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Characterization Of The Role For NMNAT, Kinase Cascades, And Purines In Axonal Degeneration

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CHARACTERIZATION OF THE ROLE FOR NMNAT, KINASE CASCADES, AND PURINES IN AXONAL DEGENERATION

by

Craig Adam Press

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ABSTRACT OF THE DISSERTATION

Characterization of the role for Nmnat, kinase cascades, and purines in axonal degeneration

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Axonal degeneration is a prominent feature of many neurological disorders including Parkinson's disease (PD), motor neuron disease, inherited, diabetic, and drug-induced peripheral neuropathies. It is now thought that axonal degeneration is an active process, due in large part to studies of the *Wld*^{s} mutant mouse, which undergoes delayed Wallerian degeneration in response to axonal injury. *Wld*^{s} mice have slower disease progression in numerous models of neurodegenerative diseases. The *Wld*^{s} mutation results in the production of a chimeric protein that containing nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1). Increased expression of Nmnat isoforms are sufficient for axonal protection *in vitro* and *in vivo*. We attacked axonal degeneration from three directions. First, a common finding in neurodegenerative disorders with axonal degeneration is mitochondrial dysfunction. We found that mitochondrial inhibition, via rotenone, induced profound axonal degeneration in dorsal root ganglia neurons; however, this degeneration was delayed by expression of Nmnat. Nmnat decreased axonal accumulation and sensitivity to reactive oxygen species, but did

not affect the rate of ATP loss. Second, it has also been demonstrated that inhibition of the mixed lineage kinases (MLKs) can inhibit not only neuronal death, but loss of axonal terminals in models of neurological disorders, including PD. We found that the loss of dual leucine zipper kinase (DLK) or inhibition of its downstream target, JNK, decreases the rate of axonal degeneration in vitro. Finally, purine nucleosides are known to have trophic effects on neurons and can stimulate axonal growth and regeneration. Guanosine has been used *in vivo* to decrease injury in both models of stroke and spinal cord injury. We found that both adenosine and guanosine robustly slow axonal degeneration *in vitro* while inosine does not. Adenosine is protective when added previous to, immediately following, or up to 6 hr after the injury suggesting that it has a local mechanism of action on components of axonal degeneration likely downstream of JNK activation. The protection lasts several days, but is halted by removal of adenosine demonstrating its necessity during protection. This armamentarium of axonal degeneration inhibitors will provide new avenues for understanding disease and therapy development.

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CHAPTER 1

Introduction: Background and Significance

Neurodegenerative disorders and axonal degeneration

Neurodegenerative disorders and axonal degeneration

Axonal degeneration is theorized to be a causative factor in many neurodegenerative diseases. Diseases such as Parkinson's Disease (PD), Alzheimer's (AD), Amyotrophic Lateral Sclerosis(ALS), Charot-Marie Tooth (CMT), Multiple Sclerosis (MS), mechanical nerve injury, diabetic neuropathy, and drug induced neuropathies all have a pathology that includes degeneration of the axon (Coleman and Perry, 2002). These diseases affect millions of people a year and place a heavy burden on both individuals afflicted with the illness and upon society. PD, the second most common neurodegenerative disorder, affects as many as one million people in the United States and approximately 40,000 people are diagnosed with PD yearly in the U.S. (Weintraub et al., 2008b). The incidence of PD increases with aging and will only continue to rise as the number of individuals over the age of 65 increases. Annually the cost of PD is upwards of $25 billion in the US alone (Foundation, 2006). Alzheimer's disease, the most common neurodegenerative disorder, affects more than 4.5 million Americans, a number which has doubled since 1980 (Hebert et al., 2003). It is imperative for the research and medical system to discover new treatments and better management strategies to support patients with neurodegenerative diseases. One aspect of research and development seeks to target the axonal degeneration common to many neurological disorders. Unfortunately, the mechanism that underlies the process of axonal degeneration is not well understood.

Parkinson's Disease

Parkinson's disease is characterized by bradykinesia (slow movement), rigidity, resting tremor, and postural instability (Weintraub et al., 2008b). These symptoms take a heavy toll on patients, their family, friends and caregivers. There are treatments available, such as L-DOPA, for example, that decrease the severity of the disease in the initial stages; however, in advanced disease, these treatments are ineffective and have significant side-effects including dyskinesias (Weintraub et al., 2008a). In patients that are resistant to medication, a subset can be treated with deep brain stimulation (DBS). DBS involves the insertion of electrodes into the subthalamic nucleus and relieves the severity of the motor symptoms of PD. None of the available treatments slow the progression of disease.

The first signs of degeneration in PD appear in the synapses and their supporting axons and dendrites. Protein accumulations, known as Lewy bodies, are characteristic of PD and present in neurites early in the disease (Conforti et al., 2007). The hallmark of PD is degeneration of the neurons located in the substantia nigra. These neurons extend axons to the striatum where the neurotransmitter dopamine is released. In pathological studies the decrease in striatal dopamine is more striking than that in the substantia nigra, suggesting that axonal loss is more rapid than cell loss (Hornykiewicz, 1966). An animal model of PD also demonstrates early loss of axonal terminals preceding neuronal cell loss (Betarbet et al., 2000). These data suggest that axonal degeneration is a component of PD and may be the initial site of injury. Much of the previous work on axonal injury and neurodegeneration focused on PD and models of PD. This literature provides a framework to discuss axonal degeneration.

Description of what is known about axonal degeneration

Neurodegenerative disease is commonly associated with neuronal cell death, but it is increasingly apparent that axonopathy is also a major component of many neurodegenerative diseases including ALS, PD, and AD (Coleman, 2005). One model of axonal degeneration derives from studies of anoxic axonal injury, which leads to mitochondrial failure, decreased levels of ATP, reduced Na^+/K^+ ATPase activity, and increased axonal Na^+ and Ca^{2+} (Stys et al., 1992). These changes are followed by calpain activation and eventual axonal protein degradation (Fig. 1) (Coleman, 2005). While this is the general mechanism of axonal degeneration following anoxic injury, it is not at all clear that the same events necessarily occur after other variations of axonal injury or during other neurodegenerative disease conditions. The field would greatly benefit from a detailed study of the events after axonal severing in culture. With new techniques including time lapse imaging of axons that can be maintained in culture over hours to days these important questions can begin to be addressed in a comprehensive fashion.

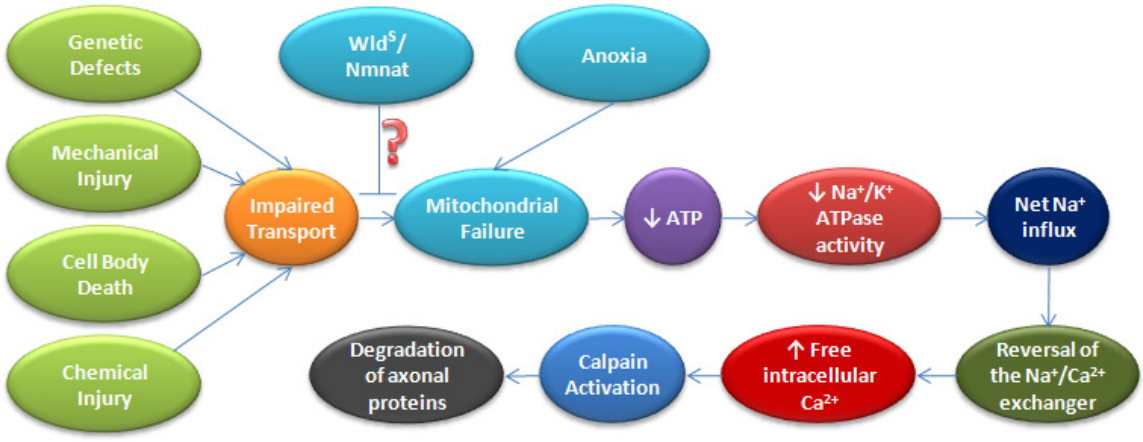

Figure 1. A schematic of the pathway leading to axonal degeneration. Adapted from Coleman Nat. Rev. Neuroscience 2005.

Wld^s mouse

It was not until recently that axonal degeneration was, like apoptosis, understood to be an active process (Raff et al., 2002). The key discovery was a spontaneous mutant mouse, *Wld*^{s}, that exhibited slow Wallerian degeneration (Lunn et al., 1989). Wallerian degeneration is the process of axonal deterioration that occurs after the transection of a nerve. It was quickly realized that halting Wallerian degeneration could be a mechanism to protect against other forms of axonal degeneration. Axonal degeneration is delayed by the *wld*^{s} mutation in a wide range of disease models, such as the *pmn* mice, a model of motor neuron degeneration, vincristine treatment, a model of chemotherapy induced neuropathy, and models of PD (Wang et al., 2001b; Ferri et al., 2003; Sajadi et al., 2004; Hasbani and O'Malley K, 2006), suggesting that a common 'degenerative' pathway is activated by these insults that can be inhibited by this mutation. It is important to note that the axonal degeneration pathway has been demonstrated, repeatedly, to be separate from cellular apoptosis. *Wld*^{s} does not inhibit cell body apoptosis after trophic factor withdrawal and halting apoptotic mechanisms does not prevent Wallerian degeneration directly (Deckwerth and Johnson, 1994; Sagot et al., 1995; Burne et al., 1996; Finn et al., 2000; Whitmore et al., 2003).

Role of Nmnat in Wlds protection

The *Wld*^{s} mutant mouse harbors a triplication of a gene fusion that produces a chimeric protein composed of the N-terminal 70 amino acids of ubiquitination factor 4b, a unique 18 residue linker region and the full length nicotinamide mononucleotide

adenylyltransferase 1 (Nmnat1) protein (Conforti et al., 2000). Using dorsal root ganglion (DRG) neuronal cultures, we previously demonstrated that the Nmnat1 portion of the Wld^S protein is the moiety responsible for axonal protection (Araki et al., 2004). Additionally, it was demonstrated that the enzymatic product of Nmnat1, nicotinamide adenine dinucleotide (NAD^+), could slow degeneration in culture, but to a lesser extent. Many studies in both rodents and *Drosophila* have confirmed the role for Nmnat in axonal protection, but not axonal pruning, a normal elimination of axons during development (Hoopfer et al., 2006; Macdonald et al., 2006; Zhai et al., 2006).

The original analysis of the *Wld*^{S} gene product described expression predominately in the nucleus *in vivo* (Mack et al., 2001). This led to the hypothesis that Nmnat1 functioned through an indirect mechanism to protect axons since, after transection, nuclear-derived changes in protein would not be available to the severed axons. This early hypothesis was further bolstered by the connection to Sirt1; the activity of Sirt1 activity was necessary for NAD^+ to be protective (Araki et al., 2004). Contrary to this, our lab has demonstrated that overexpression of Nmnat1 with a mutated nuclear localization signal is at least as potent as wildtype Nmnat1 (Sasaki et al., 2006). Two additional isoforms of Nmnat1 were found, Nmnat2 and Nmnat3 (Raffaelli et al., 2002; Zhang et al., 2003; Yalowitz et al., 2004). Nmnat3 was found to be active and localized to the mitochondria (Berger et al., 2005). We went on to demonstrate that Nmnat3 was also effective at protecting axons from post-transection degeneration. As a further test that the subcellular localization was not a limiting factor, we created a form of Nmnat3 that localized to the nucleus and found that it was effective at delaying degeneration after axotomy (Sasaki et al., 2006).

There has been some controversy in the literature about the role of Nmnat1 in the *Wld*^{s} mouse (Wang et al., 2005; Conforti et al., 2006). Attempts to genetically engineer transgenic mice that express high levels of Nmnat1 in dorsal root ganglia have been largely unsuccessful in our lab. These mice were reportedly sensitive to axonal degeneration due to sciatic nerve lesion (Conforti et al., 2006; Yahata, 2007). However, it was recently reported that transgenic expression of Nmnat3 does provide axonal protection *in vivo* and that mutation of the enzymatic site of Nmnat in the *Wld*^{s} protein destroys its ability to protect *in vivo* (Yahata, 2007). We believe that these data showing protection by multiple isoforms of Nmnat, independent verification in additional species and the new transgenic mice are ample evidence supporting Nmnat as a protective factor.

It has been suggested that the mechanism of protection for Nmnat or *Wld*^{s} is due to their ability to maintain the ATP and NAD^{+} concentration in degenerating axons (Ikegami and Koike, 2003; Wang et al., 2005). Interestingly, in healthy mice expressing the *Wld*^{s} mutation, increased levels of NAD^{+} were not detected (Mack et al., 2001). In unpublished data, we have also been unable to detect increased levels of NAD^{+} in cultured neurons expressing increased levels of Nmnat. These studies have shown that Nmnat does not drastically alter the level of NAD^{+} or ATP at the baseline. Studies show that the levels of ATP and NAD^{+} drop as axons degenerate after injury (Ikegami and Koike, 2003; Wang et al., 2005). These studies do nothing to address the primary insult; it is no surprise that degenerated neurites have less ATP than those that are not degenerating. To understand the role of Nmnat overexpression and its ability to halt degeneration in such a dramatic fashion necessitates a rigorous, systematic approach to find the metabolic, signaling or enzymatic pathways that Nmnat alters.

Mitochondrial dysfunction in neurodegenerative disorders

Mitochondrial dysfunction caused by mutations or toxic agents has been implicated in many neurodegenerative diseases, including those associated with axonal degeneration (Lin and Beal, 2006). It results in a host of cellular abnormalities including decreased ATP synthesis, disrupted mitochondrial fusion and transport, and the increased generation of reactive oxygen species (ROS) (Lin and Beal, 2006; Baloh et al., 2007). The association of mitochondrial dysfunction with neurodegenerative diseases is perhaps strongest in Parkinson's Disease (PD), which has been associated with mutations in mitochondria-associated proteins (e.g. DJ-1, PINK-1) (Martin, 2006) and ingestion of 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), an inhibitor of mitochondrial Complex I (Langston et al., 1983; Betarbet et al., 2000).

It is generally accepted that environmental factors play an important role in the pathogenesis of PD (Sherer et al., 2002a); in particular, there is a strong association between PD and exposure to pesticides, including many which inhibit mitochondrial Complex I (Semchuk et al., 1992; Gorell et al., 1998; Priyadarshi et al., 2000; Priyadarshi et al., 2001; Gorell et al., 2004; Ascherio et al., 2006; Brown et al., 2006). Further studies have shown that the pesticide rotenone leads to dopaminergic neuron loss and PD-like symptoms in animals (Betarbet et al., 2000). The rotenone model shows a similar pathology to human PD where striatal terminals (axons) are lost prior to detectable neuronal cell death (Hornykiewicz, 1966; Betarbet et al., 2000). The role for axonal degeneration was further supported when it was shown that the *Wld*^{s} mouse had decreased

loss of striatal terminals, but not cell bodies, in two acute models of PD (MPTP and 6-OHDA) (Sajadi et al., 2004; Hasbani and O'Malley K, 2006).

This crucial role for mitochondrial dysfunction in neurodegenerative diseases led us to explore its role in axonal degeneration in a culture model of PD. In Chapter 2 we present our results showing that rotenone induces axonal degeneration in culture which is slowed by Nmnat expression. This protection was related to decreased accumulation of ROS rather than maintenance of ATP. Furthermore, we found that Nmnat could prevent axonal degeneration from exogenous oxidants placing Nmnat downstream of ROS initiated axonal degeneration.

Known role of mixed linage kinases in neurodegenerative diseases

An interesting story suggesting a role for mixed lineage kinases in axonal degeneration began in 1998 when a new drug, CEP-1347, was found to inhibit the activation of c-Jun N-terminal Kinase (JNK) and inhibit motor neuron apoptosis *in vitro* (Maroney et al., 1998). Shortly thereafter, it was found that the mechanism by which CEP-1347 functioned involved inhibition of the activity of a family of MAPKKK's known as the mixed lineage kinases (MLK): MLK1-3, dual leucine zipper kinase (DLK), and leucine zipper kinase (LZK) (Maroney et al., 2001). The ability for CEP-1347 to inhibit apoptosis was studied in the well established model of trophic factor withdrawal from superior cervical ganglia (SCGs). CEP-1347 inhibited both cytochrome c release from mitochondria and the neuron's "competence to die" in response to cytochrome c release while simultaneously maintaining metabolism and protein synthesis. CEP1347

thus maintained "not only survival but the 'quality of life' of neurons" (Harris et al., 2002).

Due to its neuronal protective effect, CEP-1347 and an additional MLK inhibitor (CEP -11004) were tested in two models of PD, 6-hydroxydopamine (6-OHDA) and MPTP. In both of these paradigms, axonal degeneration occurs to a significant degree in the striatum but the inhibition of MLKs protected both striatal terminals and neurons in the substantia nigra (Saporito et al., 1999; Ganguly et al., 2004). Using another model of PD *in vitro* where cultures of midbrain neurons were treated with methamphetamine, CEP-1347 inhibited both neurite loss and cell death even when added shortly after treatment with methamphetamine was initiated (Lotharius et al., 2005). As was the case with rotenone treatment of DRGs, this neurite loss preceded cell death and was associated with ROS damage, which was reduced by CEP-1347. In an effort to determine if inhibition of the MLK pathway with genetic means could inhibit neurodegeneration *in vivo*, adeno-associated virus carrying a dominant negative DLK (dnDLK) was injected into the striatum of mice that were also treated with 6-OHDA. The mice demonstrated reduced neuronal cell death, although there was no protection of the striatal terminals (Chen et al., 2008).

In chapter 3 the role for MLKs in axonal degeneration, in particular DLK, is explored. In the first report of a loss of function mutation resulting in delayed axonal degeneration, we show that flies lacking DLK have slowed Wallerian degeneration. We go on to show *in vitro* that mouse DRGs lacking DLK exhibit slowed axonal degeneration and this effect can be recapitulated by JNK inhibitors. *In vivo*, DLK mutant mice also have delayed axonal degeneration after sciatic nerve transection. These results

suggest that MLKs and JNK activation play a role in regulating axonal degeneration and present a new therapeutic target for neurodegenerative disorders featuring axonal degeneration.

Known roles for purines and nucleosides in neurodegeneration and cell death

During hypoxic injuries such as occurs during a stroke, it is known that adenosine is released and accumulates in the brain as ATP is consumed without restoration by oxidative phosphorylation (Fredholm et al., 2001). Purines have been demonstrated to have neuroprotective effects in cultures models of hypoxia and to stimulate axonal outgrowth (Bocklinger et al., 2004). For example, cerebellar granular neurons treated with rotenone to induce both cell death and reduce axonal outgrowth were protected by the addition of adenosine, guanosine or inosine to the cultures with reductions in the number of neurons lost and an increase in the number of neurite bearing cells. Further, the authors commented that in unpublished work they have data supporting a nucleoside transport mediated mechanism for this effect. Later work attempted to link this protection to both Hif1-α and the MAPK 42/44 pathways although it was not clear if these results were a result of increased sensitivity to hypoxia by neurons lacking Hif1-α or MAPK24/44 (Tomaselli et al., 2008; zur Nedden et al., 2008). Guanosine was also shown to reduce apoptosis during treatment of a neuroblastoma cell line with the mitochondrial toxin, MPP^+ (Pettifer et al., 2007). Guanosine was protective when added either before, with or after treatment with MPP^+, effects which appeared to be mediated

via the PI-3K signaling pathway. These previous experiments stimulated us to explore the role of purines and nucleosides in axonal protection. Data showing that adenosine and guanosine, but not inosine play a protective role in axonal degeneration are presented here in Chapter 4. These small molecules were effective after injury perhaps indicating therapeutic potential.

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CHAPTER 2

Nmnat delays axonal degeneration caused by mitochondrial and oxidative stress

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Nmnat delays axonal degeneration caused by mitochondrial and oxidative stress

Abbreviated title: Nmnat prevents oxidative damage

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Abstract

Axonal degeneration is a prominent feature of many neurological disorders that are associated with mitochondrial dysfunction, including Parkinson's disease (PD), motor neuron disease, and inherited peripheral neuropathies. Studies of the *Wld*^{s} mutant mouse, which undergoes delayed Wallerian degeneration in response to axonal injury, suggest that axonal degeneration is an active process. *Wld*^{s} mice also have slower axonal degeneration and disease progression in numerous models of neurodegenerative disease. The *Wld*^{s} mutation results in the production of a chimeric protein that contains the full length coding sequence of nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1), which alone is sufficient for axonal protection *in vitro*. To test the effects of increased Nmnat expression on axonal degeneration induced by mitochondrial dysfunction, we examined dorsal root ganglia (DRG) neurons treated with rotenone. Rotenone induced profound axonal degeneration in DRG neurons; however, this degeneration was delayed by expression of Nmnat. Nmnat-mediated protection resulted in decreased axonal accumulation and sensitivity to reactive oxygen species (ROS) but did not affect the change in the rate of rotenone-induced loss in neuronal ATP. Nmnat also prevented axonal degeneration caused by exposure to exogenous oxidants and reduced the level of axonal ROS after treatment with vincristine, further supporting the idea that Nmnat promotes axonal protection by mitigating the effects of ROS.

Introduction

Mitochondrial dysfunction caused by mutations or toxic agents has been implicated in many neurodegenerative diseases, including those associated with axonal degeneration (Lin and Beal, 2006). It results in a host of cellular abnormalities including decreased ATP synthesis, disrupted mitochondrial fusion and transport, and the increased generation of reactive oxygen species (ROS) (Lin and Beal, 2006; Baloh et al., 2007). The association of mitochondrial dysfunction with neurodegenerative diseases is perhaps strongest in Parkinson's Disease (PD), which can be associated with mutations in mitochondria-associated proteins (e.g. DJ-1, PINK-1) (Martin, 2006) and ingestion of 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), an inhibitor of mitochondrial Complex I (Langston et al., 1983; Betarbet et al., 2000). It is generally accepted that environmental factors play an important role in the pathogenesis of PD (Sherer et al., 2002a); in particular, there is a strong association between PD and exposure to pesticides, including many which inhibit mitochondrial Complex I (Semchuk et al., 1992; Gorell et al., 1998; Priyadarshi et al., 2000; Priyadarshi et al., 2001; Gorell et al., 2004; Ascherio et al., 2006; Brown et al., 2006). Further studies have shown that the pesticide rotenone leads to dopaminergic neuron loss and PD-like symptoms in animals (Betarbet et al., 2000).

