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A DLK AND JNK DEPENDENT AXON SELF-DESTRUCTION PROGRAM PROMOTES WALLERIAN DEGENERATION

by

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ABSTRACT OF THE DISSERTATION

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Axon loss is a debilitating consequence of a wide range of neurological conditions. As axons degenerate, they go through a stereotyped sequence of morphological changes termed Wallerian degeneration. It has long been hypothesized that there is an active axonal breakdown program, conceptually similar to apoptosis, which underlies Wallerian degeneration. However, the molecular pathways that accomplish this program in neurons have remained elusive.

We demonstrate that dual leucine kinase (DLK) promotes degeneration of severed axons in *Drosophila* and mice, and its target JNK promotes degeneration locally in axons as they commit to degenerate. This pathway also promotes degeneration after chemotherapy exposure, and thus may be a component of a general axon self-destruction program.

We also show that SCG10, an axonal target of JNK, is rapidly lost after a variety of axonal insults. In healthy axons, we found that SCG10 is continuously degraded and then replaced by axonal transport. This baseline SCG10 turnover requires JNK and the proteasome. Following injury, axonal transport is blocked and SCG10 cannot be replenished distal to the injury. Continuous axonal transport and degradation of an axonal maintenance factor is a potential mechanism by which distal axons sense their healthy connection to the cell body.

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Chapter One: Introduction

Abstract

Axon degeneration underlies many common neurological disorders. Studies of the Wlds mouse, a strain with delayed axon degeneration, show that an internal axon self-destruction program orchestrates axon breakdown. This program is triggered by a range of clinically important insults. Though conceptually similar to apoptosis and developmental axon pruning, pathological axon self-destruction is mechanistically distinct. Signaling pathways directing axon self-destruction are great therapeutic targets, but they are poorly understood. We found that dual leucine kinase (DLK) promotes degeneration of severed axons in *Drosophila* and mice, and its target c-jun N-terminal kinase (JNK) promotes degeneration locally in axons as they commit to degenerate. This pathway also promotes degeneration after chemotherapy exposure, and thus may be a component of a general axon self-destruction program.

Axonal injury can trigger apoptosis, axon regeneration, and axon degeneration. DLK and JNK are important for all three potential injury responses. Depending on the setting, DLK and JNK can kill neurons, stimulate axon regeneration, or trigger axon self-destruction. The diverse functions of DLK and JNK, their rapid involvement after injury, and their local axonal activation, suggest that DLK and JNK may be important early sensors of axonal injury.

Wallerian Degeneration

Axon degeneration contributes to many of the most prevalent and debilitating neurological disorders (Coleman and Perry, 2002). Axon loss is a hallmark of disorders such as diabetes, glaucoma, and chemotherapy-induced neurotoxicity. Debilitating neurodegenerative diseases such as Alzheimer's and Parkinson's Disease exhibit substantial axon loss early in the disease progression. The great length of many axons makes them particularly vulnerable to mechanical injury, and axon degeneration following trauma is a major cause of disability. Axon degeneration is an active and highly regulated process, yet the intrinsic, neuronal program promoting degeneration is poorly understood. What causes axons to degenerate, and how can this be prevented?

In 1850, Augustus Waller described the "alterations which take place in the elementary fibres of the nerve after they have been removed from their connection with the brain," notably fragmentation, and he correctly concluded that "what we term functional diseases of the nerves are in reality owing to certain organic and physical changes in the tubular fibre, which it will be the province of the microscope to ascertain" (Waller, 1850). Indeed, over one hundred and fifty years of microscopy has revealed that disconnecting axons from their cell bodies triggers a pathological progression featuring mitochondrial dysfunction, blockages of axonal transport, cytoskeletal breakdown, and focal swellings. Ultimately, axons fragment and surrounding cells clear the debris. Following Waller's landmark descriptive study and call to action, degeneration of the distal segment of transected peripheral axons, Wallerian degeneration, became the model of choice for investigating

mechanisms of axon degeneration. The discovered morphological changes are not specific to Wallerian degeneration: most degenerating axons show similar changes regardless what triggers the degeneration and where the axons are in the nervous system (Coleman, 2005).

Lessons from the Wlds Mouse

Axons Self-Destruct

After many years of descriptive work, two decades of rapid progress in the axon degeneration field began with the discovery of a mouse strain with delayed Wallerian degeneration (Wlds) (Lunn et al., 1989). Wlds mice led to the fundamental insight that an active axon self-destruction program orchestrates the pathological progression first described by Waller. This was surprising as the popular view of Wallerian degeneration was as an inflammatory attack on injured axons by the immune system and glia (Rosen and Gordon, 1987). However, changes in the immune response cannot be responsible for Wlds protection: bone marrow transplants from Wlds to wildtype mice do not protect axons, and replacement of Wlds bone marrow with wildtype does not diminish Wlds protection (Perry et al., 1990). Nor can surrounding glia account for the protection as wildtype axons are not protected when grown within nerve sheaths grafted from Wlds mice (Glass et al., 1993). Thus, Wlds protection is conferred by changes in axons themselves.

Axonal protection in Wlds mice results from the expression of the chimeric Wlds protein. This protein consists of seventy N-terminal amino acids of the ubiquitin assembly protein Ube4b, the entire sequence of nicotinamide

mononucleotide adenylyl transferase (NMNAT), and an intervening linker sequence (Mack et al., 2001). Expression of this single protein allows axons severed from their cell bodies to maintain metabolic activity (Deckwerth and Johnson, 1994), cytoskeletal integrity, and action potentials for up to three weeks (Tsao et al., 1994). Thus, a starvation model of Wallerian degeneration is ruled out: Wallerian degeneration does not result from a general interruption of energy and supplies from the soma. The alternative is an active model. There must be a Wlds-sensitive program that breaks down axons from the inside.

A General Self-Destruction Program

Delaying degeneration of axons detached from their cell bodies has little clinical utility. Thus, determining the mechanisms of Wallerian degeneration will be most useful if they also apply to other forms of axon degeneration. Studies of the Wlds mouse show that Wallerian degeneration has mechanistic overlap with many forms of axon degeneration. Wlds protects axons in response to a variety of mechanical and chemical insults and in many genetic models of human neuropathies. Wlds axons are slow to degenerate after exposure to vincristine, a chemotherapeutic agent that disrupts microtubules and has the dose-limiting side effect of painful sensory neuropathy (Wang et al., 2000). Wlds protects two models of periphery neuropathies with primary defects in axon structure and function: the progressive motor neuronopathy (pmn) mouse and the gracile axonal dystrophy (gad) mouse (Ferri et al., 2003; Mi et al., 2005). It is also protective in the P0 mouse, which has a myelination defect (Samsam et al., 2003).

Wlds also protects against lesions to the central nervous system. Glaucoma is the second leading cause of blindness, and axons are protected by Wlds in mouse glaucoma models (Howell et al., 2007). Wlds protects axons in response to immune attacks on myelin in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (MS) (Kaneko et al., 2006). Parkinson's disease (PD), a common neurodegenerative disease, results in substantial axon loss, and axons are protects in two mouse models of PD (Sajadi et al., 2004; Hasbani and O'Malley, 2006). Wlds modestly protects axons in a mouse model of amyotrophic lateral sclerosis (ALS) (Fischer et al., 2005).

Taken together, studies of Wlds show that axons degenerate using a common Wlds-sensitive breakdown program in response to insults ranging from genetic disruptions of the axonal cytoskeleton in peripheral nerves to autoimmune attacks on the CNS. Axons are apparently not protected by Wlds in some models of human disease (Vande Velde et al., 2004; Rose et al., 2008; Gültner et al., 2009). However, the overriding message from studies of Wlds is that there is a common pathway underlying many forms of axon degeneration. This common axon self-destruction pathway is an excellent therapeutic target.

Axon Degeneration and Apoptosis

Many neurological disorders result from the combined loss of axons, cell bodies, and synapses. In order to maintain neurological function, therapies must each of these elements. Here the promise of Wlds is less extraordinary. Axon self-destruction is conceptually and morphological similar to apoptosis: both use an

internal program to breakdown the cytoskeleton, package cellular remnants into vesicles, and then stimulate surround cells to clear the debris. However, early studies of Wlds proved that axon degeneration and neuronal apoptosis are mechanistically distinct. Trophic factor withdrawal triggers both axon degeneration and cell body apoptosis. Surprisingly, Wlds protects axons from loss of trophic support, but it leaves cell bodies to their normal demise (Deckwerth et al., 1994).

The dichotomy between programmed cell death and axon degeneration has disappointing therapeutic implications, but it is very instructive. It shows us that neurons have at least two independent or divergent self-destruction programs that can be triggered by similar insults. This result has been confirmed in multiple animal models of human disease. Wlds protects axons but not cell bodies after mechanical injury (Adalbert et al., 2006; Wang et al., 2006), after direct increases in intraocular pressure to mimic glaucoma (Beirowski et al., 2008), and after chemical insults that model Parkinson's disease (Sajadi et al., 2004; Hasbani et al., 2006). Complementary studies assessing the role of neuronal apoptosis signaling pathways in axon degeneration also show that axon self-destruction and cell body self-destruction are distinct. Though there are important exceptions described below, in most cases inhibiting apoptosis does not delay axon degeneration. Mice with mutations in two important effectors of apoptosis, bak and bax, are protected from neuronal apoptosis but not axon degeneration (Whitmore et al., 2003).

Axon degeneration and neuronal apoptosis are clearly implemented by distinct programs, but the distinction between axon breakdown and synapse loss is

less clear. Synapse loss is delayed by Wlds in some animal models of human disease. Wlds protects synapses in response to mechanical peripheral axon injury (Mack et al., 2001), CNS injury (Gillingwater et al., 2006), and genetic lesions that disrupt axonal function (Ferri et al., 2003) and myelination (Samsam et al., 2003). This is exciting from a therapeutic perspective because protecting axon loss is not beneficial if synapses are left to die. Despite the promise of Wlds to protect both axons and synapses, there is also room for doubt. Wlds protection of synapses is shorter-lived than Wlds protection of axons. And in some cases, Wlds protects axons and apparently confers no benefit to synapses (Gillingwater et al., 2002; Mi et al., 2005). It is unclear if synapses and axons sometimes use the same Wlds-sensitive breakdown program, or if the axon breakdown program triggers or accelerates an independent synapse breakdown program.

The pmn mouse is an important exception to the general rule that neuronal apoptosis unfolds independently of axon and synapse loss. Pmn is a mouse model of human neuropathies such as ALS and SMA that involve loss of cell bodies, axons, and synapses (Schmalbruch et al., 1991). A mutation in a tubulin chaperone disrupts microtubule assembly and causes pmn mice to progressively lose their axons and cell bodies (Bommel et al., 2002; Martin et al., 2002). Inhibiting apoptosis blocks pmn cell body loss but has no effect on axon loss and does not increase lifespan (Sagot et al., 1995). Thus, cell body death and axon degeneration are implemented by separate programs. However, when pmn mice are crossed with Wlds mice, lifespan is extended and synapses, axons, and cell bodies are all spared (Ferri et al., 2003). Since multiple lines of evidence show that Wlds does not directly inhibit

neuronal apoptosis, this result indicates that cell body loss in pmn mice is secondary to axon degeneration. Therefore, in some diseases axon degeneration may activate an independently executed neuronal apoptosis program. This may explain a discrepancy between Wlds impact on two glaucoma models. When glaucoma is modeled by directly increasing intraocular pressure, Wlds protects axons but retinal ganglion cells still undergo apoptosis (Beirowski et al., 2008). However, in a genetic glaucoma model Wlds protects both axons and cell bodies (Howell et al., 2007). Once again, these data show that cell body death does not require the axon degeneration program, but in some disease settings cell body loss may be triggered by axon degeneration. In diseases where the primary insult is to axons, therapies designed to block axon degeneration may therefore have the secondary benefit of protecting cell bodies.

Axon Degeneration and Axon Pruning

Cell body, axon, and synapse breakdown pathways must be distinct, or at least compartmentalized, during development. The immature nervous system abounds with extra neurons, axons, and synapses. As the system matures, the surplus components are pruned. Different elements of a single neuron can have wildly different fates (Luo and O'Leary, 2005). Branches of an axon can degenerate without triggering breakdown of other branches of the same axon, and without triggering cell death. Synapses formed by terminals of a single axon branch can retract without affecting the axon branch itself or other synapses formed by that

same branch. So perhaps it should come as no surprise that apoptosis, axon degeneration, and synapse loss have different sensitivities to Wlds.

Pathological axon loss could conceivably result from the unfortunate reactivation of the developmental axon pruning program. Developmental axon pruning could provide an easy explanation of the normal physiological function of the Wlds-sensitive axon self-destruction pathway. It therefore came as a surprise that developmental axon pruning is insensitive to Wlds. Axon pruning proceeds smoothly in flies and mice that over-express Wlds (Hoopfer et al., 2006). Thus, pathological axon loss is not equivalent to a reactivation of axon pruning. This may not hold true for the glial role in axon degeneration and axon pruning: inhibiting glial clearance of axons delays both injury induced axon loss and developmental pruning (Hoopfer et al., 2006; MacDonald et al., 2006). However, within the axons themselves, there are certainly important differences in the pathways that orchestrate pathological axon loss and axon pruning. There may be entirely independent axonal programs. Alternatively, a similar pathway may be triggered by a Wlds-dependent signal pathologically, and by a Wlds-insensitive signal developmentally.

Why Self-Destruct?

Is there a benefit to Wlds-sensitive axon degeneration? Rapid axon breakdown may protect the CNS against neurotropic viruses and also help to clear the way for axon regeneration after injury. Theiler's murine encephalomyelitis virus (TMEV) is a neurotropic virus that causes no clinical symptoms in C57BL/6

mice. In contrast C57BL/6 mice harboring Wlds are devastated by TMEV. Infection leads to CNS inflammation and severe paralysis (Tsunoda et al., 2007). Wlds mice develop a much higher CNS viral titer than controls, and degeneration of infected axons in controls may be important to prevent viral dissemination throughout the CNS.

Wlds mice also have defects in axon regeneration after injury. The regenerative capacity of Wlds mice is not compromised: damaged peripheral axons eventually regenerate and reach their targets in Wlds mice (Lunn et al., 1989). However, they regenerate slowly (Bisby and Chen, 1990; Brown et al., 1992). The delay likely results from the failure to remove the damaged axons rather than a slow regenerative response. In support of this, Wlds regeneration commences after the delayed clearance of damaged axons (Brown et al., 1994). Furthermore, growth promoting genes are up-regulated just as quickly in Wlds mice as in controls (Gold et al., 1993, 1994). When regenerating axons reach their targets, levels then decline in both wildtype and Wlds mice. However, Wlds axons take longer to reach their targets and the decline in expression levels is concomitantly delayed (Bisby et al., 1995).

Delayed regeneration in Wlds mice may involve more than physical obstruction. Blocking transcription in injured nerve stumps accelerates Wlds regeneration to wildtype rates (Court and Alvarez, 2000). Thus, there may be a transcription-dependent Schwann cell response that halts the entry of regenerating axons until degenerating axons are removed. In Wlds, the extended waiting period

comes at a price: nerve injury elicits dramatically more cell body death in Wlds mice compared to controls with typically speedy Wallerian degeneration (Chen and Bisby, 1993). Thus, rapid axon self-destruction may clear the way for regenerating axons to swiftly reach their targets and relay trophic factors back to their cell bodies.

NMNAT Blocks Axon Degeneration

There have been many conceptual advances since Waller's description of axon degeneration. We now know that Wallerian degeneration is orchestrated by an active program that works within axons and breaks down axons in response to a variety of clinically important insults. This program is distinct from the apoptosis program and developmental axon pruning. It is an essential therapeutic target because protection of cell bodies is ineffective if axons are loss. Furthermore, in some cases cell body and synapse loss are secondary to axon loss. Despite how far we have come since 1850, we still do not understand the molecular basis of Wallerian degeneration.

One approach to elucidating the endogenous pathways underlying Wallerian degeneration is to try to pinpoint where Wlds interrupts the program. Establishing which component of Wlds confers axonal protection is an essential first step. Though still controversial, the bulk of the evidence shows that NMNAT confers protection and the Ube4b fragment may help localize or stabilize the Wlds protein.

In vitro, over-expression of NMNAT alone potently protects axons against axotomy and the chemotherapeutic agent vincristine (Araki et al., 2004). Initial

attempts to protect axons *in vivo* by expressing NMNAT in transgenic mice were unsuccessful (Conforti et al., 2007). However, when NMNAT is targeted to the cytosol, it protects axons *in vivo* in mice (Sasaki, Vohra, Baloh, et al., 2009). Thus, NMNAT is sufficient to protect axons *in vitro* and *in vivo* and the remaining Wlds sequence may stabilize NMNAT or target it to the crucial subcellular compartment.

How does NMNAT inhibit the endogenous axon self-destruction program? NMNAT is an enzyme that synthesizes nicotinamide adenine dinucleotide (NAD+), a critical metabolite with wide-ranging cellular functions. NMNAT may protect axons by maintaining NAD+. In support of this hypothesis, most studies show that NMNAT enzymatic activity is required for NMNAT to confer protection *in vitro* and *in vivo* (Conforti et al., 2007; Sasaki, Vohra, Lund, et al., 2009). Additionally, direct application of NAD+ and NAD+ metabolites protects axons, as do a variety of manipulations that increase NAD+ synthesis (Araki et al., 2004; Sasaki et al., 2006; Press and Milbrandt, 2009).

In a surprising departure from the NAD+ model, NAD+ levels are apparently not increased by NMNAT over-expression (Sasaki, Vohra, Lund, et al., 2009). Furthermore, inhibition of an enzyme that provides NMNAT with a substrate for NAD+ synthesis does not block NMNAT axonal protection (Sasaki, Vohra, Lund, et al., 2009). Additionally, while direct NAD+ application is protective, it is not as potent as NMNAT over-expression. Given the importance of NMNAT enzymatic activity for protection, these results suggest that NMNAT may have additional enzymatic functions. However, one study challenges the importance of NMNAT

enzymatic altogether. Over-expression of an apparently enzymatically dead NMNAT protects *Drosophila* from neuron death induced by the spinocerebellar ataxia 1 protein (Zhai et al., 2008). In this capacity, NNMAT may act as a chaperone that helps to degrade the toxic protein.

The Axon Self-Destruction Program

Determining the precise mechanism of NMNAT protection will likely reveal the endogenous axon breakdown program, but our current understanding of NMNAT is too incomplete to speculate on exactly what it will show us. However, we may have the outlines of a picture of the endogenous pathway if we consider the morphological changes that precede axon fragmentation and the known genetic insults, toxins, and drugs that suppress or enhance axonal degeneration. The key events that precede irreversible axon fragmentation include mitochondrial and metabolic disruption, dysregulation of ion gradients, axonal transport defects, regulated cytoskeletal breakdown, and clearance of axonal debris by surrounding cells. Each of these steps may be a cause or consequence of axon degeneration. However, there is evidence that each plays a causal role. Drugs that target ion channels and regulated proteolysis delay axon degeneration. And many of the mutations that cause axon degeneration in mouse models and inherited human neuropathies map to genes involved in axonal transport and glial function.

