *C. elegans*. Although we did not observe sprouting in either of the two other classes of motor neurons we examined, it remains a mystery how widespread activity-dependent phenomena are found in *C. elegans* synaptic development. As a free-living soil nematode, *C. elegans* probably develops under a vast array of environmental conditions. Although ultrastructural analysis of *C. elegans* suggests that the wiring of most neurons is relatively stereotypic, this connectivity data may not represent the normal variability in synaptic contacts in *C. elegans*, since they were assembled from a limited number of animals grown under ideal conditions. Alternatively, only a small fraction of synaptic connections might use or require activity-dependent mechanisms to be efficiently generated. Regardless of the extent to which connections are influenced by activity, our work demonstrates that mechanisms to induce such changes are present in *C. elegans.*

Synaptic development probably requires cell-cell recognition among the interacting partners. However, retrograde signaling mechanisms that modulate synaptic development, maintenance, and both functional and structural plasticity, may have evolved largely after synaptic signaling, or coevolved with it. Conventional evolutionary trees have nematodes diverging from the vertebrate lineage before arthropods, mollusks or crustaceans. Molecular data both supporting (Sidow and Thomas, 1994; Mushegian et al., 1998) and challanging this view (Aguinaldo et al., 1997; Mushegian et al., 1998) have been recently published. Despite the uncertainty concerning the evolutionary relationships of nematodes to other invertebrates, the discovery of synaptic activity-dependent morphological plasticity in *C. elegans* suggests that these mechanisms were already established early in the metazoan lineage. Further study of the properties of different synaptic connections in simple organisms should provide critical insight into how these intricate signaling structures developed during evolution.

Motor neuron sprouting in other systems

A wide variety of perturbations will induce sprouting, branching, overgrowth and similar phenomenon in motor neurons. In mouse, mutants lacking components of the acetylcholine receptor assembly apparatus such as MuSK, agrin and rapsyn exhibit defects in synapse assembly and extensive motor axon branching and overgrowth (DeChiara et al., 1996; Gautam et al., 1996; Gautam et al., 1995). Additionally, overexpression of the growth factor GDNF will induce hyperinnervation of muscle and application of certain growth factors will promote sprouting (Caroni and Grandes, 1990). Partial denervation or a muscle activity blockade will induce robust sprouting in mouse (Brown et al., 1981; Holland and Brown, 1980). In *Drosophila*, motor axon sprouting can also be induced by denervation of muscle. Whether these perturbations all utilize the same fundamental molecular pathways to induce changes in motor neuron structure is unclear. The process we have observed exhibits some similarities to those observed in vertebrates, and most closely parallels the activity blockade-induced terminal sprouting (Duchen and Strich, 1968; Holland and Brown, 1980). Nevertheless, there are substantial differences between the two systems. First, sprouting at the vertebrate neuromuscular junction occurs more robustly as the animal matures (Hopkins et al., 1985; Thompson and Jansen, 1977). By contrast, we observed sprouting in developing, but not mature, animals. Secondly, we only observed sprouting of one class of motor neurons, while sprouting occurs at a wide variety of muscle synapses in mouse (Brown et al., 1981). How the distinctive properties of the systems will translate into similarities and differences at the mechanistic level awaits a molecular identification of the signaling components in one of the systems.

Developmental regulation of sprouting

Analysis of the temporal regulation of sprouting using temperature-sensitive mutants in choline acetyltransferase suggests that there is a time window during development when the SAB neurons are capable of responding to reduction of activity by sprouting. This period overlaps with very active neuromuscular synaptogenesis during *C. elegans* development. At hatching, first larval stage animals innervate 81 body wall muscles using 16 cholinergic and 6 GABAergic motor neurons. In the late first larval stage, an additional 40 cholinergic and 13 GABAergic motor neurons are born (Sulston and Horvitz, 1977), approximately tripling the number of motor neurons. In the same period, an additional 14 body wall muscle cells are also born and intercalate into the previously existing muscle quadrants. Thus, at this time both developing motor neurons and mature motor neurons establish new synaptic contacts on previously innervated muscles and newly added muscle cells.

A mechanism which senses synaptic activity could ensure that previously innervated muscle cells and new muscles are innervated with similar efficacy by the different classes of motor neurons.

Remodeling in an activity-dependent manner during critical windows in development represents a common theme in many organisms. For example, in the mammalian visual system, eye closure results in a shift of the eye preference of most cortical neurons in favor of the open eye only when the deprivation occurs during the first few months in neonatal life (Hubel and Wiesel, 1970). In *Drosophila*, reduction of neural activity induces collateral sprouting of neurons innervating body wall muscle; the neural activity must be reduced during late embryogenesis and the first larval instar to promote these changes (Jarecki and Keshishian, 1995). A brief window of activity-dependent remodeling allows for functional tuning of synaptic contacts, without undue exposure to change in synaptic connections that might result from lifelong plasticity. Mechanisms underlying the existence of these critical periods are still unclear. In *C. elegans*, genetic screens for mutants in which the critical window extends into adulthood could provide one approach to address this question.

What mechanisms regulate sprouting?

Our results suggest that muscle is involved in the decision to sprout in response to the reduction of activity. A muscle-derived signal has also been proposed to play a role in the partial denervation-induced axonal sprouting in vertebrates (Brown et al., 1981). However, the molecular nature of the signal has not been identified. A number of different cellular components capable of signaling are upregulated by denervation in mammals including insulin-like growth factors 1 and 2, two factors capable of inducing collateral sprouting both in vitro and in vivo (Caroni, 1993). Other molecules such as NCAM, CGRP and GAP-43, whose expression is regulated by neural activity, may also be involved in the sprouting process (Covault and Sanes, 1985; Piehl et al., 1998). Indeed, overexpression of GAP-43 in neurons induces spontaneous sprouting of motor neurons (Aigner et al., 1995). Cellular factors required for sprouting in *C. elegans* have not been identified. Nevertheless, our studies suggest a retrograde signal that acts locally, since sprouting of one axonal branch of SABD was not influenced by the sprouting of the other branch (data not shown). Several avenues are now available for further characterization of the molecular mechanism underlying sprouting in the worm. First, the role of *C. elegans* proteins implicated in other synaptic regulatory mechanisms can be examined. For example, type II calcium- and calmodulin-dependent protein kinase II activity is required for maintaining the normal density of glutamatergic synapses in *C. elegans* (Rongo and Kaplan, 1999), and could play a role in SAB development. The *C. elegans* homologs of vertebrate components that modulate sprouting can now be directly assessed using reverse genetic techniques. Furthermore, screens designed to identify genes that disrupt the ability of sprouting in an activity-compromised genetic background provide a genetic approach to develop further insights into the mechanisms regulating these processes.

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