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Targeting NMNAT1 to Axons and Synapses Transforms Its Neuroprotective Potency In Vivo

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Axon and synapse degeneration are common components of many neurodegenerative diseases, and their rescue is essential for effective neuroprotection. The chimeric Wallerian degeneration slow protein (WldS) protects axons dose dependently, but its mechanism is still elusive. We recently showed that WldS acts at a non-nuclear location and is present in axons. This and other recent reports support a non-nuclear NMNAT1 relative to WldS also needs to be established in vivo. Because the N-terminal portion of WldS (N70) localized to axons, we hypothesized that it mediates the trafficking of the NMNAT1 portion. To test this, we substituted N70 with an axonal targeting peptide derived from amyloid precursor protein, and fused this to NMNAT1 with disrupted nuclear targeting. In transgenic mice, this transformed NMNAT1 from a molecule unable to inhibit Wallerian degeneration, even at high expression levels, into a protein more potent than WldS, able to preserve injured axons for several weeks at undetectable expression levels. Preventing NMNAT1 axonal delivery abolished its protective effect. Axonally targeted NMNAT1 localized to vesicular structures, colocalizing with extranuclear WldS, and was cotransported at least partially with mitochondria. We conclude that axonal targeting of NMNAT activity is both necessary and sufficient to delay Wallerian degeneration, and that promoting axonal and synaptic delivery greatly enhances the effectiveness.

Introduction

Axonal degeneration is a primary or contributory component of many neurodegenerative diseases and a promising therapeutic target (Raff et al., 2002; Saxena and Caroni, 2007). Studies of the mutant mouse WldS (Wallerian degeneration slow) (Lunn et al., 1989) established axonal degeneration as a tightly regulated process. In this strain, the chimeric WldS protein delays the progressive degradation of experimentally transected nerves (Wallerian degeneration) (Waller, 1850), preserving axons and synapses in a nonlinear, dose-dependent fashion (Mack et al., 2001). Importantly, WldS ameliorates axonopathy in models of various neurodegenerative conditions, such as progressive motor neuronopathy, Charcot–Marie–Tooth disease, Parkinson’s disease, and glaucoma (Ferri et al., 2003; Samsam et al., 2003; Sajadi et al., 2004; Howell et al., 2007; Beirowski et al., 2008), opening new avenues for therapeutic approaches.

The mechanism of WldS protection remains largely elusive at the molecular level. WldS arose from a de novo triplication, which links the N-terminal 70 aa of the ubiquitination factor Ube4b (N70) to the nuclear NAD+ -synthesizing enzyme nicotinamide mononucleotide adenylyltransferase (NMNAT1), via a unique 18 aa sequence (Wld18) (Conforti et al., 2000). Despite its abundance in the nucleus, we recently detected WldS in axons, where it accumulates after nerve constriction (Beirowski et al., 2009). Since axonally transported proteins typically accumulate at constriction sites (Cavalli et al., 2005), WldS may be transported, either directly or by associating with vesicles or organelles. Moreover, redistribution of WldS to cytoplasm strikingly enhanced axonal and synaptic protection (Beirowski et al., 2009), possibly by increasing the delivery to axons and synapses where WldS may function locally.

Further support for this model comes from studies of the NMNAT1 portion of WldS. The intrinsic catalytic activity of WldS is required for the phenotype in vitro and in vivo (Araki et al., 2004; Jia et al., 2007; Avery et al., 2009; Conforti et al., 2009). Nuclear NMNAT1 is not sufficient to confer axon protection in mice and has only a weak effect in Drosophila and in primary neuronal culture relative to WldS (Conforti et al., 2007; Avery et al., 2009; Sasaki et al., 2009b; Yahata et al., 2009). If NMNAT1 is shifted outside the nucleus (cyt-NMNAT1), the protection in culture is enhanced and cyt-NMNAT1 can also protect axons in vivo when overexpressed at least 15-fold more than WldS (Sasaki et al., 2009b). However, it remains unclear whether near-physiological levels of
NMNAT1 are sufficient to protect injured axons in mice, and whether the critical site is axonal or somatic.