The Role of Ca^{2+}

Nerve injury triggers leads to an increase in axonal cytosolic Ca^{2+} concentration. Trauma to the plasma membrane may trigger an initial Ca^{2+} spike (Mata et al., 1986). Subsequent increases in axonal Na^{+}, potentially due to a decrease in energy to run the Na+/K+ ATPase, may further boost Ca^{2+} by decreasing, or even reversing, the driving force of the Na^{+}/Ca^{2+} exchanger (LoPachin and Lehning, 1997). Disruptions of Ca^{2+} homeostasis can be toxic to neurons and lead to cell death. Increased Ca^{2+} may also promote axon degeneration. Ca^{2+} channel blockers delay axon degeneration in response to multiple insults (Stys, 2005). Na^{+} channels blockers are also protective and this may be due to the resulting decrease in Ca^{2+} influx (Bechtold et al., 2005).

One mechanism of Ca^{2+} toxicity may be the activation of calpains, Ca^{2+} dependent proteases. Calpain inhibition delays some elements of Wallerian degeneration. Immediately distal to the cut site, the axonal stump rapidly retracts. This retraction is blocked by calpain inhibitors (Kerschensteiner et al., 2005). Calpain inhibitors also decrease trauma induced axonal beading (Kilinc et al., 2009) and cytoskeleton degradation (George et al., 1995).

The Role of the UPS

The ubiquitin proteasome system (UPS) also contributes to axon degeneration. The posttranslational addition of an ubiquitin tag can target a protein to the proteasome for degradation. Pharmacological inhibition of this highly regulated protein breakdown pathway delays some aspects of degeneration.

Proteasome inhibitors are effective *in vitro* to delay degeneration after axotomy of superior cervical ganglion axons, and *in vivo* to delay degeneration after optic nerve injury (Zhai et al., 2003). These inhibitors delayed the axonal swelling and cytoskeletal breakdown that occur within the first day after axotomy.

Interestingly, proteasome inhibitors delay the microtubule depolymerization that ensues before tubulin degradation (Zhai et al., 2003). Thus, the UPS may play a more specific role than general degradation of axonal proteins. It could target and degrade essential regulators of axon structure and function. As described below, we have discovered that axon injury leads to the rapid proteasomal degradation of the microtubule regulator superior cervical ganglion-10 (SCG10).

The Role of Glia and the Immune Response

Wlds and pharmacological inhibitors of axon degeneration show that an active program within the axon involving regulated proteolysis drives axon breakdown. However, the signaling pathways that orchestrate this program are poorly understood. Identification of loss of function mutants with delayed Wallerian degeneration would definitively show that Wallerian degeneration is an active process, similar to apoptosis, and would point to potential therapeutic targets. As with apoptosis, large scale genetic screens for suppressors and enhancers of axonal degeneration may be required to bring the self-destruction program into focus. While these are underway, we can consider the known genetic mutations that delay axon degeneration.

In *Drosophila*, loss of function of Draper delays axon degeneration after axotomy (Hoopfer et al., 2006; MacDonald et al., 2006). Draper is required in glia to promote axon degeneration. It is a glial receptor that may recognize degenerating axons and activate glia to clear the debris. The glial response also involves the UPS as over-expression of UPS inhibitors in glia also delays WD in *Drosophila* (Hoopfer et al., 2006).

Glia may do more than clear axon debris. In Draper mutants and after glial UPS inhibition, axon fragmentation itself is delayed. This suggests that activated glia may accelerate the internal axon self-destruction pathway and then clear the resulting axon fragments. Conversely, glia to axon signaling may inhibit the axon self-destruction pathway in the absence of injury and disease. Many inherited mouse and human peripheral neuropathies map to genes involved in glial function (Nave and Trapp, 2008). In these mouse models, axon degeneration is delayed by Wlds (Samsam et al., 2003). Thus, depending on the circumstances, glial can likely repress or activate the internal axon self-destruction program.

In mammals, axonal injury activates both glia and the immune system. Similar to the results from flies, blocking glial catabolic pathways delays axon breakdown in mice (De et al., 2003). Axon degeneration is also delayed by inhibiting the immune response to injury. Disruptions the multi-functional complement system attenuate axon degeneration (Rosen et al., 1987; Ramaglia et al., 2007), as do mutations of molecules that recruit immune cells (Kirsch et al.,

2009; Sakakima et al., 2009), mutations of immune cell receptors (Boivin et al., 2007), and mutations of perforins (Howe et al., 2007).

Signaling Molecules Required for Axon Self-Destruction: DLK and JNK

While a number of genes have been identified that attenuate the glial and immune responses to axonal injury, far less is known about the genes required to orchestrate axonal degeneration from within. Loss of function mutations of translation elongation factor (eEF1A2) dramatically delays axon degeneration in mice (Murray et al., 2008). It is unknown if eEF1A2 acts in neurons or other cells to promote axon degeneration. However, there is evidence that protein translation may increase during the early stages of axon degeneration. After peripheral nerve transection, Schwann cells transfer ribosomes directly to axons (Court et al., 2008). This greatly increases the number axonal ribosomes and may increase local protein translation in injured axons. Follow up studies with eEF1A2 may reveal a translation dependent axon self-destruction pathway.

Some components of the internal pathways that orchestrate developmental axon pruning have been identified. Given the morphological similarities between axon pruning and pathological axon degeneration, these may also play a role in axon degeneration. Though Wlds does not block developmental pruning, the two pathways may still overlap. Wlds may block a pathology specific step that is upstream of the common pathway, or a parallel pathway that is only required for pathological axon degeneration.

Disruption of p75 neurotrophin receptor (p75NTR) blocks competitive sympathetic axon pruning in rats and mice (Singh et al., 2008). In an interesting twist, winning axons secrete brain-derived neurotrophic factor (BDNF). This factor activates p75NTR on the losing axon and triggers degeneration. BDNF release is not associated with axonal injury and disease, so this exact developmental mechanism is unlikely to pathological axonal degeneration. However, it remains to be determined if the downstream consequences of p75NTR activation are shared by developmental axon pruning and pathological axon degeneration.

Nerve growth factor (NGF) withdrawal induces developmental axon pruning, and loss of trophic support may contribute to pathological axon loss later in life. Developmental NGF withdrawal leads to both axon loss and neuronal apoptosis (Nikolaev et al., 2009). This is accelerated by an N-terminal fragment of amyloid precursor protein (APP). NGF withdrawal triggers axonal APP cleavage and the shed N-terminal APP fragment then activates death receptor 6 (DR6) on the axonal membrane. DR6 directs neuronal apoptosis via caspase 3 and axon fragmentation via caspase 6. The link between an important disease molecule and axon breakdown is exciting, and a receptor mediated pathway is a good therapeutic target, but it is unclear if this NGF withdrawal paradigm is relevant to pathological axon degeneration. Inhibitors of APP cleavage do block cell body death and white matter damage after traumatic brain injury (Loane et al., 2009). However, as discussed in Chapter 3, APP cleavage and function is not required for axotomy-induced axon degeneration.

The APP/DR6 results show that a common trigger can use divergent effectors to execute axon loss and neuronal apoptosis. Thus, we might revisit the idea that apoptosis and pathological axon degeneration are entirely distinct. It is true that two central players in neuronal apoptosis, bax and bak, are not required for axon degeneration. Furthermore, Wlds potently blocks axon degeneration but apparently has no direct effect on apoptosis. However, apoptosis and axon degeneration could share an upstream pathway that diverges into an apoptosis pathway requiring bax and bak, and a Wlds-sensitive axon degeneration pathway.

C-jun N-terminal kinase (JNK) is a mitogen activated protein kinase (MAPK) with a range of functions in the nervous system including promoting apoptosis (Bogoyevitch and Kobe, 2006). JNK is present in axons and it is activated by axonal injury (Cavalli et al., 2005). MAPKs are at the bottom of a three tiered kinase cascade: MAP3Ks phosphorylate MAP2Ks, and MAP2Ks subsequently phosphorylate and activate MAPKs. Dual leucine kinase (DLK) is a MAP3K present in axons that leads to the activation of JNK. DLK and JNK are activated by diverse cellular insults and fit the profile of potential components of the axon self-destruction pathway.

As described in Chapter 2, we tested the hypothesis that DLK promotes axon degeneration using a *Drosophila* olfactory receptor neuron (ORN) axotomy model, and we found that axon degeneration is delayed in DLK mutant flies (Miller et al., 2009). Furthermore, we found that DLK acts within neurons to promote degeneration.

To determine if DLK promotes axon degeneration in mammals, we transected sciatic nerves of DLK knockout mice (Miller et al., 2009). We found that DLK knockouts have delayed axon degeneration *in vivo.* Using dorsal root ganglion (DRG) cultures in which non-neuronal cells are eliminate, we found that DLK is required within mammalian neurons for normal axon degeneration.

Wlds shows that there is likely a common pathway that orchestrates axon degeneration in response to multiple insults. To determine if DLK promotes degeneration in response to multiple insults, we exposed DLK-deficient DRG axons to vincristine, a chemotherapeutic drug whose dose-limiting side effect in patients is painful sensory neuropathy (Miller et al., 2009). We found that DLK-deficient axons are significantly protected from vincristine-induced fragmentation suggesting that DLK operates in a general axon breakdown program.

To determine whether DLK's axonal target JNK promotes axon degeneration, we inhibited JNK in the DRG axotomy model using wildtype cultures (Miller et al., 2009). We found that JNK inhibition significantly delays axon degeneration. Thus JNK, like DLK, acts within neurons to promote axon degeneration.

JNK could promote each of the potential phases of the axon self-destruction program: competence, commitment, and execution. If JNK's primary role were to promote competence to degenerate, then JNK activity should be required prior to axotomy. This is not the case: applying the JNK inhibitor twenty-four hours prior to axotomy and then removing it just before axotomy is not protective (Miller et al., 2009). In contrast, JNK inhibition started concurrently with axotomy was

protective. Thus, JNK promotes axon fragmentation after the competence period and acts within the severed distal axon segment.

JNK could commit axons to degenerate during the delay between injury and breakdown, or it could operate during the subsequent execution phase of axon breakdown. To test whether JNK activity is required during the execution phase, we added the JNK inhibitor three hours after axotomy, which is approximately nine hours before the onset of fragmentation (Miller et al., 2009). This treatment schedule spans the transition from the proposed commitment phase to the execution phase and the entire execution phase itself. Continuous JNK inhibition beginning three hours post-axotomy did not delay axon fragmentation. Therefore, JNK activity is not required during the execution phase of axon fragmentation. Rather, JNK activity is required during the early response to injury that commits the axon to breakdown hours later.

Converging lines of evidence suggested that there is a general internal axon self-destruction program activated by injury and disease, but its molecular components were unknown. We found that the MAP3K DLK and its downstream MAPK JNK are important elements of such a program. Disrupting this pathway delays axon fragmentation in response to both axotomy and the neurotoxic chemotherapeutic agent vincristine. Thus, a common self-destruction program may promote axon breakdown in response to diverse insults, and so may be targetable in multiple clinical settings.

JNK works within axons as they commit to degenerate early after injury. Superior cervical ganglion-10 (SCG10) is an axonal JNK target that regulates microtubule dynamic instability (Riederer et al., 1997; Antonsson et al., 1998). As described in Chapter 4, we found that SCG10 is rapidly lost after multiple axonal insults. In healthy axons, we found that SCG10 is continuously degraded and then replaced by axonal transport. This baseline SCG10 turnover requires JNK and the proteasome. Following injury, axonal transport is blocked and SCG10 cannot be replenished distal to the injury. Continuous axonal transport and degradation of an axonal maintenance factor is a potential mechanism by which distal axons sense their healthy connection to the cell body. Since such a factor would be lost distal to a cut site, but not proximal, this could be a simple mechanism to selectively disinhibit the axon self-destruction pathway distal to an injury.

On the Other Side of the Cut

Neurons have three potential responses to axonal injury: axon degeneration, axon regeneration, and cell body apoptosis. We found that DLK and JNK promote the degeneration of axon segments disconnected from their cell bodies. DLK and JNK play a role in both axon regeneration and neuronal apoptosis. Many neurons, especially in the CNS, undergo apoptosis after axonal injury. JNK knockout mice suffer from decreased apoptosis after a variety of insults (Borsello and Forloni, 2007). Expression of dominant negative forms of DLK also prevents neuronal apoptosis (Xu et al., 2001; Chen et al., 2008). Peripheral neurons often survive axonal insults and their axons regenerate. Surprisingly, DLK and JNK are also

required for axon regeneration after injury . JNK activates a transcriptional program that promotes axon regeneration in peripheral neurons. DLK and JNK may also act locally in the axon to promote regeneration. Depending on the setting, DLK and JNK can kill neurons, stimulate axon regeneration, or trigger axon self-destruction. The diverse functions of DLK and JNK, their rapid involvement after injury, and their local axonal activation, suggest that DLK and JNK may be important early sensors of axonal injury.

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Chapter Two:

A DLK-Dependent Axon Self-Destruction Program Promotes Wallerian Degeneration

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Abstract

Axon degeneration underlies many common neurological disorders. Injured axons follow a self-destruction program, termed Wallerian degeneration, which appears active and well regulated. However, the signaling pathways that activate and carry out this program are unknown. Here we show that the MAP3K DLK and its downstream target JNK promote Wallerian degeneration. Genetic deletion of the *Drosophila* DLK ortholog *wallenda* in flies, genetic deletion of DLK in mice, and pharmacological inhibition of JNK all decrease Wallerian degeneration. JNK activity is required within axons as they commit to degenerate shortly after injury, rather than during the pre-injury competence or execution phases of degeneration. Disruption of this pathway also decreases axon degeneration induced by vincristine, a chemotherapeutic agent whose dose-limiting side effects include neuropathy. The identification of a signaling pathway that promotes axon degeneration in response to multiple insults may open new avenues for the development of therapies aimed at minimizing axon loss and the resulting neurological disability.

Introduction

Axons degenerate in a range of neurological disorders including mechanical injury, chemotherapy-induced neuropathy, hereditary neuropathies, glaucoma, diabetes, and neurodegenerative diseases such as Alzheimer's Disease and Parkinson's Disease (Coleman and Perry, 2002; Luo and O'Leary, 2005; Saxena and Caroni, 2007). Despite the diversity of insults that lead to axon loss, axons tend to degenerate along a stereotyped pathological progression. The orderliness of this breakdown process, termed Wallerian degeneration and first described in the 1850s, suggests that it is active and well regulated (Coleman, 2005; Raff et al., 2002). Hence, axon loss, like apoptotic cell death, may be a salutary response to dysfunction when appropriately deployed, but also a cause of disease when misapplied. In support of the hypothesis that axon degeneration is active, pharmacological inhibition of the ubiquitin proteasome system (Hoopfer et al., 2006; MacInnis and Campenot, 2005; Zhai et al., 2003), calpain proteases (George et al., 1995; Spira et al., 2003; Touma et al., 2007; Wang et al., 2004), and trypsin-like proteases (Ikegami et al., 2004) decreases Wallerian degeneration. Preventing rises in Ca^{2+} can also be protective (Stys, 2005), suggesting that Ca^{2+} may trigger signal transduction cascades that promote axon degeneration. In addition, over-expression of the chimeric Wlds protein (Mack et al., 2001) and its component nicotinamide mononucleotide adenylyltransferase (Araki et al., 2004; Wang et al., 2005) each delay axon degeneration in response to a variety of insults. Axon

degeneration is therefore likely active, and diverse neuronal insults may trigger a common self-destruction mechanism within the axon.

If axon loss is a regulated process, then it should be delayed by loss-of-function mutations in the pathways that promote it. Such pathways likely operate within the axon itself to orchestrate breakdown, and identifying the elements of these pathways may help to reveal what drives axon loss in disease. Loss-of-function mutations that delay the final step of axon loss, phagocytosis of axon fragments, have been identified (Dailey et al., 1998; Liu et al., 1999; MacDonald et al., 2006). However, these mutations affect cells surrounding the axon that promote its clearance, rather than the internal axon breakdown pathway. Hence, the internal neuronal pathways that orchestrate axon breakdown in injury and disease remain unidentified.

Which signaling pathways are likely to promote axon breakdown? Degenerating axons show many of the same pathological features as apoptotic neurons, and the search for the molecular underpinnings of axon degeneration began with the effectors of neuronal apoptosis (Coleman, 2005; Sievers et al., 2003). However, pharmacologic treatments and genetic lesions that block apoptosis do not block axon degeneration (Finn et al., 2000; Sagot et al., 1995; Whitmore et al., 2003). Therefore, the analogy of axon degeneration to apoptosis apparently breaks down at the molecular level.

Candidate components of axon breakdown pathways should be present in axons and activated by diverse cellular insults. One such candidate is dual leucine kinase (DLK), a mitogen-activated protein kinase kinase kinase (MAP3K) involved in axonal transport (Horiuchi et al., 2007), axon pathfinding (Hirai et al., 2006), neuronal migration (Hirai et al., 2006), and neuronal apoptosis (Xu et al., 2001). Interestingly, one of DLK's downstream targets, the mitogen-activated protein kinase (MAPK) c-Jun N-terminal kinase (JNK), is activated locally in axons following injury (Cavalli et al., 2005). We tested the hypothesis that DLK is a component of the molecular pathway that promotes axon degeneration. Using cultured dorsal root ganglion neurons, we demonstrate that DLK promotes axon degeneration in response to both axotomy and vincristine, a chemotherapeutic drug whose dose-limiting side effects include neuropathy (Casey et al., 1973). We show that the downstream kinase JNK promotes axon degeneration locally in the severed axons as they commit to degenerate shortly after injury. Finally, we demonstrate that *in vivo* Wallerian degeneration is inhibited in DLK loss-of-function mutants from both *Drosophila* and mice. Hence, a DLK-dependent axon self-destruction pathway promotes degeneration in response to multiple insults.

Results & Discussion

We tested the hypothesis that DLK promotes axonal degeneration using a well-established *Drosophila* model that displays molecular and pathological similarities to mammalian Wallerian degeneration (Hoopfer et al., 2006; MacDonald et al., 2006). Green fluorescent protein (GFP) was expressed in a subpopulation of olfactory receptor neurons (ORNs) that have peripheral cell bodies in the antennae and axons that extend into antennal lobes of the brain and across a midline commissure (**Fig. 1a**). To induce degeneration, the antennae are surgically removed, thereby severing the cell bodies in the antennae from the axons remaining in the brain. We severed ORN axons from both wildtype flies and mutants lacking the *Drosophila* ortholog of DLK, *wallenda* (*wnd*) (Collins et al., 2006). Most wild-type axons degenerate within twenty-four hours as scored by the absence of the ORN axon commissure (**Fig. 1b**). In contrast, twenty-four hours after axotomy *DLK/wnd* mutant axons are significantly preserved (**Fig**. **1c**), with the commissure visible in 33 out of 49 *DLK/wnd* mutant flies compared to 14 out of 46 wildtype flies ($p < 0.005$; X^2, DOF = 2) (**Fig. 1e)**. *DLK/wnd* is therefore required for normal axon degeneration in *Drosophila*.