Neurodegenerative disease is commonly associated with neuronal cell death, but it is becoming increasingly apparent that axonopathy is also a major component of many neurodegenerative diseases including amyotrophic lateral sclerosis (ALS), PD, and Alzheimer's disease (AD) (Coleman, 2005). Unfortunately, the molecular mechanisms underlying axonal degeneration remain poorly characterized. One model of axonal

degeneration derives from studies of anoxic injury to axons, which leads to mitochondrial failure, decreased levels of ATP, reduced Na^+/K^+ ATPase activity, and increased axonal Na^+ and Ca^{2+} (Stys et al., 1992). These changes are followed by calpain activation and eventual axonal protein degradation (Coleman, 2005). Studies of the *Wld*^{s} mutant mouse, which exhibits slow axonal Wallerian degeneration in response to nerve injury, have led to the conclusion that axonal degeneration, like apoptosis, is an active process (Lunn et al., 1989; Raff et al., 2002). Axonal degeneration is delayed by the *wld*^{s} mutation in a wide range of disease models, such as the *pmn* mice, a model of motor neuron degeneration, vincristine treatment, a model of chemotherapy induced neuropathy, or MPTP, a model of PD (Wang et al., 2001b; Ferri et al., 2003; Hasbani and O'Malley K, 2006), suggesting that a common 'degenerative' pathway is activated by these insults that can be inhibited by this mutation.

The *Wld*^{s} mutant mouse harbors a triplication of a gene fusion that produces a chimeric protein composed of the N-terminal 70 amino acids of ubiquitination factor 4b, an unique 18 residue linker region and the full length nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1) protein (Conforti et al., 2000). Using dorsal root ganglion (DRG) neuronal cultures, we previously demonstrated that the Nmnat1 portion of the Wld^s protein is the moiety responsible for axonal protection (Araki et al., 2004). Many studies in both rodents and Drosophila have confirmed these results (Hoopfer et al., 2006; Macdonald et al., 2006; Zhai et al., 2006), while other investigators have suggested the Ube4b fragment may modulate the Nmnat protective effect (Wang et al., 2005; Conforti et al., 2006). Notably, we have found that Nmnat3, which is localized to mitochondria, also provides axonal protection from mechanical injury (Sasaki et al., 2006). In the

present study, we show that increased Nmnat protein expression effectively delayed rotenone-induced axonal degeneration in DRG neuronal cultures. Nmnat3 expression decreased ROS accumulation in neurons treated with rotenone, but had little effect on the rate of ATP loss in these cells. We also demonstrate that Nmnat3 can prevent axonal damage resulting from exogenous application of oxidants, suggesting that Nmnat expression prevents axonal degeneration by decreasing the accumulation or damage caused by ROS generated during mitochondrial and/or oxidative injury. We show that vincristine, a known cause of axonal degeneration that is preventable by Nmnat (Araki et al., 2004), induces ROS and that this increase can be prevented by Nmnat. Finally, we demonstrate that antioxidants are sufficient for axonal protection from rotenone-induced axonal degeneration, further supporting the idea that Nmnat-mediated protection occurs via mitigating ROS-mediated damage.

Materials and Methods

Reagents. All reagents are from Sigma-Aldrich (St. Louis, MO) unless otherwise noted.

Dissociated DRG cultures. DRGs were dissected from embryonic day 14.5 (E14.5)-15.5 Sprague-Dawley rat embryos. DRGs were dissociated with collagenase followed by trypsin and subsequent triteration through a fire polished glass pipette. Cells were seeded onto Matrigel (BD Biosciences, San Jose, CA) coated 24-well dishes and grown in DMEM containing 10% FBS and penicillin and streptomycin, 3 μM aphidicolin or, alternatively, in neurobasal with B27 (Invitrogen, Carlsbad, California) and 25 ng/ml nerve growth factor followed 24 hr later by the addition of 1 μM 5-fluoro-2′-deoxyuridine and 1 μM uridine (Harlan Bioproducts, Indianapolis, IN). After 4 days in

culture, all of the neuronal cultures were maintained in DMEM containing 10% FBS, penicillin and streptomycin, 1 μM 5-fluoro-2′-deoxyuridine and 1 μM uridine and NGF.

Construction of Lentiviral Expression Plasmids. The GFP plasmid (FUGW) was a gift from D. Baltimore (California Institute of Technology, Pasadena, CA). Nmnat1 (FCIV-Nmnat1) and Nmnat3 (FCIV-Nmnat3) were described previously (Sasaki et al., 2006). Hexahistidine-tagged-Nmnat1 (FUW-Nmnat1) and Nmnat3 (FUW-Nmnat3) (Araki et al., 2004; Sasaki et al., 2006) were cloned into the FUW plasmid without GFP (gift from D. Baltimore) to produce lentiviral transfer vectors without GFP expression for studies utilizing ROS-sensitive fluorescent dyes.

Lentiviral Infection of DRG neurons. Lentiviruses expressing transgenes were generated as previously described (Araki et al., 2004). For infection of DRG neurons, lentivirus (10^5 to 10^6 infectious units) was added to an individual well of a 24 well plate containing approximately $5x10^4$ neurons. Transgene expression from the lentivirus was allowed to proceed for 4-10 days prior to using the infected neurons for experiments. Viral infection and transgene expression was monitored, where applicable, by monitoring the GFP reporter via fluorescent microscopy.

Neuronal Cell Body Death. Neurons were treated with rotenone or vehicle control and monitored for cell body damage by ethidium homodimer exclusion from Biotium (Hayward, CA). Ethidium homodimer was added to the cultures at the indicated time at a final concentration of 100 nM. For each condition, 4 fields with an average of 35 cells/well in each of 4 wells were examined using phase contrast and red fluorescence merged images. Data was collected from 2 independent experiments.

Morphometric analysis of axonal degeneration. Dissociated DRG neurons were treated with the indicated chemical agents and imaged using phase contrast microscopy with a 40x lens at the indicated time points. A grid was created over each image using NIH Image J software using the grid plugin (line area= 100,000). The cell counting plugin was used to score each neurite. Degenerating and healthy axons were counted in at least 3 high power field per image (~20 neurites) for each well (the observer was blinded to the condition). Axonal segments were considered degenerated if they showed evidence of swellings and/or blebbing, n≥6 wells per condition from duplicate experiments

Analysis of ATP Levels. ATP assays were performed on neuronal cultures seeded at the same density lysed in 1% TritonX-100, 0.1M Tris pH 7.8 and 0.5mM EDTA. Cell lysates were used in a luciferase-based ATP assay (Promega, Madison, WI) where ATP is expressed as percent of control based on arbitrary units of luminescence (A.U.) and then converted to μM ATP based on a standard curve (n≥6 wells per condition, results are from 2 independent experiments) (Chang et al., 2003). ATP was measured under all conditions at the end of the treatment period.

Quantitation of ROS Levels. ROS was measured using CM-H_2DCFDA dye at 1.85 μM (Molecular Probes, Carlsbad, California). The dye was added to neurons in serum-free DMEM without phenol red for 1 hr and the cells were washed twice with PBS. Fields containing exclusively axons were identified using phase contrast microscopy. These fields were imaged using epifluorescence with a FITC filter and equal exposure times on a Eclipse TE300 microscope with a 40x Nikon Pan-Fluor lens (Nikon, Tokyo, Japan). The mean intensity of the field minus the minimum intensity (to remove the background fluorescence of the field) was recorded, 3 fields per well were imaged from 3

wells per condition. Results are from 2 independent experiments. All conditions were measured at the end of the treatment period.

Visualization of mitochondrial potential. The methodology we used has been previously described (Ikegami and Koike, 2003). Briefly, DRG cultures were treated with either vehicle control, or rotenone for 6 hrs. Cultures were then changed to Phenol-red free DMEM that contained the mitochondrial potential sensitive dye Mitotracker Red CMXRos (final concentration 25 nM) (Molecular Probes, Carlsbad, California) and then co-stained with Calcein-AM (final concentration 500nM) (Molecular Probes, Carlsbad, California), a small fluorescein-based molecule that is cleaved by non-specific esterases and trapped inside the cell, to visualize the axons. Cultures were treated with 100 μM FCCP for 1 hr prior to imaging and during exposure to the Mitotracker Red and calcein-AM dyes to depolarize the mitochondrial membrane. Cultures were incubated at 37^{o} for 30 min and then washed twice with Phenol-red free DMEM and visualized by fluorescence microscopy using the FITC filter for calcein-AM and the Rhodamine filter for Mitotracker Red.

Quantitation of antioxidant enzyme expression. RNA was collected from dissociated rat DRGs after 14 DIV. Five wells from a 24 well plate were pooled and three pools for each condition were collected. RNA was prepared using Trizol (Invitrogen, Carlsbad, CA). First-strand cDNA templates were prepared using 1 μg of RNA template from each pool using standard methods. Quantitative reverse transcription qRT-PCR was performed by monitoring in real time the increase in fluorescence of the SYBR-green dye on a TaqMan 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The expression levels were normalized to 18s rRNA to account for variations between the

levels of total cDNA template across different samples. Each qRT-PCR experiment was performed in triplicate using 3 pools of cDNA template. The primers used are as follows glutathione peroxidase 1 (GPX1) (Fernandez-Gomez et al., 2006) forward 5'-GCAGATACACCAGGCGCTTT-3' reverse 5'-GGCTTCTATATCGGGTTCGA-3', catalase (CATA) forward 5'-GCACACTTTGACAGAGAGCGG-3' reverse 5'-CTTTGCCTTGGAGTATCTGGTAATATC-3', superoxide dismutase 1 (SOD1) forward 5'- TCAGGAGAGCATTCCATCATTG-3' reverse 5'-CAGCATTTCCAGTCTTTGTACTTTCTT-3', SOD2 forward 5'-CACAGCATTTTCTGGACAAACC-3' reverse 5'-CCTTAAACTTCTCAAAAGACCCAAAG-3', 18s forward 5'-CGCCGCTAGAGGTGAAATTCT-3' reverse 5'-CGGCTACCACATCCAAGGAA-3'

Statistics. ANOVA was used followed by individual comparisons with Student's two-tailed, unpaired t-tests. Comparisons were considered significant with a $P>0.05$ after Bonferroni's correction for multiple comparisons. Values are reported as mean ± SEM unless otherwise noted.

RESULTS

Rotenone treatment of DRG neurons results in axonal degeneration

The expression of *Wld*^{S}, Nmnat1 or Nmnat3 in neurons protects against axonal degeneration resulting from damage due to mechanical, genetic, or chemical injury (Gillingwater and Ribchester, 2001; Wang et al., 2001a; Wang et al., 2001b; Ferri et al., 2003; Samsam et al., 2003; Araki et al., 2004; Gillingwater et al., 2004; Mi et al., 2005; Sasaki et al., 2006; Zhai et al., 2006). The association between mitochondrial dysfunction

and axonal degeneration in multiple neurological disorders encouraged us to investigate whether Nmnat expression could protect axons from damage caused by mitochondrial dysfunction. We used DRG sensory neurons, which we previously utilized (Araki et al., 2004; Sasaki et al., 2006) in our studies of Wld^{S}- and Nmnat-mediated axonal protection after mechanical injury, to study axonal degeneration caused by mitochondrial inhibitors. We tested the effects of rotenone, a lipophilic Complex I inhibitor that can be used in non-dopaminergic neurons (Betarbet et al., 2000; Sherer et al., 2003), on DRG neurons in concentrations ranging from 0.1 to 10 μM. We assessed axonal degeneration using phase contrast microscopy after rotenone addition and found that 1 to 5 μM rotenone caused significant axonal degeneration by 24 hr that continued to progress until by 72 hr most axons had largely degenerated (Fig. 1A, B and data not shown).

To determine whether axonal degeneration preceded cell death in neurons exposed to rotenone, we performed a time course experiment in which we monitored cell body damage using an ethidium homodimer exclusion assay in conjunction with microscopic analysis of axonal degeneration. After 72 hr, when 100% of axonal segments showed signs of degeneration, 51% survival of cell bodies were still intact (Fig. 1E). These results, which indicate that axons are more susceptible to rotenone toxicity than neuronal cell bodies, are consistent with previous reports (Betarbet et al., 2000; Testa et al., 2005). Additionally, as soma membrane permeability clearly lagged behind axonal degeneration, these results also demonstrate that cell death was not a prerequisite for axonal degeneration.

Nmnat proteins protect axons and cell bodies from rotenone toxicity

To determine if Nmnat activity could protect axons and/or cell bodies against rotenone-induced mitochondrial inhibition, we infected DRG neurons with lentivirus expressing either GFP alone or hexahistidine-tagged Nmnat1 or Nmnat3 along with GFP. After infection, when >95% of neurons express GFP (as monitored by fluorescence microscopy), Nmnat transgene expression was clearly demonstrable by Western analysis (Fig. 1C). Similarly infected neuronal cultures were treated with 2.5 μM rotenone and axonal degeneration as well as cell death were monitored using phase contrast microscopy and ethidium homodimer exclusion, respectively. While neurons expressing GFP alone manifested extensive axonal degeneration within 72 hr, axons of Nmnat-expressing neurons showed no evidence of degradation until 96 hr after rotenone addition (Fig. 1A, B, D). Due to the fact that the rotenone site of action and Nmnat3 localization are both in the mitochondria, Nmnat3 was used for the remainder of the experiments. The axonal protection afforded by Nmnat proteins in neurons treated with mitochondrial inhibitors, in addition to previous work demonstrating protection against mechanical injury or vincristine exposure, suggests that Nmnat is acting at a point in the degeneration process that is common to multiple types of damage.

To confirm that rotenone inhibition of Complex I was specifically responsible for the axonal degeneration in these experiments, we treated DRG cultures with two additional Complex I inhibitors, pyridaben (2.5 μM) and fenpyroximate (2.5 μM) (Sherer et al., 2007). We found that both of these Complex I inhibitors also caused severe axonal degeneration by 24 hr. Furthermore, we found that Nmnat3 expression could clearly prevent this axonal degeneration, just as it had inhibited axonal degeneration caused by

rotenone (Suppl. Fig. 1). Nmnat expression also had a modest protective effect on cell death, although it was less impressive than the protection provided to axons. For example, while 51% of neurons expressing GFP alone excluded ethidium homodimers after 72 hr of rotenone treatment, 68% of Nmnat3-expressing neurons were viable at 72 hr (Fig. 1E). It should be noted that at 72 hr there is almost complete protection of axons even though 32% of the cells no longer exclude ethidium homodimers (a late marker of cell death). This dichotomy is similar to results obtained using axotomy or growth factor deprivation, and emphasizes that the mechanism of axonal protection is different from (and perhaps independent of) those involved in the prevention of cell death.

Nmnat3 prevents oxidant-induced axonal degeneration

The accumulation of ROS as a result of mitochondrial inhibition has been implicated in the pathogenesis of several neurodegenerative diseases (Lin and Beal, 2006). Excessive ROS production has multiple detrimental effects such as DNA damage, oxidation of proteins that leads to their subsequent inhibition, and the oxidation of lipids that creates toxic products including 4-hydroxynonenal (4-HNE) (Geller et al., 2001; Sherer et al., 2002b; Duarte et al., 2005; Keeney et al., 2006; Ramachandiran et al., 2007). Since it is well known that rotenone induces an increase in ROS, we hypothesized that rotenone damage is initiated by ROS and that Nmnat might protect against ROS damage caused by exposure to exogenous oxidants. To test this idea, we treated DRG neurons infected with lentivirus expressing either GFP alone or Nmnat3 with 100-300 μM hydrogen peroxide (H_2O_2). In cultures infected with GFP alone there was significant dose-dependent axonal degeneration after 24 hr (Fig 2A). In contrast, axonal

degeneration from H_2O_2 exposure was inhibited in neurons expressing Nmnat3 (Fig. 2A). To confirm the ability of Nmnat to prevent oxidant-induced damage, we also examined the effects of the organic oxidant tert-butyl hydroperoxide (TBHP) . In GFP-expressing (control) neurons, TBHP caused extensive axonal degeneration within 48 hr (Suppl. Fig. 2), whereas neurons expressing Nmnat3 showed no evidence of TBHP-mediated axonal degeneration during this time period (Suppl. Fig. 2). These data demonstrate that Nmnat can prevent axonal degeneration triggered by ROS, and suggest that Nmnat-mediated protection is accomplished by decreasing the toxicity of ROS produced either endogenously or exogenously.

Protection of oxidant-mediated axonal damage by Nmnat suggested that it might act via increasing neuronal expression of antioxidant proteins, including superoxide dismutase, glutathione peroxidase, and/or catalase. (Thiruchelvam et al., 2005; Fernandez-Gomez et al., 2006; Jung et al., 2007). We used qRT-PCR to compare their mRNA levels in Nmnat3-expressing vs. control DRG neurons. However, we found no significant differences in the RNA levels of these genes (Fig. 2C), suggesting that Nmnat-mediated protection does not involve transcriptional regulation of these enzymes.

Nmnat3 expression decreases axonal ROS levels

The axonal protective activity of Nmnat3 against ROS supplied by exogenous oxidant or generated intracellularly via inhibition of mitochondrial Complex I, led us to hypothesize that Nmnat expression may reduce rotenone-induced ROS accumulation and/or toxicity. To test this idea, we measured relative ROS levels in axons using a ROS-sensitive fluorescent dye, CM-H_2DCFDA, and found that ROS levels were significantly

increased in axons 6 hr after rotenone treatment (Fig. 3A, B). The level of ROS continued to rise until 96 hr after rotenone treatment, when the severely degenerated state of most axons in the cultures precluded a quantitative analysis of axonal ROS levels (Fig. 3A). Interestingly, the increase in ROS, which is detectable after 6 hr of rotenone treatment, substantially preceded any observable axonal degeneration (Fig. 3A, B).

To determine the effects of Nmnat expression on rotenone-induced ROS accumulation, neurons were infected with Nmnat3 lentivirus that lacks the GFP reporter (to avoid interference with the CM-H_2DCFDA fluorescence). The infected neurons were treated with rotenone (or vehicle) and ROS levels were measured 6, 48 or 96 hr later. We found that neurons expressing Nmnat3 had statistically significant lower axonal ROS levels after rotenone treatment compared to controls (Fig. 3A, B), and that this decrease in ROS was correlated with the slower axonal degeneration observed in these neurons.

Mitochondrial dysfunction is often accompanied by a decrease in mitochondrial membrane potential (Nicholls, 2004), and others have suggested that dissipation of mitochondrial membrane potential precedes axonal degeneration and that the degeneration can be blocked if the mitochondrial membrane potential is preserved (Ikegami and Koike, 2003; Nicholls, 2004; Yang et al., 2007). To determine if rotenone caused a loss of mitochondrial membrane potential, we stained cultures with Mitotracker Red, a mitochondrial potential dependent dye. However, consistent with other studies with rotenone (Johnson-Cadwell et al., 2007; Marella et al., 2007), we did not observe a collapse of mitochondrial membrane potential after rotenone treatment (6 hr) at a time when rotenone induced increases in ROS are already observed (Fig. 3C). These observations demonstrate that Nmnat expression decreases ROS accumulation,

suggesting that inhibition of ROS accumulation and/or toxicity is a principle mechanism by which Nmnat proteins protect against rotenone-induced axonal degeneration.

Nmnat3 inhibits ROS accumulation in vincristine treated neurons

In addition to its axonal protective effects after mechanical injury or mitochondrial inhibition, Wld^s and Nmnat proteins protect against vincristine-mediated axonal degeneration (Wang et al., 2001b; Araki et al., 2004). Recent studies have shown that vincristine toxicity in cancer cells may be mediated by ROS, and that treatment with antioxidants can inhibit vincristine-induced cell death (Groninger et al., 2002; Tsai et al., 2007). We therefore tested whether vincristine treatment resulted in increased axonal ROS, and whether Nmnat axonal protection of vincristine-treated neurons was associated with decreased ROS accumulation. We used CM-H_2DCFDA fluorescence to monitor ROS levels in DRG neurons treated with 0.04 μM vincristine for 0, 6, 24 and 48 hrs. Vincristine caused a modest, but significant, increase in ROS after 6 hr (Fig. 4A, B) that continued to increase as axonal degeneration became apparent at 24 and 48 hr after vincristine addition. In contrast, Nmnat3-expressing neurons treated with vincristine showed decreased accumulation of ROS at 24 and 48 hr (Fig 4A, B). Cultures were maintained for up to 96 hr to demonstrate that Nmnat3 protected against vincristine-mediated axonal degeneration. (Fig. 4C). While the primary effect of vincristine is microtubule disruption, these results suggest that it also stimulates ROS production and that ROS generation may play a role in vincristine axonal toxicity. Taken together, these data suggest that Nmnat-mediated axonal protection against a variety of insults results from its ability to inhibit ROS-mediated damage or signaling. This would imply, as suggested by others, that ROS-stimulated processes could be a point of convergence for

multiple pathways that culminate in axonal degeneration (Arundine et al., 2004; Alexandre et al., 2006; Holtz et al., 2006).

Nmnat3 mediated axonal protection occurs despite decreases in neuronal ATP levels

Cells treated with inhibitors of mitochondrial electron transport have decreased levels of ATP along with an increased accumulation of ROS. Several studies have indicated that increased ROS levels, and not the loss of ATP, are the proximal causes for neuronal death induced by mitochondrial inhibition (Sherer et al., 2003; Watabe and Nakaki, 2006). However, others have shown that Nmnat1-mediated axonal protection involves the maintenance of axonal ATP levels (Wang et al., 2005). While dampening the increases in ROS accumulation are clearly an important feature of Nmnat-mediated protection, we also investigated the effects of Nmnat expression on ATP levels in DRG neurons. ATP levels of DRG neurons treated with rotenone for various lengths of time were measured using an ATP-dependent luciferase-based assay. We found that rotenone caused a 55.3% and 83.4% decrease in ATP levels in DRG neurons treated for 6 and 96 hr, respectively (Fig. 5 A).