We report here that targeting NMNAT1 to axons greatly enhances its protective potential in vivo. We show for the first time that even a marginal increase in NMNAT1 level becomes robustly protective as long as NMNAT1 is delivered to axons, and that preventing axonal entry abolishes this effect. Finally we find that axonally targeted NMNAT1 colocalizes with extranuclear Wld^* and it is transported in coordinated movement with mitochondria.

Materials and Methods

Plasmids and generation of transgenic mice. An NMNAT1 construct with disrupted nuclear targeting, termed ΔNLS NMNAT1, was PCR amplified from the previously reported ΔNLS Wld^* construct, carrying the R215A mutation (Beirowski et al., 2005). The FLAG reverse primer (BamHI site underlined, mutated stop codon in bold, FLAG tag italicized) and WldS-FLAG reverse (Wilbrey et al., 2008) using WldS cDNA as template.

For ΔNLS NMNAT1-FLAG: 5′-TAGATCCCAAGCTTAACTTCTG-CCCATGGAACCTTAC-3′ (Conforti et al., 2007).

For Exon7 MN-ΔNLS NMNAT1-FLAG: 5′-ATCCCAAGCTTAACTTCTG-CCCATGGAACCTTAC-3′ (Conforti et al., 2007). Aphidicolin (2 μM) was added to dissociated and explant DRG cultures to reduce the number of non-neuronal cells. Neurites were axiomed with a scalpel at DIV 7 and phase–contrast pictures of the same field were taken immediately (0 d) and 1, 3, and 6 d after cutting. An Olympus IX81 microscope coupled to a PC running ISIS imaging software was used for image acquisition. A second cut was performed at the same location 3 d after the first axotomy to avoid regeneration and DRGs were rinsed with fresh medium. Explants were collected at DIV 13 for Western blot analysis.

Treatments with microtubule destabilizing agent. Vincristine (Sigma) and nocodazole (Calbiochem) were diluted in DMSO and added to the cultures at the final concentrations of 0.04 μM, and 5 or 20 μg/ml, respectively, at the indicated times (see Fig. 7). DMSO was used for control cultures. Medium with fresh drugs was replaced every 24 h.

Assessment of in vivo axonal preservation. We used expression of the YFP-H transgene for longitudinal imaging of YFP-labeled axons in sciatic and tibial nerve, an established method for morphological assessment of axonal preservation (Feng et al., 2000; Beirowski et al., 2004; Beirowski et al., 2005; Conforti et al., 2009). Mice were anesthetized with isoflurane (Abbot Animal Health) and subcutaneous injection of 2.5 μg of buprenorphine hydrochloride (Vetergesic, Alstoe Lt. Animal Health) and unilateral sciatic nerve lesion was performed as described by Beirowski et al. (2009); the contralateral side served as control. A 5 mm segment of sciatic nerve was removed to prevent regeneration complicating the analysis of the distal stump at longer lesion durations (14–35 d). Seven, fourteen, and thirty-five days after surgery, mice were humanely killed and 1.5–cm-long nerve distal stumps removed. The lesion site was inspected to confirm that the proximal and distal stumps had remained separated. Nerves were immersion-fixed in 4% PFA and 0.1M PBS 20–48 h later for immunofluorescence and high-resolution confocal imaging. DRG explants from E14.5–E16.5 embryos were dissected and plated as described by Conforti et al. (2007). Avidinyl (2 μM) was added to dissociated and explant DRG cultures to reduce the number of non-neuronal cells. Neurites were dried off with a scalpel at DIV 7 and phase–contrast pictures of the same field were taken immediately (0 d) and 1, 3, and 6 d after cutting. An Olympus IX81 microscope coupled to a PC running ISIS imaging software was used for image acquisition. A second cut was performed at the same location 3 d after the first axotomy to avoid regeneration and DRGs were rinsed with fresh medium. Explants were collected at DIV 13 for Western blot analysis.

