

Axons break in animals lacking β -spectrin

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Axons and dendrites can withstand acute mechanical strain despite their small diameter. In this study, we demonstrate that β -spectrin is required for the physical integrity of neuronal processes in the nematode *Caenorhabditis elegans*. Axons in β -spectrin mutants spontaneously break. Breakage is caused by acute strain generated by movement because breakage can be prevented by

paralyzing the mutant animals. After breaking, the neuron attempts to regenerate by initiating a new growth cone; this second round of axon extension is error prone compared with initial outgrowth. Because spectrin is a major target of calpain proteolysis, it is possible that some neurodegenerative disorders may involve the cleavage of spectrin followed by the breakage of neural processes.

Introduction

Neuronal processes can be one million times as long as they are wide. This elongated shape places extraordinary demands on cell integrity when axons or dendrites are placed under strain. In the vertebrate peripheral nervous system, axons are exposed to strains generated by length changes during movement (Phillips et al., 2004). Strain has also been proposed to assist in wiring the central nervous system (Van Essen, 1997) and to underlie axon extension in response to growth cone migration (Lamoureux et al., 1992). Strains may also result from external forces, such as impact experienced in traumatic head injury. However, the mechanism for the elasticity of axons and dendrites is unknown, as is the response of neurons to breaks caused by the loss of elasticity.

How do neurons maintain their structural integrity when challenged by mechanical strain? In this study, we demonstrate that β -spectrin is essential for neuronal strain resistance. The spectrin-based membrane skeleton is a cytoskeletal structure that is found in most cells, including neurons. The membrane skeleton is primarily composed of α/β -spectrin heterodimers, which associate with each other and with short actin filaments to form a 2D mesh. This submembranous structure anchors ion channels and other transmembrane proteins in the plasma membrane (Bennett and Baines, 2001). In neurons, spectrin is found at high levels in growth cones, and the injection of spectrin peptides prevents neurite extension in cultured cells (Sobue and Kanda, 1989; Sihag et al., 1996). These data suggest that the spectrin-based membrane skeleton functions in growth cone behavior. However, in erythrocytes, the spectrin-based membrane skeleton is required for membrane integrity (Lux and Palek, 1995). In theory, the spectrin scaffold could allow stretching of the long and thin extensions of neurons.

A role for the membrane cytoskeleton in neuronal structural integrity can be tested by characterizing β -spectrin mutants. In the nematode *Caenorhabditis elegans*, β -spectrin is encoded by a single gene, *unc-70* (Hammarlund et al., 2000). *unc-70* mutants exhibit axonal morphology defects that range from truncated processes to elaborate branching (Hammarlund et al., 2000; Moorthy et al., 2000). These defects in axonal anatomy are consistent with the proposed role of spectrin in neuronal development. These defects are also consistent with a loss of axonal integrity caused by mechanical stress (Moorthy et al., 2000; Bennett and Baines, 2001). In this study, we demonstrate that β -spectrin is required specifically to maintain normal axon morphology. Spectrin function is dispensable for neuronal development and axon migration. However, the loss of β -spectrin results in spontaneous breaks in neuronal processes. These results suggest that β -spectrin is an essential contributor to neuronal strain resistance.

Results and discussion

β -Spectrin is dispensable for axon outgrowth

To determine whether β -spectrin functions in axon outgrowth, we imaged growth cones of DD motor neurons in wild-type and β -spectrin mutant embryos (Fig. 1). In all experiments, only the progeny of homozygous null mutants were analyzed so that there was no maternal contribution of β -spectrin. The DD motor neurons send commissural axons from the ventral side to the dorsal side of the worm during embryogenesis (White et al., 1976; Sulston et al., 1983). To reach its target muscles in the dorsal quadrant, each DD growth cone pioneers a distinct path across the epidermis rather than following a preexisting axon tract. In wild-type animals, migrating DD growth cones display

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Abbreviation used in this paper: GABA, γ -aminobutyric acid.

four stereotypical shapes at specific points in their migratory trajectory (Fig. 1 E; Knobel et al., 1999). A growth cone is round and has radial projections when migrating across open epidermis. When a growth cone encounters the dorsal muscle quadrant, it spreads laterally into an anvil shape and extends multiple fingers between the muscle and epidermis toward the dorsal nerve cord. Finally, when a growth cone reaches the dorsal cord, it retracts all protrusions along the commissure and extends anterior and posterior processes along the cord.