To test whether Nmnat3 promoted axonal protection via prevention of these rotenone-induced decreases in ATP, we treated Nmnat3-expressing DRG neurons with rotenone and monitored ATP levels. Nmnat3-expressing neurons had a small increase in basal ATP levels (Control: 100 ± 1.3 % ATP vs. Nmnat3: 118 ± 2.7 % ATP). Rotenone treatment caused a rapid fall in ATP levels in both control and Nmnat3-expressing neurons, but ATP levels were always slightly higher in Nmnat3-expressing neurons (Fig. 5 A). We also calculated the rate of ATP loss for both control and Nmnat3-expressing

neurons. During the first 6 hr, ATP loss was rapid in both GFP- and Nmnat3-expressing neurons (GFP: 9.2 ± 0.39 % ATP/hr and Nmnat3: 9.6 ± 0.39 % ATP/hr). The rate of ATP loss slowed at later time points, but was very similar in GFP- and Nmnat3-expressing neurons (0.3 ± 0.07 % ATP/hr and (0.17) ± 0.19 % ATP/hr, respectively). The initial decrease in ATP is likely due to the inhibition of oxidative phosphorylation by rotenone, while the later and slower loses of ATP are presumably due to axonal and cellular degeneration and death. Thus, even though axons of Nmnat3-expressing neurons were intact, their ATP levels decreased to a similar degree as those of degenerating axons of control neurons. (Fig. 1B, 2A, 5A).

Although the differences in ATP levels between Nmnat3-expressing and control neurons did not correlate well with the extent of axonal degeneration, ATP levels were slightly higher when Nmnat3 was overexpressed. To further investigate the possibility that ATP levels were key to axonal degeneration, we treated cultures with tetrodotoxin, (TTX, a voltage-gated sodium channel blocker). TTX treatment should serve to increase neuronal ATP levels as it will inhibit the influx of sodium and negate the need to expend ATP for maintaining axonal membrane potential via the Na^+/K^+ ATPase (Stys, 2004). In accord, DRG cultures treated for 6 hr with TTX had a 20% increase in ATP (Fig. 5B). In cultures treated with rotenone and TTX simultaneously, the presence of TTX dramatically decreased the rotenone-stimulated drop in ATP (Fig. 5B). Despite this effect on ATP levels, TTX had no effect on rotenone-induced axonal degeneration (Fig. 5B and data not shown). These data indicate that increasing the ATP levels via inhibition of voltage stimulated sodium influxes does not inhibit axonal degeneration.

To further explore the relationship between ATP levels and axonal degeneration, we treated DRG neurons with 2-deoxyglucose (2-DG), which inhibits glycolysis by blocking glucose uptake and phosphorylation but does not interfere with the mitochondrial electron transport chain or increase ROS levels (Sherer et al., 2003). Treatment with 2-DG resulted in a 37.0 ± 5.3% decrease in ATP after 24 hr, but there was no evidence of axonal degeneration after 48 hr or increased ROS levels (Fig. 5C, data not shown). Even long-term treatment of DRG neurons with 2-DG (18 days) did not cause axonal degeneration, although the low ATP level observed at 24 hr remained throughout the treatment period (data not shown).

Antioxidants inhibit rotenone-mediated axonal degeneration without altering ATP levels.

The suppression of ROS accumulation by Nmnat proteins is reminiscent of that observed in cells treated with antioxidants, whose utility in preventing axonal degeneration secondary to mitochondrial inhibition has been suggested by others (Sherer et al., 2003; Testa et al., 2005; Sherer et al., 2007). To determine whether antioxidants can protect against rotenone-induced axonal degeneration, we administered vitamin E (α-tocopherol) in doses from 125 μM to 1000 μM (corresponding to 0.052 IU/ml to 0.47 IU/mL) to DRG neurons treated with rotenone and assessed axonal degeneration. We found that while axonal degeneration in control cultures (rotenone alone) began within 24-48 hr, axons from neurons treated with vitamin E remained intact for up to 120 hr (Fig. 6A, B). To determine if vitamin E also affected ATP levels, perhaps by directly blocking the actions of rotenone, we measured ATP levels at various times after rotenone

treatment. As previously observed in Nmnat3-expressing neurons, vitamin E did not significantly suppress the initial loss of ATP (Fig. 6C), however as axons began to breakdown (in the untreated neurons) a difference in ATP levels was observed.

Taken together, these results indicate that a decrease in ATP levels is insufficient to cause axonal degeneration. Furthermore, the degree of ATP loss, unlike ROS levels, did not correlate with axonal degeneration temporally or with Nmnat3 expression, suggesting that inhibition of ROS initiated axonal degeneration rather than maintenance of ATP levels is the primary driver of Nmnat3-mediated protection against rotenone-induced axonal degeneration.

Discussion

Elucidating the mechanism and role of axonal degeneration in neurodegeneration is an important step in developing strategies for preventing and treating neurodegenerative diseases. We recently demonstrated that Nmnat1 and Nmnat3 expression can prevent axonal degeneration against a variety of insults including microtubule disruption and mechanical injury (Sasaki et al., 2006). This protection requires the enzymatic activity of Nmnat that converts nicotinamide mononucleotide (NMN) to nicotinamide adenine dinucleotide (NAD^+) (Araki et al., 2004; Berger et al., 2005), although recent studies in Drosophila neurodegeneration have suggested additional Nmnat activities may also be important for synaptic protection (Zhai et al., 2006). The importance of mitochondrial dysfunction in neurological disorders involving axonal degeneration, and the ability of the *Wld*^{s} mutation to protect against MPTP-induced parkinsonism (Hasbani and O'Malley K, 2006), suggested that Nmnat proteins

might prevent degeneration caused by mitochondrial inhibition. Here, we demonstrated that Nmnat expression robustly inhibits rotenone-induced degeneration of DRG axons and provides modest protection to the neuronal soma. In exploring the mechanism of this protection, we found that Nmnat expression did not affect the rate of decrease in ATP levels caused by rotenone-mediated mitochondrial inhibition. Further, we found that 50-60% losses of ATP were neither necessary or sufficient for axonal degeneration. However, Nmnat proteins inhibited axonal degeneration caused by external oxidants and decreased the accumulation of axonal ROS during rotenone and vincristine treatment, suggesting that they protect axons by reducing ROS accumulation or toxicity.

Interestingly, studies with the acute 6-OHDA and MPTP rodent PD models showed that the *Wld*^{s} mutation provided robust protection against TH^{+} fiber loss but little or no protection of dopaminergic neuronal cell bodies (Sajadi et al., 2004; Hasbani and O'Malley K, 2006). These *in vivo* results are consistent with our findings, and suggest that Nmnat-mediated protection is focused primarily on axons. The effects of *Wld*^{s} have not been tested in the chronic rotenone model of PD that more closely mimics the degenerative progression observed in PD patients since this model only works in rats (Hornykiewicz, 1966; Betarbet et al., 2000; Richter et al., 2007); however, our results suggest that increasing Nmnat expression in this model may rescue axonal function and thereby prevent dopaminergic neuron death.

ROS has been implicated in the pathogenesis of neurodegenerative diseases, in particular PD, as well as in the deficits that occur in normal aging (Lin and Beal, 2006). For example, Complex I inhibition and oxidative damage to mitochondrial proteins have been observed in brains of PD patients, changes that can be replicated by treatment of

isolated mitochondria with rotenone (Keeney et al., 2006). Indeed, antioxidants provide protection for dopaminergic neurons in the MPTP, 6-OHDA, rotenone and genetic models of PD *in vivo* and *in vitro* (Schulz et al., 1995; Matthews et al., 1999; Bahat-Stroomza et al., 2005; Testa et al., 2005; Wang et al., 2006). In addition, RNAi-mediated inhibition of Drosophila PINK1, a kinase mutated in familial PD, resulted in losses of dopaminergic neurons and eye defects. These deficits could be rescued by expression of superoxide dismutase or treatment with the antioxidant vitamin E, suggesting that ROS was responsible for the damage (Wang et al., 2006). Moreover, organotypic midbrain cultures treated with rotenone display loss of TH^+ fibers, increased protein oxidation, and to a much lesser extent cell body shrinkage; deficits that are consistent with an axonopathy in which axonal degeneration precedes cell body loss. Similar to our findings, these deficits appear to involve ROS as they could be prevented by co-administration with vitamin E (Testa et al., 2005). Taken together, these results suggest that halting ROS-induced damage can prevent the damage caused by mitochondrial dysfunction.

Inhibition of the electron transport chain can cause apoptosis in human dopaminergic cells (Watabe and Nakaki, 2006). Further study showed that ATP loss could not account for the induction of apoptosis in this model; instead, ROS production was necessary and sufficient to promote apoptosis of these cells. Axonal degeneration was not directly addressed in that study, however the results are consistent with the idea that moderate mitochondrial inhibition causes damage primarily via excess ROS production rather than through abnormalities associated with energetic deficits (i.e. decreased ATP) (Bao et al., 2005). On the other hand, some studies have suggested that

preventing axonal ATP loss is a crucial aspect of axonal protection by Nmnat (Ikegami and Koike, 2003; Wang et al., 2005). However, our results indicate that the rate of ATP loss is comparable in control and Nmnat-expressing neurons as well as vitamin E treated neurons, suggesting that this is not the mechanism by which Nmnat protects against axonal degeneration. Furthermore, neurons treated with 2-DG for prolonged periods of time have up to 40% lower levels of ATP but show no evidence of axonal degeneration, providing additional support for the idea that decreases in ATP levels are unlikely to be the only driver of axonal degeneration. Finally, we showed that treatment with tetrodotoxin could mitigate rotenone-induced decreases in ATP yet could not prevent axonal degeneration. It appears that moderate ATP losses are insufficient for axonal degeneration, however lower ATP levels could affect axonal stability by increasing neuronal susceptibility to ROS-mediated damage.

Others have shown that the Wld^s protein maintains the mitochondrial membrane potential in vinblastine-treated SCG neurons and they suggested that this may play a role in Wld^s mediated axonal protection (Ikegami and Koike, 2003). Our results indicate that rotenone causes an increase in ROS without a collapse of the mitochondrial membrane potential. It is possible that rotenone does cause small changes in the mitochondrial membrane potential, but the physiological significance of small changes in mitochondrial membrane potential are unclear (Nicholls, 2004; Nicholls et al., 2007).

We cannot exclude the possibility that ROS is simply a trigger to initiate axonal degeneration, much like axonal severing, and that Nmnat protects axons by blocking downstream targets of this signaling cascade. It also possible that ROS accumulation is secondary to the initiation of this degeneration pathway; however, this is less likely since

axonal degeneration induced by exogenous oxidants can also be blocked by Nmnat expression.

Furthermore, Nmnat expression can protect axons from diverse insults, suggesting that the axonal damage in many of these paradigms may ultimately involve ROS, or ROS-mediated signaling. While mitochondria are considered to be the major source of ROS, other sources of ROS production include membrane-bound NADPH-oxidase (NOx), a source of oxidative products induced in neurons by signaling intermediates, and xanthine oxidase, which produces ROS in neurons in response to hypoxia and glucose deprivation (Ibi et al., 2006; Abramov et al., 2007). Other insults not directly linked to mitochondrial dysfunction, including taxol, vincristine and mechanical injury, also induce ROS and neurodegeneration (Hall, 1987; Groninger et al., 2002; Arundine et al., 2004; Holtz et al., 2006; Jérôme Alexandre, 2006; Tsai et al., 2007).

The mechanism by which Nmnat proteins prevent the accumulation and/or toxicity of ROS is unclear. It does not appear that transcriptional changes for previously identified antioxidant genes are involved (Gillingwater et al., 2006b). Interestingly, the production and usage of NAD^+ and its metabolites can play important roles in antioxidant responses (Mack et al., 2001). One possibility is that Nmnat proteins increase NAD^+ availability without increasing NADH breakdown via oxidation, thus providing more NADH for conversion to NADPH, a cofactor for many ROS scavenging enzymes (Elizabeth and Karam, 2003). Alternatively, Nmnat proteins may have additional activities, such as antioxidant activity, much like peroxidases or dismutases. An undiscovered function of Nmnat, in addition to its role in NAD^+ biosynthesis, could explain why Nmnat enzymatic activity is not required to block vacuole formation and

neurodegeneration in the Drosophila retina (Zhai et al., 2006). However, this is inconsistent with observations indicating that NAD^+ and NAD^+ precursors as well as other enzymes involved in NAD^+ biosynthesis can also provide axonal protection after axotomy (Araki et al., 2004; Wang et al., 2005; Sasaki et al., 2006). Identifying how increased Nmnat expression limits ROS damage may yield clues as to how it promotes axonal protection in general, as well as opening up new avenues for understanding how ROS causes axonal degeneration.

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Figure Legends

Figure 1. Nmnat expression protects against rotenone-mediated axonal degeneration in DRG neurons. A, B. DRG neurons were treated with rotenone (2.5 μM) for the indicated time and phase contrast microscopy was used to assess axonal degeneration. Degeneration is subtle after 24 hr, but note the extensive axonal damage caused by rotenone by 48 hr. Nmnat1 and 3-expressing neurons do not show axonal degenerative changes until after 96 hr. (A) Scale Bar = 20 μm (B) Scale bar = 100 μm. **C**. Western-blot analysis of lysates of DRG neurons infected with lentivirus expressing GFP alone, Nmnat1 or Nmnat3 using a monoclonal antibody to the hexahistidine tag. **D**. Quantification of rotenone induced axonal degeneration at 0, 24, 48, and 72 hr in DRG neurons expressing GFP or Nmnat3 (4 fields per well, 6 wells per condition from duplicate experiments) (see Materials and Methods). **E**. Quantification of surviving DRG neurons after treatment with rotenone for 24, 48 or 72 hr using ethidium homodimer exclusion (6 wells per condition from duplicate experiments) (*=P<0.01 compared to GFP-expressing neurons at 0 hr ; **=P<0.01 compared to rotenone-treated GFP-expressing neurons at corresponding time point; Error bars=±SEM)

Figure 2. Nmnat activity delays axonal degeneration induced by treatment with the oxidant H_2O_2. **A**. Quantification of axonal degeneration induced by H_2O_2 treatment. DRG neurons were treated with the indicated dose of H_2O_2 and axonal degeneration was monitored after 24 hr using phase contrast microscopy (*=P<0.01 compared to GFP infected cultures, 3 fields per well, 6 wells per condition from duplicate experiments, Error Bars= ±SEM). **B**. Representative images of DRG axons treated with 0-300 μM

H_2O_2 after 24 hr and visualized by phase contrast microscopy. **C**. Expression of genes encoding antioxidant enzymes. Quantitative RT-PCR analysis was performed using mRNA templates prepared from DRG neurons infected with GFP or Nmnat3 for 10 DIV. The mRNA levels of the antioxidant genes examined were not significantly altered by Nmnat3 (n=3, each sample represents 5 pooled wells, $p>0.1$ in all comparisons, data from a representative experiment)

Figure 3. Nmnat expression in DRG neurons decreases rotenone-mediated ROS accumulation. **A**. Representative images of DRG neurons treated with rotenone or vehicle for 0, 6, 48, 96 hr. ROS levels were monitored using the ROS-sensitive dye CM-H_2DCFDA and fluorescence microscopy (left) and axonal degeneration was monitored using phase contrast microscopy (right) (Scale bar= 20 μm). **B**. Quantification of ROS levels in DRG neurons treated as above. Rotenone-treated DRG neurons expressing Nmnat3 showed decreased CM-H_2DCFDA dye fluorescence (i.e. ROS) compared to control neurons. ROS levels do increase in Nmnat3-expressing neurons when axonal degeneration becomes visible (96 hr) (#=$P<0.05$ compared to time zero of each condition *=$P<0.05$ compared to control culture at corresponding time point, n=18 fields from duplicate experiments, mean ± SEM). **C**. Visualization of mitochondrial membrane potential. Neuronal cultures were treated with DMSO (control), FCCP for 1 hr, or rotenone for 6 hr and then Mitotracker Red (mitochondrial membrane potential-dependent dye) and Calcein-AM (intracellular dye to stain axons) were added and visualized by fluorescence microscopy. FCCP decreases the staining of Mitotracker Red consistent with a drop mitochondrial membrane potential, whereas the staining in

rotenone-treated neurons showed minimal changes compared to control neurons. (Representative images from 3 fields per well, 3 wells per condition, duplicated experiments. Scale Bar = 20 μm)

Figure 4. Nmnat expression in DRG neurons inhibits vincristine-mediated increases in ROS levels. **A**. DRG neurons were treated with vincristine (0.04 μM) or vehicle and ROS levels were measured at the indicated time points using the ROS-sensitive dye CM-H_2DCFDA and visualization with fluorescence (left) and phase contrast microscopy (right) (Scale bar= 20 μm). **B**. Quantification of ROS levels in DRG neurons demonstrated that neurons expressing Nmnat3 had decreased CM-H_2DCFDA fluorescence (i.e. ROS) (#=P<0.05 compared to control at time zero *=P<0.05 compared with vehicle treated control cultures, n=18 fields from duplicate experiments, mean ± SEM). **C**. DRG neurons (control vs. Nmnat3-expressing) were treated with vincristine for 96 hr to demonstrate the axonal protection afforded by Nmnat3. Representative images are shown.

Figure 5. Nmnat3 does not slow rotenone-induced decreases in ATP levels. **A**. DRG neurons expressing Nmnat3 or GFP were treated with rotenone (2.5 μM) for the indicated times and ATP levels were determined using a luciferase-based assay. Note that Nmnat3 expression had minimal effects on the rate of ATP loss (*=P<0.05 compared to control at the indicated time, n=6 wells per condition read in duplicate from duplicate experiments). **B**. Left) DRG neurons were treated with 1 mM TTX for 6 hr with or without 2.5 μM rotenone and ATP levels were determined. TTX increased ATP in both conditions

(*=P<0.05 compared to non-TTX control, **=P<0.05 compared to non-TTX and non-rotenone control, n=6 wells per condition read in duplicate from 2 independent experiments, Error bars=±SEM).control). Right) Axonal degeneration was observed in the absence or presence of TTX at 24 hr. **C**. Left) DRG neurons were treated with 2-DG (20 mM) for 24 hr and ATP levels were measured (*=P<0.05, n≥6, Error bars=±SEM). Right) DRG neurons were treated with 2-DG (20 mM) for 48 hr. Representative phase contrast images show no evidence of axonal degeneration.

Figure 6. Vitamin E prevents axonal degeneration, but does not affect initial ATP losses. **A**. DRG neurons were treated with rotenone (2.5 μM) and the indicated doses of vitamin E. Axonal degeneration was quantified after 6 days of treatment using images obtained by phase contrast microscopy (*=P<0.05 compared to control, n=6 wells per condition from duplicate experiments, Error bars=±SEM). B. DRG neurons were treated with rotenone (2.5 μM) in the presence or absence of vitamin E (1 mM) and axonal degeneration was assessed by phase contrast microscopy. While axons in cultures treated with rotenone only were visibly damaged by 48 hr, neurons treated with rotenone and vitamin E were intact at 120 hr. **C**. DRG cultures were treated with rotenone (2.5 μM) in the presence or absence of vitamin E (1 mM) and ATP levels were measured at the indicated time points. Vitamin E failed to prevent the initial loss of ATP (*=P<0.05, n≥6 wells from duplicate experiments, Error bars=±SEM).

Supplemental Figure 1. Nmnat expression protects against Complex I inhibitor-mediated axonal degeneration in DRG neurons. DRG neurons were treated with

pyridaben (Pyr), or fenpyroximate (Fen) (2.5 μM) for 24 hr time and phase contrast microscopy was used to assess axonal degeneration. (n=6 wells per condition from duplicate experiments).

Supplemental Figure 2. Nmnat slows TBHP induced axonal degeneration. Representative images of DRG neuronal axons treated with 50 μM TBHP after 48 hr and visualized by fluorescence microscopy. (Top) GFP fluorescence (Bottom) Phase contrast microscopy.