DLK/Wnd could act within neurons themselves to promote breakdown after injury or it could act within surrounding cells to promote axon clearance. To distinguish between these possibilities, we attempted to rescue the degeneration phenotype by expressing DLK/Wnd only in the GFP-expressing subpopulation of ORNs. The DLK/Wnd expressing axons from these otherwise *DLK/wnd* mutant flies

are not preserved twenty-four hours after axotomy, with the commissure visible in 3 out of 20 flies (**Fig**. **1d,e**) (there is no preservation of axons compared to wildtype flies, $p > 0.25$, and there is suppression of axon preservation compared to complete *DLK/wnd* mutants, $p < 0.005$; X^2, DOF = 2). Thus, DLK/Wnd functions in an internal neuronal pathway that promotes axon degeneration.

To determine if DLK promotes axon degeneration in mammals, we used the *in vitro* dorsal root ganglion (DRG) axotomy model (Araki et al., 2004). We cultured embryonic mouse DRGs for fourteen to sixteen days to allow the axons to extend, severed the axons with a micro-scalpel, and evaluated degeneration of the distal axon segment. Within twenty-four hours, wild-type axons distal to the transection deteriorate from smooth and continuous axon processes to rough and irregular axon fragments (contrast **Fig**. **2a** and **b**). To quantify the extent of axon degeneration in the distal axon segments we measured the fraction of total axonal area occupied by axon fragments (degeneration index, DI). We axotomized DRG axons from DLK-deficient mice and littermate controls and found that DLK-deficient axons were significantly preserved. Twenty-four hours after axotomy, DLK-deficient axons have a DI decreased by 65% ± 3.3% (s.e.m) relative to wildtype littermate axons (contrast **Fig**. **2e** and **2b**) ($p<0.001$, Student's t-test; similar results were found with DRGS taken from two independently generated DLK knockout mouse lines). This delay in fragmentation persists for forty-eight hours (**Fig**. **4**). DLK therefore promotes axon degeneration in flies and mice. Furthermore, since

non-neuronal cells are eliminated in this DRG culture system, DLK must operate within mammalian neurons themselves to promote axon breakdown.

A range of insults relevant to human disease lead to axon degeneration resembling axotomy-induced degeneration, suggesting that axon breakdown in response to diverse stimuli may be driven by a common pathway (Coleman, 2005; Coleman and Perry, 2002). To determine if DLK participates in such a common pathway, we assessed the response of DLK-deficient DRG axons to vincristine, a chemotherapeutic drug whose side effects in patients includes neuropathy (Casey et al., 1973). In cultured DRG neurons, vincristine triggers axon degeneration that is morphologically similar to that induced by axotomy (Wang et al., 2000) (**Fig**. **2c**). After vincristine exposure, DLK-deficient axons are significantly preserved compared to wildtype axons (contrast **Fig**. **2f** and **2c**) (the DI of DLK knockout axons is 59% ± 6.3% (s.e.m) less than vincristine treated littermate control axons; $p < 0.002$, Student's t-test). Hence, DLK promotes axon degeneration in response to both axotomy and neurotoxin exposure, suggesting that it operates in a general axon breakdown program that is activated by a range of insults.

Disrupting DLK interferes with an internal axon degeneration pathway and delays degeneration *in vitro*. *In vivo* however, external factors such as the immune response may dominate and overwhelm the protective effects of blocking the internal pathway. We therefore determined if disrupting DLK reduces Wallerian degeneration *in vivo* in adult mice. We used sciatic nerve transection, a simple and well-characterized *in vivo* model of axon degeneration. Fifty-two hours post-

transection, the distal segments of adult wild-type mice degenerate (Beirowski et al., 2004), whereas distal segments of DLK-deficient transected sciatic nerves are largely intact (**Fig. 2g-l**). We counted non-collapsed axon profiles in Toluidine blue stained cross-sections of sciatic nerves and found that compared to wildtype mice, DLK-deficient mice have more than twice the number of remaining axon profiles (DLK-deficient mice have 208% ± 22% (s.e.m) axon profiles per μm^2 relative to wildtype littermates, $p<0.007$, Student's t-test, n = 4 wildtype animals and n = 5 DLK-deficient animals). The spared axon profiles are not empty myelin sheaths: transmission electron microscopy reveals axons rich in microtubules and mitochondria (**Fig. 2l, Fig. 5**). Thus, normal axon degeneration *in vivo* in adult mice requires DLK.

DLK is a MAP3K that can lead to the activation of JNK and p38 via intermediary MAP2Ks (Gallo and Johnson, 2002). To determine whether either downstream target promotes axon degeneration, we inhibited JNK and p38 in the DRG axotomy model. We treated wildtype DRG cultures with the JNK inhibitor SP600215 and the p38 inhibitor SB203580 and found that inhibition of JNK, but not p38, protects transected axons from degeneration, and a significant delay in fragmentation persists for over forty-eight hours (**Fig. 3a-e; Fig. 4**). Thus JNK, like DLK, acts within neurons to promote degeneration.

How does JNK promote axon degeneration? Axon degeneration is hypothesized to comprise at least three distinct phases – competence to degenerate, much of which is determined transcriptionally before axotomy; commitment to

degenerate, which occurs in the substantial delay period between injury and axon fragmentation; and the execution phase, when axons fragment (Saxena and Caroni, 2007). If JNK's primary role is to promote competence to degenerate, for instance by promoting the expression of pro-degenerative factors that are activated after injury, then JNK activity should be required prior to axotomy. We found that this is not the case: applying the JNK inhibitor twenty-four hours prior to axotomy and then removing it just before axotomy is not protective (**Fig**. **3f**). In contrast, JNK inhibition started concurrently with axotomy is protective (**Fig**. **3g**). Thus, JNK activity promotes axon degeneration after the competence period and it works within the severed distal axon segment itself.

One hallmark of Wallerian degeneration is the substantial delay between the onset of axonal injury and the execution of rapid axon breakdown. Axons may commit to degenerate during this delay period and JNK could be involved in this commitment step or in the subsequent execution phase of axon breakdown. To test whether JNK activity is required during the execution phase, we added the JNK inhibitor three hours after axotomy, which is approximately nine hours before the onset of fragmentation. This treatment schedule should span the transition from the proposed commitment phase to the execution phase as well as the entire execution phase itself. We found that continuous JNK inhibition beginning three hours post-axotomy does not decrease axon degeneration twenty-four hours post-axotomy (**Fig**. **3h**). Therefore, JNK activity is not required during the execution phase of axon

fragmentation. Rather, JNK activity is required in the early response to injury that commits the axon to degenerate hours later (contrast **Fig**. **3g** and **3h**).

Axon degeneration is a shared feature of many neuropathological conditions. Converging lines of evidence suggest that there is a general internal axon self-destruction program, but its molecular components are unknown. We now show that the MAP3K DLK and its downstream MAPK JNK are important elements of an internal axon degeneration program. Genetic deletion of *DLK/wnd* in flies, DLK in mice, and pharmacological inhibition of JNK all diminish axotomy-induced Wallerian degeneration. We also demonstrate that axons commit to degenerate shortly after axotomy and long before fragmentation, and that this commitment phase is regulated by JNK. In addition, disruption of this pathway decreases axon degeneration in response to both axotomy and the chemotherapeutic agent vincristine, whose dose-limiting side effects include neuropathy. Thus, a common axon self-destruction program may orchestrate axon breakdown in response to multiple insults, and so may be targetable in multiple clinical settings.

Axonal injury may trigger neuronal apoptosis, axon regeneration, and/or axon degeneration. JNK has a previously described role promoting neuronal apoptosis and axon regeneration (Lindwall et al., 2004; Xia et al., 1995). The findings presented here that JNK also promotes axon degeneration establish that

JNK is a common and crucial component of the three major neuronal responses to axonal injury.

Material & Methods

Drosophila Wallerian degeneration

We assessed Wallerian degeneration in adult control and *wnd* mutant flies following established methods (Hoopfer et al., 2006; MacDonald et al., 2006). We expressed green fluorescent protein (GFP) in a subpopulation of olfactory receptor neurons (ORN) using Or47b-Gal4 and UASmcd8GFP (Bloomington Stock Center). We used *wnd* mutants trans-heterozygous for two independently generated mutant *wnd* alleles *wnd1* and *wnd3* as described (Collins et al., 2006). A UAS-*wnd* transgene (Collins et al., 2006) was used to express Wnd in ORNs. Flies of all genotypes used have an ORN commissure before axon severing. We severed ORN axons by surgically removing the third antennal segments bilaterally with forceps. Flies were then kept at 25°C for 24 hours. Fly heads were then removed and fixed in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) and 0.1% Triton-X100 (Amresco) in PBS for 3 hours at 4°C. Brains were then removed and washed in PBS with 0.1% Triton-X100 for 16-18 hours at 4°C. Brains were stained with rabbit derived A488-conjugated anti-GFP (Invitrogen) for 16-18 hours at 4°C. Brains were then rinsed with PBS with 0.1% Triton-X100 and mounted in 70% glycerol (Amresco) in PBS. Images were acquired using standard confocal microscopy and degeneration was scored based on the presence or absence of the commissure connecting the two antennal lobes formed by ORN axons (*2*). Quantification was

done blind to genotype. Statistical significance was determined using the X^2 test with two degrees of freedom (three genotypes were compared).

DLK mutant mice

Two independently generated DLK mutant mouse lines were used in this study. We previously described a strong DLK hypomorph developed from a BayGenomics genetrap ES cell line (Bloom et al., 2007). We also developed a conditional DLK knockout by flanking the exons that encode DLK's kinase domain with LoxP sites (**Fig**. S1) using homologous recombination as previously described (Bloom et al., 2007). The neo-cassette was flanked by FRT sites (**Fig**. **6**) and was removed by breeding the targeted DLK mice to mice expressing flip1recombinase under the human actinB promoter (The Jackson Laboratory). Animals are genotyped using PCR with the primers: 5'-GGAAAGGTGTGGCCCTGGCTGGCTTGGAAG-3' and 5'-CAGGTGCAGCAAGATCTGTCGGAATGATGG-3.' The DLK Western blot (**Fig**. **6**) was carried out as previously described (Bloom et al., 2007).

We generated a constitutive DLK knockout allele by breeding conditional DLK mice to a cre-recombinase line with germline expression (zp3-cre) (The Jackson Laboratory). The genetrap DLK mutant, constitutive DLK mutant, and conditional DLK mutant with the mutation induced *in vitro* using lentiviral mediated cre-recombinase expression as previously described (Araki et al., 2004) were all used for the DRG cultures presented in **Fig**. **2a-f** and **Fig. 4** with similar results

found for all mutant alleles. We used the genetrap DLK strong hypomorphic mutant for the *in vivo* experiments presented in **Fig. 2g-l** because the constitutive DLK mutant is perinatal lethal, consistent with previous findings (Hirai et al., 2006). Animals were housed in the Washington University Mouse Genetics Core and all mouse experiments were approved by the Washington University School of Medicine Animal Studies Committee.

Mouse DRG culture preparation and treatment

DRGs were cultured from embryonic day 12.5-14.5 mice in twenty-four well plates (Corning) coated with poly-d-lysine (Sigma) and laminin (Sigma). Cultures were grown for 14-16 days before axotomy or drug treatment in 500µL serum free medium consisting of Neurobasal (Invitrogen) containing penicillin and streptomycin and supplemented with 2% B27 (Invitrogen), 25 ng/ml nerve growth factor, and 1 µM 5-fluoro-2'-deoxyuridine and 1 µM uridine (Harlan Bioproducts) to eliminate dividing non-neuronal cells. For cultures derived from crosses of heterozygous mutant parents (DLK genetrap mutants or DLK constitutive knockouts) one intact DRG explant was cultured per well. For all other cultures, all DRGs of a given litter were combined, trypsinized for 20 minutes at 37°C, triturated in medium, and seeded at a density of 1 DRG per well in 2 µL medium for forty minutes at 37°C before the addition of 500µL medium. Wildtype cultures in **Fig. 3** and **Fig. 4** were derived from CD1 mice (Charles River). DRGs were axotomized

using a micro-scalpel. All drugs were dissolved in DMSO (Sigma) and the controls were treated with this vehicle. Vincristine was used at 0.04 μM, SP600125 (Biomol) at 15 μM, and SB203580 (Biomol) at 20 μM.

Quantifying DRG axon degeneration

Live DRG cultures were imaged using phase contrast and a 20X objective. 3-4 non-overlapping images were taken per well with a field of view of approximately 0.15cm^2. Images were taken 24 hours post-axotomy or 48 hours after vincristine addition. The degeneration index (DI) was measured using a macro developed in ImageJ (National Institutes of Health, USA). Phase contrast images are binarized to measure the total area occupied by axons (including both continuous axons and degenerated fragments), and the area of degenerated axon fragments is detected using the particle analyzer algorithm of ImageJ. The DI is the area occupied by degenerated axon fragments divided by the total axon area. This automated method enabled us to sample an order of magnitude greater image area than by manual analysis. The mean DI of each culture well was calculated by averaging the DIs of three to four non-overlapping images from that well. Using this analysis, non-axotomized wildtype cultures have a DI of 0.15 ± 0.023 (sem) before axotomy and a DI of 0.67 ± 0.26 (sem) 24 hours post-axotomy. There is no significant increase in DI three hours post-axotomy (DI = 0.11 ± 0.018; n = 3; $p > 0.15$ compared to non-cut, Student's t-test). Unless noted, there was an $n \geq 7$ axotomized DRG cultures or

vincristine-treated DRG cultures per genotype or drug treatment condition and each result was obtained from multiple experiments. Quantification was done blind to genotype or treatment group. DI's are presented in the text normalized to the indicated control DI for each independent experiment. When only two conditions or genotypes were compared, Student's t-test was used to determine statistical significance. When more than two were compared, we used ANOVA with posthoc Tukey means comparison.

Mouse *in vivo* sciatic nerve transection

Adult DLK genetrap mutant animals and littermate controls were anesthetized with isofluorane. A small incision was made unilaterally to expose the sciatic nerve. The sciatic nerve was transected with fine surgical scissors and the incision was then sutured. After 52 hours, the animals were sacrificed using CO_2 and the sciatic nerves were removed bilaterally (distal to the transection of the transected nerve) and fixed 16-18 hours at 4°C in 4% PFA and 2.5% glutaldehyde in 0.1M cacodylate buffer. Approximately 2 mm long sciatic nerve stumps were post-fixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour at RT and then embedded in resin as follows. Nerves were dehydrated through an ethanol series, then in propylene oxide and then overnight in a 1:1 mix of propylene oxide in Epon 812 (hard formulation; Ted Pella Inc.) under -5 inches Hg vacuum. The next day, the samples were changed into fresh Epon resin, left on a rotator for several hours, and

then placed in fresh Epon in coffin molds. The resin was cured at 60 °C for 48 hours and sectioned with a diamond knife (Micro Star Technologies) on a Leica EM UC6 ultramicrotome (Leica Microsystems). Sections were taken at 500 nm for Toluidine blue staining and 70 nm for electron microscopy (EM). Sections for EM were transferred to grids, stained with filtered 5% uranyl acetate in methanol for 10 minutes, washed, dried, and stained for 2 minutes in filtered lead citrate. Pictures were taken on a Hitashi H-7500 TEM using 70 kV accelerating voltage. Axon density (axons/μm^2) was determined by counting the total number of axons with non-collapsed sheaths in the tibial division of the sciatic nerve and dividing by the area. Quantification was done blind to genotype. Statistical significance was determined using Student's t-test.

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Author Contributions

B.R.M. designed and conducted all of the experiments and co-wrote the manuscript. C.P. helped to design and to conduct the *in vitro* DRG experiments. R.W.D. contributed to the electron microscopy analysis. Y.S. developed the degeneration index algorithm. J.M. supervised the project. A.D. supervised the project and co-wrote the manuscript.

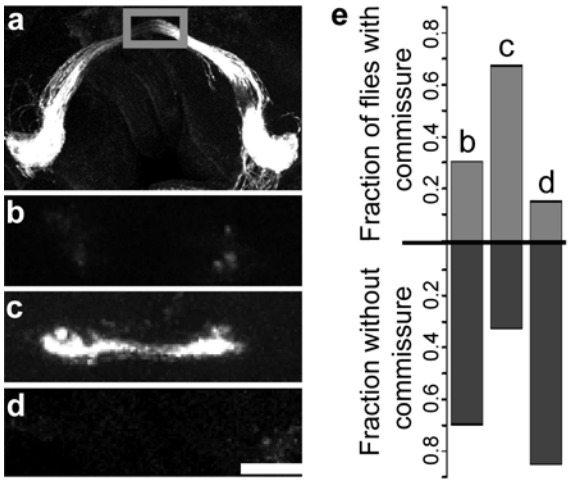

Figure 1: Neuronal Wnd promotes axon degeneration in *Drosophila*.

(**a**) GFP was expressed in a subpopulation of ORNs using *Or47b-Gal4* to visualize ORN axons. Before severing, the commissure shown in the boxed region was present in all genotypes. (**b,c**) Higher-power images of the commissural region taken 24 h after axotomy showed that no axons remained in the commissure of most wild-type flies (14 of 46 flies had a commissure, **b**) and that axons were significantly preserved in the commissure of most *wnd* mutant flies (33 of 49 flies had a commissure, **c**). This reflects a delay of degeneration, as remaining commissures were thinner than non-axotomized commissures and *wnd* mutant flies reached wild-type levels of degeneration after 48 h (data not shown). (**d**) When Wnd was

exclusively expressed in the GFP-expressing subpopulation of ORNs of otherwise *wnd* mutant flies, no axons were detected in the commissure of most flies (3 out of 20 flies had a commissure). Thus, Wnd promotes degeneration cell autonomously. (**e**) Fraction of flies of each genotype with and without an ORN commissure. Axons were significantly preserved in genotype C compared with both genotype B and genotype D ($P < 0.005$) and axons in genotype D were not preserved compared with genotype B ($P > 0.25$; X^2, two degrees of freedom). Scale bar represents 25 μm (**a**) and 10 μm (**b–d**).