We found that growth cones in β -spectrin mutant animals displayed normal morphology and that changes in the shape of the growth cone were observed at the correct point of the migratory trajectory (Fig. 1, A–D). Quantitation of these structures in an age-matched cohort of embryos demonstrated that there was a similar distribution of shapes in our wild-type and mutant

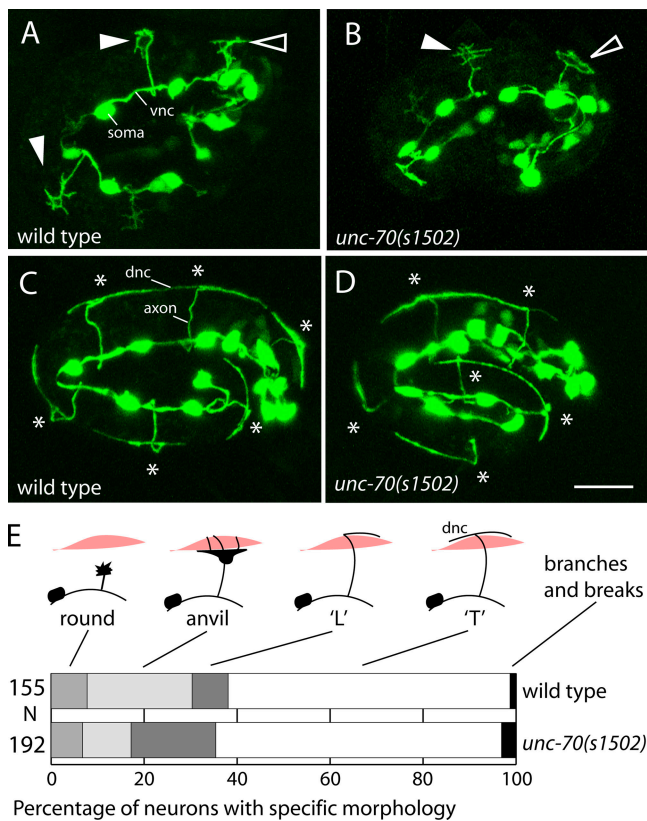


Figure 1. Neurons that lack β -spectrin develop normally. (A–D) Representative images of migrating DD growth cones in wild-type (A and C) and *unc-70(s1502)* (B and D) embryos. Embryos are curled up in the egg, and the soma of the DD neurons are along the ventral nerve cord (vnc); commissural axons extend to the dorsal nerve cord (dnc). GABA motor neurons are visualized by expressing GFP under the promoter of the vesicular GABA transporter gene *unc-47* (*oxIs12[Punc-47::GFP]*; McIntire et al., 1997). Bar, 10 μ m. (A and B) Growth cones extend across the epidermis. Closed arrowheads indicate growth cones migrating near the midline, displaying a rounded shape with radial protrusions. Open arrowheads indicate growth cones that have reached the dorsal muscle quadrant. These growth cones flatten and send protrusions to the dorsal cord. (C and D) Slightly older embryos extend processes along the dorsal nerve cord. Asterisks indicate commissures that have reached the dorsal nerve cord and are beginning to extend anterior and posterior processes along the nerve cord. (E) Percentage of growth cones with specific morphologies in wild-type and *unc-70(s1502)* embryos. Wild type, $n = 155$ neurons; *unc-70(s1502)*, $n = 192$ neurons.

samples (Fig. 1 E; see Quantitation of embryonic DD neuronal phenotypes). Finally, we found that a similar fraction of neurons had successfully completed their migration (formed T shapes on the dorsal cord) in wild-type and mutant embryos (fraction of neurons forming T shapes: wild type = 61%; *unc-70(s1502)* = 61%; $P = 0.91$ in a two-tailed Fisher's exact test; Fig. 1 E). Together, these experiments demonstrate that β -spectrin is not essential for normal axon outgrowth of the DD neurons.

To determine whether β -spectrin is dispensable for the extension of other neuronal processes, we examined the morphology of sensory dendrites, interneurons, and acetylcholine motor neurons in newly hatched *unc-70* mutants (Fig. 2 C). These neurons were essentially normal. Commissures could be distinguished in all animals ($n = 20$), and the dorsal and ventral nerve cords appeared intact. The dendritic processes of the sensory neurons were also similar to the wild type. Thus, in general, the extension of axons and dendrites does not require β -spectrin.