Assessment of in vitro axonal preservation. For transection experiments on DRG explant cultures (see Fig. 4), phase-contrast images (20× magnification) of axotomized neurites were taken at 0, 1, 3, and 6 d. Brightness and contrast were adjusted for an entire 0.3 mm × 0.3 mm square from each image using Adobe Photoshop to obtain similar background intensities. Images were further processed using NIH ImageJ software similar to the method described by Sasaki et al. (2009a). Accordingly, images were binarized so that axons appear black. The degree of axonal neurite intensity and contrast were adjusted for an entire 0.3 mm × 0.3 mm square from each image using Adobe Photoshop to obtain similar background intensities. Images were further processed using NIH ImageJ software similar to the method described by Sasaki et al. (2009a). Accordingl

3• (Beirovski et al., 2009), and double-hemizygous ΔN16 Wld^*YFP-H mice (line 1) (Conforti et al., 2009). Triple-heterozygous tg-Wld^*Wld/H YFP-H mice express levels of Wld^* protein similar to that in homozygous natural Wld^- mice and display a similarly retarded time course of axon degeneration (Beirovski et al., 2005).

All animal work was performed in accordance with the Animals (Scientific Procedures) Act (1986) under Project Licenses PPL 80/1778 and PPL 80/2254.

Genotyping. For mouse genotyping by Southern blotting, genomic DNA extracts were digested with BamHI and HindIII (Invitrogen) at 37°C overnight, and run on 0.8% agarose gels in TBE. The gels were denatured in 0.4 M NaOH, and hybridized with a probe of radioactive Wld^- cDNA. Posthybridization washes were performed at a stringency of 0.1× SSC, 0.1% SDS. Radioactive filters were exposed to x-ray film for 24 h or longer at ~80°C.

Cell culture. Culture and transfection of hippocampal neurons were described previously (Wilbrey et al., 2008) and dorsal root ganglion (DRG) neurons were processed in a similar way. In brief, dissociated neurons from E14.5–E16.5 embryos were plated on coated glass coverslips or IBIDI μ–dishes, and transfected at DIV 5–3. Neurons were cut with a scalp to test axonal preservation 24 h after transfection or fixed with 4% paraformaldehyde (PFA) in 0.1 M PBS 20–48 h later for immunofluorescence and high-resolution confocal imaging. DRG explants from E14.5–E16.5 embryos were dissected and plated as described by Conforti et al. (2007). Avidinyl (2 μM) was added to dissociated and explant DRG cultures to reduce the number of non-neuronal cells. Neu-

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continuity was scored using the Particle Analyzer tool with size (in square pixels) set at 100 to infinity. This algorithm measures the number of black pixels in continuous axons and excludes axons with high degree of fragmentation. We normalized it for the total number for black pixels (total axonal area) in the field to obtain a protection index (PI) value. Typically an image of intact axons has a PI value around 1 and an image of fragmented axons has a PI value around 0.5. A PI around 0 occurs when axons detach from the dish (such fields where excluded from the quantification) or when only debris remains that is smaller than the minimum set size (100 square pixels). Results from C57BL/6 embryos and Ax-NMNAT1-negative embryo littermates were grouped together as wild-type (WT) control. For each genotype, images from 3–10 dishes were quantified (2–3 dishes/dish). DRG explant cultures in presence of microtubule destabilizing agents (see Fig. 7) were cut and imaged in the same way. Axonal protection of dissociated DRG neurons was tested by cutting single neurons that coexpressed EGFP and Ax-NMNAT1-mCherry. Neurons were imaged with an Olympus IX81 microscope, and only the ones coexpressing green and red fluorescence were selected to be axotomized. The percentage of axons that did not develop extensive varicosities or fragmentation 48 h after cut was quantified (=2D), in presence or absence of microtubule destabilizing agents (see Fig. 7) (10–20 neurons/condition, 2–4 dishes tested).

