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A local mechanism mediates NAD-dependent protection of axon degeneration

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Axon degeneration occurs frequently in neurodegenerative diseases and peripheral neuropathies. Important insight into the mechanisms of axon degeneration arose from findings that the degeneration of transected axons is delayed in Wallerian degeneration slow (Wlds) mice with the overexpression of a fusion protein with the nicotinamide adenine dinucleotide (NAD) synthetic enzyme, nicotinamide mononucleotide adenylyltransferase (Nmnat1). Although both Wld^s and Nmnat1 themselves are functional in preventing axon degeneration in neuronal cultures, the underlying mechanism for Nmnat1- and NAD-mediated axon protection remains largely unclear. We demonstrate that NAD levels decrease in degenerating axons and that preventing this axonal NAD decline efficiently protects axons from degeneration. In support of a local protective mechanism, we show that the degeneration of axonal segments that have been separated from their soma could be prevented by the exogenous application of NAD or its precursor nicotinamide. Furthermore, we provide evidence that such Nmnat1/NAD-mediated protection is primarily mediated by their effects on local bioenergetics. Together, our results suggest a novel molecular pathway for axon degeneration.

Introduction

In addition to neuronal cell death, axon degeneration is a major morphological change observed in peripheral neuropathies as well as neurodegenerative diseases like Alzheimer's disease (Stokin et al., 2005) and amyotrophic lateral sclerosis (Fischer et al., 2004). Although cell death represents the irreversible late phase of degenerative processes, axonal degeneration usually occurs much earlier, and often precedes or correlates closely with clinical symptoms such as cognitive decline. However, the underlying mechanisms for the degeneration of neuronal processes remain largely elusive.

A useful model for studying the mechanism of axon degeneration is the self-destructive process observed at the distal portion of a transected axon after injury, termed Wallerian degeneration (Waller, 1850). Studies in rodents have established that degeneration of distal axons occurs within 24 to 48 h after axotomy (Coleman and Perry, 2002; Raff et al., 2002). Interestingly, it appears that an apoptosis-independent mechanism is involved in Wallerian degeneration (Raff et al., 2002), as such degenerative processes do not seem to involve activation of the caspase family of cysteine proteases (Finn et al., 2000). In addition, Wallerian degeneration can be mechanistically separated from the apoptosis triggered by NGF deprivation in sympathetic neurons (Deckwerth and Johnson, 1994; Zhai et al., 2003; Brunet et al., 2004).

Initial insight into the molecular mechanisms of Wallerian degeneration came with the discovery of a spontaneously occurring mutant mouse strain, C57BL/Wld^s, whose axons survived for as long as weeks after transection (Lunn et al., 1989; Glass et al., 1993). Genetic studies have attributed this slow Wallerian degeneration phenotype to the overexpression of a fusion protein (Wld^s) that consists of the full-length nicotinamide mononucleotide adenylyltransferase (Nmnat1), an enzyme required for both the de novo and salvage pathways of nicotinamide adenine dinucleotide (NAD) biosynthesis, and a short region of a ubiquitin assembly protein, UFD2 (Conforti et al., 2000; Mack et al., 2001). In cultured neurons, although inhibiting the activity of the ubiquitin proteosome system can slow down Wallerian degeneration (Zhai et al., 2003), a recent study demonstrated that overexpressing Nmnat1 alone could also prevent the axon degeneration

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Abbreviations used in this paper: AMPK, AMP-activated protein kinase; DRG, dorsal root ganglion; HSV, Herpes simplex virus; NAD, nicotinamide adenine dinucleotide; Nmnat1, nicotinamide mononucleotide adenylyltransferase; Wlds, Wallerian degeneration slow.

