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NMN Deamidase Delays Wallerian Degeneration and Rescues Axonal Defects Caused by NMNAT2 Deficiency In Vivo

Highlights

- NMN deamidase delays Wallerian degeneration in vivo in mice and zebrafish
- NMN deamidase reduces NMN accumulation in transected nerves
- NMN deamidase protects axons against non-injury insults
- NMN deamidase rescues axonal defects and lethality in NMNAT2-deficient mice

Authors

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In Brief

Di Stefano et al. show that the NMN-consuming bacterial enzyme NMN deamidase delays degeneration of transected axons in vivo in both mice and zebrafish. It also rescues axonal outgrowth defects and perinatal lethality in mice lacking NMNAT2. These findings support the proposed pro-degenerative effect of NMN accumulation in axons.

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NMN Deamidase Delays Wallerian Degeneration and Rescues Axonal Defects Caused by NMNAT2 Deficiency In Vivo

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SUMMARY

Axons require the axonal NAD-synthesizing enzyme NMNAT2 to survive. Injury or genetically induced depletion of NMNAT2 triggers axonal degeneration or defective axon growth. We have previously proposed that axonal NMNAT2 primarily promotes axon survival by maintaining low levels of its substrate NMN rather than generating NAD; however, this is still debated. NMN deamidase, a bacterial enzyme, shares NMN-consuming activity with NMNAT2, but not NAD-synthesizing activity, and it delays axon degeneration in primary neuronal cultures. Here we show that NMN deamidase can also delay axon degeneration in zebrafish larvae and in transgenic mice. Like overexpressed NMNATs, NMN deamidase reduces NMN accumulation in injured mouse sciatic nerves and preserves some axons for up to three weeks, even when expressed at a low level. Remarkably, NMN deamidase also rescues axonal outgrowth and perinatal lethality in a dose-dependent manner in mice lacking NMNAT2. These data further support a pro-degenerative effect of accumulating NMN in axons in vivo. The NMN deamidase mouse will be an important tool to further probe the mechanisms underlying Wallerian degeneration and its prevention.

INTRODUCTION

Axon degeneration is a widely recognized hallmark of many neurodegenerative disorders and axonopathies, including peripheral neuropathies, Parkinson's disease, multiple sclerosis, and others [1, 2]. Therefore, understanding the molecular mechanisms causing axon destruction will have significant therapeutic implications.

Wallerian degeneration, the degeneration of the distal axon stump following injury [3], shares both morphological and mechanistic features with the axon pathology in several neurodegenerative disorders [2]. Recent studies revealed a crucial role for the endogenous mammalian nicotinamide mononucleotide adenylyltransferase (NMNAT) isoform NMNAT2 in axon survival [4]. NMNAT2 is actively transported along the axon, but, due to a short half-life [4, 5], its levels in transected axons decline prior to any visible sign of fragmentation, suggesting it may be a trigger for axon degeneration [4]. Any of the three natural NMNAT isoforms or the slow Wallerian degeneration protein (WLD^S) (an aberrant fusion protein with NMNAT activity [6]) can robustly delay Wallerian degeneration when present at sufficient levels in axons [2]. This is likely achieved by maintaining axonal NMNAT enzymatic activity after loss of endogenous NMNAT2 through increased levels and/or greater relative stability of the introduced proteins [2, 4]. The findings that specific depletion of NMNAT2 in neuronal primary culture is sufficient to initiate WLD^S-sensitive degeneration [4] and that mice lacking NMNAT2, which develop severe axonal defects and die at birth, can be rescued by WLD^S expression [7] are also consistent with this model.

More recently, other endogenous regulators of Wallerian degeneration have emerged, but NMNAT2 loss appears to be a critical, early event in a conserved axon degeneration pathway upstream of other core regulators, including the pro-degenerative sterile alpha and TIR motif containing 1 (SARM1) protein [2, 8–12]. However, exactly how maintaining NMNAT activity promotes axon survival has still not been fully resolved [8]. We have proposed a model, based on pharmacological and genetic evidence, where accumulation of the NMNAT substrate, nicotinamide mononucleotide (NMN), an expected consequence of

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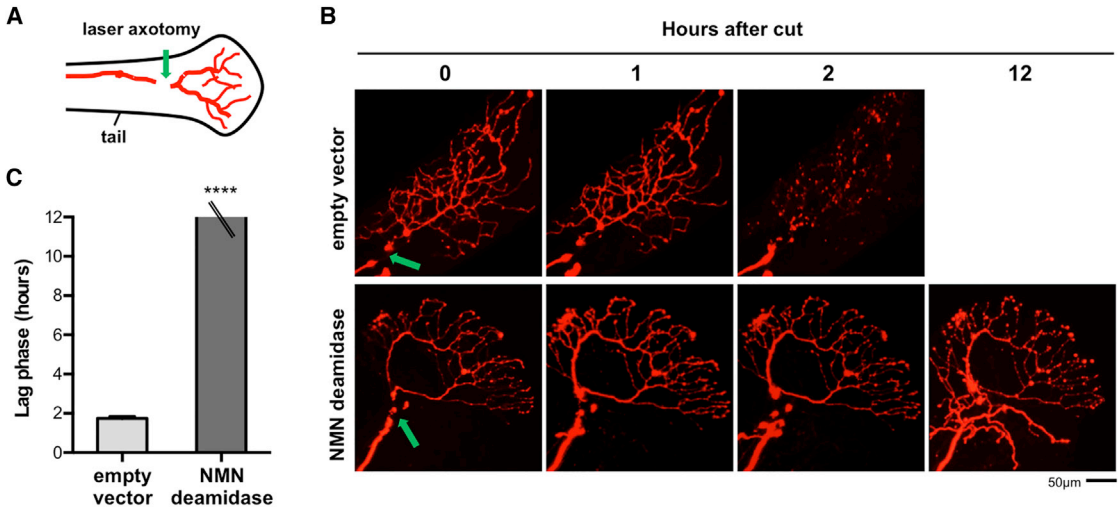


Figure 1. NMN Deamidase Blocks Wallerian Degeneration in Zebrafish Larvae

(A) Schematic representation of larval zebrafish tail showing the location of the sensory (Rohon-Beard) axon and the site of injury (green arrow).

(B) One-cell-stage emrbyos were injected with plasmids for DsRed and NMN deamidase or empty vector. Time-lapse fluorescent images show DsRed-expressing axons in empty vector or NMN deamidase embryos after cut (green arrow shows the location of cut site). Axons have degenerated within 2 hr in the empty vector control, whereas NMN deamidase-expressing axons are preserved up to at least 12 hr.

(C) Length of the lag phase (time between the cut and the first sign of degeneration) in control or NMN deamidase-expressing axons (mean ± SEM; empty vector n = 9, NMN deamidase n = 8; Student's t test, ****$p < 0.0001$).

NMNAT2 depletion, promotes Wallerian degeneration [11, 13]. A cornerstone of the model was the finding that expression of the *E. coli* enzyme NMN deamidase, for which the only known activity is conversion of NMN to its deamidated form, nicotinic acid mononucleotide (NaMN) [14], confers neurite protection in primary neuronal cultures comparable to that produced by WLD^S or stable NMNATs [13]. Crucially, both WLD^S/NMNATs and NMN deamidase are able to scavenge NMN, but, unlike WLD^S/NMNATs, NMN deamidase lacks intrinsic NAD synthesis activity.

Here we have used zebrafish larvae and a transgenic mouse line to test whether *E. coli* NMN deamidase can delay axon degeneration in vivo as it does in primary neuronal cultures. We have also tested whether it can protect axons from insults that do not involve physical injury, including whether it can rescue axonal defects in mice lacking NMNAT2. In all cases, we find that NMN deamidase expression confers strong protection, consistent with a pro-degenerative role for NMN accumulation in axons in vivo.

