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INVESTIGATION OF THE MOLECULAR BASIS UNDERLYING A MULTI-STEP MODEL OF AXON INJURY RESPONSES

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ABSTRACT

Neurons are susceptible to a range of genetic and environmental insults. The ability to survive and recover from these insults is critical to maintaining a healthy and functional nervous system. The goal of my study is to identify the molecular and cellular mechanisms behind two axon injury responses, self-protection and axon regeneration, and study the relationship between the two.

I have established a conditioning lesion assay in *Drosophila* larval sensory neurons to identify an endogenous protective response. I have discovered that traumatic axon injury protects dendrites against a secondary injury, and this protection requires DLK-JNK-fos-dependent upregulation of microtubule dynamics. Furthermore, the microtubule nucleation protein γTub23C is important for injury-induced microtubule dynamics and protection. Strikingly, this microtubule-based protection is activated in two types of chronic stresses including expressing poly-Q neurodegenerative disease proteins and compromising kinesin-3-mediated axonal transport. In both scenarios, microtubule dynamics antagonize long-term neurodegeneration. Therefore, neurons may utilize upregulated microtubule dynamics as a general survival strategy to resist a variety of acute and chronic neuronal insults.

Nicotinamide mononucleotide adenylyltransferases (Nmnats) are well-established neuroprotective factors. I have found that endogenous Nmnat is required for axon injury-induced protection. Genetic analysis suggests that Nmnat is subject to positive regulation by the DLK-JNK-fos pathway and negative regulation by a caspase cascade downstream of mitochondrial fission. Together, these results reveal a high degree of signaling complexity in Nmnat regulation.

Why is Nmnat so tightly regulated? Protection is an early axon injury response and axon regeneration occurs after its inactivation. Hyperactivation of Nmnat caused by JNK and fos overexpression or reduction of the initiator caspase Dronc, however, results in abnormal

protection in conjunction with axon regeneration defects. These defects can be rescued by reducing Nmnat. This suggests that (1) completion of neuroprotection is necessary for axon regeneration; (2) coordination between protection and axon regeneration relies on a tight control of Nmnat activity.

In summary, my results suggest that axon injury responses can be divided into multiple steps, such as protection and regeneration, and each step involves distinct regulatory mechanisms. As a beginning effort to untangle the complex regulatory networks of this multi-step model, I have uncovered that the DLK pathway protects neurons through Nmnat and microtubules early after axon injury, and later inactivates Nmnat through mitochondrial fission and caspases to turn protection off and allow axons to regenerate.

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PREFACE

The content in Chapter 2 has been published in Proc Natl Acad Sci U S A 2012 109 (29): 842-7. doi: 10.1073/pnas.1121180109. In Chapter 2, Figure 1 was prepared by Juan Tao. Figure S2A, Figure S3A and B were prepared by Michelle Stone and Melissa Rolls. Figure S6A was prepared by Juan Tao and Melissa Rolls. Melissa Rolls and I wrote the paper together. In Chapter 3, Figure 5G was prepared by Michelle Stone. Melissa Rolls and I wrote the paper together. Michelle Stone and Kyle Gheres generated the ppk-Wlds fly line, performed the neighbor axotomy assay and wrote the corresponding methods. Our collaborators Xin Xiong and Catherine Collins generated the GFP-tagged Nmnat fly lines.

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To my parents and my Nobel Prize winner

Chapter 1

Introduction

Neurons play fundamental roles in almost all our daily activities. Even the simplest tasks involve complicated neuronal activities, for example, photoreceptor neurons are essential for vision; sensory and motor neurons enable coordinated body movement; and decision-making and memory would be impossible without neuronal networks in the brain. Most mature neurons develop multiple dendrites and a single axon to receive and send information, respectively. Due to the long length and slender morphology, axons are susceptible to acute injuries such as trauma and stroke, as well as a number of chronic insults including neurotoxins, neurodegenerative diseases, and diabetic neuropathy. Remarkably, many neurons are built with self-protective and self-repair strategies to deal with adverse situations. Manipulating these strategies will likely ameliorate the devastating consequences of neurodegeneration. In this thesis project, I aimed to gain a better knowledge of the molecular mechanisms of axon regeneration and protection induced by axon injury, with a particular focus on the roles of microtubules, the dual leucine zipper kinase (DLK) pathway, and nicotinamide mononucleotide adenylyltransferases (Nmnats). In the first part of the introduction, I will give an overview of the roles of microtubules and DLK in axon regeneration. In the second part, I will discuss the roles of DLK and Nmnats in neuroprotection.

Axon regeneration

Axons in the central nervous system (CNS) have limited regeneration after injury compared to their counterparts in the peripheral nervous system (PNS). The lack of regeneration is attributed

to growth inhibitors in the environment and diminished capacity of intrinsic growth (reviewed in Liu et al., 2011b). CNS injury induces myelin-associated inhibitory factors and generates an astrocyte-based scar. The glial scar not only forms a physical barrier but also secretes inhibitory molecules that prevent axon regrowth, including chondroitin sulphate proteoglycans (CSPGs) and intermediate filament proteins (reviewed in Yiu and He, 2006). Targeting the identified inhibitory factors achieves initial success *in vitro*, however, it largely fails to improve central axon regeneration *in vivo* (reviewed in Liu et al., 2011b). This has led researchers to investigate intrinsic mechanisms that play an equally critical role.

Microtubules in axon regeneration

Microtubules are a major component of the neuronal cytoskeleton and play important roles in maintaining the health and function of axons. Organelles (e.g., mitochondria and ribosomes), proteins (e.g., sodium and potassium channel subunits), and presynaptic vesicles are transported into the axon, and materials that are destined for recycling and degradation need to be transported back to the cell body. Both of these processes require the use of stable microtubules as tracks (reviewed in Hirokawa et al., 2010). Accordingly, axon integrity is undermined when microtubules become destabilized by drug treatment (Zhai et al., 2003), and by mutations in tubulin-specific chaperone e (Tbce) (Martin et al., 2002) or cytosolic carboxypeptidase (CCP1) (Rogowski et al., 2010). Therefore, microtubule integrity is essential for neuronal maintenance.

Microtubule stabilization, on the other hand, is important for axon outgrowth during development. Early studies by Gary Banker's group characterized five stages of neuronal polarization using cultured hippocampal neurons. According to their model, stage 2 neurons have multiple immature and morphologically equivalent neurites and it is not until stage 3 that one of the neurites shows accelerated elongation compared to others and later becomes the axon (Dotti et

al., 1988; reviewed in Neukirchen and Bradke, 2011). Subsequent studies suggest that microtubule stability may play an instructive role in determining which neurite becomes the future axon. First, microtubule stability starts to increase selectively in the to-be-axon neurite and later in the specified axon. Second, treating neurons with taxol at a dose that promotes microtubule polymerization is sufficient to induce multiple axons (Witte et al., 2008).

Microtubule stabilization is also important for axon regeneration. In mouse cortical neurons and dorsal root ganglion (DRG) neurons, stabilizing microtubules by inhibiting histone deacetylase 6 (HDAC6), promotes cell survival and axon regeneration in response to oxidative stress (Rivieccio et al., 2009). In *C. elegans* neurons, the tubulin carboxypeptidase (CCPP-6) is required for axon regeneration presumably by generating delta-2 tubulin (Ghosh-Roy et al., 2012), a highly stable form of tubulin (reviewed in Janke and Kneussel, 2010). In addition, taxol-mediated moderate microtubule stabilization promotes CNS axon regeneration by preventing axon retraction and by decreasing scar formation (Erturk et al., 2007; Hellal et al., 2011). Together, these studies suggest a positive role of microtubule stabilization in axon regeneration.

However, caution should be taken given the fact that microtubules exhibit distinct variations in stability throughout different regions of the axon. For example, formation of growth cones is a conserved feature in regenerating axons in mammals and *C. elegans* (Erturk et al., 2007; Hammarlund et al., 2009). Microtubule dynamics in growth cones are particularly dynamic (Ahmad et al., 1993), especially in the peripheral domain, thus allowing axons to swiftly respond to guidance cues during navigation. In contrast, microtubules in the central domain and shaft are bundled and less dynamic so that they are optimal for transporting and delivering vesicles and organelles for axon extension (reviewed in Dent and Gertler, 2003; Schaefer et al., 2002). It is thus conceivable that indiscriminate microtubule stabilization is detrimental to long-term axon regeneration and neuronal function in general. In support of this idea, two recent studies suggest that a novel microtubule deacetylase HDAC5 is necessary for axon regeneration. HDAC5

regulates regeneration in two ways: (1) by enhancing local microtubule dynamics in growth cones; and (2) by altering gene transcription (Cho and Cavalli, 2012; Cho et al., 2013). In summary, moderate, local and temporal control of microtubule stability is key to achieving successful axon regeneration.

It remains largely unknown whether microtubule stability plays roles other than regeneration. My colleague Michelle Stone made the observation that the number of growing microtubules in dendrites and the cell body is dramatically up-regulated one day after axon injury (Stone et al., 2010), which is referred to as increased microtubule dynamics. In chapter 2, I will reveal the molecular mechanism and function of injury-induced microtubule dynamics.

DLK in axon regeneration

Mitogen-activated protein kinase (MAPK) cascades are conserved signaling transduction pathways in all eukaryotes. Phosphoryl groups from the serine/threonine kinase MAPKKKs are relayed to MAPKKs and MAPKs before reaching substrates (reviewed in Gallo and Johnson, 2002) giving rise to signaling complexity and specificity. A group of MAPKKKs share the MAPKs JNK and p38 as substrates, thus are collectively named mixed-lineage kinases (MLKs) (reviewed in Gallo and Johnson, 2002). Dual leucine zipper kinase (DLK) is a conserved MLK that is named DLK-1 in *C. elegans*, and wallenda or wnd in *Drosophila* (Collins et al., 2006; Yan et al., 2009). Reported functions of DLK include but are not limited to synaptic growth (Collins et al., 2006), degeneration of DRG neurons during development (Ghosh et al., 2011), axon degeneration after injury (Miller et al., 2009), axon regeneration (Itoh et al., 2009), neuroprotection (Xiong and Collins, 2012), and migration of pyramidal neurons and axon formation (Hirai et al., 2011; Hirai et al., 2006). It remains a challenge to explain how DLK carries out these seemingly unrelated and even opposite functions, but extensive efforts have been

made in recent years to reveal downstream mechanisms. With regard to axon regeneration, DLK seems to control injury responses in both the axon and the cell body.

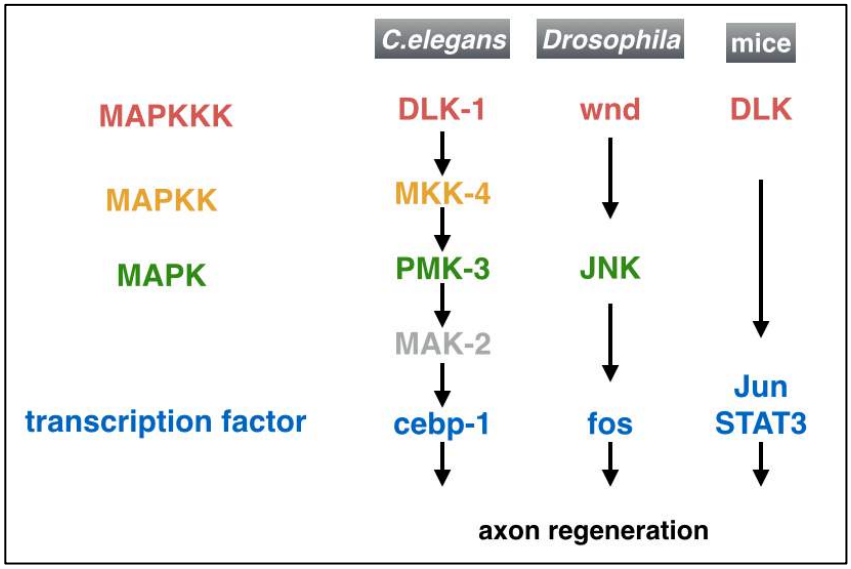

Figure 1. A summary of identified mechanisms underlying DLK-mediated axon regeneration.

A local role for DLK in axons

The pro-regenerative role for DLK was initially discovered in *C. elegans* by examining motor axon regeneration in a beta-spectrin mutant background, and was confirmed using laser axotomy (Hammarlund et al., 2009). The authors found that a conserved MAPK pathway, consisting of the MAPKKK DLK-1, the MAPKK MKK-4 and the MAPK PMK-3, is important for axon regeneration (Figure 1). Based on the defects in growth cone formation in DLK-1 mutants, the authors proposed that one way DLK-1 promotes axon regeneration is by regulating microtubule stability in growth cones. Their observations were soon corroborated by another group (Yan et al., 2009). Furthermore, the second study identified the MAPK-activated protein kinase MAK-2 downstream of PMK-3. PMK-3 activation stabilizes cebp-1 mRNA and thus increases its local translation in the injured axon (Figure 1). DLK-1 is also required for elevated

microtubule growth in axons close to the injury site (Ghosh-Roy, et al., 2012). Therefore, some aspects of DLK-mediated axon regeneration are through local signaling within the injured axon. However, as I will discuss in the next part, several recent studies point out that DLK supports axon regeneration primarily through nuclear remodeling.

DLK regulates transcription in the cell body

The cell body plays a paramount role in supporting persistent axon regeneration. Retrograde transport of injury signals coding the length of the lost axon and severity of the injury must be sent to the cell body to generate proper injury responses (reviewed in Abe and Cavalli, 2008; Cho et al., 2013; Moore et al., 2009; Raivich et al., 2004). For example, a distal axotomy induces regeneration from the remaining axon while an injury close to the cell body may transform a dendrite into a new axon (Figure 2) (Bradke and Dotti, 2000; Dotti and Banker, 1987; Stone et al., 2010; Takahashi et al., 2007).

MAPKs are a class of well-established retrograde signals. Upon peripheral nerve injury, locally phosphoactivated MAPK Erks bind to the vimentin-importin-dynein complex, and travel along microtubules towards the cell body to regulate regeneration (Perlson et al., 2005). Another MAPK, JNK, is also transported toward the cell body in response to injury as a result of increased interaction of dynein-dynactin and JNK interacting protein 3 (JIP3) (Cavalli et al., 2005). Multiple studies have identified DLK as an activator of JNK, and the transcription factors fos and jun as the primary effectors downstream of JNK. In *Drosophila* motor and sensory neurons, axon injury activates the DLK-JNK-fos pathway, and inactivating any member in the pathway prevents axon regeneration (Figure 1) (Stone et al., 2010; Xiong et al., 2010). Therefore, DLK may promote axon regeneration by directing retrograde transport of JNK, leading to transcriptional alteration. In cultured neurons, it is demonstrated that apoptosis induced by nutrient deprivation

requires c-Jun phosphorylation downstream of DLK/JIP3/JNK in the distal axon compartment (Watkins et al., 2013). Furthermore, peripheral nerves do not regenerate in DLK knockout mice, in part due to defects in phosphorylating c-Jun and retrograde transport of the transcription factor STAT3 (Figure 1) (Shin et al., 2012). Altogether, these findings provide strong evidence that nuclear reprogramming is a major mechanism of DLK-mediated injury responses in neurons.

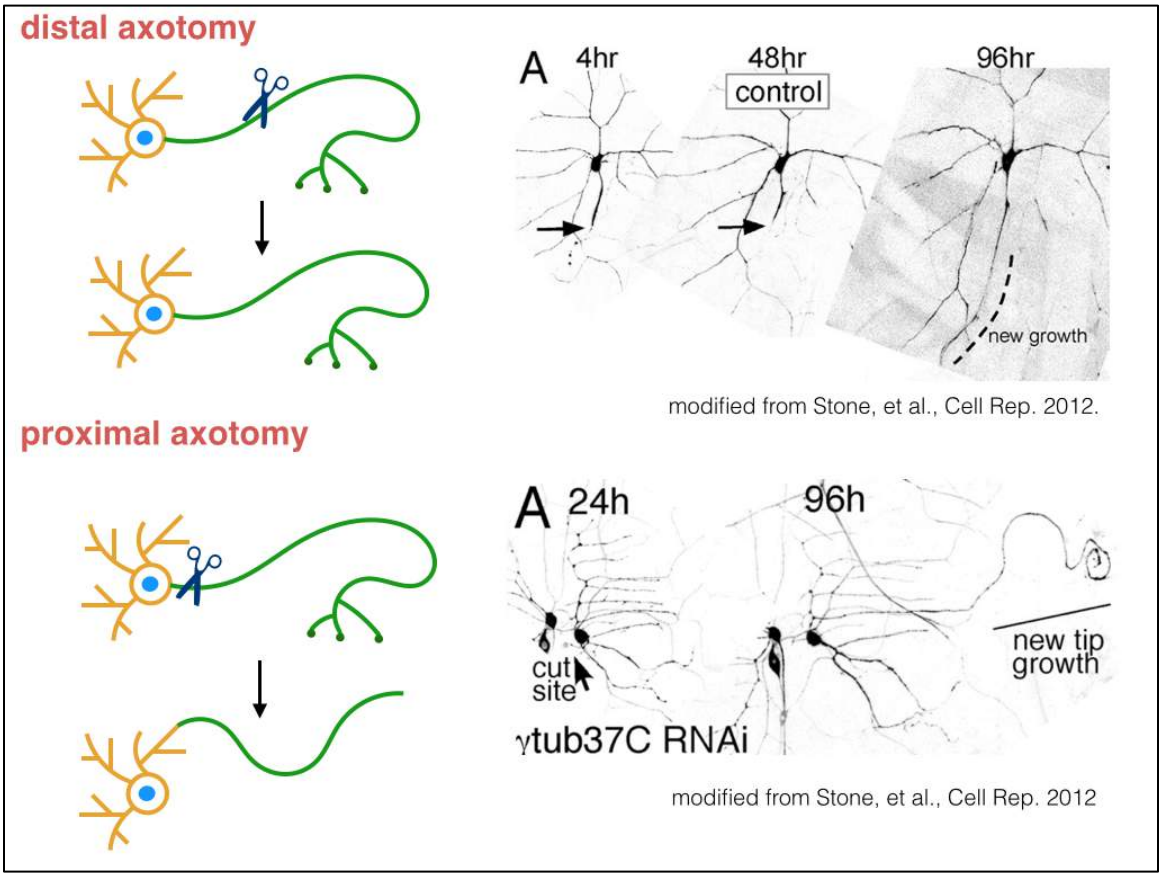

Figure 2. Two types of axon regeneration.

Left, schematic illustration showing that the axon regenerates from the proximal axon in response to a distal injury, whereas a proximal injury leads to regeneration from a dendrite. Right, representative images of the two types of axon regeneration in *Drosophila* sensory neurons. Arrows indicate site of axon injury.

As mentioned above, DLK is a master regulator of multiple injury responses but the underlying mechanisms remain mysterious. Indeed, our investigation of this molecule reveals that

it is responsible for inducing both positive and negative regulation of axon injury responses through transcriptional regulation. In Chapter 3, we attempt to reconcile these results in a multi-step axon injury response model.

Neuroprotection

In comparison to axon regeneration, neuroprotection is a general term that describes the beneficial effect of delaying or blocking neurodegeneration in the entire neuron, or specific compartments, such as dendrites and axons. Neuroprotection can be achieved by inhibiting pro-degeneration molecules (e.g., protein proteases (Zhai et al., 2003) and caspases (Schoenmann et al., 2010)), but the intertwined and sometimes interdependent nature of the known pro-degeneration pathways makes it difficult to save neurons by targeting selective molecules. An alternative approach may be to generally increase the resistance of neurons against degeneration, with the prospect of preventing disease progression at asymptomatic stages. Neuronal resistance can be upregulated by boosting protective mechanisms that are activated spontaneously in adverse conditions. For example, neurons that are preconditioned by a nonlethal stress stimulus are more capable of combating a second lethal insult (reviewed in Iadecola and Anrather, 2011). It is conceivable that understanding the cellular and molecular mechanisms of preconditioning-induced protection will greatly facilitate the design of novel therapy for patients who suffer from stroke and neurodegenerative disorders. In this part of the introduction, I will first briefly introduce the role of DLK in preconditioning-induced neuroprotection. Second, I will introduce another class of neuroprotective factors, nicotinamide mononucleotide adenylytransferases (Nmnats). Several mechanisms have been proposed to explain Nmnat-mediated neuroprotection, and new ones are continuously emerging. In contrast to the extensive mechanistic study, the

influence of Nmnats on regeneration is not clear. This is an equally important question and should not be overlooked, especially before drug development and clinical trials. Major findings in these two research directions will be the focus of the second part of the introduction.

Preconditioning-induced neuroprotection and the role of DLK pathway

Various triggers, including global and focal ischemia, excitotoxicity, reactive oxygen species, and cytokines, can activate endogenous protective mechanisms in neurons (reviewed in Dawson and Dawson, 2006). Researchers have found that nerve crush induces protective effects in a rat model of amyotrophic lateral sclerosis (ALS) by delaying and reducing degeneration of motoneurons (Franz et al., 2009), suggesting that neurons can also be conditioned by axon injury. Furthermore, the proximal axon becomes more resilient to injury after a distal axotomy (Xiong and Collins, 2012). Based on these two studies, it seems likely that axon injury may elicit general protection across different types of insults, including chronic stress and acute injury. In the second paper, the authors found that wnd, the *Drosophila* DLK, positively regulates axon lesion-induced protection. The protection is abolished when wnd is reduced or when the transcription factor fos is inactivated, indicating that the wnd-mediated protection is executed at the transcriptional level (Xiong and Collins, 2012). In Chapter 2, I will present our discovery that different types of axon preconditioning converge on a common pathway to protect dendrites. In the following study, I have characterized the protective pathway in greater detail and the results surrounding DLK and Nmnats are particularly intriguing. These results will be presented in Chapter 3.

Exogenous Nmnats provide neuroprotection

Nicotinamide mononucleotide adenylytransferases (Nmnats) are recognized as powerful and conserved neuroprotective molecules. In both invertebrate and vertebrate models, overexpression of Nmnats delays neurodegeneration by injury, oxidative stress, energy deprivation, neurotoxins, and several types of neurodegenerative diseases (Ali et al., 2012; Araki et al., 2004; Bhattacharya et al., 2012; Press and Milbrandt, 2008; Shen et al., 2013; Tao and Rolls, 2011; Zhai et al., 2008b). The idea that Nmnats may be relevant in treating neurodegeneration has driven extensive research efforts in the past decade to elucidate the underlying mechanisms of their protective effect. Progresses have been made, however, many discrepancies remain to be resolved.

Functions, localization and regulation of Nmnats

Nmnats catalyze the biochemical reaction that converts nicotinamide mononucleotide (NMN) and nicotinic acid mononucleotide (NaMN) to nicotinamide adenine dinucleotide (NAD+) (Figure 3) (reviewed in Imai and Guarente, 2014). Three Nmnats (Nmnat1 in the nucleus, Nmnat2 in the cytosol with suggested Golgi-localization, and Nmnat3 in mitochondria) exist in the mammalian genome (Berger et al., 2005), while *Drosophila* has only one Nmnat (Zhai et al., 2006). The distinct distribution of Nmnats may be important for local generation of NAD+ to meet temporal and spatial demands in different cellular compartments (Figure 3). Two classes of NAD+ consumers are present in the nucleus: Poly ADP-ribose polymerases (PARPs) and sirtuins. In general, PARPs sense DNA damage and use NAD+ to repair DNA through protein post-translational modification (reviewed in Schreiber et al., 2006). Nuclear sirtuins deacetylate transcription factors such as PGC-1α and foxo, and thus contribute to mitochondrial

biogenesis, fatty acid metabolism and oxidative stress (Brunet et al., 2004; reviewed in Houtkooper and Auwerx, 2012; Lagouge et al., 2006). Mitochondrial NAD^+ provides the substrate for sirtuin-dependent protein deacetylation, as well as functions as a key co-enzyme in glycolysis, the TCA cycle, and ATP synthesis. Cytosolic NAD^+ fuels the production of cyclic ADP-ribose (cADPR) to engage in Ca^{2+} signaling pathways (reviewed in Stein and Imai, 2012). In summary, Nmnat-mediated NAD^+ generation plays key roles in multiple steps of cell metabolism.

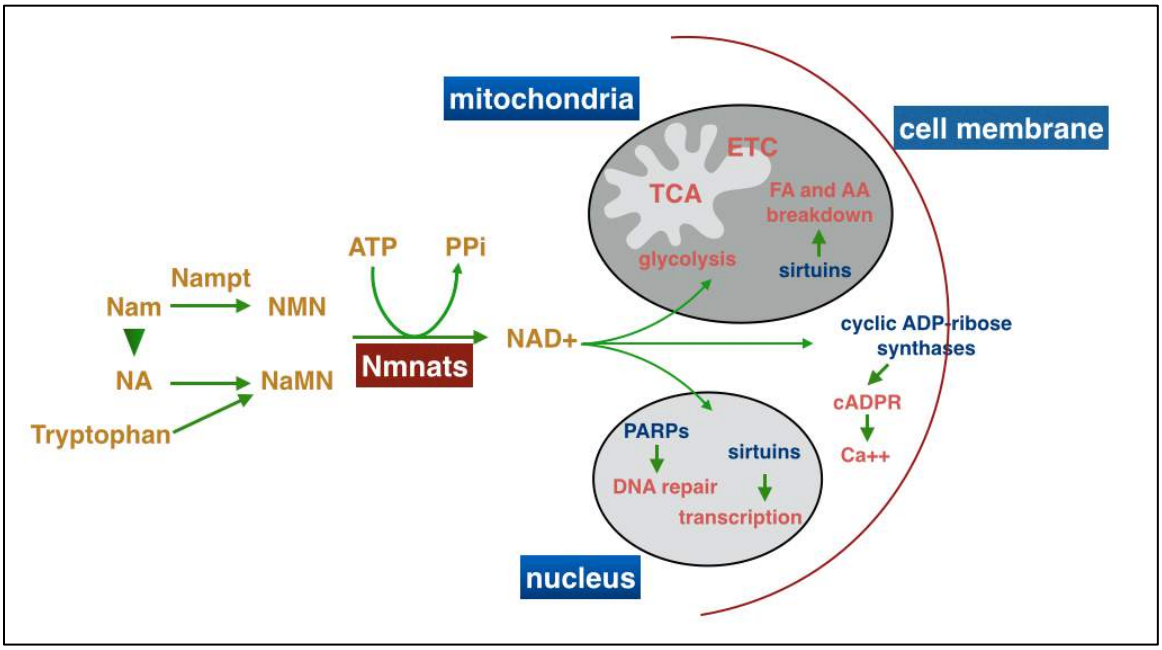

Figure 3. Nmnat generates NAD+ to engage in energy metabolism and adaptive responses.

Nam, nicotinamide. NA, nicotinic acid. NMN, nicotinamide mononucleotide. NaMN, nicotinic acid mononucleotide. TCA, tricarboxylic acid cycle. ETC, electron transfer chain. FA, fatty acids. AA, amino acids.

The importance of Nmnats in metabolic homeostasis is underlined by loss-of-function studies in animal models. Loss of Nmnat causes severe degeneration of photoreceptor neurons in adult flies (Zhai et al., 2006). At the larval stage, knockdown of Nmnat causes dendrite retraction (Wen et al., 2011b) and sensitizes dendrites to hypoxia in class IV da neurons (Wen et al., 2013). Mice without Nmnat1 or Nmnat2 die at embryonic stage or at birth (Ali et al., 2013d), and

knockdown of Nmnat2 in cultured neurons results in neurite degeneration (Gilley and Coleman, 2010). Together, these findings highlight a neuronal survival role for Nmnats.

Not much is known about how Nmnat level and activity is regulated. The transcription of *Drosophila* Nmnat is increased upon heat stress and hypoxia in a heat shock factor (HSF)-dependent manner (Ali et al., 2011). The E3 ubiquitin ligase Phr1 controls Nmnat2 protein stability in mammalian neurons. The presence of Phr1 in axons (Lewcock et al., 2007) determines Nmnat2 destabilization in severed axons and the subsequent axon degeneration (Babetto et al., 2013). This mechanism is conserved in *Drosophila* neurons and is mediated by Hiw (Xiong et al., 2012). In contrast to distal axons, the amount of Nmnat is increased in the proximal axon. This is correlated with an increase in the stability of the proximal axon to a second injury (Xiong et al., 2010). Whether the accumulated Nmnat is required for axon stabilization, however, remains to be determined.

In summary, Nmnats are housekeeping genes and play fundamental roles in energy metabolism and stress adaptation. In Chapter 3, I will demonstrate my study on the role of endogenous Nmnat in conditioning-induced neuroprotection and its upstream regulatory pathways.

Proposed mechanisms of Nmnat-mediated neuroprotection

The existing literature suggests at least four mechanisms in regard to where and how Nmnats protect neurons:

1. Nmnats increase NAD^+ and the nucleus is the site of action.
2. Nmnats function locally (perhaps in mitochondria) and enhance mitochondrial function.
3. Nmnats reduce NMN accumulation in degenerating neurites.
4. Nmnats function like a chaperone.

Although some of the proposed mechanisms may be debatable, these studies provide invaluable insights in revealing the complexity of Nmnat-regulated signaling and justify the necessity of future investigations. In the next few paragraphs, I will summarize the representative findings that lead to each model.

1. Nmnats increase NAD^+ and the nucleus is the site of action.

The primary role for Nmnat as an NAD^+ biosynthetic enzyme leads to the hypothesis that NAD^+ is responsible for Nmnat-induced protection. Consistently, the enzymatic activity of Nmnats is essential for neuroprotection, as the full length Nmnat1 blocks degeneration after axon transection while an NAD^+ synthesis-incompetent version of Nmnat1 shows limited protection (Araki et al., 2004; Avery et al., 2009; Conforti et al., 2009). However, the contribution of NAD+ is less clear. First, Nmnat1 does not alter NAD^+ level compared to control neurons (Araki et al., 2004; Sasaki et al., 2009b). Second, exogenous NAD^+ is effective at protection if applied at a very high level, perhaps because of the immediate breakdown once into cells. However, the level of protection is not comparable to that of Nmnat1 (Araki et al., 2004; Sasaki et al., 2009b; Wang et al., 2005). In support of a nuclear site of function: (1) Nmnat1 is localized in the nucleus (Araki et al., 2004); (2) the nuclear sirtuin SIRT1 is necessary for NAD^+-induced protection (Araki et al., 2004); (3) transcription profiles are drastically different between control and Wlds mice (Barrette et al., 2010). Wlds is a fusion protein consisting of the entire Nmnat1gene, the N-terminal 70 amino acids of Ube4b and a unique linker. Wlds axons become resistant to degeneration mainly because of Nmnat1 (reviewed in Coleman, 2005; Coleman et al., 1998).

2. Nmnats function locally (perhaps in mitochondria) and enhance mitochondrial function.

The nuclear mechanism does not explain the protective effect of a cytoplasmic Nmnat1 mutant (Sasaki et al., 2009a) or an axonally targeted Nmnat1 (Babetto et al., 2010; Beirowski et al., 2009). In addition, Wlds is detected outside the nucleus (Avery et al., 2012b; Beirowski et al., 2009). Furthermore, other studies find that sirtuins are neither required nor sufficient to confer

protection (Avery et al., 2009; Wang et al., 2005). In contrast, adding NAD^+ 5h post axon transection provides the same level of protection as before transection. Together with the observation that Wlds, Nmnat1 and NAD^+ application delays the loss of ATP in degenerating axons, the authors propose a local role for Nmnat-mediated protection (Wang et al., 2005). Consistent with the influence on ATP, it is found that overexpression of the mitochondrial Nmnat3 also induces strong protection (Avery et al., 2012; Sasaki et al., 2006; Yahata et al., 2009). Subsequent studies suggest that Nmnats may improve ATP synthesis (Yahata et al., 2009), prevent accumulation of oxidative species (O'Donnell et al., 2013), maintain mitochondrial motility and buffer calcium influx (Avery et al., 2012b). Altogether, mitochondria seem to be a major target of Nmnats.

3. Nmnats reduce NMN accumulation in degenerating neurites.

Despite the mysterious role for NAD+, authors of (Di Stefano et al., 2014) presented amble evidence that accumulation of NMN, the substrate of Nmnat, in severed axons is responsible for axon degeneration. Consistently, reducing NMN synthesis by targeting Nampt has protective effects *in vitro* and *in vivo*. The accumulation of NMN is likely due to fast degradation of the labile Nmnat2 (Gilley and Coleman, 2010) by the ubiquitin ligase Phr1 (Babetto et al., 2013).

4. Nmnats function like a chaperone.

While the majority of the above studies use axotomy to untangle the protective mechanisms of Nmnats, it is possible that different mechanisms are utilized under other settings. In support of this idea, although the enzymatic activity of Nmnats is critical for it to protect against injury-induced degeneration, an enzymatically dead Nmnat is competent in antagonizing degeneration in *Drosophila* models of spinocerebellar ataxia 1 (SCA1), a type of poly-Q disease, and in tauopathy (Ali et al., 2012; Zhai et al., 2008). One proposed mechanism involves enhanced

clearance of protein aggregates by a chaperone-like activity of Nmnat (Zhai et al., 2006; Zhai et al., 2008). Whether mammalian Nmnats have chaperone activity requires further study.