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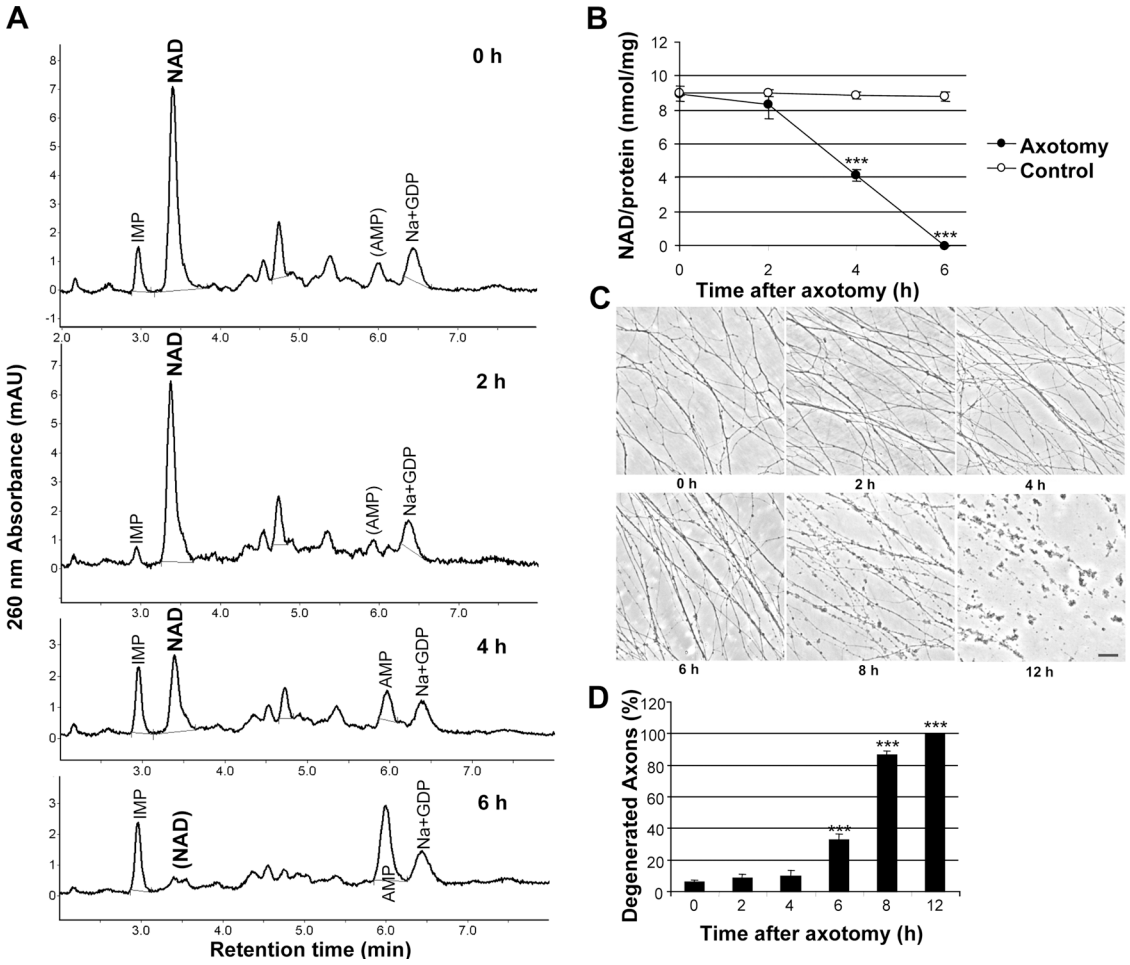

Figure 1. **Reduced NAD levels in degenerating axons.** (A) HPLC chromatograms of the axonal segments undergoing Wallerian degeneration. DRG explants cultured for 5 d were subjected to axonal transection, and the acidic extracts of axonal segments were prepared at the indicated time-points after transection and analyzed by HPLC. The chromatograms containing NAD and a few other pyridine compounds, including IMP, AMP, and nicotinic acid (Na) plus GDP from the same amounts of axonal extracts are shown. The absorbance (mAU) was monitored at 260 nm. (B) Quantification of NAD levels in control and transected axons. NAD levels were normalized against total axonal protein contents and expressed as nmol/mg. The results are presented from six independent experiments. Statistical analysis was done by *t* test. ***, $P < 0.001$. (C) Representative phase images of axons undergoing Wallerian degeneration taken at indicated time-points after transection. Bar, 50 μm. (D) Quantification of axon degeneration results. Statistical analysis was done by *t* test. ***, $P < 0.001$.

triggered by mechanical or chemical insults (Araki et al., 2004). In addition, this study further suggested that the protective effect of Nmnat1 is mediated by a NAD-dependent nuclear deacetylase Sirt1 (Araki et al., 2004), a mammalian homologue of yeast sir2 that has been implicated in regulating life span in yeast (Blander and Guarente, 2004). Recent studies have demonstrated that in mammalian cells this NAD-dependent deacetylase activity of Sirt1 could regulate death/survival decision in response to stress by down-regulating the p53 tumor suppressor gene (Luo et al., 2001; Vaziri et al., 2001) and the FOXO transcription factors (Brunet et al., 2004; Motta et al., 2004). However, previous studies have shown that NAD levels are not enhanced in the tissues of Wallerian degeneration slow (Wlds) mice (Mack et al., 2001) or in cultured neurons overexpressing Wlds or Nmnat1 (Araki et al., 2004), raising the question as to how Wld^s and Nmnat1 are involved in axon degeneration. In this study, we provide evidence arguing for the existence of a Sirt1-independent local mechanism that is involved in NAD-mediated protection.