RESULTS

NMN Deamidase Blocks Wallerian Degeneration in Zebrafish Larvae

Many events occurring in Wallerian degeneration are evolutionarily conserved [15–19]. We previously found that the NAMPT inhibitor FK866 delays Wallerian degeneration in zebrafish larvae, likely through inhibition of NMN accumulation and a consequent rise in Ca^{2+} [11, 13]. Therefore, we first asked whether expression of NMN deamidase can protect axons in vivo using this relatively simple vertebrate model system.

NMN deamidase was transiently expressed (along with DsRed) in trigeminal and Rohon-beard somatosensory neurons (Figure 1A). Axons were cut 48–54 hr post-fertilization using two-photon laser axotomy. Control larvae displayed a normal rate of axon degeneration after injury (expressed as lag phase [18]), usually completed within 2 hr under these experimental conditions (Figures 1B and 1C; Movie S1). In contrast, NMN deamidase blocked injury-induced axon degeneration up to at least 12 hr (Figures 1B and 1C; Movie S2), similar to the previously reported effects of WLD^S [18]. A few axons (n = 3) were followed up to 24 hr and were found to remain intact (Movie S2). Axon regrowth from the site of injury confirmed successful axon transection and that the zebrafish larvae were viable for the duration of the experiment. Interestingly, quick axonal regeneration following laser axotomy in NMN deamidase-expressing neurons occurred despite the presence of the preserved distal stump (Movie S2), contrasting what is seen in nerves in Wld^S mice [20, 21]. It may be that there are fewer physical restrictions influencing regrowth in the zebrafish larvae, but, as previously described [18], the regenerating axons still avoided the distal stump, suggesting the regeneration process may still be affected.

These data indicate that NMN deamidase can confer strong in vivo protection against Wallerian degeneration in a vertebrate system.

Generation and Biochemical Characterization of an NMN Deamidase Transgenic Mouse

Next, we generated a transgenic mouse expressing *E. coli* NMN deamidase, fused to EGFP, under the control of the β-actin promoter (see the Experimental Procedures). We obtained four positive founder mice that showed no overt phenotype up to at least 1 year (mice were not aged further). All four founders were fertile, but only one (14209) transmitted the NMN deamidase transgene to fertile offspring. Although we were able to detect the presence of transgene mRNA by RT-PCR (Figure 2A), EGFP-tagged NMN deamidase was undetectable using fluorescence microscopy,

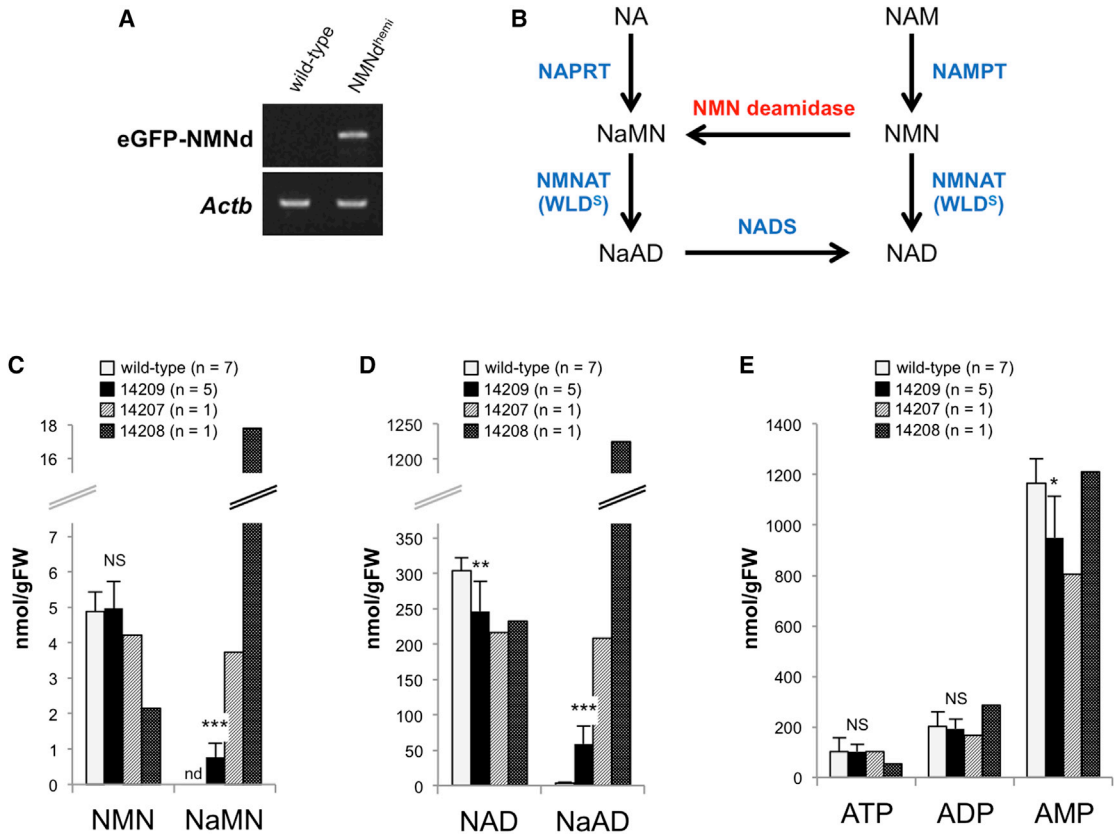


Figure 2. NMN Deamidase Expression Increases Steady-State Levels of NaMN and NaAD

(A) RT-PCR analysis of eGFP-NMN deamidase and *Actb* (sample control) mRNA in E18.5 brain of wild-type or NMNd^{hemi} embryos (representative of n = 3).

(B) Schematic representation of NAD biosynthetic pathways and point at which bacterial NMN deamidase will act in mammalian cells (NA, nicotinc acid; NAM, nicotinamide; NaMN, nicotinic acid mononucleotide; NMN, nicotinamide mononucleotide; NaAD, nicotinic acid adenine dinucleotide; NAD, nicotinamide adenine dinucleotide; NAPRT, nicotinc acid phosphoribosyltransferase; NAMPT, nicotinamide phosphoribosyltransferase; NMNAT, nicotinamide mononucleotide adenylyltransferase; NADS, NAD synthase).

(C–E) Mononucleotide (C, NMN and NaMN), dinucleotide (D, NAD and NaAD), and nucleotide (E; ATP, ADP, and AMP) levels in brain of wild-type, 14209-derived NMNd^{hemi} mice, and NMN deamidase founders 14207 and 14208 (nd, non-detectable). Data are expressed as nanomoles per gram fresh weight (nmol/gFW) (mean ± SD; Student's t test, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$; NS, non-significant).