In summary, it is possible that the protection by Nmnats depends on one or several of the proposed mechanisms. My study on the protective pathway-induced after axotomy has led to the discovery of a surprising relationship between Nmnat and mitochondria and a new target of Nmnat, which will be covered in Chapter 3.

The influence of Wlds/Nmnats on axon regeneration

In mammals, the lack of robust axon regeneration is accompanied by slow axon degeneration in the CNS. While peripheral axons degenerate within about one to two weeks, this process takes months and even years to complete for central axons (reviewed in Vargas and Barres, 2007). The difference is largely attributed to inefficient phagocytosis of myelin inhibitory molecules in the CNS. For example, schwann cells decrease myelin expression and swiftly degrade myelin debris after axon injury in the PNS (Stoll et al., 1989; Trapp et al., 1988). In contrast, the CNS myelinating glia oligodendrocytes undergo apoptosis at an early stage of axon degeneration (Beattie et al., 2002) and thus cannot effectively execute myelin debris clearance. Activation of macrophages is impeded in the CNS because of the nearly impermeable blood brain barrier. In the absence of the professional phagocytes, microglia take the responsibility of removing myelin-associated inhibitors in the CNS, but at a slow pace (reviewed in Vargas and Barres, 2007).

Question arises as to whether delayed axon degeneration results in regeneration failure. Several early studies showed that mouse sensory and motor neurons expressing Wlds regenerate at a reduced rate than control axons but can eventually reach target tissues (Bisby and Chen, 1990; Brown et al., 1992), suggesting that Wlds has moderate regeneration inhibitory function.

An independent study using zebrafish nicely demonstrates that although the speed of axon regeneration is not altered in the presence of Wlds, the new axon is forced to reroute due to the persistent distal stump (Martin et al., 2010), suggesting that Wlds may interfere with target reinnervation.

It is widely assumed that the moderate regeneration defect in Wlds neurons is due to the persistent axon stump, which either serves as a physical barrier or sends repulsive cues to the cell body and/or new axons, but solid evidence is missing (Bisby and Chen, 1990; Brown et al., 1992). Alternatively, it may be possible that Wlds functions in the cell body to alter the capacity of regeneration. In support of this hypothesis, gene expression in Wlds and wild-type sciatic nerves differ dramatically (Barrette et al., 2010). Furthermore, injury-induced induction of CCL2, a chemokine involved in macrophage recruitment, is found in wild-type but not Wlds mice (Niemi et al., 2013). Last, a recent study provides direct evidence showing that Wlds functions in the cell body rather than axons to inhibit axon regeneration. The authors examined neurite outgrowth in explanted DRG neurons after nerve transection and found shorter neurites in neurons derived from Wlds mice (Niemi et al., 2013). Wlds and Nmnat delays injury-induced axon degeneration in *Drosophila* sensory neurons (Tao and Rolls, 2011). We tested whether and how Wlds and Nmnat inhibit axon regeneration using the same type of neurons. Our results reveal a novel mechanism supporting the cell body hypothesis that will be shown in Chapter 3.

References

Abe, N., and Cavalli, V. (2008). Nerve injury signaling. Curr Opin Neurobiol *18*, 276-283.
Ahmad, F.J., Pienkowski, T.P., and Baas, P.W. (1993). Regional differences in microtubule dynamics in the axon. J Neurosci *13*, 856-866.
Ali, Y.O., Li-Kroeger, D., Bellen, H.J., Zhai, R.G., and Lu, H.C. (2013). NMNATs, evolutionarily conserved neuronal maintenance factors. Trends Neurosci *36*, 632-640.
Ali, Y.O., McCormack, R., Darr, A., and Zhai, R.G. (2011). Nicotinamide mononucleotide adenylyltransferase is a stress response protein regulated by the heat shock factor/hypoxia-inducible factor 1alpha pathway. The Journal of biological chemistry *286*, 19089-19099.
Ali, Y.O., Ruan, K., and Zhai, R.G. (2012). NMNAT suppresses tau-induced neurodegeneration by promoting clearance of hyperphosphorylated tau oligomers in a Drosophila model of tauopathy. Human molecular genetics *21*, 237-250.
Araki, T., Sasaki, Y., and Milbrandt, J. (2004). Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. Science *305*, 1010-1013.
Avery, M.A., Rooney, T.M., Pandya, J.D., Wishart, T.M., Gillingwater, T.H., Geddes, J.W., Sullivan, P.G., and Freeman, M.R. (2012). WldS prevents axon degeneration through increased mitochondrial flux and enhanced mitochondrial Ca2+ buffering. Curr Biol *22*, 596-600.
Avery, M.A., Sheehan, A.E., Kerr, K.S., Wang, J., and Freeman, M.R. (2009). Wld S requires Nmnat1 enzymatic activity and N16-VCP interactions to suppress Wallerian degeneration. J Cell Biol *184*, 501-513.
Baas, P.W., and Lin, S. (2011). Hooks and comets: The story of microtubule polarity orientation in the neuron. Developmental neurobiology *71*, 403-418.
Babetto, E., Beirowski, B., Janeckova, L., Brown, R., Gilley, J., Thomson, D., Ribchester, R.R., and Coleman, M.P. (2010). Targeting NMNAT1 to axons and synapses transforms its neuroprotective potency in vivo. J Neurosci *30*, 13291-13304.
Babetto, E., Beirowski, B., Russler, E.V., Milbrandt, J., and DiAntonio, A. (2013). The Phr1 ubiquitin ligase promotes injury-induced axon self-destruction. Cell Rep *3*, 1422-1429.
Barkus, R.V., Klyachko, O., Horiuchi, D., Dickson, B.J., and Saxton, W.M. (2008). Identification of an axonal kinesin-3 motor for fast anterograde vesicle transport that facilitates retrograde transport of neuropeptides. Mol Biol Cell *19*, 274-283.
Barres, B.A., and Raff, M.C. (1993). Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. Nature *361*, 258-260.
Barrette, B., Calvo, E., Vallieres, N., and Lacroix, S. (2010). Transcriptional profiling of the injured sciatic nerve of mice carrying the Wld(S) mutant gene: identification of genes involved in neuroprotection, neuroinflammation, and nerve regeneration. Brain Behav Immun *24*, 1254-1267.
Beattie, M.S., Harrington, A.W., Lee, R., Kim, J.Y., Boyce, S.L., Longo, F.M., Bresnahan, J.C., Hempstead, B.L., and Yoon, S.O. (2002). ProNGF induces p75-mediated death of oligodendrocytes following spinal cord injury. Neuron *36*, 375-386.
Beirowski, B., Babetto, E., Gilley, J., Mazzola, F., Conforti, L., Janeckova, L., Magni, G., Ribchester, R.R., and Coleman, M.P. (2009). Non-nuclear Wld(S) determines its neuroprotective efficacy for axons and synapses in vivo. J Neurosci *29*, 653-668.
Berger, F., Lau, C., Dahlmann, M., and Ziegler, M. (2005). Subcellular compartmentation and differential catalytic properties of the three human nicotinamide mononucleotide adenylyltransferase isoforms. J Biol Chem *280*, 36334-36341.
Bhattacharya, M.R., Gerdts, J., Naylor, S.A., Royse, E.X., Ebstein, S.Y., Sasaki, Y., Milbrandt, J., and DiAntonio, A. (2012). A model of toxic neuropathy in Drosophila reveals a role for MORN4 in promoting axonal degeneration. J Neurosci *32*, 5054-5061.

Bisby, M.A., and Chen, S. (1990). Delayed wallerian degeneration in sciatic nerves of C57BL/Ola mice is associated with impaired regeneration of sensory axons. Brain research *530*, 117-120.
Bradke, F., and Dotti, C.G. (2000). Differentiated neurons retain the capacity to generate axons from dendrites. Curr Biol *10*, 1467-1470.
Brown, M.C., Lunn, E.R., and Perry, V.H. (1992). Consequences of slow Wallerian degeneration for regenerating motor and sensory axons. Journal of neurobiology *23*, 521-536.
Brunet, A., Sweeney, L.B., Sturgill, J.F., Chua, K.F., Greer, P.L., Lin, Y., Tran, H., Ross, S.E., and Mostoslavsky R, C.H., Hu LS, Cheng HL, Jedrychowski MP, Gygi SP, Sinclair DA, Alt FW, Greenberg ME. (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011-2015.
Cavalli, V., Kujala, P., Klumperman, J., and Goldstein, L.S. (2005). Sunday Driver links axonal transport to damage signaling. The Journal of cell biology *168*, 775-787.
Cho, Y., and Cavalli, V. (2012). HDAC5 is a novel injury-regulated tubulin deacetylase controlling axon regeneration. The EMBO journal *31*, 3063-3078.
Cho, Y., Sloutsky, R., Naegle, K.M., and Cavalli, V. (2013). Injury-induced HDAC5 nuclear export is essential for axon regeneration. Cell *155*, 894-908.
Coleman, M. (2005). Axon degeneration mechanisms: commonality amid diversity. Nat Rev Neurosci *6*, 889-898.
Coleman, M.P., Conforti, L., Buckmaster, E.A., Tarlton, A., Ewing, R.M., Brown, M.C., Lyon, M.F., and Perry, V.H. (1998). An 85-kb tandem triplication in the slow Wallerian degeneration (Wlds) mouse. Proc Natl Acad Sci U S A *95*, 9985-9990.
Collins, C.A., Wairkar, Y.P., Johnson, S.L., and DiAntonio, A. (2006). Highwire restrains synaptic growth by attenuating a MAP kinase signal. Neuron *51*, 57-69.
Conde, C., and Cáceres, A. (2009). Microtubule assembly, organization and dynamics in axons and dendrites. Nat Rev Neurosci *10*, 319-332.
Conforti, L., Wilbrey, A., Morreale, G., Janeckova, L., Beirowski, B., Adalbert, R., Mazzola, F., Di Stefano, M., Hartley, R., Babetto, E., *et al.* (2009). Wld S protein requires Nmnat activity and a short N-terminal sequence to protect axons in mice. J Cell Biol *184*.
Dawson, V.L., and Dawson, T.M. (2006). Mining for survival genes. Biochem Soc Trans *34*, Pt6.
Dent, E.W., and Gertler, F.B. (2003). Cytoskeletal dynamics and transport in growth cone motility and axon guidance. Neuron *40*, 209-227.
Di Stefano, M., Nascimento-Ferreira, I., Orsomando, G., Mori, V., Gilley, J., Brown, R., Janeckova, L., Vargas, M.E., Worrell, L.A., Loreto, A., *et al.* (2014). A rise in NAD precursor nicotinamide mononucleotide (NMN) after injury promotes axon degeneration. Cell Death Differ.
Dotti, C.G., and Banker, G.A. (1987). Experimentally induced alteration in the polarity of developing neurons. Nature *330*, 254-256.
Dotti, C.G., Sullivan, C.A., and Banker, G.A. (1988). The establishment of polarity by hippocampal neurons in culture. J Neurosci *8*, 1454-1468.
Erturk, A., Hellal, F., Enes, J., and Bradke, F. (2007). Disorganized microtubules underlie the formation of retraction bulbs and the failure of axonal regeneration. J Neurosci *27*, 9169-9180.
Franz, C.K., Quach, E.T., Krudy, C.A., Federici, T., Kliem, M.A., Snyder, B.R., Raore, B., and Boulis, N.M. (2009). A conditioning lesion provides selective protection in a rat model of Amyotrophic Lateral Sclerosis. PLoS One *4*, e7357.
Gallo, K.A., and Johnson, G.L. (2002). Mixed-lineage kinase control of JNK and p38 MAPK pathways. Nat Rev Mol Cell Biol *3*, 663-672.
Ghosh, A.S., Wang, B., Pozniak, C.D., Chen, M., Watts, R.J., and Lewcock, J.W. (2011). DLK induces developmental neuronal degeneration via selective regulation of proapoptotic JNK activity. J Cell Biol *194*, 751-764.

Ghosh-Roy, A., Goncharov, A., Jin, Y., and Chisholm, A.D. (2012). Kinesin-13 and tubulin posttranslational modifications regulate microtubule growth in axon regeneration. Developmental cell *23*, 716-728.
Gilley, J., and Coleman, M.P. (2010). Endogenous Nmnat2 is an essential survival factor for maintenance of healthy axons. PLoS Biol *8*, e1000300.
Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E.M., and Bastiani, M. (2009). Axon regeneration requires a conserved MAP kinase pathway. Science *323*, 802-806.
Hellal, F., Hurtado, A., Ruschel, J., Flynn, K.C., Laskowski, C.J., Umlauf, M., Kapitein, L.C., Strikis, D., Lemmon, V., Bixby, J., *et al.* (2011). Microtubule stabilization reduces scarring and causes axon regeneration after spinal cord injury. Science *331*, 928-931.
Hirai, S., Banba, Y., Satake, T., and Ohno, S. (2011). Axon formation in neocortical neurons depends on stage-specific regulation of microtubule stability by the dual leucine zipper kinase-c-Jun N-terminal kinase pathway. J Neurosci *31*, 6468-6480.
Hirai, S., Cui de, F., Miyata, T., Ogawa, M., Kiyonari, H., Suda, Y., Aizawa, S., Banba, Y., and Ohno, S. (2006). The c-Jun N-terminal kinase activator dual leucine zipper kinase regulates axon growth and neuronal migration in the developing cerebral cortex. J Neurosci *26*, 11992-12002.
Hirokawa, N., Niwa, S., and Tanaka, Y. (2010). Molecular motors in neurons: transport mechanisms and roles in brain function, development, and disease. Neuron *68*, 610-638.
Houtkooper, R.H., and Auwerx, J. (2012). Exploring the therapeutic space around NAD+. J Cell Biol *199*, 205-209.
Iadecola, C., and Anrather, J. (2011). Stroke research at a crossroad: asking the brain for directions. Nat Neurosci *14*, 1363-1368.
Imai, S., and Guarente, L. (2014). NAD+ and sirtuins in aging and disease. Trends Cell Biol *24*, 464-471.
Itoh, A., Horiuchi, M., Bannerman, P., Pleasure, D., and Itoh, T. (2009). Impaired regenerative response of primary sensory neurons in ZPK/DLK gene-trap mice. Biochem Biophys Res Commun *383*, 258-262.
Janke, C., and Kneussel, M. (2010). Tubulin post-translational modifications: encoding functions on the neuronal microtubule cytoskeleton. Trends Neurosci *33*, 362-372.
Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., *et al.* (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell *127*, 1109-1122.
Lewcock, J.W., Genoud, N., Lettieri, K., and Pfaff, S.L. (2007). The ubiquitin ligase Phr1 regulates axon outgrowth through modulation of microtubule dynamics. Neuron *56*, 604-620.
Liu, K., Tedeschi, A., Park, K.K., and He, Z. (2011). Neuronal intrinsic mechanisms of axon regeneration. Annu Rev Neurosci *34*, 131-152.
Martin, N., Jaubert, J., Gounon, P., Salido, E., Haase, G., Szatanik, M., and Guénet, J.L. (2002). A missense mutation in Tbce causes progressive motor neuronopathy in mice. Nat Genet *32*, 443-447.
Martin, S.M., O'Brien, G.S., Portera-Cailliau, C., and Sagasti, A. (2010). Wallerian degeneration of zebrafish trigeminal axons in the skin is required for regeneration and developmental pruning. Development *137*, 3985-3994.
Miller, B.R., Press, C., Daniels, R.W., Sasaki, Y., Milbrandt, J., and DiAntonio, A. (2009). A dual leucine kinase-dependent axon self-destruction program promotes Wallerian degeneration. Nat Neurosci *12*, 387-389.
Moore, D.L., Blackmore, M.G., Hu, Y., Kaestner, K.H., Bixby, J.L., Lemmon, V.P., and Goldberg, J.L. (2009). KLF family members regulate intrinsic axon regeneration ability. Science *326*, 298-301.

Neukirchen, D., and Bradke, F. (2011). Neuronal polarization and the cytoskeleton. Semin Cell Dev Biol *22*, 825-833.
Niemi, J.P., DeFrancesco-Lisowitz, A., Roldán-Hernández, L., Lindborg, J.A., Mandell, D., and Zigmond, R.E. (2013). A critical role for macrophages near axotomized neuronal cell bodies in stimulating nerve regeneration. J Neurosci *33*, 16236-16248.
O'Donnell, K.C., Vargas, M.E., and Sagasti, A. (2013). WldS and PGC-1alpha regulate mitochondrial transport and oxidation state after axonal injury. J Neurosci *33*, 14778-14790.
Perlson, E., Hanz, S., Ben-Yaakov, K., Segal-Ruder, Y., Seger, R., and Fainzilber, M. (2005). Vimentin-dependent spatial translocation of an activated MAP kinase in injured nerve. Neuron *45*, 715-726.
Press, C., and Milbrandt, J. (2008). Nmnat delays axonal degeneration caused by mitochondrial and oxidative stress. J Neurosci *28*, 4861-4871.
Raivich, G., Bohatschek, M., Da Costa, C., Iwata, O., Galiano, M., Hristova, M., Nateri, A.S., Makwana, M., Riera-Sans, L., Wolfer, D.P., *et al.* (2004). The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. Neuron *43*, 57-67.
Rivieccio, M.A., Brochier, C., Willis, D.E., Walker, B.A., D'Annibale, M.A., McLaughlin, K., Siddiq, A., Kozikowski, A.P., Jaffrey, S.R., L., T.J., *et al.* (2009). HDAC6 is a target for protection and regeneration following injury in the nervous system. Proc Natl Acad Sci U S A *106*, 19599-19604.
Rogowski, K., van Dijk, J., Magiera, M.M., Bosc, C., Deloulme, J.C., Bosson, A., Peris, L., Gold, N.D., Lacroix, B., Bosch Grau, M., *et al.* (2010). A family of protein-deglutamylating enzymes associated with neurodegeneration. Cell *143*, 564-578.
Sasaki, Y., Araki, T., and Milbrandt, J. (2006). Stimulation of nicotinamide adenine dinucleotide biosynthetic pathways delays axonal degeneration after axotomy. J Neurosci *26*, 8484-8491.
Sasaki, Y., Vohra, B.P., Baloh, R.H., and Milbrandt, J. (2009a). Transgenic mice expressing the Nmnat1 protein manifest robust delay in axonal degeneration in vivo. J Neurosci *29*, 6526-6534.
Sasaki, Y., Vohra, B.P., Lund, F.E., and Milbrandt, J. (2009b). Nicotinamide mononucleotide adenylyl transferase-mediated axonal protection requires enzymatic activity but not increased levels of neuronal nicotinamide adenine dinucleotide. J Neurosci *29*, 5525-5535.
Schaefer, A.W., Kabir, N., and Forscher, P. (2002). Filopodia and actin arcs guide the assembly and transport of two populations of microtubules with unique dynamic parameters in neuronal growth cones. J Cell Biol *158*, 139-152.
Schoenmann, Z., Assa-Kunik, E., Tiomny, S., Minis, A., Haklai-Topper, L., Arama, E., and Yaron, A. (2010). Axonal degeneration is regulated by the apoptotic machinery or a NAD+-sensitive pathway in insects and mammals. J Neurosci *30*, 6375-6386.
Schreiber, V., Dantzer, F., Ame, J.C., and de Murcia, G. (2006). Poly(ADP-ribose): novel functions for an old molecule. Nat Rev Mol Cell Biol *7*, 517-528.
Shen, H., Hyrc, K.L., and Goldberg, M.P. (2013). Maintaining energy homeostasis is an essential component of Wld(S)-mediated axon protection. Neurobiol Dis *59*, 69-79.
Shin, J.E., Cho, Y., Beirowski, B., Milbrandt, J., Cavalli, V., and Diantonio, A. (2012). Dual leucine zipper kinase is required for retrograde injury signaling and axonal regeneration. Neuron *74*, 1015-1022.
Stein, L.R., and Imai, S. (2012). The dynamic regulation of NAD metabolism in mitochondria. Trends Endocrinol Metab *23*.
Stoll, G., Griffin, J.W., Li, C.Y., and Trapp, B.D. (1989). Wallerian degeneration in the peripheral nervous system: participation of both Schwann cells and macrophages in myelin degradation. J Neurocytol *18*, 671-683.

Stone, M.C., Nguyen, M.M., Tao, J., Allender, D.L., and Rolls, M.M. (2010). Global up-regulation of microtubule dynamics and polarity reversal during regeneration of an axon from a dendrite. Mol Biol Cell *21*, 767-777.
Takahashi, D., Yu, W., Baas, P.W., Kawai-Hirai, R., and Hayashi, K. (2007). Rearrangement of microtubule polarity orientation during conversion of dendrites to axons in cultured pyramidal neurons. Cell Motil Cytoskeleton *64*, 347-359.
Tao, J., and Rolls, M.M. (2011). Dendrites have a rapid program of injury-induced degeneration that is molecularly distinct from developmental pruning. J Neurosci *31*, 5398-5405.
Trapp, B.D., Hauer, P., and Lemke, G. (1988). Axonal regulation of myelin protein mRNA levels in actively myelinating Schwann cells. J Neurosci *8*, 3515-3521.
Vargas, M.E., and Barres, B.A. (2007). Why is Wallerian degeneration in the CNS so slow? Annu Rev Neurosci *30*, 153-179.
Wang, J., Zhai, Q., Chen, Y., Lin, E., Gu, W., McBurney, M.W., and He, Z. (2005). A local mechanism mediates NAD-dependent protection of axon degeneration. J Cell Biol *170*, 349-355.
Wang, M., Wu, Y., Culver, D.G., and Glass, J.D. (2001). The gene for slow Wallerian degeneration (Wld(s)) is also protective against vincristine neuropathy. Neurobiol Dis *8*, 155-161.
Watkins, T.A., Wang, B., Huntwork-Rodriguez, S., Yang, J., Jiang, Z., Eastham-Anderson, J., Modrusan, Z., Kaminker, J.S., Tessier-Lavigne, M., and Lewcock, J.W. (2013). DLK initiates a transcriptional program that couples apoptotic and regenerative responses to axonal injury. Proc Natl Acad Sci U S A *110*, 4039-4044.
Wen, Y., Parrish, J.Z., He, R., Zhai, R.G., and Kim, M.D. (2011). Nmnat exerts neuroprotective effects in dendrites and axons. Mol Cell Neurosci *48*, 1-8.
Wen, Y., Zhai, R.G., and Kim, M.D. (2013). The role of autophagy in Nmnat-mediated protection against hypoxia-induced dendrite degeneration. Molecular and cellular neurosciences *52*, 140-151.
Witte, H., Neukirchen, D., and Bradke, F. (2008). Microtubule stabilization specifies initial neuronal polarization. J Cell Biol *180*, 619-632.
Xiong, X., and Collins, C.A. (2012). A conditioning lesion protects axons from degeneration via the Wallenda/DLK MAP kinase signaling cascade. J Neurosci *32*, 610-615.
Xiong, X., Hao, Y., Sun, K., Li, J., Li, X., Mishra, B., Soppina, P., Wu, C., Hume, R.I., and Collins, C.A. (2012). The Highwire ubiquitin ligase promotes axonal degeneration by tuning levels of Nmnat protein. PLoS biology *10*, e1001440.
Xiong, X., Wang, X., Ewanek, R., Bhat, P., Diantonio, A., and Collins, C.A. (2010). Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. The Journal of cell biology *191*, 211-223.
Yahata, N., Yuasa, S., and Araki, T. (2009). Nicotinamide mononucleotide adenylyltransferase expression in mitochondrial matrix delays Wallerian degeneration. J Neurosci *29*, 6276-6284.
Yan, D., Wu, Z., Chisholm, A.D., and Jin, Y. (2009). The DLK-1 kinase promotes mRNA stability and local translation in C. elegans synapses and axon regeneration. Cell *138*, 1005-1018.
Yiu, G., and He, Z. (2006). Glial inhibition of CNS axon regeneration. Nat Rev Neurosci *7*, 617-627.
Zhai, Q., Wang, J., Kim, A., Liu, Q., Watts, R., Hoopfer, E., Mitchison, T., Luo, L., and He, Z. (2003). Involvement of the ubiquitin-proteasome system in the early stages of wallerian degeneration. Neuron *39*, 217-225.
Zhai, R.G., Cao, Y., Hiesinger, P.R., Zhou, Y., Mehta, S.Q., Schulze, K.L., Verstreken, P., and Bellen, H.J. (2006). Drosophila NMNAT maintains neural integrity independent of its NAD synthesis activity. PLoS biology *4*, e416.
Zhai, R.G., Zhang, F., Hiesinger, P.R., Cao, Y., Haueter, C.M., and Bellen, H.J. (2008). NAD synthase NMNAT acts as a chaperone to protect against neurodegeneration. Nature *452*, 887-891.

Chapter 2

Axon injury and stress trigger a microtubule-based neuroprotective pathway

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Abstract

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 proteins SCA1, SCA3 and huntingtin. In all cases dynamics was 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 polyglutamine-induced neurodegeneration. Thus increased microtubule dynamics can delay short-term injury-induced degeneration, and in the case of polyglutamine 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.

Introduction

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 then 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 either extends the stump of the existing axon, or grows a new axon from a dendrite (Chen et al., 2007; Liu et al., 2011a; Wang and Jin, 2011).

In addition to the regenerative response, axon injury can cause other less well-understood changes, including in distant regions of the cell. 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 (Hoffman, 2010; Silver, 2009). In Drosophila sensory neurons, axon injury causes cytoskeletal changes in the entire dendrite arbor, specifically the number of growing microtubules is upregulated (Stone et al., 2010). In neither case is the physiological significance of the response completely clear. 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's disease, 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 (Haass and Selkoe, 2007). One such set of misfolded protein diseases is CAG-repeat or polyglutamine (poly-Q) repeat diseases (La Spada and Taylor, 2010) including Huntington's 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 (Tao and Rolls, 2011). 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 neurons (see Supplemental Materials and Methods for information about these neurons) in intact animals, and tracked dendrite clearance after severing at subsequent timepoints.

When dendrites of the ddaE neuron were severed immediately after axons, all dendrites were cleared by 18h after injury (Figure 1A and B) as in neurons without prior axon injury (Tao and Rolls, 2011). However, when axons were severed 8 or 24h before dendrite severing, more than half of dendrites remained 18h after they were cut from the cell body (Figure 1 A 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 48h, the stabilization effect was reduced. We also tested whether this stabilization pathway could act on a much larger dendritic arbor. The ddaE neuron (Figure 1A) has a small arbor and ddaC has a large arbor, but was also stabilized by prior axon injury (Figure S2G). In these experiments axons were severed near the cell body. When ddaC axons were severed 50 microns or more from the cell body, dendrite clearance after later dendrite severing was also delayed (Figure S1). Thus both proximal and distal axotomy resulted in a cellular response that slowed injury-induced dendrite degeneration.

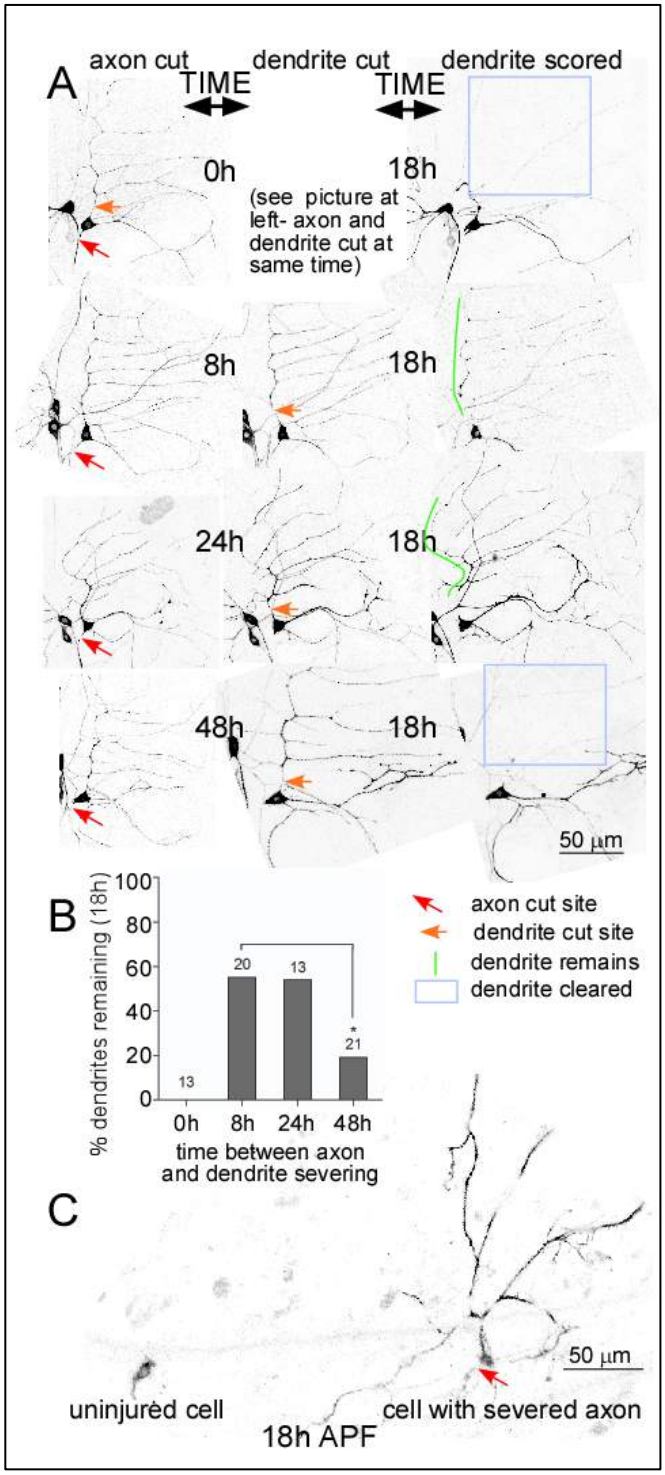

Figure 1. Axon injury delays subsequent dendrite degeneration.

(A and B) Class I da neurons were labeled by using 221-Gal4 to drive expression of EB1- GFP. Whole larvae were mounted on slides for live imaging, and axons of the ddaE neurons were severed where indicated at time 0. The comb-like dendrite that extends dorsally from these neurons was severed immediately afterward (Top) or after letting the animal recover for 8, 24, or 48 h. During the recovery period animals were returned to their normal media. In all cases, the presence of the comb dendrite was scored 18 h after it was severed from the cell body. Example images are shown in A and quantification is shown in B. Numbers above the bars in the graph are number of neurons, one per animal, that were analyzed. A Fisher exact test was used to determine significance. Throughout the figures, the same notation for P values is used: *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 (toward bottom), but no dendrites are seen dorsal to the cell body. In contrast, its neighbor, at right, has an elaborate dendrite arbor.

We next considered two possibilities: (1) that axon injury might turn on a response to specifically protect the neuron against subsequent physical trauma, or (2) 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 dendritic arborization neurons are pruned during metamorphosis and then regrow dendrite arbors into the remodeled adult body wall (Shimono et al., 2009). 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 18h after pupariation had initiated. In all cases in which axons were injured first (9/9) dendrites remained (Figure 1C). All dendrites of uninjured cells in these animals (about 10 per animal) were successfully pruned. Thus axon injury stabilized dendrites against two different types of short-term degeneration, 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 upregulated in dendrites after axon injury (Stone et al., 2010). To test whether this change in microtubule dynamics might be related to dendrite stabilization, we compared the timing of these two responses after axon injury. 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 (Jiang and Akhmanova, 2011), and so the number of comets is a readout of the number of growing microtubules. The number of comets in dendrites peaked at 24h after axon injury and decreased thereafter (Figure 2A). This timing is similar to that of dendrite stabilization (Figure 1B).