FIGURE 1

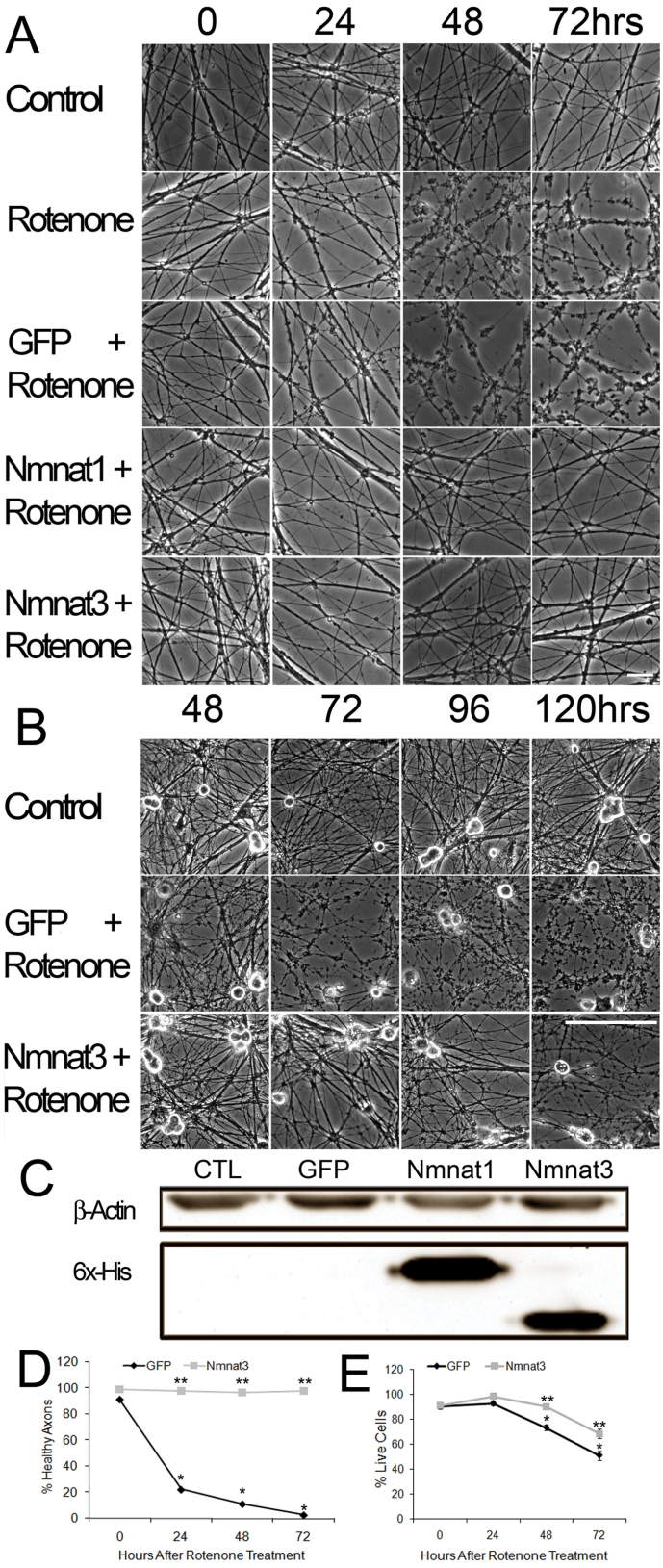

A
0
24
48
72hrs
Control
Rotenone
GFP + Rotenone
Nmnat1 + Rotenone
Nmnat3 + Rotenone
B
48
72
96
120hrs
Control
GFP + Rotenone
Nmnat3 + Rotenone
C
CTL
GFP
Nmnat1
Nmnat3
β-Actin
6x-His
D
GFP
Nmnat3
% Healthy Axons
Hours After Rotenone Treatment
E
GFP
Nmnat3
% Live Cells
Hours After Rotenone Treatment

FIGURE 2

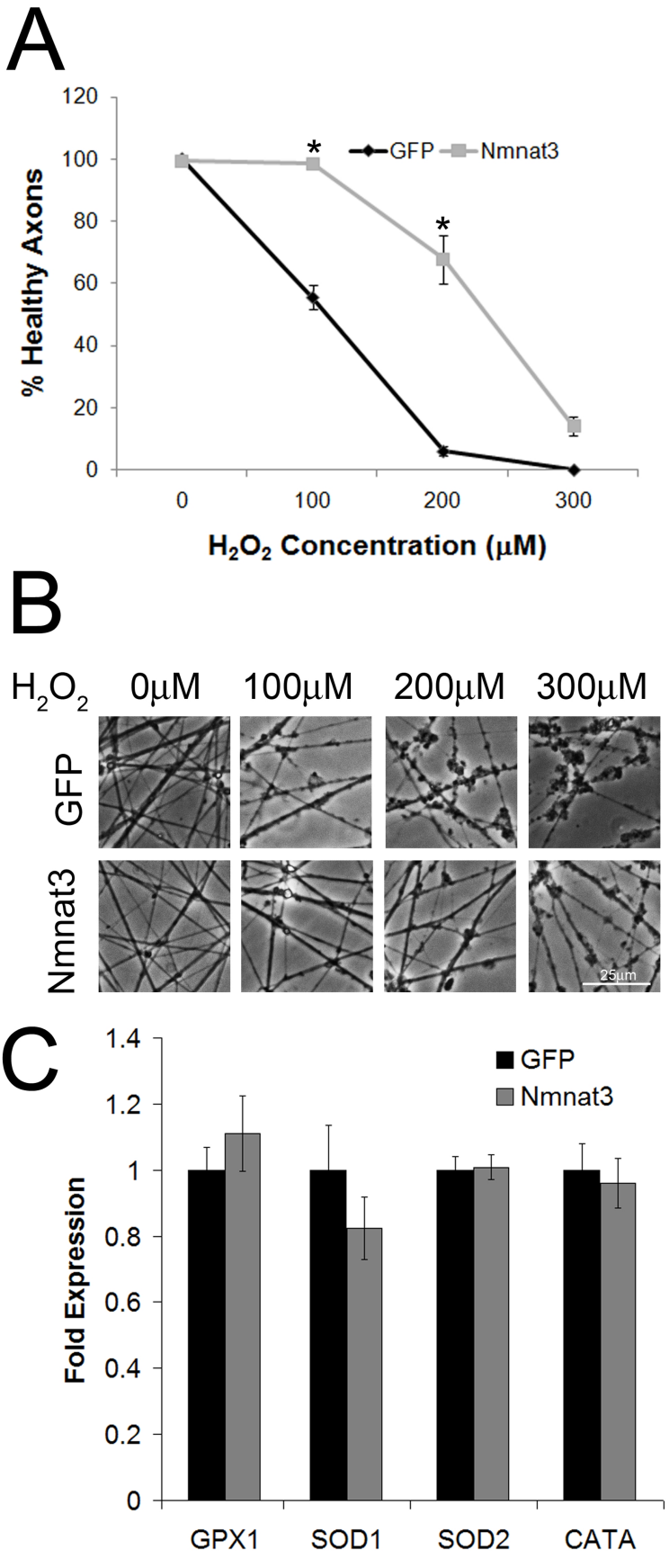
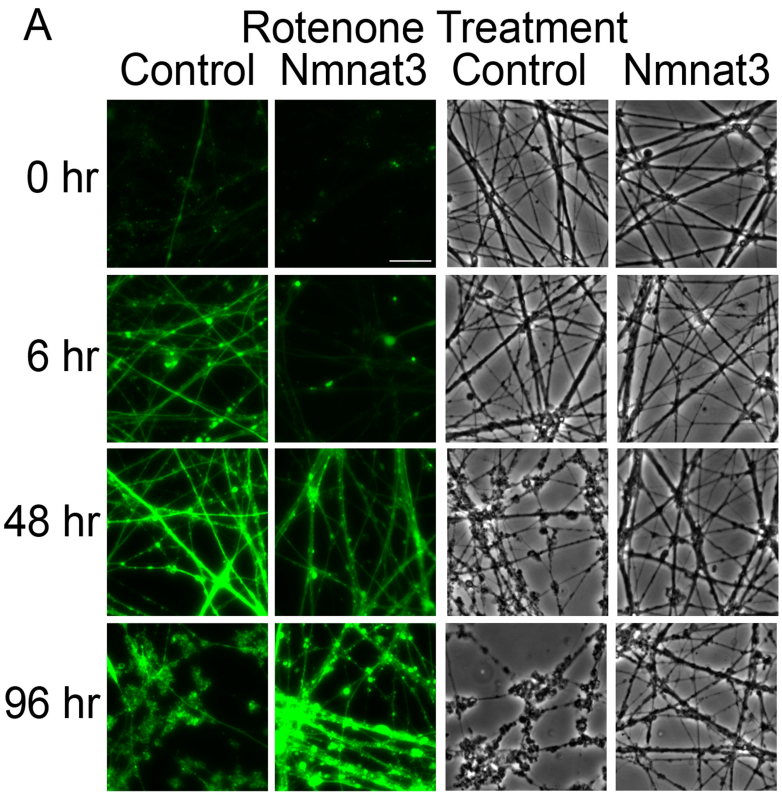

A
120
100
80
60
40
20
0
% Healthy Axons
*
*
GFP
Nmnat3
0
100
200
300
H2O2 Concentration (μM)
B
H2O2
0μM
100μM
200μM
300μM
GFP
Nmnat3
25μm
C
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Fold Expression
GFP
Nmnat3
GPX1
SOD1
SOD2
CATA

FIGURE 3



B

ROS accumulation

Condition	*0 Hours*	*6 Hours*	*48 Hours*	*96 Hours*
Control	100 ± 6.64	464.86 ± 22.45$^{\#}$	626.18 ± 49.62$^{\#}$	-
Nmnat3	84 ± 5.54	236 ± 17.76 $^{*\#}$	362.79 ± 49.55 $^{*\#}$	814.85 ± 90.17$^{\#}$

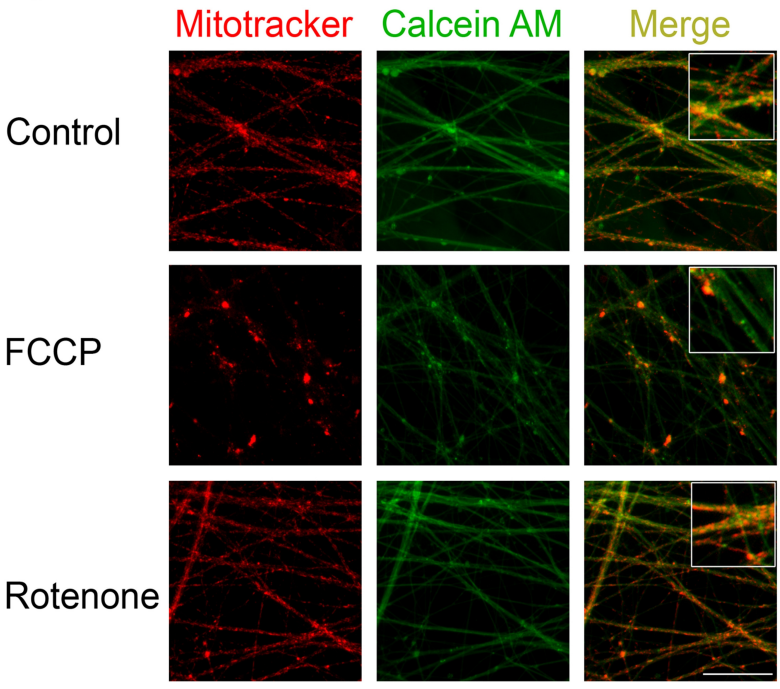
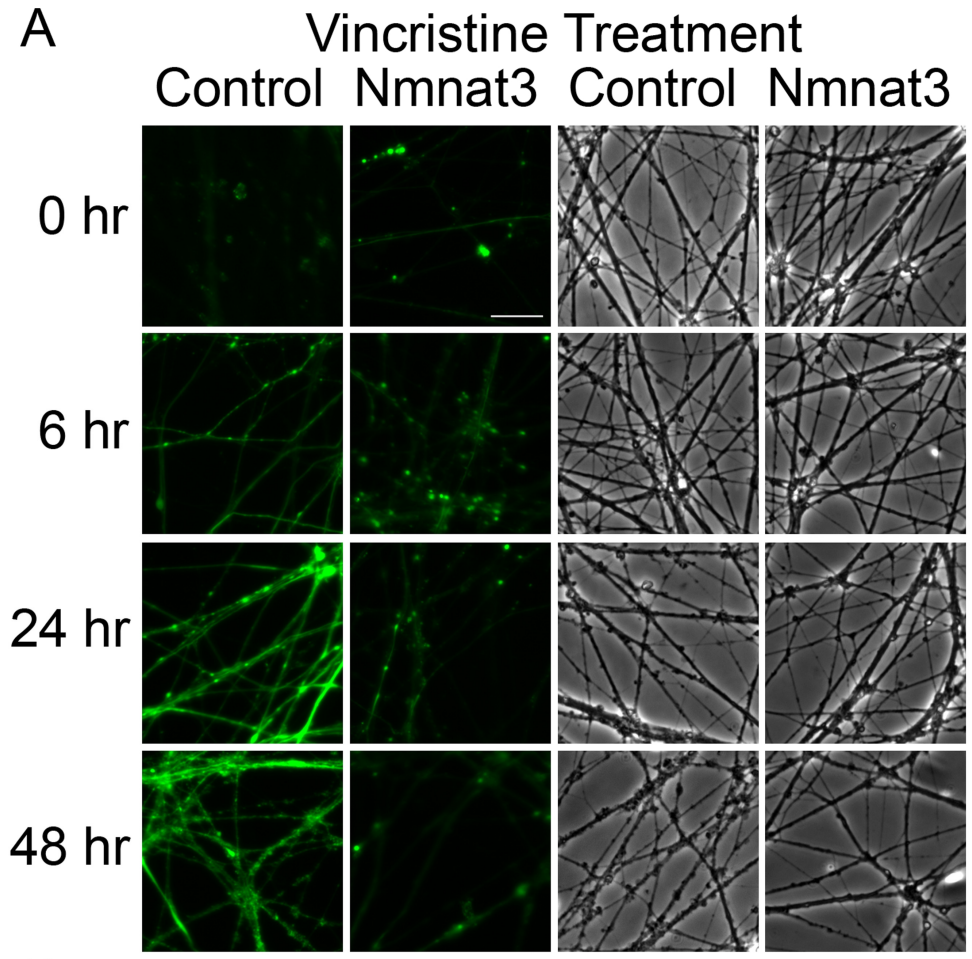


FIGURE 4



B

ROS accumulation

Condition	*0 Hours*	*6 Hours*	*24 Hours*	*48 Hours*
Control	100 ± 4.66	176.7 ± 11.212#	343.54 ± 34.31#	383.74 ± 38.55 #
Nmnat3	106.0 ± 9.7	141.41 ± 8.96	144.16 ± 9.78 *	140.79 ± 17.3 *

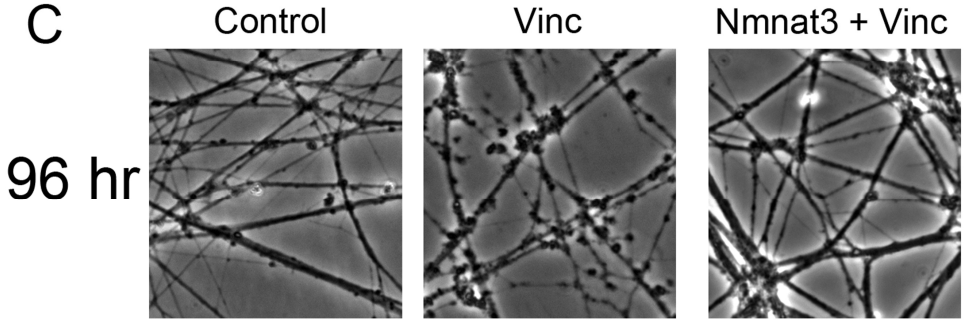


FIGURE 5

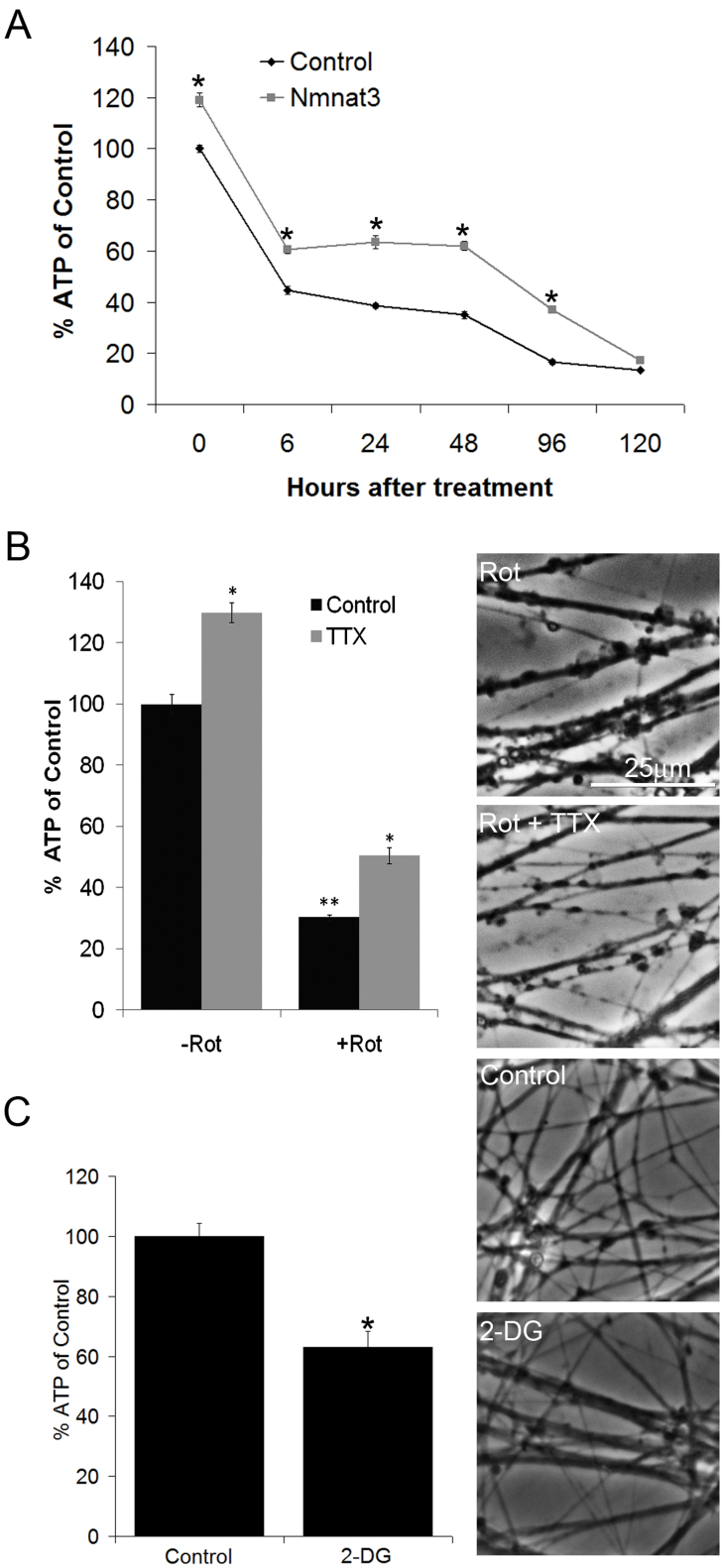
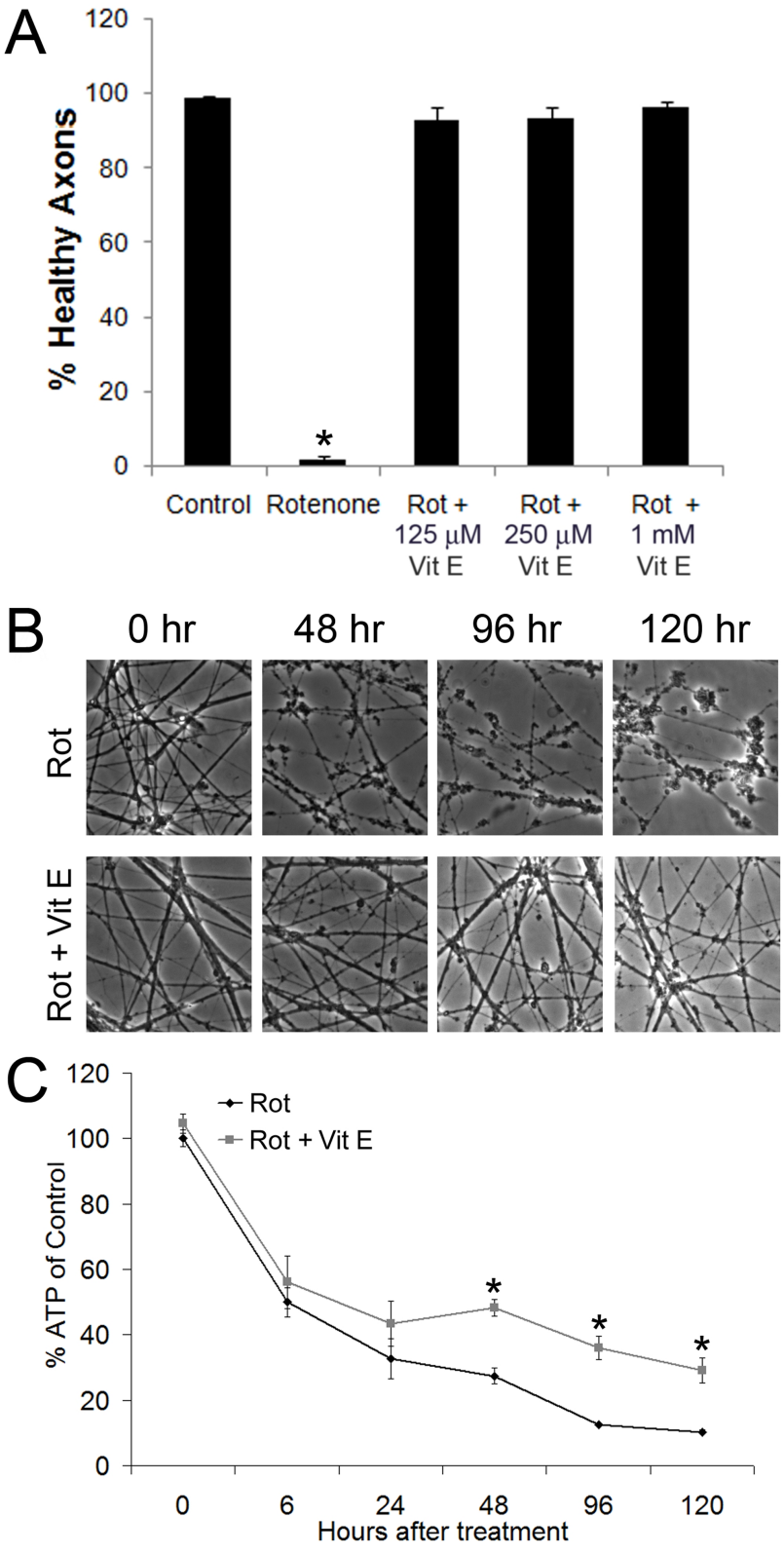
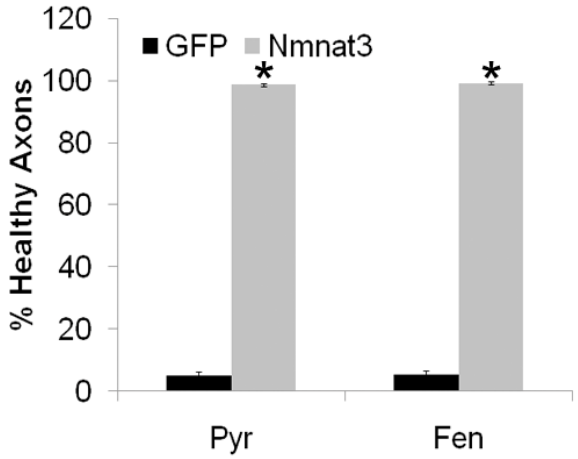

A
140
120
100
80
60
40
20
0
% ATP of Control
Control
Nmnat3
*
*
*
*
*
0
6
24
48
96
120
Hours after treatment
B
140
120
100
80
60
40
20
0
% ATP of Control
Control
TTX
*
**
*
-Rot
+Rot
Rot
25μm
Rot + TTX
C
120
100
80
60
40
20
0
% ATP of Control
*
Control
2-DG
Control
2-DG

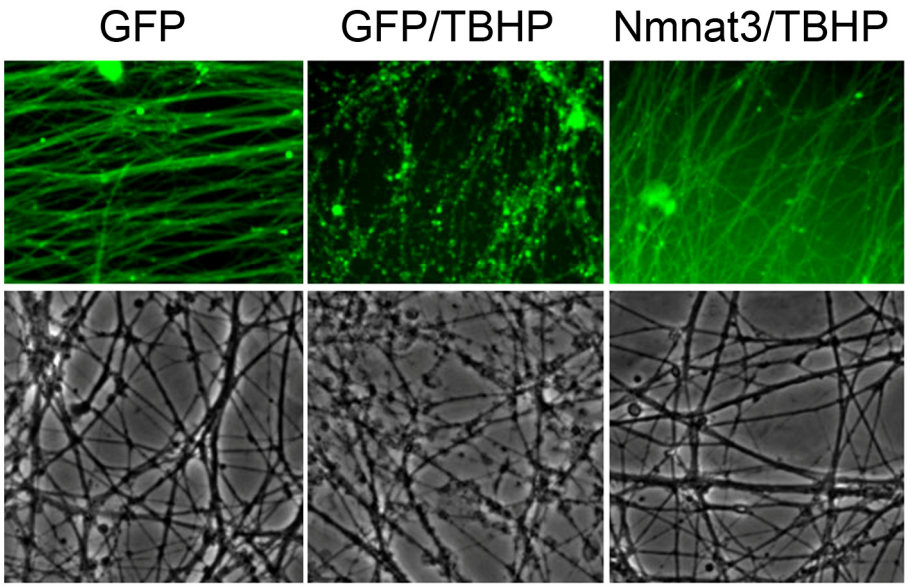
FIGURE 6


A
120
100
80
60
40
20
0
% Healthy Axons
*
Control
Rotenone
Rot + 125 µM Vit E
Rot + 250 µM Vit E
Rot + 1 mM Vit E
B
0 hr
48 hr
96 hr
120 hr
Rot
Rot + Vit E
C
120
100
80
60
40
20
0
% ATP of Control
Rot
Rot + Vit E
*
*
*
0
6
24
48
96
120
Hours after treatment

SUPPL. 1



SUPPL. 2



CHAPTER 3

Wallerian Degeneration is an Active Process Promoted by DLK and JNK

This manuscript is in preparation for publication

Bradley R. Miller, **Craig Press**, Richard W. Daniels, Jeffrey Milbrandt, Aaron DiAntonio. Wallerian Degeneration is an Active Process Promoted by DLK and JNK

C.P. was directly involved in the work presented in Figures 2 and 3 including the initial *in vitro* mouse experiments that demonstrated that DLK is required for normal axonal degeneration in mammals. The DLK knockout mice were developed by B.R.M. in A.D.'s laboratory. **C.P.** contributed to the cultures utilized in Figure 3 and applied the vehicle and drugs used in Figure 3A-C. **C.P.** provided intellectual input to every part of the project including manuscript preparation. R.W.D. assisted in the preparation, sectioning and EM of the nerves in Figure 4. B.R.M., a graduate student in A.D's laboratory, was involved in all aspects of the work presented in this chapter, including manuscript preparation. The *in vitro* mouse work was carried out in the laboratory of J.D.M.