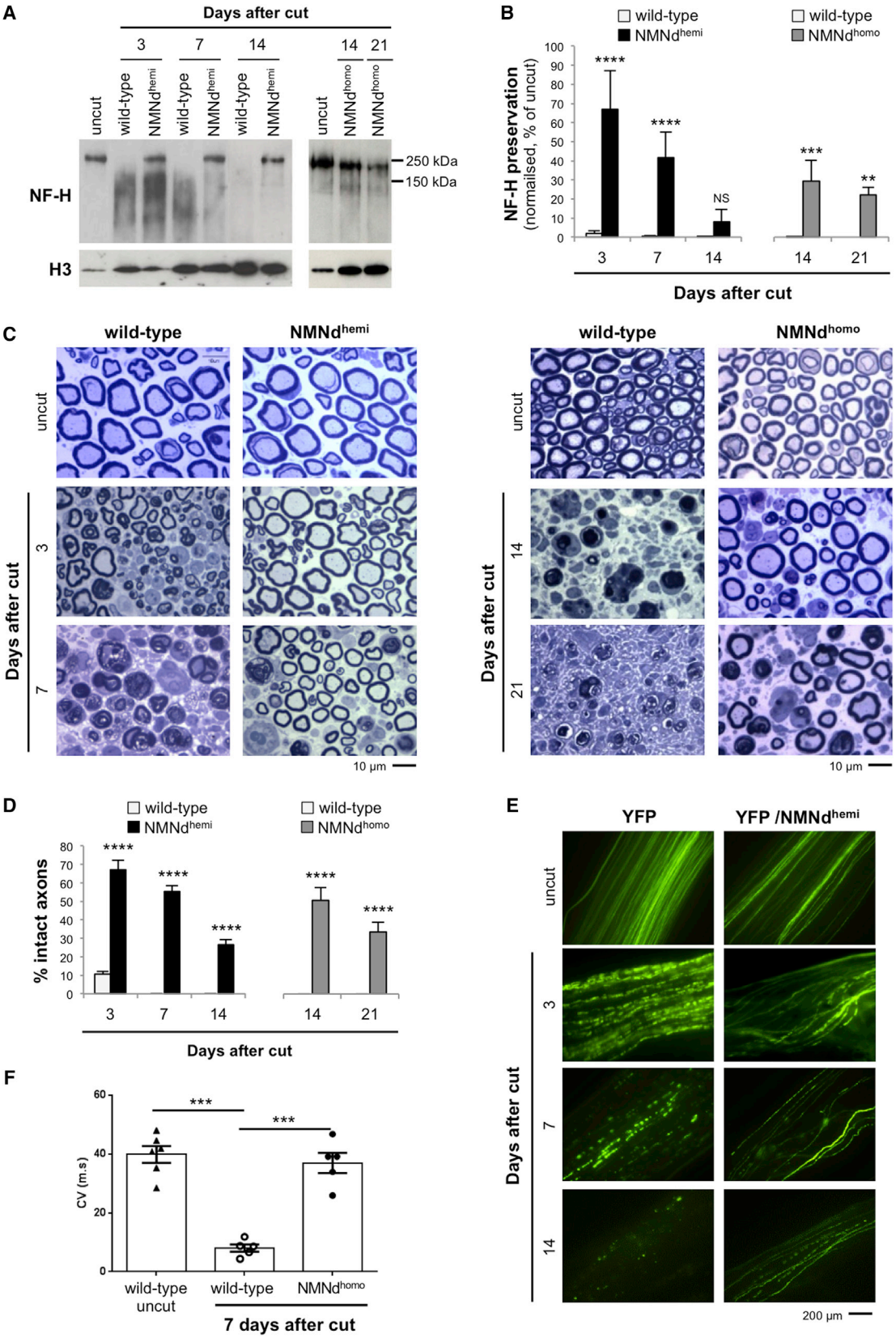
immunofluorescence, or by immunoblotting for EGFP (data not shown), suggesting extremely low expression of the protein. However, NMN deamidase activity was detectable in brain extracts. Interestingly, mean enzyme activity in founder 14209 and its hemizygous offspring (0.007 ± 0.001 mU/mg) was one or two orders of magnitude lower than in the other founders, which were unable to efficiently transmit the transgene (0.069 and 0.612 mU/mg protein in founders 14207 and 14208, respectively). Enzyme activity was essentially undetectable in wild-type mice ($\leq$0.002 mU/mg).

Notably, brain levels of NMN negatively correlated with NMN deamidase activity in the founders (Figure 2C), whereas NaMN and the corresponding dinucleotide NaAD, which were only reliably detectable in transgenic mice, both positively correlated with activity (Figures 2C and 2D). These are predicted consequences of NMN deamidase activity on the NAD biosynthetic pathway (Figure 2B), and they match previously reported nucleotide determinations in cultured dorsal root ganglion (DRG) neurons exogenously expressing NMN deamidase [22]. NAD levels did not appear to correlate as strongly with NMN deamidase activity (Figure 2D), perhaps indicating that the generation of NAD from NaAD (via NAD synthase) can balance any loss of production from NMN (via NMNAT) (Figure 2B). ATP, ADP, and AMP levels appeared largely unaffected by NMN deamidase expression (Figure 2E).

NMN Deamidase Expression Confers Morphological and Functional Preservation of Transected Axons

We previously reported that NMN deamidase expression confers protection against injury-induced axon degeneration in primary neuronal cultures similar to WLD^S [11, 13]. Here, using four complementary methods, we assessed whether this protective effect was reproducible in vivo by comparing rates of Wallerian degeneration in wild-type mice with those in hemizygous (NMNd^{hemi}) or homozygous (NMNd^{homo}) transgenic mice.

First, we found that degradation of neurofilament heavy chain (NF-H) in tibial nerves after sciatic nerve lesion, as determined by immunoblot analysis, was delayed in NMNd^{hemi} and



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NMNd^{homo} mice for up to 2 or 3 weeks, respectively (Figures 3A and 3B; Figure S1C). Second, light microscopy revealed morphological preservation of significant numbers of transected myelinated sciatic nerve axons for at least 2 weeks after lesion in NMNd^{hemi} mice and for greater than 3 weeks in NMNd^{homo} mice (Figures 3C and 3D; Figure S1D). Third, structural continuity of YFP-labeled axons in lesioned sciatic nerves from NMNd^{hemi} mice additionally hemizygous for the YFP-H transgene was also preserved for up to 2 weeks (Figure 3E). Finally, we used electromyography to assess functionality of severed axons, and we found that conduction velocity was fully preserved in NMNd^{homo} mice at 7 days after sciatic nerve lesion (Figure 3F). Crucially, structural preservation of axons after sciatic nerve lesion was also seen in independent NMN deamidase founder mice (Figures S1A, S1B, and S1E), thereby ruling out the possibility that the protective phenotype in the founder 14209-derived line is the result of disruption of another gene at the site of transgene integration. While the majority of axons in transected nerves from NMNd^{hemi} and NMNd^{homo} mice were strongly protected, a small proportion appeared to degenerate rapidly. This likely reflects variation in NMN deamidase expression between different axons, although we cannot rule out this subpopulation was instead dying via an alternative mechanism. Interestingly, variable protection of individual fibers is also seen in transected *Wld*^{S} nerves [6].

Together, these data show that the NMN scavenger enzyme NMN deamidase delays Wallerian degeneration in mice. The level of protection is approaching that seen in *Wld*^{S} mice [6], despite NMN deamidase being unable to synthesize NAD directly. Crucially, where NMNd^{hemi} and NMNd^{homo} mice were compared directly, we saw greater and prolonged protection of axons in the NMNd^{homo} mice (Figures 3A–3D; Figure S1C). Enzymatic activity and deamidated nucleotide levels were higher in NMNd^{homo} mice than in NMNd^{hemi} mice (data not shown), in keeping with an expected higher expression level of the bacterial enzyme, indicating a dose-dependent protective effect for NMN deamidase similar to that seen for WLD^{S} [6, 23].

NMN Deamidase Prevents NMN Accumulation in Injured Sciatic Nerves

We have previously shown that NMN accumulates in transected wild-type sciatic nerves prior to degeneration of their axons, reasoning that this is likely due to rapid loss of axonal NMNAT2 and a subsequent failure to convert NMN to NAD [13]. In transected nerves from NMNd^{hemi} mice, we instead observed a reduction in the rate of accumulation of NMN, consistent with the activity of the bacterial enzyme, coupled to a compensatory increase in NaMN (Figures 4A and 4B). Accumulation of NaMN in the transected nerves from NMNd^{hemi} mice, like NMN accumulation in wild-type nerves, is an anticipated consequence of an early loss of NMNAT2, since conversion of NaMN to NaAD, like the conversion of NMN to NAD, requires NMNAT activity (Figure 2B). Similarly, modestly declining levels of NAD in both the wild-type and NMNd^{hemi} transected nerves, and of NaAD in the NMNd^{hemi} nerves (Figures 4C and 4D), are also realistic outcomes of loss of axonal NMNAT2 prior to frank degeneration of axons. This metabolic profiling of pyridine mononucleotides and dinucleotides in transected sciatic nerves is consistent with the hypothesis that NMN deamidase delays Wallerian degeneration by preventing NMN accumulation.