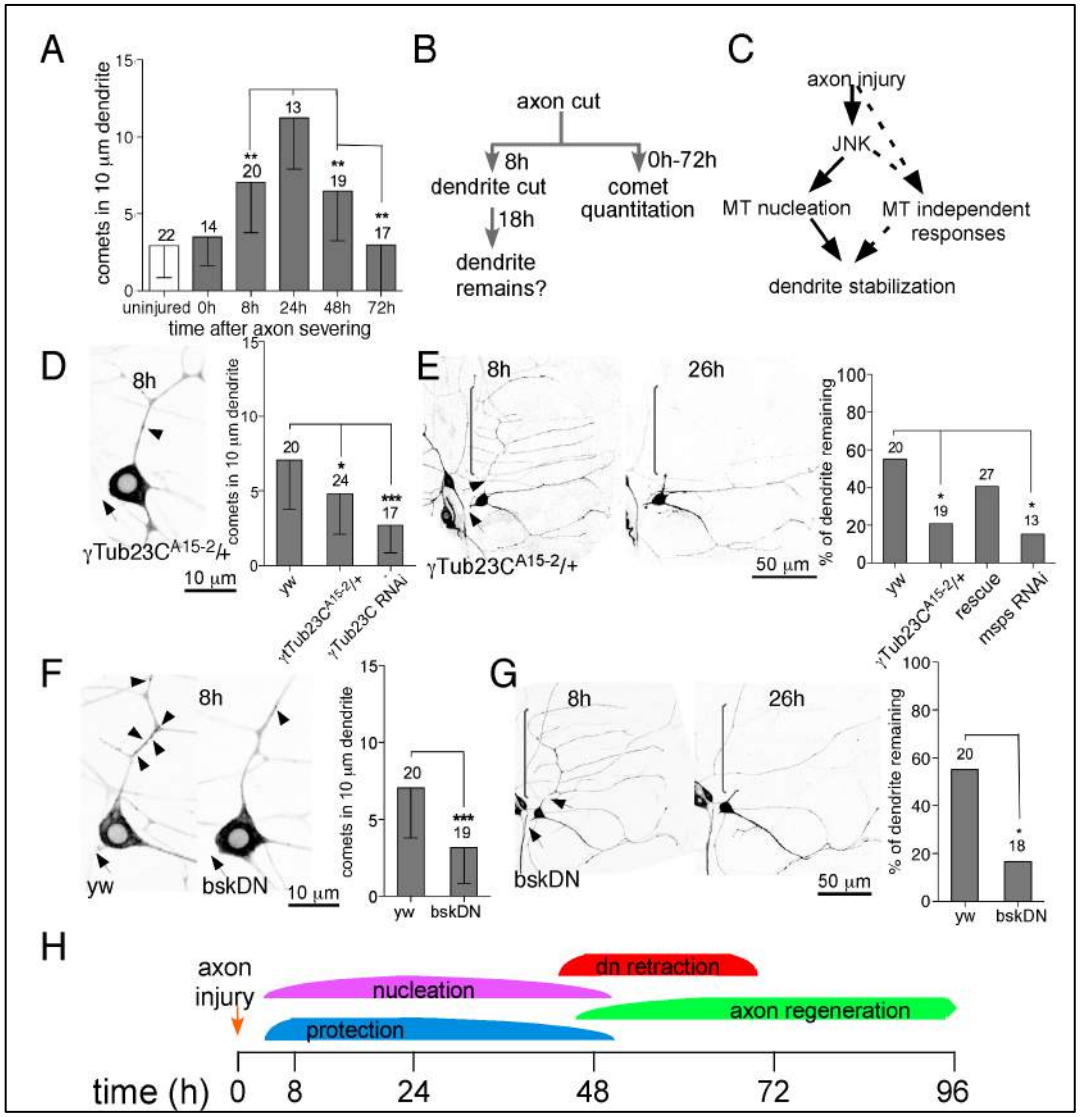

Figure 2. Injury-induced microtubule dynamics and dendrite protection require γ-tubulin and JNK signaling.

(A) 221-Gal4 was used to drive EB1-GFP expression in class I neurons. Axons of ddaE were severed at time 0. Movies of EB1-GFP dynamics were acquired at different times after axon severing. The sum of EB1-GFP comets in a 10-µm length of the dendrite is presented in the graph.

(B and C) A schematic of experiments in this figure is shown in B, and C shows a summary of results in the figure.

(D) A tester line containing 221-Gal4 and EB1-GFP, and dicer2 for RNAi experiments, transgenes was crossed to either control (*yw*) or *γTub23C* mutant flies or γTub23C RNAi flies. In larval progeny, ddaE axons were severed at time 0 and comet number in dendrites was assayed 8 h later.

(E) Genotypes used were similar to those in D. For the rescue experiment, γTub23C-GFP was added into the $\gamma Tub23C^{A15\text{-}2}$/+ background. For all genotypes, axons were severed at 0 h, the

dorsal comblike dendrite was severed 8 h later, and presence of the severed dendrite was scored 18 h after that.

(F and G) A tester line containing 221-Gal4 and EB1-GFP was crossed to control (*yw*) or UAS-bskDN–containing flies. EB1-GFP comet number was monitored in dendrites 8 h after axotomy (F), and dendrite clearance after sequential axon and dendrite severing is also shown (G).

(H) Timeline summarizes the results here and in Figure S3 is shown. Error bars in all figures indicate SD.

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 the number of 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 using the Gal4-UAS system. Compared to a control hairpin targeting γTub37C, a maternal γ-tubulin that does not play a major role in somatic cells (Wiese, 2008), only the hairpin targeting γTub23C, the major somatic nucleation protein, reduced comet number in the cell body 24h after axon injury (Figure 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*$^{A15\text{-}2}$/+) 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 Figure S2D); after injury the increase in microtubule dynamics in dendrites was reduced at 8h (Figure 2D) and 24h (Figure S2E). When we tested heterozygotes for dendrite stabilization after axon injury (see experiment schematic in Figure 2B), we found that it was

impaired compared to control (*yw*) animals (Figure 2E). To make sure that the phenotype was due to reduction of γTub23C levels and was cell-autonomous, we performed a rescue experiment with GFP-tagged γTub23C (Figure 2E). We were not able to confirm this result with γTub23C RNAi (Figure S2F), perhaps partial knockdown of γTub23C was simply not enough to abrogate protection. Consistent with this idea, protection was also not disrupted 24h after axon injury in the *γTub23C* heterozygous background (Figure S2F). We therefore confirmed that regulation of microtubules was involved in dendrite stabilization using an independent approach to disrupt microtubule growth. Msps, or XMAP215, is proposed to be a microtubule polymerase (Brouhard et al., 2008), and RNAi targeting msps eliminates EB1-GFP comets in neurons (Stone et al., 2010). We therefore used msps RNAi to perturb the behavior of neuronal microtubule plus ends. This condition blocked the protective effect of axon injury on dendrites in ddaE (Figure 2E) and ddaC (Figure S2G) neurons. We conclude that global changes in microtubule dynamics induced by axon injury play a functional role in dendrite stabilization.

Increased microtubule dynamics and dendrite stabilization after axon injury depend on JNK signaling.

JNK signaling is implicated in axon injury response in many systems (Hammarlund et al., 2009; Itoh et al., 2009; Nix et al., 2011; Stone et al., 2010; Xiong et al., 2010). We therefore hypothesized that JNK signaling would be required for increased microtubule dynamics and for dendrite stabilization. To test this hypothesis we compared both responses in control (yw) neurons and neurons that expressed a dominant-negative form of the Drosophila JNK protein bsk (bskDN), which blocks axon injury signaling in Drosophila motor neurons (Xiong et al., 2010). Expression of bskDN dampened the microtubule number increase after axon injury (Figure 2F), and also reduced the protective effect of axon injury on dendrites (Figure 2G). These results are

consistent with JNK signaling being upstream of both increased microtubule nucleation and dendrite stabilization after axon injury (Figure 2C). We also tested whether elevation of JNK signaling by overexpression of bsk in uninjured neurons was sufficient to either increase microtubule dynamics or stabilize dendrites and found that it was not (Figure S2B and C). Thus while JNK signaling is required for these injury responses, it most likely requires other pathways that are activated by injury to generate a response.

Axon regeneration is not dependent on upregulation of microtubule dynamics.

One of the major injury responses downstream of JNK is axon regeneration. We therefore wished to determine the relationship between 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 (Figure S3A and B). Similarly, regeneration after distal axotomy of ddaC was normal in γTub23C RNAi neurons (Figure 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 (Linda et al., 1992; Sumner and Watson, 1971; Tao and Rolls, 2011; Yawo, 1987). We monitored dendrite shape in ddaC neurons after distal axotomy and found that fine dendrite branches were trimmed back after distal axon severing (Figure S3D) just as in mammals. Most of this trimming occurred between 24 and 72h after axotomy, and was

unchanged when γTub23C levels were reduced (Figure S3D and S4). To compare timing of the responses to axon injury a timeline is shown in Figure 2H.

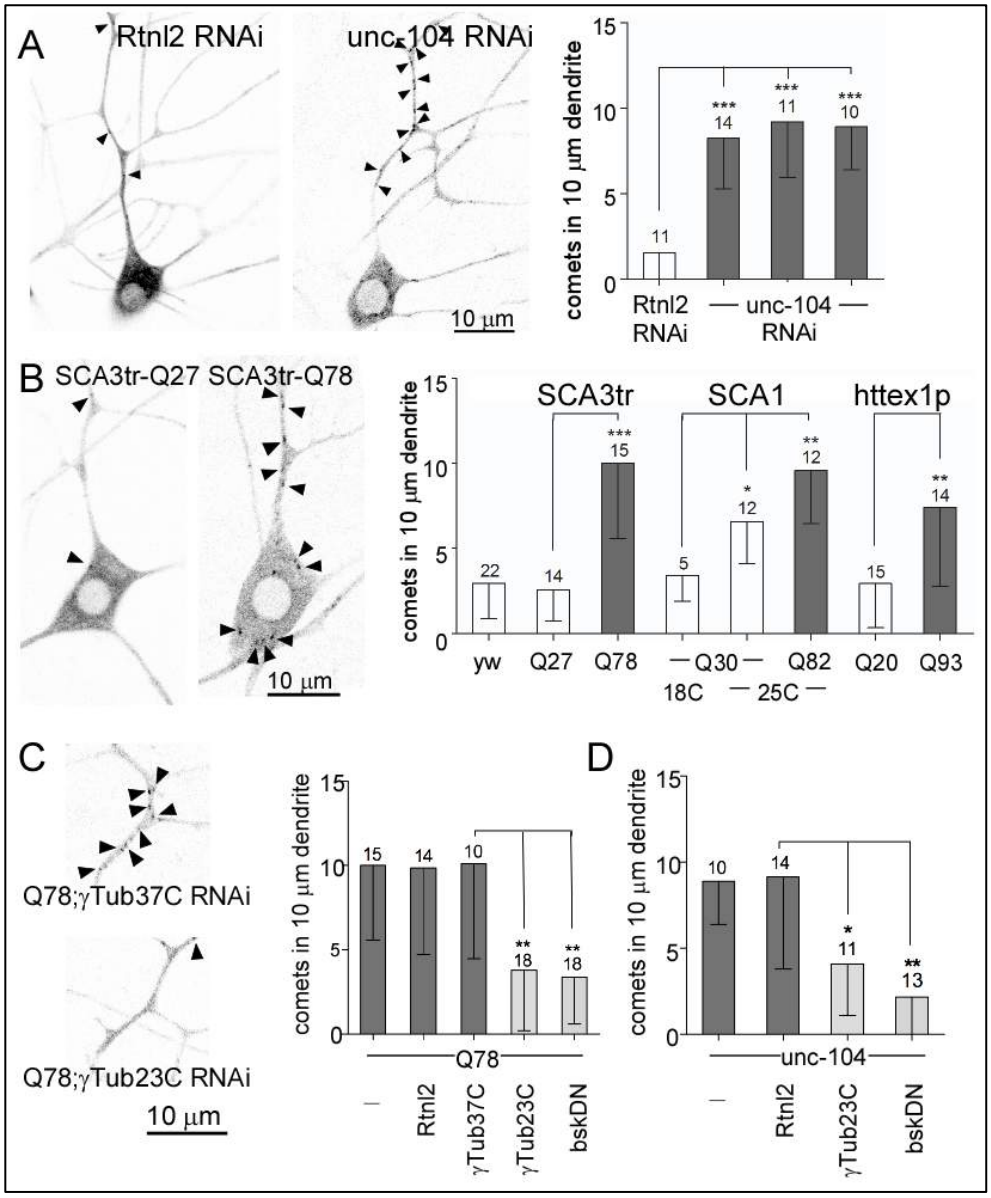

Figure 3. Reduction of unc-104 or expression of expanded poly-Q proteins triggers nucleation-based increases microtubule dynamics.

(A) ddaE neurons expressing EB1-GFP, dicer2, and either unc-104 or Rtnl2 RNAi hairpins were imaged in whole larvae. Single frames from movies are shown. The cell body and part of the comb dendrite is visible. Arrowheads indicate EB1-GFP comets. Comets in a region of the dendrite were counted, and averages are shown. Each of the unc-104 bars in the graphs represents data obtained from animals expressing a different RNA hairpin.

(B) Human proteins containing short or long poly-Q repeats were expressed in ddaE neurons together with EB1-GFP. All data were obtained at 25 °C unless indicated otherwise.

(C and D) The SCA3tr-Q78 transgene or unc-104 hairpin was expressed together with a control RNA hairpin (γTub37C or Rtnl2), a hairpin targeting the major nucleation protein in somatic cells, γTub23C, or bskDN. In all graphs, numbers above the bars indicate the number of neurons analyzed. Significance was calculated by using unpaired t tests. Error bars indicate SD.

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 expression of poly-Q proteins. In both cases we assayed microtubule dynamics and dendrite stabilization to determine whether a microtubule-based neuroprotective program was activated.

We found fortuitously that RNAi hairpins targeting unc-104 caused a dramatic increase in the number of EB1-GFP comets in dendrites (Figure 3A and Movie 1) similar to that seen after axon injury. Comet number in these cells was compared to that in neurons expressing a control hairpin (Rtnl2). Unc-104 has previously been linked to synaptic vesicle transport (Barkus et al., 2008; Hall and Hedgecock, 1991; Pack-Chung et al., 2007) and thus might cause stress. To test whether other long-term axon stress might cause changes in dendrite microtubules, we assayed microtubule dynamics in neurons that expressed proteins used to model neurodegenerative disease in Drosophila.

When human expanded poly-Q proteins are expressed in fly eyes they can cause degeneration (Fernandez-Funez et al., 2000; Steffan et al., 2001; Warrick et al., 2005). We expressed three matched pairs of short and expanded poly-Q proteins in Drosophila sensory neurons. Expression of a truncated version of spinocerebellar ataxia 3 with a long poly-Q repeat

(SCA3tr-Q78), SCA1 with long repeat (SCA1-Q82), or a truncated huntingtin protein with long repeat (httex1p-Q93) resulted in increased microtubule dynamics (Figure 3B and Movie 2). For SCA3 and htt the neurons expressing the short poly-Q proteins had similar comet numbers to control cells. For SCA1-Q30 expressing cells comet number was slightly elevated at the normal incubation temperature (25C) consistent with previous results indicating the Q30 form has some deleterious effects (Fernandez-Funez et al., 2000). Both unc-104 RNAi and expression of SCA3tr-Q78 caused reduced complexity of the highly branched class IV ddaC neuron (Figure S5). The effect of SCA3tr-Q78 on cell morphology is similar to that reported in a recent paper (Lee et al., 2011).

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 to controls (Figure 3C and D and Movies 3 and 4). We conclude that, like axon injury, reduction of the unc-104 motor or expression of expanded poly-Q proteins caused neurons to upregulate 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 that did not express a long poly-Q protein, most

dendrites were cleared 18h after severing from the cell body (Figure 4A and C). In contrast, over half of dendrites in ddaE neurons expressing SCA3tr-Q78 were still present 18h after removal from the cell body (Figure 4B and C). Expression of SCA3tr-Q78 also delayed degeneration in ddaC cells (Figure S6A), and similar results were obtained with unc-104 RNAi (Figure S6).

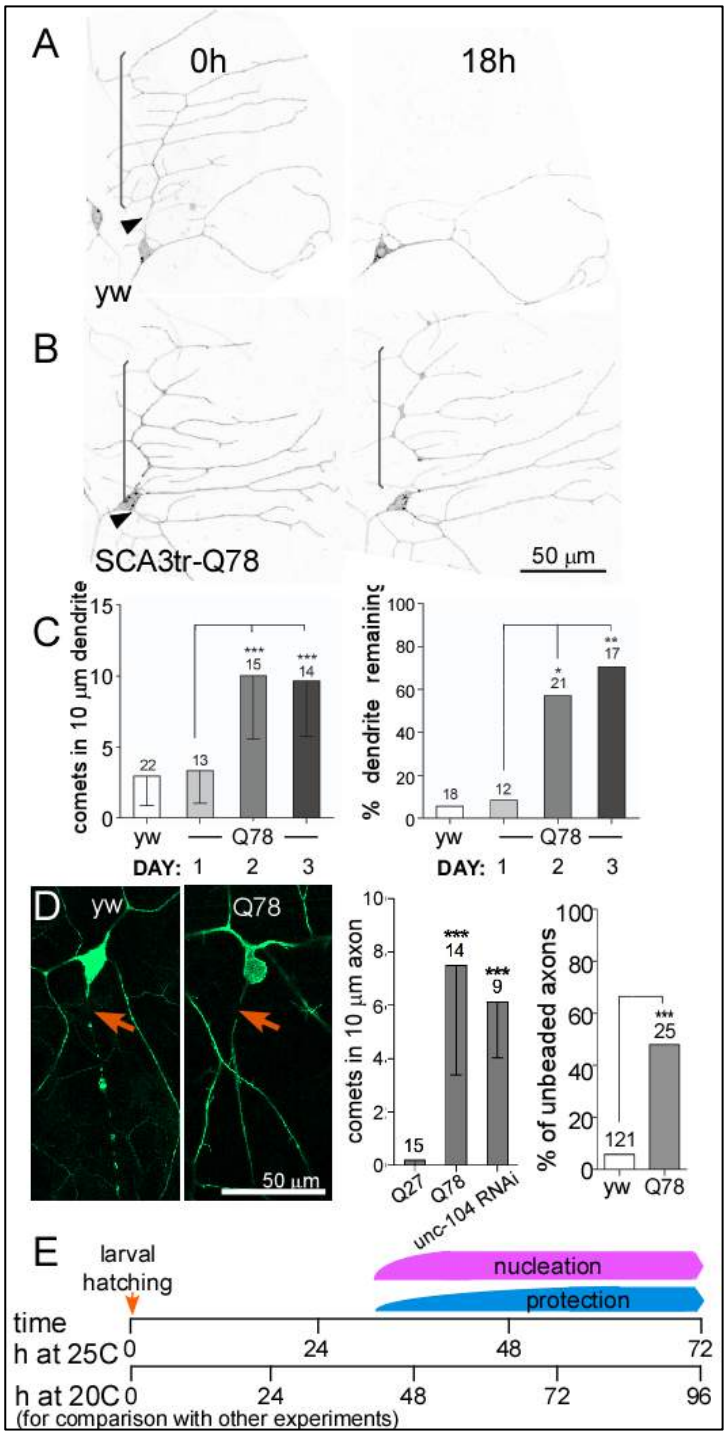

Figure 4. Expression of SCA3tr-Q78 delays injury-induced dendrite degeneration.

(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.

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 grown at 25C were assayed 1, 2 and 3 days after hatching (Figure 4C). 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 (Figure 4C and E).

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 (Figure 4D), 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 either a control RNA hairpin (Rtnl2) or γTub23C RNAi had complex dendrite arbors that increased number of branches during larval life (Figure 5). Similarly, neurons expressing SCA3tr-Q78 with Rtnl2 RNA hairpins had complex dendrite arbors that increased in branching during larval life (Figure 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 (Figure 5 and S7A). Strikingly similar results

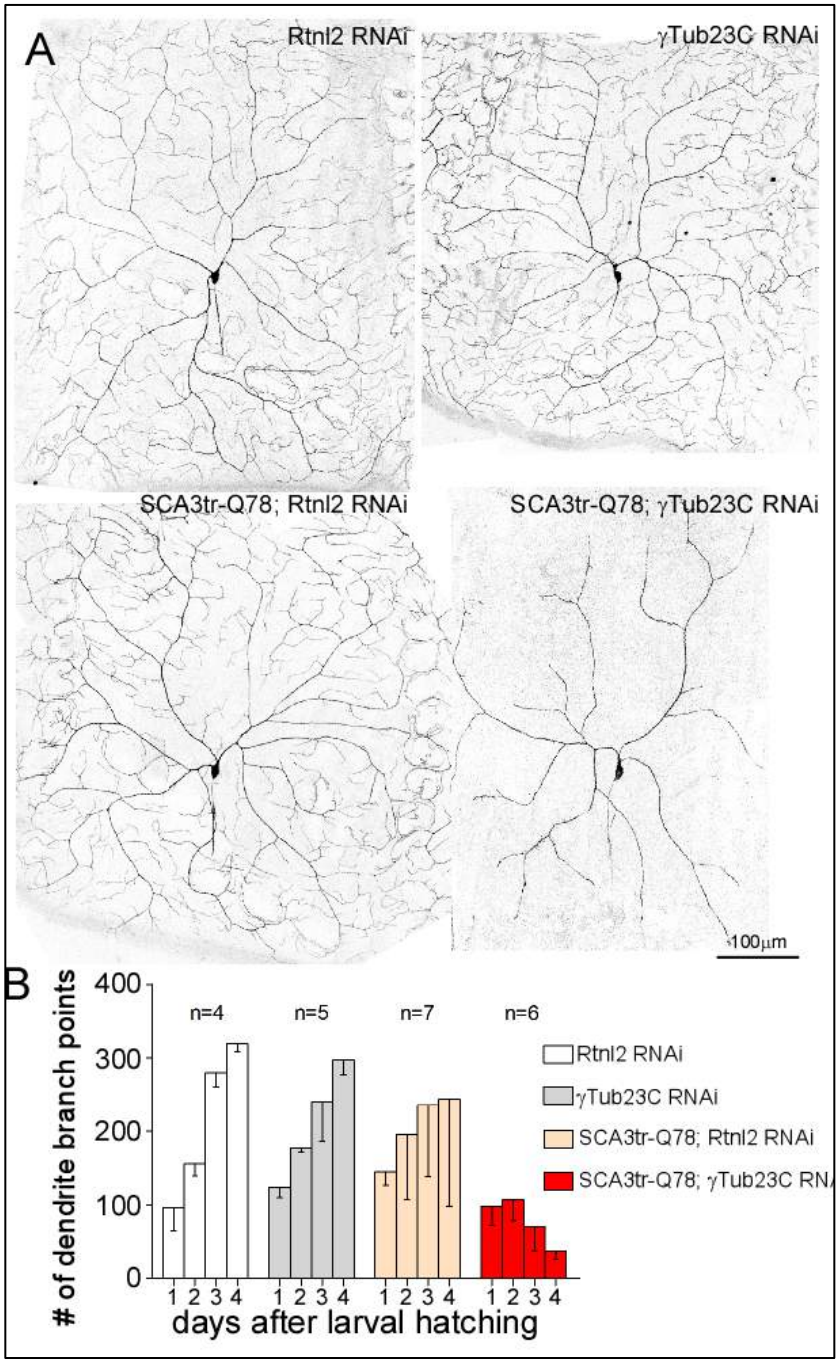

Figure 5. Reduction of microtubule nucleation increases dendrite degeneration in SCA3tr-Q78–expressing neurons.

(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.

were found when unc-104 RNA hairpins were paired either with a control RNA hairpin or γTub23C hairpin (Figure 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 (Figure S5). This is most likely because all of 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 (Lu and Vogel, 2009). Expression of SCA3tr-Q27 or γTub23C RNA hairpins in photoreceptors did not disrupt eyes (Figure 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 (Figure S8). This result is consistent with poly-Q proteins turning on a stabilization pathway that counteracts progressive degeneration (diagram in Figure 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, trigger 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 48h 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 (Xiong and Collins, 2012). Although dependence on JNK could not be tested in this model, an upstream JNK regulator, wallenda, was required (Xiong and Collins, 2012) 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 both increased microtubule dynamics and dendrite stabilization over the several days available to assay before animals pupated. The extended activation of this pathway suggested that it might be able to protect against long-term degeneration. When we tested the idea that an endogenous microtubule-based stabilization program turned on by SCA3tr-Q78 might protect cells from the deleterious effects of this protein, we found support for it in both da neurons and eyes: in both cases SCA3tr-Q78-induced degeneration was more severe when microtubule nucleation was partially reduced.

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 both 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 (MacDonald et al., 2006; Tao and Rolls, 2011) In support of this idea, microtubule disassembly has been proposed to be an early step in both Wallerian axon degeneration (Luo and O'Leary, 2005; Zhai et al., 2003) and developmental pruning of dendrites (Watts et al., 2003). In fact, for the developmental pruning pathway, a putative microtubule severing protein is required at an early step (Lee et al., 2009). 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 is 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. This would allow treatment to begin at an earlier timepoint. Identification of this pathway also offers some novel ideas about therapies for neurodegenerative disease. Turning on this pathway earlier in patients that 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 (Stone et al., 2010). For information about genotypes and additional experimental details see SI Materials and Methods

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References

Barkus, R.V., Klyachko, O., Horiuchi, D., Dickson, B.J., and Saxton, W.M. (2008). Identification of an axonal kinesin-3 motor for fast anterograde vesicle transport that facilitates retrograde transport of neuropeptides. Mol Biol Cell *19*, 274-283.

Brouhard, G.J., Stear, J.H., Noetzel, T.L., Al-Bassam, J., Kinoshita, K., Harrison, S.C., Howard, J., and Hyman, A.A. (2008). XMAP215 is a processive microtubule polymerase. Cell *132*, 79-88.

Chen, Z.L., Yu, W.M., and Strickland, S. (2007). Peripheral regeneration. Annu Rev Neurosci *30*, 209-233.

Fernandez-Funez, P., Nino-Rosales, M.L., de Gouyon, B., She, W.C., Luchak, J.M., Martinez, P., Turiegano, E., Benito, J., Capovilla, M., Skinner, P.J., *et al.* (2000). Identification of genes that modify ataxin-1-induced neurodegeneration. Nature *408*, 101-106.

Haass, C., and Selkoe, D.J. (2007). Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer's amyloid beta-peptide. Nat Rev Mol Cell Biol *8*, 101-112.

Hall, D.H., and Hedgecock, E.M. (1991). Kinesin-related gene unc-104 is required for axonal transport of synaptic vesicles in C. elegans. Cell *65*, 837-847.

Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E.M., and Bastiani, M. (2009). Axon regeneration requires a conserved MAP kinase pathway. Science *323*, 802-806.

Hoffman, P.N. (2010). A conditioning lesion induces changes in gene expression and axonal transport that enhance regeneration by increasing the intrinsic growth state of axons. Exp Neurol *223*, 11-18.

Itoh, A., Horiuchi, M., Bannerman, P., Pleasure, D., and Itoh, T. (2009). Impaired regenerative response of primary sensory neurons in ZPK/DLK gene-trap mice. Biochem Biophys Res Commun *383*, 258-262.

Jiang, K., and Akhmanova, A. (2011). Microtubule tip-interacting proteins: a view from both ends. Curr Opin Cell Biol *23*, 94-101.

La Spada, A.R., and Taylor, J.P. (2010). Repeat expansion disease: progress and puzzles in disease pathogenesis. Nat Rev Genet *11*, 247-258.

Lee, H.H., Jan, L.Y., and Jan, Y.N. (2009). Drosophila IKK-related kinase Ik2 and Katanin p60-like 1 regulate dendrite pruning of sensory neuron during metamorphosis. Proc Natl Acad Sci U S A *106*, 6363-6368.

Lee, S.B., Bagley, J.A., Lee, H.Y., Jan, L.Y., and Jan, Y.N. (2011). Pathogenic polyglutamine proteins cause dendrite defects associated with specific actin cytoskeletal alterations in Drosophila. Proc Natl Acad Sci U S A *108*, 16795-16800.

Linda, H., Cullheim, S., and Risling, M. (1992). A light and electron microscopic study of intracellularly HRP-labeled lumbar motoneurons after intramedullary axotomy in the adult cat. J Comp Neurol *318*, 188-208.

Liu, K., Tedeschi, A., Park, K.K., and He, Z. (2011). Neuronal Intrinsic Mechanisms of Axon Regeneration. Annu Rev Neurosci.

Lu, B., and Vogel, H. (2009). Drosophila models of neurodegenerative diseases. Annu Rev Pathol *4*, 315-342.

Luo, L., and O'Leary, D.D. (2005). Axon retraction and degeneration in development and disease. Annu Rev Neurosci *28*, 127-156.

MacDonald, J.M., Beach, M.G., Porpiglia, E., Sheehan, A.E., Watts, R.J., and Freeman, M.R. (2006). The Drosophila cell corpse engulfment receptor Draper mediates glial clearance of severed axons. Neuron *50*, 869-881.

Nix, P., Hisamoto, N., Matsumoto, K., and Bastiani, M. (2011). Axon regeneration requires coordinate activation of p38 and JNK MAPK pathways. Proc Natl Acad Sci U S A *108*, 10738-10743.
Pack-Chung, E., Kurshan, P.T., Dickman, D.K., and Schwarz, T.L. (2007). A Drosophila kinesin required for synaptic bouton formation and synaptic vesicle transport. Nat Neurosci *10*, 980-989.
Shimono, K., Fujimoto, A., Tsuyama, T., Yamamoto-Kochi, M., Sato, M., Hattori, Y., Sugimura, K., Usui, T., Kimura, K., and Uemura, T. (2009). Multidendritic sensory neurons in the adult Drosophila abdomen: origins, dendritic morphology, and segment- and age-dependent programmed cell death. Neural Dev *4*, 37.
Silver, J. (2009). CNS regeneration: only on one condition. Curr Biol *19*, R444-446.
Steffan, J.S., Bodai, L., Pallos, J., Poelman, M., McCampbell, A., Apostol, B.L., Kazantsev, A., Schmidt, E., Zhu, Y.Z., Greenwald, M.*, et al.* (2001). Histone deacetylase inhibitors arrest polyglutamine-dependent neurodegeneration in Drosophila. Nature *413*, 739-743.
Stone, M.C., Nguyen, M.M., Tao, J., Allender, D.L., and Rolls, M.M. (2010). Global up-regulation of microtubule dynamics and polarity reversal during regeneration of an axon from a dendrite. Mol Biol Cell *21*, 767-777.
Sumner, B.E., and Watson, W.E. (1971). Retraction and expansion of the dendritic tree of motor neurones of adult rats induced in vivo. Nature *233*, 273-275.
Tao, J., and Rolls, M.M. (2011). Dendrites have a rapid program of injury-induced degeneration that is molecularly distinct from developmental pruning. J Neurosci *31*, 5398-5405.
Wang, Z., and Jin, Y. (2011). Genetic dissection of axon regeneration. Curr Opin Neurobiol *21*, 189-196.
Warrick, J.M., Morabito, L.M., Bilen, J., Gordesky-Gold, B., Faust, L.Z., Paulson, H.L., and Bonini, N.M. (2005). Ataxin-3 suppresses polyglutamine neurodegeneration in Drosophila by a ubiquitin-associated mechanism. Mol Cell *18*, 37-48.
Watts, R.J., Hoopfer, E.D., and Luo, L. (2003). Axon pruning during Drosophila metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. Neuron *38*, 871-885.
Wiese, C. (2008). Distinct Dgrip84 isoforms correlate with distinct gamma-tubulins in Drosophila. Mol Biol Cell *19*, 368-377.
Xiong, X., and Collins, C.A. (2012). A conditioning lesion protects axons from degeneration via the Wallenda/DLK MAP kinase signaling cascade. J Neurosci *32*, 610-615.
Xiong, X., Wang, X., Ewanek, R., Bhat, P., Diantonio, A., and Collins, C.A. (2010). Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. J Cell Biol *191*, 211-223.
Yawo, H. (1987). Changes in the dendritic geometry of mouse superior cervical ganglion cells following postganglionic axotomy. J Neurosci *7*, 3703-3711.
Zhai, Q., Wang, J., Kim, A., Liu, Q., Watts, R., Hoopfer, E., Mitchison, T., Luo, L., and He, Z. (2003). Involvement of the ubiquitin-proteasome system in the early stages of wallerian degeneration. Neuron *39*, 217-225.

Supplemental Information

SI figures

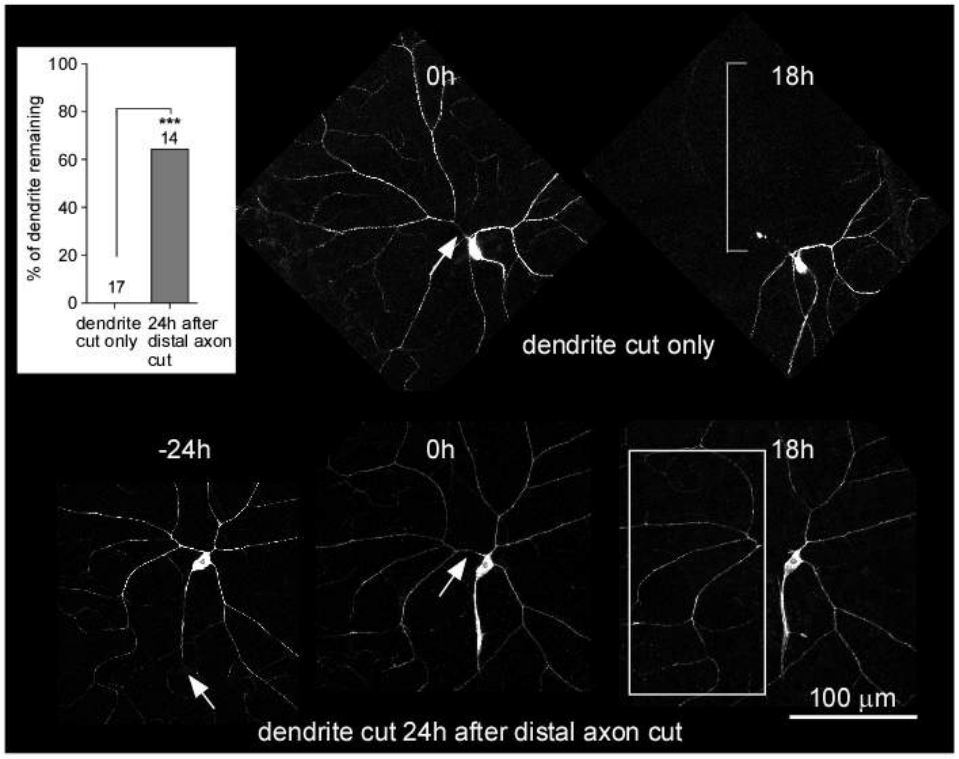

Figure S1. Previous distal axotomy delays injury-induced dendrite degeneration.

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.