**NMNAT enzyme activity assay.** Brains were divided in half sagittally, snap frozen in liquid nitrogen, and kept at −80°C. One hemisphere was used for NMNAT activity assay and the other was used for Western blot analysis. NMNAT assay was performed as described previously (Mack et al., 2001) with minor modifications. Tissue was Ultra-Turrax homogenized in 5 volumes of ice-cold buffer [50 mM HEPES, pH 7.4, 0.5 mM EDTA, 1 mM MgCl₂, 1 mM DTT, and protease inhibitor (Mini-protean reagent, Invitrogen)]. NMNAT activity assay was performed at 37°C in a 0.15 ml reaction mixture containing 50 mM Tris-HCl, pH 7.5, 5 mM nicotinamide mononucleotide (NMN), 3 mM ATP, 20 mM MgCl₂, 20 mM NaF, and an appropriate aliquot of brain homogenate. The reaction was started by adding 5 mM NMN and stopped by the addition of a half-volume of ice-cold 1.2 M HClO₄. After 10 min at 0°C, the mixture was started by adding 5 mM NMN and stopped by the addition of a half-volume of ice-cold 1.2 M HClO₄. After 10 min at 0°C, the mixture was centrifuged and 100 µl of supernatant was neutralized by the addition of 26 µl of 0.8 M K₂CO₃. NMNAT activity was calculated by reverse-phase HPLC identification (C18 column) and quantification of the product (NAD⁺). One unit of enzyme was defined as the amount capable of producing 1 µmol of NAD⁺ per minute at 37°C. Specific NMNAT activity was obtained by normalization with protein content calculated by Bradford assay (Bio-Rad).

**Assessment of in vivo preservation of neuromuscular junctions.** Mice were killed by cervical dislocation and the sciatic nerve was removed by sharp dissection. Brains were divided in half sagittally, cryosectioned and processed as positive controls. Sections (20 µm) were fixed in 4% PFA, 0.1M PBS for 10 min, permeabilized with 1% Triton X-100, blocked, and immunostained with mouse anti-FLAG antibody (1:2000) (Sigma F3165) overnight followed by 1 h incubation with secondary Alexa647-goat anti-FLAG antibody (1:200) (Invitrogen), both diluted in 5% PBS in PBS. In hippocampal cultures, dendrites were counterstained with chicken anti-MAP-2 antibody (1:30,000) (AbCam) and secondary Alexa647-goat anti-chicken antibody (Invitrogen). Proximal axons were stained with rabbit anti-Ankyrin G antibody (1:400) (Santa Cruz Biotechnology) and secondary Alexa488-goat anti-rabbit antibody (Invitrogen).

**Cryosections and cell culture samples** were mounted in Vectashield mounting medium containing DAPI for nuclear counterstaining (Vector Laboratories) and imaged using the Zeiss LSM 510 Meta Confocal system in Multi-track mode to avoid fluorescence bleed-through. Z-series were merged using projection algorithms from Zeiss LSM Software Release 3.2.

**Western blotting and subcellular fractionation.** Western blotting of total brain homogenates (see Fig. 2) was performed as described by Conforti et al. (2007) with minor modifications. Brains were homogenized in 5 volumes of RIPA buffer, high-speed supernatant was further centrifuged at 2000 × g for 5 min, and concentrated brain homogenate was loaded for SDS-PAGE and blotted to PVDF membranes. After overnight blocking, mouse anti-FLAG antibody (1:2000) (Sigma F3165) overnight followed by 1 h incubation with secondary Alexa647-goat anti-FLAG antibody (1:200) (Invitrogen), both diluted in 5% PBS in PBS. In hippocampal cultures, dendrites were counterstained with chicken anti-MAP-2 antibody (1:30,000) (AbCam) and secondary Alexa647-goat anti-chicken antibody (Invitrogen). Proximal axons were stained with rabbit anti-Ankyrin G antibody (1:400) (Santa Cruz Biotechnology) and secondary Alexa488-goat anti-rabbit antibody (Invitrogen).
antibody Wld18 (Samsam et al., 2003), all followed by the appropriate secondary IgG-HRP-conjugated antibody. Protein concentration was measured by Bradford assay (Bio-Rad) and similar amount of protein from all fractions was used for immunoblots.