NMN Deamidase Protection Is Neuron Specific and Effective against Insults that Do Not Involve Physical Injury

Exogenous expression of NMN deamidase in primary superior cervical ganglion (SCG) neurons is sufficient to confer a slow Wallerian degeneration phenotype [13]. To confirm that the axon-protective effect of transgene-expressed NMN deamidase is also neuron specific, we cultured SCGs and DRGs from NMNd^{hemi} and NMNd^{homo} mice and assessed protection of cut neurites. We found that SCG and DRG neurites were both strongly protected against injury-induced degeneration in the transgenic cultures and that this was dose dependent (Figures 5A and 5B; Figures S3A and S3B). Protection appeared stronger in DRG neurites than in SCG neurites. Interestingly, the exogenous addition of NMN to transected NMNd^{homo} SCG neurites had limited toxic effect on the protective phenotype, reflecting the ability of NMN deamidase to scavenge exogenous NMN (Figures 5A and 5B). This contrasts the toxic effect of NMN on transected neurites protected with FK866, where the synthesis of the NMN is inhibited but where a scavenging system for the exogenously added NMN is not present [13].

We next tested the relative resistance of NMNd^{homo} SCG neurons to two different pro-degenerative insults, vincristine toxicity and trophic factor deprivation, both of which trigger WLD^{S}-sensitive axon degeneration without physical injury [24–26]. In both cases, we observed substantial neurite protection in the NMNd^{homo} cultures relative to controls (Figures 5C–5F).

These data confirm that NMN deamidase functions within neurons and that it inhibits an axon degeneration pathway that can be activated by several types of insult, including those not involving physical injury.

Figure 3. NMN Deamidase Expression Confers Morphological and Functional Preservation of Transected Axons

(A) Western blot showing reduced neurofilament heavy chain (NF-H) degradation in tibial nerve at the indicated times after sciatic nerve transection. Degradation is delayed in NMNd^{hemi} and NMNd^{homo} mice compared to wild-type controls.

(B) Quantification of NF-H band intensity after normalization to histone 3 (H3) in wild-type, NMNd^{hemi}, and NMNd^{homo} mice. Less nerve lysate from uncut nerve was used per lane to avoid overloading and progressively higher amounts loaded for cut nerves (as degeneration increased) to better visualize the intact NF-H and smear of degradation products. We corrected for this by normalizing to the loading control band and expressing values as a percentage of the normalized value in uncut nerve (mean ± SD; n = 3–4; two-way ANOVA followed by Bonferroni post hoc test, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$; NS, non-significant).

(C and D) Light microscopy images of sciatic nerves from wild-type, NMNd^{hemi}, and NMNd^{homo} mice at the indicated time points after cut (C) and quantification of the percentage of intact axons (D) (mean ± SD; n = 3; two-way ANOVA followed by Bonferroni post hoc test, $^{****}p < 0.0001$).

(E) Fluorescent images of sciatic nerves from YFP and YFP/NMNd^{hemi} mice at the indicated time points after cut. Axon continuity is better preserved in the NMN deamidase-expressing axons.

(F) Conduction velocities measured in uncut nerves or 7 days after cut via stimulation of sciatic nerve to evoke electromyographic activity (mean ± SEM; n = 5–6; one-way ANOVA, $^{***}p < 0.001$). See also Figure S1.

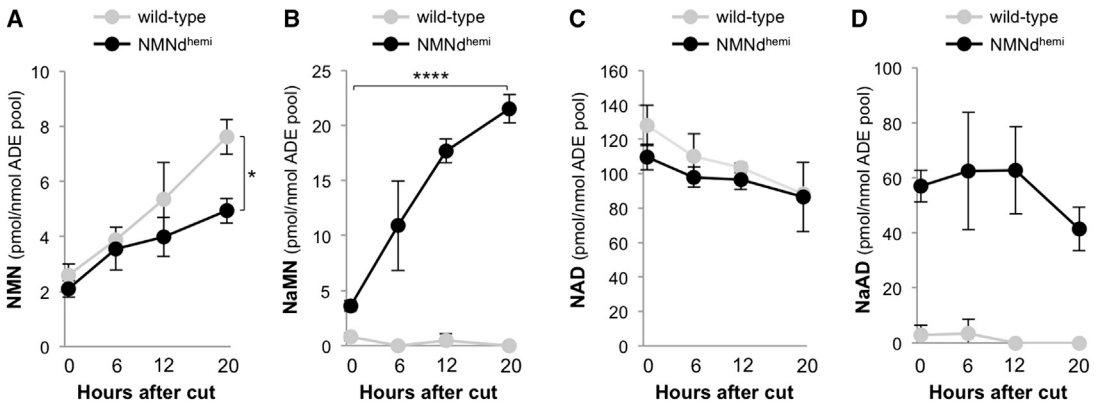


Figure 4. NMN Deamidase Reduces NMN Accumulation in Injured Sciatic Nerves

(A–D) Pyridine mononucleotide (A and B, NMN and NaMN) and pyridine dinucleotide (C and D, NAD and NaAD) levels in sciatic nerves of wild-type and NMNd^{hemi} mice at the indicated time points after cut. Data are normalized to the total adenylate (ADE) pool (ATP + ADP + AMP) as a measure of nucleotide yield (mean ± SD; n = 5 nerves per two independent measurements per time point; two-way ANOVA followed by Bonferroni post hoc test, *p < 0.05). ADE pool remained stable in nerves up to 20 hr after cut (data not shown). The levels of the nucleotides in the uncut contralateral nerves remained relatively stable (Figure S2) and their average represents time 0. See also Figure S2.

NMN Deamidase Rescues Axonal Defects in NMNAT2-Deficient Mice

Mice homozygous for the *Nmnat2*^{gtE} gene trap allele lack detectable NMNAT2 expression and die at birth, with widespread axon truncation caused by an underlying axon outgrowth defect [7]. WLD^{S} expression or an absence of SARM1 can rescue the axon extension defect, allowing mice to survive into adulthood, indicating that the underlying defect shares aspects of its mechanism with Wallerian degeneration [7, 10]. Consistent with an involvement of NMN in promoting axon degeneration [13], we previously found that blocking NMN synthesis in *Nmnat2*$^{gtE/gtE}$ DRG cultures with NAMPT inhibitor FK866 partially rescues neurite extension [10]. However, technical limitations meant more pronounced or prolonged rescue could not be achieved. We therefore assessed whether NMN deamidase expression in NMNAT2-deficient mice can restore axon extension and promote their survival.