Results and discussion

As a first step in examining the role of NAD in Wallerian degeneration, we used an HPLC method to measure the concentration of NAD and its derivatives in degenerating axons. Acidic lysates were prepared from axonal segments collected at different time points after transecting the axons from cultured dorsal root ganglia (DRG) explants (Fig. 1, A and B), and were resolved by reverse-phase chromatography. At 4 h post-axotomy when the transected axons were still morphologically indistinguishable from their uncut controls (Fig. 1, C and D), NAD levels began to decrease. At 6 h after axotomy, when early morphological signs of degeneration started to develop (Fig. 1, A–D), NAD levels declined to almost undetectable. The observed NAD decline is not due to leakage across the axonal membrane, as other small molecules such as nicotinic acid (Na) and GDP did not decrease, whereas inosine 5-monophosphate (IMP) and AMP increased in the degenerating axons

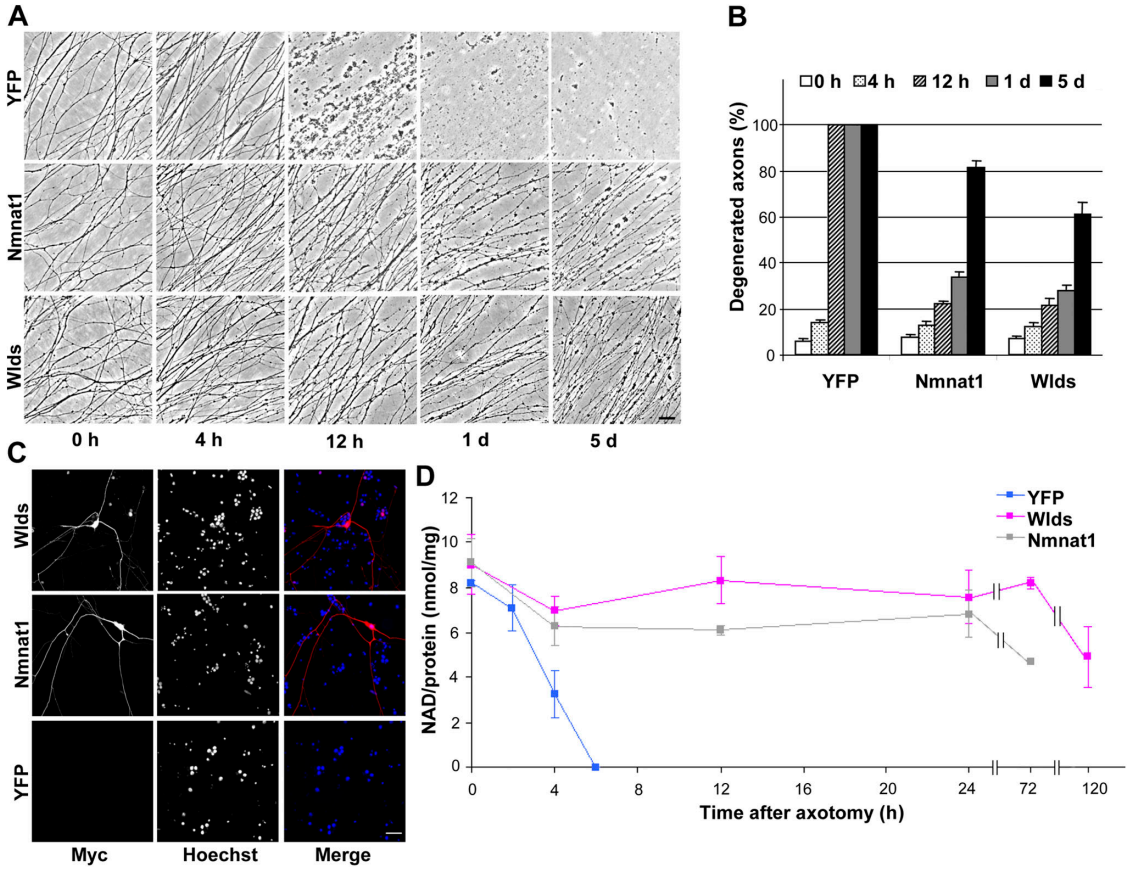

Figure 2. **Nmnat1 and Wlds delay both NAD decrease and Wallerian degeneration.** (A and B) Wallerian degeneration is delayed by overexpression of Nmnat1 or Wlds protein. DRG explants were cultured for 5 d and infected with HSVs expressing YFP, Nmnat1, or Wlds 24 h before axotomy. Representative phase images (A) were taken from transected axonal segments at indicated time-points after transection, and the extents of axon degeneration were quantified base on phase images (B). Statistical analysis was done by two-way ANOVA using GraphPad Prism software. $P < 0.001$ for both Nmnat1 and Wlds vs. YFP. Bar, 50 μm. (C) Localization of overexpressed Nmnat1 and Wlds proteins. Dissociated DRG neurons were cultured for 5 d and infected with HSVs expressing YFP or myc-tagged Nmnat1 or Wlds protein (MOI = 0.1). 24 h after infection the neurons were fixed and stained with antibodies against myc (left panels) and Hoechst (middle panels). Bar, 100 μm. (D) Both Wlds and Nmnat1 overexpression delay the decline of NAD levels in degenerating axons. The DRG explants were infected with HSVs expressing YFP, Nmnat1, or Wlds 24 h before axonal transection. Axonal segments collected at the indicated time-points were analyzed by HPLC to measure NAD levels. Statistical analysis was done by two-way ANOVA using GraphPad Prism software. $P < 0.001$ for both Nmnat1 and Wlds vs. YFP.