Axonal defects arise in mature neurons

Because axon and dendrite outgrowth does not require β -spectrin, neuronal defects that were previously observed in animals lacking β -spectrin must occur after outgrowth is complete. We confirmed the accumulation of commissural defects in neurons that had completed development by documenting DD axon morphology at three time points: during embryogenesis, just after hatching, and at 24 h after hatching (Fig. 2 A; see Quantitation of larval DD neuronal phenotypes). We found that neurons with defects—wandering, branched, or broken commissures—were rarely observed in wild-type animals at any time point. Defects were also rare in β -spectrin mutant embryos (fraction of neurons with defects: wild type = 1.3%; *unc-70(s1502)* = 3.1%; $P = 0.31$ in a two-tailed Fisher's exact test). However, β -spectrin mutant animals accumulated defects with time. At hatching, the percentage of neurons with defects had increased to 26% ($P < 0.0001$ compared with embryos in a two-tailed Fisher's exact test). At 24 h after hatching, this percentage had further increased to 60% ($P < 0.0001$ compared with hatching in a two-tailed Fisher's exact test).

The defects that accumulated in β -spectrin mutants were of several types, none of which were ever observed in wild-type animals (Fig. 2 B). First, we observed broken axon commissures that were detached from their process in the dorsal cord. These were clearly breaks rather than retractions, as both the proximal and the corresponding distal fragments could be identified as belonging to a single neuron. Second, we observed postembryonic growth cones. Finally, we observed aberrant branching. The percentage of all three types of defects increased between hatching and at 24 h after hatching (broken axons, 12 to 21%; growth cones, 2 to 17%; and branching, 13 to 22%).

To determine whether the processes of other neurons also accumulated defects, we examined the morphology (in adult *unc-70* mutants; $n = 20$) of acetylcholine phasmid neurons, interneurons, and motor neurons (Fig. 2, C and D). In contrast to newly hatched animals of this genotype, commissures were never visible, and all animals displayed defects in the dorsal cord and sensory dendrites. Thus, β -spectrin prevents the

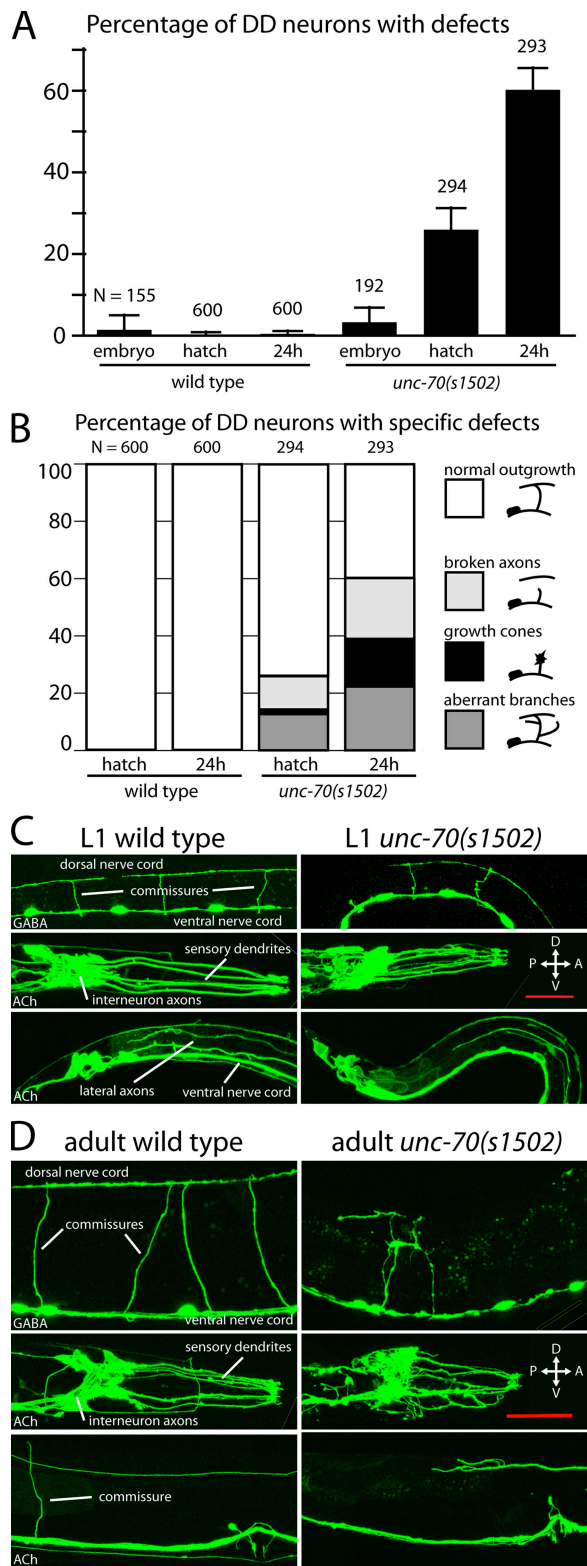


Figure 2. Neuronal defects accumulate in animals that lack β -spectrin. (A) Percentage of DD neurons with defects in wild-type *oxls12* and *unc-70(s1502)*; *oxls12* animals. Defects were scored in embryos, in larvae just after hatching, and in larvae 24 h after hatching. Bars represent percentage, and error bars represent 95% confidence interval. (B) Percentage of DD neurons with specific defects in wild-type *oxls12* and *unc-70(s1502)*; *oxls12* animals. Defects were scored just after hatching and at 24 h after hatching. (C) Representative L1 larvae from wild-type (left) and *unc-70(s1502)* (right) animals that express GFP in the GABA nervous system