We first assessed rescue of the axon defect in newborn *Nmnat2*$^{gtE/gtE}$;NMNd^{hemi} pups (lacking NMNAT2 but expressing NMN deamidase). Although *Nmnat2*$^{gtE/gtE}$;NMNd^{hemi} pups still died during the first post-natal day, they lacked the characteristic hunched posture of *Nmnat2*$^{gtE/gtE}$ pups that die at birth (Figure 6A). Consistent with this rescue of gross morphology, we found that distal phrenic nerve branches, which are absent from diaphragms of embryonic day (E)18.5 *Nmnat2*$^{gtE/gtE}$ embryos, were present in *Nmnat2*$^{gtE/gtE}$;NMNd^{hemi} embryos (Figure 6C). In addition, neurite outgrowth in E18.5 DRG and SCG explant cultures was significantly rescued (Figures 6D and 6E). *Nmnat2*$^{gtE/gtE}$;NMNd^{hemi} DRG neurite outgrowth actually matched that of wild-type and NMNd^{hemi} controls. In contrast, rescue of *Nmnat2*$^{gtE/gtE}$;NMNd^{hemi} SCG neurite outgrowth was substantial, but less complete (including no rescue of a small subpopulation of neurites). Interestingly, this is consistent with the marginally weaker protection against Wallerian degeneration in SCG cultures compared to DRG cultures, and it may reflect different levels of transgene expression in different neuronal types. Crucially, the *Nmnat2*^{gtE} allele remained effectively silenced when NMN deamidase was expressed (Figure 6B), excluding the possibility that changes in *Nmnat2* silencing are responsible for the observed rescue.

To assess whether higher doses of NMN deamidase can confer improved rescue, we inter-crossed *Nmnat2*$^{+/gtE}$;NMNd^{hemi} mice. These crosses produced a number of mice lacking NMNAT2 and positive for the NMN deamidase transgene that survived beyond weaning up to at least 2–3 months of age (Figure 6F). Our current genotyping methods cannot distinguish NMNd^{hemi} mice from NMNd^{homo} mice with complete certainty, but the fact that no *Nmnat2*$^{gtE/gtE}$;NMNd^{hemi} pups from *Nmnat2*$^{+/gtE}$ × *Nmnat2*$^{+/gtE}$;NMNd^{hemi} crosses survived past the first post-natal day (Figure 6F) strongly suggests the viable NMNAT2-deficient/NMN deamidase-positive mice from the *Nmnat2*$^{+/gtE}$;NMNd^{hemi} inter-crosses were homozygous for the NMN deamidase transgene (*Nmnat2*$^{gtE/gtE}$;NMNd^{homo} mice). Interestingly, this implies dose-dependent rescue, similar to that conferred by WLD^{s} in the same model [7], matching the dose-dependent protection of axotomized axons (see above).

The ability of NMN deamidase expression to rescue axon defects in *Nmnat2*$^{gtE/gtE}$ mice and promote their survival in a dose-dependent manner provides strong support for a key involvement of NMN accumulation in the axon truncation phenotype caused by NMNAT2 deficiency.

DISCUSSION

Here we have demonstrated that NMN deamidase is able to delay Wallerian degeneration in vivo, thereby extending previous findings obtained in primary neuronal cultures [11, 13, 22]. It can do this in two disparate vertebrate species and seemingly also when expressed at very low levels. Neurites in primary cultures of neurons from NMN deamidase transgenic mice also show strong protection following vincristine treatment and nerve growth factor (NGF) withdrawal, both of which are insults that do not involve physical injury. Strikingly, NMN deamidase

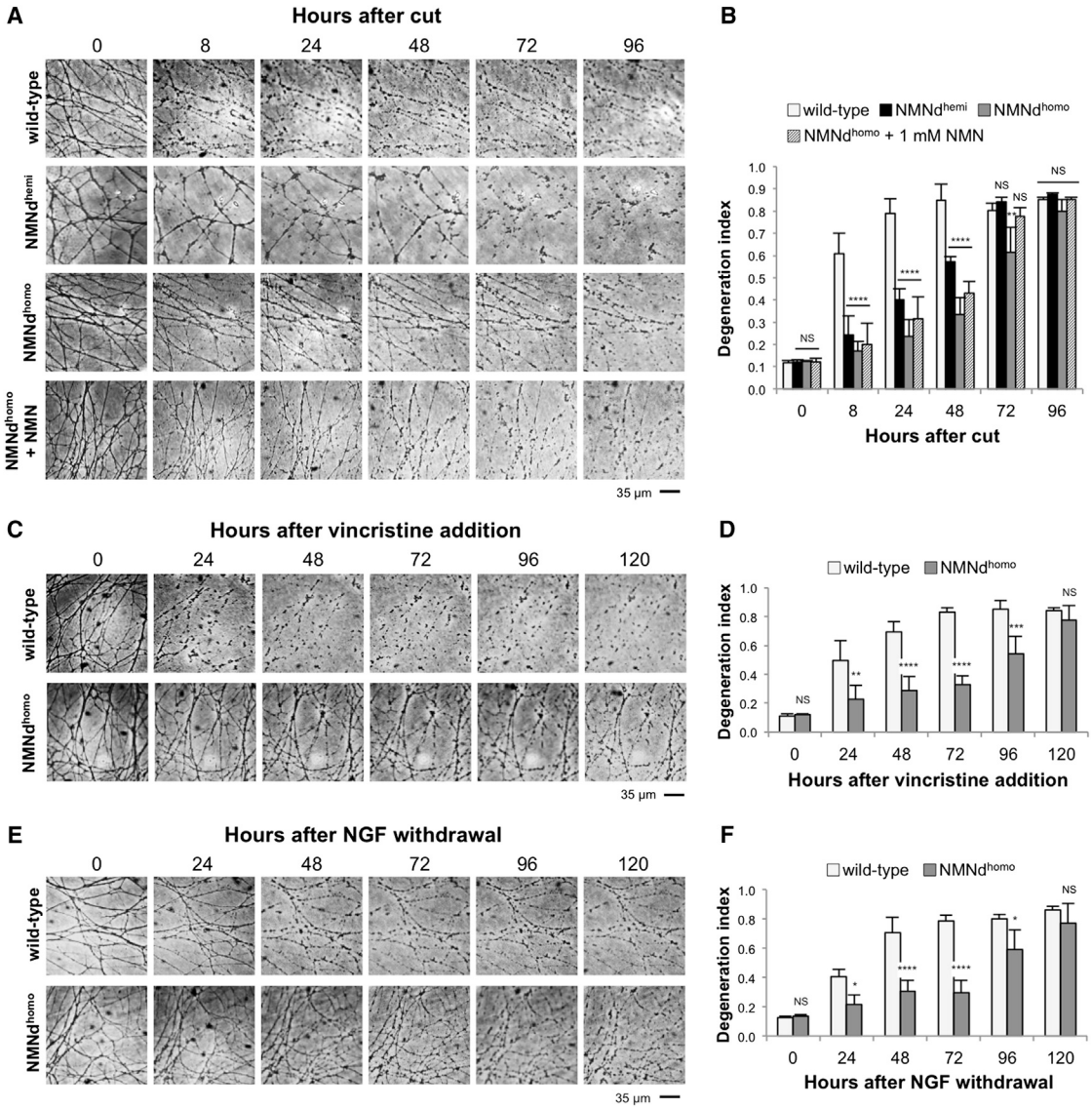


Figure 5. NMN Deamidase Protection Is Neuron Specific and Effective against Various Toxic Insults

(A) Representative bright-field images of transected neurites in wild-type, NMNd^{hemi}, and NMNd^{homo} SCG cultures. Transected neurites expressing NMN deamidase show morphological continuity several days after axotomy.

(B) Quantification of the degeneration index in experiments described in (A).

(C) Representative bright-field images of neurites in wild-type and NMNd^{homo} SCG cultures at the indicated time points after 20 nM vincristine addition.

(D) Quantification of the degeneration index in experiments described in (C).

