Axon injury and stress trigger a microtubule-based neuroprotective pathway

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Axon injury elicits profound cellular changes, including axon regeneration. However, the full range of neuronal injury responses remains to be elucidated. Surprisingly, after axons of Drosophila dendritic arborization neurons were severed, dendrites were more resistant to injury-induced degeneration. Concomitant with stabilization, microtubule dynamics in dendrites increased. Moreover, dendrite stabilization was suppressed when microtubule dynamics was dampened, which was achieved by lowering levels of the microtubule nucleation protein γ-tubulin. Increased microtubule dynamics and global neuronal stabilization were also activated by expression of expanded polyglutamine (poly-Q) proteins SCA1, SCA3, and huntingtin. In all cases, dynamics were increased through microtubule nucleation and depended on JNK signaling, indicating that acute axon injury and long-term neuronal stress activate a common cytoskeleton-based stabilization program. Reducing levels of γ-tubulin exacerbated long-term degeneration induced by SCA3 in branched sensory neurons and in a well established Drosophila eye model of poly-Q–induced neurodegeneration. Thus, increased microtubule dynamics can delay short-term injury-induced degeneration, and, in the case of poly-Q proteins, can counteract progressive longer-term degeneration. We conclude that axon injury or stress triggers a microtubule-based neuroprotective pathway that stabilizes neurons against degeneration.

Many animals generate a single set of neurons that must function for the entire life of the individual. Each neuron typically has a single axon that transmits signals to other neurons or output cells such as muscle. As axons can extend long distances, they are at risk for injury, and, if the single axon is damaged, the cell can no longer function. Many neurons thus mount major responses to axon injury. The best characterized of these responses is axon regeneration, the process in which a neuron extends the stump of the existing axon or grows a new axon from a dendrite (1–3).

In addition to the regenerative response, axon injury can cause other less well-understood changes. For example, in mammalian dorsal root ganglion cells, injury of the peripheral axon causes a transcriptional response that increases the capacity of the central axon to regenerate if it is subsequently injured (4, 5). In Drosophila sensory neurons, axon injury causes cytoskeletal changes in the entire dendrite arbor, specifically the number of growing microtubules is up-regulated (6). In this study, we investigated the functional significance of the cytoskeletal changes in the dendrite arbor. We present results that suggest the altered microtubule dynamics in dendrites acts to stabilize them, and thus axon injury seems to trigger a neuroprotective pathway that acts on the rest of the cell. However, this neuroprotective pathway is turned on only transiently after axon injury and subsides as axon regeneration initiates.

Axon injury is a very acute neuronal stress. Neurons are also subject to a variety of long-term stresses that have major implications for human health. For example, many forms of neurodegenerative disease, including Alzheimer’s and Parkinson diseases, manifest after long periods in which the neurons survive under stress. These long-term stresses include accumulation of misfolded proteins or protein aggregates inside or outside the cell (7). One such set of misfolded protein diseases is CAG-repeat or polyglutamine (poly-Q) repeat diseases (8), including Huntington disease and many forms of spinocerebellar ataxia (SCA). In these diseases stretches of CAG nucleotides in the coding region of specific proteins are expanded in the genome. This results in proteins with long poly-Q spans, which, over time, cause neurodegeneration.

Quite unexpectedly, we found several chronic stresses, including expression of long-poly-Q–containing proteins, induced the same type of cytoskeletal changes as axon injury. We therefore hypothesized that long-term axon stress might trigger the same type of microtubule-based stabilization pathway as acute axon stress. We found evidence to support this hypothesis by examining long-term degeneration in neurons that expressed poly-Q proteins. In this assay, increased microtubule dynamics acted to slow the course of degeneration. The microtubule-based stabilization pathway we describe thus represents an endogenous neuroprotective response to axon stress. This neuroprotective response is turned on transiently after axon injury and for longer periods of chronic stress.

Results

Axon Injury Stabilizes Dendrites. To determine whether axon injury might turn on a pathway to stabilize distant regions of a neuron, we developed an assay to probe dendrite stability after axon injury. We previously showed that dendrites of Drosophila larval sensory neurons are cleared rapidly after they are severed from the cell body (9). We reasoned that, if axon injury turned on a stabilization pathway, this might slow down dendrite degeneration after severing. To test this idea, we used a pulsed UV laser to sever axons of GFP-labeled Drosophila dendritic arborization (da) neurons (SI Materials and Methods includes information about these neurons) in intact animals, and tracked dendrite clearance after severing at subsequent time points.