Time-lapse imaging. Transfected dissociated DRG and hippocampal neurons were imaged as described previously (Gilley and Coleman, 2010) within 24 h after transfection, and immediately after treatment with MitoTracker Green FM (Invitrogen), as per manufacturer protocol. Wide-field epifluorescence images were captured at 1 Hz. After acquisition dishes were moved to a Zeiss LSM510 confocal and stacks acquired.

Statistical analysis. Data are presented as mean ± SD. Student t test was performed for statistical analysis; group comparison was considered not statistically significant (NS) if \( p > 0.05 \), or highly significant (***) if \( p < 0.01 \).

Results
A C-terminal peptide from the amyloid precursor protein can target NMNAT1 to axons in vitro
We hypothesized that N70 delivers NMNAT1, which is normally a nuclear protein (Schweiger et al., 2001; Berger et al., 2005), to

Figure 1. Confocal z-projections showing the targeting of NMNAT1 to axons. A, Immunostained hippocampal neurons transfected with FLAG-tagged native NMNAT1 (NMNAT1-FLAG), FLAG-tagged NMNAT1 with disrupted nuclear localization (ΔNLS NMNAT1-FLAG), and FLAG-tagged ΔNLS NMNAT1 N-terminally fused to a peptide from exon 7 of SMN protein (Exon7 SMN-ΔNLS NMNAT1-FLAG) or to a peptide from the AICD of APP (AICD-ΔNLS NMNAT1-FLAG). Anti-FLAG immunocytochemistry labeled the overexpressed NMNAT1 variants (red). On the right, 20× images show that AICD-ΔNLS NMNAT1-FLAG redistributes to distal axons most efficiently. Cell body insets are shown at 63× magnification on the left. Anti-ankyrin G identified proximal axons (green), and anti-MAP-2 marked dendrites (pink); nuclei were counterstained with DAPI (blue). Superimposed images of overexpressed NMNAT1 variants and ankyrin G are shown in the merge panel. Arrows point to axons. B, The AICD-ΔNLS NMNAT1-FLAG construct was named Ax-NMNAT1 (axonally targeted NMNAT1) and selected for the generation of transgenic mice. The N-terminal axonal targeting sequence, evolutionarily conserved amino acids are highlighted in yellow.
xons and synapses in Wld<sup>5</sup> mice, where it prolongs their survival after injury. In transfected hippocampal cultures, we detected N70 exclusively outside the nucleus, and it was abundant in neurites. In contrast, we found overexpressed NMNAT1 only in the nucleus (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). This is in line with previous reports showing presence of full-length Ube4b in the cytoplasm and axons in vivo (Mack et al., 2001; Fang et al., 2005). The unique 18 aa sequence of Wld<sup>5</sup> (Wld18) did not affect distribution (supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Next, we fused several reported axonal targeting peptides N-terminally to NMNAT1 that had been mutated in its nuclear localization sequence (ΔNLS NMNAT1). The aim was to identify an axonal targeting tag that would mimic the N70-mediated targeting of NMNAT1. We tested the peptides GFPQNKKEGRCS from exon 7 of the survival motor protein (SMN) (Zhang et al., 2003), the cytoplasmic targeting sequence (MEM) MLCCMMRTKQKEKNDEDQKI from growth cone-associated protein (GAP-43) (Zuber et al., 1989), and GYENPTYKFQEQM from amyloid precursor protein intracellular domain (AICD) (Satpute-Krishnan et al., 2006). For comparative immunofluorescence all constructs were also FLAG tagged at their C terminus.

The efficiency of these peptides in targeting ΔNLS NMNAT1 to axons was compared in transfected hippocampal cultures. Axons were identified by morphological criteria and by immunostaining with the proximal axonal marker anti-ankyrin G (Kordeli et al., 1995). Only neurons with low-to-medium level of immunosignal were analyzed to avoid mislocalization due to overexpression. The 15-aa-long AICD sequence of APP (AICD-ΔNLS NMNAT1-FLAG) was the most effective in redistributing ΔNLS NMNAT1 to the axon, especially to distal regions (Fig. 1A). Although a little AICD-ΔNLS NMNAT1-FLAG was also present in MAP-2-positive dendrites, significantly more diffuse or dendritically stained was visible without targeting (ΔNLS NMNAT1-FLAG) or in presence of the targeting sequence from exon 7 of SMN protein (Exon7 SMN-ΔNLS NMNAT1-FLAG). The MEM sequence of GAP-43 caused toxicity and was therefore not examined further. Thus, the AICD-ΔNLS NMNAT1-FLAG construct (Fig. 1B) was selected for microinjection to produce axonally targeted NMNAT1 (Ax-NMNAT1) mice.