(Fig. 1 A). By a similar approach of alkaline extraction (Micheli and Sestini, 1997) it has been difficult for us to detect NADH in the axonal extracts, presumably due to the low abundance of NADH and the high NAD/NADH ratio reported in mammalian cells (Zhang et al., 2002; Kasischke et al., 2004). Thus, we have focused this study on analyzing NAD concentrations. A similar NAD decline was also observed in vincristine-treated DRG neurons (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200504028/DC1), another commonly used axon degeneration model (Wang et al., 2001a; Araki et al., 2004). These results imply that a decrease in axonal NAD is common to degenerating axons triggered by both mechanical and chemical insults.

Next, we examined whether overexpression of Wlds or Nmnat1 could prevent the NAD decrease in degenerating axons. To do this, we made recombinant herpes simplex viruses (HSVs) to express either Wlds fusion protein or the full-length Nmnat1 in cultured DRG explants and dissociated neurons (Fig. 2). Consistent with a recent study (Araki et al., 2004), we found that both Wlds and Nmnat1 proteins significantly slowed down the degenerative process of transected axonal segments (Fig. 2, A and B). Neither Wlds nor Nmnat1 significantly increased neuronal NAD levels in intact neurons (unpublished data). Yet, overexpression of either protein profoundly delayed the NAD decline in transected axons (Fig. 2 D) in a temporal pattern similar to their protective effects on axonal degeneration (Fig. 2, A and B). The fact that both Nmnat1 and Wlds prevent the decrease in NAD levels in transected axonal segments implies that these overexpressed proteins may enhance the NAD-synthesizing activity in transected axons. Further supporting this hypothesis are the observations that the overexpressed Wlds and Nmnat 1 proteins were present not only in the nuclei of DRG neurons, but also in the axons as determined by both immunostaining (Wang et al., 2001b) and Western blotting (Wang et al., 2001b). Thus, our results imply that overexpressed Wlds/Nmnat1 proteins could exert their protective effects by synthesizing NAD locally in axons and preventing NAD decline that resulted from transection.

To definitively determine whether preventing the local decrease in NAD levels could result in axon protection, we used