postdevelopmental accumulation of defects in neuronal processes, including sensory neurons as well as motor neurons, in processes oriented on the anterior-posterior axis as well as those on the dorsal-ventral axis, and in dendrites as well as axons.

Neurons that lack β -spectrin break, regenerate, and break again

The breaks, growth cones, and branches in the neuronal processes of β -spectrin mutants could be the result of at least two different functions of β -spectrin. First, β -spectrin could act to prevent breaks, and growth cones and branches could be a consequence of breaking. Alternatively, β -spectrin could act to inhibit growth in mature neurons, and breaks and branches could be a consequence of initiating a new growth cone on an established process. To distinguish between these possibilities, we performed longitudinal studies in which we observed 12 individual animals at 24-h intervals (Fig. 3). In these studies, 25 individual nerve processes (that were initially normal) were later found to be broken. In some cases, we observed neurons that broke, regrew, and then broke again at a later time point (Fig. 3 C). Subsequent breaks occur at different locations along the axon. Although breaks are most easily observed in the commissure, breaks can also be observed in the nerve cords. Importantly, new growth cones were never observed on unbroken processes in these experiments (or in hundreds of additional single observations of β -spectrin mutants). Rather, growth cones appeared only on the proximal end of newly broken axons. Furthermore, every broken process in which the proximal end could be identified (21/25 breaks) reinitiated growth. Breakage and reinitiation of growth were observed at all larval stages. Thus, the primary defect in mutant axons is that they break, and the appearance of growth cones and abnormal branches is a secondary effect of breakage. These results demonstrate that β -spectrin is required to prevent spontaneous breaks in neuronal processes.

Although neurons in animals that lack β -spectrin are capable of sprouting new growth cones, regrowth is error prone in comparison with initial outgrowth and generates pathfinding errors and aberrant branching (Fig. 3 C). However, these defects are not likely caused by the lack of β -spectrin. A large fraction of wild-type *C. elegans* neurons that have been artificially severed also fail to regrow normally, demonstrating that abnormal regrowth is a property of regenerating neurons rather than of spectrin mutants (Yanik et al., 2004). Thus, neurons that lack β -spectrin have a specific defect in strain resistance.

from the integrated transgene *oxls12(Punc-47:GFP)* (top, DD3-5) or in the acetylcholine nervous system from the transgene *oxEx81(Pacr-5:GFP)* (middle, nerve ring and phasmids; bottom, posterior). Although *unc-70* animals are dumpy, their neuronal morphology is only slightly defective. Unbranched and unbroken processes are visible in the dorsal, ventral, and lateral tracts, commissures, and phasmids. Dorsal, up; anterior, right. Bar, 10 μ m. (D) Representative adult wild-type (left) and *unc-70(s1502)* (right) animals that express GFP in the GABA nervous system from the integrated transgene *oxls12(Punc-47:GFP)* (top, DD and VD neurons) or in the acetylcholine nervous system from the transgene *oxEx81(Pacr-5:GFP)* (middle, nerve ring and phasmids; bottom, posterior and vulval region). In the *unc-70* animals, most of the dorsal processes are missing, as are most of the commissures, and the remaining processes are extensively branched and broken. Dorsal, up; anterior, right. Bar, 25 μ m.