Expression of Ax-NMNAT1 transgene at low doses

We established six Ax-NMNAT1 hemizygous transgenic lines from seven founders (lines 1–4, 6–7) by breeding to YFP-H mice (Feng et al., 2000) for convenient assessment of Wallerian degeneration (Beировski et al., 2004). Western blotting of brain homogenates revealed low levels of axonally targeted NMNAT1 protein in Ax-NMNAT1 mice using anti-FLAG antibody, that increased approximatively twofold in line 2 when bred to homozygosity (Fig. 2A). Anti-NMNAT1 antibody 183 (Conforti et al., 2000) demonstrated that levels of Ax-NMNAT1 were substantially lower than Wld<sup>5</sup> in Wld<sup>5</sup> heterozygotes and NMNAT1 in NMNAT1-overexpressing transgenic mice (Conforti et al., 2007), respectively (Fig. 2B). In hemizygous line 1 Ax-NMNAT1 mice protein expression was below the detection limit, and only faintly visible in concentrated DRG samples from homozygous mice (see Fig. 4C). Because enzymatic NMNAT activity is essential for Wld<sup>5</sup> neuroprotection (Araki et al., 2004; Jia et al., 2007; Conforti et al., 2009; Sasaki et al., 2009a) and measurements of NMNAT enzyme activity also allow quantitative assessment of transgenic protein levels (Conforti et al., 2007; Beировski et al., 2009), we assayed total NMNAT activity in brains from Ax-NMNAT1 mice. Line 1 samples showed activity similar to wild-type (WT) littermates, further indicating that transgene expression in this line is very low. Activity in line 2 was only slightly increased and remained significantly lower than that in Wld<sup>5</sup> heterozygotes (Fig. 2C). This confirmed that the fusion protein was enzymatically active and the specific values of NMNAT enzyme activity correlate with protein expression levels as detected by Western blotting. The absence of specific immunofluorescence on brain cryosections (supplemental Fig. 2, available at www.jneurosci.org as supplemental material) further confirms the lack of high transgene expression. The efficacy of the FLAG antibody was validated using controls from FLAG-tagged wild-type (WF) and frame-shifted (DF) superoxide dismutase transgenic mice (Watanabe et al., 2005) (supplemental Fig. 2, available at www.jneurosci.org as supplemental material).
Robust axon protection \textit{in vivo} and \textit{in vitro}\textsuperscript{1}

An amount of Wld\textsuperscript{5} protein comparable to this level of axonally targeted NMNAT1 would be unlikely to grant axonal protection in Wld\textsuperscript{5} transgenics or at best have only a weak effect (Mack et al., 2001). Strikingly however, both Ax-NMNAT1 lines showed robust axonal protection, as did all four other Ax-NMNAT1 lines, which expressed the fusion protein at levels similar to or lower than line 2 (data not shown). Seven and fourteen days after transection, most axons were still intact in the distal stump of the sciatic nerve as assessed by YFP longitudinal imaging (Fig. 3). In clear contrast, all axons from WT and NMNAT1-overexpressing mice were completely fragmented as early as 3 d after nerve lesion as previously reported (Beirowski et al., 2004; Conforti et al., 2007), despite the higher transgene expression level in the latter.