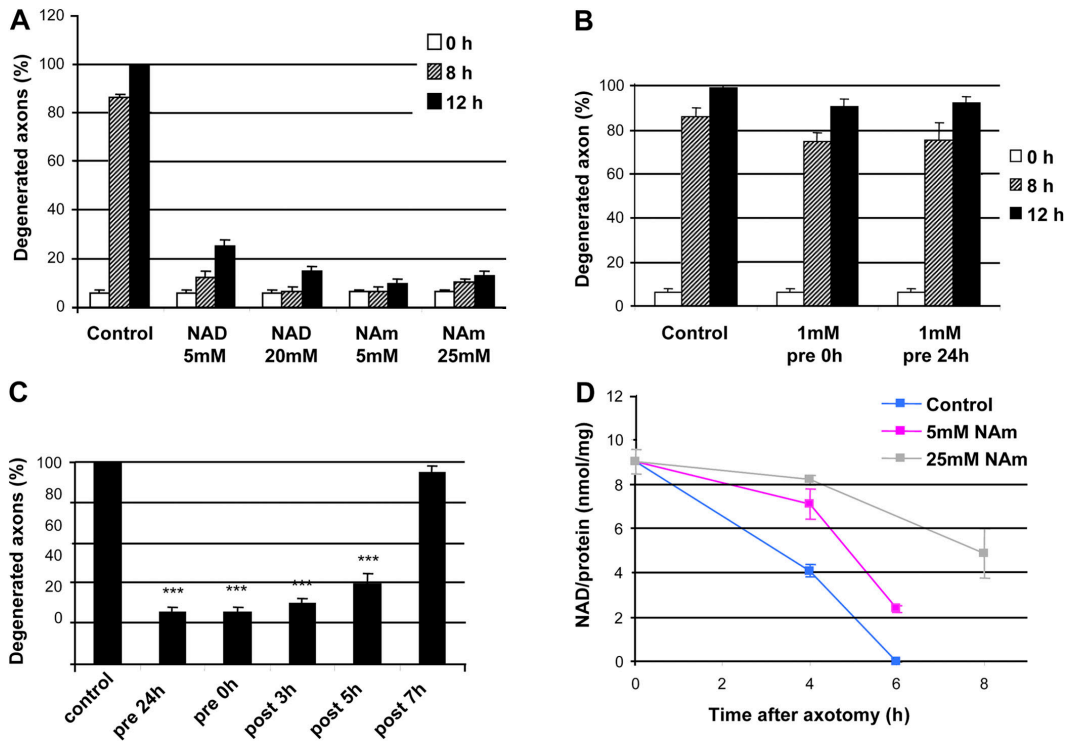

Figure 3. **Exogenously provided NAD and nicotinamide delay Wallerian degeneration in soma-free axonal segments.** (A) Wallerian degeneration of soma-separated axonal segments is delayed by NAD and nicotinamide (NAm) without pretreatment. DRG explants cultured for 5 d were subjected to axonal transection and the ganglia were removed. At the same time, indicated concentrations of NAD or nicotinamide were added to the medium of the axonal segments only. Quantification of axon degeneration was performed from phase images taken at different time-points after transection. Statistical analysis was done by two-way ANOVA using GraphPad Prism software. $P < 0.001$ for NAD (5 mM, 20 mM), NAm (5 mM, 25 mM) vs. control. (B and C) Quantification of the protective effects of 1 mM (B) and 5 mM (C) NAD added at indicated time-points. Pre 24h: NAD added 24 h before transection; pre 0h: NAD added at the same time as transection; post 3, 5, 7 h: NAD added 3, 5, and 7 h post-transection, respectively. Quantification was performed from phase images taken at indicated time-points (B) or at 12 h after transection (C). Statistical analysis: two-way ANOVA in B: $P < 0.001$ for NAD 1 mM pre 0 h and 1 mM pre 24 h vs. control; $P > 0.05$ for NAD 1 mM pre 0 h vs. pre 24 h; *t* test in C: ***, $P < 0.001$. (D) Nicotinamide (NAm) attenuates NAD decrease in soma-free axonal segments. DRG explants cultured for 5 d were subjected to axonal transection and the ganglia were removed. At the same time, indicated concentrations of nicotinamide were added to the medium with axonal segments only. The axonal samples were collected at indicated time-points after transection and subjected to HPLC analysis. The results from three independent experiments are presented and statistical analysis was done by two-way ANOVA using GraphPad Prism software. $P < 0.001$ for 5 mM NAm and 25 mM NAm vs. control.

soma-free axonal segments that have been separated from their cell bodies in the explants as models to examine the protective effects of different pharmacological treatments. It has been shown that NAD exogenously applied to the culture medium of DRG neurons can be transported across the axonal membrane (Araki et al., 2004). Thus, we first examined the protective effects of different concentrations of NAD added to the culture before or after axon transection and removal of ganglia. Consistent with previous observations (Araki et al., 2004), we found that pretreating DRG explant cultures with NAD 24 h before transection delayed axon degeneration (Fig. 3, B and C). However, unlike the NAD concentrations (0.1–1 mM) used by Araki et al. (2004), we found that only higher concentrations (1–20 mM) of NAD could result in significant protective effects. Surprisingly, we found that the same concentrations of NAD also efficiently prevented axon degeneration when added at the time of axon transection (Fig. 3 A), strongly suggesting that locally supplied NAD is sufficient to prevent axon degeneration.

To further assess the local contribution to this NAD-dependent protection, we compared the protective effects of 1 and 5 mM NAD provided at different time points before or after axon transection. As shown in Fig. 3 (B and C), we found that axons pretreated with NAD for 24 h before transection were similarly protected as the axons treated with same concentrations of NAD added at the time of transection, and even a few hours after transection (Fig. 3 C). These results suggest that NAD-mediated protection acts primarily through a local mechanism in the axons, arguing against a significant contribution of NAD-induced transcription and other nuclear events. In this respect, our results are inconsistent with those shown by Araki et al. (2004), possibly due to the different concentrations of NAD used in our studies (1–20 mM) as compared with those by Araki et al. (0.1–1 mM). As additional support for a local protective effect of NAD, we found that nicotinamide, a precursor of NAD synthesis, had a similar protective effect even when applied at the time of axonal transection (Fig. 3 A) or 3 h after transection (unpublished data). As expected, HPLC analysis indicated that both exogenously applied NAD and nicotinamide delayed the decrease of axonal NAD levels triggered by transection (unpublished data; Fig. 3 D). Together, these results suggest that NAD could affect the process of axon degeneration through a local protective mechanism.