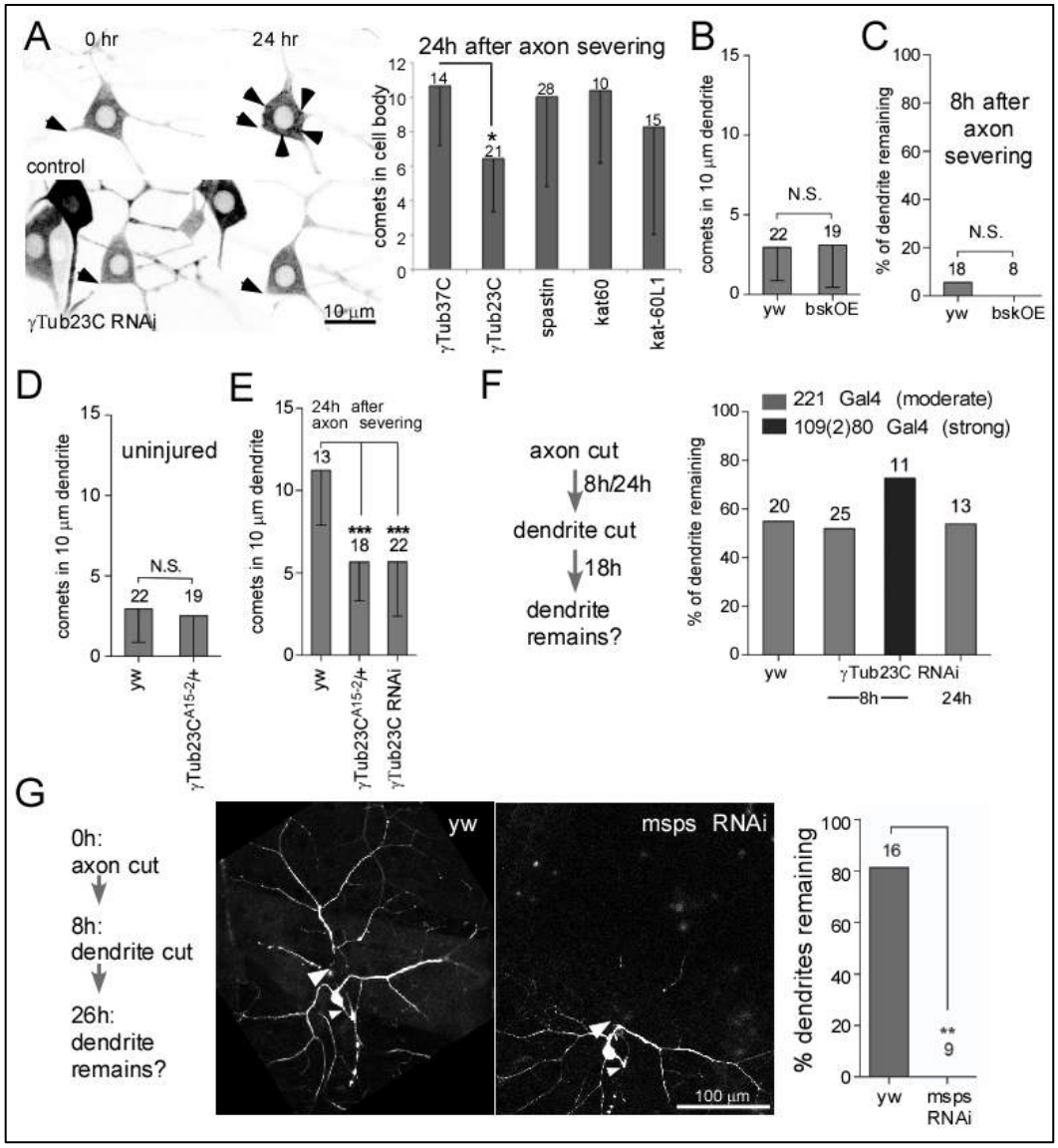

Figure S2. Further characterization of the microtubule-based program induced by axon severing.

(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 *γTub23C*$^{A15\text{-}2}$ 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 Figure 2D, except for the time after axon injury at which comet number was counted. In Figure 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 Figure 2E. At 8 h after axon injury, dendrite protection was reduced in the $\gamma Tub23C^{A15\text{-}2}$ heterozygous background (Figure 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 (Figure 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 (Figure 2E) or ddaC neurons as shown here. Numbers above the bars are numbers of neurons assayed. Significance was determined with a Fisher exact test.

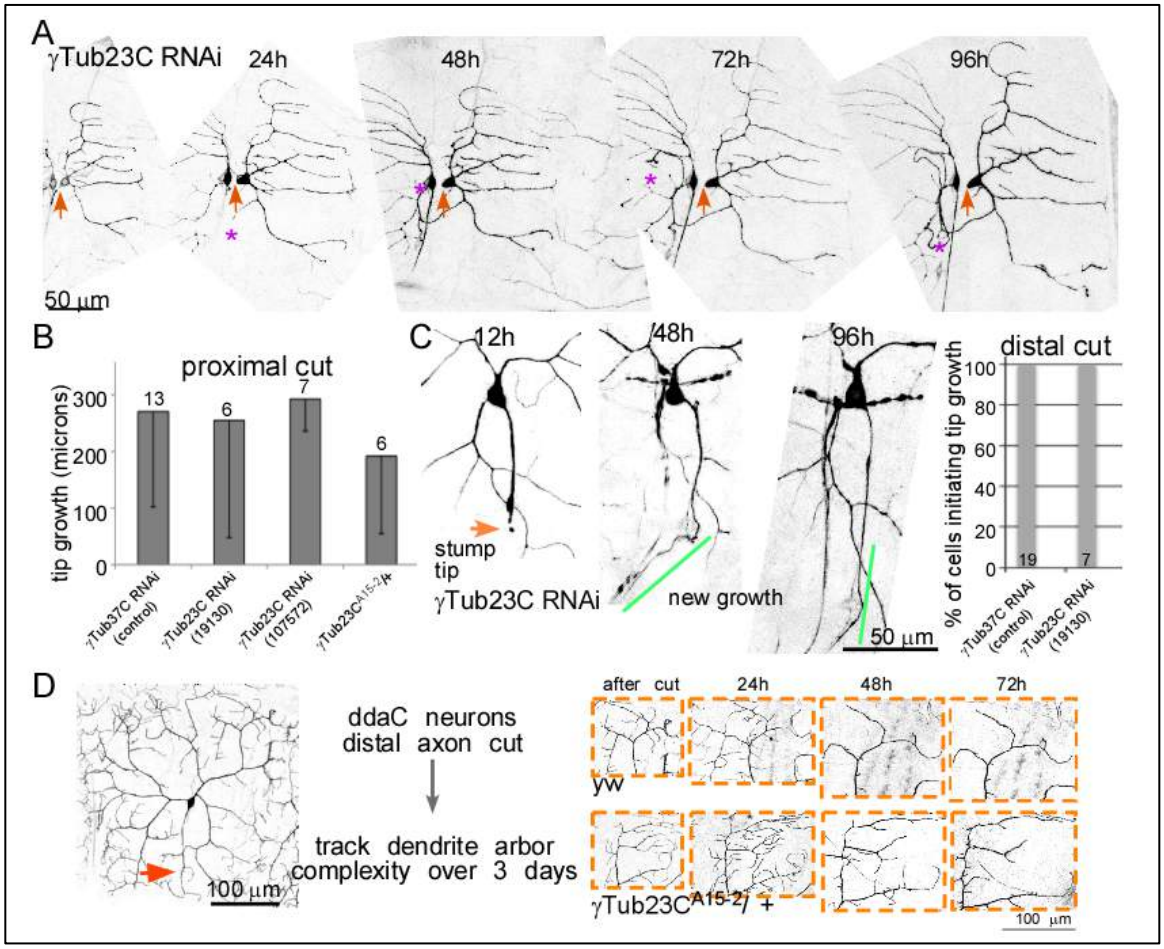

Figure S3. Axon regeneration and dendrite simplification is normal in neurons with reduced levels of γTub23C.

(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*$^{A15\text{-}2}$ heterozygote are shown. In both cases, dendrite termini were lost.

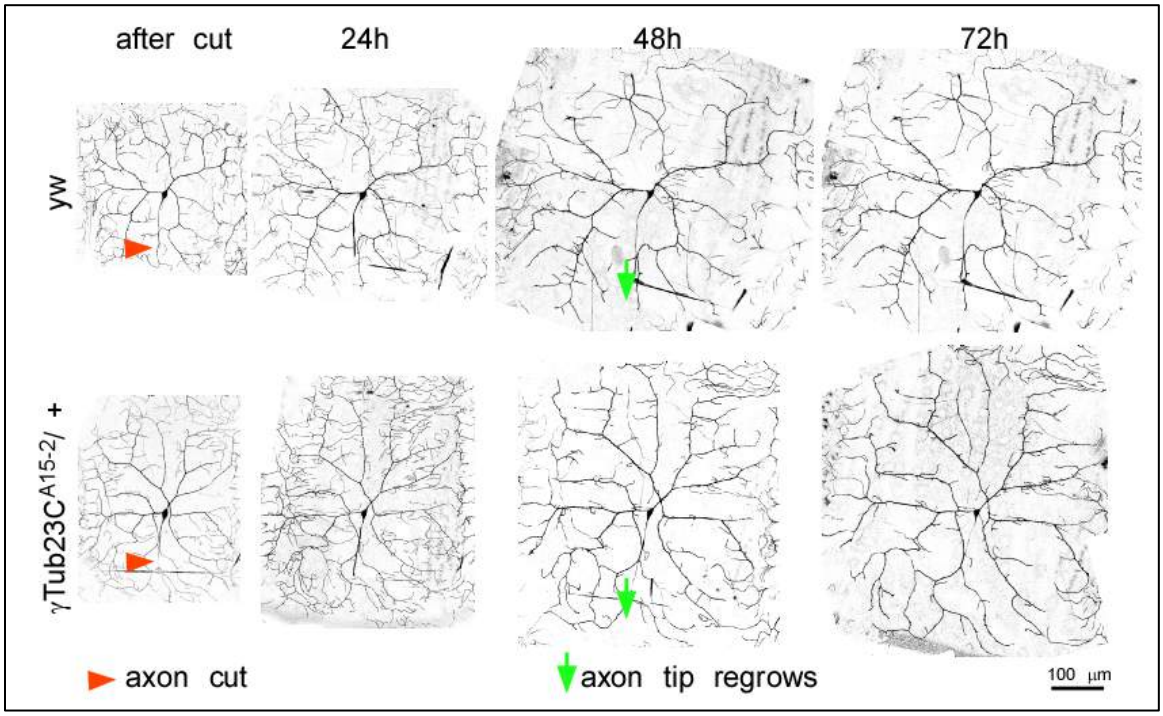

Figure S4. Dendrites retract after distal axotomy in control and *γTub23C* heterozygous neurons.

This figure is a companion to Figure S3D. Small regions of dendrite arbors are shown in Figure S3D for simplicity; the full ddaC arbor is shown over time after axon severing here for reference.

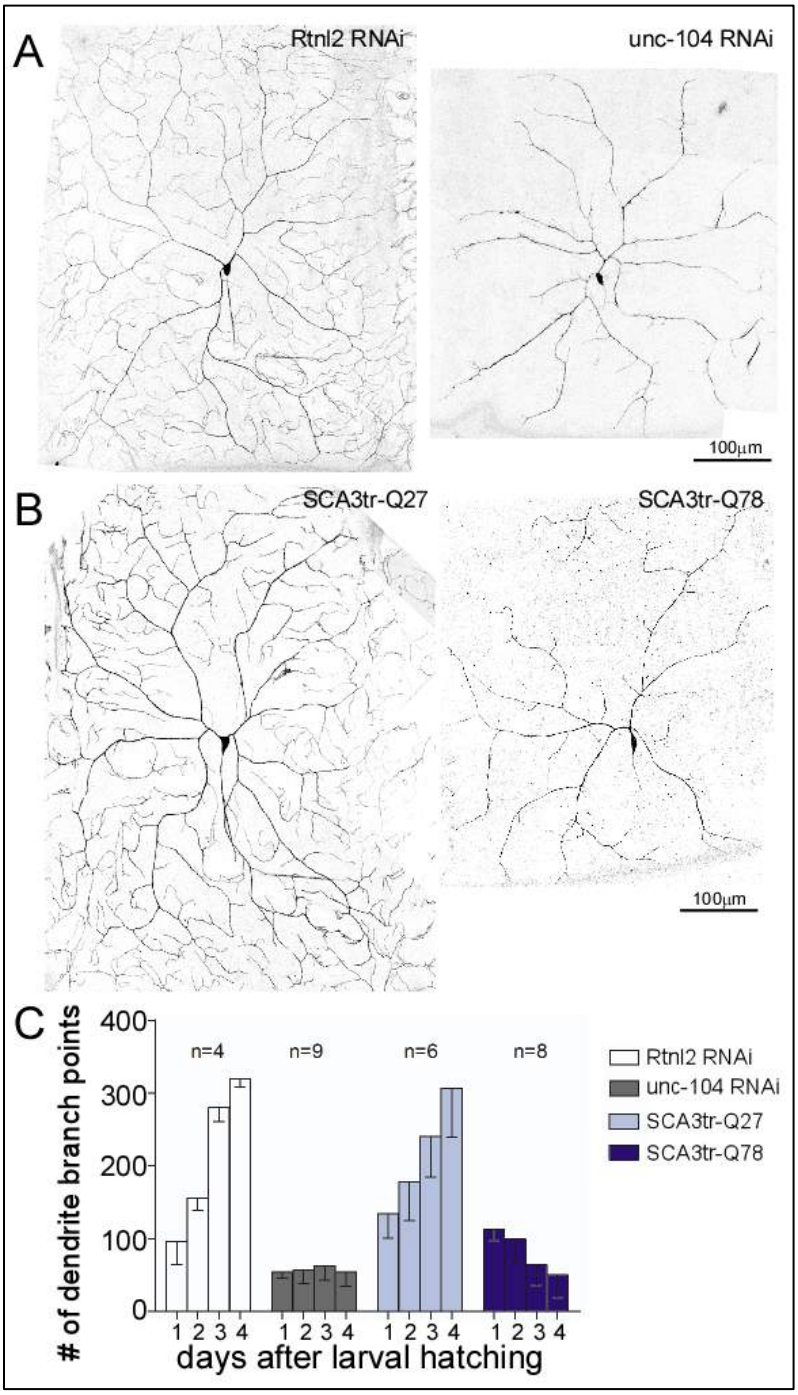

Figure S5. Reduction of an axonal motor or expression of SCA3tr-Q78 reduces dendrite complexity of ddaC.

(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.

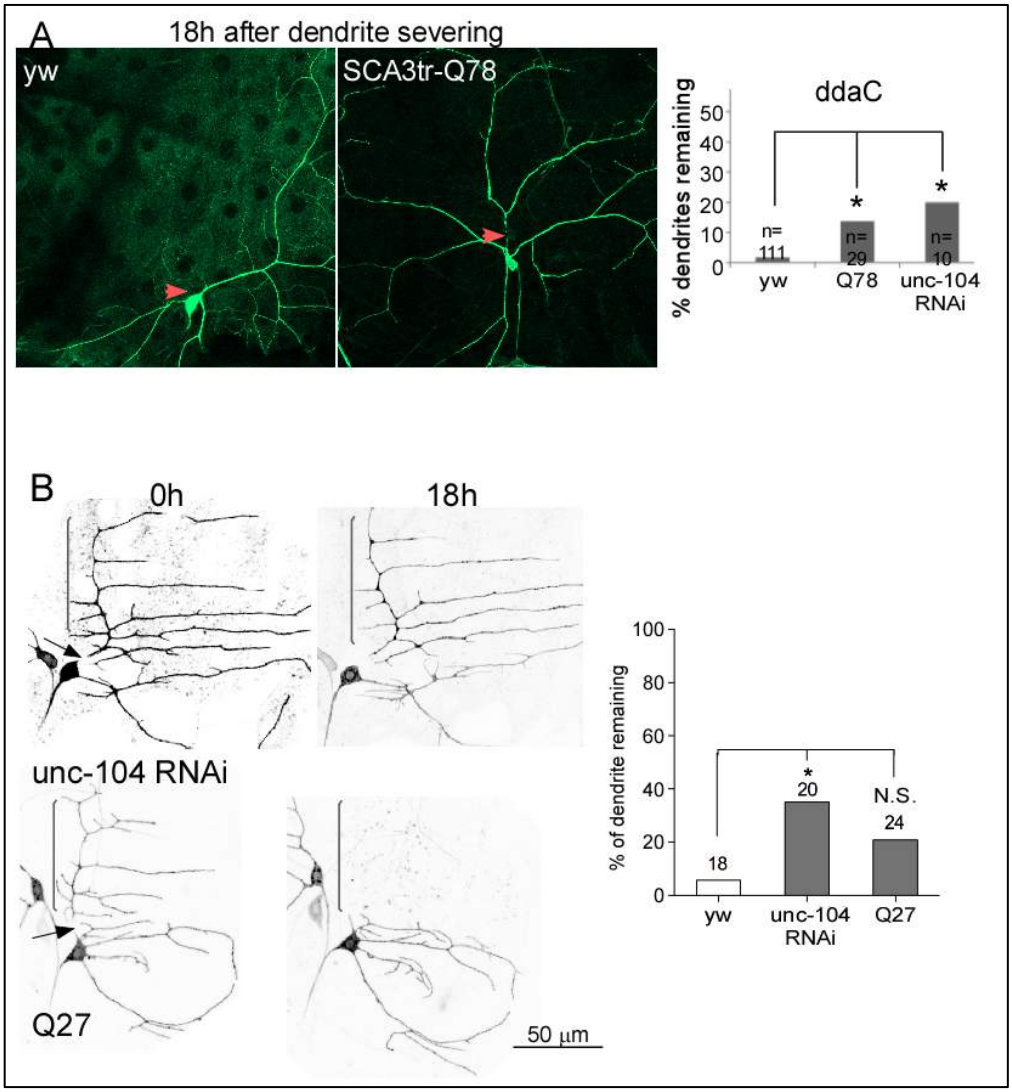

Figure S6. SCA3tr-Q78 delays degeneration in ddaC, as does unc-104 RNAi; unc-104 RNAi also delays degeneration in ddaE.

(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.

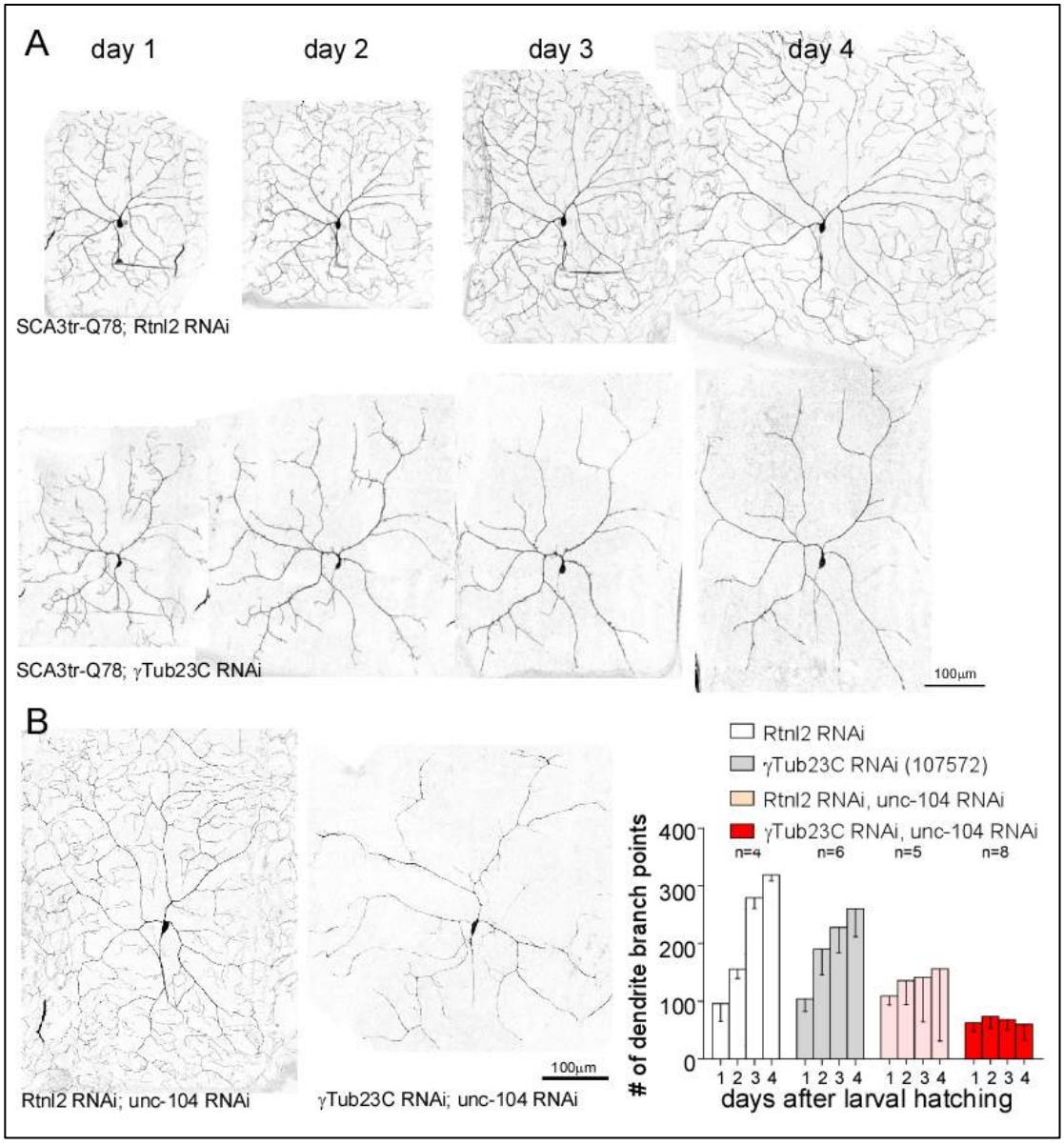

Figure S7. Expression of hairpin RNAs targeting γTub23C worsens degeneration in neurons expressing SCA3tr-Q78 or unc-104 RNA hairpins.

(A) This is a companion to Figure 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.

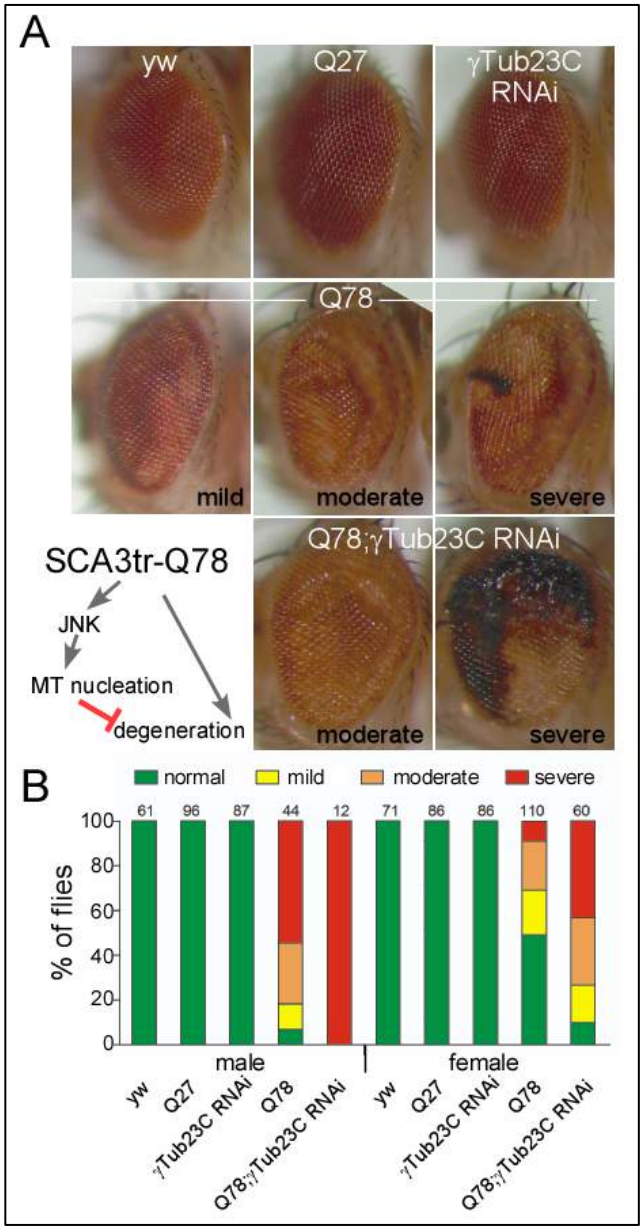

Figure S8. Global degeneration induced by SCA3tr-Q78 in eyes is countered by microtubule nucleation.

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.

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 (Grueber et al., 2002). They respond to a variety of stimuli and act as proprioceptors and nociceptors, sending their axons back to the central nervous system (Hughes and Thomas, 2007; Hwang et al., 2007). Their internal organization is similar to other Drosophila neurons (Rolls, 2011; Stone et al., 2008), 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 (Stone et al., 2010; Tao and Rolls, 2011). 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 (33320), unc-104 (47171, 23465, 23464), γTub23C (19130, 107572), γTub37C (25271), and msps (21982). Human disease transgenes 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 ((Fernandez-Funez et al., 2000); Baylor College of Medicine, Houston, TX), and UAS-httex1p-Q20/Q93 from Leslie M. Thompson ((Steffan et al., 2001); 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 was injected into fly embryos followed by standard procedures to generate transgenic flies.) EB1-GFP, mCD8-RFP, or mCD8-GFP were used to visualize the overall cell morphology driven by cell type-specific Gal4s, including 221 and 109(2)80 for class I ddaE neurons, and 477 for class IV ddaC. Dicer2 was included in all RNAi experiments to achieve a higher knockdown efficiency. The γTub23CA15-2/CyO, actin-GFP; 221Gal4, UAS-EB1-GFP/TM3, ftz –lacZ fly line was used to introduce one copy of γtub23C-null allele, in which background a UAS-γTub23C-GFP (C9.1 on II) was expressed for the rescue experiment. The tester line APPLGFP; longGMR-Gal4 was used to express polyglutamine proteins in fly retina photoreceptor neurons. The APPL-GFP labels endogenous β-amyloid protein precursor-like with GFP, which does not interfere with Gal4/UAS expression system.

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-d 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 and Axon Injury.

The injury-induced dendrite degeneration assay was performed in ddaE and ddaC neurons as described (Tao and Rolls, 2011). 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. A “whirlpool” screen was performed in ddaE cells to study changes in microtubule dynamics after proximal axotomy. 109(2)80 Gal4 was used to drive EB1-GFP and dicer2. This tester line was crossed to different Vienna Drosophila RNAi Center RNAi lines. At 24 h after proximal axon transection, movies of EB1 comets in cell bodies of the injured cells were acquired as described (Stone et al., 2010). 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 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 (Williams et al., 2006), 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 γTub23CA15-2 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 40Å~ oil immersion objective, and the images were pieced together as montages using Acdsee 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 numbers 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.

SI movies

http://www.pnas.org/content/109/29/11842.long?tab=ds

Movie S1. Targeting the axonal kinesin unc-104 increases the number of growing microtubules in dendrites.

Movie S2. Expression of SCA3tr-Q78, but not SCA3tr-Q27, increases the number of growing microtubules in neurons.

Movie S3. The core microtubule nucleation, γTub23C, is required to increase microtubule dynamics in response to SCA3tr-Q78.

Movie S4. γTub23C is required to increase microtubule dynamics in response to reduction of unc-104.

Movie S5. JNK signaling is required to increase microtubule dynamics in SCA3tr-Q78–expressing neurons.

Movie S6. JNK signaling is required to increase microtubule dynamics when unc-104 levels are reduced by RNAi.

SI references

Rolls, M.M. (2011). Neuronal polarity in Drosophila: Sorting out axons and dendrites. Dev Neurobiol *71*, 419-429.
Fernandez-Funez, P., Nino-Rosales, M.L., de Gouyon, B., She, W.C., Luchak, J.M., Martinez, P., Turiegano, E., Benito, J., Capovilla, M., Skinner, P.J., *et al.* (2000). Identification of genes that modify ataxin-1-induced neurodegeneration. Nature *408*, 101-106.
Grueber, W.B., Jan, L.Y., and Jan, Y.N. (2002). Tiling of the Drosophila epidermis by multidendritic sensory neurons. Development *129*, 2867-2878.
Hughes, C.L., and Thomas, J.B. (2007). A sensory feedback circuit coordinates muscle activity in Drosophila. Mol Cell Neurosci *35*, 383-396.
Hwang, R.Y., Zhong, L., Xu, Y., Johnson, T., Zhang, F., Deisseroth, K., and Tracey, W.D. (2007). Nociceptive neurons protect Drosophila larvae from parasitoid wasps. Curr Biol *17*, 2105-2116.
Rolls, M.M. (2011). Neuronal polarity in Drosophila: Sorting out axons and dendrites. Dev Neurobiol *71*, 419-429.
Stone, M.C., Roegiers, F., and Rolls, M.M. (2008). Microtubules Have Opposite Orientation in Axons and Dendrites of Drosophila Neurons. Mol Biol Cell *19*, 4122-4129.
Stone, M.C., Nguyen, M.M., Tao, J., Allender, D.L., and Rolls, M.M. (2010). Global up-regulation of microtubule dynamics and polarity reversal during regeneration of an axon from a dendrite. Mol Biol Cell *21*, 767-777.
Williams, D.W., Kondo, S., Krzyzanowska, A., Hiromi, Y., and Truman, J.W. (2006). Local caspase activity directs engulfment of dendrites during pruning. Nat Neurosci *9*, 1234-1236.

Chapter 3

Mitochondria and Caspases Tune Nmnat-Mediated Stabilization to Promote Axon Regeneration

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Abstract

When axons are severed, regeneration is required to reconnect the neuron to its targets. Neuroprotection is a rapid response to axon injury that precedes regeneration and makes the uninjured regions of the cell resistant to degeneration. To determine whether mitochondria promote neuroprotection we depleted them from dendrites. Surprisingly, dendrites became more resistant to degeneration when they contained fewer mitochondria. Suppression of

neuroprotection by mitochondria requires mitochondrial fission and subsequent caspase activity. While mitochondria and caspases negatively regulate neuroprotection, a DLK/JNK/fos cascade is required to activate it. Downstream of both positive and negative regulatory arms, the enzyme Nmnat is the central mediator of neuroprotection. Nmnat is required for stabilization, and acts upstream of microtubule dynamics. Neuroprotection normally ends about 48h after axon injury as regeneration is initiated. Overexpresson of JNK, fos extends neuroprotection and dampens axon regeneration. Regeneration defects were rescued by reducing Nmnat. We conclude that the canonical DLK, JNK, fos axon regeneration cascade turns on neuroprotection as an early response to axon injury. Neuroprotection is mediated by Nmnat and allows the rest of the cell to resist degeneration. However, if neuroprotection is not downregulated by caspase activity, axon regeneration is reduced. This data suggests that neuroprotection and regeneration are incompatible, and that therapeutic strategies to boost one or the other response may have unanticipated negative consequences.

Introduction

The ability of neurons to survive injury, misfolded proteins, hypoxic stress and other deleterious conditions allows the nervous system to function for a lifetime without large-scale production of new neurons. Neuronal survival strategies buy the cells time to maintain or regain function. For example, neurons may remain non-functional for weeks, months or years after axonal trauma. Their survival allows axon regeneration to take place, and eventually, if an appropriate target is reached the cells may again function.

Preconditioning is a transient survival strategy triggered by a stressful, but sublethal, event. For example, when blood flow to a region of the brain is transiently reduced, the effects of a subsequent ischemic stroke are not as severe (Dirnagl et al., 2003; Stetler et al., 2014). Tissue-

level preconditioning seems to have an immediate phase, and then a longer-term transcription-dependent phase (Dirnagl and Meisel, 2008; Dirnagl et al., 2003) and is proposed to be a very general stress response mechanism.

Preconditioning has also been described at a single cell level. In Dorsal Root Ganglion (DRG) neurons, severing the peripheral axon enables the central axon for regeneration (Silver, 2009). The initial peripheral lesion triggers transcriptional changes in the cell body that are proposed to facilitate subsequent regeneration of the central axon (Smith and Skene, 1997; Ylera et al., 2009). In *Drosophila* models of conditioning lesion in sensory and motor neurons, axon severing turns on a stabilization pathway that is measured by resistance to degeneration after a subsequent injury (Chen et al., 2012; Xiong and Collins, 2012). Dendrite stabilization is downstream of dual leucine zipper kinase (DLK) (Xiong and Collins, 2012) and c-Jun N-terminal Kinase (JNK) (Chen et al., 2012). DLK is a MAP kinase kinase kinase, and JNK is the downstream MAP kinase, which play central roles in the regulatory cascade that initiates axon regeneration in nematodes, flies and mammals (Hammarlund et al., 2009; Shin et al., 2012; Xiong et al., 2010; Yan et al., 2009). DLK/JNK are therefore implicated in regulation of both axon regeneration and preconditioning-induced neuroprotection in response to axon injury. One possibility is that these two responses are mediated by the same set of effectors activated by DLK/JNK.