Abstract

Axon degeneration underlies many common neurological disorders and is a prevalent cause of disability. Injured axons follow an apparently active and well regulated self-destruction program, termed Wallerian degeneration, but the genes required for this program have not been previously identified. We show that DLK and its downstream target JNK are components of a pathway that promotes Wallerian degeneration. Genetic deletion the *Drosophila* DLK ortholog, Wnd, decreases Wallerian degeneration *in vivo*. Wallerian degeneration is diminished in DLK mutant mice after axotomy of DRG axons *in vitro* and following sciatic nerve transection *in vivo*. Pharmacological inhibition of JNK during the first three hours of axotomy, well before axon fragmentation begins, is necessary and sufficient to decrease axon degeneration, suggesting that this pathway acts within neurons as injured axons commit to degenerate, rather than controlling the neuron's pre-injury competence to degenerate or the execution phase of the degeneration program. Axon degeneration induced by vincristine, a chemotherapeutic agent whose dose limiting side effects include neuropathy, is also decreased in DLK mutant mice. Thus, diverse insults may activate a common DLK dependent axon degeneration program. The identification of a signaling pathway that promotes axon degeneration opens new avenues for the development of therapies aimed at minimizing axon loss and the resulting neurological disability.

Introduction

Axon degeneration is a common feature of many neurological ailments including mechanical injury, exposure to neurotoxins, hereditary neuropathies, glaucoma, and neurodegenerative diseases such as Alzheimer's Disease and Parkinson's Disease[1-3]. Axon loss is a direct cause of neurological impairment and it also often proceeds and promotes cell body dysfunction and death. Despite the diversity of insults that lead to axon loss, axons tend to degenerate following a stereotyped progression of morphological changes. This breakdown process, termed Wallerian degeneration and first described in the 1850s, is hypothesized to be an active and highly regulated process[4,5]. Yet, no loss-of-function mutants with diminished Wallerian degeneration have been identified and the genetic underpinnings of axon degeneration remain unknown. What causes axons to degenerate in such a stereotyped fashion? There may be a common axon self-destruction program that is triggered by a wide range of insults. In support of this hypothesis, pharmacological inhibition of the ubiquitin proteasome system[6-8], calpain proteases[9-12], and trypsin-like proteases[13] decreases Wallerian degeneration, suggesting that regulated protein degradation may relieve an inhibitory restraint on the degenerative mechanism. Preventing rises in Ca^{2+} can also be protective[14], suggesting that Ca^{2+} may trigger signal transduction cascades that promote axon degeneration. Finally, over-expression of the chimeric protein Wld^{s} [15] and nicotinamide mononucleotide adenylyltransferase (Nmnat)[16] dramatically delays axon degeneration in response to multiple insults, providing strong evidence that axon degeneration is not due to passive deterioration of the severed axon following its separation from the neuronal cell body. Thus, the degenerative process is

likely active, and diverse neuronal insults may trigger a common self-destruction mechanism within the axon. However, the molecular pathway that promotes axon degeneration is unknown.

Which signal transduction pathways are likely to mediate Wallerian degeneration? Wallerian degeneration is conceptually similar to apoptosis: each appears to be an intrinsic process that is primed and waiting for a triggering stimulus to activate the execution phase. Degenerating axons exhibit microtubule depolymerization, altered axonal transport, mitochondrial dysfunction, increased reactive oxygen species, phosphatidylserine exposure, membrane blebbing, and axon fragmentation[1-5]. Many of these pathological changes are shared with neuronal apoptosis. Despite these similarities, current experimental data suggest that the molecular pathways are distinct. A variety of manipulations that block neuronal apoptosis do not prevent Wallerian degeneration[17-19]. However, the final stages of apoptosis and Wallerian degeneration, the phagocytosis of cellular debris by surrounding cells, share some common molecular features. Loss-of-function mutations that delay clearance of cellular debris also interfere with the clearance of axon fragments and thereby delay some aspects of Wallerian degeneration[8,20,21]. Importantly, these mutations do not directly affect the pathways within axons that promote degeneration. Hence, the intrinsic neuronal pathways that orchestrate axon breakdown in injury and disease remain unidentified.

Loss of DLK/Wnd inhibits axonal degeneration in *Drosophila*

Many insults, such as trophic withdrawal and axotomy, can induce both neuronal apoptosis and axon degeneration. Although inhibition of the effectors of neuronal apoptosis does not decrease axon degeneration, some upstream regulators of these

effectors are highly expressed in axons and are activated by axonal insults. These are good candidates as components of the axon degeneration pathway. One such molecule is dual leucine kinase (DLK), a mitogen activated protein kinase kinase kinase (MAP3K) involved in axonal transport[22], axon pathfinding[23], neuronal migration[23], and neuronal apoptosis[24]. 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[25]. We hypothesized that DLK is a component of the molecular pathway that promotes axon degeneration. We tested this hypothesis using a well-established *Drosophila* axon degeneration model[8,26]. We expressed green fluorescent protein (GFP) in a subpopulation of olfactory receptor neurons (ORNs). ORN cell bodies are located peripherally in the antennae, and their axons extend into the brain and terminate in glomeruli of both the ipsilateral and contralateral antennal lobes, which are connected by a commissure (Fig. 1A). To sever these axons and induce axon degeneration, the antennae are surgically removed. In this paradigm, most wild-type axons degenerate within 24 hours, and this degeneration can be delayed by expressing the Wld^{s} [8] in the ORNs. The degree of degeneration is scored based on the presence or absence of the commissure formed by the ORN axons connecting the two lobes[8]. Mutants in the *Drosophila* ortholog of DLK, Wnd, have substantially diminished axon degeneration compared to control flies (Fig. 1B,C). The commissure was visible in 4 out of 32 control flies and 18 out of 27 Wnd/DLK mutant flies (Chi-square, $p<0.001$). Wnd/DLK is therefore required for normal axon degeneration in *Drosophila*.

Loss of DLK/Wnd inhibits axonal degeneration in mouse DRG cultures

Having demonstrated that Wnd/DLK promotes axon degeneration in flies, we wished to determine if DLK has a similar activity in mammals. To assess degeneration, we used the *in vitro* dorsal root ganglion (DRG) axotomy model. We cultured embryonic mouse DRGs for 14-16 days to allow their axons to radiate from the central core of cell bodies before severing the axons with a micro-scalpel. After 24 hours, transected wild-type axons are dramatically degenerated. The initially smooth and continuous axonal processes become rough and irregular axon fragments (Fig. 2). We quantified the extent of axon breakdown by measuring the fraction of axonal area occupied by fragmented axons (degeneration index, DI). When cultures from DLK mutants and littermate controls were axotomized, degeneration of the mutant axons was significantly diminished (Fig. 2). This result was obtained using two independently generated DLK mutant mouse lines[9]. Because non-neuronal cells are eliminated in this DRG culture system, DLK must be required within neurons themselves for the normal axon degenerative response. The identification of loss-of-function mutants with decreased axon degeneration in both flies and mice demonstrates that axon degeneration is an active process driven in part by an evolutionary conserved pathway that includes DLK.

Axon degeneration is triggered by a range of insults in addition to axotomy that are relevant to human disease. Inhibitors of axotomy induced degeneration often also decrease axon loss in mouse models of these diseases, suggesting that a common axon self-destruction pathway is employed[1,4]. To investigate whether DLK participates in such a common pathway, we assessed the response of DLK mutant DRG axons to vincristine toxicity. Vincristine is a chemotherapeutic drug that inhibits microtubule formation, and

whose dose-limiting side effects in patients include peripheral neuropathy due to axon degeneration[27]. In cultured DRG neurons, applying vincristine triggers an axon degeneration that is morphologically similar to that induced by axotomy[28] (Fig. 2). DLK mutant axons are protected from vincristine toxicity, with a significantly decreased degenerative response relative to control axons (Figure 2). Hence, DLK promotes axon degeneration in response to both axotomy and neurotoxin exposure. This result suggests that DLK is a component of a common axon self-destruction pathway utilized by both axotomy and vincristine induced axon degeneration.

Inhibition of JNK slows axonal degeneration mouse DRG cultures

DLK is a MAP3K that activates the MAP kinases JNK and/or p38 in a variety of systems[29]. To determine whether the axon degeneration pathway requires either JNK or p38, we used pharmacological inhibitors of each MAP kinase in the DRG axotomy model. Wild-type DRG cultures were treated with the JNK inhibitor SP600215 and the p38 inhibitor SB203580. Inhibition of JNK, but not p38, decreased degeneration of DRG axons following axotomy (Figure 3B,C). Thus JNK, like DLK, is a component of an intrinsic pathway that promotes axon 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[3]. 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 would be required prior to axotomy. We found

that this is not the case since application of the JNK inhibitor 24 hours prior to axotomy followed by a wash just before axotomy is not protective (Figure 3D). In contrast, JNK inhibition started concurrently with axotomy is protective (Figure 3E). JNK therefore does not control the axon's pre-injury competence to degenerate, but instead it is required in the severed axon itself to promote axon degeneration.

One hallmark of Wallerian degeneration is the substantial delay between the onset of axonal injury and the initiation of rapid axon breakdown, suggesting that a signaling pathway commits the axon to a breakdown program during this delay. To assess whether JNK is involved in the commitment or execution phase of axon breakdown, we added the JNK inhibitor 3 hours after axotomy, which is approximately 12 hours before substantial fragmentation begins. We found that JNK inhibition beginning 3 hours post-axotomy, and continuing for the rest of the experiment in order to span the transition to the execution phase, does not decrease axon degeneration (Figure 3F). Thus, JNK inhibition during the execution phase is not sufficient to decrease degeneration. We next wished to determine if inhibiting JNK only during the early phase is sufficient to decrease axon degeneration. When the JNK inhibitor is added concurrently with the axotomy and then washed off 3 hours post-axotomy, axon degeneration is decreased (Figure 3G). Thus, JNK activity during this early period, the hypothesized commitment phase, is both necessary and sufficient to promote axon degeneration.

Mice lacking DLK/Wnd have slowed axonal degeneration *in vivo*

There are many differences between *in vitro* and *in vivo* models of axon degeneration, and manipulations that diminish degeneration *in vitro* do not always show the same effect *in vivo*[30]. We therefore investigated whether DLK plays a role in axon

degeneration *in vivo* in mice. Sciatic nerve transection is a simple and well-characterized *in vivo* model of Wallerian degeneration. Most wild-type axons degenerate within 52 hours post-transection[31] (Fig. 4). In the DLK mutants, however, many axons are spared (Fig. 4). We find a more than two-fold increase in the number of non-collapsed axonal sheaths from sciatic nerve sections of DLK mutants distal to the axotomy site. In wild-type axons distal to the axotomy, electron microscopic analysis reveals degenerating myelin sheaths, collapsed axonal sheaths, and few recognizable mitochondria or microtubules in the remaining axons. In the DLK mutants, these pathological features are much less prominent, with less degeneration of the surrounding myelin and many more axons containing mitochondria and microtubules (Fig. 4). The finding that DLK is required *in vivo* for normal axon degeneration validates the relevance of our *in vitro* results and demonstrates that the DLK pathway may be a new potential therapeutic target for mitigating axon degeneration.

Conclusion

Axon degeneration is a shared feature of many neuropathological conditions. The protective effects of Wld^{S}/Nmnat over-expression and proteasome inhibition suggest that there is an intrinsic axon self-destruction program, but the components of this program had not been previously identified. We have now shown that the MAP3K DLK and its downstream MAPK JNK are critical factors in the axon degeneration program. Genetic deletion of Wnd/DLK in flies, DLK in mice, and pharmacological inhibition of JNK all diminish axotomy-induced Wallerian degeneration. Thus, the conceptually and morphologically similar processes of apoptosis and axon degeneration share some molecular components. This JNK pathway acts within neurons as injured axons commit

to degenerate, rather than controlling the neuron's pre-injury competence to degenerate or the execution phase of the degeneration program. Inhibition of this pathway also decreases axon degeneration in response to the chemotherapeutic agent vincristine, whose dose-limiting side effects include neuropathy, suggesting that a common axon self-destruction program orchestrates axon breakdown in response to multiple insults. The identification of a signaling pathway that promotes axon degeneration opens new avenues for the development of therapies aimed at minimizing axon loss and the resulting neurological disability.

Materials and Methods

Drosophila Wallerian degeneration

We assessed Wallerian degeneration in adult control and Wnd mutant flies following established methods[8,26]. We expressed green fluorescent protein (GFP) in a subpopulation of olfactory receptor neurons (ORN) using Or47bGal4 and UASmcd8GFP (Bloomington Stock Center). We used Wnd mutants *wnd1/wnd2* as described[32]. 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[8]. Statistical significance was determined using the Chi-square test.

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[9]. We also developed a conditional DLK knockout by flanking the exons that encode the kinase domain with LoxP sites using homologous recombination. We generated a constitutive DLK knockout allele by breeding these mice to a cre-recombinase line with germline expression (zp3-cre[33]; 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 described[16] were all used for the DRG cultures presented in Fig. 2. We used the genetrap DLK strong hypomorphic mutant for the *in vivo* experiments presented in Fig. 3 because the constitutive DLK mutant is perinatal lethal.

Mouse DRG culture preparation, treatment, and analysis

DRGs were cultured from embryonic day 12.5-14.5 mice in 24 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 40 minutes at 37°C before the addition of 500μL medium. Wild-type cultures in Fig. 3 were made 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.

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 program written in NIH ImageJ that calculates the fraction of axonal area occupied by fragmented axons. This enabled us to sample an order of magnitude greater area than by manual analysis. The mean DI of each well was calculated by averaging the DIs of the 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). There is no significant increase in DI 3 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. DI's are presented in the text normalized to the indicated

control DI for each independent experiment. When only 2 conditions or genotypes were compared, Student's t-test was used. When more than 2 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 trasection 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 500nm for Toluidine blue staining and 70 nm for 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. Statistical significance was determined using Student's t-test.

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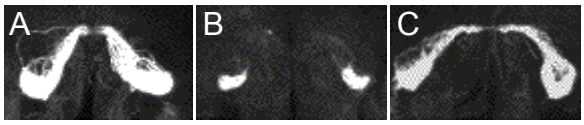
FIGURE 1

Fig 1: Wallerian degeneration is delayed in Wnd/DLK mutant flies.



A. Non axotomized ORN axons expressing GFP.
B. Degenerated WT axons 24hrs post-axotomy.
C. Wnd/DLK mutant axons 24hrs post-axotomy.

FIGURE 2

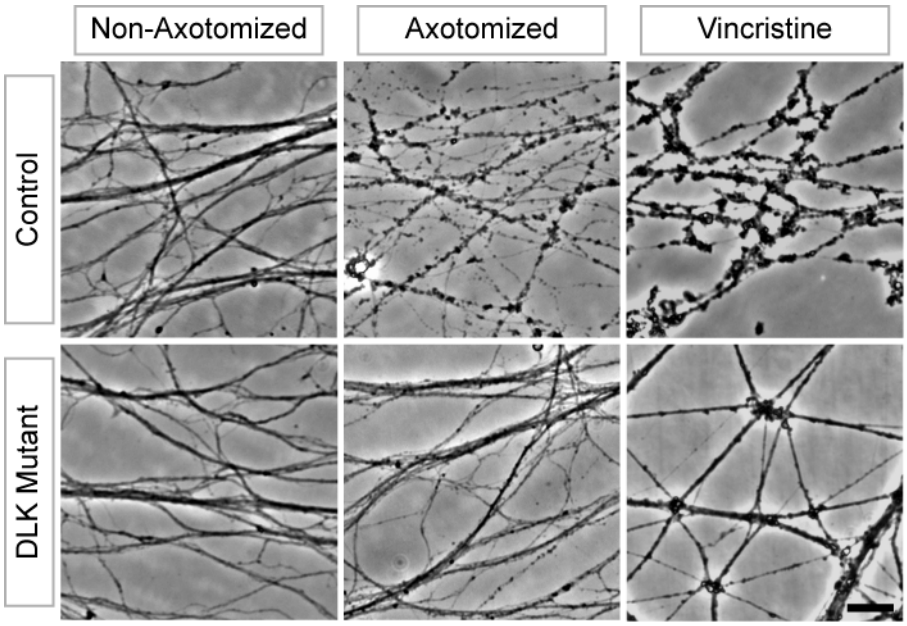


Fig. 2. Normal axon degeneration in response to axotomy and vincrisine requires DLK. Phase contrast images of DRG axons from DLK mutants and littermate controls. Axotomized DLK mutant axons have a 65% ± 3.2% (sem) decrease in degeneration index (DI) relative to controls ($p<0.0001$, Student's t-test) 24 hours post-axotomy. Vincristine treatment induces 59% ± 6.3% (sem) less DI in DLK mutants relative to controls ($p<0.002$, Student's t-test) after 48 hours. Scale bar = 20 μm.

FIGURE 3

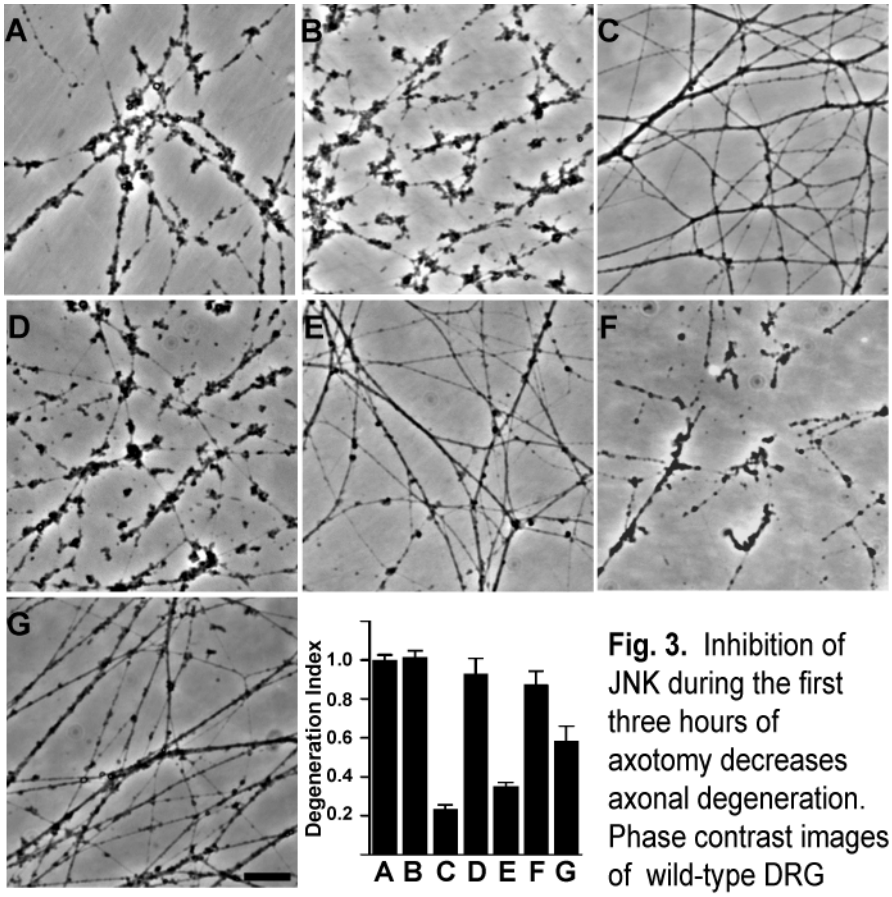


Fig. 3. Inhibition of JNK during the first three hours of axotomy decreases axonal degeneration. Phase contrast images of wild-type DRG axons 24 hours post-axotomy. Unless noted, vehicle and inhibitors were added 24 hours pre-axotomy and left on for the duration of the experiment. (A) Vehicle (DMSO). (B) SB203580 (P38 inhibitor). (C) SP600215 (JNK inhibitor). (D) SP600215 added 24hr pre-axotomy, and removed just before axotomy. (E) SP600215 added concurrently with axotomy. (F) SP600215 added 3 hours post-axotomy.(G) SP600215 added concurrent with axotomy and removed 3 hours post-axotomy. Conditions C, E, and G are significantly less degenerated than A ($p<0.001$, ANOVA, posthoc Tukey's means comparison, bars are sem). Scale bar = 20 μm.