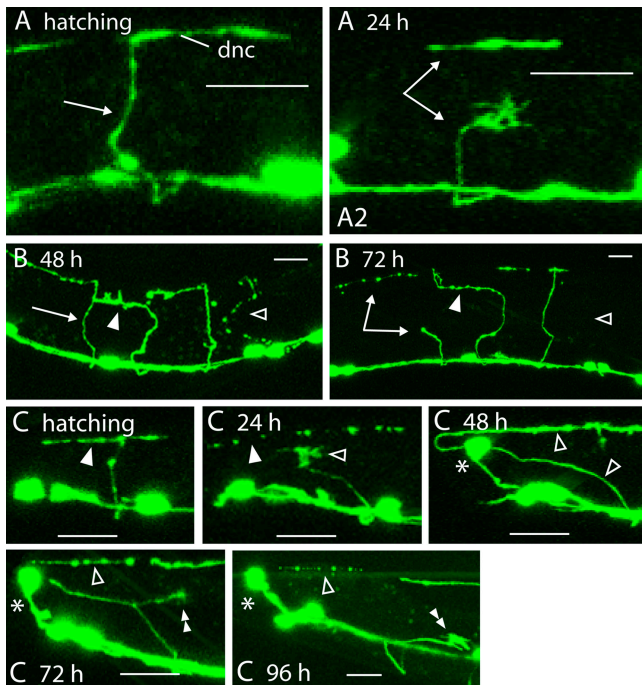


Figure 3. Individual axons break and reinitiate growth in neurons that lack β -spectrin. (A) A representative *unc-70(s1502)* animal imaged immediately after hatching and again after an additional 24 h. Arrows indicate the DD4 axon, which has reached the dorsal nerve cord (dnc) correctly at hatching. At 24 h, this axon has broken, retracted, and initiated a new growth cone (bottom arrow), leaving behind a fragment in the dorsal nerve cord (top arrow). (B) A single *unc-70(s1502)* animal imaged 48 h after hatching and again after an additional 24 h. Arrows indicate a D-type axon, which is essentially normal at 48 h and has broken and retracted at 72 h, leaving a fragment in the dorsal nerve cord. However, this neuron has not initiated a new growth cone. Closed arrowheads indicate a second D axon, which appears to be actively growing at 48 h (presumably as a result of an earlier break) and to have reached the dorsal nerve cord at 72 h. Open arrowheads indicate a third D axon, which appears to be degenerating at 48 h, presumably as a result of a break near the ventral nerve cord. This axon has completely disappeared at 72 h. (C) A single *unc-70(s1502)* animal imaged immediately after hatching and again every 24 h; the DD6 neuron is shown. At hatching, the DD6 neuron has normal morphology: its commissure reaches the dorsal nerve cord and extends along it in both directions (closed arrowhead). At 24 h, the axon has broken, and the distal end is in the process of degenerating (closed arrowhead). The proximal end has initiated a new growth cone (open arrowhead). At 48 h, the secondary growth has reached the dorsal cord, albeit by an aberrant posterior route (open arrowheads). [The new dorsal and posterior neuron is DVB [asterisks], which arises postembryonically and sends an axon ventrally to join the ventral nerve cord]. At 72 h, the second axon has broken, and the distal end has begun to degenerate (open arrowhead). The proximal end has again initiated a new growth cone (closed double arrowhead). At 96 h, degeneration of the second axon is almost complete (open arrowhead). The third growth cone has failed to reach the dorsal nerve cord and instead has extended only a short distance anterolaterally (closed double arrowhead). Note that this last image is slightly rotated compared with the preceding four images. These animals express GFP in the GABA nervous system from the integrated transgene *oxls12[Punc-47:GFP]* and express β -spectrin in the skin from the integrated transgene *oxls95[Ppdi-2:UNC-70]*. Bars, 10 μ m.

Breaks are caused by a movement-generated strain

Breaks in neuronal processes that lack β -spectrin could be the result of two potential functions of β -spectrin in neurons. First, β -spectrin might be involved in the addition of membrane to axons during growth of the organism (Morris, 2001).

Because worms increase in length and circumference during development, failure to insert membrane into axons could cause them to break. Alternatively, β -spectrin might protect neurons against the acute strains caused by movement. To distinguish between these possibilities, we assayed axotomy in β -spectrin mutant animals that were paralyzed. *unc-54* encodes a muscle myosin (MacLeod et al., 1981), and RNAi of this gene results in completely paralyzed animals. Furthermore, RNAi of *unc-54* in β -spectrin mutants results in animals that are longer than controls (Hammarlund et al., 2000). Thus, if movement is causing axotomy, RNAi of this muscle myosin should alleviate the phenotype because the animals move less. Conversely, if growth is causing axotomy, *unc-54*/myosin RNAi should exacerbate the phenotype because the animals are longer.