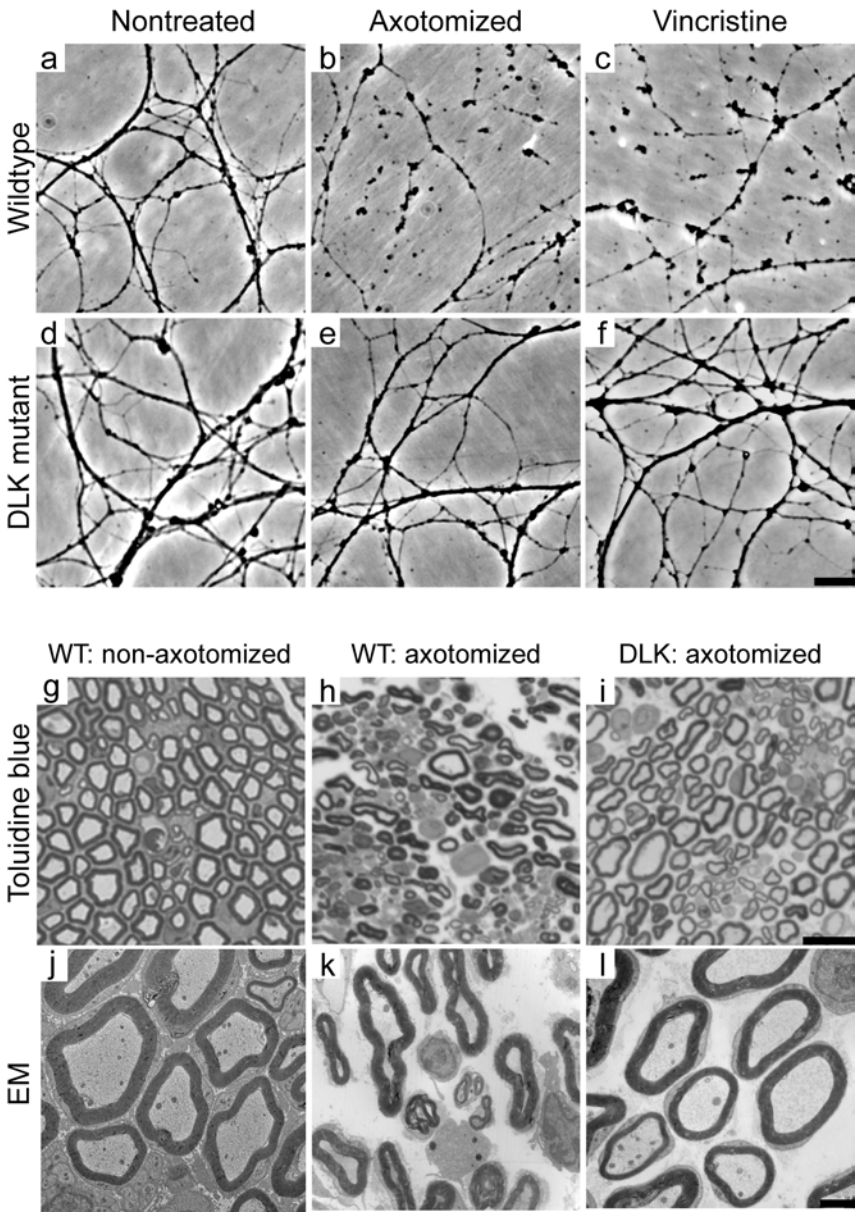

Figure 2: Normal axon degeneration in mice requires DLK *in vitro* and *in vivo*.

(**a–f**) Phase contrast images of DRG axons from littermate wild-type (**a–c**) and DLK mutant embryos (**d–f**). DLK-deficient axons had a 65 ±3.2% (s.e.m.) degeneration

decrease relative to controls ($P < 0.001$, Student's *t* test) 24 h after axotomy (**b,e**). Vincristine induced 59 ±6.3% (s.e.m.) less degeneration in DLK mutants than in littermate controls after 48 h ($P < 0.002$, Student's *t* test; **c,f**). (**g–l**) *In vivo* sciatic nerve transections in adult mice. Sciatic nerve cross-sections distal to the transection site were stained with Toluidine blue (**g–i**) or imaged by electron microscopy (**j–l**). Axons were significantly preserved in DLK-deficient nerves 52 h post-axotomy (208 ± 22% axon profiles per μm^2 compared with wild-type nerves, $P < 0.007$, Student's *t* test, $n = 4$ wild-type animals and 5 DLK mutant animals; **h,i**). Electron microscopy showed that these profiles contained axons with mitochondria and a cytoskeletal network (**j–l**; **Fig. 4**). Scale bars represent 20 μm (**a–i**) and 2 μm (**j–l**). All mouse experiments were approved by the Washington University School of Medicine Animal Studies Committee.

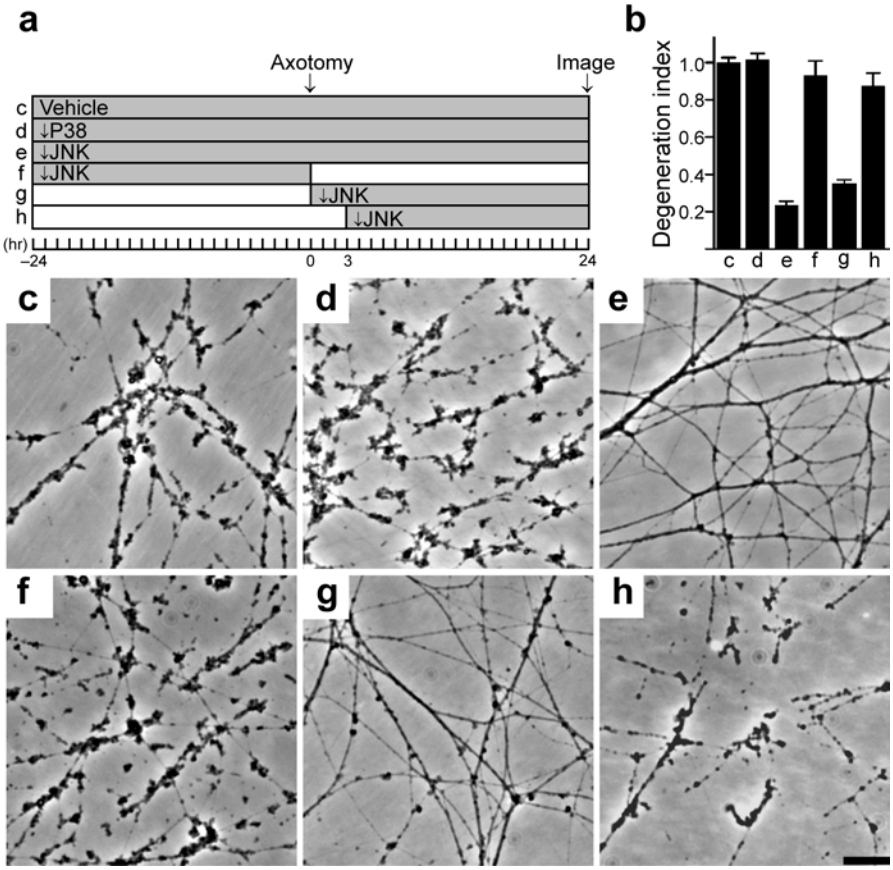

Figure 3: JNK promotes Wallerian degeneration and is critical in the first 3 h after axotomy.

(**a**) Shaded areas designate the drug treatment period. ↓P38 refers to the P38 inhibitor SB203580 (20 μM) and ↓JNK refers to the JNK inhibitor SP600125 (15 μM). (**b**) Degeneration index of the conditions shown in **c–h**. (**c–h**) Phase-contrast images of axotomized wild-type DRG axons with the indicated drug treatment. Shown are wild-type DRG axons distal to the axotomy 24 h post-axotomy. Conditions **e** and **g** showed significantly less degeneration than conditions **c**, **d**, **f** and

h ($P < 0.001$, ANOVA and *post hoc* Tukey test). Error bars represent s.e.m. Scale bar represents 20 μm.

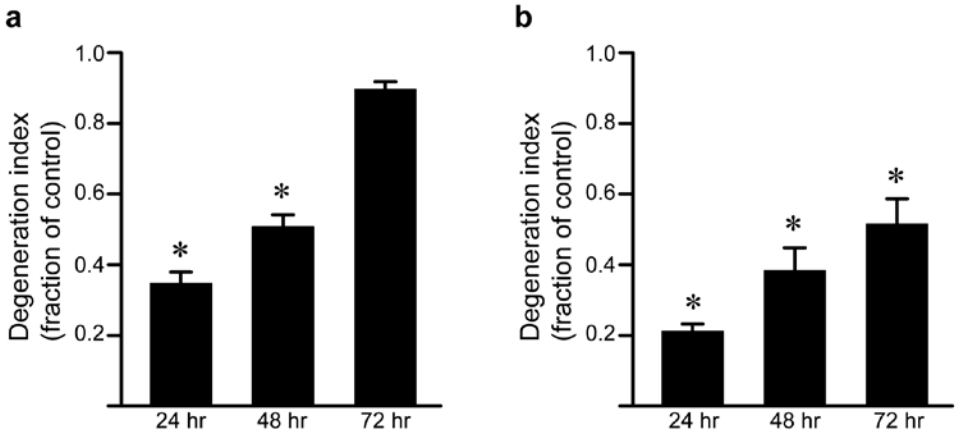

Figure 4: Time-course of axon fragmentation after axotomy.

(**a**) DLK-deficient DRG cultures have significantly less axon fragmentation than wild-type littermate cultures at 24 hours and 48 hours post-axotomy. (**b**) Wild-type DRG cultures treated with JNK inhibitor (SP600125, 15μM) have significantly less axon fragmentation than vehicle treated cultures at 24 hours, 48 hours, and 72 hours post-axotomy. (∗) designates a significant decrease in fragmentation compared to (a) wild-type littermate cultures at the indicated time-point or (**b**) vehicle treated wild-type cultures at the indicated time-point ($p < 0.001$; Student's t-test. $n \geq 4$ per condition). Error bars are s.e.m.

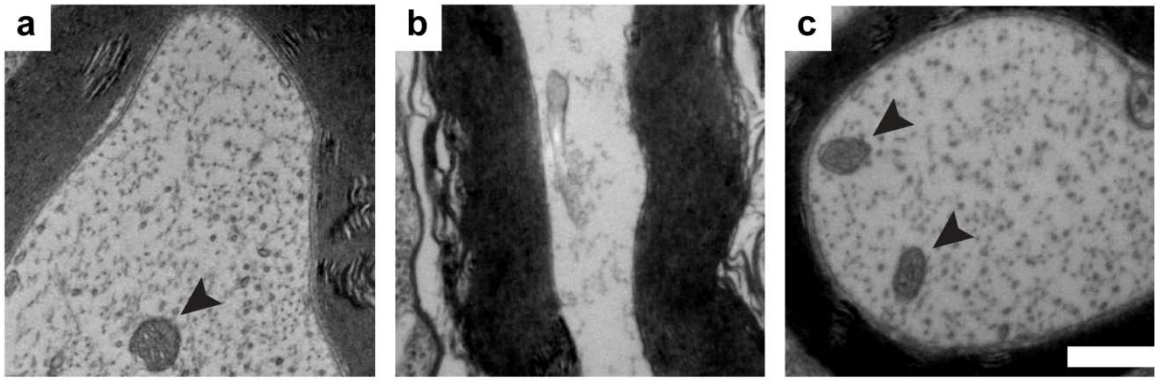

Figure 5: EM analysis shows preserved axons in DLK mutant mice fifty-two hours after sciatic nerve transection.

Cross-sections of (**a**) non-transected wild-type sciatic nerves show axons containing mitochondria (arrowheads) and a cytoskeletal network. (**b**) These features are absent in degenerated axons of transected wild-type littermate controls, and (**c**) these features are present in preserved axons of DLK-deficient nerves. Scale bar represents 500 nm.

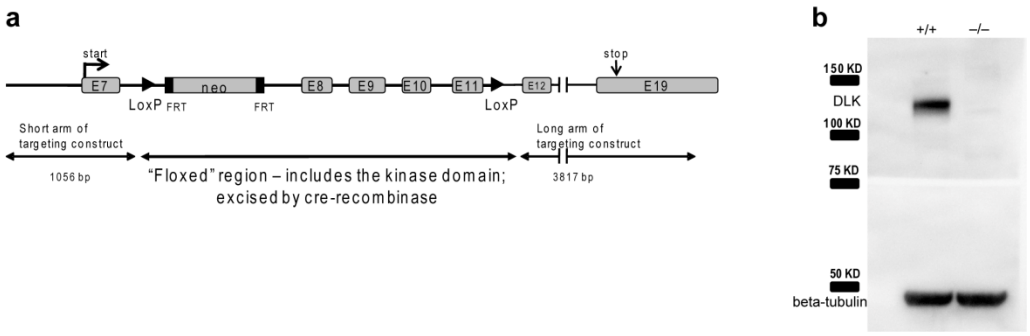

Figure 6: Conditional DLK knockout mouse.

(**a**) Schematic of the targeted DLK locus. (**b**) Western blot of brain preparations from (–/–) constitutive DLK knockouts generated from the conditional DLK knockout as described in the Methods and (+/+) wild-type littermate controls.

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Chapter Three:

The Requirement for APP Cleavage Defines Distinct Mechanisms of Axon Degeneration

This chapter is a subsection of a larger manuscript in preparation:

Requirement for APP cleavage and caspase 6 activity defines distinct mechanisms of axonal degeneration. Bhupinder P.S. Vohra, Yo Sasaki, Bradley R Miller, Jufang Chang, Aaron DiAntonio, and Jeffrey Milbrandt

The work presented here consists of experiments performed by B.R.M. and designed in collaboration with all authors. All authors contributed to the writing.

Abstract

Axon degeneration is a hallmark of many prevalent and debilitating neurological disorders. Diverse experimental insults trigger a similar morphological progression of axon breakdown that may be driven by a common axon self-destruction pathway. Nerve growth factor withdrawal accelerates this degeneration program by inducing the shedding of an N-terminal APP fragment that binds the TNF receptor DR6 to activate caspase 6. In contrast, we find that axotomy triggers axon degeneration that is independent of APP cleavage. Expression of NMNAT and inhibition of JNK is protective in both paradigms. Hence, distinct neuronal insults work through unique molecular pathways before converging on a common, NMNAT-sensitive axonal degeneration program that involves JNK.

Introduction

Why is axon degeneration a common element of a diverse set of neurological disorders? A favorite hypothesis is that a wide range of neuronal insults triggers a general axon self-destruction program. In support of this hypothesis, over-expression of Wlds, and its component NMNAT, block degeneration in response to many distinct stimuli (Araki et al., 2004; Sasaki, Vohra, Baloh, et al., 2009). Furthermore, although the intrinsic pathways that drive degeneration are poorly understood, calcium influx, regulated protein degradation, and JNK activation by DLK each can promote degeneration in multiple contexts (Coleman, 2005; Miller et al., 2009).

A general molecular pathway leading from the initial insult to the effectors of axon breakdown has remained elusive. One such candidate pathway was recently described for axon degeneration following trophic factor withdrawal. Upon removal of nerve growth factor (NGF), axonal APP is cleaved and a shed N-terminal APP fragment activates the tumor necrosis factor (TNF) receptor DR6 leading to caspase 6 activation (Nikolaev et al., 2009). Inhibition of each of these events delays degeneration after trophic factor withdrawal. The discovery of a pathway initiated by a receptor-mediated event on the axonal membrane and leading to the activation of a potential effector of axon breakdown could have broad clinical significance. Inhibition of APP cleavage protects cell bodies and axons *in vivo* after traumatic brain injury (Loane et al., 2009). Thus, the APP/DR6/caspase pathway may orchestrate a general axon degeneration program.

Results & Discussion

Is the APP/DR6/caspase 6 pathway a universal driver of axon degeneration or is it specific to NGF-withdrawal? To test directly the generality of the APP/DR6/caspase 6 pathway we used two distinct triggers of axon degeneration: NGF withdrawal and axotomy in dorsal root ganglion (DRG) neurons. After each of these insults, we inhibited the pathway at the level of APP cleavage and APP function. Inhibition of APP cleavage using a BACE inhibitor blocked degeneration after NGF-withdrawal. A function-blocking antibody against the shed N-terminal APP fragment was also protective. Over-expression of NMNAT and inhibition of JNK both protect axons after NGF-withdrawal.

Neither the BACE inhibitor nor the N-terminal APP function-blocking antibody protected axons following axotomy. Therefore, APP is necessary for axon degeneration following trophic withdrawal but not following mechanical insults in this system. In contrast, both over-expression of NMNAT and inhibition of JNK protect axons following axotomy. Thus axons have distinct mechanisms promoting degeneration in response to diverse insults.

Axonal degeneration is a hallmark of many debilitating neurological disorders. Identifying the mechanisms that drive such degeneration may lead to treatments to block or delay axon loss. The recent identification of the APP/DR6/Caspase 6 pathway was a benchmark in the field because it suggests

rational targets for novel therapeutic interventions. We now demonstrate that this APP/Caspase 6 pathway is not a general trigger of axonal degeneration, but rather defines the pathway from trophic factor withdrawal to the activation of the degeneration program. Our results suggest that multiple stimulus-dependent pathways link neuronal insults to a common JNK-dependent, NMNAT-sensitive axon degeneration pathway.

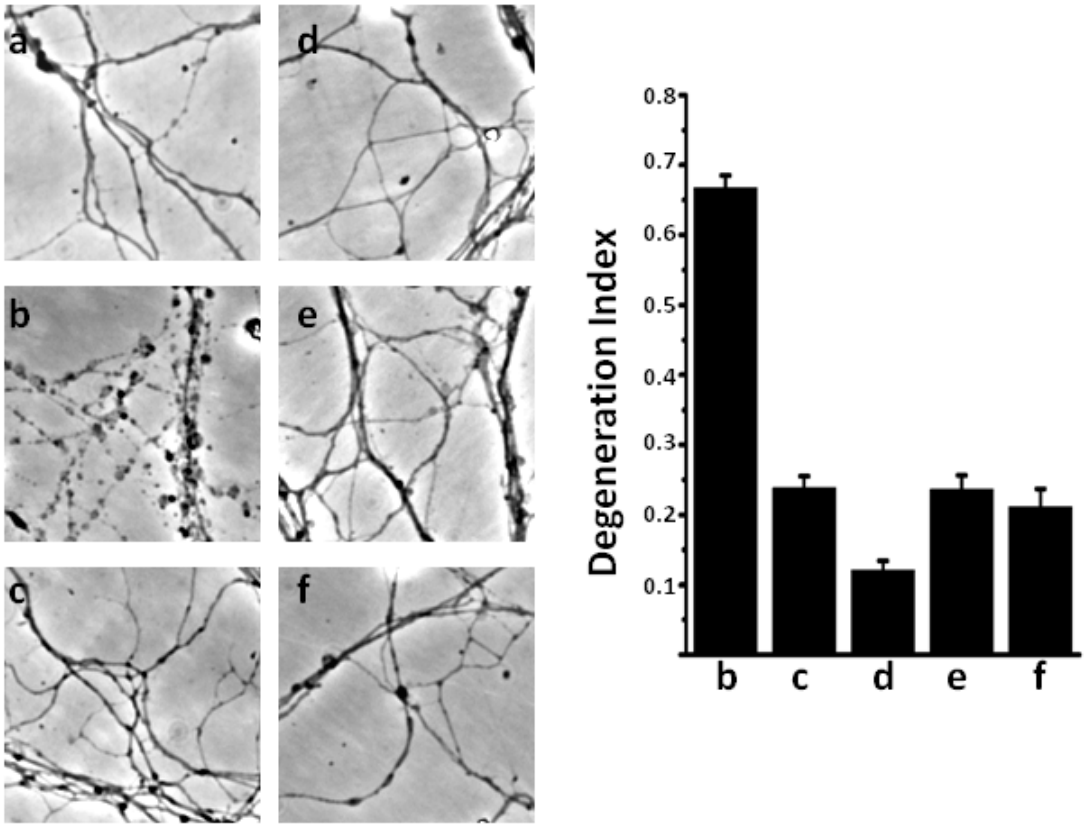

Figure One: NGF withdrawal induced axon degeneration requires JNK, APP cleavage, and is NMAT-sensitive.