FIGURE 4

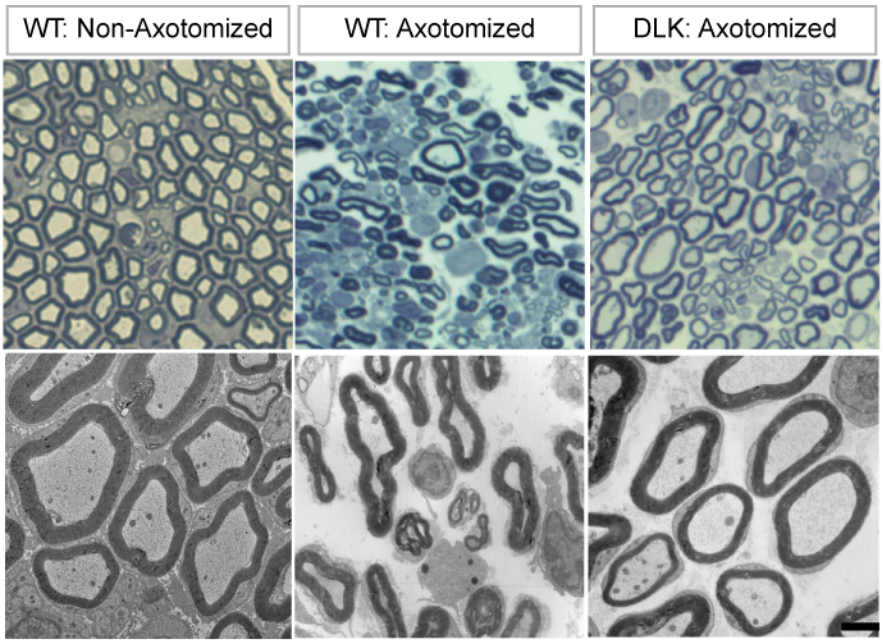


Fig. 4. DLK mutant mice have decreased Wallerian degeneration *in vivo*. Sciatic nerve cross-sections distal to the axotomy stained with Toluidine blue (top row) or imaged by EM (bottom). 52 hours post-axotomy, DLK mutants have 208% ± 22% (se) axons per μm^2 compared to wild-type ($p<0.007$, t-test, n = 4 wild-type sciatic nerves and 5 DLK mutant sciatic nerves). By EM, mitochondria and microtubules are abundant in the axotomized DLK mutants but not in the axotomized wild-types. EM scale bar = 2 μm.

CHAPTER 4

Purines prevent axotomy induced degeneration by a local mechanism

This manuscript is in preparation for publication

Craig Press, Jeffrey Milbrandt. Purines prevent axotomy induced degeneration by a local mechanism

C.P. was involved in all aspects of the work presented in this chapter including manuscript preparation.

Abstract

Axonal degeneration is a key component of neurodegenerative diseases. Our current understanding of axonal degeneration is based on observations after anoxic injury and mechanical severing. The discovery of inhibitors of the degenerative pathway including overexpression of nicotinamide mononucleotide adenylyltransferase (Nmnat), treatment with nicotinamide adenine dinucleotide (NAD^+), and resveratrol have suggested that there is an active pathway, like apoptosis, that can be inhibited. We have discovered that the purine nucleosides adenosine and guanosine, but not inosine are able to slow mechanically induce axonal degeneration in culture. Addition of adenosine, either prior to or up to 6hr after axonal injury, was protective. Furthermore, adenosine was necessary during the protective period to maintain protection. These findings suggest that endogenous purines may play a role in regulating axonal degeneration and present a new therapeutic target for neurodegenerative diseases with axonal degeneration.

Introduction

Neurodegenerative diseases including Parkinson's Disease (PD), Alzheimer's (AD), Lou Gehrig's Disease or Amyotrophic Lateral Sclerosis(ALS), Charot-Marie Tooth (CMT), Multiple Sclerosis (MS), mechanical nerve injury, diabetic neuropathy, and drug induced neuropathies have a pathology that includes axonal degeneration and affect millions of people (Coleman and Perry, 2002; Coleman, 2005). Unfortunately, the molecular mechanisms that underlie axonal degeneration and the factors that regulate it are poorly understood. Recently, axonal degeneration has been discovered to be an active process much like apoptosis (Raff et al., 2002). This understanding is a result of the study of a mutant mouse, *Wld*^{s}, which manifests slowed axonal degeneration (Lunn et al., 1989) . The *Wld*^{s} mouse is resistant to a number of mouse models of neurodegenerative disease both genetic and toxin induced (Wang et al., 2002; Ferri et al., 2003; Samsam et al., 2003; Sajadi et al., 2004; Mi et al., 2005; Gillingwater et al., 2006a; Hasbani and O'Malley K, 2006). The diversity of diseases that affect axonal degeneration and are slowed in the *Wld*^{s} mouse suggest that there is a common pathway to axonal degeneration that can be manipulated (Coleman, 2005).

The mutation responsible for the *Wld*^{s} phenotype is a tandem triplication of a gene fusion containing the N-terminal 70 amino acids of ubiquitination factor 4b (Ube4b/Ufd2a), an 18 unique amino acid linking region and the full length coding region of nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1) (Conforti et al., 2000).

The effects of *Wld*^{s} have been replicated in an *in vitro* with cultures of mouse dorsal root ganglia (DRG) and sympathetic ganglia (Deckwerth and Johnson, 1994; Wang et al., 2005; Conforti et al., 2006). Subsequently, it was shown Nmnat1 (or

Nmnat3) overexpression is sufficient in cultures to slow axonal degeneration from a variety of insults and that this protection was dependent upon the enzymatic activity of Nmnat (Araki et al., 2004; Wang et al., 2005; Sasaki et al., 2006; Press and Milbrandt, 2008).

Nmnat is known to convert nicotinamide mononucleotide (NMN) to nicotinamide adenine dinucleotide (NAD^+) using NMN and ATP as substrates (Berger et al., 2005). NAD^+ was shown to be able to slow axonal degeneration after axonal severing *in vitro* and this effect is hypothesized to have a nuclear effect in one case and a local effect it the other depending on concentration and the culture conditions (Araki et al., 2004; Wang et al., 2005). Interestingly, other precursors to NAD^+, including NMN, were also able to stimulate axonal protection *in vitro* and *in vivo* (Kaneko et al., 2006; Sasaki et al., 2006). It has recently been reported that purine nucleosides were able to delay neuronal cell death and increase neurite outgrowth in response to rotenone (Bocklinger et al., 2004). These two findings spurred us to explore the potential for other NAD^+ derivative nucleotides to provide axonal protection. We used *in vitro* DRG axonal cultures that were axotomized as a model of Wallerian degeneration. Here we report that adenosine slows axonal degeneration in a dose dependent fashion. Adenosine is protective when either added prior to axotomy or several hours after injury. We go on to show that exogenous adenosine is necessary for the maintenance of the protective effect. Finally, we demonstrate that in addition to adenosine, guanosine is protective at similar concentrations while inosine is not.

Materials and Methods

Reagents: All reagents are from Sigma-Aldrich unless otherwise noted

Culture of DRGs: Tissue culture plates are coated with poly-D-lysine and laminin (Invitrogen, Carlsbad, California). Plates are initially coated with 250 µl of 0.1 mg/ml poly-D-lysine (sigma) solution for overnight. Then poly-D-lysine solution was removed and wells were washed twice with 500 µl of water and place in a culture hood until dry. 250 µl of 2 to 5 µg/ml of mouse laminin solution was added to each well and incubated for 1 to 2 hours. The laminin solution was then removed and the plates were dried in the culture hood prior seeding.

DRGs were collected from CD1 mouse embryos at the gestation days between e12.5 and e13.5. Approximately 45-50 DRGs are removed from each embryo. DRGs from 6 embryos were collected into single 1.5 ml microfuge tube containing DMEM for 6 24-well plates. After centrifugation (2,000 x *g*, briefly) the supernatant was removed and 500 µl of solution containing 0.05% trypsin and 0.02% EDTA was added and incubated at 37°C for 15 min. After the incubation DRGs were triturated by using 1000 µl pipette until the DRG clumps are disrupted. The cell suspension was centrifuged (2,000 x *g*, briefly) and supernatant was removed and suspended in 500 µl of complete media containing, Neurobasal media (Invitrogen) containing 0.02% B27 (Invitrogen) and 50 ng/ml of NGF (2.5S; Harlan Bioproducts, Indianapolis, IN). Cell suspension was centrifuged again (2,000 x *g*, briefly), the supernatant was removed and complete media was added to the cell pellet at the ratio of 50 µl to one dissected embryo. Two micro liter of cell suspension was placed as a drop slightly below the center of each 24 well coated with poly-D-lysine and laminin and incubated at 37°C with 5% CO_2 for 1 hour. After the

attachment of cells, 500 µl complete media plus 1 µM 5-fluoro-2′-deoxyuridine, and 1 µM uridine was added to each well. The 125ul of media was removed and 150ul was replaced each 4 to 5 days with media to account for evaporation. In this condition, DRG cell bodies are clustered within 3 to 5 mm diameter in the lower part of wells and axons are extending in radially. Axons are severed by a micro-scalpel (Fine Science Tools, Foster City, CA) after 14 days in vitro (DIV).

Quantification of axonal degeneration. Axonal degeneration was quantified as described elsewhere (Sasaki and Milbrandt, 2008). Briefly, after axonal severing, phase contrast images were taking with an inverted microscope with a 20x objective (Eclipse TE 300; Nikon). For each wells, 3-4 random fields of distal axons were imaged by using CCD camera (Cool SNAP ES; Nikon) and Metamorph software (Molecular Devices) with 40 ms exposure time. Images were adjusted for brightness and background intensity by the auto-level function and converted to 8-bit in Adobe Photoshop (Adobe, San Jose, CA) and analyzed by Image J (NIH). To obtain the total area of axon, images were binarized. Non degenerated axons have continuous tracts, while unhealthy degenerating axons were fragmented and beaded showing up as aggregates. The total axonal area was determined by the total number of detected pixels after the imaged was binarized. Degenerated axons were detected using the particle analyzer of Image J as small particles, while healthy axonal area was represented by large continuous areas. The degeneration index (DI) is the ratio of fragmented axon area to total axon area. ≥20 fields were evaluated per condition combined from multiple independent experiments and the data presented are adjusted representative images from repeated experiments.

Statistics: All comparisons were analyzed by student's t-tests with Bonferroni's correction for multiple comparisons.

Results

Exogenous adenosine slows Wallerian degeneration

Purine nucleosides have been show to have trophic effects on neurons and support axonal growth (Bocklinger et al., 2004). This led us to explore the effects of exogenous purine nucleosides on axonal degeneration. To address this question we used a previously developed model of *in vitro* axonal degeneration. Mouse DRGs were cultured from E13.5 embryos by spotting the neurons in an isolated drop on a culture dish from which axons grow out of to form a halo of axons. The cultures were maintained for 14 DIV and severed using a micro-scalpel. Using the percent of axonal area that was fragmented we determined a degenerative index (DI). Higher numbers (0.0-1.0) indicate more fragmentation and thus degeneration. After 24 hr the axons exhibit a significant amount of swelling, beading and fragmentation (DI=0.62±0.02) (Fig. 1). To determine the effect of adenosine on axonal degeneration we added a range of adenosine concentrations to cultures 24hr prior to axotomy. We monitored the axonal degeneration over 96 hr following axotomy. Adenosine was protective after 24 hr at all doses including 1.25mM when added 24hr prior to axonal injury (1.25 mM DI=0.28± 0.02, 2.5 mM DI=0.17± 0.01, 5 mM DI=0.16± 0.01, 10 mM DI=0.13± 0.01) (Fig. 1). There was a dose dependent increase in the duration of protection where 2.5 mM, 5.0 mM and 10 mM adenosine were protective for 48hr, 72hr and 96hr respectively (Fig. 1).

We compared this level of protection to two compounds known to inhibit axonal degeneration, 5 mM NAD^+ and the JNK inhibitor SP600125 (15 μM) (Araki et al., 2004; Wang et al., 2005; Miller, 2008). Compared to NAD^+ treatment and JNK inhibition, adenosine was a more potent inhibitor of axonal degeneration at 48 hr after injury (5 mM NAD^+ DI=0.54±0.04, JNK inhibitor DI=0.47±0.06 vs. 10 mM adenosine DI=0.19±0.01) (Fig. 1). These data demonstrate that adenosine is the most efficacious small molecule inhibitor of axonal degeneration known to date and that the duration of this effect is dose dependent.

Adenosine is protective both pre- and post- axotomy

To determine if the effects of adenosine are mediated by a local mechanism or via actions in the cell body and/or nucleus we treated cultures with 10 mM adenosine 24 hr prior to, immediately afterwards, or 6hr after injury and measured the degeneration index for 72 hr after the injury. Treatment at all times both pre- and post-axotomy were protective after 24 hr (Fig. 2). The level of protection was dependent, in a significant way, on the time of the treatment with pretreatment (DI=0.13±0.01) giving the strongest protection, followed by immediate treatment (DI=0.18±0.01) and treatment 6 hr after injury being slightly weaker (DI=0.23±0.02), but better than no treatment (DI=0.64±0.02). This time dependent effect was also apparent after 48 hr when pretreatment (DI=0.12±0.01) was stronger than 6 hr post-axotomy (DI=0.43±0.04) (Fig. 2). 72 hr after the injury all treatments were less fragmented than control (DI=0.66±0.03), however pretreatment (DI=0.21±0.02) with adenosine was significantly better than either post-axotomy treatments (Post-axotomy DI=0.51±0.03 and 6 hr post-

axotomy DI=0.54±0.03) (Fig. 2). These data demonstrate that adenosine has a local mechanism of protection and can be a potent modulator of axonal degeneration even when applied several hours after injury. Importantly, this rules out a role for transcriptional changes since the axon is not able to synthesize new message after injury.

Adenosine is necessary after injury to maintain protection

While it was clear that adenosine was sufficient to slow axonal degeneration even when added many hours after injury it was not clear if adenosine was only needed during a critical window (i.e. the first 3-6 hr after injury) or if it was needed during the entire period of protection. To determine if adenosine was necessary to maintain protection after the initial 24 hr when most axons would have normally degenerated (Fig. 1), we treated cultures with 10 mM adenosine 24 hr prior to injury and 24 hr after injury we replaced it with media either containing or lacking 10 mM adenosine three times. We then followed the axonal degeneration 24 hr after the wash step. Removing adenosine from the media resulted in rapid degeneration within 24 hr (Fig. 3). Cultures that were washed with media lacking adenosine were significantly more fragmented (DI=0.64±0.02) than those with washed media still contained adenosine (DI=0.19±0.01). Cultures without adenosine for the duration were still significantly worse than either condition (DI=0.73±0.02) (Fig. 3). This suggests that adenosine is necessary for the duration of protection rather than during a critical early time window.

Guanosine, but not inosine slows Wallerian degeneration

Knowing that adenosine was protective, we explored the protective potential of additional purine nucleosides including guanosine and inosine. Due to the limited solubility of guanosine we were only able to test up to 2.5 mM in warmed media. We compared the effects of pretreatment with 2.5 mM guanosine and adenosine 24 and 48 hr after axonal injury. 2.5 mM Guanosine slowed axonal degeneration after 24 hr (Control DI=0.68±0.02 vs. Guanosine DI=0.30±0.02), but not 48 hr (Control DI=0.69±0.02 vs. Guanosine DI=0.71±0.06) (Fig. 4A). However, inosine up to 10 mM added 24 hr prior to axotomy was not able to slow axonal degeneration at 24 hr after injury, in fact it was significantly worse than control (Control DI=0.64±0.02 vs. Inosine DI=0.73±0.02) (Fig. 4B). This data suggests that adenosine and guanosine are protective and that this mechanism is not likely via conversion to inosine by extracellular enzymes, although it does not exclude intracellular metabolism.

Discussion

Following evidence that suggests that NAD^+ precursors are able to slow axonal degeneration (Sasaki et al., 2006) and evidence that purine nucleosides have trophic effects on *in vitro* neuronal cultures (Bocklinger et al., 2004), we investigated the potential role for other metabolites in axonal degeneration following mechanical injury *in vitro*. Adenosine added exogenously to cultures robustly delayed axonal degeneration and is the most efficacious chemical inhibitor amongst those we have tested date (Fig. 1). This protection was apparent up to 96 hr post injury and the duration of protection was dose dependent (Fig. 1). Adenosine protection was present when added up to 6 hr post

axotomy and was dependent upon maintenance of adenosine in the media during the protective period (Fig. 2, 3). Finally, we also demonstrated that guanosine was protective at similar doses to adenosine, while inosine was not protective (Fig. 4). It should be noted that the effect of adenosine and guanosine could be independent or based on the same mechanism.

These results suggest that extracellular or exogenous purine nucleosides play a role in the timing of axonal degeneration in a local, non-transcription dependent manner. This is potentially important since adenosine is well known to accumulate after neuronal injury, including stroke, and its metabolism is altered after peripheral injury (Sawynok and Liu, 2003; Stone et al., 2007). Adenosine has at least two major modes of action 1) receptor mediated effects and 2) intracellular interactions via membrane transport (Fredholm et al., 2001).

Adenosine has four known G-coupled protein receptors, A_1, A_{2A}, A_{2B}, and A_3 receptors. It is thought that A1 and A3 receptors are inhibitory acting through G_i and G_o, while A2 receptors are excitatory acting through Gs, G_{olf}, and G_q (Sawynok and Liu, 2003). The best studied and well characterized receptors are the A_1 and A_{2A} receptors. A_1 receptor agonists and A_{2A} receptor antagonists are known to spare striatal terminals and act as a neuroprotectant against the MPTP model of PD (Lau and Mouradian, 1993; Pierri et al., 2005). This suggests that selectively activating the A_1 receptor while inhibiting the A_{2A} receptor would be beneficial in axonal protection. From *in vivo* data we know that adenosine has analgesic effects after spinal injuries that appear to be a result of A_1 stimulation (Sawynok and Liu, 2003). The data concerning the role of receptor A3 is less clear and suggests both neuroprotective and destructive roles

(Abbracchio and Cattabeni, 1999). Interestingly, we have preliminary data that shows that theophylline, a relatively non-selective adenosine receptor blocker (although weakly at A_3), does not inhibit adenosine mediated protection.

The second major known mechanism by which purines affect cells is through transport via 2 classes of transporters. Purines nucleosides can be transported via either equilibrative (ENT1 - ENT4) or concentrative (CNT1 – CNT5) mechanisms (Podgorska et al., 2005). Purines have been shown to enhance neurite outgrowth in a number of cellular systems that were shown to be, or thought to be, dependent on equilibrative transport dependent and activation of protein kinase N (PKN) (Benowitz et al., 1998, Bocklinger et al., 2004). Using two equilibrative transport inhibitors, dipyridamole and NBTI, increased axonal growth caused by guanosine and inosine was blocked. Further, they demonstrated in PC-12 cells that the PNK inhibitor and purine analog, 6-thioguanine decrease inosine induced axonal growth. In contrast to our work, these studies have seen that inosine is in fact more potent than adenosine. It is known that adenosine is converted to inosine through the activity of adenosine deaminase (ADA) since inhibition of ADA blocked adenosine induced axonal growth (Benowitz et al., 1998). These differences may be due to cell type differences, different culture media or a completely different or novel mechanism.

The different equilibrative transports are blocked to a different extent by the different transport inhibitors. The two best characterized are ENT1 and ENT2. In humans ENT1 is termed the "es" transporter due to its sensitivity to NBTI, while ENT2 is the "ei" transporter since it is less sensitive to NBTI (Podgorska et al., 2005). Initial reports suggest that mouse mENT1 is sensitive to the three inhibitors, dipyridamole,

NBTI, and dilazep, while mENT2 is insensitive to both NBTI and dilazep (Kiss et al., 2000). Unfortunately, the drugs were not tested to their full range of concentrations thus the insensitivity can only be stated as a relative insensitivity. We have preliminary data suggesting that treatment of cultures with adenosine and dilazep or dipyridamole inhibits the adenosine induced protection, while NBTI did not have a consistent inhibitory effect. Dilazep and dipyridamole appeared to induce morphological changes that can be best described as swellings in the DRGs when added alone, although they did not induce rapid degeneration. This may suggest that adenosine needs to be transported to provide protection.