Using the *Drosophila* sensory neuron model for preconditioning, we investigate the effectors mediating neuroprotection downstream of DLK/JNK, and the relationship between neuroprotection and axon regeneration. One hallmark of neuroprotection is a dramatic increase in microtubule dynamics (Chen et al., 2012). As microtubule growth is GTP-dependent, this neuroprotective pathway may be regulated by mitochondria, which have been suggested to play a central role in brain preconditioning (Correia et al., 2010). We therefore started by investigating the role of mitochondria in neuroprotection. Surprisingly, we find that, rather than promoting

neuroprotection, mitochondria have an inhibitory role in this process through caspase regulation. Moreover, although regeneration and neuroprotection are downstream of the same kinase cascade, neuroprotection antagonizes regeneration. These results are unexpected, but fit together into a multi-step model of response to axon injury downstream of DLK/JNK that includes initial activation of neuroprotection, which must be tempered by caspases for subsequent axon regeneration.

Results

Mitochondria negatively regulate neuroprotection.

We previously showed that in *Drosophila* sensory neurons, severing an axon with a pulsed UV laser turns on a stabilization pathway (Chen et al., 2012). This is measured by the ability of dendrites to resist injury-induced degeneration. Dendrites normally degenerate completely within 18h after injury (Figure 1A). However, if axons are damaged 8h prior to dendrite injury, the severed dendrites are stabilized and take more than 18h to fragment (Figure 1A' and B). Stabilization is maximal at 8-24h and tapers off at 48h after axon injury (Chen et al., 2012) (Figure 1A''). Thus conditioning-induced neuroprotection is a transient response to axon injury.

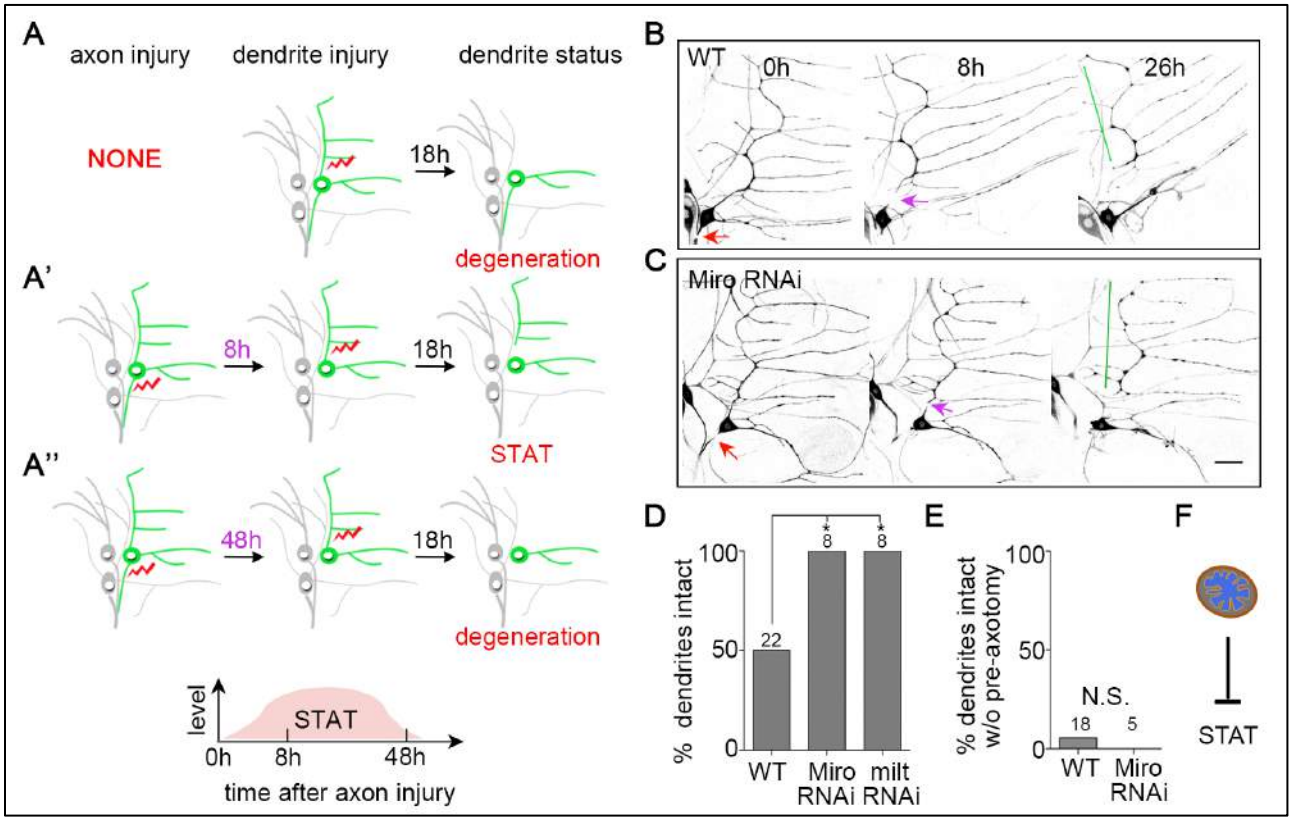

Figure 1. Reducing mitochondria in dendrites increases protection.

(A-A'') A schematic of the protection assay is shown. (A) Without pre-axon injury, dendrites degenerate within 18h after injury. (A') An axon injury 8h prior to dendrite injury induces neuroprotection so that dendrite degeneration is delayed. (A'') Neuroprotection is reduced 48h after axon injury. UV-induced injury (red lightning bolts) was performed in ddaE neurons (green). Other neurons labeled by 221-Gal4, which was used in most experiments to drive expression, are drawn in grey.

(B-D) Sequential axon and dendrite injury as illustrated in Figure 1A' was performed in wide-type (WT) and Miro RNAi neurons. Neurons were labeled with EB1-GFP under the control of 221-Gal4. Red arrows indicate site of axon injury; purple arrows, dendrite injury. Green lines mark stabilized dendrites. In D, the numbers of neurons analyzed for each genotype are indicated on the bars. A Fisher's exact test was used to determine statistical significance. * $p<0.05$. The scale bars is 20 μm

(E) Dendrite severing without pre-axon injury was performed in control and Miro RNAi neurons. Dendrite presence was assayed 18h after severing. The numbers of neurons analyzed are indicated on the bars. Fisher's exact test was used to determine statistical significance. Scale bars: 20 μm

(F) A summary model of the data in the figure.

To understand how neuroprotection might be regulated, we considered the dramatic upregulation of microtubule dynamics that is a key feature of neuroprotection (Chen et al., 2012). As microtubule growth is GTP-dependent, this increase in the number of growing microtubules may require additional energy production. We therefore hypothesized that mitochondria might positively regulate neuroprotection.

To assess the role of mitochondria in neuroprotection, we depleted mitochondria from dendrites using RNAi-mediated knockdown of the mitochondrial Rho-GTPase Miro, which is required for mitochondrial transport in neurons (Glater et al., 2006; Guo et al., 2005). We have previously shown that Miro RNAi reduces the number of mitochondria in dendrites of ddaE neurons, the same cells used here (Tao and Rolls, 2011). Based on our hypothesis that mitochondria positively regulate neuroprotection, we predicted that neuroprotection would be reduced in neurons that expressed Miro RNAi hairpins.

To our surprise, rather than suppressing neuroprotection, Miro RNAi increased it (Figure 1C and D). Targeting milton, which recruits Miro to mitochondria (Glater et al., 2006; Guo et al., 2005), also increased neuroprotection (Figure 1D). To make sure that this increase was due to an alteration in the neuroprotection response and not a difference in timing of dendrite degeneration, we measured dendrite degeneration without first cutting the axon. In this case Miro RNAi had no and dendrites degenerated normally (Figure 1E). These data suggested that rather than promoting neuroprotection, mitochondria negatively regulate this injury response (Figure 1F).

Axon injury induces Drp1-mediated mitochondrial fission.

As a negative role for mitochondria in neuroprotection was unexpected, we analyzed mitochondrial shape and motility in dendrites after axon injury to try to understand what role mitochondria might play after axon injury. Both mito-GFP (mitochondria) and mCD8-RFP (cell

membrane) were expressed under the control of 221-Gal4, which is a driver for Class I dendritic arborization neurons including ddaE.

We first measured motility of mitochondria in the dendrite arbor. In uninjured neurons 3.4% of mitochondria moved more than a micron in a one minute imaging window. 8h after axon injury, the number of moving mitochondria increased to 13% (Figure S1A and B). This observation is consistent with a vertebrate study showing a peripheral conditioning lesion increases mitochondrial transport in the central axon of dorsal root ganglion neurons (Mar et al., 2014). However, how this might relate to negative regulation of neuroprotection was not clear.

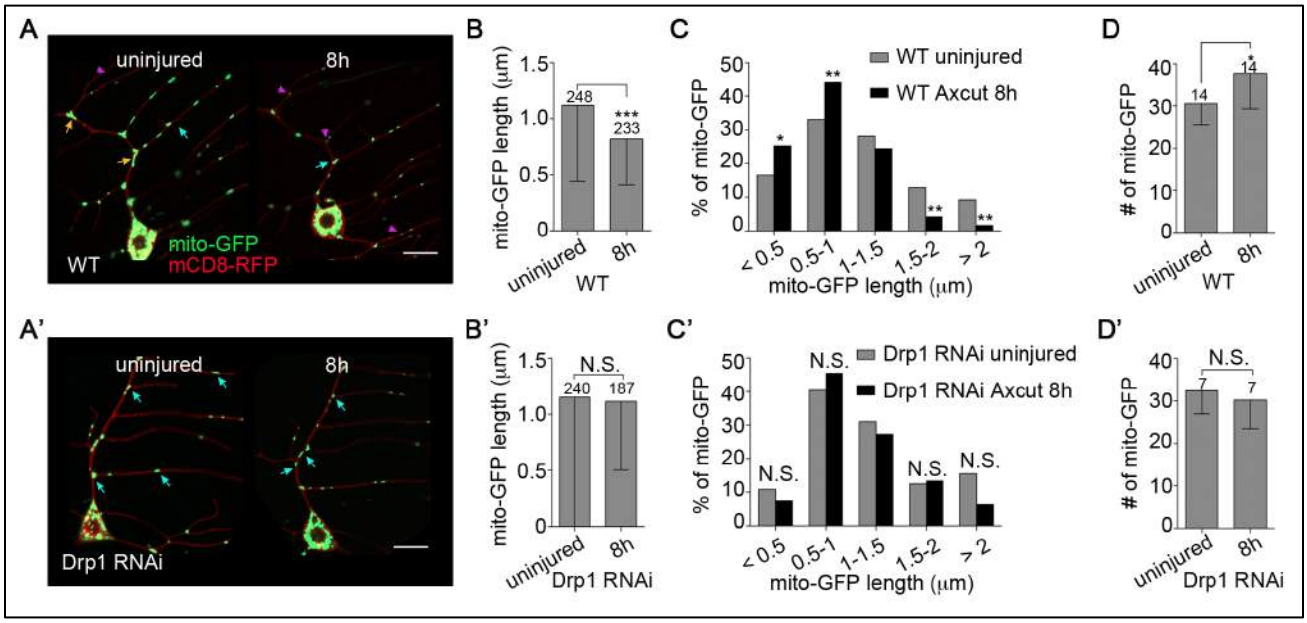

Figure 2. Axon injury triggers Drp1-dependent mitochondrial fission in dendrites.

(A and A') Representative images of dendritic mitochondria before and after axon injury in wide-type (A) and Drp1 RNAi neurons are shown. (A') mito-GFP and mCD8-RFP were coexpressed in ddaE neurons under the control of 221-Gal4 in order to visualize mitochondria and the cell membrane, respectively. Orange, blue and magenta arrows indicate long, medium and short mitochondria. The scale bars are 10 μm.

(B-D') The length, length distribution, and total number of mitochondria in wide-type (B-D) and Drp1 RNAi neurons (B'-D') before and after axon injury were measured. Statistical significance was determined using an unpaired t test (B and B'), a Fisher's exact test (C and C'), or a paired t test (D and D'). *P<0.05, **P<0.01, ***P<0.001, N.S. not significant. Error bars represent the standard deviation (SD). For B and C the same set of mitochondria was analyzed, and total numbers of mitochondria are indicated on the bars in B. At least 9 animals were used for each condition. (D and D') The numbers of neurons analyzed are indicated on the bars.

We next analyzed mitochondrial morphology and number. After injury, the average length of mitochondria decreased significantly (Figure 2A and B). After axon injury more short and fewer long mitochondria were present (Figure 2C). These length changes suggested mitochondrial fission might be upregulated after axon injury. Consistent with this idea the total number of mitochondria in dendrites was higher after axon injury than in uninjured neurons (Figure 2D).

To test whether the changes in mitochondrial length and number after axon injury were due to an increase in mitochondrial fission, we used RNAi to target Drp1, a dynamin-related GTPase that mediates mitochondrial fission (Westermann, 2010). Drp1 RNAi hairpins were expressed in class I neurons under control of 221-Gal4, together with Dicer2 and mitochondrial and plasma membrane markers. We found that Drp1 RNAi blocked the injury-induced changes in mitochondrial length and number (Figure 2A'-D') without affecting them in uninjured cells (Figure S1C). We conclude that axon injury triggers increased mitochondrial fission in a Drp1-dependent manner.

Mitochondrial fission and caspases negatively regulate neuroprotection

To determine whether the increase in mitochondrial fission induced by axon injury was related to downregulation of neuroprotection by mitochondria, we assayed neuroprotection in Drp1 RNAi neurons. As in Miro and milton RNAi neurons, neuroprotection was dramatically increased when Drp1 was targeted by RNAi (Figure 3A and B). Drp1 RNAi did not influence the normal time course of dendrite degeneration (Figure S2). These results suggested that mitochondrial fission, rather than the mitochondria themselves, negatively regulates

neuroprotection. Consistent with this idea, overexpression of Drp1 reduced neuroprotection (Figure 3B).

Drp1-mediated mitochondrial fission occurs during apoptosis in *Drosophila* (Goyal et al., 2007) as well as in other organisms (Wang and Youle, 2009). In mammals and in *C. elegans* this fission is upstream of caspase activation (Cassidy-Stone et al., 2008; Jagasia et al., 2005). Because of this connection between mitochondrial fission and caspase activation, we hypothesized that caspases might inhibit neuroprotection downstream of mitochondria.

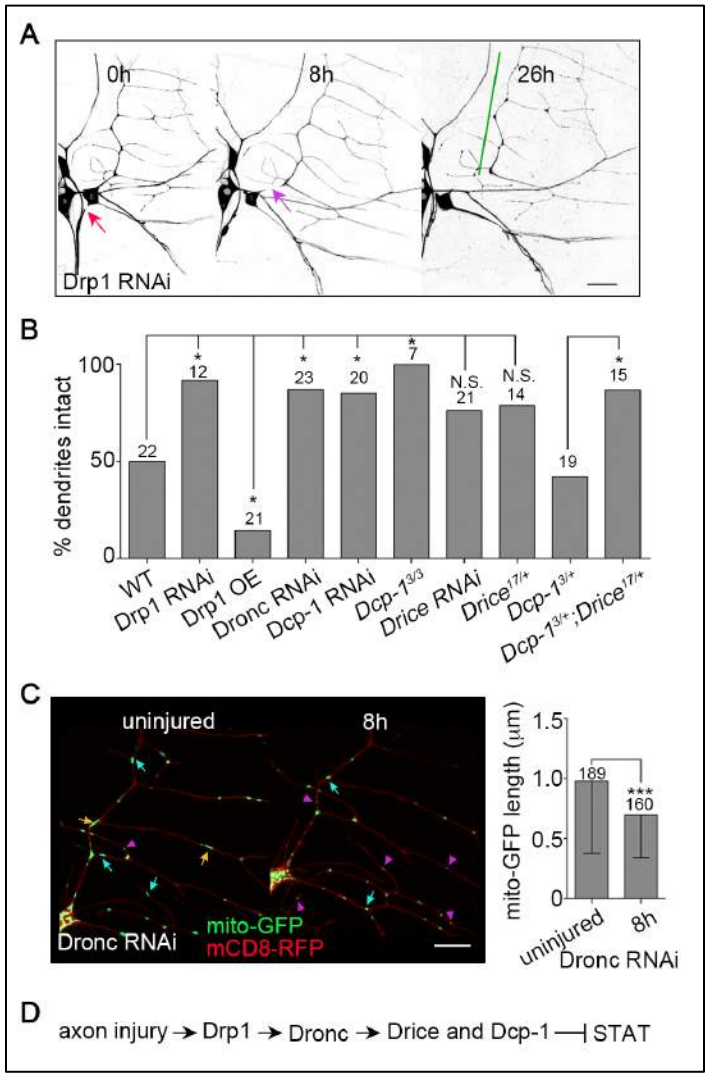

Figure 3. Drp1 and caspases inhibit neuroprotection.

(A and A') Representative images of dendritic mitochondria before and after axon injury in wide-type (A) and Drp1 RNAi neurons are shown. (A') mito-GFP and mCD8-RFP were coexpressed in ddaE neurons under the control of 221-Gal4 in order to visualize mitochondria and the cell membrane, respectively. Orange, blue and magenta arrows indicate long, medium and short mitochondria. The scale bars are 10 μm.

(B-D') The length, length distribution, and total number of mitochondria in wide-type (B-D) and Drp1 RNAi neurons (B'-D') before and after axon injury were measured. Statistical significance was determined using an unpaired t test (B and B'), a Fisher's exact test (C and C'), or a paired t test (D and D'). *P<0.05, **P<0.01, ***P<0.001, N.S. not significant. Error bars represent the standard deviation (SD). For B and C the same set of mitochondria was analyzed, and total numbers of mitochondria are indicated on the bars in B. At least 9 animals were used for each condition. (D and D') The numbers of neurons analyzed are indicated on the bars.

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To test this hypothesis we expressed large RNA hairpins to target the initiator caspase Dronc and effector caspases Dcp-1 and Drice. Neurons expressing Dronc or Dcp-1 RNA hairpins had high levels of neuroprotection, similar to neurons with Drp1 and Miro hairpins (Figure 3B). The results with Drice RNAi were inconclusive, perhaps because of insufficient knockdown. We confirmed the involvement of caspases in negative regulation of neuroprotection by using a strong loss-of-function allele $Dcp\text{-}1^{3}$ (Laundrie et al., 2003). Neuroprotection was also increased in this background (Figure 3B). Introduction of one mutant copy of Drice, $Drice^{17}$ (Xu et al., 2006), into heterozygous $Dcp\text{-}1^{3}$ mutant animals significantly enhanced neuroprotection (Figure 3B) indicating both effector caspases are likely to be involved in negative regulation of neuroprotection.

Although in *C. elegans* and mammals, mitochondrial fission and Drp1 act upstream of caspases (Cassidy-Stone et al., 2008; Jagasia et al., 2005), in *Drosophila* the effector caspase Dcp-1 can regulate mitochondrial shape and function (DeVorkin et al., 2014). To determine whether caspases acted upstream or downstream of mitochondrial fission in neuroprotection, we assayed mitochondrial fission in neurons expressing hairpin RNAs targeting Dronc. Mitochondrial length still decreased in response to axon injury in Dronc RNAi neurons (Figure 3C) consistent with caspases acting downstream of mitochondrial fission in negative regulation of neuroprotection (Figure 3D).

Nmnat is required for neuroprotection

So far we have shown that rather than positively regulating neuroprotection, mitochondrial fission negatively regulates neuroprotection through caspases. There must also be

factors that positively regulate neuroprotection in response to axon injury. The enzyme Nmnat is a good candidate for a positive player as it has been implicated in neuroprotection in a variety of scenarios (Ali et al., 2013a).

In addition to its role as a stress response factor, Nmnat is required for normal dendrite maintenance (Wen et al., 2011a). We therefore first sought to determine whether reducing Nmnat increased the rate of injury-induced dendrite degeneration. Dendrite morphology was scored at different time points following dendrite severing in control and Nmnat RNAi neurons. Beading, fragmentation and clearance of dendrites proceeded with similar timing in both genotypes (Figure 4A-C). We conclude that partial knockdown of Nmnat does not change the time course of dendrite degeneration in the absence of a conditioning axon injury. We also checked whether neurons with reduced Nmnat levels could still regenerate in response to axon injury. Axon regeneration was normal in Nmnat RNAi neurons (Figure S3A). Neurons with reduced Nmnat were therefore healthy enough to mount new outgrowth, and able to sense that the axon had been injured.

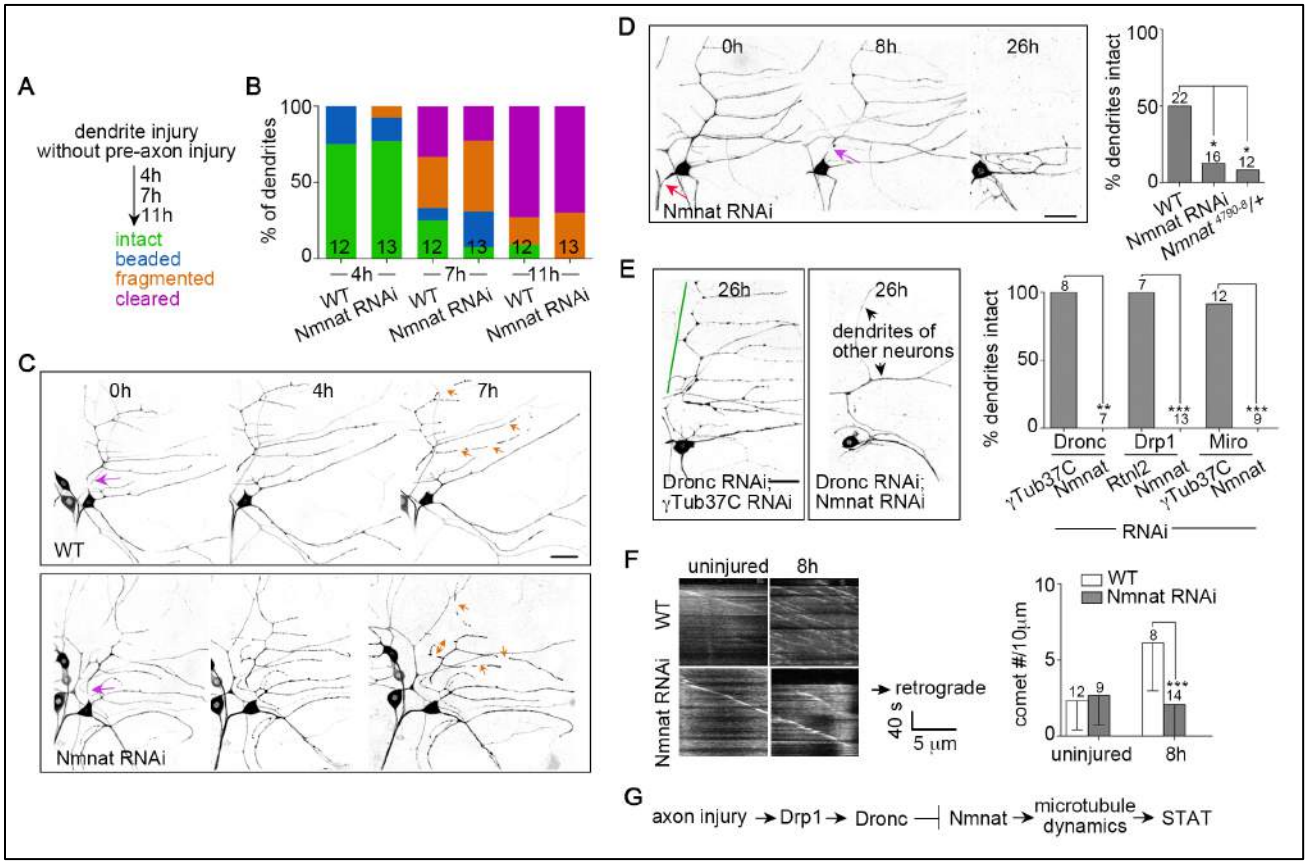

Figure 4. Nmnat is required for neuroprotection.

(A and A') Representative images of dendritic mitochondria before and after axon injury in wide-type (A) and Drp1 RNAi neurons are shown. (A') mito-GFP and mCD8-RFP were coexpressed in ddaE neurons under the control of 221-Gal4 in order to visualize mitochondria and the cell membrane, respectively. Orange, blue and magenta arrows indicate long, medium and short mitochondria. The scale bars are 10 μm.

(B-D') The length, length distribution, and total number of mitochondria in wide-type (B-D) and Drp1 RNAi neurons (B'-D') before and after axon injury were measured. Statistical significance was determined using an unpaired t test (B and B'), a Fisher's exact test (C and C'), or a paired t test (D and D'). *P<0.05, **P<0.01, ***P<0.001, N.S. not significant. Error bars represent the standard deviation (SD). For B and C the same set of mitochondria was analyzed, and total numbers of mitochondria are indicated on the bars in B. At least 9 animals were used for each condition. (D and D') The numbers of neurons analyzed are indicated on the bars.

To determine whether Nmnat was required for neuroprotection, we performed sequential axon and dendrite injury. Nmnat RNAi eliminated the stabilizing effect of axon injury on dendrites (Figure 4D). To confirm that this loss of neuroprotection was due to reduction of Nmnat, we performed the same experiment in animals heterozygous for a *Nmnat* null allele (Zhai

et al., 2006). Loss of one copy of *Nmnat* also resulted in complete loss of neuroprotection (Figure 4D). This phenotypic similarity between Nmnat RNAi and *Nmnat* heterozygous animals is consistent with antibody staining of Nmnat RNAi neurons. In these neurons, Nmnat staining intensity in the cell body was a little less than 50% of control neurons (Figure S3B). These results indicate that endogenous Nmnat is required to increase dendrite stability after axon conditioning.

Based on our previous results (Chen et al., 2012; Xiong and Collins, 2012), we suspected that Nmnat-mediated neuroprotection would affect axons as well as dendrites. In the experiments above, the ddaE axon was severed near the cell body so axon stabilization could not be tested. Severing individual ddaE axons more distally is challenging as they bundle with other labeled axons. In contrast, the axon of the Class IV dendritic arborization neuron, ddaC, can be labeled in isolation with the ppk-Gal4 driver. Without a distal conditioning lesion, most axons severed from the cell body were beaded 12h later (Figure S3C). In contrast, if a distal conditioning lesion was first performed, very few axon middle stumps were beaded within 12h (Figure S3C). When Nmnat was targeted by RNAi, the stabilizing effect of a distal axon injury was no longer observed (Figure S3C). These results indicate that Nmnat-dependent neuroprotection is a global response to axon injury.

We further tested the requirement of Nmnat for neuroprotection by crossing in RNA hairpins targeting Miro, Drp1 and Dronc. Even with the increased levels of neuroprotection provided by targeting any of these negative regulators, Nmnat RNAi completely eliminated the stabilizing effect of axon injury (Figure 4E). We conclude that Nmnat is a critical regulator of neuroprotection, and negative regulation of neuroprotection by caspases acts upstream of Nmnat.

Although Nmnat is a critical regulator of neuroprotection, we did not see large changes in amount or distribution of endogenous Nmnat after injury using immunofluorescence (Figure S4F). This may be because small changes in levels or activity of Nmnat are sufficient to mediate neuroprotection. Because endogenous Nmnat was barely detectable by immunofluorescence, we

also generated transgenic flies to overexpress GFP-tagged Nmnat. Two splice forms of Nmnat exist in *Drosophila*. Nmnat-A contains a nucleus localization sequence (NLS) while Nmnat-B does not. Note that our GFP-Nmnat-B is generated from HA-Nmnat [28] but lacks the N-terminal 31 amino acids as annotated in Flybase, and thus is referred to as Nmnat-B-deltaN. In ddaE sensory neurons, GFP-Nmnat-A is detected primarily in the nucleus (Figure S4B). The ratio of nuclear to cytoplasmic Nmnat-A signal changed in response to axon injury in wide-type but not Dronc RNAi neurons (Figure S4B), indicating at minimum that Nmnat-A is responsive to axon injury signals. GFP-Nmnat-B-deltaN has a wider distribution but does not respond to injury (Figure S4B).

We previously found that microtubule dynamics is dramatically upregulated in dendrites after axon injury in sensory neurons [29], which acts to stabilize dendrites against degeneration [8]. As Nmnat is required for neuroprotection, we wished to test its relationship to microtubule dynamics. When we compared induction of microtubule dynamics by axon injury in control and Nmnat RNAi neurons, we found that the increase in number of growing microtubules was sensitive to Nmnat reduction (Figure 4F). This suggests that increases in microtubule dynamics in response to axon injury require Nmnat. It seems unlikely, however, that microtubule dynamics is the sole effector of Nmnat as dampening microtubule dynamics does not block neuroprotection as strongly or consistently as reducing Nmnat (Figure 4D and E and (Chen et al., 2012)).

Dronc tunes Nmnat-mediated neuroprotection to promote regeneration

So far we have shown that neuroprotection is mediated by Nmnat, in part through increased microtubule dynamics. This stabilizing response to injury is also negatively regulated by caspases downstream of mitochondrial fission (Figure 4G). While increased stability is likely to help neurons to survive in damaged tissue, the reason for limiting neuroprotection through

caspase activity is not obvious. However, the timing of events triggered by axon regeneration suggested a hypothesis. Neuroprotection is maximal 8-24h after axon injury in ddaE neurons (Figure 1A-A'' and (Chen et al., 2012)), while axon regeneration typically begins 24-48h after injury in these cells (Stone et al., 2010). We therefore hypothesized that turning down the early neuroprotection response might promote subsequent regeneration.

To test whether uncontrolled neuroprotection might inhibit regeneration, we compared regeneration in control and Dronc RNAi neurons. When ddaE neurons are axotomized close to the cell body axon regeneration proceeds by converting a dendrite into a growing axon (Stone et al., 2010). In control neurons the average amount of new axon growth 96h after injury was over 200 microns (Figure 5A and F). In Dronc RNAi neurons average growth was less than 100 microns (Figure 5B and F). Dronc activity therefore promotes regeneration, perhaps by limiting neuroprotection.

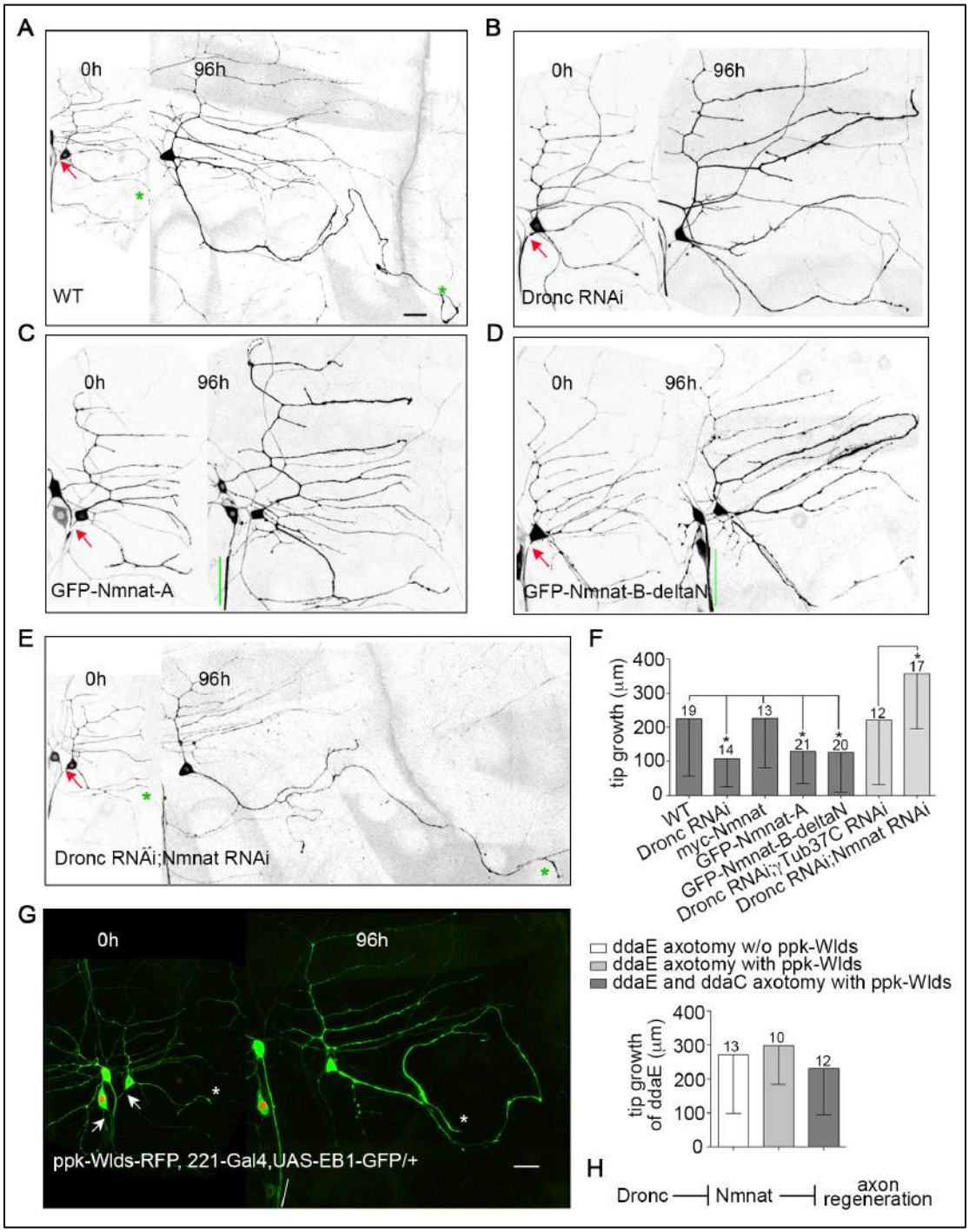

Figure 5. Dronc deficiency and Nmnat overexpression suppress axon regeneration.