We found that RNAi of *unc-54*/myosin largely rescued the neuronal defects of animals lacking β -spectrin (Fig. 4; see section RNAi in Materials and methods). *unc-70* L4-stage larvae had a mean of 4.8 axonal defects per animal. In contrast, *unc-70* L4-stage larvae animals treated with *unc-54* double-stranded RNA had a mean of 1.3 defects per animal ($P < 0.0001$ compared with control plasmid in a two-tailed *t* test). RNAi of *unc-54*/myosin not only reduced truncated and broken axons but also reduced the incidence of growth cones and branching. The lack of new growth cones further supports the idea that excess and aberrant neuronal growth in animals that lack β -spectrin is a secondary effect of breakage rather than hypertrophic axon growth. RNAi of *unc-54*/myosin had no effect on the nervous system of wild-type animals. To demonstrate that the suppression of axon breakage was the result of paralysis rather than a specific effect of *unc-54* perturbation or the process of RNAi, we tested the effect of *unc-22* mutations. *unc-22* encodes the muscle protein Twitchin, and mutant animals are unable to initiate coordinated movement or deep body bends (Benian et al., 1989). We found that the genetic loss of Twitchin in the β -spectrin mutant background resulted in paralyzed animals with fewer neuronal defects than β -spectrin mutants alone (number of normal commissures: *unc-70* = 2.7 ± 0.4 ; *unc-70*; *unc-22* = 6.7 ± 0.5 ; $n = 10$ each; $P < 0.0001$ in a two-tailed *t* test). Thus, neurons in animals that lack β -spectrin are sensitive to strain caused by movement rather than growth. These data suggest that β -spectrin does not function in the process of neuronal membrane addition during organismal growth. Rather, β -spectrin protects neurons against breakage caused by movement-induced strain.

Neuronal elasticity in health and disease

In *C. elegans*, β -spectrin is not essential for many neuronal functions, including growth cone migration and axon elongation. Disorganization of axon architecture is caused by breaks in axons followed by error-prone outgrowth. Similar to the worm mutants, β -spectrin mutants in *Drosophila melanogaster* exhibit disorganized axon tracts (Garbe et al., 2006). Garbe et al. (2006) note that pioneer neurons initially grow out normally but that later, axon projections exhibit midline crossing defects. The loss of β II-spectrin in mice also results in nervous system defects, and the homozygous mutants die in utero (Tang et al., 2003). It is interesting to speculate that these defects could

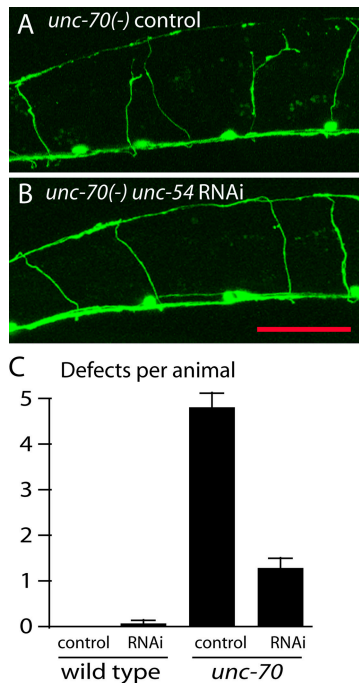


Figure 4. *unc-54* (muscle myosin) RNAi rescues neuronal defects in *unc-70* animals. All animals are expressing GFP in the GABA nervous system from the integrated transgene *oxls12[Punc-47:GFP]*. (A) Representative *unc-70[s1502]; oxls12* animal raised on bacteria containing an empty RNAi feeding plasmid (control). (B) Representative *unc-70[s1502]; oxls12* animal raised on bacteria containing an *unc-54* RNAi feeding plasmid. Bar, 50 μ m. (C) Comparison of the number of neuronal defects per animal. Wild-type *oxls12* and *unc-70[s1502]; oxls12* animals were treated with an empty RNAi vector (control) or were depleted of UNC-54 myosin (*unc-54* RNAi). Bars represent the mean, and error bars represent SEM. $n = 15$ wild-type control, 15 wild-type *unc-54* RNAi, 36 *unc-70* control, and 44 *unc-70 unc-54* RNAi.

be caused by breaks followed by error-prone outgrowth as in the worm.

In *C. elegans*, breaks occur in processes of β -spectrin mutants because of sensitivity to acute strain generated by muscle contraction. Neuronal processes may be particularly vulnerable to membrane lesions compared with other cells because a membrane rupture of such a slender process is more likely to result in a break that cannot be repaired by conventional membrane repair (McNeil and Kirchhausen, 2005). Thus, β -spectrin may be particularly important in neurons for strain resistance. Consistent with this, muscle and epithelial cell membranes do not appear to degenerate in *C. elegans* animals that lack β -spectrin, and β -spectrin is expressed more strongly in neurons than other tissues (Hammarlund et al., 2000; Moorthy et al., 2000). The elastic properties of cells may arise from the intrinsic ability of spectrin to reversibly unfold in response to mechanical pulling (Rief et al., 1999). Red blood cells must withstand deformation and shear as they pass through arterioles, and β -spectrin is required for this membrane elasticity (Lux and Palek, 1995; Lee and Discher, 2001). Similarly, neurons can exhibit a remarkable amount of elasticity, stretching up to 65% of their length without breaking (Smith et al., 1999). Thus, a role for spectrin in neuronal elasticity is consistent with its known function in membrane elasticity in erythrocytes.