Phase contrast images of DRG axons. (**a**) Vehicle treated without NGF withdrawal. (**b–f**) 24 hour NGF withdrawal. (**b**) Vehicle treated. (**c**) NMNAT expression. (**d**) JNK inhibition (SP600125, 15 μM). (**e**) BACE inhibition (OM99-2, 10 μM). (**f**) Function blocking antibody against N-terminal APP fragment (anti N-APP, 20 μg/mL). Axons in conditions **c–f** are significantly protected from NGF withdrawal induced axon degeneration compared to vehicle treated condition **b** ($p < 0.01$, ANOVA).

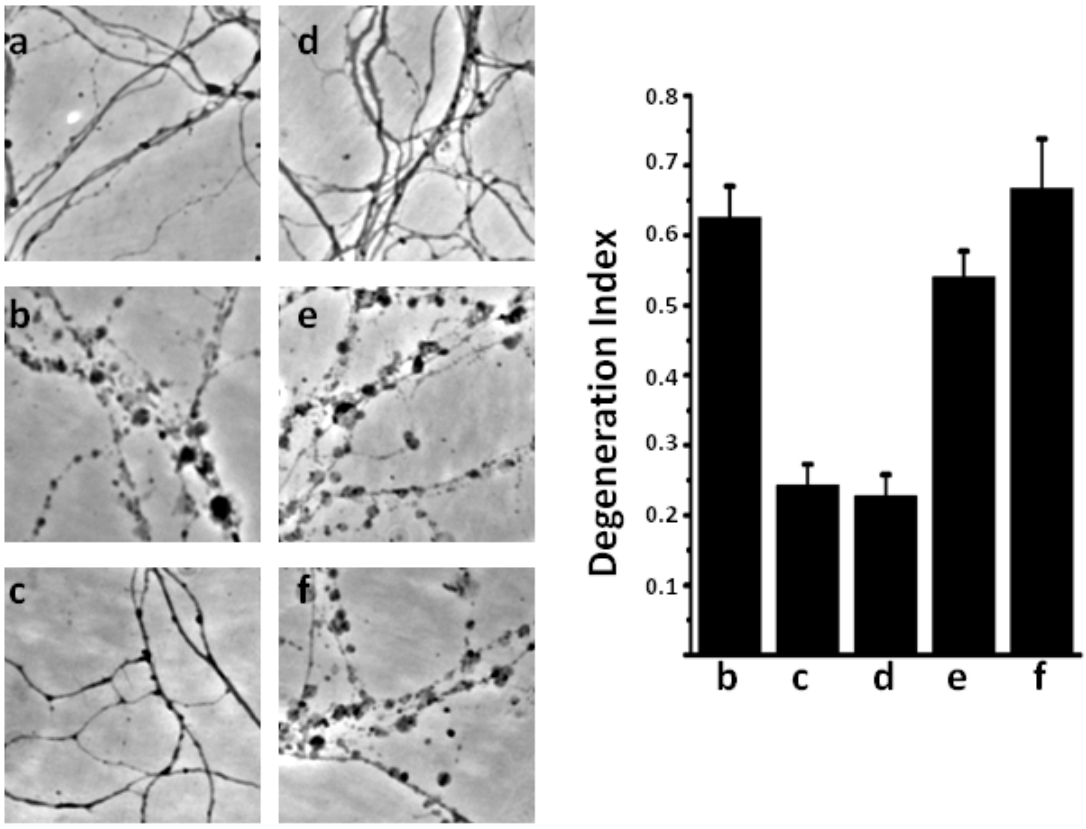

Figure Two: Axotomy induced axon degeneration requires JNK and is NMAT-sensitive, but does not require APP cleavage.

Phase contrast images of DRG axons. (**a**) Vehicle treated without axotomy. (**b–f**) 6 hour axotomy. (**b**) Vehicle treated. (**c**) NMNAT expression. (**d**) JNK inhibition (SP600125, 15 μM). (**e**) BACE inhibition (OM99-2, 10 μM). (**f**) Function blocking antibody against N-terminal APP fragment (anti N-APP, 20 μg/mL). Only conditions **c** and **d** are significantly protected from axotomy induced axon degeneration compared to vehicle treated condition **b** ($p < 0.01$, ANOVA).

Materials & Methods

DRG Cultures:

DRGs were cultured from embryonic day 12.5-14.5 CD1 mice (Charles River) in twenty-four well plates (Corning) coated with poly-d-lysine (Sigma) and laminin (Sigma). Cultures were grown for 5-6 days in 500μL serum free medium consisting of Neurobasal (Invitrogen) containing penicillin and streptomycin and supplemented with 2% B27 (Invitrogen), 25 ng/ml nerve growth factor, and 1 μM 5-fluoro-2'-deoxyuridine and 1 μM uridine (Harlan Bioproducts) to eliminate dividing non-neuronal cells. They were then subjected to NGF withdrawal or axotomy with a microscalpel.

NGF Withdrawal:

NGF containing media was removed and neurons were washed with NGF-depleted media twice. Neurons were then grown in NGF-depleted neurobasal/B27 media containing goat anti-NGF antibody (1:1000).

Inhibitors, Function Blocking APP Antibody Treatment, NMNAT expression:

In all experiments, DMSO vehicle, inhibitors, or function blocking antibody were added to the medium at the time of NGF withdrawal or axotomy. We used the JNK inhibitor (SP600125, Biomol) at 15 μM, and the BACE inhibitor (OM99-2, Calbiochem) at 10 μM. The function blocking APP antibody was an (anti-N-APP polyclonal antibody, Fisher scientific) was used at 20μg/ml. NMNAT-cherry lentivirus was designed and produced as previously described (Sasaki, Vohra, Lund, et al., 2009).

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Chapter Four:

Axonal Injury Induces Rapid JNK-Dependent SCG10 Degradation

Introduction

Many of the most prevalent and debilitating neurological disorders share the common feature of axonal degeneration. Axon loss is a hallmark of disorders such as diabetes, glaucoma, and chemotherapy-induced neurotoxicity. Debilitating neurodegenerative diseases such as Alzheimer's and Parkinson's disease exhibit substantial axon loss early in the disease progression. And axonal degeneration following trauma is a major cause of disability, as the great length of many axons makes them particularly vulnerable to mechanical injury. Recent studies demonstrate that axonal degeneration is an active and highly regulated process, yet the intrinsic, neuronal mechanism promoting degeneration is poorly understood. What causes axons to degenerate, and how can this be prevented?

Why is axonal degeneration a common element of a diverse set of neurological disorders? A favorite hypothesis is that a general active axon self-destruction program is triggered by a wide range of insults. Converging lines of evidence support this hypothesis. Overexpression of deubiquitinating enzymes and incubation with proteasome inhibitors, both of which inhibit the ubiquitin proteasome system (UPS), delay axonal degeneration (Zhai et al., 2003; Hoopfer et al., 2006). This suggests that regulated protein degradation may relieve an inhibitory restraint on the degenerative mechanism. Preventing rises in Ca^{2+} is also protective in a variety of contexts, suggesting that Ca^{2+} may trigger signal transduction cascades that promote axonal degeneration (Stys, 2005). Finally, expression of the chimeric protein Wlds and its component NMNAT dramatically delay Wallerian degeneration, providing strong evidence that axonal degeneration

is not merely due to starvation of the severed axon following its separation from the neuronal cell body (Mack et al., 2001; Araki et al., 2004).

What orchestrates axon breakdown from within? In *Drosophila* and mice, dual leucine kinase (DLK) promotes axon self-destruction (Miller et al., 2009). Disruption of DLK delays axon breakdown in response to axotomy and vincristine, a chemotherapy with the dose-limiting side effect of painful peripheral neuropathy. DLK is a mitogen activated protein kinase kinase kinase (MAP3K) that can lead to the activation of the MAPK c-jun N-terminal kinase (JNK). JNK promotes degeneration locally in distal axons as they commit to degenerate shortly after injury (Miller et al., 2009).

How does JNK promote axon degeneration? JNK is a kinase with over fifty known substrates with diverse neuronal functions (Bogoyevitch and Kobe, 2006). Superior cervical ganglion-10 (SCG10) is an axonal JNK target that regulates microtubule dynamic instability (Riederer et al., 1997; Antonsson et al., 1998). It is expressed throughout the mammalian nervous system and its expression correlates with neuronal differentiation and neurite outgrowth (Stein, Mori, et al., 1988; Oishi et al., 2002). SCG10 is a member of the stathmin family and it binds microtubules with its stathmin-like domain (Riederer et al., 1997). Stathmin-like domains bind tubulin α-β heterodimers and hold them in a curved state that resembles depolymerizing microtubules (Gigant et al., 2000). When SCG10 binds microtubules, it promotes microtubule catastrophe: the sharp transition between

microtubule growth and microtubule contraction. Consequently, it frees new tubulin for subsequent rounds of microtubule growth.

Though at the molecular level SCG10 triggers microtubule catastrophes, at the level of the whole axon SCG10 encourages growth. SCG10 was discovered in a screen for transcripts induced by nerve growth factor (NGF), a potent stimulator of neurite outgrowth (Stein, Orit, et al., 1988). SCG10 enhances NGF stimulated neurite outgrowth (Gong et al., 2008) and SCG10 over-expression increases axon outgrowth *in vitro* (Riederer et al., 1997; Morii, Shiraishi-Yamaguchi, et al., 2006). Furthermore, SCG10 knockdown decreases axon outgrowth (Morii, Shiraishi-Yamaguchi, et al., 2006; Li et al., 2009). However, extreme SCG10 over-expression triggers neurite retraction (Morii, Shiraishi-Yamaguchi, et al., 2006). Thus, there is a target level of SCG10 induced microtubule catastrophes, above physiological baseline, that maximizes neurite outgrowth. Levels above or below this target compromise axon outgrowth.

How do microtubule catastrophes enhance axon outgrowth? Catastrophes may free tubulin for subsequent rounds of growth in dynamic regions of the axon. Also, alternating rounds of expansion and contraction may allow microtubules to efficiently explore intracellular space and find areas of the axonal membrane receiving growth promoting signals (Holy and Leibler, 1994).

SCG10 is targeted to growth cones (Stein, Mori, et al., 1988) and it may promote axon outgrowth by maintaining the growth cone cytoskeleton in a dynamic state. Within growth cones, SCG10 associates with dynamic tyrosinated

microtubules (Poulain and Sobel, 2007). In support of a direct role for SCG10 in promoting growth cone dynamism, growth cones become more dynamic with moderate SCG10 over-expression (Morii, Shiraishi-Yamaguchi, et al., 2006). Conversely, SCG10 knockdown results in expanded and relatively static growth cones (Morii, Shiraishi-Yamaguchi, et al., 2006; Poulain et al., 2007). Microtubule dynamics may feedback on SCG10 as pharmacological stabilization of growth cones causes SCG10 to redistribute to the axon shaft (Di Paolo, Lutjens, Osen-Sand, et al., 1997).

Along with promoting growth cone dynamics and axon outgrowth, SCG10 may help to guide axons through the nervous system to their targets. For instance, Ephrin, a generally repulsive axon guidance molecule, may steer axons by regulating SCG10. Ephrin triggers SCG10 loss from growth cones and increases growth cone pausing (Suh et al., 2004). Increased pausing may be a direct result of SCG10 loss: direct SCG10 disruption with function blocking antibodies results in similar growth cone pausing (Suh et al., 2004).

Axons are dynamic developmentally, during learning in the adult, and after injury. Studies of SCG10 expression suggest that it may play a role in each of the processes. During the critical period visual system development SCG10 is highly expressed in the lateral geniculate nucleus and V1. This expression is dependent on neuronal activity and it is enhanced by brain derived neurotrophic factor (BDNF) (Higo et al., 2000; Imamura et al., 2006). BDNF and neural activity are both crucial

for axonal plasticity during visual system development (Tropea et al., 2009) and they may exert their effects in part by regulating SCG10.

In the adult brain, axons are dynamic after LTP induction (Nikonenko et al., 2003). SCG10 levels increase presynaptically in the hippocampus three hours after stimuli that induce LTP (Peng et al., 2004). Increased SCG10 in axon terminals may promote LTP-triggered axon remodeling.

After injury, peripheral nervous system (PNS) axons regenerate considerable distances and central nervous system (CNS) axons sprout locally (Cafferty et al., 2008). SCG10 may promote axon growth in the injured PNS and CNS. After peripheral nerve transection, SCG10 expression increases and SCG10 accumulates at the proximal end of the cut site (Mason et al., 2002). Interestingly, the increase in SCG10 at the cut site precedes the increase in SCG10 transcription (Voria et al., 2006). This suggests that injury triggers SCG10 transport to the cut site or SC10 accumulates at the cut site because its baseline transport is interrupted. The increase in SCG10 expression is transient and SCG10 transcript levels return to baseline after regenerating axons reach their targets (Iwata et al., 2002; Mason et al., 2002). This pattern of expression is similar to that of genes known to promote axonal regeneration (Raivich and Makwana, 2007).

In the CNS, SCG10 expression increases after cortical stroke (Carmichael et al., 2005), an insult that induces CNS axon sprouting. SCG10 levels also increase after transection of olfactory receptor neuron axons (Camoletto et al., 2001; Pellier-Monnin et al., 2001), CNS axons with unique regenerative capacity. Furthermore,

there is a prolonged increase in SCG10 expression when CNS axons are induced to regenerate by placing peripheral nerve grafts into the brain (Mason et al., 2002).

Studies of SCG10 expression support a role for SCG10 in axon dynamics during developmental plasticity, adult learning, and axonal regeneration. However, loss-of-function studies are needed to determine if SCG10 is functionally important for any of these processes.

SCG10 is a potent inducer of microtubule catastrophes and its level, localization, and activity are likely tightly regulated. As described above, SCG10 transcription is regulated by trophic factors and growth promoting stimuli. SCG10 localization is regulated by palmitylation, and SCG10 activity is regulated phosphorylation and through interactions with small GTPases. Finally, as is described below, we found that SCG10 is highly sensitive to proteasomal degradation.

SCG10 is routed through the Golgi to vesicles that continue on to neurites and growth cones (Stein, Mori, et al., 1988; Di Paolo, Lutjens, Pellier, et al., 1997). In order to get to the Golgi, SCG10 requires an N-terminal domain. This domain is missing from cytosolic stathmin family members, and it is sufficient to target cytosolic proteins to the golgi membrane (Di Paolo, Lutjens, Pellier, et al., 1997). There are two palmitoylated cysteines in SCG10's N-terminal domain and both are required to target SCG10 to the Golgi (Lutjens et al., 2000). Non-palmitoylated SCG10 never gets to the growth cone, so this post-translational modification is presumably important for SCG10's neuronal function. It is not known if membrane

targeting is important for SCG10 function beyond its role in localization. However, one can speculate the SCG10 may be important for transducing signals at the growth cone plasma membrane into changes in microtubule dynamics.

Phosphorylation effectively counteracts SCG10's known function by decreasing its affinity for tubulin heterodimers (Antonsson et al., 1998). SCG10 is phosporylated on four serines (Antonsson et al., 1998). In cell free systems, SCG10 can be phosphorylated by extracellular regulated kinase (ERK), cyclin dependent kinase (Cdk), c-Jun N-terminal kinase (JNK), p38, and protein kinase A (PKA) (Antonsson et al., 1998; Neidhart et al., 2001). With the exception of JNK, described below, it is unknown if any of these kinases phosphorylates SCG10 *in vivo.* The stimuli that trigger SCG10 phosphorylation are also poorly understood, though it has been reported that kainite leads to NMDA-dependent SCG10 phosphorylation (Morii, Yamada, et al., 2006). NGF withdrawal also increases SCG10 phosphorylation (Neidhart et al., 2001).

JNK is the best characterized SCG10 regulator. JNK directly phosphorylates SCG10 *in vitro* and SCG10 isolated from JNK knockout mice is hypo-phosphorylated (Tararuk et al., 2006). Neuronal expression of mutant forms of SCG10 that cannot be phosphorylated by JNK leads to a decrease in neurite outgrowth. It also causes a decrease in tubulin fluorescent recovery after photobleaching (FRAP) (Tararuk et al., 2006). Expression of constitutively active non-phosphorylatable SCG10 may decrease microtubule dynamics and thereby decrease neurite outgrowth. Expression of dominant negative forms of JNK results in a similar shortening of

neurites and decrease in tubulin FRAP, consistent with the model that JNK phosphorylation of SCG10 promotes neurite outgrowth (Tararuk et al., 2006). In further support of this model, expression of phospho-mimetic SCG10 suppresses the effects of expressing the dominant negative JNK.

The finding that JNK inactivation of SCG10 promotes neurite outgrowth is at odds with the previous findings that SCG10 over-expression increases neurite outgrowth and SCG10 knockdown decreases neurite outgrowth. Does SCG10 enhance or suppress neurite outgrowth? The answer depends on SCG10 levels. Moderate wildtype SCG10 over-expression increases axon outgrowth, while more extreme SCG10 over-expression results in axon retraction (Morii, Shiraishi-Yamaguchi, et al., 2006). Over-expressing a form of SCG10 that JNK cannot inactivate may be similar to more extreme SCG10 over-expression. The same is true for direct JNK inactivation. Thus, JNK keeps SCG10 activity within a growth promoting level.