(A-F) Axon regeneration after proximal axotomy assayed in the genotypes indicated. EB1-GFP was expressed in ddaE neurons under the control of 221-Gal4. Red arrows indicate the site of axon injury. Stars mark the tip of the dendrite that converts to an axon before and after injury. Green lines mark cut off axons that have not degenerated. The scale bar is 20 mm.

Numbers of neurons analyzed are indicated on the bars in F. Statistical significance was determined by an unpaired t test. Error bars represent SD. *P<0.05.

(G) A persistent axon stump (white line) was generated by severing the axon of a ddaC neuron expressing Wlds-RFP (red nucleus). Axon regeneration was measured in the neighboring ddaE neuron. Stars show the tip of the new axon before and after growth. The scale bar is 20 μm. An unpaired t test was used to determine statistical significance. Error bars represent SD.

(H) A summary model is shown.

To determine whether reduced regeneration in Dronc RNAi neurons could be due to overactive Nmnat, we overexpressed GFP-Nmnat-A, GFP-Nmnat-B-deltaN, myc-Nmnat (MacDonald et al., 2006), and the Wlds protein (MacDonald et al., 2006), which includes mouse Nmnat1 and 70 additional amino acids (Coleman and Freeman, 2010). The two GFP-tagged Nmnat transgenes suppressed axon regeneration (Figure 5C, D and F), as did the Wlds protein (Figure S4A). The myc-tagged Nmnat did not have a significant effect in this assay (Figure 5F). One possible explanation may be the first 70 nucleotides in Nmnat-B-deltaN but not myc-Nmnat is important, or different expression levels. Nmnat overexpression had a similar effect on regeneration to Dronc loss, consistent with the idea that Nmnat might be the target of Dronc.

To determine whether Nmnat was the critical inhibitor of regeneration downstream of Dronc, we paired Dronc RNAi with Nmnat RNAi to see if reducing Nmnat would rescue the Dronc RNAi phenotype. To correctly control the experiment and keep overall Gal4-driven transgene number the same, we paired Dronc RNAi with γTub37C RNAi, which we have previously used as a control RNAi (Chen et al., 2012). The addition of the control transgene reduced the effect of Dronc RNAi on regeneration (Figure 5F), probably through dilution of Gal4 among additional UAS-controlled transgenes. However, in neurons in which Dronc RNAi was paired with Nmnat RNAi, regeneration was significantly enhanced compared to the matched control (Figure 5E and F). This result is consistent with Dronc promoting regeneration by curbing Nmnat activity. The exuberant regeneration observed in the Dronc; Nmnat double RNAi suggests that Dronc may target additional proteins that promote regeneration.

There are two places where excessive Nmnat could dampen regeneration: either in the cut off axon or the rest of the cell. Slow axon degeneration induced by Wlds overexpression is associated with slow regeneration in a variety of cell types and contexts (Bisby and Chen, 1990; Chen and Bisby, 1993; Martin et al., 2010; Niemi et al., 2013a). The most frequent explanation for this is that the persistent axon stump inhibits regeneration either directly or through a secreted signal. To determine whether extra Nmnat/Wlds might inhibit axon regeneration through a persistent axon stump, we expressed the Wlds protein in a cell next to the one in which we assayed regeneration. This approach enabled generation of a persistent stump near a cell body that did not itself express extra Wlds or Nmnat (Figure 5G). RFP-tagged Wlds was expressed in ddaC neurons using the ppk promoter to directly drive it, rather than the binary Gal4-UAS expression system. Gal4-UAS expression was used to label the ddaC and ddaE neurons with GFP. When we severed axons of both the Wlds-expressing cell (ddaC) and wild-type ddaE, the ddaC axon persisted as expected (Figure 5G) Even though this stump is as close to the ddaE cell body as its own stump would be, regeneration was not reduced (Figure 5G). Thus excessive Wlds or Nmnat within the regenerating cell itself, rather than in the axon stump, likely inhibits regeneration.

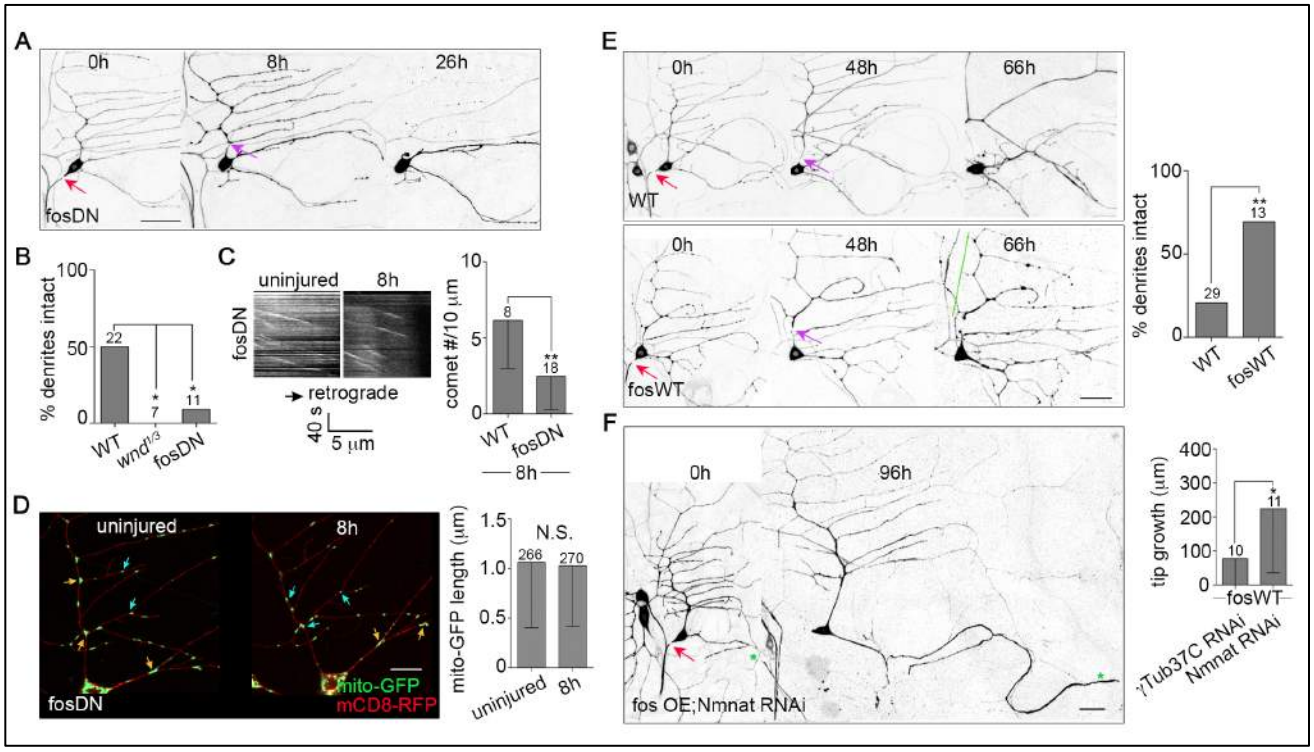

Figure 6. DLK/fos signaling coordinates neuroprotection and axon regeneration.

(A and B) Neuroprotection assays were performed in EB1-GFP-labeled ddaE neurons with different genetic backgrounds: control, fosDN-expressing, or *wnd* mutant. The scale bar is 50 μm. The numbers of neurons analyzed are indicated on the bars. Statistical significance was determined with a Fisher's exact test. *P<0.05

(C) Kymographs of EB1-GFP comets in fosDN-expressing neurons with and without axon injury. The number of comets per 10 μm segment of dendrite was counted. Numbers of neurons analyzed are indicated on the bars. Statistical significance was determined by an unpaired t test. **P<0.01

(D) Mitochondrial length was measured in fosDN-expressing neurons. The scale bar is 10 μm. Orange and blue arrows indicate long and medium mitochondria, respectively. Numbers on the graph are total number of mitochondria measured. 8 cells were used in each condition to gather this data. Statistical significance was determined by an unpaired t test. Error bars represent SD.

(E) Neuroprotection assays were performed in control and fos overexpressing neurons as in A, except that 48h elapsed between axon injury and dendrite injury rather than the 8h used elsewhere. The scale bar is 50 μm. The numbers of neurons analyzed are indicated on the bars. A Fisher's exact test was used to determine statistical significance. **P<0.01.

(F) Regeneration assays were performed in neurons overexpressing fos paired with a control RNAi hairpin (γTub37C) or Nmnat RNAi. The scale bar is 20 μm.

Positive and negative regulation of neuroprotection occurs downstream of conserved axon injury signals

In animals including mice, nematodes and flies DLK is required for neurons to regenerate in response to axon injury (Hammarlund et al., 2009; Shin et al., 2012; Xiong et al., 2010; Yan et al., 2009). Activation of DLK triggers a kinase cascade that includes JNK and results in fos-mediated transcription (Xiong et al., 2010). The major output of this signaling pathway is axon regeneration (Rishal and Fainzilber, 2014). However, as it is activated soon after axon injury, we hypothesized that it may also turn on neuroprotection.

To determine whether the DLK pathway is upstream of neuroprotection, we performed sequential axon and dendrite injury assays in two different backgrounds. To reduce levels of DLK (also called wallenda, or wnd in *Drosophila*) signaling, we used *wnd* hypomorphic alleles and a fos dominant negative (fosDN) transgene. The fosDN and *wnd* mutant combination block injury signaling (Xiong et al., 2010) and regeneration (Stone et al., 2014). In both *wnd* mutant and fosDN backgrounds neuroprotection was not triggered by axon injury (Figure 6A and B). Thus, DLK and fos are required not only to initiate axon regeneration, but also for the earlier neuroprotection response. We further tested this requirement by examining microtubule dynamics in the presence of the fosDN transgene. Like neuroprotection, increased microtubule dynamics was blocked by fosDN (Figure 6C). We conclude that the DLK/fos pathway acts upstream of neuroprotection and upregulation of microtubule dynamics.

A pro-apoptosis role for DLK has been established in mice during neuronal development (Ghosh et al., 2011) and after nerve injury (Watkins et al., 2013). Interestingly, DLK regulates cell death- and regeneration-associated gene expression in the same cell type (Watkins et al., 2013). This lead us to test whether the DLK/fos pathway might also be upstream of the mitochondria/Dronc pathway that negatively regulates neuroprotection. Mitochondrial length was visualized before and after axon injury in the presence of the fosDN transgene. Unlike control

neurons (Figure 2A and B), no decrease in mitochondrial length was observed in fosDN neurons (Figure 6D). This result suggests that fos is required not only to turn on neuroprotection, but also to dampen it through mitochondrial fission.

To test the hypothesis that fos controls induction of neuroprotection soon after axon injury and then turns it down to facilitate regeneration, we tested whether fos overexpression might extend the time course of neuroprotection after axon injury and block regeneration. In control neurons neuroprotection peaks at 8-24h after axon injury and is almost completely gone by 48h (Figure 6E). In contrast, when fos was overexpressed neuroprotection remained high at 48h (Figure 6E). When fos was overexpressed regeneration was also blocked (Figure 6F). Although fos likely has many targets, Nmnat RNAi in the background of fos overexpression eliminated the regeneration block (Figure 6F). This result suggests that fos overexpression blocks regeneration by prolonging neuroprotection. We conducted similar experiments with overexpressed bsk, the JNK homolog in *Drosophila*. Like fos, bsk overexpression blocked regeneration in a Nmnat-dependent manner (Figure S5). We conclude that DLK, JNK and fos activate neuroprotection and mitochondrial fission after axon injury. If neuroprotection is not balanced by caspase activity triggered by mitochondrial fission then regeneration is limited.

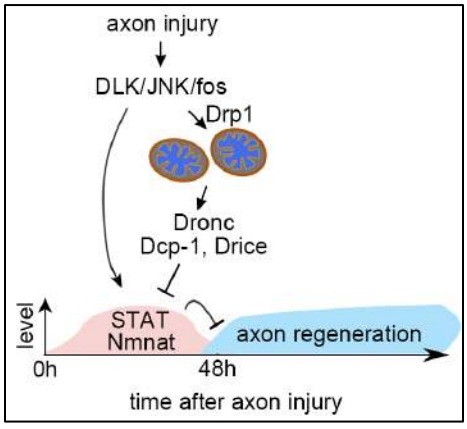

Figure 7. A model for sequential axon injury responses initiated by DLK signaling.

Axon injury activates the DLK kinase cascade that includes JNK. The AP-1 transcription factor fos controls early injury responses that include neuroprotection and mitochondrial fission. Neuroprotection is mediated by Nmnat, which, if unchecked dampens subsequent regeneration. Caspases acting downstream of mitochondrial fission counteract neuroprotection. By 48h after axon injury, neuroprotection has been reduced to baseline and axon regeneration proceeds. If neuroprotection is not turned off, then axon regeneration is suppressed.

Discussion

Our results lead to a model (Figure 7) in which axon injury triggers several opposing responses downstream of the initial DLK/JNK/fos signaling cascade. One early output of this conserved injury response pathway is neuroprotection, a global stabilization of the parts of the neuron still connected to the cell body. The central mediator of neuroprotection is the neuroprotective protein Nmnat. One Nmnat effector is the dramatic increase in microtubule dynamics observed after axon injury. As axon damage is likely to be accompanied by disturbances in the surrounding tissue, making the cell more resistant to degeneration by turning on neuroprotection is likely to help the neuron survive the initial trauma.

DLK/JNK/fos injury signaling also triggers Drp1-mediated mitochondrial fission in the first few hours after axon injury, and this leads to dampening of neuroprotection by caspases. We envision positive and negative regulation of neuroprotection balancing one another in different ways through time after injury. Eventually the negative pathway must outweigh the positive or regeneration is dampened by persistent neuroprotection (Figure 7). It is possible that the timing of this balance shift is controlled by additional signals that report whether the environment is conducive for regeneration.

This model suggests that rather than DLK/JNK/fos directly regulating regeneration, this signaling pathway kicks off a multi-step response to axon injury that includes regeneration as a relatively late event. Indeed, although this pathway is known as the conserved axon regeneration pathway, it first turns on a response that inhibits regeneration. Although this idea is surprising, the model helps explain several previous findings.

In mammals (Soares et al., 2001) and flies (Xiong et al., 2010) the AP-1 transcription factor fos is activated soon after axon injury. However, a different AP-1 transcription factor, jun, that is activated later in mammals (Soares et al., 2001), has been more clearly linked to axon

regeneration (Patodia and Raivich, 2012; Raivich et al., 2004). In many contexts fos and jun form a heterodimer (Kockel et al., 2001), but the difference in timing in mammals (Soares et al., 2001) and role of fos, but not jun, in early injury-induced transcription in flies (Xiong et al., 2010) suggests that they do not work together after axon injury. Our data suggests that fos orchestrates the injury responses that precede regeneration, rather than regeneration itself. Thus we hypothesize that fos and jun act sequentially in the injury response, with fos directing neuroprotection and jun directing regeneration.

Our results also shed some light on the role of caspases in axon regeneration. A study in C. elegans indicated that caspases promote axon regeneration (Pinan-Lucarre et al., 2012), which is surprising considering their involvement in self-destruct programs like apoptosis and axon degeneration. We confirm that in *Drosophila* caspases are pro-regenerative. In addition, we were able to show that this effect is not through a direct role in regeneration, but because caspases regulate Nmnat; when Nmnat is reduced then caspases are no longer needed to promote regeneration.

A negative role for mitochondria in neuroprotection was also surprising. However, this again made sense once caspase-mediated regulation of Nmnat was identified as the key downstream effector. It is likely that mitochondria also have positive roles in neuroprotection as increased microtubule dynamics is a hallmark of this response, and is energetically expensive.

The role of Nmnat in injury responses has been difficult to classify simply as either positive or negative. Its ability to prevent injury-induced Wallerian degeneration, as well as to act as an endogenous neuroprotective factor (Ali et al., 2013a) has led to the idea that it is a purely positive influence on neuronal health. However, the myriad ways in which it can be regulated (Ali et al., 2013a) suggest that it is useful only in exactly the right dose. Indeed we show that it when its regulation is disrupted, Nmnat inhibits a different type of neuronal resilience: axon

regeneration. Thus upregulation of Nmnat as a potential therapeutic strategy to counteract neurodegeneration could have negative outcomes due to dampened regeneration.

While our experiments support the idea that endogenous Nmnat is a central regulator of neuronal stability, the way it exerts this effect remains unclear. Nmnat is an enzyme that uses ATP and NMN (nicotinamide mononucleotide) to make NAD+. Protective effects of endogenous or overexpressed Nmnat have been proposed to be due to maintenance of high NAD levels (Araki et al., 2004; Sasaki et al., 2006; Wang et al., 2005), keeping levels of the precursor NMN low (Di Stefano et al., 2014), acting as a chaperone (Zhai et al., 2006; Zhai et al., 2008), and through maintaining mitochondrial integrity or function (Avery et al., 2012; Fang et al., 2014; Fang et al., 2012). We now show that Nmnat also acts upstream of increased microtubule dynamics after axon injury, but we do not rule out that other Nmnat functions are important during neuroprotection. In fact it seems likely that this is the case as reducing microtubule dynamics has a weaker effect on neuroprotection (Chen et al., 2012) than reduction of Nmnat itself.

In conclusion, we propose a model in which DLK signaling initiates key injury responses before axon regeneration begins. These responses include upregulation of Nmnat-mediated neuroprotection and mitochondrial fission. Mitochondrial fission counteracts neuroprotection through caspase activation. Although this early response is downstream of the core axon regeneration kinase cascade, it actually inhibits regeneration if unchecked (Figure 7). This multi-step model of injury responses downstream of DLK helps explain the function of caspases in promoting regeneration and also why fos acts downstream of DLK without being as closely linked to regeneration as jun. We anticipate that understanding the transition between early injury responses and regeneration itself will suggest strategies for promoting axon regeneration without overactivating neuroprotection, which would, in turn, dampen regeneration. A more complete understanding of the relationship between neuroprotection and regeneration is essential to designing any therapeutic approach to either stabilize neurons or to enhance regeneration.

Acknowledgements

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References

Ali, Y.O., Li-Kroeger, D., Bellen, H.J., Zhai, R.G., and Lu, H.C. (2013). NMNATs, evolutionarily conserved neuronal maintenance factors. Trends in neurosciences *36*, 632-640.

Araki, T., Sasaki, Y., and Milbrandt, J. (2004). Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. Science *305*, 1010-1013.

Avery, M.A., Rooney, T.M., Pandya, J.D., Wishart, T.M., Gillingwater, T.H., Geddes, J.W., Sullivan, P.G., and Freeman, M.R. (2012). Wld(S) Prevents Axon Degeneration through Increased Mitochondrial Flux and Enhanced Mitochondrial Ca(2+) Buffering. Current biology : CB.

Bisby, M.A., and Chen, S. (1990). Delayed wallerian degeneration in sciatic nerves of C57BL/Ola mice is associated with impaired regeneration of sensory axons. Brain research *530*, 117-120.

Cassidy-Stone, A., Chipuk, J.E., Ingerman, E., Song, C., Yoo, C., Kuwana, T., Kurth, M.J., Shaw, J.T., Hinshaw, J.E., Green, D.R., *et al.* (2008). Chemical inhibition of the mitochondrial division dynamin reveals its role in Bax/Bak-dependent mitochondrial outer membrane permeabilization. Developmental cell *14*, 193-204.

Chen, L., Stone, M.C., Tao, J., and Rolls, M.M. (2012). Axon injury and stress trigger a microtubule-based neuroprotective pathway. Proc Natl Acad Sci U S A *109*, 11842-11847.

Chen, S., and Bisby, M.A. (1993). Impaired motor axon regeneration in the C57BL/Ola mouse. The Journal of comparative neurology *333*, 449-454.

Coleman, M.P., and Freeman, M.R. (2010). Wallerian degeneration, wld(s), and nmnat. Annu Rev Neurosci *33*, 245-267.

Collins, C.A., Wairkar, Y.P., Johnson, S.L., and DiAntonio, A. (2006). Highwire restrains synaptic growth by attenuating a MAP kinase signal. Neuron *51*, 57-69.

Correia, S.C., Santos, R.X., Perry, G., Zhu, X., Moreira, P.I., and Smith, M.A. (2010). Mitochondria: the missing link between preconditioning and neuroprotection. Journal of Alzheimer's disease : JAD *20 Suppl 2*, S475-485.

Deng, H., Dodson, M.W., Huang, H., and Guo, M. (2008). The Parkinson's disease genes pink1 and parkin promote mitochondrial fission and/or inhibit fusion in Drosophila. Proc Natl Acad Sci U S A *105*, 14503-14508.

DeVorkin, L., Go, N.E., Hou, Y.C., Moradian, A., Morin, G.B., and Gorski, S.M. (2014). The Drosophila effector caspase Dcp-1 regulates mitochondrial dynamics and autophagic flux via SesB. The Journal of cell biology *205*, 477-492.

Di Stefano, M., Nascimento-Ferreira, I., Orsomando, G., Mori, V., Gilley, J., Brown, R., Janeckova, L., Vargas, M.E., Worrell, L.A., Loreto, A., *et al.* (2014). A rise in NAD precursor nicotinamide mononucleotide (NMN) after injury promotes axon degeneration. Cell death and differentiation.

Dirnagl, U., and Meisel, A. (2008). Endogenous neuroprotection: mitochondria as gateways to cerebral preconditioning? Neuropharmacology *55*, 334-344.

Dirnagl, U., Simon, R.P., and Hallenbeck, J.M. (2003). Ischemic tolerance and endogenous neuroprotection. Trends in neurosciences *26*, 248-254.

Fang, C., Decker, H., and Banker, G. (2014). Axonal transport plays a crucial role in mediating the axon-protective effects of NmNAT. Neurobiology of disease *68*, 78-90.

Fang, Y., Soares, L., Teng, X., Geary, M., and Bonini, N.M. (2012). A Novel Drosophila Model of Nerve Injury Reveals an Essential Role of Nmnat in Maintaining Axonal Integrity. Curr Biol.

Ghosh, A.S., Wang, B., Pozniak, C.D., Chen, M., Watts, R.J., and Lewcock, J.W. (2011). DLK induces developmental neuronal degeneration via selective regulation of proapoptotic JNK activity. J Cell Biol *194*, 751-764.

Glater, E.E., Megeath, L.J., Stowers, R.S., and Schwarz, T.L. (2006). Axonal transport of mitochondria requires milton to recruit kinesin heavy chain and is light chain independent. J Cell Biol *173*, 545-557.

Goyal, G., Fell, B., Sarin, A., Youle, R.J., and Sriram, V. (2007). Role of mitochondrial remodeling in programmed cell death in Drosophila melanogaster. Developmental cell *12*, 807-816.

Guo, X., Macleod, G.T., Wellington, A., Hu, F., Panchumarthi, S., Schoenfield, M., Marin, L., Charlton, M.P., Atwood, H.L., and Zinsmaier, K.E. (2005). The GTPase dMiro is required for axonal transport of mitochondria to Drosophila synapses. Neuron *47*, 379-393.

Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E.M., and Bastiani, M. (2009). Axon regeneration requires a conserved MAP kinase pathway. Science *323*, 802-806.

Iijima-Ando, K., Hearn, S.A., Shenton, C., Gatt, A., Zhao, L., and Iijima, K. (2009). Mitochondrial mislocalization underlies Abeta42-induced neuronal dysfunction in a Drosophila model of Alzheimer's disease. PLoS One *4*, e8310.

Jagasia, R., Grote, P., Westermann, B., and Conradt, B. (2005). DRP-1-mediated mitochondrial fragmentation during EGL-1-induced cell death in C. elegans. Nature *433*, 754-760.

Kockel, L., Homsy, J.G., and Bohmann, D. (2001). Drosophila AP-1: lessons from an invertebrate. Oncogene *20*, 2347-2364.

Laundrie, B., Peterson, J.S., Baum, J.S., Chang, J.C., Fileppo, D., Thompson, S.R., and McCall, K. (2003). Germline cell death is inhibited by P-element insertions disrupting the dcp-1/pita nested gene pair in Drosophila. Genetics *165*, 1881-1888.

MacDonald, J.M., Beach, M.G., Porpiglia, E., Sheehan, A.E., Watts, R.J., and Freeman, M.R. (2006). The Drosophila cell corpse engulfment receptor Draper mediates glial clearance of severed axons. Neuron *50*, 869-881.

Mar, F.M., Simões, A.R., Leite, S., Morgado, M.M., Santos, T.E., Rodrigo, I.S., Teixeira, C.A., Misgeld, T., and Sousa, M.M. (2014). CNS axons globally increase axonal transport after peripheral conditioning. J Neurosci *34*, 5965-5970.

Martin, S.M., O'Brien, G.S., Portera-Cailliau, C., and Sagasti, A. (2010). Wallerian degeneration of zebrafish trigeminal axons in the skin is required for regeneration and developmental pruning. Development *137*, 3985-3994.

McGurk, L., and Bonini, N.M. (2012). Protein interacting with C kinase (PICK1) is a suppressor of spinocerebellar ataxia 3-associated neurodegeneration in Drosophila. Hum Mol Genet *21*, 76-84.

Niemi, J.P., DeFrancesco-Lisowitz, A., Roldan-Hernandez, L., Lindborg, J.A., Mandell, D., and Zigmond, R.E. (2013). A critical role for macrophages near axotomized neuronal cell bodies in stimulating nerve regeneration. The Journal of neuroscience : the official journal of the Society for Neuroscience *33*, 16236-16248.

Patodia, S., and Raivich, G. (2012). Role of transcription factors in peripheral nerve regeneration. Front Mol Neurosci *5*, 8.

Pinan-Lucarre, B., Gabel, C.V., Reina, C.P., Hulme, S.E., Shevkoplyas, S.S., Slone, R.D., Xue, J., Qiao, Y., Weisberg, S., Roodhouse, K., *et al.* (2012). The core apoptotic executioner proteins CED-3 and CED-4 promote initiation of neuronal regeneration in Caenorhabditis elegans. PLoS biology *10*, e1001331.

Raivich, G., Bohatschek, M., Da Costa, C., Iwata, O., Galiano, M., Hristova, M., Nateri, A.S., Makwana, M., Riera-Sans, L., Wolfer, D.P., *et al.* (2004). The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. Neuron *43*, 57-67.

Rishal, I., and Fainzilber, M. (2014). Axon-soma communication in neuronal injury. Nature reviews Neuroscience *15*, 32-42.

Sasaki, Y., Araki, T., and Milbrandt, J. (2006). Stimulation of nicotinamide adenine dinucleotide biosynthetic pathways delays axonal degeneration after axotomy. The Journal of neuroscience : the official journal of the Society for Neuroscience *26*, 8484-8491.

Shin, J.E., Cho, Y., Beirowski, B., Milbrandt, J., Cavalli, V., and Diantonio, A. (2012). Dual leucine zipper kinase is required for retrograde injury signaling and axonal regeneration. Neuron *74*, 1015-1022.

Silver, J. (2009). CNS regeneration: only on one condition. Curr Biol *19*, R444-446.

Smith, D.S., and Skene, J.H. (1997). A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. The Journal of neuroscience : the official journal of the Society for Neuroscience *17*, 646-658.

Soares, H.D., Chen, S.C., and Morgan, J.I. (2001). Differential and prolonged expression of Fos-lacZ and Jun-lacZ in neurons, glia, and muscle following sciatic nerve damage. Experimental neurology *167*, 1-14.

Stetler, R.A., Leak, R.K., Gan, Y., Li, P., Zhang, F., Hu, X., Jing, Z., Chen, J., Zigmond, M.J., and Gao, Y. (2014). Preconditioning provides neuroprotection in models of CNS disease: paradigms and clinical significance. Progress in neurobiology *114*, 58-83.

Stone, M.C., Albertson, R.M., Chen, L., and Rolls, M.M. (2014). Dendrite injury triggers DLK-independent regeneration. Cell reports *6*, 247-253.

Stone, M.C., Nguyen, M.M., Tao, J., Allender, D.L., and Rolls, M.M. (2010). Global up-regulation of microtubule dynamics and polarity reversal during regeneration of an axon from a dendrite. Mol Biol Cell *21*, 767-777.

Tao, J., and Rolls, M.M. (2011). Dendrites have a rapid program of injury-induced degeneration that is molecularly distinct from developmental pruning. J Neurosci *31*, 5398-5405.

Wang, C., and Youle, R.J. (2009). The role of mitochondria in apoptosis*. Annual review of genetics *43*, 95-118.

Wang, J., Zhai, Q., Chen, Y., Lin, E., Gu, W., McBurney, M.W., and He, Z. (2005). A local mechanism mediates NAD-dependent protection of axon degeneration. The Journal of cell biology *170*, 349-355.

Watkins, T.A., Wang, B., Huntwork-Rodriguez, S., Yang, J., Jiang, Z., Eastham-Anderson, J., Modrusan, Z., Kaminker, J.S., Tessier-Lavigne, M., and Lewcock, J.W. (2013). DLK initiates a transcriptional program that couples apoptotic and regenerative responses to axonal injury. Proc Natl Acad Sci U S A *110*, 4039-4044.

Wen, Y., Parrish, J.Z., He, R., Zhai, R.G., and Kim, M.D. (2011). Nmnat exerts neuroprotective effects in dendrites and axons. Molecular and cellular neurosciences *48*, 1-8.

Westermann, B. (2010). Mitochondrial dynamics in model organisms: what yeasts, worms and flies have taught us about fusion and fission of mitochondria. Seminars in cell & developmental biology *21*, 542-549.

Xiong, X., and Collins, C.A. (2012). A conditioning lesion protects axons from degeneration via the Wallenda/DLK MAP kinase signaling cascade. J Neurosci *32*, 610-615.

Xiong, X., Wang, X., Ewanek, R., Bhat, P., Diantonio, A., and Collins, C.A. (2010). Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. The Journal of cell biology *191*, 211-223.

Xu, D., Wang, Y., Willecke, R., Chen, Z., Ding, T., and Bergmann, A. (2006). The effector caspases drICE and dcp-1 have partially overlapping functions in the apoptotic pathway in Drosophila. Cell Death Differ *13*, 1697-1706.

Yan, D., Wu, Z., Chisholm, A.D., and Jin, Y. (2009). The DLK-1 kinase promotes mRNA stability and local translation in C. elegans synapses and axon regeneration. Cell *138*, 1005-1018.

Ylera, B., Erturk, A., Hellal, F., Nadrigny, F., Hurtado, A., Tahirovic, S., Oudega, M., Kirchhoff, F., and Bradke, F. (2009). Chronically CNS-injured adult sensory neurons gain regenerative competence upon a lesion of their peripheral axon. Current biology : CB *19*, 930-936.

Zhai, R.G., Cao, Y., Hiesinger, P.R., Zhou, Y., Mehta, S.Q., Schulze, K.L., Verstreken, P., and Bellen, H.J. (2006). Drosophila NMNAT maintains neural integrity independent of its NAD synthesis activity. PLoS biology *4*, e416.

Zhai, R.G., Zhang, F., Hiesinger, P.R., Cao, Y., Haueter, C.M., and Bellen, H.J. (2008). NAD synthase NMNAT acts as a chaperone to protect against neurodegeneration. Nature *452*, 887-891.

Materials and Methods

Drosophila stocks

The following RNAi fly strains were used in this study: Rtnl2 (33320) (Chen et al., 2012), γTub37C (25271) (Chen et al., 2012), Miro (106683) (Tao and Rolls, 2011), milt (41508) (Iijima-Ando et al., 2009), Dronc (23035) (Tao and Rolls, 2011), Dcp-1 (107560) and Drice (28065) (McGurk and Bonini, 2012) from the Vienna Drosophila RNAi Center, and Drp1 (27682), Nmnat (29402) (Fang et al., 2012) from the Bloomington Drosophila Stock Center (BDSC). All RNAi transgenes were coexpressed with UAS-Dcr2 to increase knockdown efficiency. Other lines include 221-Gal4, ppk-Gal4, UAS-mito-GFP (BDSC 8443), UAS-EB1-GFP, UAS-mCD8-RFP, UAS-Drp1 (Deng et al., 2008), UAS-Wlds (MacDonald et al., 2006), $Nmnat^{delta4790\text{-}8}$ (Zhai et al., 2006), $Drice^{17}$ (Xu et al., 2006), $Dcp\text{-}1^{3}$ (Laundrie et al., 2003), UAS-fosWT (BDSC 7213), UAS-bsk-A.Y (BDSC 6407), wnd^{1}, wnd^{3}, and UAS-fosDN (Collins et al., 2006).

Live imaging, injury and image procession

Fly embryos were collected at 20C overnight and aged at 25C for 2 or 3 days before imaging. Larvae of appropriate sizes were mounted between an agarose slide and a coverslip. A pulsed UV laser was used to injure dendrites and axons of ddaE neurons expressing EB1-GFP or mCD8-RFP under the control of 221-Gal4. Confocal images were acquired using a Zeiss LSM510 with a 63 x oil objective (NA1.4) right after injury. Larva were then kept in a food cap at 20C for various time periods and were then reimaged using the Olympus FV1000 confocal microscope equipped with a 60 x oil objective (NA1.42). To study axon regeneration, ddaE neurons were axotomized close to the cell body and reimaged after 96h. To determine levels of neuroprotection, the primary dendrite was injured 8 or 48h after axon injury. Dendrite status was determined 18h later and categorized as degeneration or stabilization. To monitor the progression of dendrite degeneration, neurons were imaged at 4h, 7h and 11h after dendrite injury. Maximal intensity projections were generated using ImageJ software, and were aligned and processed using PhotoShop software.