When dendrites of the ddaE neuron were severed immediately after axons, all dendrites were cleared by 18 h after injury (Fig. 1A and B), as in neurons without previous axon injury (9). However, when axons were severed 8 or 24 h before dendrite severing, more than half of dendrites remained 18 h after they were cut from the cell body (Fig. 1A and B). This result is consistent with the hypothesis that axon injury stabilizes dendrites. When the time between axon and dendrite severing was increased to 48 h, the stabilization effect was reduced. We also tested whether this stabilization pathway could act on a much larger dendritic arbor. The ddaE neuron (Fig. L4) has a small arbor and ddaC has a large arbor, but was also stabilized by previous axon injury (see Fig. S2G). In these experiments, axons were severed near the cell body. When ddaC axons were severed 50 μm or more

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subsequent physical trauma or (i) that axon injury might turn on a general protective pathway. We therefore used animals heterozygous for a null mutation in Tub23C, the major somatic nucleation protein, reduced comet formation so that normal cellular functions would be unaffected. We therefore used animals heterozygous for a null mutation in Tub23C and had normal numbers of dynamic microtubules (shown for heterozygote in Fig. S2D); after injury, the increase in microtubule dynamics in dendrites was reduced at 8 h (Fig. 2D) and 24 h (Fig. 2E). When we tested heterozygotes for dendrite stabilization after axon injury (experiment schematic shown in Fig. 2B), we found that it was impaired compared with control (%P < 0.05, **P < 0.005, and ***P < 0.0001). (C) Class IV neurons were labeled with the membrane marker mCD8-GFP under control of ppk-Gal4. The axon of a single ddaC neuron per animal was severed shortly before the onset of pupariation. Pupae were imaged 18 h after pupa formation (APF), and the presence of dendrites was scored. In each animal (n = 9), only the injured cell retained dendrites. An example of two neighboring cells in a pupa is shown. The cell body at left has an axon emerging ventrally (to-ward bottom), but no dendrites are seen dorsal to the cell body. In contrast, its neighbor, at right, has an elaborate dendrite arbor.

from the cell body, dendrite clearance after later dendrite severing was also delayed (Fig. S1). Thus, both proximal and distal axotomy resulted in a cellular response that slowed injury-induced dendrite degeneration.

We next considered two possibilities: (i) that axon injury might turn on a response to specifically protect the neuron against subsequent physical trauma or (ii) that axon injury might turn on a more general stabilization pathway. To test for a general stabilization pathway, we asked whether axon injury stabilized dendrites against developmental degeneration. Many of the da neurons are pruned during metamorphosis and then regrow dendrite arbors into the remodeled adult body wall (10). To test whether axon injury could delay pruning of the ddaC neuron, we severed axons as larvae were preparing to pupariate. We then assayed for complete clearance of dendrites 18 h after pupariation had initiated. In all cases in which axons were injured first (n = 9), dendrites remained (Fig. 1C). All dendrites of uninjured cells in these animals (approximately 10 per animal) were successfully pruned. Thus, axon injury stabilized dendrites against both injury-induced degeneration and developmental pruning, suggesting that it turns on a general protective pathway.

Microtubule Nucleation Plays a Role in Dendrite Stabilization. We found previously that the number of growing microtubules is up-regulated in dendrites after axon injury (6). To test whether this change in microtubule dynamics might be related to dendrite stabilization, we compared the timing of these two responses. We assayed the number of dynamic microtubules by expressing the microtubule plus-end-binding protein EB1 tagged with GFP and assaying EB1 comet number before and after axon injury. Each EB1-GFP comet marks the plus end of a growing microtubule (11), and so the number of comets is a readout of the number of growing microtubules. The number of comets in dendrites peaked at 24 h after axon injury and decreased thereafter (Fig. 2A), similar to dendrite stabilization (Fig. 1B).

To further test the relationship between microtubule dynamics and dendrite stabilization, we had to identify the machinery that regulated microtubules in response to axon injury. We hypothesized that either new nucleation of microtubules or severing of existing microtubules could lead to an increase in growing microtubule plus ends labeled by EB1-GFP. We therefore tested whether reduction of nucleation or severing proteins by RNAi could block injury-induced microtubule dynamics. Large RNA hairpins were expressed in the ddaE neurons together with EB1-GFP by using the Gal4-UAS system. Compared with a control hairpin targeting γTub37C, a maternal γ-tubulin that does not play a major role in somatic cells (12), only the hairpin targeting γTub23C, the major somatic nucleation protein, reduced comet number in the cell body 24 h after axon injury (Fig. S2A). We concluded that nucleation contributed to increased microtubule dynamics in response to axon injury.

To determine whether microtubule dynamics played a functional role in dendrite stabilization, we performed sequential axon and dendrite severing in genetic backgrounds with reduced nucleation. We wished to only partially reduce microtubule nucleation so that normal cellular functions would be unaffected. We therefore used animals heterozygous for a null mutation in γTub23C (γTub23C<413-2>+/+) or expressed RNA hairpins to reduce γTub23C. Uninjured neurons in both cases appeared normal and had normal numbers of dynamic microtubules (shown for heterozygote in Fig. S2D); after injury, the increase in microtubule dynamics in dendrites was reduced at 8 h (Fig. 2D) and 24 h (Fig. 2E). When we tested heterozygotes for dendrite stabilization after axon injury (experiment schematic shown in Fig. 2B), we found that it was impaired compared with control (i.e.,yw) animals (Fig. 2E). To make sure that the phenotype was caused by reduction of γTub23C levels and was cell-autonomous, we performed a rescue experiment with GFP-tagged γTub23C (Fig. 2E). We were not able to confirm this result with γTub23C RNAi (Fig. S2F), perhaps because partial knockdown of γTub23C was simply not enough to abrogate protection. Consistent with this idea, protection was also not disrupted 24 h after axon injury in the γTub23C heterozygous background (Fig. S2F). We therefore confirmed that regulation of microtubules was involved in dendrite stabilization by using an independent approach to disrupt microtubule growth. msp5, or XMAP215, is proposed to be
A microtubule polymerase (13), and RNAi targeting mps eliminates EB1-GFP comets in neurons (6). We therefore used mps RNAi to perturb the behavior of neuronal microtubule plus ends. This condition blocked the protective effect of axon injury on dendrites in ddaE (Fig. 2E) and ddaC (Fig. S2G) neurons. We conclude that global changes in microtubule dynamics and dendrite stabilization are activated by injury to generate a response.