Next, we examined axonal preservation 35 d after axotomy, a time point at which native Wld\textsuperscript{5} no longer preserves axons. Continuous axons were consistently present in distal stumps of sciatic nerve from Ax-NMNAT1 L2 mice and in tibial nerves of both Ax-NMNAT1 lines, similar to the preservation by extranuclear Wld\textsuperscript{5} (ΔNLS Wld\textsuperscript{5}) (Fig. 3 and supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Regeneration was ruled out in the distal stump by imaging the cut site, where all the intact axons originated from end bulbs (likely resulting from continued retrograde axonal transport), which mark the lesion site (supplemental Fig. 3, available at www.jneurosci.org as supplemental material). Moreover, degeneration in Ax-NMNAT1 nerves was clearly dose-dependent. Accordingly, in Ax-NMNAT1 line 3, which showed transgene instability, the degree of axonal protection correlated to protein expression level in each mouse of the same progeny (supplemental Fig. 4, available at www.jneurosci.org as supplemental material).

The comparison of Ax-NMNAT1 and ΔNLS Wld\textsuperscript{5} lines with similar transgene expression levels indicated a stronger axon sparing activity of Ax-NMNAT1. Mice from line 1 of the ΔNLS Wld\textsuperscript{5} strain express undetectable level of transgenic protein (Beirowski et al., 2009), analogous to Ax-NMNAT1 line 1, but their tibial nerve axons show clearly more extensive fragmentation 14 d after lesion (supplemental Fig. 5, available at www.jneurosci.org as supplemental material).

Thus, Ax-NMNAT1 protects axons more robustly than Wld\textsuperscript{5}, even when expressed at substantially lower levels. Moreover, these data indicate that Ax-NMNAT1 confers higher axoprotective potency than extranuclear Wld\textsuperscript{5} \textit{in vivo}. The efficacy was also evident \textit{in vitro} using neurons from Ax-NMNAT1 mice (Fig. 4). We evaluated neurite survival up to 6 d after cutting in cultured DRG explants from hemizygous embryos of Ax-NMNAT1 line 1 and 2 both by identification of beading and fragmentation (Fig. 4A) and by quantitative software-based analysis of axonal continuity (Fig. 4B). For this purpose, we developed a formula that quantifies the degree of axonal protection, which we termed PI. PI calculates the area occupied by continuous axons versus the total axonal area in each imaged field and is near to 1 when all axons are intact. As fragmentation progresses after axotomy PI decreases, dropping to

![Figure 3](https://www.jneurosci.org/app/figures/30/40/30485/30485g03.jpg)
levels around 0.5 within the first 24 h for WT or DRGs from homozygous NMNAT1-overexpressing transgenic mice. By contrast, Ax-NMNAT1 neurites from both lines remained unfragmented for at least 6 d after cutting, similar to WldS and \( \Delta NLS \) Wld\(^{S}\) neurites, with a PI constantly close to 1 (Fig. 4B).

We confirmed high NMNAT1 expression in DRGs from NMNAT1 homozygotes, whereas Ax-NMNAT1 expression was low in homozygous cultures from line 2 (Fig. 4C). Remarkably, similar to the \textit{in vivo} findings, Ax-NMNAT1 protein was detectable in Ax-NMNAT1 line 1 DRGs only if cultured from homozygotes (Fig. 4C), but remained below detection limit in hemizygous cultures (data not shown).

Thus, whereas overexpressed nuclear NMNAT1 does not protect axons, axonally targeted NMNAT1 delays Wallerian degeneration similar to extranuclear Wld\(^{S}\) \textit{in vivo} and \textit{in vitro}, despite the greatly reduced expression level.

Axonally targeted NMNAT1 localizes outside the nucleus \textit{in vivo} at low levels

Because the protective action is determined by extranuclear Wld\(^{S}\) (Beirowski et al., 2009) and is dose dependent (Mack et al., 2001), we compared extranuclear expression levels between Ax-NMNAT1 and other strains. Commercially available anti-NMNAT1 antibodies could barely detect native NMNAT1 in mouse lumbar spinal cord cryosections (Conforti et al., 2007). Thus, we based our comparison on Western blots of fractionated brain homogenates immunoblotted with antibody 183, which we previously used for successful detection of NMNAT1 and Wld\(^{S}\) (Conforti et al., 2007). We confirmed that Ax-NMNAT1 protein is redistributed outside the nucleus in line 2 samples (Fig. 5), and found levels similar to the ones from the variant \( \Delta N16 \) Wld\(^{S}\), which does not confer axon protection (Conforti et al., 2009). Densitometric quantification of cytoplasmic levels revealed that axonally targeted NMNAT1 is expressed ~24.4\% lower than Wld\(^{S}\), and ~96.5\% lower than \( \Delta NLS \) Wld\(^{S}\) in the respective strains. Nevertheless, axonal preservation in Ax-NMNAT1 line 2 mice is greater than in Wld\(^{S}\) and similar to \( \Delta NLS \) Wld\(^{S}\) mice from line 3 (Fig. 3), suggesting that axonal delivery of NMNAT1 markedly increases its efficacy.