Neighbor axotomy assay

ppk-Wlds-td flies were crossed with 221Gal4,UAS-EB1-GFP flies and stored at 20C. Apple caps were collected from mating bottles every 24hrs. Embryos were then transferred to food vials and aged at 25C for 48hr. Control data consisted of axotomies to just the class I ddaE neurons, which were then assayed for regeneration. The experimental data consisted of dual axotomies to both the ddaE and ddaC neurons and regeneration of the ddaE was assayed.

Quantification and statistics

GraphPad Prism 6 software was used to generate graphs and perform statistic analysis. Data were plotted as mean $\pm$ SD. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Axon regeneration: One dendrite usually extends and converts into a new axon by 96h in response to a proximal axon injury. We measured the length of the specified dendrite at 0h (R0h) and 96h (R96h), and a nearby non-regenerating dendrite as the reference of dendrite development

(NR0h an NR96h) using the NeuronJ plugin in the ImageJ software. The formula R96h-R0h*NR96h/NR0h was used to calculate growth of the new axon tip. The average values from at least 10 neurons were compared with unpaired t test. Dendrite stabilit: Statistical significance was determined using Fisher's exact test.

Mitochondrial imaging and analysis

Live imaging of mitochondria was performed by expressing UAS-mCD8-RFP and UAS-mito-GFP under the control of 221-Gal4. Images were taken at 1 frame/s using a 63x objective and 2x zoom. Injury-induced mitochondrial behavior changes were analyzed in dendrites in a 66.8 μm x 66.8 μm region close to the cell body. The template matching plugin in ImageJ was used to minimize larval body movement artifact. Mitochondrial length was measured along the longest dimension of mito-GFP using the Measure tool in ImageJ. To further analyze changes in length, mitochondria were grouped according to the length of mito-GFP, and the percentage of each group was calculated before and after injury. Fisher's exact test was used to determine statistical significance.

Microtubule dynamics

To measure microtubule dynamics in dendrites, we imaged neurons for at least 100 frames (1 frame/2s) using Olympus FV1000 with a 60 x objective at zoom 3, and counted the total number of EB1-GFP in a 10 μm dendrite segment close to the cell body from 3 in-focus frames. Only comets moving in 3 consecutive frames were included for quantification. The reslice tool of ImageJ was used to generate kymographs with 1 pixel spacing and the resulting kymographs were converted to 8 bit so that the trajectory of EB1-GFP appeared in white.

Generation of GFP-Nmnat flies

The coding sequence of Drosophila Nmnat isoform A was amplified from a cDNA clone using forward primer 5'-CCGGAATTCATGATTGTGAAAATCAGCTGGCCCAAG-3' and reverse primer 5'-ATATGCGGCCGCCTAAAGTTGCACTTGGGAAATC-3'.

The coding sequence of Drosophila Nmnat isoform B was amplified from the UAS-Nmnat.HA construct (Zhai et al., 2006) using forward primer: 5'-CCGGAATTCATGTCAGCATTCATCGAGGAAAC-3' and reverse primer: ATATGCGGCCGCTCAAGAGTCGCATTCGGTCGGAG.

Both forward primers contain an EcoRI site and reverse primers contain a NotI site. The amplified sequences were cloned into a pUAST-GFP vector, respectively, and the resulting constructs were injected into fly embryos to generate several transgenic flies. UAS-GFP-Nmnat-A4 and UAS-GFP-Nmnat-B-deltaN8 lines were used in this study.

Generation of ppk-Wlds-td flies

The coding sequence of Wlds was amplified from genomic DNA isolated from UAS-Wlds flies (MacDonald et al., 2006) with the forward primer 5'-ACTTGCAGATCTCAAAACATGGAGGAGCTGAGCGCTGAC-3' containing a Bgl2 restriction site, and the reverse primer 5'-CATCGAACTAGTCAGAGTGGAATGGTTGTGCTT-3' containing a Spe1 restriction site. The pCasper-ppk-EGFP plasmid was used as a PCR template for the ppk promotor with the forward primer 5'-ACATGCATGCAAGAGTTGGCAACAGGAG-3' including an SphI restriction site, and the reverse primer 5'-CCGGAATTCGTACCCTAGAGGATCAGC-3'containing an EcoRI site. The PCR products of ppk promotor and Wlds were cloned into the vector pUAST::tdC. The final construct was injected into fly embryos to generate transgenic fly lines.

Supplemental Information

SI figures

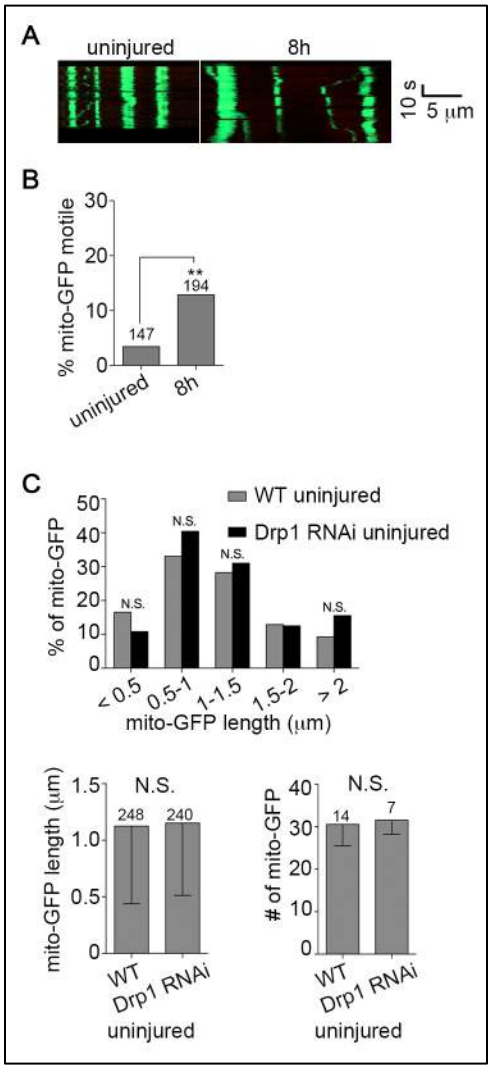

Figure S1. Characterizing mitochondrial motility and length in dendrites after axon injury.

(A) Kymographs of mito-GFP in dendrites of wide-type ddaE neurons before and 8h after axon injury. The X- and Y-axes represent distance and time, respectively.

(B) The percentage of motile mitochondria increases after axon injury. The numbers on the graph bars are the total number of mitochondria. Data were obtained from six neurons. Statistical significance was determined with a Fisher's exact test. **P<0.01.

(C) Mitochondrial length was quantitated in uninjured Drp1 RNAi neurons. Data of uninjured neurons from Figure 2B-D' were included in the graphs here to give a direct comparison between wide-type and Drp1 RNAi neurons.

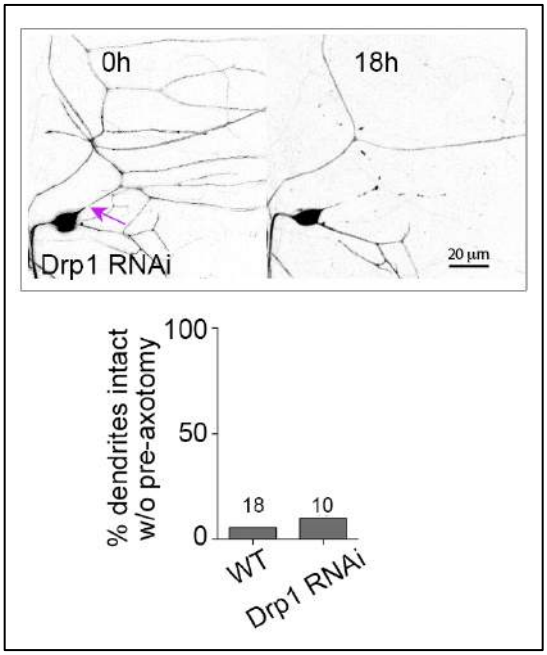

Figure S2. Drp1 is dispensable for dendrite degeneration.

Dendrites of ddaE neurons labeled with EB1-GFP were severed and degeneration was assayed 18h later. The numbers of neurons analyzed are indicated on the bars. Statistical significance was determined by a Fisher's exact test.

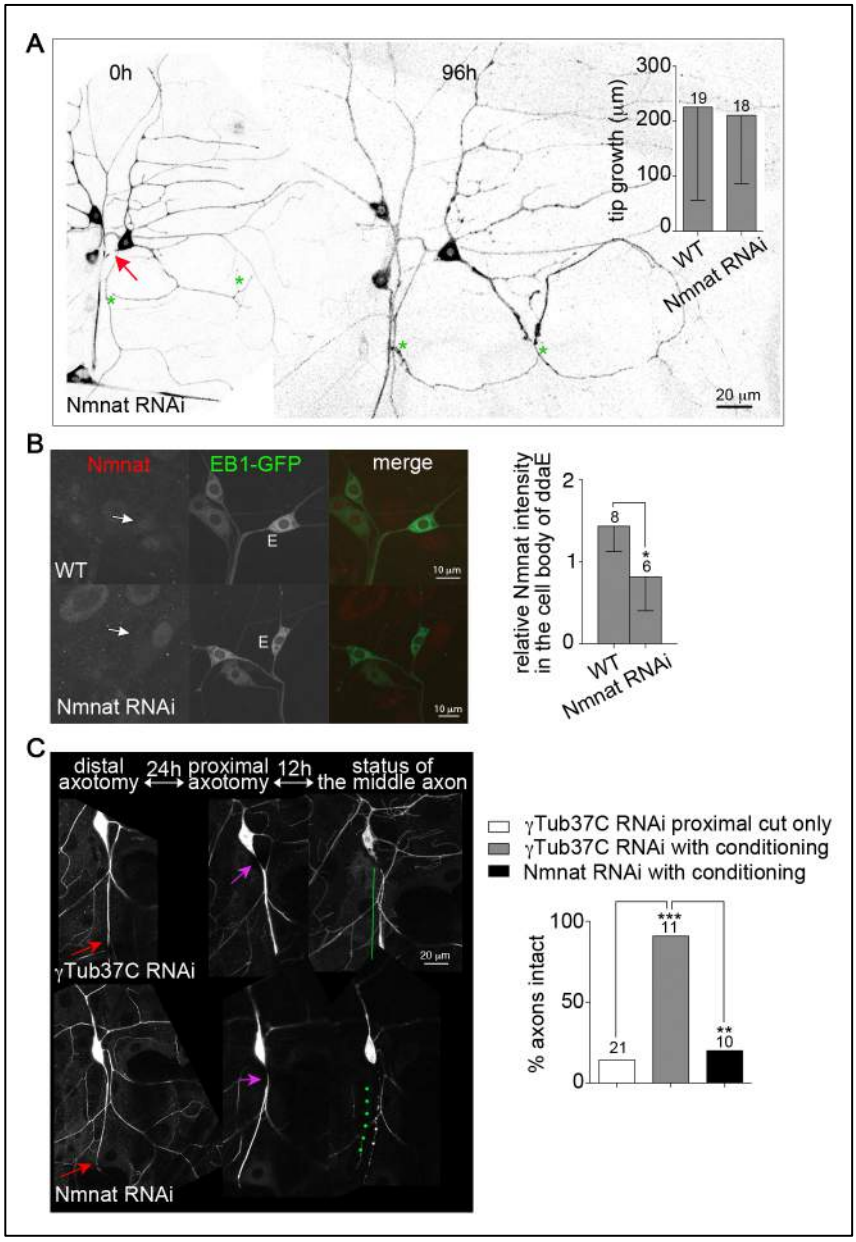

Figure S3. Phenotypes of Nmnat RNAi neurons.

(A) Axon regeneration was assayed in Nmnat RNAi neurons. The red arrow shows the site where the axon was cut. The green stars indicate the tips of the dendrite that was converted into a regenerating axon before and after growth. The numbers of neurons analyzed are indicated on the bars. Statistical significance was determined by an unpaired t test. Error bars represent SD.

(B) Control larvae and larvae in which Nmnat RNAi hairpins were expressed under control of 221-Gal4 were fileted, fixed and immunostained for endogenous Nmnat. EB1-GFP was also expressed under control of 221-Gal4. The average Nmnat intensity in the cell body was normalized to background and averaged from 8 neurons in 3 animals for WT and 6 neurons in 3

animals for Nmnat RNAi. Arrows and “E” point to the cell body of ddaE neurons. Statistical significance was determined by an unpaired t test. Error bars represent SD. *P<0.05.

(C) The axon of ddaC neurons expressing mCD8-GFP was first injured (red arrows) close to bipolar neurons. 24h later, the axon was injured closer to the cell body (purple arrows), and axon status was determined 12h later. γTub37C RNAi was used as a control. The green line marks an intact axon and the fragmented line, a beaded axon. The numbers of neurons analyzed are indicated on the bars. Statistical significance was determined with a Fisher’s exact test. **P<0.01, ***P<0.001

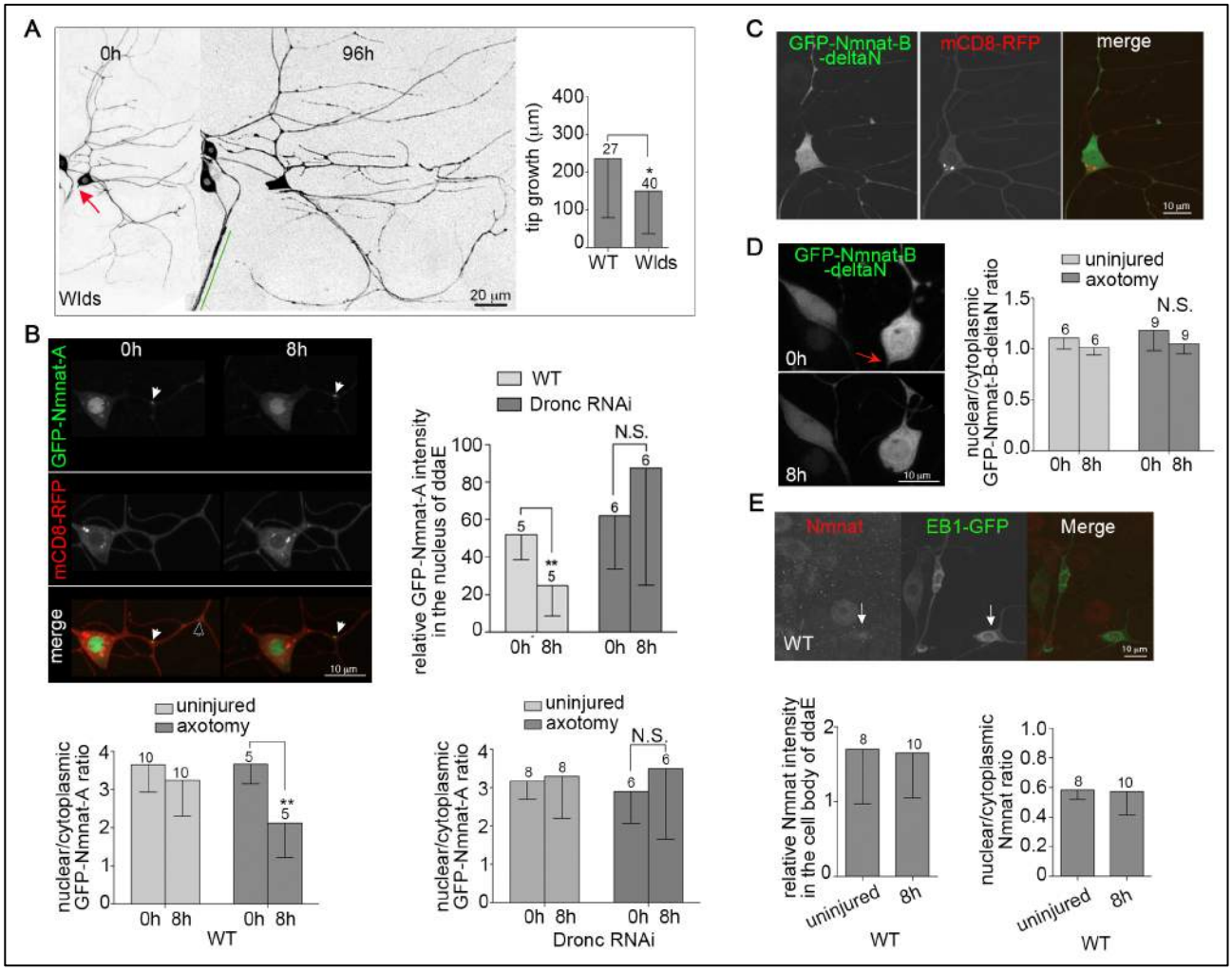

Figure S4. Nmnat localization in uninjured and injured neurons.

(A) Axon regeneration assays were performed in control and Wlds-expressing neurons. The number of animals tested for each genotype is indicated on the bars in the graph. An example image for Wlds overexpression is shown. The red arrow shows where the axon was severed and the green line marks the cut off axon that is still intact 96h after injury. An unpaired t test was used to determine statistical significance. Error bars represent SD. *P<0.05.

(B) GFP-Nmnat-A and mCD8-RFP were coexpressed under 221-Gal4 control and axons of ddaE neurons were severed. The average intensity of GFP-Nmnat-A in the nucleus and cytoplasm was measured in different genotypes. The numbers of neurons analyzed are indicated on the bars. Statistical significance was determined by a paired t test. Error bars represent SD. *P<0.05

(C and D) GFP-Nmnat-B-deltaN was expressed in ddaE neurons together with mCD8-RFP to mark cell membranes. Average intensities in the nucleus and cytoplasm were measured with and without injury.

(E) Neurons were labeled with EB1-GFP driven by 221-Gal4 and axon injury was performed close to the cell body. 8h after axon injury, larval filets were prepared for Nmnat

immunostaining. The arrow indicates the cell body of ddaE 8h after axon injury. Fluorescence intensity was measured in injured and uninjured neurons. In the left graph this was normalized to background in the image. The number of neurons is indicated on each bar, and these were gathered from 3 filet preps in each case. Statistical significance was determined by unpaired t test. Error bars represent SD.

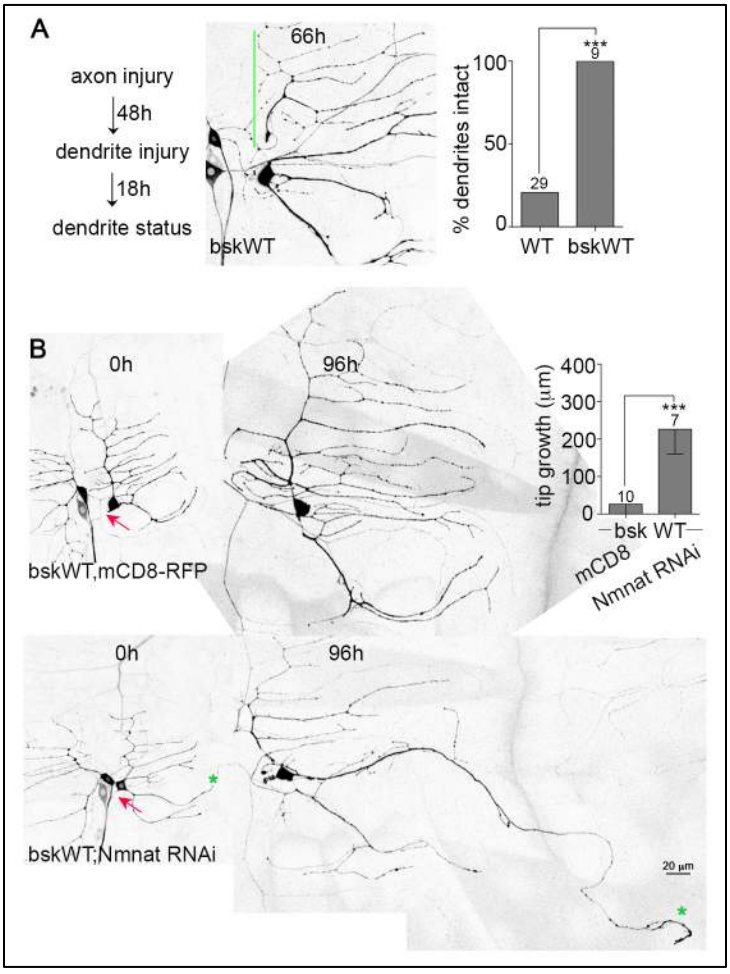

Figure S5. Effects of JNK (bsk) overexpression on neuroprotection and axon regeneration.

(A) A 48h neuroprotection assay was performed in bsk-overexpressing neurons. The green line indicates stabilized dendrites. Statistical significance was determined by a Fisher's exact test and the numbers on the bars indicate numbers of neurons analyzed. ***P<0.001

(B) Axon regeneration assays were performed in neurons overexpressing bsk paired with either mCD8-RFP or Nmnat RNAi. UAS-mCD8-RFP was expressed as a control for Nmnat RNAi to rule out Gal4 dilution effect. Statistical significance was determined by an unpaired t test. Error bars represent SD. ***P<0.001

SI methods

Immunofluorescence of endogenous Nmnat

Third instar larva expressing EB1-GFP driven by 221-Gal4 were dissected in Schneider's media and fixed in 4% PFA for 45 min, incubated in blocking solution (0.2% Triton X-100, 10 mM glycine, 1% BSA in PBS) for 10min. Fillets were incubated with anti-Nmnat polycolonal antibody generated in guinea pig (Zhai et al., 2006) at 1:1000 overnight, washed in blocking solution for 1h, and with Rhodamine-conjugated goat anti-guinea pig antibody (1:50, Jackson ImmunoResearch Laboratories Inc.) at room temperature for 1h.

Nmnat intensity was measured in the cell body, nucleus and cytoplasm of ddaE neurons defined by EB1-GFP fluorescence and was normalized to background. The average intensity was calculated based on at least five neurons from three fillets.

Mitochondrial motility

Imaging was performed using a 63x objective and 2x zoom. Mitochondria in all dendrite branches in this filed were quantified. Motility was determined by counting the number of mitochondria that move over 1μm along dendrites in a one-minute imaging window. Kymographs were generated using the reslice tool in ImageJ with 1 pixel spacing.

Axon stability

A distal axotomy was performed in ddaC neurons expressing mCD8-GFP under the control of ppk-Gal4. 24h later, the injured axon was severed again close to the cell body and the status of the middle axon was determined after 12h. neuroprotection was measured by the ratio of intact axons, which showed no signs of beading or fragmentation.

GFP-Nmnat levels

GFP-Nmnat-A and B-deltaN were expressed in ddaE neurons together with mCD8-RFP under the control of 221-Gal4. GFP intensity was measured in the cell body, the nucleus and

cytoplasm and was normalized to background. The normalized GFP intensity was used to calculate the average intensity in the nucleus and the average nuclear/cytoplasmic ratio. At least five neurons were analyzed. Statistic significance was determined using paired t test.

Chapter 4

Conclusions

Neurons use axons to communicate with distal targets. Because of their unique structure, axons are extremely vulnerable to acute injury and age-related diseases. In these situations, the continuity and function of axons are frequently compromised, resulting in neuronal dysfunction and death. Remarkably, some neurons are capable of adapting to axon damage by inducing self-protective and repair responses. Understanding how these responses are regulated may help develop new treatments for patients who suffer from stroke, traumatic injury and neurodegenerative disorders. In this study, I have demonstrated that *Drosophila* larval sensory neurons are an ideal model system for studying the mechanisms of multiple injury responses. Using this system, I have found that axon injury triggers an endogenous protective response before axon regeneration. I have identified Nmnat and ©Tub23C as specific regulators of injury-induced protection, whereas the DLK pathway is required for protection and regeneration. Most importantly, I have found that DLK indirectly regulates regeneration by inactivating Nmnat-dependent protection. Together, these results suggest the importance of a transient activation of DLK and Nmnat in determining the timing of two axon injury responses. In addition, my work suggests that neurons use ©Tub23C-mediated upregulation of microtubule dynamics to combat neurodegeneration caused by compromising axon transport and expressing expanded polyQ proteins.

Drosophila sensory neurons as an ideal system to study multiple axon injury responses

Recent research in invertebrate models has contributed significantly to our understanding of the mechanisms of axon injury responses. For example, the *spectrin* mutant worm strain and laser axotomy-based approach serve as excellent screening platforms for identifying molecules necessary for axon regeneration (Chen et al., 2011; Hammarlund et al., 2009), and *Drosophila* olfactory receptor neurons (ORNs) have been used to successfully uncover novel axon degeneration regulators (Neukomm et al., 2014; Osterloh et al., 2012). These systems, however, are limited in their ability to study additional aspects of injury responses. For example, axons of *C. elegans* neurons remain after injury and thus are not useful for understanding axon degeneration, and the ORN system is not suitable for studying axon regeneration because the injury procedure removes the neuronal cell bodies.

Drosophila larval sensory neurons have several advantages as a model system for studying injury responses. First, these neurons are amenable to live imaging and time-course experiments. Second, injury responses can be followed at single neuron levels after a precise laser-induced injury. Third, this system is versatile in studying multiple injury responses, including axon and dendrite degeneration, conditioning-induced neuroprotection, and two types of axon regeneration (proximal and distal). Last, it is feasible to study the relationship between different injury responses in this system, such as protection and regeneration.

The *Drosophila* sensory neuron system is extremely useful for genetic and cell biology analysis, however it is difficult to use for performing classical biochemical assays due to the challenges of extracting these neurons and small sample size. For example, although Nmnat immunoreactivity increases in crushed larval nerves (Xiong and Collins, 2012), the same Nmnat antibody works poorly in individual neurons (our data and personal communication with Dr. Grace Zhai).

Furthermore, our ability to study axon regeneration is hindered by the short life span of larva. Within the available time window, we can observe axon extension but not target reinnervation, a sign of successful axon regeneration. As a result, it remains to be determined whether the reduced tip growth at 96h reflects a mere delay or blockage of axon regeneration. One way to distinguish these two possibilities may be to generate long-lived larva using the temperature-sensitive ecd mutants (Garen et al., 1977). An alternative is assessing regeneration in adult flies. Existing adult models for studying axon regeneration include explanting brains and ablating wing nerves (Ayaz et al., 2008; Soares et al., 2014). Wing nerve transection injures over 200 axons at a time and induces responses characteristic of vertebrate systems, including axon degeneration, regrowth, scar formation and hemocyte mobilization. These conserved features make wing nerve transection an attractive system to study how neurons and other cell types interact in response to axon injury.

Cultured mammalian neurons have been widely used to study axon degeneration and regeneration. A unique feature of cultured neurons is that researchers can determine where the gene of interest functions by using compartmentalized chambers (Cho and Cavalli, 2012; Schoenmann et al., 2010; Zhai et al., 2003), and when it functions by varying the timing of manipulations (Araki et al., 2004; Wang et al., 2005b). One concern of using cultured neurons, however, is whether they are mature and functional. It is also important to keep in mind that the lack of additional cell types, such as phagocytes, may yield different outcomes than *in vivo* systems.

The research on axon regeneration from invertebrates and cultured neurons ultimately needs to be validated in higher systems. Traditional studies in vertebrates have been hindered by the lack of high-resolution imaging techniques and the long time needed for generating transgenic animals. Recent advances in imaging technology has made it possible to follow single DRG axon regeneration repetitively in living mice (Bareyre et al., 2011), and even allows whole-brain

imaging (Chung and Deisseroth, 2013). It is thus conceivable that these imaging tools may greatly facilitate our understanding of how mammalian neural circuits are altered in injury and disease settings. Furthermore, with the maturation of many genome-editing techniques such as CRISPR/CAS9 (Dance, 2015), the mechanistic study of neuronal injury responses in higher organisms may become much faster than before.

DLK-mediated microtubule dynamics represent a novel and broad protective response

Larval sensory neurons make a great system to study cytoskeletal organization in different neuronal compartments. Dendrites and axons are enriched with stable microtubules that support long-range transport of materials. Consistent with this idea, *Drosophila* neurons do not have many actively growing microtubules (Stone et al., 2008). It is thus striking to see a 4-fold increase in microtubule dynamics after axotomy (Chapter 2 Figure 2) (Stone et al., 2010). We sought to understand the mechanism of this dramatic change. We have found that ©Tub23C, the key microtubule nucleation protein, is important for elevating microtubule growth in the cell body (Chapter 2 Figure S2A) and dendrites (Chapter 2 Figure 2D), and microtubule nucleation is increased as a result of activating the DLK-JNK-fos pathway. This discovery adds a novel function of this MAPK pathway in addition to axon degeneration, apoptosis, axon regeneration, and conditioning effects (Miller et al., 2009; Nix et al., 2011; Watkins et al., 2013; Xiong and Collins, 2012). How does the DLK-JNK-fos pathway interpret different injury signals to generate proper injury responses? Are these responses executed using common mechanisms? To answer these questions, it is necessary to know when and how DLK is activated, whether different JNK isoforms and scaffold proteins such as JIP1 and JIP3 have distinct roles, what substrates are regulated by fos, and how their expression changes over time. Answers to these questions may provide entry points to understand this multi-faceted pathway in greater detail.

We originally hypothesized that the elevated microtubule growth is important for axon regeneration. This hypothesis is based on the finding that a population of dynamic microtubules in growth cones is important for elongation of new axons (Cho and Cavalli, 2012; Erturk et al., 2007). However, reducing ©Tub23C using RNAi and a heterozygous null allele, ©*Tub23C*$^{A15\text{-}2}$, inactivates injury-induced microtubule dynamics but does not affect axon regeneration, suggesting upregulated microtubule dynamics does not play a role in axon regeneration.

In addition to microtubule dynamics, axon injury also changes microtubule polarity in dendrites (Stone et al., 2010). Microtubule polarity is opposite in fly dendrites and axons (Stone et al., 2008). In response to a close axotomy, axons regenerate from a dendrite. During regeneration, the polarity of microtubules in the selected dendrite becomes mixed and is subsequently reversed (Stone et al., 2010). Failed polarity reversion is associated with impaired axon regeneration, as observed in kinesin-2 deficient neurons (Mattie et al., 2010) and when ©Tub23C is reduced by the transheterozygotes of ©*Tub23C*$^{A15\text{-}2}$ with a hypomorph allele, ©*Tub23C*$^{A14\text{-}9}$ (Nguyen unpublished data). Therefore, changes in microtubule polarity may be necessary for axon regeneration after a proximal injury.

My study reveals that upregulated microtubule dynamics play an important role in conditioning-induced neuroprotection. Inactivation of microtubule dynamics by reducing ©Tub23C and inhibiting JNK suppresses dendrite protection. Surprisingly, the same mechanism is required for inducing microtubule dynamics under two non-traumatic types of neuronal insults: expressing pathogenic human poly-Q disease proteins (SCA1, SCA3 and Huntington's disease) and interfering with axonal transport of presynaptic vesicle precursors. In both cases, neurons show dendrite complexity defects. The defects are exacerbated by reducing γTub23C. γTub23C knockdown also makes degeneration of adult photoreceptor neurons more severe, without interfering with neuronal development. Together, these results suggest that microtubule dynamics

may represent a general self-protective strategy to ameliorate neuronal destruction in different adverse situations. Although dendrite protection triggered by axon injury seems transient (to accommodate axon regeneration), it is persistently active from the larval stage to adult under the other two conditions. Notably, subsequent studies suggest that axon injury increases microtubule dynamics in *C. elegans* (Ghosh-Roy et al., 2012) and mice (Kleele et al., 2014). A mouse model of amyotrophic lateral scelerosis (ALS) shows a similar increase in microtubule growth (Kleele et al., 2014), further demonstrating the generality of this type of cytoskeletal reorganization. In the future, it may be important to test whether the same regulatory mechanisms identified in *Drosophila* are used in other models.

Mitochondrial dynamics antagonizes neuroprotection

Mitochondria play critical roles in energy production, nutrient metabolism, stress adaptation and cell death. These functions are influenced by the shape and size of mitochondria, which is determined by the relative activity of fission and fusion, or mitochondrial dynamics. Neurons are particularly sensitive to changes in mitochondrial dynamics. Dysregulation of mitochondrial dynamics has been implicated in a variety of neurodegenerative diseases. For example, inhibiting Drp1 ameliorates mitochondrial fragmentation and neuronal death in animal models of Huntington's disease (Grohm et al., 2012; Song et al., 2011), glutamate excitotoxicity and ischemia (Grohm et al., 2012).