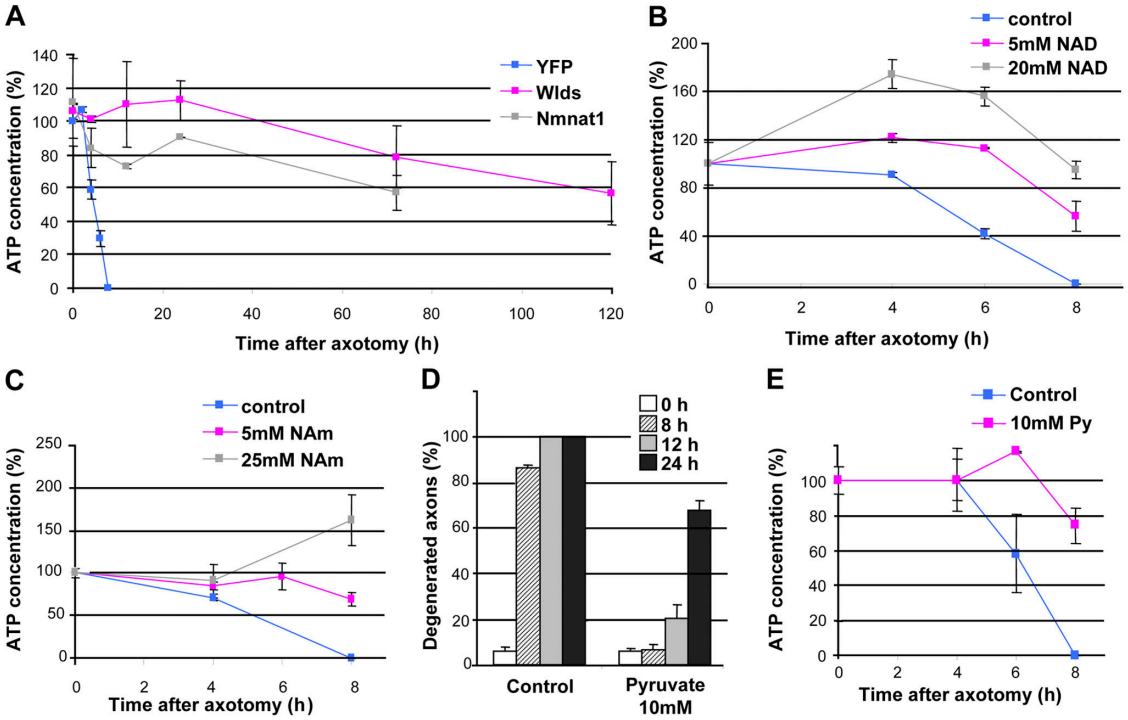

Figure 4. **Impaired ATP metabolism in degenerating axons.** (A) Both Wlds and Nmnat1 delay the decrease of ATP levels in transected axons. 24 h before axonal transection, DRG explant cultures were infected with HSVs expressing YFP, Wlds, or Nmnat1. The axonal segments were collected at the indicated times-points after transection and subjected to HPLC analysis. Axonal ATP levels in the axons expressing Wlds and Nmnat1 are expressed as the percentages of that in control YFP-expressing axons (also for B and C). The results from three independent experiments are presented and statistical analysis was done by two-way ANOVA using GraphPad Prism software. $P < 0.001$ for Wlds and Nmnat1 vs. YFP controls. (B and C) Exogenously provided NAD (B) and nicotinamide (C) delay the decrease of ATP levels in soma-free axonal segments. Indicated concentration of NAD (B) or nicotinamide (NAm) (C) was added to the cultures at the same time when the axonal transection was performed. The axonal segments were collected at the indicated time-points after transection and subjected to HPLC analysis. The results from three independent experiments are presented, and statistical analysis was done by two-way ANOVA using GraphPad Prism software. $P < 0.001$ for 5 and 20 mM NAD and 25 mM NAm vs. controls; $P < 0.01$ for 5 mM NAm vs. control. (D) Methyl-pyruvate slows down Wallerian degeneration. 10 mM of methyl-pyruvate was added to the cultures at the same time when the axonal transection was performed. Quantification of axon degeneration was performed from phase images taken at indicated time-points after transection. Statistical analysis was done by two-way ANOVA using GraphPad Prism software. $P < 0.001$ for methyl-pyruvate vs. control. (E) Methyl-pyruvate delays the decline of axonal ATP levels. 10 mM methyl-pyruvate (Py) was added to the cultures at the time of axon transection. The axonal segments were collected at the indicated time-points after transection and subjected to HPLC analysis. The results from three independent experiments are presented and statistical analysis was done by two-way ANOVA using GraphPad Prism software. $P < 0.001$ for 10 mM methyl-pyruvate vs. controls.

Although Sirt1 has been suggested to be a mediator of this NAD-dependent protection (Araki et al., 2004), nicotinamide, a known Sirt1 inhibitor (Luo et al., 2001), could protect axons from degeneration. On the other hand, NAD is known to be essential for ATP-synthesizing redox reactions that are involved in glycolysis and oxidative phosphorylation (Shibata et al., 1991). In particular, cytosolic NAD is required in the glycolytic pathway for the conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate. Thus, upon cytosolic NAD depletion, glucose can no longer be converted to the pyruvate needed to fuel oxidative phosphorylation in mitochondria. Considering the significant energy demand of various physiological functions in axons, such as maintenance of membrane potential and axonal transport, it is conceivable that reduced levels of NAD may impair axonal energy production, thus triggering the onset of axon degeneration. In support of this, we found that axonal ATP levels decreased in parallel with NAD levels by HPLC analysis of degenerating axons (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200504028/DC1). As an additional indicator, we found that the phosphorylation of AMP-activated protein kinase (AMPK), known to be activated by an increase in the cellular AMP/ATP ratio (Kahn et al., 2005), is also induced in degenerating axonal segments (Fig. S2 B). On the other hand, both overexpression of Wlds/Nmnat1 (Fig. 4 A) and exogenously providing NAD (Fig. 4 B) or nicotinamide (Fig. 4 C) delayed the decrease of ATP levels in transected axons separated from their somas.