Heteromeric Gproteins and Rho family GTPases help to transduce signals at the plasma membrane into rearrangements of the cytoskeleton by directly regulating microtubules (Roychowdhury and Rasenick, 2008) and by regulating microtubule associated protein proteins such as stathmin family members (Witte and Bradke, 2008). Two regulators of Gprotein signaling, RGS6 and RGSZ1, antagonistically control SCG10. RGS6 binds SCG10, increases its microtubule destabilizing activity, and potentiates its ability to promote NGF-induced PC12 differentiation (Liu et al., 2002). RGSZ1 binds SCG10 and inhibits its ability to depolymerize microtubules (Nixon et al., 2002). The Rho GTPase Rnd1 binds SCG10

and promotes axon extension (Li et al., 2009). Rnd1's growth promoting affects require SCG10. Thus, kinases, heteromeric Gproteins, and Rho GTPases control neuronal microtubule dynamics by regulating SCG10.

SCG10 levels are also dynamically regulated. As mentioned above, SCG levels increased by NGF and decreased by Ephrin. Increases in direct microtubule stabilizers such as taxol and tau also decrease axonal SCG10 levels (Vega et al., 2006). This requires calpain proteases and may potentiate microtubule stabilization. Taxol is a chemotherapeutic agent that causes peripheral neuropathy (Lee and Swain, 2006) and increases in tau are associated with axonopathy in Alzheimer's disease and numerous other tauopathies (Brunden et al., 2009). SCG10 levels may also change in other disease states. A single nucleotide polymorphism directly upstream of SCG10 is associated with early onset sporadic Creutzfeldt-Jakob disease, a devastating prion disease (Mead et al., 2009). And, in and *in vitro* model of prion disease, exposure of cultured neurons to Rocky Mountain Laboratory scrapie induces an over ninety percent decrease in SCG10. Thus, SCG10 levels are regulated during normal development and are possibly affected by disease.

We hypothesized that JNK may promote axon degeneration by inactivating SCG10. We found that axotomy leads to a rapid degradation of SCG10. Within two hours of axotomy, there is a nearly ninety percent reduction in SCG10. Proteasome inhibitors block this degradation and preferentially preserve higher molecular weight SCG10. JNK inhibitors partially block SCG10 degradation and preferentially preserve lower molecular weight SCG10. Furthermore, the JNK phosphorylation

sites are required in SCG10 for rapid degradation after injury. Thus, direct phosphorylation of SCG10 by JNK may target axonal SCG10 for proteasomal degradation.

Surprisingly, we found that SCG10 is rapidly degraded and then replaced by axonal transport in healthy axons. In healthy axons, proteasome inhibition leads to a doubling of SCG10 within two hours, and axonal transport inhibitors cause a nearly ninety percent decrease in SCG10 within two hours. Thus, axonal SCG10 likely has a half-life of approximately one hour and is continuously replenished by axonal transport.

Baseline SCG10 turnover requires JNK, and the loss of SCG10 after axotomy may simply be a continuation of normal baseline SCG10 turnover. Continuous axonal transport and degradation of an axonal maintenance factor is a potential mechanism by which distal axons sense their healthy connection to the cell body.

Results

We used mouse dorsal root ganglion (DRG) cultures to test the hypothesis that JNK inactivates SCG10 after axonal injury. We axotomized DRG axons after nine days *in vitro* (DIV) and assessed SCG10 in the distal axon segments detached from their cell bodies. Three hours after axotomy, we prepared protein from distal axons segments and control axons. This is approximately six hours before axotomized nine DIV axons begin to degenerate. Using Western blotting, we found that SCG10 levels are dramatically decreased within three hours of axotomy in axons distal to the cut site (**Fig.1A**). We confirmed this using immunofluorescence staining and found that SCG10 is decreased to 13% ± 3% of its baseline level at this early post-axotomy time point ($p < 0.01$, ANOVA) (**Fig.1B**).

We found that rapid degradation of SCG10 requires the proteasome. Treatment with a proteasome inhibitor concurrently with axotomy largely blocked SCG10 degradation: SCG10 levels were significantly greater after axotomy and proteasome treatment compared to axotomy and vehicle treatment, maintaining 75% ± 11% of its baseline level ($p < 0.01$, ANOVA) (**Fig.1B**). Treatment with a JNK inhibitor partially blocked SCG10 degradation after axotomy: SCG10 level with JNK inhibitor treatment and axotomy was maintained at 47% ± 12% of its baseline level, greater than triple the level after axotomy with vehicle treatment ($p < 0.01$, ANOVA) (**Fig.1B**).

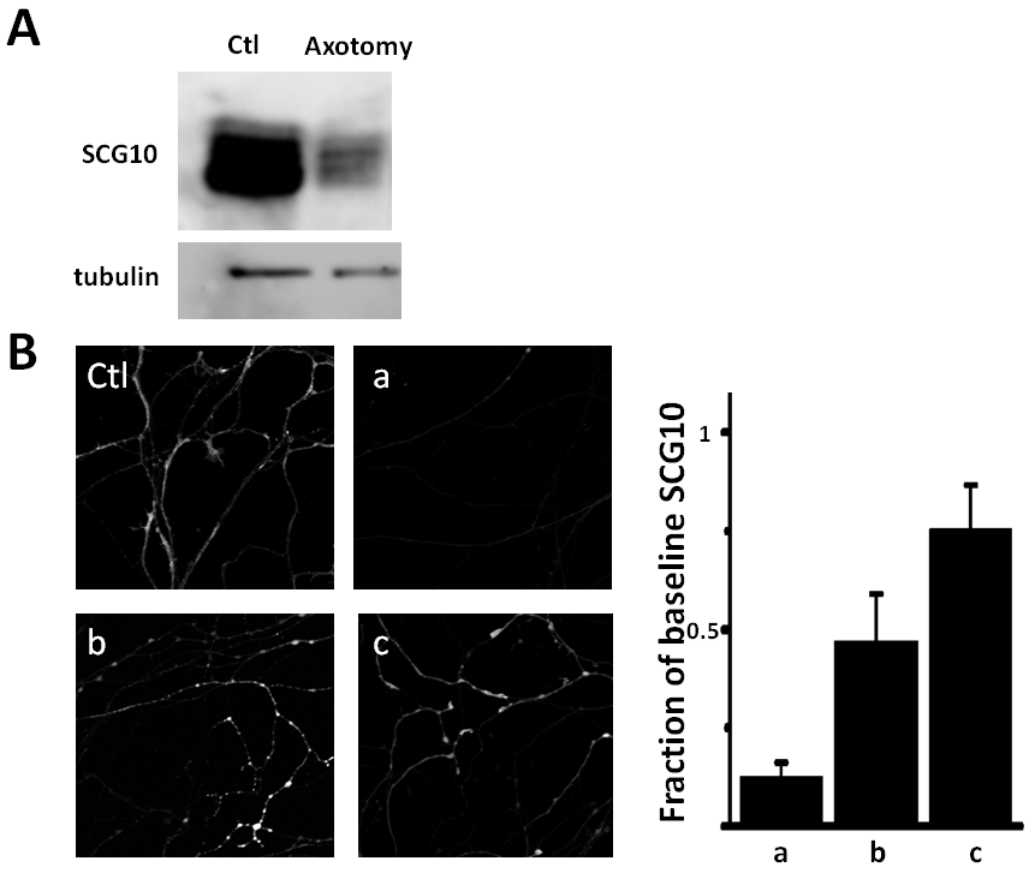

Figure One: Axotomy causes a rapid loss of axonal SCG10 distal to the cut. This is partially blocked by JNK inhibition and proteasome inhibition.

A. Western blot for SCG10 and tubulin from control DRG axons and axons distal to an axotomy three hours post-transection.

B. Immunofluorescent staining and quantification of SCG10 in control DRG axons and (**a**-**c**) axons distal to an axotomy three hours post-transection. (**a**) Vehicle treated. (**b**) JNK inhibitor (SP600125, 15 μM). (**c**) Proteasome inhibitor (MG132, 10 μM). Three hours post-axotomy, conditions **b** and **c** had significantly more SCG10 than condition **a** ($p < 0.01$, ANOVA).

Active JNK could directly phosphorylate SCG10 and target it to the proteasome for degradation. Alternatively, JNK could trigger other cellular processes after axotomy that expose SCG10 to the proteasome or increase proteasome activity. In support of direct JNK phosphorylation of SCG10, the proteasome inhibitor preferentially preserves a high molecular weight SCG10 band and the JNK inhibitor preferentially preserves a lower molecular weight SCG10 (**Fig. 2**).

To further explore the connection between JNK and SCG10 degradation, we used lentivirus to express EGFP-tagged SCG10 constructs in DRG neurons. We expressed wildtype SCG10 and SCG10-AA in which the serines phosphorylated by JNK were replaced with non-phosphorylatable alanines. We axotomized DRGs expressing SCG10-WT and DRGs expressing SCG10-AA. We found that SCG10-WT is degraded substantially more than SCG10-AA (**Fig. 3**). Thus, SCG10 phosphorylation by JNK, or another kinase utilizing JNK phosphorylation sites, is important for SCG10 degradation after axonal injury.

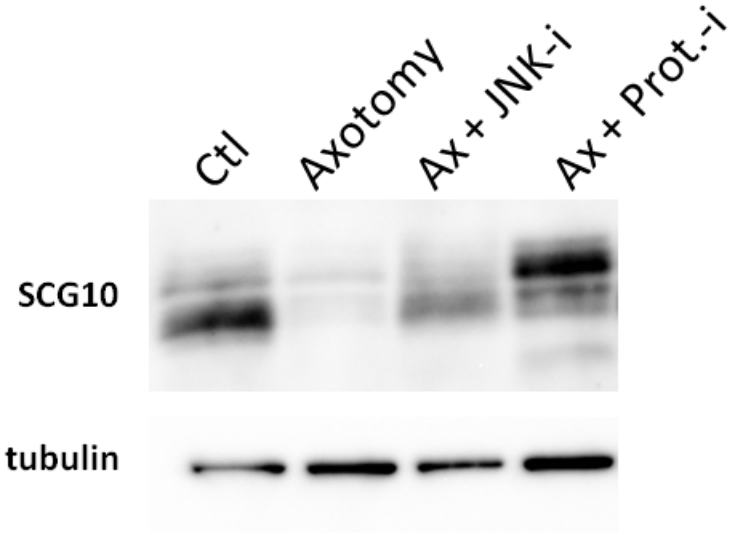

Figure Two: After axotomy, JNK inhibition preferentially preserves lower molecular weight SCG10 and proteasome inhibition preferentially preserves higher molecular weight SCG10.

Western blot for SCG10 and tubulin from control DRG axons and axons distal to an axotomy three hours post-transection in the presence of vehicle, JNK inhibitor (SP600125, 15 μM), or proteasome inhibitor (MG132, 10 μM).

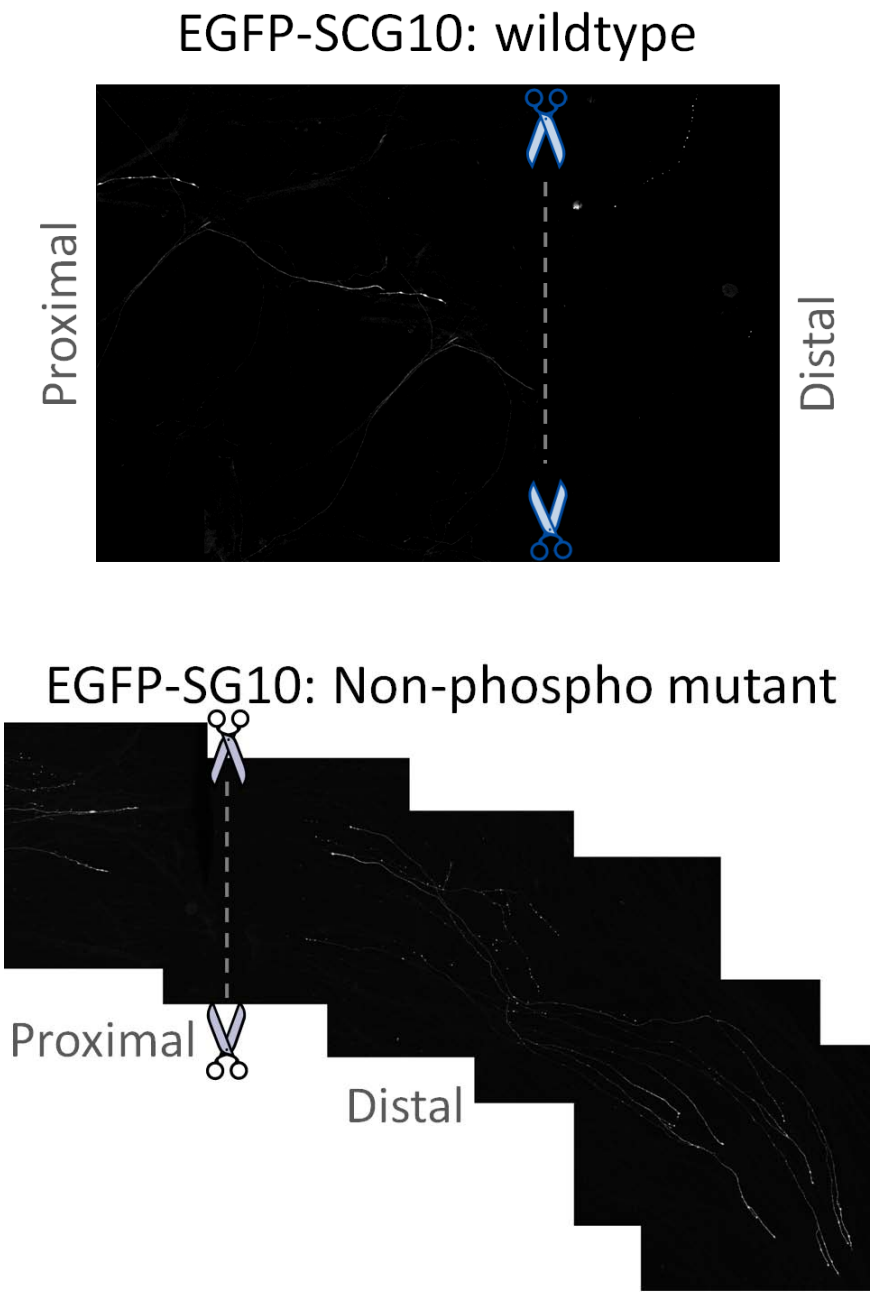

Figure Three: Mutations of JNK phosphorylation sites on SCG10 block SCG10 degradation after axotomy. DRG axons expressing either EGFP-SCG10-WT, or non-phosphorylatable EGFP-SCG10, three hours post-axotomy. A small fraction of neurons in each well express the SCG10 constructs resulting in sparse labeling.

Axotomy could trigger rapid SCG10 degradation. Alternatively, axonal SCG10 could be continuously degraded and replenished by axonal transport. Interruption of axonal transport by axotomy would prevent the resupply of SCG10 and SCG10 levels would drop. If axotomy simply reveals baseline SCG10 degradation, then treatment of healthy axons with a proteasome inhibitor should cause a spike in SCG10 levels. We treated healthy axons with a proteasome inhibitor and we found that SCG10 levels nearly double within three hours (180% ± 7% of control levels, $p < 0.01$, ANOVA) (**Fig. 4**). Thus, the proteasome likely continuously and rapidly degrades SCG10 in healthy axons.

If the same mechanism underlies baseline SCG10 degradation and SCG10 degradation after axotomy, then treatment of healthy axons with a JNK inhibitor should cause a spike in SCG10 levels. We treated healthy axons with a JNK inhibitor and we found that SCG10 levels are significantly increased within three hours (140% ± 20% of control levels, $p < 0.01$, ANOVA). Thus, the JNK likely promotes SCG10 degradation in both healthy and axotomized axons (**Fig. 4**).

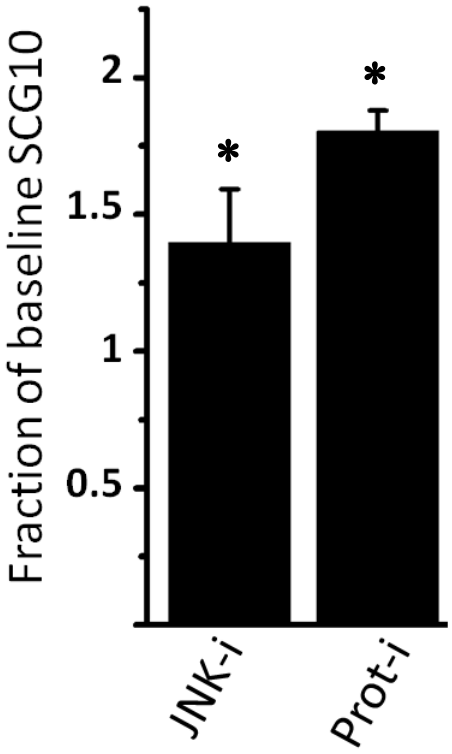

Figure Four: Inhibition of JNK and the proteasome causes a rapid increase in SCG10 levels in otherwise healthy axons.

Quantification of SCG10 levels in DRG axons treated for three hours with JNK inhibitor (SP600125, 15 μM) or proteasome inhibitor (MG132, 10 μM). SCG10 was measured by immunofluorescence staining, and values are normalized to SCG10 levels in vehicle treated axons. (*****) denotes a significant increase of axonal SCG10 relative to vehicle treated axons ($p < 0.01$)

We next determined the time course of SCG10 increase after proteasome inhibition and SCG10 decrease after interruption of axonal transport. If the decrease in SCG10 after injury reflects baseline SCG10 degradation, then these manipulations should increase and decrease SCG10 with similar kinetics as the decrease in SCG10 after injury. Alternatively, if axotomy triggers an increase in SCG10 degradation, then the rates should differ. We treated otherwise healthy axons with a proteasome inhibitor or vincristine, a microtubule destabilizer the blocks fast axonal transport (Park et al., 2008). We compared the change in SCG10 levels after these treatments with the change in SCG10 after axotomy (**Fig. 5**). We found that proteasome inhibition and axotomy cause complementary changes in SCG10 levels with very similar kinetics. By two hours, the proteasome causes a doubling of SCG10 and axotomy causes an 87% ± 2% decline. Blocking axonal transport with vincristine and axotomy cause a similar decline in SCG10 after two hours, with vincristine causing an 85% ± 1% decrease. However, compared to axotomy, vincristine has an approximately thirty minute delay before it causes a decrease in SCG10 levels. This could mean that axotomy accelerates SCG10 degradation. Alternatively, it could take vincristine thirty minutes to effectively block axonal transport. We favor the later interpretation because thirty minutes proteasome inhibition and thirty minutes of axotomy cause nearly equal and opposite changes in SCG10 levels. This further supports the model that loss of SCG10 after injury is a consequence of baseline SCG10 turnover.