My work is consistent with a negative role of excessive mitochondrial fission on neuronal integrity. Axon injury increases the number and decreases the length of mitochondria in dendrites, which can be rescued by Drp1 knockdown (Chapter 3 Figure 2), suggesting that axon injury either increases mitochondrial fission, decreases fusion, or both in dendrites. Knockdown of Drp1 and Miro enhances neuroprotection, suggesting that mitochondrial fission negatively

influences dendrite stability. It is known that during apoptosis, mitochondrial fission activates caspases by providing docking sites for pro-apoptotic factors (Abdelwahid et al., 2007; Frank et al., 2001). In support of this idea, I found that several caspases inhibit conditioning-induced protection (Chapter 3 Figure 3). Non-apoptotic roles of capspases have been identified in dendrite pruning, axon guidance, and long-term depression (reviewed in Hyman and Yuan, 2012). Now I have shown that the caspase cascade is capable of regulating dendrite stability. What is the mechanism? It has been proposed that local activation may be key to the non-killing functions of caspases. Based on my results, I speculate that capsases are activated at the sites of mitochondrial fission. I have attempted to determine the location of active caspases using immunocytochemistry (anti-caspase3 and anti-cPARP antibodies) and a fluorescent reporter (apoliner). Although these reporters have been used to detect caspase activity in cells undergoing apoptosis (Bardet et al., 2008; Williams et al., 2006), I did not find evidence of caspase activation in individual da neurons (Appendix Figure 3). The lack of caspase signals may be attributed to multiple factors. Most importantly, mitochondria in dendrites of da neurons are short, and the level of mitochondrial dynamics, and perhaps caspase activity, is low and transient. Hence, these reporters may not be sensitive enough. Instead, they may work well in cultured mammalian neurons that have tubular mitochondrial networks (Grohm et al., 2012).

Our model positions JNK and fos upstream of mitochondrial fission and caspases. This fits with the pro-apoptosis role of the DLK pathway in mammalian model systems (Watkins et al., 2013). It may be interesting to see whether the expression and localization of mitochondrial fission and fusion proteins (Drp1, FZO, mitofusins, and OPA1) (reviewed in Detmer and Chan, 2007) are regulated by DLK, JNK and fos.

Novel regulatory mechanisms and target of Nmnat

The *Drosophila* Nmnat responds to heat and hypoxia by increasing transcription (Ali et al., 2011), suggesting that endogenous Nmnat may be stress-inducible and protective. My work shows that Nmnat is required for the conditioning effect that stabilizes dendrites and axons against injury in sensory neurons. It remains to be determined whether this is mediated by changes in the activity, level, or subcellular localization of Nmnat. Immunostaining of endogenous Nmnat does not yield much information, perhaps due to suboptimal antibody sensitivity at single cell levels. Exogenous Nmnat-A, however, shows a significant decrease in the nucleus after injury (Chapter 3 Figure S4). The function of this change is not clear at present, but it is worth emphasizing that similar changes have been observed in an independent study. In motoneurons, nerve crush leads to a disappearance of nuclear Nmnat. Overactivating DLK triggers a similar change. Conversely, DLK loss-of-function mutants increase nuclear Nmnat (Xiong et al., 2012 and unpublished data). These biochemical data are consistent with our model in which Nmnat functions downstream of DLK and suggest that DLK may regulate the level or nuclear export of endogenous Nmnat.

Although it has been proposed that Nmnats protect neurons by improving mitochondrial function, I found that Nmnat-mediated dendrite protection does not depend on mitochondria. In fact, I have provided strong genetic evidence that Nmnat functions downstream of the mitochondrial fission-induced caspase cascade (Chapter 3 Figure 4E). These results generate interesting questions such as whether Nmnat is a caspase substrate and whether it becomes inactive due to caspase-mediated procession. I have attempted to examine whether the protein stability of Nmnat changes during apoptosis in cultured fly S2 cells (Appendix Figure 4). The results are not conclusive and in-depth experiments are needed. On the other hand, I have found that Nmnat is required for increasing microtubule dynamics after axon injury. In summary, my

work suggests some new regulators and a new effector of Nmnat. An immediate question following this work is whether the NAD^+ synthetic ability of Nmnat is necessary for protection and microtubule dynamics. Generation of enzymatically inactive Nmnat fly lines will help to answer this question. Alternatively, investigation of NAD^+ consumers may give a hint. My results showed that RNAi based-knockdown of Parp1, sirt2 and sir2 does not affect dendrite protection (Appendix Table 1), but the reliability of these preliminary data should be verified using mutant approaches.

Step-specific roles of axon injury response regulators

It is well established that Nmnats/Wlds have neuroprotective functions, but it is not clear whether they can regulate other neuronal damage responses. My results in Chapter 3 suggest that although the activity of Nmnat is critical for the early protection, unrestricted Nmnat activity inhibits axon regeneration. Thus, a transient activation of Nmnat is required to coordinate multiple injury responses. Likewise, JNK and fos mediate early protection but overexpression of JNK and fos leads to extended or high protection and inhibit axon regeneration (Chapter 3 Figure 6E, F and Figure S5). The JNK phosphatase puc is induced at 24h after laser axotomy (Appendix Figure 1). This result indirectly suggests that JNK activity is downregulated at later timepoints after injury.

The AP-1 transcription factors fos and jun are well-established regulators of tissue development and stress responses. As bZIP family members, fos and jun share common features such as containing an N-terminal transactivation domain and a bZIP domain for dimerization and DNA binding. Upon stimuli, phosphoactivated fos and jun acquire the ability to form homodimers or heterodimers, and subsequently bind to a TPA-responsive element (TRE) motif to regulate gene expression (Angel and Karin, 1991).

The model in Chapter 3 suggests that fos activity is required for protection and its inactivation turns on axon regeneration. In contrast, I have found that jun specifically regulates axon regeneration. First, knockdown of jun does not alter the rate of protection (Figure 1A) nor does it have any affect on induction of an AP-1 activity reporter after injury (Figure 1B). Second, reducing jun inhibits axon regeneration while overexpressing it has the opposite affect (Figure 2). Together, these results suggest that jun may be induced later than fos to promote axon regeneration.

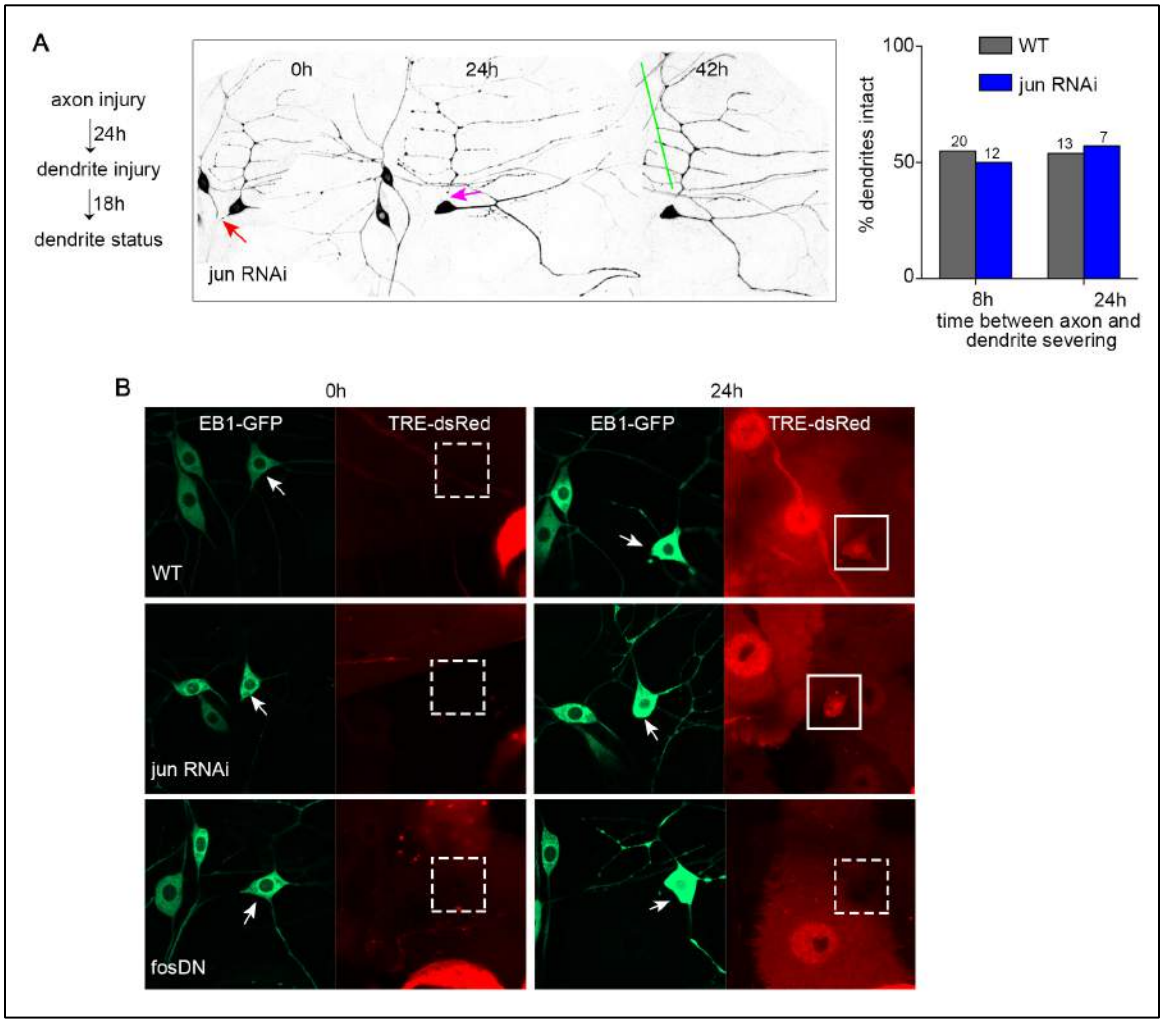

Figure 1. Jun is not required for early axon injury responses.

A. Conditioning-induced dendrite protection is not affected by reducing jun.
B. TRE-dsRed is strongly induced in injured neurons after 24h and the induction is blocked by fosDN but not jun RNAi. Arrows indicate axotomized ddaE neurons. Solid squares: cell bodies showing increased TRE signals. Dashed squares: no TRE signals.

Previous studies have shown that fos and c-Jun are required for axon regeneration in flies and mice, respectively (Raivich et al., 2004; Xiong et al., 2010). My results support these findings and further point out that fos and jun do not play identical roles in this process. Intriguingly, studies in vertebrates suggest that the transcript levels of Jun family members show a delayed but persistent increase after peripheral nerve injury, whereas fos either does not change or is induced at early stages (Ben-Yaakov et al., 2012; Jenkins and Hunt, 1991; Kenney and Kocsis, 1998; Leah et al., 1991). Therefore, it is tempting to speculate that the step-specific functions of fos and jun may be conserved in higher species.

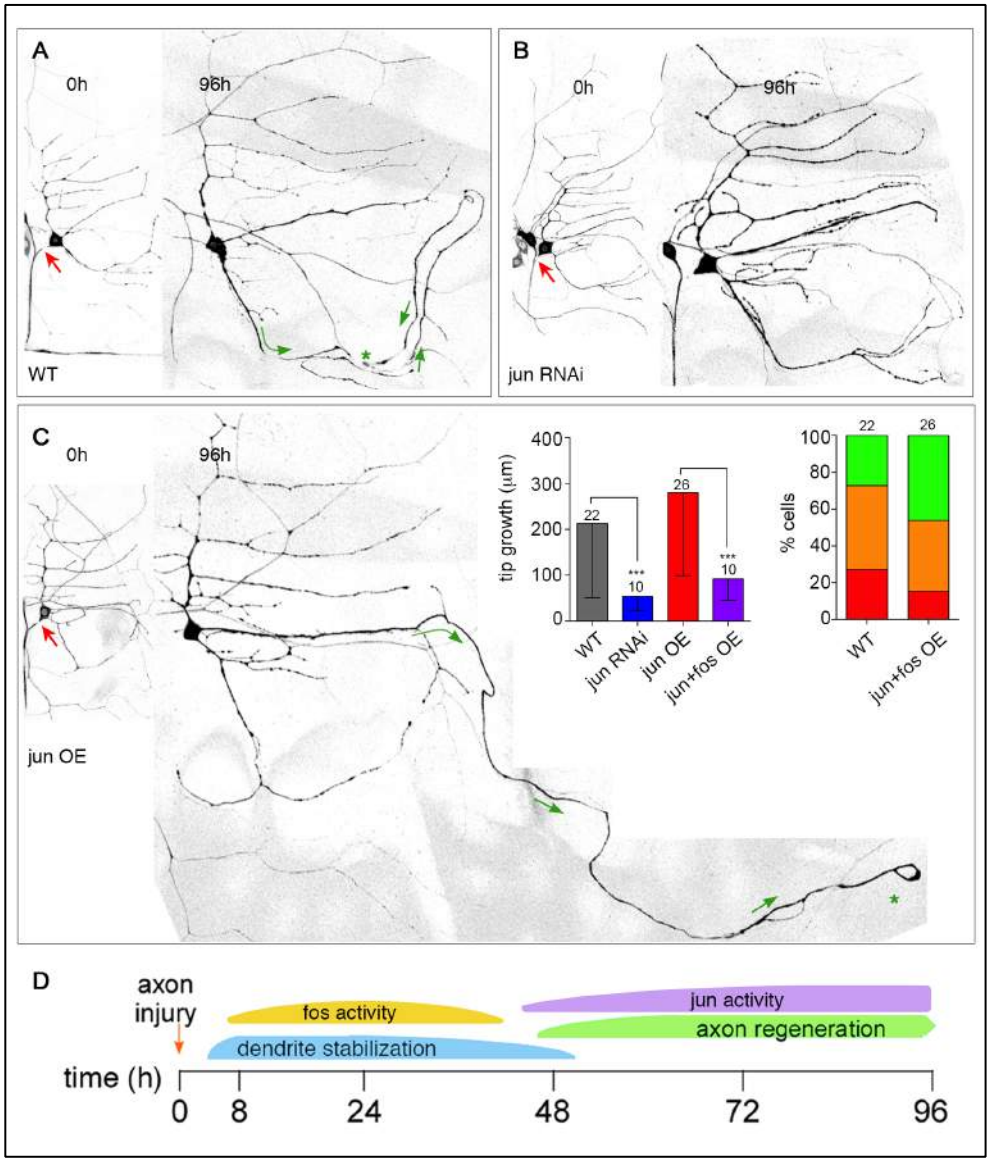

Figure 2. The influence of jun on axon regeneration.

References

Abdelwahid, E., Yokokura, T., Krieser, R.J., Balasundaram, S., Fowle, W.H., and White, K. (2007). Mitochondrial disruption in Drosophila apoptosis. Dev Cell *12*, 793-806.

Ali, Y.O., McCormack, R., Darr, A., and Zhai, R.G. (2011). Nicotinamide mononucleotide adenylyltransferase is a stress response protein regulated by the heat shock factor/hypoxia-inducible factor 1alpha pathway. The Journal of biological chemistry *286*, 19089-19099.

Angel, P., and Karin, M. (1991). The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta *1072*, 129-157.

Araki, T., Sasaki, Y., and Milbrandt, J. (2004). Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. Science *305*, 1010-1013.

Bardet, P.L., Kolahgar, G., Mynett, A., Miguel-Aliaga, I., Briscoe, J., Meier, P., and Vincent, J.P. (2008). A fluorescent reporter of caspase activity for live imaging. Proc Natl Acad Sci U S A *105*, 13901-13905.

Bareyre, F.M., Garzorz, N., Lang, C., Misgeld, T., Buning, H., and Kerschensteiner, M. (2011). In vivo imaging reveals a phase-specific role of STAT3 during central and peripheral nervous system axon regeneration. Proc Natl Acad Sci U S A *108*, 6282-6287.

Ben-Yaakov, K., Dagan, S.Y., Segal-Ruder, Y., Shalem, O., Vuppalanchi, D., Willis, D.E., Yudin, D., Rishal, I., Rother, F., Bader, M., *et al.* (2012). Axonal transcription factors signal retrogradely in lesioned peripheral nerve. EMBO J *31*, 1350-1363.

Chen, L., Wang, Z., Ghosh-Roy, A., Hubert, T., Yan, D., O'Rourke, S., Bowerman, B., Wu, Z., Jin, Y., and Chisholm, A.D. (2011). Axon regeneration pathways identified by systematic genetic screening in C. elegans. Neuron *71*, 1043-1057.

Cho, Y., and Cavalli, V. (2012). HDAC5 is a novel injury-regulated tubulin deacetylase controlling axon regeneration. The EMBO journal *31*, 3063-3078.

Detmer, S.A., and Chan, D.C. (2007). Functions and dysfunctions of mitochondrial dynamics. Nat Rev Mol Cell Biol *8*, 870-879.

Erturk, A., Hellal, F., Enes, J., and Bradke, F. (2007). Disorganized microtubules underlie the formation of retraction bulbs and the failure of axonal regeneration. J Neurosci *27*, 9169-9180.

Frank, S., Gaume, B., Bergmann-Leitner, E.S., Leitner, W.W., Robert, E.G., Catez, F., Smith, C.L., and Youle, R.J. (2001). The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell *1*, 515-525.

Ghosh-Roy, A., Goncharov, A., Jin, Y., and Chisholm, A.D. (2012). Kinesin-13 and tubulin posttranslational modifications regulate microtubule growth in axon regeneration. Developmental cell *23*, 716-728.

Grohm, J., Kim, S.W., Mamrak, U., Tobaben, S., Cassidy-Stone, A., Nunnari, J., Plesnila, N., and Culmsee, C. (2012). Inhibition of Drp1 provides neuroprotection in vitro and in vivo. Cell Death Differ *19*, 1446-1458.

Hammarlund, M., Nix, P., Hauth, L., Jorgensen, E.M., and Bastiani, M. (2009). Axon regeneration requires a conserved MAP kinase pathway. Science *323*, 802-806.

Hyman, B.T., and Yuan, J. (2012). Apoptotic and non-apoptotic roles of caspases in neuronal physiology and pathophysiology. Nat Rev Neurosci *13*, 395-406.

Jenkins, R., and Hunt, S.P. (1991). Long-term increase in the levels of c-jun mRNA and jun protein-like immunoreactivity in motor and sensory neurons following axon damage. Neurosci Lett *1*, 107-110.

Kenney, A.M., and Kocsis, J.D. (1998). Peripheral axotomy induces long-term c-Jun amino-terminal kinase-1 activation and activator protein-1 binding activity by c-Jun and junD in adult rat dorsal root ganglia In vivo. J Neurosci *18*, 1318-1328.

Kleele, T., Marinković, P., Williams, P.R., Stern, S., Weigand, E.E., Engerer, P., Naumann, R., Hartmann, J., Karl, R.M., Bradke, F., *et al.* (2014). An assay to image neuronal microtubule dynamics in mice. Nat Commun *5*, 4827.
Leah, J.D., Herdegen, T., and Bravo, R. (1991). Selective expression of Jun proteins following axotomy and axonal transport block in peripheral nerves in the rat: evidence for a role in the regeneration process. Brain Res *566*, 198-207.
Mattie, F.J., Stackpole, M.M., Stone, M.C., Clippard, J.R., Rudnick, D.A., Qiu, Y., Tao, J., Allender, D.L., Parmar, M., and Rolls, M.M. (2010). Directed Microtubule Growth, +TIPs, and Kinesin-2 Are Required for Uniform Microtubule Polarity in Dendrites. Curr Biol *20*, 2169-2177.
Miller, B.R., Press, C., Daniels, R.W., Sasaki, Y., Milbrandt, J., and DiAntonio, A. (2009). A dual leucine kinase-dependent axon self-destruction program promotes Wallerian degeneration. Nat Neurosci *12*, 387-389.
Neukomm, L.J., Burdett, T.C., Gonzalez, M.A., Züchner, S., and Freeman, M.R. (2014). Rapid in vivo forward genetic approach for identifying axon death genes in Drosophila. Proc Natl Acad Sci U S A.
Nix, P., Hisamoto, N., Matsumoto, K., and Bastiani, M. (2011). Axon regeneration requires coordinate activation of p38 and JNK MAPK pathways. Proc Natl Acad Sci U S A *108*, 10738-10743.
Osterloh, J.M., Yang, J., Rooney, T.M., Fox, A.N., Adalbert, R., Powell, E.H., Sheehan, A.E., Avery, M.A., Hackett, R., Logan, M.A., *et al.* (2012). dSarm/Sarm1 is required for activation of an injury-induced axon death pathway. Science *337*, 481-484.
Raivich, G., Bohatschek, M., Da Costa, C., Iwata, O., Galiano, M., Hristova, M., Nateri, A.S., Makwana, M., Riera-Sans, L., Wolfer, D.P., *et al.* (2004). The AP-1 transcription factor c-Jun is required for efficient axonal regeneration. Neuron *43*, 57-67.
Schoenmann, Z., Assa-Kunik, E., Tiomny, S., Minis, A., Haklai-Topper, L., Arama, E., and Yaron, A. (2010). Axonal degeneration is regulated by the apoptotic machinery or a NAD+-sensitive pathway in insects and mammals. J Neurosci *30*, 6375-6386.
Song, W., Chen, J., Petrilli, A., Liot, G., Klinglmayr, E., Y., Z., P., P., J., T., MA., P., MR., H., *et al.* (2011). Mutant huntingtin binds the mitochondrial fission GTPase dynamin-related protein-1 and increases its enzymatic activity. Nat Med *17*, 377-382.
Stone, M.C., Nguyen, M.M., Tao, J., Allender, D.L., and Rolls, M.M. (2010). Global up-regulation of microtubule dynamics and polarity reversal during regeneration of an axon from a dendrite. Mol Biol Cell *21*, 767-777.
Stone, M.C., Roegiers, F., and Rolls, M.M. (2008). Microtubules Have Opposite Orientation in Axons and Dendrites of Drosophila Neurons. Mol Biol Cell *19*, 4122-4129.
Wang, J., Zhai, Q., Chen, Y., Lin, E., Gu, W., McBurney, M.W., and He, Z. (2005). A local mechanism mediates NAD-dependent protection of axon degeneration. J Cell Biol *170*, 349-355.
Watkins, T.A., Wang, B., Huntwork-Rodriguez, S., Yang, J., Jiang, Z., Eastham-Anderson, J., Modrusan, Z., Kaminker, J.S., Tessier-Lavigne, M., and Lewcock, J.W. (2013). DLK initiates a transcriptional program that couples apoptotic and regenerative responses to axonal injury. Proc Natl Acad Sci U S A *110*, 4039-4044.
Williams, D.W., Kondo, S., Krzyzanowska, A., Hiromi, Y., and Truman, J.W. (2006). Local caspase activity directs engulfment of dendrites during pruning. Nat Neurosci *9*, 1234-1236.
Xiong, X., and Collins, C.A. (2012). A conditioning lesion protects axons from degeneration via the Wallenda/DLK MAP kinase signaling cascade. J Neurosci *32*, 610-615.
Xiong, X., Hao, Y., Sun, K., Li, J., Li, X., Mishra, B., Soppina, P., Wu, C., Hume, R.I., and Collins, C.A. (2012). The Highwire ubiquitin ligase promotes axonal degeneration by tuning levels of Nmnat protein. PLoS biology *10*, e1001440.

Xiong, X., Wang, X., Ewanek, R., Bhat, P., Diantonio, A., and Collins, C.A. (2010). Protein turnover of the Wallenda/DLK kinase regulates a retrograde response to axonal injury. The Journal of cell biology *191*, 211-223.
Zhai, Q., Wang, J., Kim, A., Liu, Q., Watts, R., Hoopfer, E., Mitchison, T., Luo, L., and He, Z. (2003). Involvement of the ubiquitin-proteasome system in the early stages of wallerian degeneration. Neuron *39*, 217-225.

Appendix

preliminary data

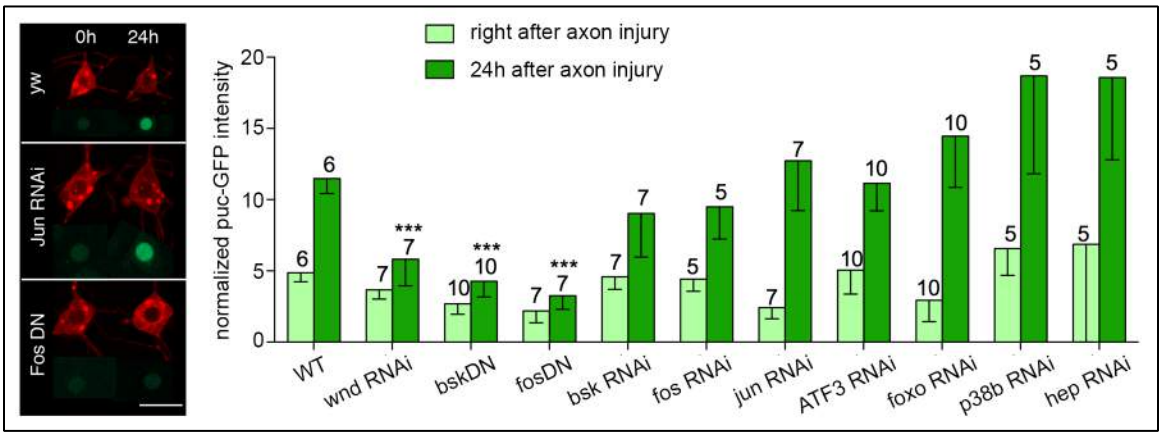

Figure 1. Screening for regulators of puc-GFP induction after axon injury.

Table 1. Candidate screen of genes regulating neuroprotection

Genotype	protection 8h after axotomy	protection 24h after axotomy
WT	11/22	7/13
crebDN	5/6	7/7
ATF2 RNAi		7/12
ATF3 RNAi		5/6
Foxo RNAi	5/7	1/9
Foxo $^{f19\text{-}5}$ OE		6/7
Akt RNAi	4/10	11/13
Sir2 RNAi	7/10	8/10
Sirt2 RNAi	7/10	5/7
Parp RNAi	5/11	
Hep RNAi	7/10	6/6
MLK2 RNAi	4/6	7/8
JIP3 RNAi	6/14	
P38a RNAi	7/17	11/13
CDK5 RNAi	2/10	6/7

Ratios indicate the number of neurons that have intact dendrites/the total number of neurons.

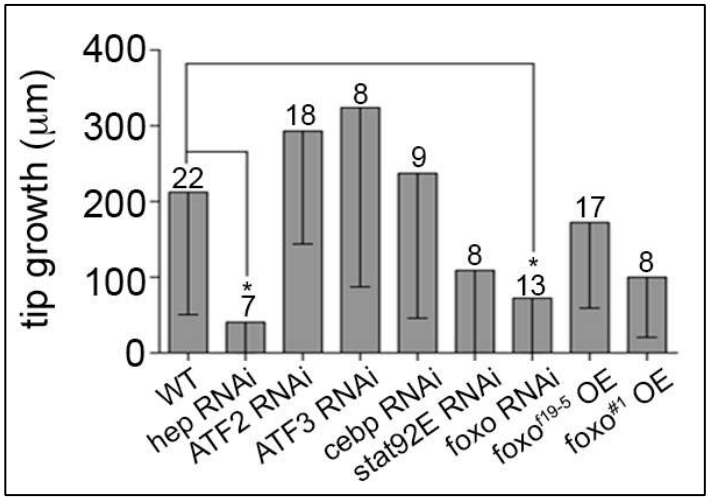

Figure 2. Screening for genes required for axon regeneration of ddaE neurons.

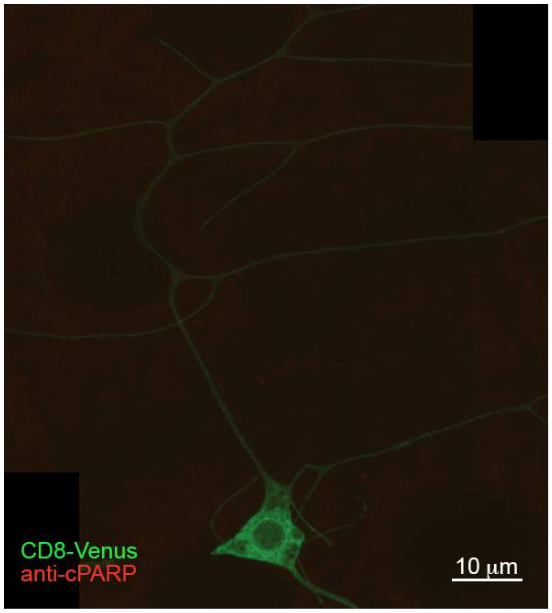

Figure 3. Absence of caspase activity in axotomized ddaE neurons.

The UAS-CD8-PARP-Venus fly line was used to detect caspase activity in axotomized ddaE neurons. The reporter line was crossed to 221Gal4, UAS-EB1-GFP, and axotomy was performed in 4 day-old larva from the cross. Fillets were prepared 8h after axon injury. The following antibodies were used: anti-cleaved PARP antibody (abcam ab2317, 1:20) and Rhodamine Red-conjugated goat anti-rabbit antibody (Jackson ImmunoResearch, 1:200).

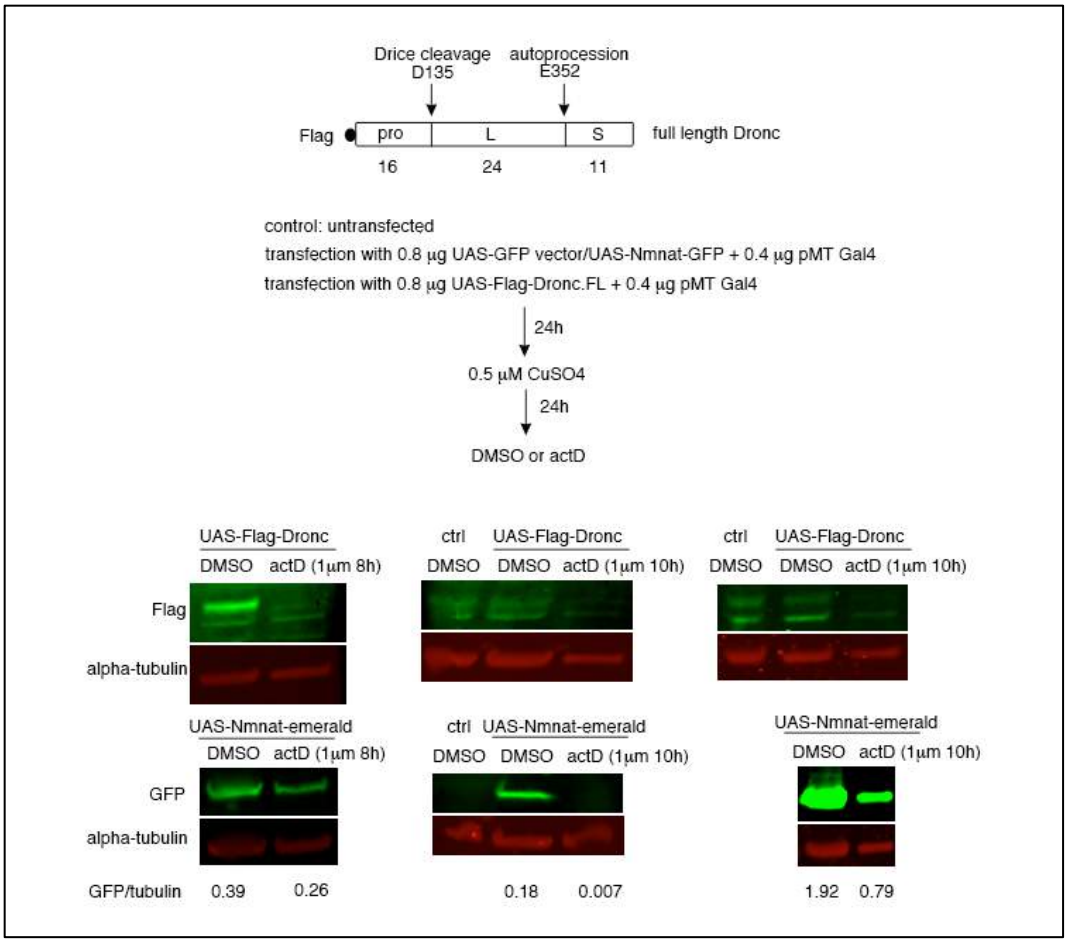

Figure 4. Changes of Nmnat in cultured S2 cells upon actinomycin D treatment.

Cell transfection was performed using Effectene (QIAGEN) following the manufacture's instructions. Primary antibodies used include: rabbit anti-GFP (abcam ab290, 1:4000), rabbit anti-Flag (sigma F7425, 1:4000) and mouse anti-alpha tubulin (DM1A 1:8000). Secondary antibodies include goat anti-mouse 680RD and goat anti-rabbit 800CW (LiCor biosciences, 1:15,000). Images were acquired using the ODYSSEY Clx Imaging System.

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Chen, L., Stone, M. C., Gheres, K.W., Xiong, X., Collins, C. A., and Rolls, M. M. Mitochondria and caspases tune Nmnat-mediated stabilization to promote axon regeneration. Submitted.
Chen, L., Stone, M.C., Tao, J., and Rolls, M.M. (2012). Axon injury and stress trigger a microtubule-based neuroprotective pathway. Proc Natl Acad Sci U S A *109*, 11842-11847.
Li, J., Sun, X., Wang, Z., **Chen, L.**, Li, D., Zhou, J., and Liu, M. (2012). Regulation of vascular endothelial cell polarization and migration by hsp70/hsp90-organizing protein. PLos One *7*, e36389.

Reviews
Chen, L., and Rolls, M.M. (2012). Microtubule deacetylation sets the stage for successful axon regeneration. EMBO J *31*, 3033-3035.

Recognition

2013-2015 American Heart Association Winter 2013 Predoctoral Fellowship
Spring 2014 Penn State University Office of Global Programs Graduate Travel Grant
2006-2009 The Third Class Scholarship at Nankai University
2012 Interviewed by the Penn State Science Journal. http://science.psu.edu/sciencejournal/archives/december-2012/features/person-to-person-li-chen