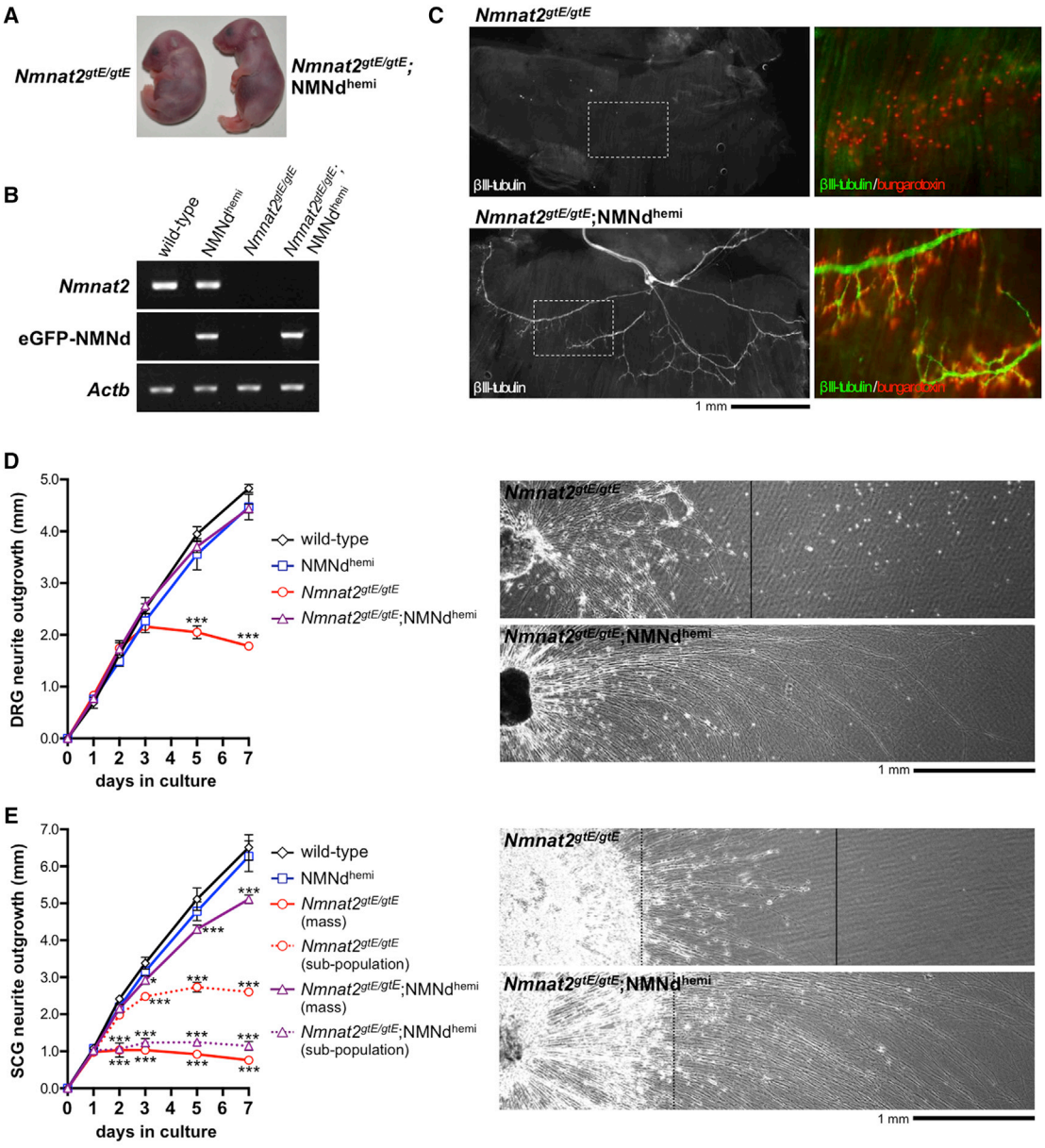
(E) Representative bright-field images of neurites in wild-type and NMNd^{homo} SCG cultures at the indicated time points after NGF withdrawal.

(F) Quantification of the degeneration index in experiments described in (E). Degeneration index in (B), (D), and (F) was calculated from three fields per sample in three independent experiments (mean ± SD; two-way ANOVA followed by Bonferroni post hoc test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$; NS, non-significant).

See also Figure S3.

expression can also rescue the severe axon outgrowth defect in mice lacking NMNAT2, which has an underlying degenerative basis [7, 10], to the extent that NMNAT2-deficient mice, rather than dying at birth, can instead survive well past weaning age if they express enough of the enzyme. Notably, all of these outcomes, including the dose dependency of the protective phenotype, mirror those previously seen with WLD^S, thereby supporting the idea that NMN deamidase influences a core step in an evolutionarily conserved WLD^S-sensitive axon degeneration pathway that is activated in a variety of situations.

When considered in isolation, all the data presented here are consistent with a model in which preventing NMN accumulation in axons after induced or constitutive depletion of NMNAT2 protects them from degeneration. We originally proposed this model in response to two observations that contradicted the view that maintaining NAD levels is instead critical for axon



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cross	viable offspring (>P1) genotype	Obs.	Exp.
Nmnat2+/gtE x Nmnat2+/gtE; NMNdhemi	wild-type	15	10.25
	NMNdhemi	16	10.25
	Nmnat2+/gtE	27	20.5
	Nmnat2+/gtE;NMNdhemi	24	20.5
	Nmnat2gtE/gtE	0	10.25
	Nmnat2gtE/gtE;NMNdhemi	0	10.25

cross	viable offspring (>P1) genotype	Obs.	Exp.
Nmnat2+/gtE; NMNdhemi x Nmnat2+/gtE; NMNdhemi	wild-type	4	6.8125
	NMNdhemi	29	13.625
	NMNdhomo		6.8125
	Nmnat2+/gtE	23	13.625
	Nmnat2+/gtE;NMNdhemi	48	27.25
	Nmnat2+/gtE;NMNdhomo		13.625
	Nmnat2gtE/gtE	0	6.8125
	Nmnat2gtE/gtE;NMNdhemi	5	13.625
	Nmnat2gtE/gtE;NMNdhomo		6.8125

Figure 6. Expression of NMN Deamidase Rescues Axon Defects in Mice Lacking NMNAT2

(A) Representative gross morphology of newborn *Nmnat2*$^{gtE/gtE}$ and *Nmnat2*$^{gtE/gtE}$;NMNd^{hemi} pups.

(B) RT-PCR analysis of *Nmnat2*, eGFP-NMN deamidase, and *Actb* (sample control) mRNA in E18.5 brain from embryos of the indicated genotypes (images representative of n = 3).

(legend continued on next page)

survival. The first was the ability of NAMPT inhibitor FK866 to delay Wallerian degeneration despite a simultaneous decline in NAD levels, while the second was our previous finding that exogenous expression of NMN deamidase in primary neuronal cultures can phenocopy WLD^S, despite there being no clear route for generating NAD from its product, NaMN, once NMNAT2 depletion has occurred [13]. We argued that the simplest way to link these observations to the strong protective effect of NMNATs (which do maintain NAD levels) was through a shared ability to keep NMN levels low.

However, a recent study has questioned this model, reporting situations where genetic and pharmacological manipulation of the NAD biosynthetic pathway in primary DRG cultures raises NMN either in uninjured neurites that remain unaffected or in injured neurites that show delayed Wallerian degeneration [22]. While these findings seem inconsistent with the NMN accumulation model, it is notable that all instances where raised NMN correlates with neurite preservation also involve substantial increases in NAD. An increase in NAD is also not universally sufficient to protect injured axons [22, 27], so it was postulated that changes in other, as yet unknown, metabolites cause axon degeneration [22]. However, the common protective mechanism of FK866, bacterial NMN deamidase, and mammalian NMNAT is more likely to be their known, shared influence on NMN than similar effects on another metabolite that has not yet been identified or shown to be similarly affected by all three manipulations. Likewise, it can be argued that a direct involvement of changes to endogenous levels of NaMN or NaAD in the protective mechanism is unlikely.