**Axon Regeneration Is Not Dependent on Up-Regulation of Microtubule Dynamics.** One of the major injury responses downstream of JNK is axon regeneration. We therefore wished to determine the relationship among axon regeneration, dynamic microtubules, and dendrite stabilization. After proximal axotomy of ddaE, axon regeneration from a dendrite was unperturbed in γTub23C heterozygotes or when RNAi was used to target γTub23C (Fig. S3A and B). Similarly, regeneration after distal axotomy of ddaC was normal in γTub23C RNAi neurons (Fig. S3C). Thus, axon regeneration does not depend on γTub23C in the same way as dendrite stabilization. Importantly, the ability of neurons to initiate axon regrowth when γTub23C levels were reduced indicates that these neurons were not generally sick or unable to respond to injury.

We also considered that altered microtubule dynamics might be required to remodel dendrites after axon injury. In mammalian neurons in vivo, dendrite simplification has been documented after axon injury (18–20). We monitored dendrite shape in ddaC neurons after distal axotomy and found that fine dendrite branches were trimmed back after distal axon severing (Fig. S3D), just as in mammals. Most of this trimming occurred between 24 and 72 h after axotomy, and was unchanged when γTub23C levels were reduced (Figs. S3D and S4). To compare timing of the responses to axon injury, a timeline is shown in Fig. 2H.

**Reduction of an Axonal Motor or Expanded Poly-Q Protein Expression Triggers Increased Microtubule Nucleation.** Thus far, we have demonstrated that axon injury turns on a pathway that transiently stabilizes dendrites after injury. This pathway seems to be turned off when regeneration begins. We also wished to know whether this type of stabilization pathway could be turned on for more extended times to perhaps protect neurons from long-term degeneration. We tested two types of chronic stress: reduced unc-104 and expanded poly-Q proteins. In both cases, we assayed microtubule dynamics or stabilize dendrites, and found that it was not (Fig. S2F and C). Thus, although JNK signaling is required for these injury responses, it most likely requires other pathways that are activated by injury.

When human expanded poly-Q proteins are expressed in fly eyes, they can cause degeneration (24–26). We expressed three matched pairs of short and expanded poly-Q proteins in Drosophila. When human expanded poly-Q proteins are expressed in fly eyes, they can cause degeneration (24–26). We expressed three matched pairs of short and expanded poly-Q proteins in Drosophila.
number was slightly elevated at the normal incubation temperature (25 °C), consistent with previous results indicating the Q30 form has some deleterious effects (26). Both unc-104 RNAi and expression of SCA3tr-Q78 caused reduced complexity of the highly branched class IV ddaC neuron (Fig. S5). The effect of SCA3tr-Q78 on cell morphology is similar to that reported in a recent study (27).

To test whether increased microtubule dynamics resulting from unc-104 RNAi and poly-Q protein expression relied on microtubule nucleation and JNK signaling, we made strains that paired SCA3tr-Q78 with control RNA hairpins (γTub37C and Rtnl2), hairpins to target γTub23C, or bskDN. We used a similar strategy for unc-104. In both cases, expression of bskDN or the hairpin targeting γTub23C reduced microtubule dynamics compared with controls (Fig. 3 C and D and Movies S3, S4, S5, and S6). We conclude that, like axon injury, reduction of the unc-104 motor or expression of expanded poly-Q proteins caused neurons to up-regulate microtubule nucleation through a JNK-dependent pathway.

Expression of an Expanded Poly-Q Protein Stabilizes Dendrites Against Injury-Induced Degeneration. The activation of a common JNK-dependent stress response pathway by axon injury and poly-Q proteins suggested that poly-Q protein expression might also stabilize dendrites. To test this idea, we again used clearance of dendrites after severing from the cell body as a reporter of dendrite stability. In control ddaE neurons, most dendrites were cleared 18 h after severing from the cell body (Fig. 4.A and C). In contrast, more than half of dendrites in ddaE neurons expressing SCA3tr-Q78 were still present 18 h after removal from the cell body (Fig. 4 B and C). Expression of SCA3tr-Q78 also delayed degeneration in ddaC cells (Fig. S6A), and similar results were obtained with unc-104 RNAi (Fig. S6).

To determine the timing of increased microtubule dynamics and dendrite stabilization in larvae that expressed poly-Q proteins, we assayed both throughout larval life. Larvae were assayed 1, 2, and 3 d after hatching (Fig. 4 C). On the first day, both microtubule dynamics and dendrite stabilization were similar to control levels, presumably because poly-Q proteins had not accumulated at high enough levels to trigger a response. Microtubule dynamics and protection levels were similar to those of injured neurons at days 2 and 3 (Fig. 4 C and D).