β -Spectrin is abundant in the vertebrate brain and is found in both the axonal and dendritic processes of all neurons (Levine and Willard, 1981; Bennett et al., 1982). Interestingly, intermediate filaments, which were long thought to function in neuronal integrity, do not play a major function in integrity but rather determine neuronal caliber (Elder et al., 1999). Thus, the elasticity of vertebrate neurons may also be determined in large part by β -spectrin and the membrane skeleton. In sick or injured neurons, activated calpain targets the spectrin-based membrane skeleton for proteolysis (Baudry et al., 1981; Czogalla and Sikorski, 2005); thus, it is possible that neuronal degeneration after traumatic injury may be caused secondarily by the loss of the spectrin membrane skeleton (Buki and Povlishock, 2006).

Finally, some inherited neuronal diseases are the direct result of mutations in β -spectrin (Bennett and Baines, 2001; Luo and O'Leary, 2005). For example, a form of inherited progressive spinocerebellar ataxia (SCA5), which affected the family of former President Abraham Lincoln, has been recently linked to mutations in β III-spectrin (Ikeda et al., 2006). Mutations in β III-spectrin are associated with a mislocalization of the glutamate transporter EAAT4 and the receptor GluR δ 2, which suggests that the disease results in a defect in protein trafficking (Ikeda et al., 2006). On the other hand, it is possible that this neurodegenerative disorder is caused by axonal breakage similar to the progressive disorder observed in *C. elegans* mutants. It is particularly interesting that spinocerebellar ataxia is marked by a delayed onset; the slow progression and variable phenotype of the disease could be caused by the slow accumulation of axonal breaks. Although we have emphasized the neuroprotective role of spectrin in the differentiated nervous system, this does not preclude a role for spectrin during development. It is possible that targeted removal of the spectrin cytoskeleton is regulated during normal development to initiate the degenerative mechanisms used to prune unneeded axons (Luo and O'Leary, 2005).

Materials and methods

Strains

All animals that were assayed were the progeny of homozygous hermaphrodites of the appropriate genotype. *unc-70[s1502]*, *unc-22[e66]*, and *oxls12* were previously described (Hedgecock et al., 1983; Benian et al., 1989; Johnsen and Baillie, 1991; McIntire et al., 1997; Hammarlund et al., 2000). *oxEx81* expresses 40 amino acids of GAP-43 fused to GFP under the control of the *acr-5* promoter (a gift of J.-L. Bessereau, Institut National de la Santé et de la Recherche Médicale, Paris, France). *oxls95* expresses UNC-70 in the hypoderm under the control of the *pdi-2* promoter (Johnstone, 2000). It was integrated by x-ray irradiation and mapped to linkage group IV. Strains were obtained from the *C. elegans* Genetics Center and were maintained at 20–23°C on plates seeded with HB101.

Quantitation of embryonic DD neuronal phenotypes

Embryos between threefold and hatching were mounted on 3% agarose pads under a coverslip and were imaged with a confocal microscope (MicroRadiance; Bio-Rad Laboratories) using a 60 \times NA 1.4 planApo lens (Nikon). Each visible neuron was scored for morphology and assigned to one of the following five classes: reached dorsal cord and formed a T; reached dorsal cord and formed an L; anvil-shaped growth cones; round growth cones with radial protrusions; and defects (breaks, aberrant branching, or wandering growth). Scoring was performed blind to genotype (wild-type *oxls12* or *unc-70[s1502]; oxls12*) and was repeated twice with similar results. For wild-type animals, 32 embryos and 155 neurons were scored, yielding the following counts: T = 94, L = 12, anvil = 35,

round = 12, and defects = 2. For *unc-70(s1502)* animals, 55 embryos and 192 neurons were scored, yielding the following counts: T = 118, L = 35, anvil = 20, round = 13, and defects = 6.

Quantitation of larval DD neuronal phenotypes

Worms of the appropriate age and genotype (wild-type *oxls12* and *unc-70(s1502)*; *oxls12*) were mounted on 3% agarose pads, paralyzed with 5 mM sodium azide in M9 under a coverslip, and imaged by epifluorescence microscopy or confocal microscopy. Each neuron was scored for defects in axonal morphology and assigned to one of the following four classes of increasing severity: intact commissure, broken commissure, ectopic growth cone, and hypertrophic branching. In a few cases, neurons displayed multiple phenotypes. To avoid double counting these neurons, the phenotypes were ordered in presumed developmental time from early to late: first, broken commissure; second, ectopic growth cone; and last, hypertrophic branching. Neurons with multiple phenotypes were then assigned to the latest class only.