To determine if adenosine and non-adenosine nucleosides can be effective as a therapy for neurodegenerative disorders it will be necessary to test their effectiveness *in vivo*. Unfortunately, due to the extremely short half life of adenosine *in vivo* (Lerman and Belardinelli, 1991)), studies have not been able to test the hypothesis that injections of adenosine would inhibit axonal degeneration. However, guanosine has been used *in vivo* against models of stroke and spinal cord injury. Guanosine injected intraperitoneally (I.P.) was able to decrease cell death in an *in vitro* model of oxygen-glucose deprivation, decrease disability and ischemic volume after middle cerebral artery occlusion (MCAO); however guanosine had no effect on the number of apoptotic cells in the ischemic penumbra (Chang et al., 2008). Guanosine injected I.P. has also been shown to reduce the severity of injury after spinal cord compression, reduce macrophage infiltration, but not astrocyte activation, reduce apoptosis in the spinal cord, and increase axonal sparing (although not in a quantitative assay) (Jiang et al., 2007). Both of these studies discuss the ability of guanosine to accumulate in the brain and be converted to guanine which

could be the protective product. Guanosine has also been used orally (at similar doses to I.P. injections) to interfere with quinolinic acid induced seizures in mice (de Oliveira et al., 2004) suggesting the possibility that supplemented water could be a route of delivery. It will be critical to determine if guanosine or adenosine analogs are able to slow axonal degeneration either after mechanical injury or in a model of neurodegeneration. Further exploration of the pathway responsible for nucleoside mediated protection will allow for better targeting of therapeutic drugs for neurodegeneration.

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Figure Legends

Figure 1

Adenosine slows axonal degeneration in a dose dependent manner. A) Axonal degeneration was monitored using phase contrast microscopy after cultures were incubated with adenosine at various concentrations (1.25mM-10mM), NAD^+ (5mM), or JNK inhibitor SP600125 (15μM). The degeneration was monitored for 96hr. B) Quantitation of the axonal degeneration using the degeneration index (*=$p \leq 0.05$ compared to control at each timepoint).

Figure 2

Adenosine slows axonal degeneration when added pre- or post-axotomy. A) Axonal degeneration was monitored using phase contrast microscopy after cultures were incubated with adenosine either 24 hr prior to, immediately after, or 6 hr after axotomy. The degeneration was monitored for 72 hr. B) Quantitation of the axonal degeneration using the degeneration index (*=$p \leq 0.05$ compared to control at each timepoint).

Figure 3

Adenosine is necessary for the maintenance of protection. A) Adenosine was added to cultures 24 hr prior to axotomy. 24 hr after axotomy, the media was replaced three times with media either containing or lacking adenosine and axonal degeneration was monitored for 24 hr. B) Quantitation of the axonal degeneration using the degeneration

index (*=p≤0.05 compared to control at each timepoint, #=p≤0.05 compared to adenosine containing media).

Figure 4

Guanosine, but not inosine slow axonal degeneration A) Guanosine (2.5 mM) or adenosine (2.5 mM) were added to cultures 24 hr prior to axotomy. The degeneration was monitored with phase contrast microscopy for 48 hr. B) Quantitation of the axonal degeneration using the degeneration index (*=p≤0.05 compared to control at each timepoint). C) Inosine (10 mM) or adenosine (10 mM) were added to cultures 24 hr prior to axotomy. The degeneration was monitored with phase contrast microscopy for 24 hr. D) Quantitation of the axonal degeneration using the degeneration index (*=p≤0.05 compared to control at each timepoint).

FIGURE 1

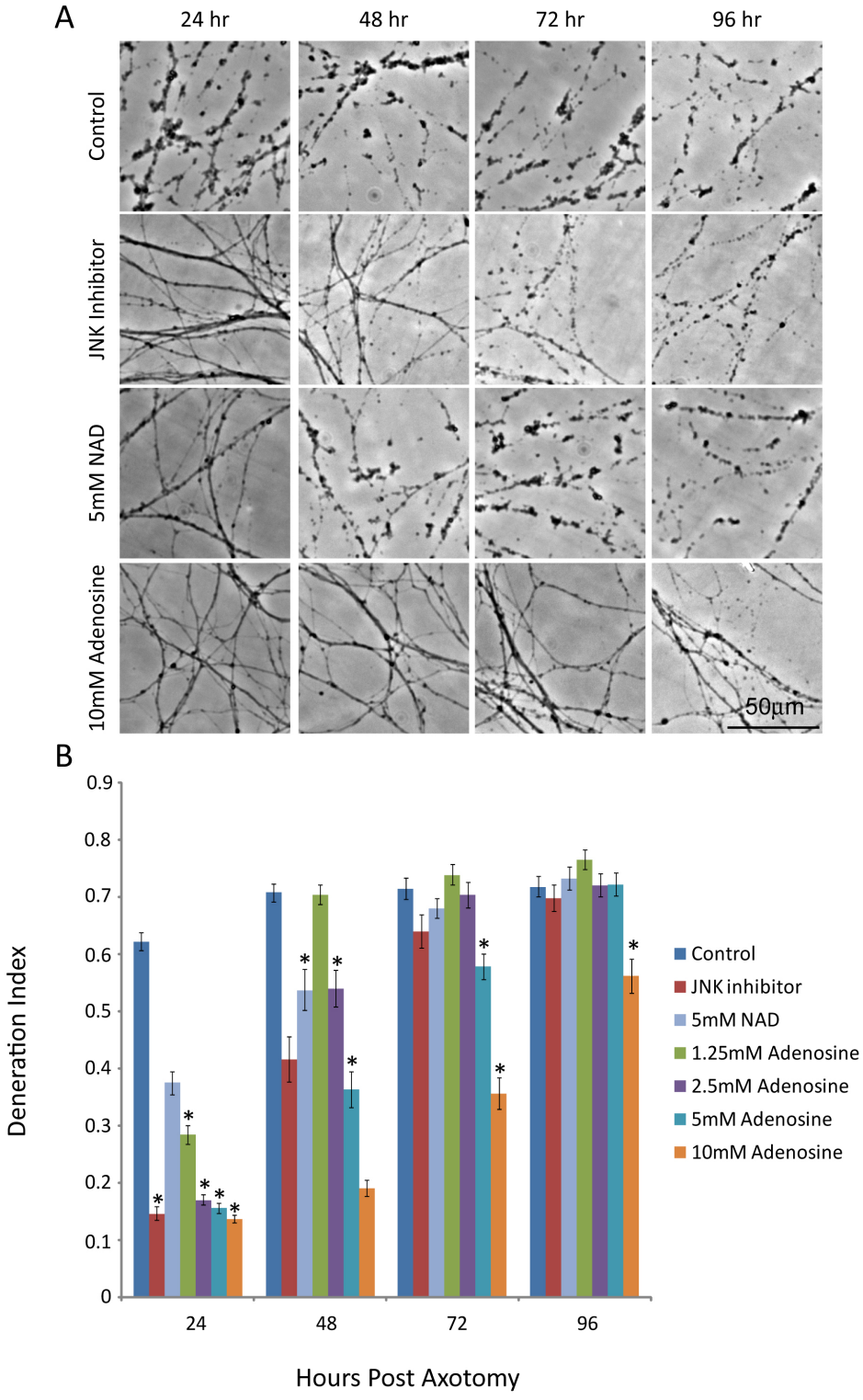

A
24 hr
48 hr
72 hr
96 hr
Control
JNK Inhibitor
5mM NAD
10mM Adenosine
50µm
B
Deneration Index
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
24
48
72
96
Hours Post Axotomy
Control
JNK inhibitor
5mM NAD
1.25mM Adenosine
2.5mM Adenosine
5mM Adenosine
10mM Adenosine

FIGURE 2

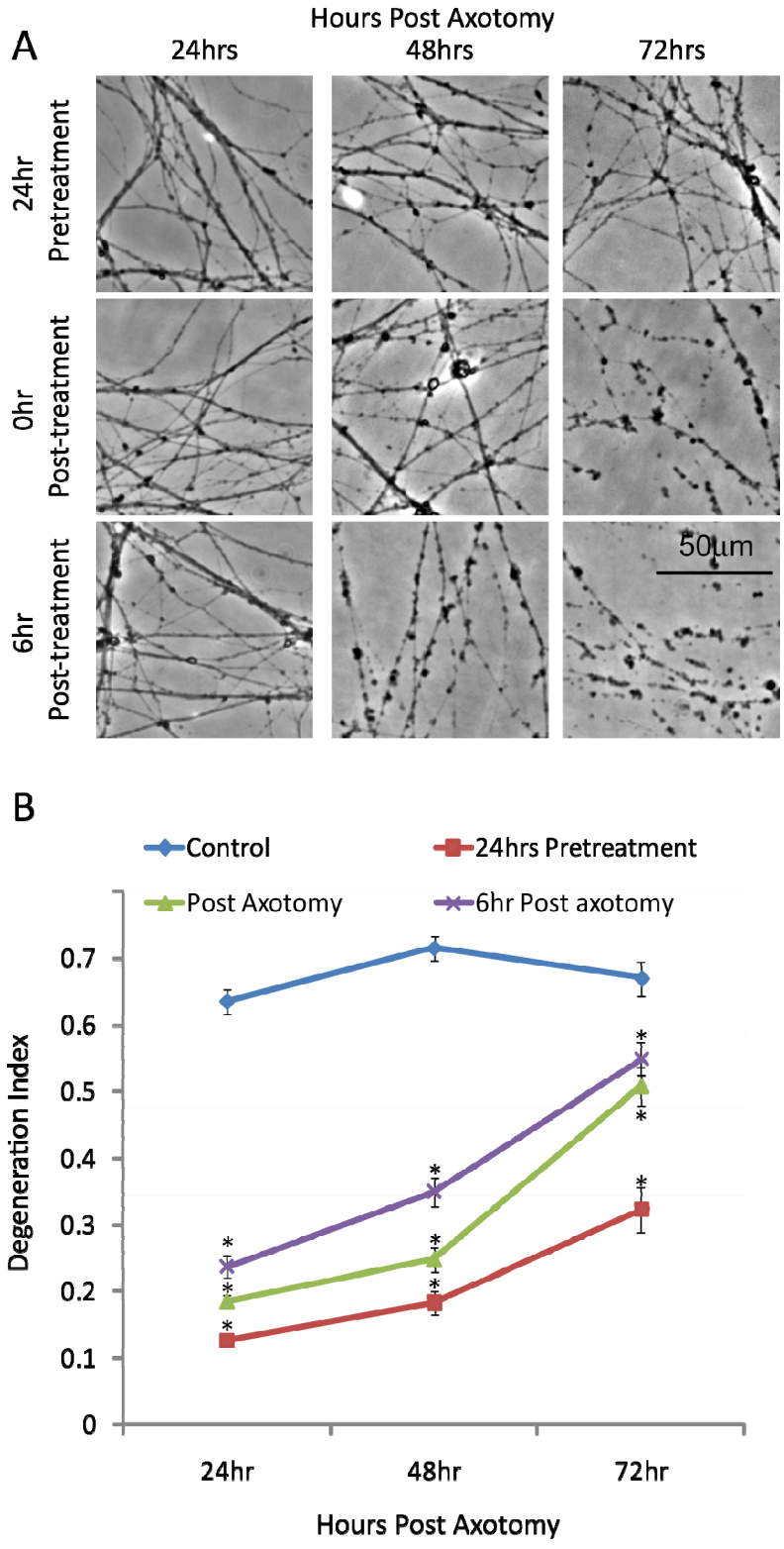

A
Hours Post Axotomy
24hrs
48hrs
72hrs
24hr Pretreatment
0hr Post-treatment
6hr Post-treatment
50µm
B
Control
24hrs Pretreatment
Post Axotomy
6hr Post axotomy
Degeneration Index
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
24hr
48hr
72hr
Hours Post Axotomy

FIGURE 3

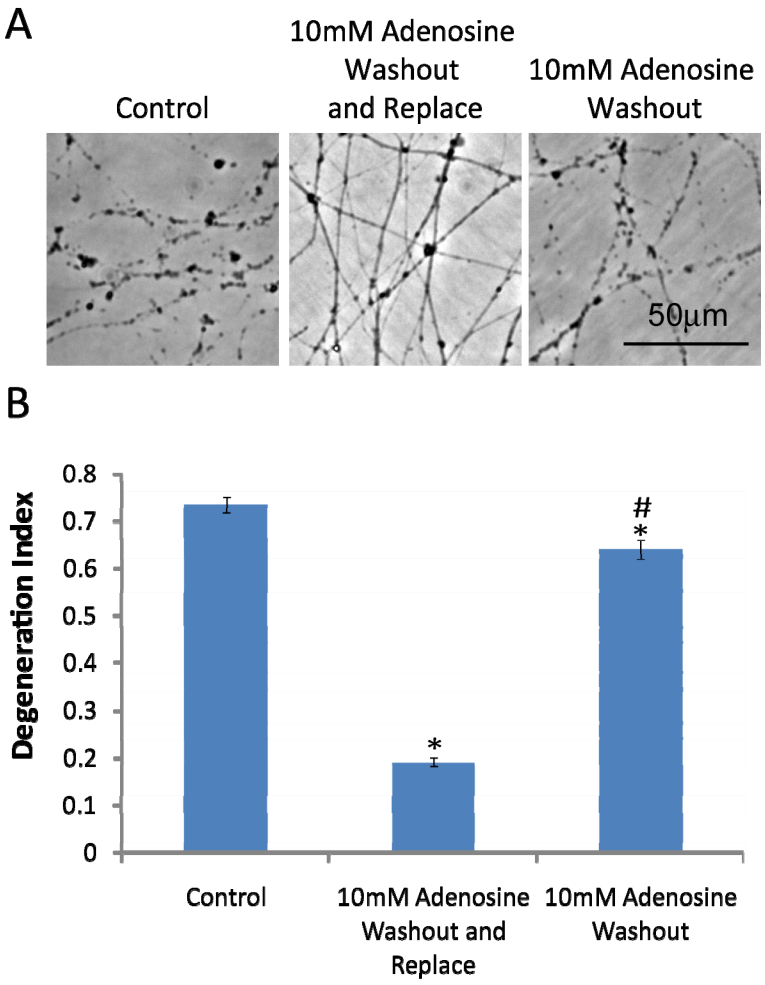
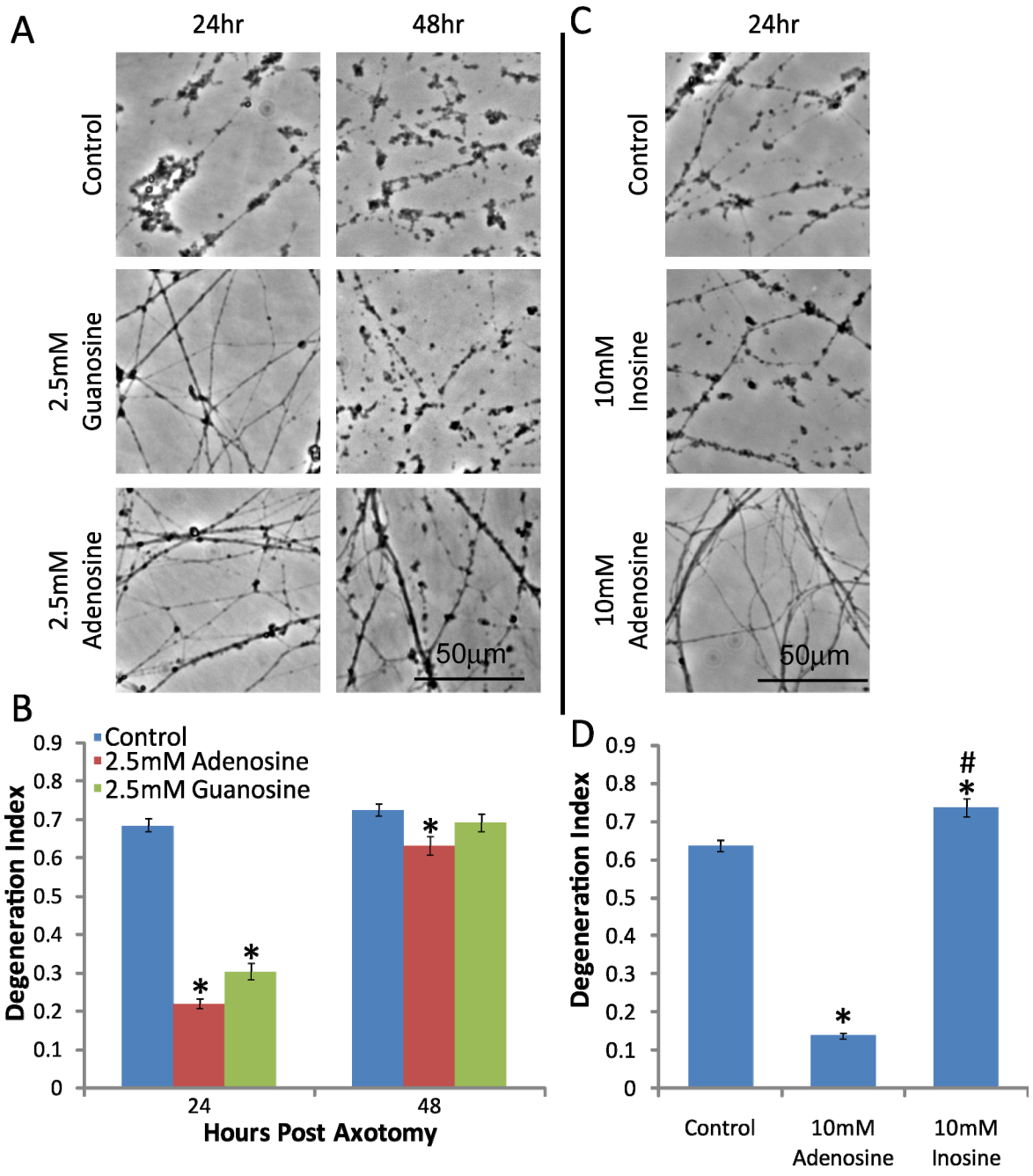

A
Control
10mM Adenosine Washout and Replace
10mM Adenosine Washout
50µm
B
Degeneration Index
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
#
*
Control
10mM Adenosine Washout and Replace
10mM Adenosine Washout

FIGURE 4


A
24hr
48hr
Control
2.5mM Guanosine
2.5mM Adenosine
50µm
C
24hr
Control
10mM Inosine
10mM Adenosine
50µm
B
Control
2.5mM Adenosine
2.5mM Guanosine
Degeneration Index
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
24
48
Hours Post Axotomy
D
Degeneration Index
Control
10mM Adenosine
10mM Inosine

CHAPTER 5

Conclusions and Future Directions

Summary of Conclusions

As our population ages, and the baby boomer generation enters into their 60's, they will begin to face the stage of life where age dependent diseases start to affect them in earnest. This includes many diseases such as a subset of neurodegenerative diseases that can, and most likely, will affect their quality of life. These diseases, including Parkinson's, Alzheimer's, Lou Gehrig's, glaucoma, diabetes, and cancer are potential targets for therapies directed at preventing axonal degeneration. As therapies for the past several decades have focused on decreasing cellular death and apoptosis with some success, an increasing number of failures has made it clear that the processes causing disabilities in these diseases also include axonal injury. By developing treatments that enhance axonal stability and function, we can not only enhance the quality of life of axons and neurons, but the patient's quality of life.

In the work presented here we have approached understanding axonal degeneration from two different angles. First, in chapter 2, using what was known previously to cause and influence axonal degeneration in models of anoxia, mechanical injury and toxin induced models of neurodegeneration we developed a cell culture model of axonal degeneration by mitochondrial inhibition. By understanding where Nmnat interferes with axonal degeneration secondary to this insult, we can narrow down our search for its underlying mechanism. Second, in chapter 3 and 4, we explored two new modifiers of axonal degeneration, DLK, and purines nucleosides. By learning how these new modulators interact with the axonal degeneration pathway and current models we can clarify the mechanisms that lead to axonal dysfunction.

We are just beginning to understand the complex field of axonal degeneration. What we have presented here are only a couple of clues or puzzle pieces that will be put into place over the next decade. As the pieces come together, it will be interesting to see how the puzzles of cancer, metabolism, cell death, and apoptosis overly this one. It will require collaborations with all of these fields to come to understand the mechanism of neurodegeneration and develop safe, effective therapies.

Nmnat mediated axonal protection

Nmnat mediated protection is related to the reduction of ROS rather than an increase in ATP

In chapter 2, we described how Nmnat expression robustly inhibits rotenone-induced degeneration of DRG axons and provides modest protection to the neuronal soma. In exploring the mechanism of this protection, we found that Nmnat expression did not affect the rate of decrease in ATP levels caused by rotenone-mediated mitochondrial inhibition. Further, we found that 50-60% losses of ATP were neither necessary nor sufficient for axonal degeneration. However, Nmnat proteins inhibited axonal degeneration caused by external oxidants and decreased the accumulation of axonal ROS during rotenone and vincristine treatment suggesting that they protect axons by reducing ROS accumulation or toxicity.

Understanding the mechanism for Nmnat mediated ROS protection

The mechanism by which Nmnat prevents the accumulation and/or toxicity of ROS is unclear. ROS accumulation causes damage in a number of neurodegenerative diseases via many mechanisms including lipid peroxidation and protein adducts (Reynolds et al., 2007). NAD^+ and its metabolites are known to play important roles in antioxidant responses (Mack et al., 2001). One possibility is that Nmnat proteins increase NAD^+ availability without increasing NADH breakdown via oxidation, thus providing more NADH for conversion to NADPH which is a cofactor for many ROS scavenging enzymes (Elizabeth and Karam, 2003). One way to explore this possibility is to establish a method using axonal cultures and small molecule detection to measure the amounts of NAD^+ metabolites in culture. Two such methods have been described using red blood cells and lymphocytes using ion-pairing HPLC (Stocchi et al., 1987; Di Pierro et al., 1995). By using a method that allows for the detection of many of the possible metabolites formed by changes in NAD^+ metabolism, we will be able to approach the problem as a complex system with multiple inputs and outputs rather than attempting to measure changes in individual metabolites without understanding how the system changes.

Alternatively, Nmnat proteins may have additional activities, such as antioxidant activity, much like peroxidases or dismutases. An undiscovered function of Nmnat, in addition to its role in NAD^+ biosynthesis, could explain why detectable Nmnat enzymatic activity is not required to block vacuole formation and neurodegeneration in the Drosophila retina (Zhai et al., 2006). For example Nmnat was shown to have chaperone activity *in vitro*, much like heat shock protein 70 (Zhai et al., 2008). However, this is

inconsistent with observations indicating that NAD^+ and NAD^+ precursors as well as other enzymes involved in NAD^+ biosynthesis can also provide axonal protection after axotomy (Araki et al., 2004; Wang et al., 2005; Sasaki et al., 2006). Furthermore, we found that by mutating the enzymatic site of Nmnat we greatly reduce its protective potential (Sasaki and Milbrandt, 2008).