For technical reasons, after axon injury, we only assayed stabilization and microtubule dynamics in dendrites. In poly-Q-expressing neurons, we were able to ask whether both responses also affected axons. Poly-Q protein expression increased the number of dynamic microtubules in axons, and delayed axon beading after severing (Fig. 4 D), suggesting that this is a very general pathway that can affect all parts of the cell.

Microtubule Nucleation Reduces Neurodegeneration Induced by Poly-Q Proteins. Thus far, we assayed neuronal stabilization by monitoring the timing of axon or dendrite disassembly after removal from the cell body. We also wished to determine whether dynamic microtubules could counteract longer-term progressive degeneration. We therefore compared dendrite retraction in SCA3tr-Q78—expressing ddaC neurons with normal and reduced levels of nucleation. Neurons expressing a control RNA hairpin (Rtnl2) or γTub23C RNAi had complex dendrite arbors that increased the number of branches during larval life (Fig. 5). Similarly, neurons expressing SCA3tr-Q78 with Rtnl2 RNA hairpins had complex dendrite arbors that increased in branching during larval life (Fig. 5). However, when the γTub23C hairpin RNA was paired with SCA3tr-Q78, dendrite complexity was reduced as larvae aged, and, in late larvae, only the main dendrite trunks were present (Fig. 5 and Fig. S7A). Strikingly similar results were found when unc-104 RNA hairpins were paired with a control RNA hairpin or γTub23C hairpin (Fig. S7B).

It is important to note that dendrite complexity was also reduced when SCA3tr-Q78 or unc-104 RNAi was expressed in the absence of additional RNA hairpins (Fig. S5). This is most likely because all the transgenes, including SCA3, rely on the Gal4-UAS expression system. The reduction in phenotype severity when additional transgenes are added is consistent with dilution of the Gal4 protein between more UAS-driven transgenes. We therefore controlled for transgene number in our experiments.

To test whether the microtubule-based stabilization pathway acted to globally protect from poly-Q–induced neurodegeneration in adult neurons, we used a well-established neurodegeneration model. Expression of human proteins that induce degeneration in Drosophila eyes has proven to be a powerful system in which to study neurodegeneration (28). Expression of SCA3tr-Q27 or γTub23C RNA hairpins in photoreceptors did not disrupt eyes (Fig. S8). Expression of SCA3tr-Q78 caused a variety of eye phenotypes including lack of pigmentation (mild), eye collapse (moderate), and appearance of completely disrupted black areas on the eye (severe). Phenotypes were slightly more severe in males than females. When microtubule nucleation was reduced in the background of SCA3tr-Q78 expression, severe
disruption of eye morphology was very common (Fig. S8). This result is consistent with poly-Q proteins turning on a stabilization pathway that counteracts progressive degeneration (diagram in Fig. S8A). We conclude that microtubule-based neuroprotection can counter degeneration in different types of neurons in larvae and adults.

Discussion

We have shown that axon injury, reduction of a synaptic vesicle motor, or expression of expanded poly-Q proteins triggers dramatic changes in the cytoskeleton. Each of these cellular stresses results in a huge increase in the number of dynamic microtubules throughout the neuron. Moreover, in all cases, this increase in dynamic microtubules is blocked if levels of a microtubule nucleation protein are reduced. We therefore propose that axon injury, as well as more long-term neuronal stresses, triggers a global cellular response that alters microtubules. This response is mediated by JNK signaling, suggesting that these three diverse stresses activate a common pathway with cytoskeletal regulation as its output.

After axon injury, the increase in microtubule dynamics was transient, and it tapered off by 48 h after the injury. Dendrite stabilization followed the same time course. This time course is also similar to protection after axon injury in a recently described model for conditioning lesion in Drosophila (29). Although dependence on JNK could not be tested in this model, an upstream JNK regulator, wallenda, was required (29), hinting that there may be mechanistic overlap between these protective pathways.

In contrast to the brief activation of protection after axon injury, expression of SCA3tr-Q78 resulted in increased microtubule dynamics and dendrite stabilization over several days. The extended activation of this pathway suggested that it might be able to protect against long-term degeneration. We found support for this idea in both da neurons and eyes: in both cases, SCA3tr-Q78–induced degeneration was more severe when microtubule nucleation was partially reduced.

Fig. 4. Expression of SCA3tr-Q78 delays injury-induced dendrite degeneration. (A–C) The comb dendrite of ddaE neurons expressing mCD8-RFP alone (A) or with SCA3tr-Q78 (B) was severed in whole living animals with a pulsed UV laser (arrowheads) at different times after larval hatching. The presence of the distal dendrite was scored 18 h later (C, Right). Example images in A and B are from larvae severed at 2 d after hatching. Comet number in uninjured neurons expressing SCA3tr-Q78 was scored (C, Left). Significance was calculated with a Fisher exact test. Numbers above bars are numbers of neurons tested. (D) Control larvae expressing mCD8-GFP under control of 477-Gal4 or flies expressing SCA3tr-Q78 in addition to mCD8-GFP had axons of the ddaC neuron severed at 0 h. Pictures are images of neurons 12 h after severing. Arrows indicate cut sites. The graph shows the percentage of animals that had smooth unbeaded axons 12 h after severing. Numbers above the bars are the numbers of animals in each group. Statistical significance was calculated with a Fisher exact test. The number of EB1-GFP comets was quantitated as in dendrites in the genotypes indicated. (E) Results from the figure are summarized in a timeline.