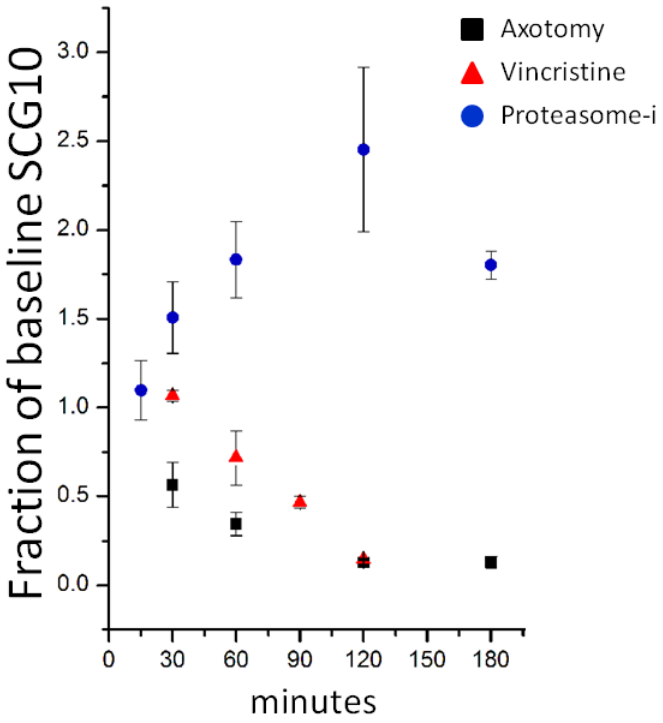

Figure Five: Timecourse of SCG10 increase in response to proteasome inhibition and SCG10 decrease in response to interruption of axonal transport by axotomy or vincristine.

SCG10 was measured by immunofluorescence staining, and values are normalized to SCG10 levels in vehicle treated axons.

Peripheral nerve transection leads to dramatically different outcomes proximal and distal to the cut site. Distal axons degenerate, and proximal axons often regenerate. However, in the first few hours after the cut, distal and proximal axons behave similarly. Within thirty minutes, both undergo a rapid calpain-dependent acute axonal degeneration (AAD) (Kerschensteiner et al., 2005), and the same signaling pathways are activated on both sides of the injury (Cavalli et al., 2005). This might be expected because the two sides of the cut receive an identical insult. After a number of hours, proximal and distal axons diverge, but this transition is poorly understood. Proximal to the cut, signals from the injury site are transported to the cell body and this leads to a transcriptional response that may orchestrate axonal regeneration (Raivich et al., 2007). However, injured axons begin to regenerate within six hours (Kerschensteiner et al., 2005), which is too rapid for retrograde transport of a signal to the cell body, gene expression, protein translation, and anterograde transport of pro-regeneration factors to the cut site. A simple model that accounts for the rapid divergence of responses proximal and distal to the cut is that an anterogradely transported molecule accumulates proximal to the cut and disappears distal to the cut. Interruption of directional transport could lead to divergent responses to an identical insult.

We axotomized DRG neurons and assessed SCG10 levels on both sides of the cut after three hours. SCG10 is rapidly lost distally, but it is preserved proximally (**Fig. 6**). This is consistent with the model that changes in SCG10 levels after injury result from the same processes that regulate SCG10 levels in uninjured axons.

Baseline SCG10 turnover and transport enables SCG10 to redistribute asymmetrically rapidly after an injury.

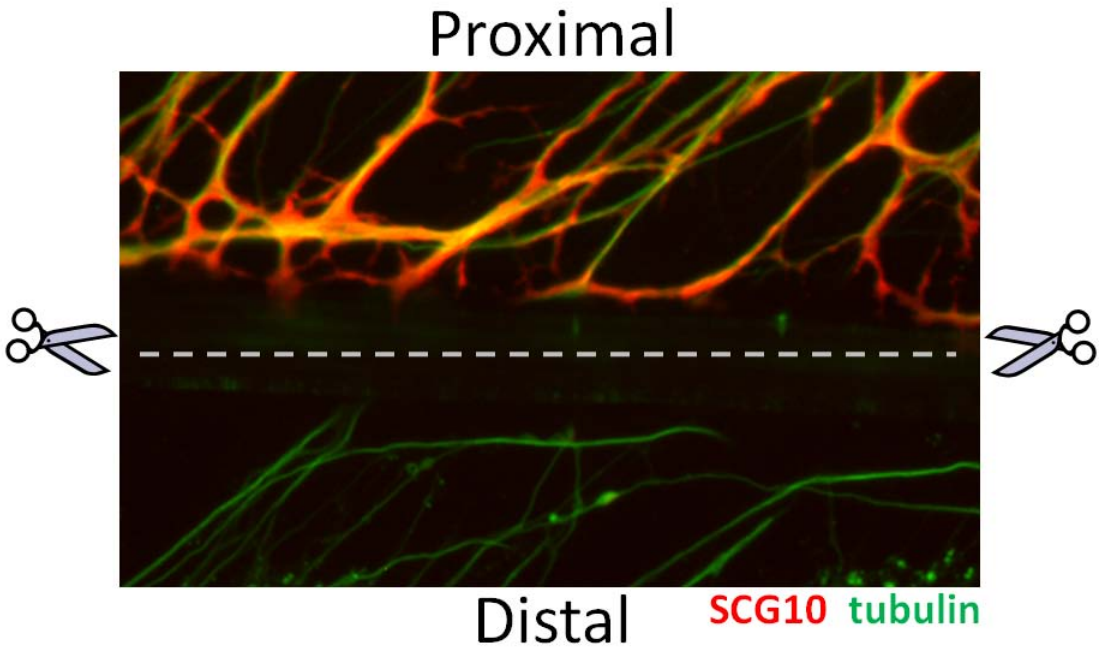

Figure Six: SCG10 is selectively lost distal to the injury site.

Immunofluorescence staining of SCG10 and tubulin in DRG axons three hours after axotomy.

Discussion

Axon degeneration is a major cause of disability and is a result of an internal axon self-destruction pathway. Inhibition of DLK and its downstream target JNK delays axon degeneration. We now show that SCG10, an axonal JNK substrate, is rapidly lost after axotomy and exposure to the chemotherapeutic agent vincristine. Within two hours, SCG10 levels decrease by nearly ninety percent. SCG10 degradation requires the proteasome, and JNK likely promotes this degradation by directly phosphorylating SCG10.

Surprisingly, injury does not trigger SCG10 degradation. Rather, SCG10 is rapidly degraded in healthy axons and replenished by axonal transport. Therefore, injuries that impede axonal transport automatically cause rapid SCG10 loss distal to the blockage. This same mechanism leads to SCG10 accumulation at the proximal end of the cut axons.

Axon degeneration distal to an axotomy is not a passive consequence of an interruption of supplies from the cell body. Expression of a single protein, Wlds, dramatically delays axon degeneration. This suggests that there is a Wlds-sensitive axon self-destruction program. Since Wlds blocks degeneration in response to a wide variety of insults, there may be a common program underlying many pathological conditions.

How does an axotomy activate the axon self-destruction program distal to the cut and allow it to remain repressed proximal to the cut? A repressor of the Wlds-sensitive axon self-destruction program may be continuously supplied by fast

anterograde axonal transport. Distal to a blockage, the repressor could be lost and the axon breakdown program could be disinhibited. Signs of axonal transport failure are found in numerous axonopathies. In particular, APP accumulates in axons in a wide range of disorders (Coleman, 2005). While it is unknown in many cases if these blockages are a cause or consequence of degeneration, they may be an important unifying event in Wlds-sensitive axon degeneration. However, we found that APP is not rapidly lost distal to an axotomy (data not shown).

For the repressor model to work without the intervention of signaling pathways activated on only one side of the injury, the repressor must be degraded at baseline. And its baseline degradation should be rapid enough to account for the quick divergence of events proximal and distal to the cut. SCG10 fits this profile. Furthermore, baseline axonal transport rapidly leads to SCG10 accumulation on the proximal end of an axotomy. Notably, increases in SCG10 promote axon extension. Thus, SCG10 is automatically lost in the axons segment destined to degenerate and automatically increased in the regenerating growth cone. As described in Chapter 6, manipulations to directly preserve SCG10 distal to a cut, or decrease SCG10 proximal to the cut, will reveal if SCG10 plays a causal role in axon degeneration and regeneration.

Materials & Methods

DRG Cultures: We prepared spot DRG cultures as described in Chapter Two and Chapter Three of this manuscript with the following exceptions. For confocal imaging, we cultured DRG neurons on plastic bottom chamber slides (Fisher). For all experiments, we cultured DRGs for seven to nine days before performing experiments.

Pharmacology: We used the JNK inhibitor SP600125 (Biomol) at 15 μM, the proteasome inhibitor MG132 (Calbiochem) at 10 μM, and vincristine at 0.05 μM for the indicated durations.

SCG10 Lentiviral Expression: Rat SCG10 tagged with EGFP in pEGFP-C1 was a gift from E. Coffey. SCG10's JNK phosphorylation sites, serine 62 and serine 73, were mutated to alanine using the QuikChange site-directed mutagenesis kit (Stratagene). Wildtype and mutant EGFP-tagged constructs were then expressed in DRG neurons using lentivirus as described in Chapter Two.

Immunofluorescent Staining and Quantification: We fixed DRG cultures in PBS with 4% paraformaldehyde and 4% sucrose for thirty minutes at room temperature. Fixed cultures were then permeablized and blocked in PBS with 10% normal goat serum and 0.1% Triton-X100 for thirty minutes at room temperature. They were then incubated overnight at 4° C in blocking solution containing the primary antibodies against SCG10 (polyclonal rabbit anti SCG10, 1:2,000, a gift from A. Sobel) and beta-tubulin (tuj1 mouse monoclonal antibody, 1:500, Chemicon). The

following day, cultures were washed three times with PBS for ten minutes at room temperature, incubated for one hour at room temperature with secondary antibodies in PBS (A488 conjugated goat anti mouse and Cy3 conjugated goat anti rabbit, both used at 1:500 and obtained from Jackson ImmunoResearch Laboratories), washed three times with PBS for ten minutes at room temperature, covered with Vectasheild mounting solution, and coverslipped.

Confocal images were obtained using standard procedures using a 20X oil objective. Three images were taken per well and an average SCG10 level was obtained for each well. To quantify SCG10 levels, we first subtracted background in the SCG10 channel by subtracting the average signal in areas containing no axons. We then measured the total SCG10 intensity and divided by axonal area as defined by tubulin staining. We tested for statistically significant differences between conditions using ANOVA and posthoc Tukey's test using Origen software.

Protein Preparation and Western Blotting: Protein was isolated from DRG axons by removing the medium, quickly rinsing with PBS, then adding 25 μL RIPA buffer containing protease inhibitors (CompleteMini, Roche) and phosphatase inhibitors (Phosphatase inhibitor cocktail 1 and 2, Sigma) to each well of a 24 well plate, incubating on ice for 30 minutes, and then removing and spinning down the lysate for 15 minutes at 4°. Supernatant was then combined with the appropriate volume of 5X Laemmli buffer and DTT (100 mM final concentration).

Samples were run on a 15% SDS-PAGE gel and transferred to a PVDF membrane. Membranes were blocked for 30 minutes at room temperature in PBS with

0.1% Tween and 5% nonfat milk, and then cut in half. The upper membrane was incubated with monoclonal mouse anti tubulin (E7, 1:200, Developmental Studies Hybridoma Bank) overnight at 4° and the lower membrane was incubated with rabbit anti SCG10 (1:2,000, gift from A. Sobel). Membranes were then were washed three times with PBS for ten minutes at room temperature, incubated for 1 hour at room temperature with the appropriate HRP conjugated secondary antibody (1:10,000, Jackson ImmunoResearch Laboratories) in PBS, washed three times with PBS for ten minutes at room temperature, developed using Immobilon Western HRP Substrate (Millipore) according to the manufacturer's instructions, and images were acquired using a ChemiDoc XRS System (Biorad).

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Chapter Five:

Loss of DLK Causes Axon Tract Defects and Axonopathy

Introduction

Dual leucine kinase (DLK) is a mitogen activated protein kinase kinase kinase (MAP3K) expressed in the mammalian nervous system (Hirai et al., 2005). As a MAP3K, it can lead to the activation of MAPKs via intermediary MAP2Ks. In invertebrates, loss of negative regulators of DLK (RPM-1 in *C. elegans* and Hiw in *Drosophila*) causes dramatic disruptions in synaptic development, and removal of DLK corrects the defects found in RPM-1 and Hiw mutants (Nakata et al., 2005; Collins et al., 2006). However, loss of DLK in a wildtype background has no effect on synaptic development. Therefore, DLK is not required for synaptic development even though it can disrupt synaptic development when over-expressed.

In mammals, disruption of Phr, the mouse homolog of RPM1 and Hiw, causes widespread defects in nervous system development (Bloom et al., 2007; Lewcock et al., 2007). However, this is not due to over-expressed DLK. We found no signs of increased DLK in Phr mutants, and loss of DLK does not suppress Phr mutant nervous system defects (Bloom et al., 2007).

What is DLK's role in the mammalian nervous system? As described in Chapter Two, DLK promotes axon self-destruction after nerve injury (Miller et al., 2009a). Other groups have found that DLK is also important for axon regeneration after injury (Hammarlund et al., 2009; Itoh et al., 2009). Thus, DLK is a central regulator of the axonal injury response.

What is DLK's function in the absence of over-expression or injury? In *Drosophila*, DLK mutants have axonal transport defects (Horiuchi et al., 2007). In mice, DLK mutants have subtle changes in neuronal migration (Hirai et al., 2006). Here we show that DLK is required for axon tract formation in mice. This is consistent with other reports of DLK function in mice (Hirai et al., 2006). We also show that DLK mutant axons have large axonal swellings and end bulbs throughout development an in adulthood. This may be due to defective axonal transport.

Results & Discussion

Anterior Commissure Defects

To determine the role of DLK in nervous system development, we generated constitutive DLK hypomorphs and conditional DLK knockouts as described in Chapter Two. Consistent with other reports (Hirai et al., 2006), we found that DLK mutant mice lack the commissural bundle and posterior branch of the anterior commissure (**Fig. 1**). Other CNS axon tracts, including the anterior branch of the anterior commissure, are intact, though somewhat reduced, in DLK mutant mice (Hirai et al., 2006). Thus, DLK is not universally required for axon outgrowth and guidance. Instead, it may play a more specific role guiding commissural axons. Interestingly, knocking out Rac-1, which requires DLK for some *in vitro* functions (Xu et al., 2001), results in a similar anterior commissure defect (Chen et al., 2007). Rac-1 operates downstream of Netrin-1, an important axon guidance molecule. Knocking out Netrin-1, and the Netrin receptor DCC, also causes anterior commissure defects (Serafini et al., 1996; Fazeli et al., 1997). DLK may operate in the pathway that transduces Netrin signaling at the growth cone plasma membrane into cytoskeletal reorganization.

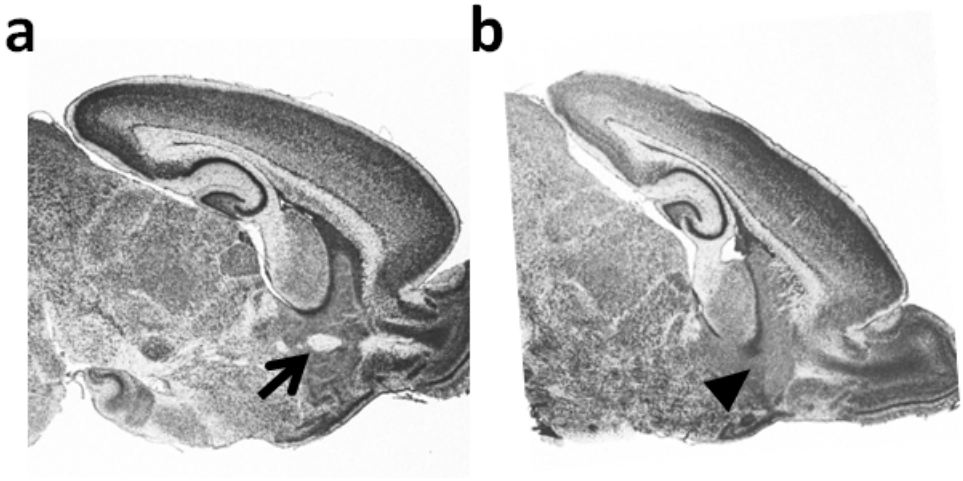

Figure One: DLK is required for anterior commissure development.

Nissl stained sagittal sections of embryonic day 18.5 brains from (**a**) wildtype and (**b**) DLK knockout. The arrow points to the wildtype anterior commissure and the arrowhead points to the location where the commissure should be in the DLK mutant.

Axonal Swellings and Endbulbs

We and others found axon tract hypoplasia in DLK knockout mice. We also found interesting defects at the level of single axons. Whole mounts of peripheral nerves taken from late embryo DLK mutants contain many morphologically normal axons. However, they also contain axons with extreme swellings and endbulbs (**Fig. 2**). These swellings are sometimes over ten times the diameter of normal axons and they accumulate anterogradely trafficked synaptic proteins. Axonal transport defects can cause protein accumulation and axonal swelling (Falzone et al., 2009). Based on the DLK swelling phenotype, and evidence that DLK is important for axonal transport in *Drosophila*, we hypothesized that DLK is required for normal axonal transport in mice. We address this issue below.

We used electron microscopy to get a closer look at the axonal swelling in DLK mutants. In longitudinal sections phrenic nerves from embryonic day 18.5 DLK mutant mice, we found many morphologically normal axons (**Fig 3a**). We also found large axonal swellings and end bulbs with accumulated vesicular organelles (**Fig 3b-d**).

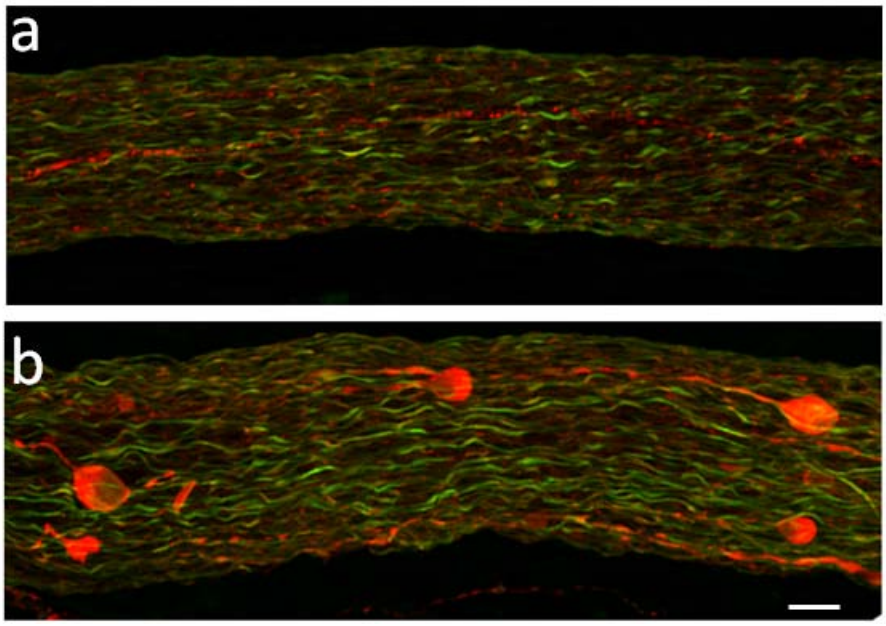

Figure Two: DLK mutant axons have swellings and endbulbs.