While we began an exploration of changes in antioxidant enzymes in response to Nmnat expression in DRGs and found that some of the major antioxidant enzymes did not have their transcriptional levels increased, a further exploration into changes in the antioxidant machinery of neurons is warranted. Transcriptional changes for previously identified antioxidant genes are likely small as they were not detected in published microarray studies or our own unpublished data (Gillingwater et al., 2006b). In mammalian tissues, key antioxidant enzymes include superoxide dismutases (SOD), catalase (CATA), glutathione peroxidases (GPX), thioredoxin (TXN) and peroxiredoxin (PRDX) complexes, and glutathione (GSR) and thioredoxin reductases (TRXR). These enzymes replenish key electron donor molecules. Many of these genes are regulated by transcriptional mechanisms (Li et al., 2007). By using quantitative RT-PCR for a subset of genes we may be able to small changes in expression of redox modifying genes in neurons expressing Nmnat. Due to the ability of Nmnat to slow axonal degeneration after severing, the transcriptional changes necessary for protection must have occurred prior to injury thus analysis could be limited to uninjured DRG neurons in the presence or absence of Nmnat expression. While transcriptional changes thus far have not been detected, it is possible that Nmnat could be regulating the expression of several enzymes to a small degree that would synergistically increase the antioxidant potential.

In addition to transcriptional regulation, antioxidant proteins are regulated at the post-transcriptional level. Some of the proteins known to be phosphorylated and regulated are SOD1, SOD2, CATA, GPX and GSR (Rhee et al., 2005). Nmnat may change the activity of some of these components, perhaps by changing the ratio of known redox molecules (NAD^+, NADH, $NADP^+$ and NADPH). A combination of approaches could be used to determine if any of the known antioxidant pathways are required for Nmnat mediated protection. 1) Using siRNA technology, key enzymes could be reduced in the presences of Nmnat overexpression. Using the rotenone model of axonal degeneration or axonal severing the relative protection could be monitored. This would allow for the detection of genes essential for Nmnat protection. However, it is important to identify those genes that may be necessary for axonal survival regardless of axonal injury. Since it is possible that a combination of enzymes, as opposed to individual enzymes, are necessary for Nmnat protection we would need to perhaps inhibit multiple pathways with multiple siRNAs. 2) By using commercially available kits we can measure antioxidant potential, and specific enzymatic activities (Oxis International Foster City, CA). Using this information we can determine if certain classes of enzymes are activated in cultured neurons expressing Nmnat. 3) Since many of enzymes that are have antioxidant potential use co-factors such as GSH and NADPH as reducing equivalents, we can measure the relative changes in GSH to GSSG and NADPH and $NADP^+$. This will allow us to determine if Nmnat changes the redox status of the neurons and thus perhaps direct the next stages of research towards classes of genes that regulate these metabolites. Identifying how increased Nmnat expression limits ROS damage may

suggest new pathways to support axonal protection and help understand the mechanisms that underlie axonal degeneration in general.

Gene Therapy Potential

The importance of mitochondrial dysfunction in neurological disorders involving axonal degeneration and the ability of the *Wld*^{s} mutation to protect against MPTP- and 6-OHDA- induced Parkinsonism (Sajadi et al., 2004; Hasbani and O'Malley K, 2006) highlight the potential to use Nmnat as an adjunctive therapy for PD. Nmnat could be delivered in a viral vector (either AAV or lentiviral) in the substantia nigra of patients with PD to enhance their axonal stability. This increase in axonal stability could, as our data suggest, enhance cell body survival. While the expression of *Wld*^{s} did not preserve the cell bodies in either the MPTP or 6-OHDA models, these are both acute models of dopaminergic neuron toxicity. To test the effects of Nmnat in a chronic model, it would be best to use the rotenone model established in rats. The chronic rotenone model of PD more closely mimics the degenerative progression observed in PD patients where the amount of axonal loss is more severe than the cell death found at each stage of disease (Hornykiewicz, 1966; Betarbet et al., 2000).

By using viral delivery a construct could be designed that would express Nmnat in combination with trophic factor delivery to further enhance cell body survival. This potent combination of axonal and cell body protection could lead to the type of synergism that is likely necessary to treat degenerative diseases that often do not present symptomatically until many of the effected neurons and axons are lost. For example, in PD it is thought that greater than 50-60% of dopaminergic neurons are when patients

present to the clinic. Recent success with AAV-derived viral vectors expressing the trophic factor neurturin in Phase I clinical trials make this possibility even more likely to succeed (Marks et al., 2008).

DLK regulates the rate of axonal degeneration

Loss of DLK function and inhibition of downstream targets inhibit axonal degeneration

In chapters 3 we explored the role for the MAPKKK pathway involving DLK to slow axonal degeneration. Based on the evidence suggesting that inhibiting that the DLK pathway is important in neurite survival in PD models both *in vitro* and *in vivo*, we examined axonal degeneration in mice and flies lacking DLK. Loss of DLK slowed axonal degeneration in a phylogenetically conserved fashion. This slowed degeneration was present *in vitro*, allowing us to examine the potential downstream target of JNK. We found that by inhibiting JNK shortly before axonal severing, but not several hours after severing, we could slow axonal degeneration. This suggests that DLK and JNK signaling are important early in axonal degeneration and regulate the speed of axonal degeneration.

Gene therapy mediated DLK protection for axonal degeneration

Our data suggests that inhibition of the MLK pathway, and in particular the DLK pathway, is a target for gene therapy treatment of neurodegenerative diseases featuring axonal degeneration. The use of small molecule inhibitors of the MLK pathway for treating neurodegenerative diseases has received enthusiastic support in the literature (Wang et al., 2004) and was the focus of a clinical trial of CEP-1347 in PD patients

(Parkinson Study Group PRECEPT Investigators, 2007). While animal trials of CEP-1347 were relatively successful, the human trial did not slow clinical progression to L-DOPA dependency. While this initial trial does prove that MLK inhibition will be ineffective since it is not clear that MLK inhibition was achieved (Burke, 2007), it does suggest that use of gene therapy via viral vectors may be a more successful method of inhibition.

Recently, dominant negative forms of DLK were delivered with use of an AAV viral vector to the dopaminergic neurons in a mouse model of PD using 6-OHDA. As mentioned in the introduction dnDLK was able in inhibit the cell death, but not the axonal degeneration. It is possible that DLK may not be able to slow axonal loss in this acute model. It is also possible that the dose of 6-OHDA tested is above the level where partial DLK inhibition could be protective in the axonal terminals. It would be worthwhile to determine if dnDLK is protective for axonal terminals at lower doses of 6-OHDA. Since the CEP inhibitors have shown that they are able to protect striatal terminals it is likely that MLKs play a role. The 6-OHDA model is a rather acute model of PD and it might be advantageous to determine if dnDLK is protective against axonal terminal loss in the chronic rotenone model of PD in rats. The chronic nature of this model could provide enhanced sensitivity for protective effects. Furthermore, it should not be overlooked that inhibition of MLKs potentially will inhibit both axonal degeneration and apoptosis. This powerful combination may be the holy grail of neurodegenerative therapies.

In fact, a study using a dominant negative c-Jun demonstrated both cell body and axonal protection after transection of the medial forebrain bundle in another model of PD

(Crocker et al., 2001). Interestingly, this suggests that either 1) the dominant negative c-Jun had a gain of function in the axons, perhaps by sequestration of JNK, or 2) loss of c-Jun transcription prior to injury resulted in the depletion of a key component of the axonal degenerative pathway. Additionally, experiments with ligated sciatic nerves show that phospho-JNK is increased and transported after axonal injury and in diabetic rat models (Middlemas et al., 2003; Cavalli et al., 2005). This further highlights the need to understand the targets of JNK in the axons during axonal degeneration.

Identification of Axonal DLK and JNK targets

Since JNK inhibition is able to slow axonal degeneration when added only minutes before severing it is unlikely that there is a transcriptional component to the protection. This suggests that JNK regulates axonal degeneration through the phosphorylation of targets. Further, it is known that phosphorylated JNK accumulates and is transported in injured sciatic nerves *in vivo* (Middlemas et al., 2003; Cavalli et al., 2005). Identification of these JNK targets is critical for understanding how JNK influences axonal degeneration and the mechanism of axonal degeneration *in vitro* and *in vivo*. The use of 2-D gels may allow for detection of multiple JNK phosphorylation targets. By the use of either radiolabeled substrates, or antibodies specific for phosphorylated epitopes on proteins we could use our DRG cultures to isolate axons from cultures that have been treated with or without JNK inhibitors prior to axonal injury at several timepoints after injury. This would allow us to develop a network of phosphorylation changes that occur after injury and how JNK inhibition changes that network.

JNK is known to have three different isoforms (Bjorkblom et al., 2008). It is not clear from the experiments done thus far if DLK inhibition prevents the activation of all three JNK isoforms or if inhibition of all of the JNK isoforms is necessary for protection. Using siRNA technology we can decrease the amount of each JNK isoform prior to axonal injury and determine if loss of any individual JNK isoforms is able to inhibit axonal degeneration. It is possible that the different JNKs have overlapping targets and thus individual knockdown may not be sufficient and may require knockdown of two or three of the isoforms. If it is possible to inhibit a single isoform this will allow for more directed targeting of therapy for neurodegenerative diseases.

Purine Nucleosides role in axonal degeneration

Adenosine and guanosine inhibit axonal degeneration

In chapter 4 we demonstrated that axonal degeneration is slowed by purine nucleosides *in vitro* after mechanical injury. We found that exogenous adenosine robustly delayed axonal degeneration and is the most efficacious chemical inhibitor amongst those we have tested to date. Where NAD^+ and JNK inhibitors are protective for ~24 hr, adenosine was able to slow axonal degeneration up to 96 hr. Importantly adenosine was not only protective when added prior to axotomy, but also when added up to 6 hr after axonal injury. While adenosine was protective for at least 96 hr, if it was removed during this protective window, the axons we would degenerate rapidly within 24 hr. This demonstrated the necessity for the continued presence of adenosine during the protective window. Finally, we also provided evidence that in addition to adenosine, guanosine was protective, while inosine was not. This could suggest some selectivity to the purines that

provide a common protective mechanism or the protection mediated by adenosine and guanosine could be independent of each other.

Proximal mechanism of adenosine mediated protection

The earliest events in adenosine mediated protection are not clear. Adenosine has at least two routes to interact with cells 1) receptor mediated effects and 2) intracellular interactions via membrane transport (Fredholm et al., 2001). Although the high concentrations (millimolar) where adenosine is effective argue against a receptor mediated mechanism which would more likely be in the micromolar range, it is necessary to test this hypothesis (Haas and Selbach, 2000; Noguchi and Yamashita, 2000). Adenosine has four known G-coupled protein receptors, A_1, A_{2A}, A_{2B}, and A_3 receptors. We have preliminary data that showing that theophylline, a relatively non-selective adenosine receptor blocker (although weakly at A_3), does not inhibit adenosine mediated protection. We also have preliminary data showing that the A_1, and A_{2A}, receptor agonists CPA, and CGS21680 do not reproduce the effects of adenosine; we have yet to test A_{2B} or A_3 receptor agonists. There are additional known agonists of each of these receptors that should be tested over a range of concentrations (Tocris Bioscience Ellisvile, MO). It is not currently clear which receptors are expressed on DRG neurons in culture, RT-PCR and immunohistochemistry could be used to determine both expression levels and subcellular localization of these receptors. Axonally located proteins would be of particular importance as it appears adenosine has a local mechanism. siRNA technology could also be used to knock down each receptor to examine its role in axonal

protection and maintenance without relying on relatively selective inhibitors that may have off-target effects.

The second major known mechanism by which purines affect cells is through intracellular action via 2 classes of transporters. Purine nucleosides can be transported via either equilibrative (ENT1 - ENT4) or concentrative (CNT1 – CNT5) transporters (Podgorska et al., 2005). We will focus on the equilibrative transporters since it has been shown that they play a role in the trophic effects of purines. Purines enhance neurite outgrowth in a number of cellular systems that was shown to be, or thought to be, dependent on equilibrative transport and activation of protein kinase N (PKN) (Benowitz et al., 1998) (Bocklinger et al., 2004). In contrast to our work, these studies have shown that inosine is in fact more potent than adenosine or guanosine. It is known that adenosine is converted to inosine through the activity of adenosine deaminase (ADA) since inhibition of ADA blocked adenosine induced axonal growth (Benowitz et al., 1998). Since inosine is ineffective against axotomy, it is unlikely that ADA inhibitors will affect adenosine mediated protection, but it should be explored. These differences may be due to different cell types, culture media or a novel mechanism.

The different equilibrative transporters are blocked to a different extent by various transport inhibitors. Early characterization showed that mouse mENT1 is sensitive to the three inhibitors, dipyridamole, NBTI, and dilazep, while mENT2 is insensitive to both NBTI and dilazep (Kiss et al., 2000). Unfortunately, the drugs were not tested to their full range of concentrations thus the insensitivity can only be stated as relative insensitivity. We have preliminary data suggesting that treatment of cultures with adenosine and dilazep or dipyridamole inhibits the adenosine induced protection, while NBTI did not

have a consistent inhibitory effect. Dilazep and dipyridamole induced morphological changes that can best be described as swellings in the DRGs when added alone, although they did not induce rapid degeneration or fragmentation. It is possible, and in fact likely, that nucleoside transport is important for the maintenance of neurons and axons. Using RT-PCR and immunohistochemistry it should be determined which transporters are expressed and their subcellular localization. It would be particularly interesting if there is differential expression of the channels on the soma and axon. The use of siRNA may also allow selective inhibition of each of the known transporters to determine if nucleoside transport is in fact necessary for adenosine mediated axonal protection.

Guanosine as a therapeutic

Due to the half-life of adenosine *in vivo* being on the order of seconds and its cardiac effects, it is impossible to use adenosine as a therapeutic or even test for efficacy (Lerman and Belardinelli, 1991). However, guanosine has been beneficial *in vivo* when injected intraperitoneally (I.P.) against models of stroke and spinal cord injury (Jiang et al., 2007; Chang et al., 2008). Guanosine has also been used orally (at similar doses to I.P. injections) to interfere with quinolinic acid induced seizures in mice (de Oliveira et al., 2004) suggesting the possibility that supplemented water could be a route of delivery. Using I.P. injections of guanosine it could be determined if there are *in vivo* protective effects when mice or rats are treated with guanosine prior to and/or immediately after sciatic nerve injury or administration of rotenone. This will determine if guanosine is able to slow axonal degeneration either after mechanical injury or in a model of neurodegeneration. The use of modulators, either receptor based or transporter

based will allow for the dissection of the *in vivo* mechanism for protection. Further exploration of the pathway responsible for nucleoside mediated protection will allow for better targeting of therapeutic drugs for neurodegeneration.

Extracellular adenosine levels during axonal degeneration

Having demonstrated that adenosine clearly slows axonal degeneration and that it is necessary to maintain this protection, we still need to determine if adenosine is sufficient to maintain the protection. The axonal degeneration is not completely prevented by treatment with adenosine as is shown by the eventual axonal degeneration. Is this degeneration due to the eventual degradation of adenosine? Another possibility is that the metabolism of adenosine leads to the build-up of metabolites that leads to some sort of end-product inhibition. Either of these would be important regardless of the mechanism of action via receptors or transport. To determine the concentration of adenosine in the media during the time course we can use HPLC to measure the amount of adenosine available immediately after addition, after axonal injury and after axonal degeneration has occurred in the presence of adenosine. If adenosine is still present at a concentration that is known to mediate protection this would suggest that adenosine is not sufficient to maintain axonal health indefinitely. The media from these cultures could be transferred to un-axotomized cultures which are then axotomized to determine if the components of protection or an inhibitor are present. The use of HPLC would also allow us to determine if adenosine treatment leads to the accumulation of any metabolites in the media. Any metabolites that are identified by spiking experiments would be tested for their ability to mediate protection or to inhibit adenosine mediated protection. Guanosine

and inosine treated cultures would provide interesting contrasts. If the same peaks appear in guanosine treated cultures, but not in inosine treated cultures this could be of significant importance and may be a regulator of axonal degeneration. However, guanosine could lead to a completely different metabolite profile which could suggest that adenosine and guanosine have different mechanisms.

Intracellular metabolism during axonal degeneration

For two reasons it will be interesting to explore the possible changes in intracellular metabolites during axonal degeneration. 1) It is possible that adenosine is transported intracellularly and is metabolized to AMP via adenosine kinase and this changing the energy status of the cells or to inosine through adenosine deaminase and altering the purine balance in the cells. 2) Nmnat or NAD^+ may also be affecting the same or similar pathways and a comparison of intracellular changes to the NAD^+ nucleotides and purine metabolites will help to determine if these are perhaps similar pathways. It is difficult to interpret the measurement of individual metabolites and their role in axonal degeneration. For example, ATP and NAD^+ have been measured during axonal degeneration and found to decrease, but is not clear that these losses correlate with axonal degeneration as the data in chapter 2 demonstrated for ATP. If neurons can lose 50% of their ATP and not degenerate acutely, how can the loss of ATP be a linear indicator of axonal degeneration? There is clearly more than one factor contributing to the balance of these metabolites.

We can begin to monitor changes in axonal metabolites during axonal degeneration by employing the armamentarium of axonal degeneration inhibitors that we

have develop here. By comparing the effects of adenosine, guanosine, inosine, NAD^+, JNK inhibitors, and Nmnat on protected axons, we can start to understand the way in which normal axons degenerate and they ways in which different protecting agents change this profile. While it is possible that all of these agents produce the same changes in metabolism, but, as they each have a different time scale of protection, this is unlikely. By observing the differences we can perhaps learn which metabolic changes are important early and which changes occur much later during protected degeneration or after adenosine or Nmnat have ceased to protect. It is possible that these metabolites will be detectable on HPLC, but it may necessitate the use of LC/MS to increase the specificity of detection (Yamada et al., 2006). We are currently working on optimizing both of these methods to detect NAD^+ nucleotides and purine metabolites.

Axonal degeneration is a multifactorial process

Time course of protection

Throughout the course of the experiments discussed in this work it has become increasingly clear that each protective agent leads to a distinct time course of protection. This is particularly clear in the case of axonal degeneration after mechanical severing. Nmnat expression can protect axons from degeneration *in vitro* for over 1 week (Araki et al., 2004). Adenosine, the best small molecule tested, is protective for up to four days or 96 hr. NAD^+ and JNK inhibition protect for 24 hr. These data alone suggest that there are different mechanisms that regulate the speed of degeneration. If they all regulated the same pathway the time course of protection would likely be similar. This is further highlighted by the fact JNK inhibition is protective only when added during in the first

three hours after axonal injury while adenosine can clearly be added up to six hours after injury.

Unfortunately, due to the need to overexpress Nmnat prior to injury we cannot be sure when Nmnat is necessary or sufficient for protection. We can compare the protection of the JNK inhibitor and adenosine in culture. What these two different stories tell us is that JNK likely acts as a trigger mechanism that happens early during degeneration. This trigger is not necessary for degeneration, but activates an accelerator pathway. In contrast, adenosine is not necessary during the early phase of axonal degeneration to act as a protecting agent. Instead, it acts as a break on the degeneration process. The earlier and stronger it is applied the more effective the protection. So while the JNK inhibitor is likely preventing a pathway from being stimulated, adenosine is likely slowing the degenerative pathway. It will be interesting to explore the possibility of combining JNK inhibition with adenosine treatment and looking for synergism. Perhaps JNK inhibition can extend the effective time window after injury for adenosine treatment.

This differential control of degeneration will allow us to understand the events that are important early in degeneration and those that are important later. A systematic and scientific approach to the problem will tell us what events are necessary for degeneration and what parts of the pathway are modulators. To begin with it will be important to start with the events that have already described in this and similar systems. Some of the factors that need to be considered are 1) membrane integrity, 2) axonal transport, 3) mitochondrial potential and function, 4) ATP levels, 5) NAD^+ levels, 6) membrane potential and Na^+/K^+ ATPase function, 7) ROS levels, 8) oxidative

metabolism, 9) intracellular Na^+, 10) intracellular Ca^{2+}, 11) cytoskeletal structure, and 12) protein degradation pathways. As axonal degeneration and fragmentation is a discrete event it will be crucial to monitor these changes in individual axons over the degenerative time course when possible. Tools to explore membrane potential and integrity, mitochondrial function and potential, ionic concentrations and ROS are already developed and need to be optimized for our culture system. A system to allow for oxygen consumption measurements while imaging neurons was recently developed to allow for the simultaneous measurements of these critical variables (Jekabsons and Nicholls, 2004). Axonal transport and cytoskeletal dynamics can be explored using fluorescently tagged markers of organelles and cytoskeletal components that have been developed in our lab (Baloh et al., 2007). Comparing how each of these markers of axonal health change dynamically during degeneration and during protection should provide a useful framework for understanding the degenerative process and developing additional inhibitors.

Is axonal degeneration comparable to apoptosis as an active pathway?

Axonal degeneration is a multifactorial process where there may be multiple factors contributing to the rate of degeneration not all being necessary or sufficient to block the entire process. The system is likely not a completely linear process, but one where effector molecules can accelerate or dampen the rate of degeneration. While the individual components of an axonal degeneration program are unknown, the ability for Nmnat to delay axonal degeneration for over a week suggests that is blocking some component of a linear process. In apoptosis, a cell body that has been "saved" by caspase

inhibitors may be metabolically alive, but it is clearly dysfunctional and may be no better than a dead neuron to an organism. Furthermore, many neurons "saved" will advance on to non-apoptotic cell death (Putcha and Johnson, 2004). The axons "saved" by Nmnat or Wld^S are excitable and functional in a sense after axonal severing, though they too will advance on to "non-Nmnat protectable" axonal degeneration (Beirowski et al., 2005). Importantly axonal severing may be the least mild of insults as disease causing genetic mutations will cause mitochondrial dysfunction, altered axonal transport, etc. and the axons will still be in a suboptimal state. Excitingly, disease models show that these saved axons are better than no axons at all by significantly slowing disease progression. While it may not reverse the physiological insult, slowing axonal degeneration may provide a therapeutic window for advanced treatment to be administered after a disease process has started thus enhancing a person's the quality of life.

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