Fig. 5. Reduction of microtubule nucleation increases dendrite degeneration in SCA3tr-Q78–expressing neurons. (A) Morphology of ddaC neurons expressing dicer2 and mCD8-GFP with hairpin RNAs only (Upper) or hairpin RNAs with SCA3tr-Q78 (Lower) was assayed over 4 d. Images from the final day are shown. (B) Complexity of ddaC dendrite arbors was assayed over time by counting the number of branch points.
Based on these results, we speculate that this pathway might function in different scenarios. In the case of axon trauma, the stabilization pathway might prevent further damage during the initial response to the injury. During long-term neurodegenerative disease, activation of this stabilization pathway could extend the time that neurons can maintain their normal structure.

We do not yet know how increased microtubule dynamics might reinforce dendrites to protect them from long- and short-term degeneration. One possibility is that nucleation of new microtubules plays a structural role. Rebuilding microtubule rods down the center of the dendrite could prevent beading, which is an early step in most types of axon and dendrite degeneration (9, 30). In support of this idea, microtubule disassembly has been proposed to be an early step in Wallerian axon degeneration (31, 32) and developmental pruning of dendrites (33). In fact, for the developmental pruning pathway, a putative microtubule severing protein is required at an early step (34). Thus, microtubule nucleation could very directly battle the microtubule disassembly that is activated during pruning or degeneration. It is also possible that microtubules could contribute to dendrite stability less directly, for example by altering intracellular transport.

If increased microtubule dynamics are a conserved general feature of neuronal response to axon stress, it could be developed into a diagnostic tool for early stages of neurodegenerative disease, perhaps before overt dysfunction becomes obvious. Identification of this pathway also offers some novel ideas about therapies for neurodegenerative disease. Turning on this pathway earlier in patients who express expanded poly-Q proteins could delay the onset of symptoms. It is possible that other neurodegenerative diseases do not activate this protective pathway. If this is the case, then strategies to activate it could result in improvement of the disease course.

**Materials and Methods**

All imaging was performed in living whole-mount larvae or pupae expressing GFP-tagged proteins in neuronal subsets. Axon and dendrite severing was performed with a pulsed UV laser as described (6). For information about genotypes and additional experimental details see SI Materials and Methods.

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Supporting Information

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SI Materials and Methods

Dendritic Arborization Neurons as a System for Studying Neuronal Injury Responses. Dendritic arborization (da) neurons have highly branched dendrite arbors that tile the body wall. Based on the complexity of arborization, these neurons can be organized into different classes, with class I neurons having the simplest arbors and class IV having the most complex (1). They respond to a variety of stimuli and act as proprioceptors and nociceptors, sending their axons back to the central nervous system (2, 3). Their internal organization is similar to other Drosophila neurons (4, 5), but they are more accessible to imaging and injury, as their cell bodies and dendrites lie near the surface of the animal in the body wall. We have previously used in vivo UV severing to study dendrite degeneration and axon regeneration in these neurons (6, 7). Class I (ddaE) and class IV (ddaC) neurons were used in this study because relatively specific Gal4 drivers exist for labeling these cell types, and because they represent the simplest (class I) and most complex (class IV) types of da neurons. Information about specific genetic backgrounds and experiments is provided later.

Drosophila Stocks and Genetic Background. Drosophila RNA interference strains targeting the following genes were obtained from the Vienna Drosophila RNAi Center: Rtnl2 (33230), unc-104 (47171, 23465, 23464), γTub23C (19130, 107572), γTub37C (25271), and msp5 (21982). Human disease genes were expressed in da or photoreceptor neurons, including UAS-SCA3tr-Q27 (8149) and UAS-SCA3tr-Q78 (8150) from the Bloomington Drosophila Stock Center, UAS-SCA1-Q30/Q82 from Juan Botas (ref. 8; Baylor College of Medicine, Houston, TX), and UAS-httex1p-Q20/Q93 from Leslie M. Thompson (ref. 9; University of California, Irvine, Irvine, CA). The UAS-bskDN and UAS-bsk.A-Y transgenic lines were obtained from the Bloomington Drosophila Stock Center. We also generated double transgenic fly lines for this study, including UAS-Rtnl2 RNAi; UAS-unc-104 RNAi, UAS-γTub23C RNAi; UAS-unc-104 RNAi, UAS-SCA3tr-Q78; UAS-γTub23C RNAi, UAS-SCA3tr-Q78; UAS-γTub37C RNAi, UAS-SCA3tr-Q78; and UAS-Rtnl2 RNAi (this RNAi line was generated in our laboratory). The pWIZ vector carrying inverted repeats of Rtnl2 gene coding sequence (this RNAi line was provided later).