Given that our new in vivo data further support a pro-degenerative role for NMN, we suggest that the best fit for all currently available data is a model involving both NMN and NAD. For example, the effects of accumulated NMN may be evident only in the context of physiological or declining NAD levels, as occurs in transected axons in the first few hours after injury [13, 22]. Given their structural relatedness, high NAD could inhibit the pro-degenerative function of NMN directly. Alternatively, accumulated NMN could activate early steps in the degeneration pathway, irrespective of NAD concentration, but raising NAD could compensate for later SARM1-dependent NAD depletion [22, 28]. Crucially, this would reconcile the NMN accumulation model [10, 11, 13] with a SARM1-dependent NAD depletion model [22, 28], and it provides a simple explanation for FK866-mediated protection of cut axons. Of note, although the protective effect of FK866 has been reported in several studies, the degree of protection achieved does vary [11, 13, 22, 27, 29]. Different sources of FK866 could account for this variability; however, in our hands, FK866 is one of the most effective pharmacological tools for delaying Wallerian degeneration, consistently protecting injured DRG neurites for up to 48 hr [13].

NMN deamidase transgenic mice have other potential uses. The ability of NMN deamidase to rescue axon defects in NMNAT2-deficient mice suggests that these mice could be a useful tool to study the putative detrimental effects of NMN accumulation in models of neurodegenerative diseases. Crucially, comparisons between the abilities of NMN deamidase and WLD^S to ameliorate symptoms in any given model should help to resolve whether the primary underlying defect is axon degeneration or a failure to produce NAD, particularly in the nucleus where WLD^S is most abundant [2]. Outside of the axon degeneration field, NMN deamidase transgenic mice could be informative with respect to general NAD metabolism. In keeping with the reported low expression levels of NAD synthase in the nervous system [30], which converts NaAD to NAD, these mice have abnormally high steady-state levels of both NaMN and NaAD. It will, therefore, be interesting to establish to what extent normal regulatory feedback loops in the NAD biosynthetic pathway [31] are altered in $NMNd^{hemi}$ and $NMNd^{homo}$ mice. Importantly, such changes might explain why the transgenic founders with higher enzyme activity failed to transmit the transgene.

In conclusion, our data strongly support an in vivo role for NMN accumulation in triggering axon degeneration both after injury and when NMNAT2 is constitutively depleted, with axon protection by WLD^S/NMNATs and NMN deamidase in both situations at least partially relying on their ability to limit NMN accumulation.

EXPERIMENTAL PROCEDURES

Animal Procedures

Animal work was carried out in accordance with the Animals (Scientific Procedures) Act, 1986, under Project Licenses PPL 70/7620 and PPL 40/3482 following the appropriate ethical review processes at the University of Nottingham and Babraham Institute. Design and generation of a transgene construct for expression of *E. coli* NMN deamidase fused to EGFP from a β-actin promoter and procedures for PCR-genotyping founder mice and their transgene-positive offspring are detailed in the Supplemental Experimental Procedures. Sciatic nerve lesions and the analysis of YFP-labeled nerves were performed as described previously [13, 32] (see also the Supplemental Experimental Procedures). Zebrafish experiments were performed as described in [11] (see also the Supplemental Experimental Procedures).

(C) βIII-tubulin immunostaining (left-hand images) revealing the presence of phrenic nerve terminal branches in a $Nmnat2^{gtE/gtE}$;$NMNd^{hemi}$ diaphragm. These are invariantly absent from $Nmnat2^{gtE/gtE}$ diaphragms. Boxed regions are magnified (right). Acetylcholine receptor (AChR) clusters are labeled by counter-staining with bungarotoxin-TRITC. Innervation of AChR clusters is only evident in the $Nmnat2^{gtE/gtE}$;$NMNd^{hemi}$ diaphragm (images representative of n = 3 $Nmnat2^{gtE/gtE}$ and n = 5 $Nmnat2^{gtE/gtE}$;$NMNd^{hemi}$ diaphragms). Innervation in $Nmnat2^{gtE/gtE}$;$NMNd^{hemi}$ diaphragms is superficially similar to that in wild-type and $NMNd^{hemi}$ controls (data not shown).

(D and E) Radial neurite outgrowth from DRG explants (D) and radial neurite outgrowth from SCG explants (E). Ganglia were taken from E18.5 embryos of the genotypes listed and outgrowth followed over 7 days in culture (mean radial extension in mm ± SEM; average of two ganglia per embryo for n = 3–5 embryos per genotype; two-way repeated-measures ANOVA with Dunnett's multiple comparisons post hoc tests of wild-type versus other groups, $^*p < 0.05$ and $^{***}p < 0.001$). Representative images of neurite outgrowth at 7 days for $Nmnat2^{gtE/gtE}$ and $Nmnat2^{gtE/gtE}$;$NMNd^{hemi}$ DRG (D) and SCG (E) cultures are shown to the right of each graph in each panel. $Nmnat2^{gtE/gtE}$;$NMNd^{hemi}$ DRG and SCG neurite outgrowth (from ganglia positioned on the left) extends beyond the right-hand edge of the images. Two populations of neurites can be differentiated in $Nmnat2^{gtE/gtE}$ and $Nmnat2^{gtE/gtE}$;$NMNd^{hemi}$ SCG cultures (E); most $Nmnat2^{gtE/gtE}$ neurites show severely retarded outgrowth (mass, solid line), with a subpopulation extending further (dashed line); most $Nmnat2^{gtE/gtE}$;$NMNd^{hemi}$ neurites instead show near-normal outgrowth (mass, solid line), while a subpopulation shows severely limited outgrowth (dashed line).

(F) Viability past post-natal day 1 (P1) for offspring from $Nmnat2^{+/gtE}$ × $Nmnat2^{+/gtE}$;$NMNd^{hemi}$ and $Nmnat2^{+/gtE}$;$NMNd^{hemi}$ × $Nmnat2^{+/gtE}$;$NMNd^{hemi}$ matings.

Statistical Analysis

Data are expressed as mean ± SEM or SD (as stated). Statistical analysis was performed using GraphPad ANOVA or Student's t test, with p values less than 0.05 being considered significant for any set of data.

All other methods are described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, Supplemental Experimental Procedures, and two movies and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.01.070.

AUTHOR CONTRIBUTIONS

M.D.S. and L.C. conceived the study. L.C., M.D.S., A.L., and J.G. designed the experiments. M.D.S. performed SCG and DRG culture and in vivo nerve injury experiments with some input from L.C. A.L. performed experiments on zebrafish larvae and data analysis. J.G. conducted experiments on *Nmnat2*$^{gtE/gtE}$/NMN deamidase mice. G.O., V.M., F.Z., and N.R. performed nucleotide measurements and NMN deamidase enzymatic activity determinations. R.P.H. and L.F.D. performed nerve conduction measurements. J.W. helped with western blots. M.G. assisted with the zebrafish model. L.C. supervised and coordinated the research. M.D.S., A.L., M.P.C., and J.G. wrote the manuscript, with some input from G.O., N.R., and L.C.