To further assess whether the glycolytic blockade resulting from cytosolic NAD depletion contributes to axon degeneration, we examined whether the degeneration of axotomized axons could be suppressed by supplying axons with the glycolytic end product pyruvate, which can be directly used for oxidative phosphorylation in mitochondria. 10 mM methyl-pyruvate, a membrane-permeable form of pyruvate, has been previously used to prevent the ATP decrease induced by DNA damage-induced NAD depletion (Zong et al., 2004). When added at the time of transection, 10 mM methyl-pyruvate could significantly delay the decline of axonal ATP levels (Fig. 4 E) and also protect axons from degeneration (Fig. 4 D). These results together suggest that local bioenergetics is an important effector of NAD depletion in axon degeneration.

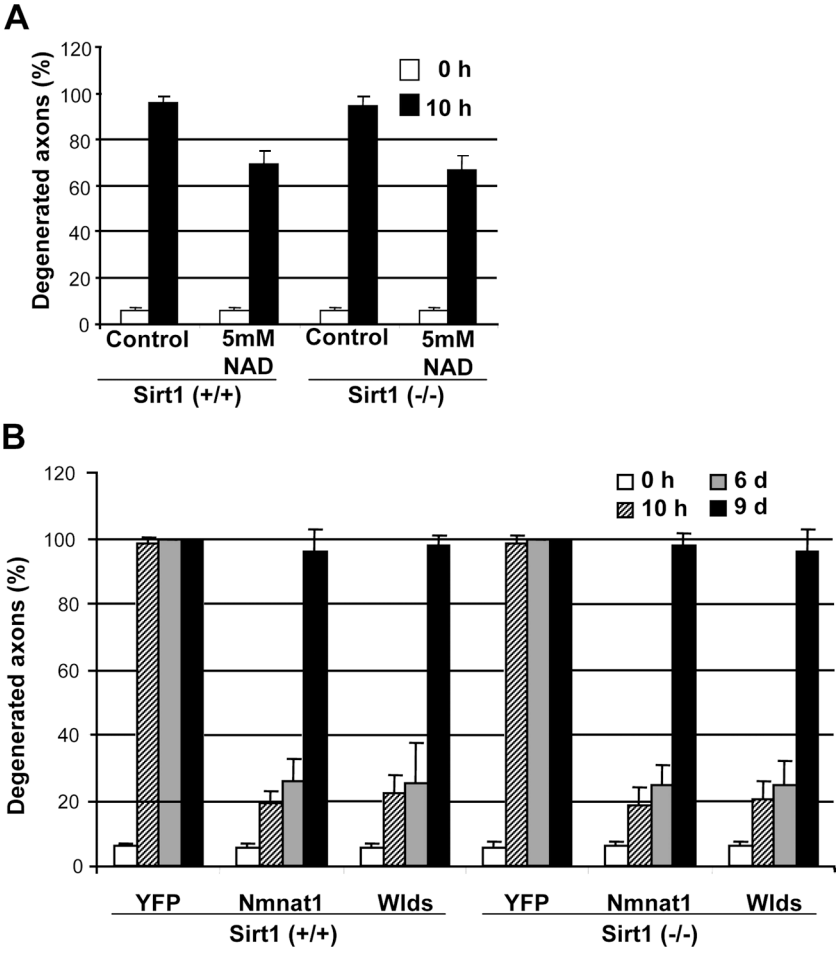

Figure 5. **Sirt1 is not required for NAD-dependent axon protection.** (A) Degeneration of DRG axons from Sirt1 (+/+) and Sirt1 (−/−) mice explant cultures. DRG explants cultured for 5 d were subjected to axonal transection. At the time of transection, 0 or 5 mM NAD was added to the medium. Quantification was performed from phase images taken 0 or 10 h after transection from duplicate experiments from at least three mice for each genotype. Statistical analysis was done by two-way ANOVA. There is no significant difference in all groups between Sirt1 (+/+) and Sirt1 (−/−). (B) The protective effects of overexpressing Nmnat1 or Wlds on Wallerian degeneration of DRG neurons from Sirt1 (+/+) and Sirt1 (−/−) mice. Virally infected DRG explants were subjected to axonal transection. Quantification was performed from phase images taken at indicated time-points after transection. Statistical analysis was done by two-way ANOVA using GraphPad Prism software. There is no significant difference in all groups between Sirt1 (+/+) and Sirt1 (−/−).

Although our results suggest a local mechanism, the nuclear deacetylase Sirt1 has recently been implicated to be the mediator of NAD-dependent axonal protection (Araki et al., 2004). By RNA interference–mediated gene silencing of each Sirt1 member (Sirt1-7) in DRG neurons, Araki et al. (2004) showed that only Sirt1 is involved in mediating the protective effects of Nmnat1 and NAD. However, we found that pretreatment with NAD 24 h before transection conferred a protective effect on axon degeneration indistinguishable from the same concentrations of NAD added at the time of transection (Fig. 3, B and C), suggesting that NAD-dependent protection is primarily mediated by local events. Furthermore, we did not observe any significant differences in the extent of Wallerian degeneration or the protective effects of Wlds/Nmnat1/NAD between neurons from wild-type and Sirt1 knockout mice (McBurney et al., 2003) (Fig. 5). Although the cause of this discrepancy in results remains unclear, it is possible that the RNA interference–mediated knockdown used by Araki et al. (2004) may also affect the expression of other important genes. In addition, neither sirtinol, a Sirt1 inhibitor, nor resveratrol, a Sirt1 activator, affected the protective effects of NAD and Wlds/Nmnat1 on axon degeneration in this same assay (unpublished data). Although we cannot rule out the possibility that other Sirt family members can compensate for the loss of Sirt1 function in Sirt1-deficient neurons, our results strongly support a local bioenergetics mechanism involved in mediating NAD-dependent protection against axon degeneration. These findings will provide new insights into designing strategies for preventing and delaying axonal degeneration in patients with Alzheimer's disease and other neurodegenerative disorders.