Axon Regeneration. To analyze axon regeneration after proximal axon severing, 221Gal4 was used to drive the expression of EB1-GFP and dicer2. This tester line was crossed to different Vienna Drosophila RNAi Center RNAi lines. At 24 h after proximal axon transaction, movies of EB1 comets in cell bodies of the injured cells were acquired as described (6). The average number of comets in three in-focus frames was used for quantitative analysis of the cell body increase in microtubule dynamics, or whirlpool.

Dendrite and Axon Injury. The injury-induced dendrite degeneration assay was performed in ddaE and ddaC neurons as described (6). In both cases, a single dendrite (comb dendrite in ddaE) was severed with a pulsed UV laser close to the cell body. An overview of the injured cell was acquired right after severing, and then the larvae was returned to food to recover at 20 °C before imaging at 18 h after injury to examine if the severed dendrite was removed. Dendrites that maintained integrity without signs of fragmentation or clearance were scored as remaining dendrites. Axon severing was performed in the same way as dendrites, and the ratio of unbeaded axons 12 h after severing was used as a readout of axon integrity. In the double cutting experiment, axon severing was performed together with dendrite severing or 8 h, 24 h, or 48 h before it.

Live Imaging of Drosophila Larvae. In most cases, fly embryos were collected on apple caps for 24 h at 20 °C before transferring to caps containing standard Drosophila media (food caps), and then were allowed to age at 25 °C for various periods of time. One-day-old larvae (48 h after egg laying) were used for all of the time-course assays requiring more than 48 h, whereas 2- or 3-day-old larvae were used for the rest. To achieve a lower expression level of SCA1-Q30, embryos were aged at 18 °C for 72 h before imaging. Whole larvae were mounted on a slide with a dry agarose cushion, and covered with a coverslip that was held in place with tape. For multiple-time point experiments, animals were released from the agarose after imaging and returned to a food cap. One neuron per animal was analyzed in all experiments. Live imaging was performed on an LSM510 confocal microscope (Zeiss) or an FV1000 confocal microscope (Olympus) at a frame rate of 2 s. ImageJ software was used to generate maximum-intensity projections and perform image analysis.

Microtubule Dynamics Analysis. To compare the number of EB1-GFP comets in different genotypes, we analyzed a 10-μm-long dendrite segment close to the cell body in ddaE neurons. The total number of EB1 comets within this area in three in-focus frames was used as an index of microtubule dynamics. Only mobile comets appearing in consecutive three frames were quantified.

Dendrite Pruning. Axon severing was performed in a larva after it emerged from the food to begin pupa formation, typically 2 to 3 h before becoming a white prepupa. Images were acquired right after severing to ensure that the axon was completely separated from its cell body. The larva was then maintained at 20 °C as it initiated pupariation. At 18 h after pupae formation, the pupal case was removed as described (10), and the presence of dendrites was scored on a confocal microscope.

Axon Regeneration. To analyze axon regeneration after proximal axon severing, 221Gal4 was used to drive the expression of EB1-GFP. Dicer2 and the hairpin RNA targeting γTub23C or γTub37C in ddaE neurons. Animals heterozygous for γTub23C142-2 were also examined without Dicer2 in the genetic background. Axons of ddaE neurons were severed close to the cell body in second instar larvae, and were immediately imaged before returning to food. Distal axon severing was performed in the same way except using ddaC neurons, and the injury was away from the cell body (~100 μm). Larvae were imaged every 24 or 48 h for as long as 96 h after axon injury. The length of the dendrite that initiated tip growth after proximal axotomy was measured right after axon
severing and at the last time point by using ImageJ software. The measurement was repeated on a nonregenerating dendrite as a readout of normal dendrite growth during larvae development. Tip growth beyond normal dendrite development was calculated. Axon regeneration in ddaC after distal axotomy was scored based on whether the axon stump initiated tip growth.

**Time-Lapse Recording of Dendrite Morphology.** To monitor dendrite morphology in ddaC neurons, 477Gal4, UAS-mCD8-GFP with or without Dicer2, depending on the presence of hairpin RNA, were crossed to different transgenic fly lines. A single ddaC neuron in early second instar larvae was followed for 4 d at 24-h time intervals without injury or 3 d after distal axon severing. The entire dendrite tree was imaged with a 40× oil immersion objective, and the images were pieced together as montages using Acobee Canvas11 software. The number of dendrite branch points was manually counted using the cell counter tool in ImageJ (http://rsbweb.nih.gov/ij/).

**Adult Drosophila Eye Degeneration.** For all genotypes studied, the same number of female virgins were crossed to male flies from APPL-GFP; longGMR-Gal4, and were raised at standard condition. The progeny from each cross were collected every day at 25 °C, and males were separated from females. Six-day-old adult flies were anesthetized with CO2 before scoring eye defects under an Olympus SZ61 light dissection microscope, and then were frozen at −20 °C. Images of fly heads were taken using SPOT Basic software connected to an Olympus SZX7 light dissection microscope.