In addition to collecting data on commissures, we also collected data on migration along the dorsal cord. We observed a substantial increase in persistent L morphologies in *unc-70* larvae. Although these morphologies are common in wild-type embryos, they are rare in newly hatched animals and were never observed in the wild type by 24 h after hatching. However, we could not distinguish whether this phenotype was independent of commissure defects and, therefore, limited our analysis to the commissures.

To determine whether degeneration also occurred in acetylcholine neurons and to ensure that degeneration was not caused by the *oxls12* transgene, we also scored commissures in acetylcholine motor neurons. *oxEx81(Pacr-5:GAP-43-GFP)* expresses GFP in the DB acetylcholine motor neurons that extend commissures to the dorsal nerve cord. In addition, *oxEx81(Pacr-5:GAP-43-GFP)* expresses GFP in the lateral CAN neuron, in the FLP and SAB neurons, and in other neurons in the head and tail. The processes of these neurons are oriented along the anterior-posterior axis unlike the dorsal-ventral commissures. We found that although some normal commissures were visible in newly hatched *unc-70(s1502)*; *oxEx81(Pacr-5:GAP-43-GFP)* L1 animals, by the L4 stage, the commissures had degenerated ($n > 10$ for each larval stage). We observed a similar degree of degeneration in the lateral processes and in the sensory dendrites. Thus, degeneration occurs in acetylcholine as well as in γ -aminobutyric acid (GABA) neurons, in processes oriented on the anterior-posterior axis as well as those on the dorsal-ventral axis, and in sensory dendrites as well as axons.

Longitudinal studies

Because *unc-70(s1502)* animals are very sick, we performed these experiments in an *unc-70(s1502)* strain that carries the *oxls95* transgene, which expresses β -spectrin under the control of a skin promoter. These animals are healthier than *unc-70(s1502)* alone but have similar neural defects. Newly hatched L1 *unc-70(s1502)*; *oxls12*; *oxls95* worms were mounted individually on 3% agarose pads under a coverslip in 5 μ l of 10 mM muscimol in M9; muscimol was used to anesthetize worms because worms recovered from these slides quickly and resumed normal behavior and growth. Images of VD and DD neurons were taken with a confocal microscope. After imaging, worms were recovered from slides and returned to small agar plates seeded with HB101. At 24-h intervals, the process was repeated for a total of 3–5 d. In all, 12 worms were analyzed.

RNAi

Animals of genotypes *oxls12* and *unc-70(s1502)*; *oxls12* were raised at 22°C on the bacterial strain HT115 containing an empty RNAi feeding plasmid (–; L4440) or an *unc-54* RNAi feeding plasmid (+; L4129; 1999 Fire Lab Vector kit; Addgene) as described previously (Kamath et al., 2001). L4 F1 hermaphrodites were selected and scored blind to genotype by counting DD and VD neuronal defects. 15 wild-type animals were scored under each condition; 44 *unc-70* animals were scored on *unc-54* RNAi, and 36 were scored on empty vector. This experiment was performed twice with similar results. The effect of RNAi on *unc-70* length was determined by measuring L4 animals at their midline. RNAi significantly increased length (L4 length of *unc-70* = 488.9 μ M, $n = 16$; L4 length of *unc-70*; *unc-54* RNAi = 555.3 μ M, $n = 18$; $P = 0.0003$ using a two-tailed t test).

We also paralyzed animals of genotypes *oxls12* and *unc-70(s1502)*; *oxls12* by introducing the *unc-22(e66)* allele. Although *unc-22(e66)* animals twitch—that is, experience minor, random contractions of individual muscles—they cannot coordinate contraction and, therefore, do

not generate deep body bends. We found that although *unc-70(s1502)*; *oxls12* animals are usually observed with deep body bends, *unc-22(e66)*; *unc-70(s1502)*; *oxls12* animals have an extended posture. To assess the effect of *unc-22*-dependent paralysis of neuronal morphology, L4 animals were scored by counting the number of commissures that extended from the ventral to the dorsal cord without wandering or branching. Counts were performed blind to genotype. We also attempted to paralyze *unc-70* animals with genetic mutations in *unc-54*, *unc-31*, and *unc-18* and by using the drug levamisole (Aurelio et al., 2002; Bulow et al., 2004). However, these treatments were lethal in combination with *unc-70(s1502)*; *oxls12*, precluding these experiments.

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