Whole mount phrenic nerves stained for neurofilament (green) and synaptophysin (red) from embryonic day 18.5 (**a**) wildtype and (**b**) DLK mutant. The DLK mutant has large axonal swellings and endbulbs containing synaptophysin. Scale bar represents 20 μm.

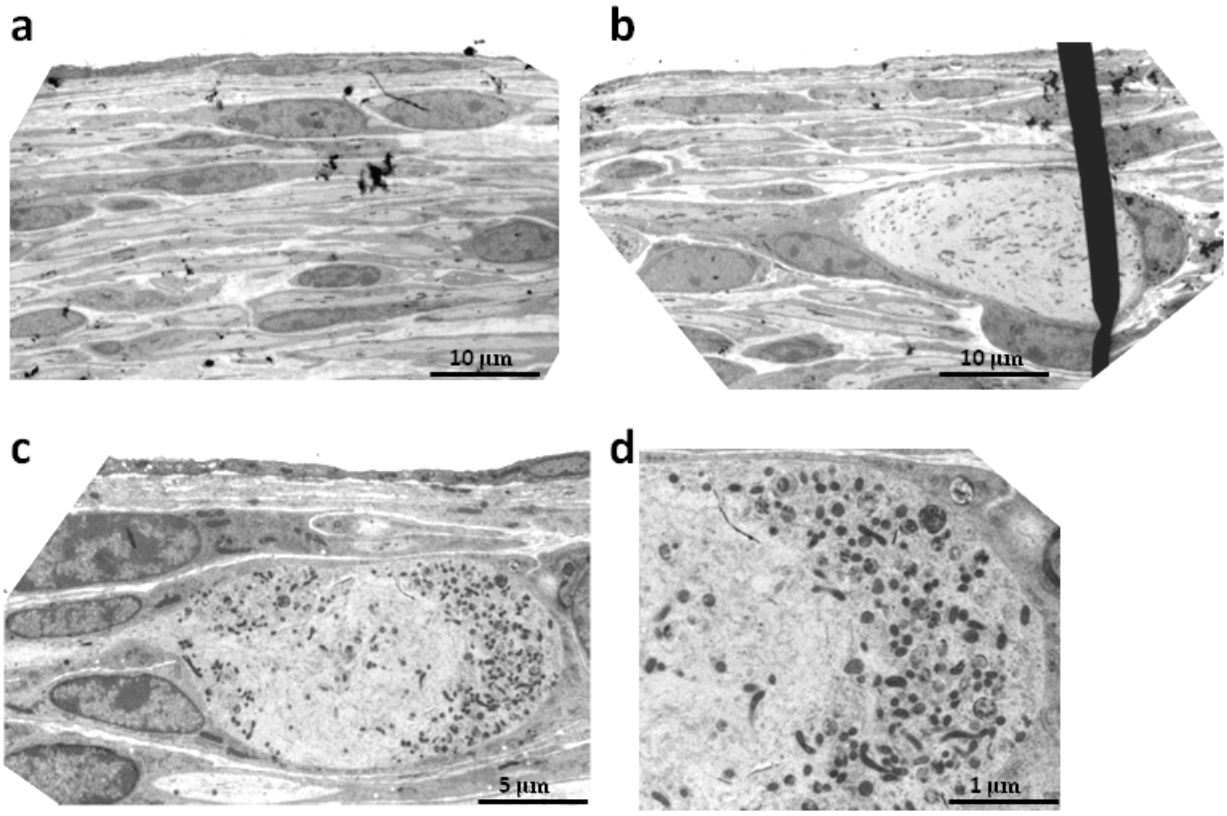

Figure Three: Electron microscopy reveals vesicular organelles accumulating in DLK mutant axon endbulbs.

Electron micrographs of longitudinal sections of the phrenic nerve from an embryonic day 18.5 DLK mutant mouse. (**a**) Examples of normal axons in the DLK mutant. (**b-d**) Examples of two DLK mutant axons with endbulbs at progressively higher magnification. **d** is an inset of **c**. The dark line in (**b**) is from the grid holding the tissue section.

DLK is expressed in axons of the mature nervous system and there could be a continual requirement for axonal DLK. Most DLK genetrap hypomorphs do not live past the perinatal stage, but a small fraction live to adulthood. We assessed axons in adult DLK genetrap hypomorphs that harbor the YFP2.2 allele and express YFP in a small subset of neurons. We found axonal swellings and endbulbs in peripheral nerves of adult DLK mutant mice (**Fig. 4)**.

DLK could be required in neurons or in supporting cells. To address this, we examined axons in adult conditional DLK mutants using Hb9-Cre to specifically knockout DLK in motor neurons. To exclusively visualize DLK knockout axons, we also crossed in an allele in which YFP is expressed only in Cre-expressing cells. We found end bulbs and swellings in DLK-deficient motor neuron axons (**Fig. 4c**). Thus, DLK is required in neurons.

Endbulbs and swellings were present in mice of all ages examined including mice over one year old. Since endbulbs are generally short-lived, these defects are probably evidence of a continuing axonopathy in DLK-deficient axons rather than holdover developmental defects. In future studies, DLK can be inducibly knocked out in adult mice to definitively address the issue of the role of DLK in axon maintenance.

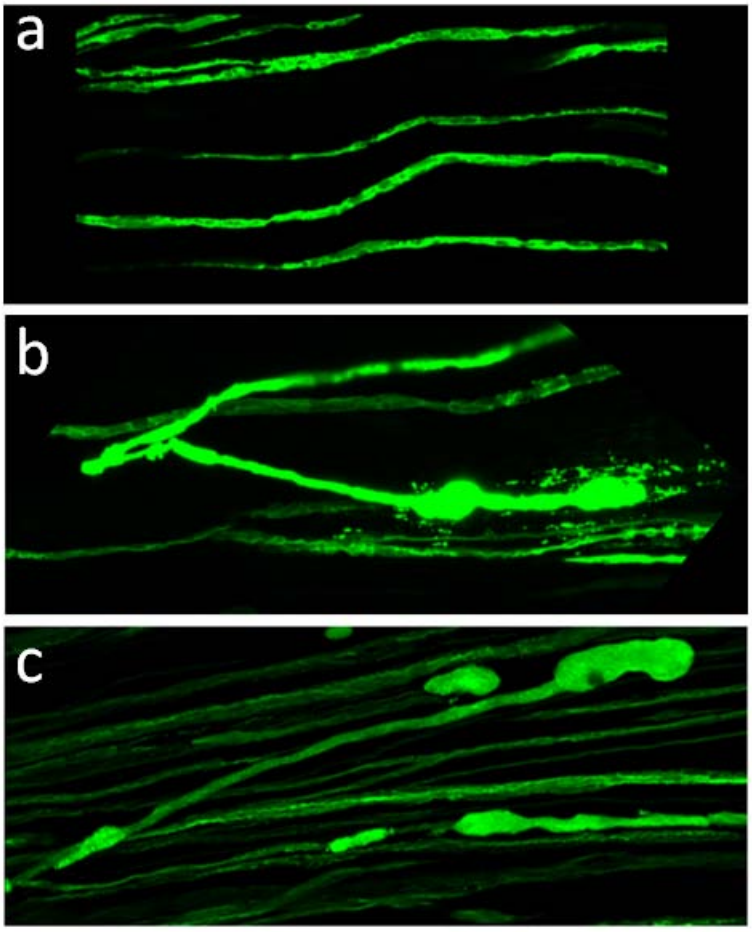

Figure Four: Swellings and endbulbs in adult DLK mutant mice.

(**a**) Whole mount of a wildtype phrenic nerve with axons sparsely labeled with YFP.

(**b**) DLK genetrap hypomorph phrenic nerve with axons sparsely labeled with YFP.

(**c**) Conditional DLK motor neuron knockout phrenic nerve. Motor neuron axons are labeled with YFP.

Axonal Transport

Why do DLK knockouts have axonal swellings and endbulbs? Axons often swell and fragment in response to interruptions of axonal transport. In Drosophila, the DLK homolog wallenda is required for axonal transport (Horiuchi et al., 2007). In mammals, MAP kinase pathways, which are downstream of DLK and other MAP3Ks, are involved in axonal transport (Inomata et al., 2003; Cavalli et al., 2005). It has not been determined which of the MAP3Ks are most relevant to axonal transport.

To determine if DLK is required for mammalian axonal transport, we used *in vivo* sciatic nerve ligation. The amyloid precursor protein (APP) is transported in peripheral axons mainly in the anterograde direction. Therefore, when the sciatic nerve is ligated, APP accumulates proximal to the ligation. Failure of APP accumulation reflects decreased axonal transport. We ligated sciatic nerves of adult DLK genetrap hypomorphs and littermate controls and we used immunofluorescent staining to visualize APP. APP levels were normalized to neurofilament levels to account for the possibility that there are substantially less axons in DLK mutants. We found a 28% ± 8% decrease in APP accumulation in DLK mutant mice compared to controls ($p < 0.05$, Student's t-test). Thus, DLK is required for normal anterograde transport in vivo in mice. DLK could play a direct role in axonal transport or axonal transport could be blocked secondary to swellings formed for some other reason. Approaches for addressing this issue are discussed in Chapter Six.

DLK helps axons respond to injuries and here we show it is also required to maintain healthy axon function. Loss of DLK results in large axonal swellings and endbulbs. These are evident in embryonic and mature DLK knockouts. This suggests that DLK is continually required to maintain axonal integrity. DLK's role may be to generally promote or regulate axonal transport. Interestingly, direct interference with the molecular motors that drive axonal transport activates DLK's downstream target JNK (Falzone et al., 2009). In healthy axons, activation of a DLK/JNK pathway at sites of moderate traffic jams may help clear the way for normal transport and prevent catastrophic transport failures and swellings.

Materials & Methods

Mice: The DLK hypomorph and conditional knockout are described in Chapter Two and (Bloom et al., 2007). Hb9-Cre and the cre-recombinase dependent YFP allele are described in (Bloom et al., 2007) To visualize individual axons as shown in **Fig. 3a,b**, we used the YFP2.2 line (Feng et al., 2000).

Histology: Preparation, Nissl staining, and imaging of brains sections was performed as described in (Bloom et al., 2007) Phrenic nerves and sciatic nerves were prepared for immunofluorescent (IF) staining as follows. They were fixed in PBS with 4% paraformaldehyde and 4% sucrose for thirty minutes at room temperature, permeablized and blocked in PBS with 10% normal goat serum and 0.1% Triton-X100 for thirty minutes at room temperature, incubated overnight at 4° C in blocking solution containing the primary antibodies, washed three times with PBS for ten minutes at room temperature, incubated for one hour at room temperature with secondary antibodies in PBS (A488 conjugated goat anti mouse and Cy3 conjugated goat anti rabbit, both used at 1:500 and obtained from Jackson ImmunoResearch Laboratories), washed three times with PBS for ten minutes at room temperature, covered with Vectasheild mounting solution, and coverslipped.

Primaries used were anti neurofilament (SMI312, 1:500), anti synaptophysin (Chemicon, 1:500), and anti APP (Chemicon, 1:500). Confocal images were obtained using standard procedures

Sciatic Nerve Ligation: Sciatic nerve ligations were carried out as described in Hanlon et al, 1997 and Cavalli et al, 2005. Briefly, adult DLK genetrap mice, and littermate controls, were be deeply anesthetized with isofluorane and a small incision will be made at midthigh level to expose the sciatic nerve. The sciatic nerve was ligated, approximately at the midpoint, with 6.0 nylon surgical thread. The incision was then closed with 9mm wound clips. After 6 hours, the animals were euthanized using carbon dioxide and the sciatic nerves were prepared for either IF as described above.

Electron Microscopy (EM): EM of phrenic nerves from embryonic day 18.5 DLK constitutive knockouts and littermate controls was performed as described in (Miller et al., 2009b).

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Chapter Six:

Conclusions and Future Directions

Conclusions

Axon loss is essential to the formation of neural circuits, but it is also a major cause of disability later in life (Luo and O'Leary, 2005). Mature axons are lost pathologically in a range of neurological disorders including mechanical injury, chemotherapy-induced neuropathy, hereditary neuropathies, glaucoma, diabetes, and neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. Pathological axon self-destruction appears to be an active process similar to developmental axon pruning, but the signaling pathways that orchestrate the degeneration of injured and diseased axons are poorly understood.

Candidate components of pathological axon degeneration pathways should be present in adult axons and activated by diverse cellular insults. One such candidate is dual leucine kinase (DLK), a mitogen-activated protein kinase kinase kinase (MAP3K). We tested the hypothesis that DLK promotes injury-induced axon degeneration using *in vivo Drosophila* olfactory receptor neuron axotomy, *in vitro* mouse dorsal root ganglion (DRG) axotomy, and *in vivo* mouse sciatic nerve transection. We found that DLK promotes axon fragmentation in all of these systems (Miller et al., 2009). Furthermore, we found that DLK is required in neurons themselves to promote axon fragmentation after injury. Thus, DLK functions in an internal neuronal pathway that promotes injury-induced axon self-destruction.

To determine if DLK promotes axon degeneration in response to multiple clinically relevant insults, we exposed DLK-deficient DRG axons to vincristine, a

chemotherapeutic drug whose dose-limiting side effects in patients include neuropathy. We found that DLK-deficient axons are significantly protected from vincristine-induced fragmentation compared to wildtype axons, suggesting that DLK operates in a general axon self-destruction program.

DLK is a MAP3K that can activate the MAPKs JNK and p38 via intermediary MAP2Ks. To determine whether either downstream kinase promotes injury-induced axon degeneration, we inhibited JNK and p38 in the mouse DRG axotomy model. Inhibition of JNK protects transected axons from fragmentation. Thus JNK, like DLK, acts within neurons to promote injury-induced axon self-destruction. Furthermore, we found that JNK activity is required during the early response to injury that commits the axon to breakdown hours later.

SCG10 is an axonal JNK target and we found that is rapidly lost after a variety of axonal insults. In healthy axons, we found that SCG10 is continuously degraded and then replaced by axonal transport. This baseline SCG10 turnover requires JNK and the proteasome. Following injury, axonal transport is blocked and SCG10 cannot be replenished distal to the injury. Continuous axonal transport and degradation of an axonal maintenance factor is a potential mechanism by which distal axons sense their healthy connection to the cell body.

DLK has wide ranging functions in axons. During development, DLK is required for axon outgrowth (Hirai et al., 2006; Eto et al., 2009), and we show that DLK is also required throughout life for axon maintenance. After injury, DLK is required for axon regeneration proximally (Yan et al., 2009; Itoh et al., 2009), and

we show that it is also required for axon degeneration distally. Future studies may reveal how this kinase performs many distinct tasks.

Future Directions

The Role of JNK in Axon Degeneration

We found that JNK inhibition delays axon degeneration. JNK is a kinase with over fifty substrates and many distinct functions (Bogoyevitch and Kobe, 2006). Which JNK targets are most important for axon degeneration? We found one good candidate in SCG10, but JNK likely regulates multiple targets. One approach figuring out what JNK does after injury is to test each JNK targets one by one for a role in axon degeneration. This is complicated by the fact JNK activates some targets and inhibits others. For targets JNK activates, one could knockdown the target and determine if that is protective. For targets JNK inactivates, one could knockdown the target and determine if this results in axon degeneration in the absence of injury, or over-express them and test for protection. Another approach is to attempt to determine which proteins undergo JNK-dependent phosphorylation after axonal injury. These could then be tested for a role in axon degeneration. This approach is technically difficult and it is complicated by the fact that JNK regulates many kinases and phosphatases.

A less substrate focused approach to figuring out what JNK does after injury is to assess which early post-injury changes are JNK-sensitive. JNK is important shortly after injury, and if JNK inhibition blocks some injury responses and not others, that could begin to reveal JNK's function.

The Role of SCG10 in Axon Degeneration and Regeneration

We found that axon transection results in a rapid loss of SCG10 distal to the injury and an accumulation of SCG10 proximal to the injury. One can speculate that loss of SCG10 promotes distal axon degeneration, and that accumulation of SCG10 promotes proximal axon regeneration. The role of SCG10 in regeneration could be approached by knocking down SCG10 in neurons and determining if this decreases their ability to regenerate axons after injury. To determine if loss of SCG10 directly contributes to axon degeneration, it would be great to determine if preserving functional SCG10 distal to an injury delays axon degeneration. This may be achievable by expressing non-phosphorylatable forms of SCG10.

DLK and Glia

JNK's downstream target c-Jun is important for Schwann cell development and the Schwann cell response to nerve injury. c-Jun inhibits Schwann cell maturation and myelination. Mature Schwann cells revert to an immature state in response to injury, and in demyelinating neuropathies, and c-Jun may be important for these processes (Parkinson et al., 2008). Thus, blocking c-Jun activation may be a good approach to stop Schwann cell reversion and demyelination. As a JNK activator expressed in glia, DLK is may be involved in the activation of c-Jun and the resulting Schwann cell reversion. This can be tested using conditional DLK knockout mice and with a glial cre-recombinase driver.

DLK and Axonal Transport

We found that DLK knockouts have axon swellings and decreased anterograde axonal transport *in vivo* when measured in bulk using sciatic nerve ligation. It is important to know if axonal transport is a primary defect, or a defect secondary to axonal swellings and endbulbs. A good starting point to address this is assessing axonal transport using live imaging in DLK-deficient axons *in vitro*. If transport is slow simply because transported goods get stuck in focally disrupted areas of the axons, then live imaging of axonal transport may be normal in morphologically normal segments of DLK-deficient axons. If DLK is directly involved in axonal transport, then DLK knockout axons may have slow transport throughout. Such a finding could lead to future studies of how mammalian DLK interacts with the axonal transport machinery.

The Role of DLK in Axon Guidance

Very little is known about how DLK is regulated *in vivo*. DLK's axon guidance defect suggests a starting point for addressing this. An anterior commissure defect is shared by knockouts of the axon guidance molecule Netrin, its receptor DCC, Rac-1, and DLK (Serafini et al., 1996; Fazeli et al., 1997; Chen et al., 2007; Hirai et al., 2006). Rac-1 is activated by DCC and *in vitro* studies suggest that Rac-1 can operate upstream of DLK (Xu et al., 2001). Thus, DLK may work in a Netrin signaling pathway that guides axons. To test this, DLK-deficient axons could be tested for responsiveness to Netrin.