![Fig. S1.](https://www.pnas.org/cgi/content/short/1121180109)

**Fig. S1.** Previous distal axotomy delays injury-induced dendrite degeneration. Dendrites of ddaC neurons were severed close to the cell body and complete clearance was assayed 18 h later (control); 17 of 17 were cleared. In another set of cells, axons were severed greater than 50 μm from the cell body and dendrites were severed 24 h later (lower row). In this case, the majority of cells had dendrites remaining 18 h later.
Further characterization of the microtubule-based program induced by axon severing. (A) The role of microtubule nucleation and severing proteins was tested in a whirlpool screen. EB1-GFP was expressed in many da neurons under control of 109(2)80 Gal4. Axons of ddaE neurons were severed at time 0 (arrows, Left), and the number of EB1-GFP comets (arrowheads, Right) in cell the cell body was quantified in single frames from movies acquired 24 h later. Only RNA hairpins targeting the somatic γTub23C significantly reduced comet number 24 h after axon injury. (B and C) To test whether activation of JNK was sufficient to drive increased microtubule dynamics and dendrite stabilization, we assayed both in neurons overexpressing bsk (bskOE) without axon injury. In neither case did extra JNK result in the same cellular response as axon injury. (D) To determine whether microtubule dynamics was reduced in neurons of uninjured γTub23CA15-2 heterozygotes, we assayed comet number in this background and a control (yw) background. The number of EB1-GFP comets in dendrites was indistinguishable in these two sets of animals. (E) The number of EB1-GFP comets in control and two different reduced nucleation backgrounds was analyzed 24 h after axon injury. This is similar to the data shown in Fig. 2D, except for the time after axon injury at which comet number was counted. In Fig. 2D, the assay was performed 8 h after axon injury, and here it is shown 24 h after axon injury. (F) The dendrite stabilization assay was performed in different genetic backgrounds at 8 h or 24 h after axon severing. This data can be compared with that in Fig. 2E. At 8 h after axon injury, dendrite protection was reduced in the γTub23C<sup>A15-2</sup> heterozygous background (Fig. 2E). However, by 24 h after axon injury, protection was no longer suppressed in this background as shown here. This is most likely because of a threshold issue—microtubule dynamics are higher at 24 h than 8 h in WT neurons (Fig. 2A)—so loss of one copy of γTub23C is not sufficient at this time. Similarly, γTub23C RNAi also did not block dendrite stabilization. (G) To reduce microtubule dynamics more strongly than in backgrounds with partial loss of γTub23C, we targeted msps by RNAi. At 8 h after axotomy, very little dendrite stabilization was observed in ddaE neurons (Fig. 2E) or ddaC neurons as shown here. Numbers above the bars are numbers of neurons assayed. Significance was determined with a Fisher exact test.
Fig. S3. Axon regeneration and dendrite simplification is normal in neurons with reduced levels of γTub23C. (A and B) Axons of ddaE neurons expressing EB1-GFP, dicer2, and hairpin RNAs, or, in the case of the γTub23C heterozygote, just EB1-GFP, were severed close (<20 μm) to the cell body. When axons are severed close to the cell body, regeneration initiates from a dendrite (1). In the example, 48 h after axon severing (arrow, site of cut), one of the dendrites started tip growth (asterisk). This dendrite continued to loop and grow over the next 2 d. Regeneration was similar in all genotypes tested, and was quantified by measuring the normalized growth from the tip of the dendrite that extended. Numbers above bars are numbers of cells, one per animal, that were tested. Error bars indicate SD. (C) The axon of ddaC neurons expressing EB1-GFP, dicer2, and different hairpin RNAs under control of 477-Gal4 was cut with a pulsed UV laser 50 to 100 μm from the cell body (arrow). In most cases, the axon stump initiated outgrowth by 48 h after injury as in the example shown. In all cases in which γTub37C (control) or γTub23C was targeted by RNAi, growth from the tip of the axon stump was initiated by 96 h. The numbers on the bars in the graph are the number of neurons analyzed for each genotype. (D) Axons of ddaC neurons expressing mCD8-GFP were severed greater than 50 μm away from the cell body (orange arrow), as axon regeneration initiates from the stump under these conditions. Confocal images of dendrites were acquired over the next 3 d. Example images of a control neuron and γTub23C A15-2 heterozygote are shown. In both cases, dendrite termini were lost.