Materials and methods

Construction and use of recombinant HSV

Full-length cDNAs of Wlds and Nmnat1 were amplified from the brain tissues of Wlds mice and confirmed by sequencing. Each construct (both wild-type and mutant Nmnat1 and Wlds with a myc tag) was then subcloned into the pHSVPrPUC vector. The resulting plasmids were transfected into 2-2 cells with FuGENE 6 (Roche). 24 h after transfection, the transfected cells were super-infected with 5dl5 HSV helper virus. The recombinant virus particles were harvested, amplified, and concentrated as described previously (Lim and Neve, 1999). Unless indicated, neuronal cultures were infected with these viruses 24 h before axotomy.

Explant cultures and axotomy

DRG explants or dissociated neurons from E16 rat embryos were cultured in medium containing fluorodeoxyuridine and uridine to eliminate nonneuronal cells. The same procedures were used for the experiments using DRGs from E12.5 to E13 embryos of Sirt1 (+/+) and Sirt1 (−/−) mice. The genotyping of the mice was performed following the procedure described in McBurney et al. (2003). For HSV infection, pilot experiments were performed to identify the number of HSVs ($\sim 3.6 \times 10^4$ HSV units per explant) that allowed most of the neurons and axons in the explants to express the protein of interest. For the DRG explant axotomy model, axons were cut through the axonal halo around each ganglion with a micro-scalpel (Fine Science Tools) and the ganglion was removed, leaving only soma-free axons in the culture dish. To observe the effects of different pharmacological perturbations on axon degeneration, indicated concentra-

tions of NAD (Sigma-Aldrich), nicotinamide (Sigma-Aldrich), or methylpyruvate (Sigma-Aldrich) were added to the cultures at the time of transection or at indicated time-points. Quantification of axon degeneration was done as described previously (Zhai et al., 2003). In brief, to quantify percentages of axons with degeneration from phase images (with beading and/or fragmented appearance), 300 axons were blindly scored per condition from duplicate explants per experiment, and from three independent experiments.

Sample preparation and HPLC analysis

Sample preparation and HPLC analysis were performed as described by Micheli and Sestini (1997) with minor modifications. In brief, after extensive washing, the soma-free axonal segments of 12 DRG explants or 2×10^5 neurons were lysed by 0.3 M ice-cold perchloric acid for 30 min. After centrifugation (10 min at 12,000 *g*), the protein content in the precipitates was measured by the BCA protein assay kit (Pierce Chemical Co.). The protein-free supernatants were then neutralized to pH 6–7 by adding 1/10 vol of ice-cold 3 M potassium bicarbonate, followed by precipitation on ice and centrifugation. The cleared samples were stored at −80°C.

For HPLC analysis, after filtration with a 0.22-mm centrifuge tube filter (Costar), each sample (100 μl) was resolved on a Luna reverse-phase C-18 column (3-μm particle size, 75 × 4.6 mm; Phenomenex). The mobile phase consisted of eluant A (0.1 M KH_2PO_4 buffer containing 6 mM tetrabutylammonium phosphate, pH 5.5) and eluant B (methanol). Each elution was performed as follows: isocratic phase at 7% solution B for 6 min, then up to 30% B for 1 min, and then back to initial conditions after 4 min. Initial conditions are restored in 10 min. The flow rate is 1 ml/min and the absorbance is monitored at 260 and 280 nm.

Western blotting

Soma-deprived axons from 12 DRG explants were directly lysed with lysis buffer (50 mM Tris-HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, and 10% glycerol). Aliquots of the resultant lysates with equal amounts of cellular proteins were resolved on 10% SDS-PAGE. The same blots were detected with antibodies against AMPK and phospho-specific AMPK (Cell Signaling).

Microscopy

Phase-contrast and fluorescence microscopy were performed at RT with a microscope (Eclipse TE300; Nikon) with 20× objective, a Plan-fluor ELWD 20×/0.45 objective (Nikon), a SPOT diagnostic camera, and SPOT advanced software. All images were imported into Adobe Photoshop 6.0 for contrast manipulation and figure assembly.

Online supplemental material

Fig. S1 shows that vincristine treatment induces NAD decline in DRG neurons. Fig. S2 shows impaired ATP metabolism in degenerating axons. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200504028/DC1.

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