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REFERENCES

1. Coleman, M. (2005). Axon degeneration mechanisms: commonality amid diversity. Nat. Rev. Neurosci. *6*, 889–898.
2. Conforti, L., Gilley, J., and Coleman, M.P. (2014). Wallerian degeneration: an emerging axon death pathway linking injury and disease. Nat. Rev. Neurosci. *15*, 394–409.
3. Waller, A. (1850). Experiments on the section of glossopharyngeal and hypoglossal nerves of the frog and observations of the alternatives produced thereby in the structure of their primitive fibres. Philos. Trans. R. Soc. Lond. B Biol. Sci. *140*, 423–429.
4. Gilley, J., and Coleman, M.P. (2010). Endogenous Nmnat2 is an essential survival factor for maintenance of healthy axons. PLoS Biol. *8*, e1000300.
5. Milde, S., Gilley, J., and Coleman, M.P. (2013). Subcellular localization determines the stability and axon protective capacity of axon survival factor Nmnat2. PLoS Biol. *11*, e1001539.
6. Mack, T.G., Reiner, M., Beirowski, B., Mi, W., Emanuelli, M., Wagner, D., Thomson, D., Gillingwater, T., Court, F., Conforti, L., et al. (2001). Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. Nat. Neurosci. *4*, 1199–1206.
7. Gilley, J., Adalbert, R., Yu, G., and Coleman, M.P. (2013). Rescue of peripheral and CNS axon defects in mice lacking NMNAT2. J. Neurosci. *33*, 13410–13424.
8. Gerdts, J., Summers, D.W., Milbrandt, J., and DiAntonio, A. (2016). Axon Self-Destruction: New Links among SARM1, MAPKs, and NAD+ Metabolism. Neuron *89*, 449–460.
9. Osterloh, J.M., Yang, J., Rooney, T.M., Fox, A.N., Adalbert, R., Powell, E.H., Sheehan, A.E., Avery, M.A., Hackett, R., Logan, M.A., et al. (2012). dSarm/Sarm1 is required for activation of an injury-induced axon death pathway. Science *337*, 481–484.
10. Gilley, J., Orsomando, G., Nascimento-Ferreira, I., and Coleman, M.P. (2015). Absence of SARM1 rescues development and survival of NMNAT2-deficient axons. Cell Rep. *10*, 1974–1981.
11. Loreto, A., Di Stefano, M., Gering, M., and Conforti, L. (2015). Wallerian Degeneration Is Executed by an NMN-SARM1-Dependent Late Ca(2+) Influx but Only Modestly Influenced by Mitochondria. Cell Rep. *13*, 2539–2552.
12. Gerdts, J., Summers, D.W., Sasaki, Y., DiAntonio, A., and Milbrandt, J. (2013). Sarm1-mediated axon degeneration requires both SAM and TIR interactions. J. Neurosci. *33*, 13569–13580.
13. Di Stefano, M., Nascimento-Ferreira, I., Orsomando, G., Mori, V., Gilley, J., Brown, R., Janeckova, L., Vargas, M.E., Worrell, L.A., Loreto, A., et al. (2015). A rise in NAD precursor nicotinamide mononucleotide (NMN) after injury promotes axon degeneration. Cell Death Differ. *22*, 731–742.
14. Galeazzi, L., Bocci, P., Amici, A., Brunetti, L., Ruggieri, S., Romine, M., Reed, S., Osterman, A.L., Rodionov, D.A., Sorci, L., and Raffaelli, N. (2011). Identification of nicotinamide mononucleotide deamidase of the bacterial pyridine nucleotide cycle reveals a novel broadly conserved amidohydrolase family. J. Biol. Chem. *286*, 40365–40375.
15. Lunn, E.R., Perry, V.H., Brown, M.C., Rosen, H., and Gordon, S. (1989). Absence of Wallerian Degeneration does not Hinder Regeneration in Peripheral Nerve. Eur. J. Neurosci. *1*, 27–33.
16. Adalbert, R., Gillingwater, T.H., Haley, J.E., Bridge, K., Beirowski, B., Berek, L., Wagner, D., Grumme, D., Thomson, D., Celik, A., et al. (2005). A rat model of slow Wallerian degeneration (WldS) with improved preservation of neuromuscular synapses. Eur. J. Neurosci. *21*, 271–277.
17. Kitay, B.M., McCormack, R., Wang, Y., Tsoulfas, P., and Zhai, R.G. (2013). Mislocalization of neuronal mitochondria reveals regulation of Wallerian degeneration and NMNAT/WLD(S)-mediated axon protection independent of axonal mitochondria. Hum. Mol. Genet. *22*, 1601–1614.
18. Martin, S.M., O'Brien, G.S., Portera-Cailliau, C., and Sagasti, A. (2010). Wallerian degeneration of zebrafish trigeminal axons in the skin is required for regeneration and developmental pruning. Development *137*, 3985–3994.
19. MacDonald, J.M., Beach, M.G., Porpiglia, E., Sheehan, A.E., Watts, R.J., and Freeman, M.R. (2006). The Drosophila cell corpse engulfment receptor Draper mediates glial clearance of severed axons. Neuron *50*, 869–881.
20. Brown, M.C., Perry, V.H., Hunt, S.P., and Lapper, S.R. (1994). Further studies on motor and sensory nerve regeneration in mice with delayed Wallerian degeneration. Eur. J. Neurosci. *6*, 420–428.
21. Chen, S., and Bisby, M.A. (1993). Long-term consequences of impaired regeneration on facial motoneurons in the C57BL/Ola mouse. J. Comp. Neurol. *335*, 576–585.
22. Sasaki, Y., Nakagawa, T., Mao, X., DiAntonio, A., and Milbrandt, J. (2016). NMNAT1 inhibits axon degeneration via blockade of SARM1-mediated NAD(+) depletion. eLife *5*, e19749.
23. Perry, V.H., Lunn, E.R., Brown, M.C., Cahusac, S., and Gordon, S. (1990). Evidence that the Rate of Wallerian Degeneration is Controlled by a Single Autosomal Dominant Gene. Eur. J. Neurosci. *2*, 408–413.
24. Wang, M., Wu, Y., Culver, D.G., and Glass, J.D. (2001). The gene for slow Wallerian degeneration (Wld(s)) is also protective against vincristine neuropathy. Neurobiol. Dis. *8*, 155–161.

25. Deckwerth, T.L., and Johnson, E.M., Jr. (1994). Neurites can remain viable after destruction of the neuronal soma by programmed cell death (apoptosis). Dev. Biol. *165*, 63–72.

26. Vohra, B.P., Sasaki, Y., Miller, B.R., Chang, J., DiAntonio, A., and Milbrandt, J. (2010). Amyloid precursor protein cleavage-dependent and -independent axonal degeneration programs share a common nicotinamide mononucleotide adenylyltransferase 1-sensitive pathway. J. Neurosci. *30*, 13729–13738.

27. Sasaki, Y., Vohra, B.P., Lund, F.E., and Milbrandt, J. (2009). Nicotinamide mononucleotide adenylyl transferase-mediated axonal protection requires enzymatic activity but not increased levels of neuronal nicotinamide adenine dinucleotide. J. Neurosci. *29*, 5525–5535.

28. Gerdts, J., Brace, E.J., Sasaki, Y., DiAntonio, A., and Milbrandt, J. (2015). SARM1 activation triggers axon degeneration locally via NAD^+ destruction. Science *348*, 453–457.

29. Shen, H., Hyrc, K.L., and Goldberg, M.P. (2013). Maintaining energy homeostasis is an essential component of Wld(S)-mediated axon protection. Neurobiol. Dis. *59*, 69–79.

30. Mori, V., Amici, A., Mazzola, F., Di Stefano, M., Conforti, L., Magni, G., Ruggieri, S., Raffaelli, N., and Orsomando, G. (2014). Metabolic profiling of alternative NAD biosynthetic routes in mouse tissues. PLoS ONE *9*, e113939.

31. Burgos, E.S., and Schramm, V.L. (2008). Weak coupling of ATP hydrolysis to the chemical equilibrium of human nicotinamide phosphoribosyltransferase. Biochemistry *47*, 11086–11096.

32. Beirowski, B., Berek, L., Adalbert, R., Wagner, D., Grumme, D.S., Addicks, K., Ribchester, R.R., and Coleman, M.P. (2004). Quantitative and qualitative analysis of Wallerian degeneration using restricted axonal labelling in YFP-H mice. J. Neurosci. Methods *134*, 23–35.