Fig. S4. Dendrites retract after distal axotomy in control and γTub23C heterozygous neurons. This figure is a companion to Fig. S3D. Small regions of dendrite arbors are shown in Fig. S3D for simplicity; the full ddaC arbor is shown over time after axon severing here for reference.
Fig. 55. Reduction of an axonal motor or expression of SCA3tr-Q78 reduces dendrite complexity of ddaC. (A) Class IV ddaC neurons were visualized by expressing mCD8-GFP. Images shown were acquired 4 d after larval hatching. Neurons also expressed dicer2 and a control (Rtnl2) hairpin RNA or a hairpin RNA targeting unc-104. (B) SCA3tr-Q27 and SCA3tr-Q78 were expressed in class IV neurons together with mCD8-GFP. Images of ddaC neurons 4 d after larval hatching are shown. (C) Images similar to those in A and B were acquired for the same set of neurons over 4 d. “N” indicates the number of neurons tracked. Between imaging, animals were returned to normal growth media. The number of dendrite branch points was counted in each image, and numbers are shown in the graph.
Fig. S6. SCA3tr-Q78 delays degeneration in ddaC, as does unc-104 RNAi; unc-104 RNAi also delays degeneration in ddaE. (A) Flies from a tester line expressing UAS-mCD8-GFP in ddaC neurons were crossed to yw (control) flies or SCA3tr-Q78 flies. A dendrite of ddaC was severed with a pulsed UV laser, and presence of the dendrite was scored 18 h later. Very few dendrites remained in control animals. The frequency of dendrites that had not degenerated by 18 h was increased when SCA3tr-Q78 or unc-104 RNA hairpins were expressed. The number of animals assayed is indicated in the graph, and statistical significance was calculated with a Fisher exact test. (B) The comb dendrite of ddaE neurons expressing either SCA3tr-Q27 or unc-104 RNA hairpins was severed at 0 h. The presence of the dendrite was scored 18 h later. Numbers above the bars are numbers of neurons tested; statistical significance was calculated with a Fisher exact test.
Fig. S7. Expression of hairpin RNAs targeting γTub23C worsens degeneration in neurons expressing SCA3tr-Q78 or unc-104 RNA hairpins. (A) This is a companion to Fig. 6A, in which only day-4 dendrite arbors are shown. Examples of ddaC neurons expressing mCD8-GFP, dicer2, SCA3tr-Q78, and hairpin RNAs targeting Rtnl2 (control) or γTub23C are shown. Images were acquired each day after larval hatching. Animals were returned to food caps between imaging sessions. (B) Example images of ddaC neurons expressing mCD8-GFP, dicer2, unc-104 hairpin RNAs, and hairpin RNAs targeting Rtnl2 (control) or γTub23C are shown 4 d after larval hatching. Number of branch points was counted at each time point. Averages of branch point number are shown on the graph; "n" indicates the number of cells analyzed for each genotype.
Fig. S8. Global degeneration induced by SCA3tr-Q78 in eyes is countered by microtubule nucleation. To induce expression of transgenes in photoreceptors the longGMR-Gal4 was crossed into different genetic background: yw flies (control) and flies with SCA3 transgenes and/OR RNAi hairpins. Example images are shown for each genotype in A. A model for induction of a protective pathway that counteracts degeneration induced by SCA3tr-Q78 is shown at lower left. (B) Flies expressing different sets of transgenes were categorized based on eye phenotype. Numbers above the bars indicate number of flies analyzed for the genotype.
Movie S1. Targeting the axonal kinesin unc-104 increases the number of growing microtubules in dendrites. Control (Rtl12) and unc-104 RNA hairpins were expressed in class I da neurons by using 221-Gal4. Dicer2 and EB1-GFP were also expressed under Gal4 control. Images of EB1-GFP comets in dendrites of ddaE were acquired every 2 s with a scanning confocal microscope. A control movie is shown (Left). Most comets move toward the cell body in the dendrites. A movie from an unc-104 RNAi neuron is also shown (Right). Many more comets are present in this cell, and they move both directions in dendrites. In all movies, dorsal direction is up, so the dendrites emerge from the top of the cell and the axon emerges from the bottom of the cell.

Movie S2. Expression of SCA3tr-Q78, but not SCA3tr-Q27, increases the number of growing microtubules in neurons. Human SCA3tr-Q78, which can cause neurodegeneration in flies, and a control nondegenerative form of the protein, SCA3tr-Q27, were expressed in class I da neurons together with EB1-GFP. SCA3tr-Q27-expressing ddaE neurons were similar to control neurons (Left; compare vs. Movie S1, Left). In contrast, neurons that expressed the long version had a dramatic increase in the number of EB1-GFP comets (Right).
Movie S3. The core microtubule nucleation, γTub23C, is required to increase microtubule dynamics in response to SCA3tr-Q78. EB1-GFP was coexpressed with SCA3tr-Q78 and a control RNAi against maternal γ-tubulin (γTub37C, Left) or an RNAi targeting the major somatic γ-tubulin (γTub23C, Right). Many fewer comets were seen in dendrites expressing γTub23C hairpins. In this movie, the axons emerge from the cell bodies at lower left.

Movie S3

Movie S4. γTub23C is required to increase microtubule dynamics in response to reduction of unc-104. EB1-GFP was coexpressed with unc-104 hairpin RNAs and a control hairpin (Left) or a γTub23C hairpin (Right). When γTub23C was targeted, many fewer comets were present in ddaE dendrites.

Movie S4
Movie S5. JNK signaling is required to increase microtubule dynamics in SCA3tr-Q78-expressing neurons. EB1-GFP was expressed with Q78 and bskDN (a dominant-negative form of the Drosophila JNK). Time-course movies of EB1-GFP comets in ddaE neurons were acquired with a confocal microscope.

Movie S5

Movie S6. JNK signaling is required to increase microtubule dynamics when unc-104 levels are reduced by RNAi. EB1-GFP was expressed with dicer2, bskDN, and unc-104 RNA hairpins. Time-course movies of EB1-GFP comets in ddaE were acquired.

Movie S6