Axonally oriented NMNAT1 preserves motor nerve terminals robustly in young and adult mice

Motor nerve terminal preservation decreases with age in homozygous spontaneous mutant and transgenic Wld\(^{S}\) mice, and is absent in Wld\(^{S}\) mice older than 3 months (Gillingwater et al., 2002). However, extranuclear Wld\(^{S}\) can protect NMJs efficiently in aged mice (Beirowski et al., 2009). Thus, as a stringent test, we measured the efficacy of axonally targeted NMNAT1 at motor nerve terminals.

Wld\(^{S}\) would not be expected to delay denervation of NMJs at these expression levels, as motor nerve terminals in Wld\(^{S}\) heterozygotes are not preserved following axotomy even at 1–2 months of age (Wong et al., 2009). First we imaged synaptic vesicle recycling (Fig. 6A) and recorded evoked action potentials by intracellular recordings in deep lumbrical and FDB muscles of 2-month-old hemizygous Ax-NMNAT1 mice from line 2 (Fig. 6B). Both the morphological and the functional evaluation revealed a robust degree of NMJ preservation 6 d after axotomy. In Ax-NMNAT1 FDB and lumbrical muscles respectively, 69\% and 67\% of motor endplates were fully labeled by AM1-44 vital staining, showing complete innervation with synaptic vesicles recycling activity in 100\% of their area 6 d after axotomy. In contrast, in Wld\(^{S}\) FDB and lumbrical muscles, only 38\% and 42\% of motor endplates retained full innervation 6 d after axotomy, in line with previous data (Gillingwater et al., 2002). In physiological record-
ings, 62.2% of fibers showed nerve evoked endplate potentials (EPPs). The extent of the NMJ preservation measured physiologically in this way was at least as strong as that of age-matched Wild^5^ mice, which full-length Wld^5^ protein was targeted away from its normal nuclear localization (Beirowski et al., 2009).

Delivery of NMNAT1 to axons is necessary for axon protection

To test whether axonal delivery of NMNAT activity is necessary for axon protection as well as sufficient, we examined the effect of blocking axonal delivery of the Ax-NMNAT1 protein. In initial experiments, we added a dileucine (LL) dendrite targeting peptide from Shal K^+^ channels—FETQHHILLHCLEKTT (Rivera et al., 2003)—but found that this was not sufficient to achieve complete exclusion from the axonal compartment in hippocampal and DRG neurons (data not shown). Considering that only very low levels of Ax-NMNAT1 are needed to protect axons (e.g., in line 1 hemizygotes), it is important to block axonal delivery more completely to test this hypothesis.

Therefore we inhibited entry of Ax-NMNAT1 into axons pharmacologically immediately after it was expressed. DRG neurons were transfected with Ax-NMNAT1-mCherry and at the same time treated with high concentration of vincristine (0.04 μM) or nocodazole (20 μg/ml), to achieve fast and complete disruption of axonal transport. Ax-NMNAT1-mCherry protein was expressed in presence of drugs, but restricted to the soma and proximal neurites (supplemental Fig. 8, available at www.jneurosci.org as supplemental material). DRGs were axotomized 24 h later (Fig. 7A,II,AIV,BI,BIV). When axonal transport was blocked before Ax-NMNAT1-mCherry protein was expressed, axonal preservation after axotomy was greatly reduced. In contrast, axonal sparing was robust if drugs were applied 24 h after transfection, at the time of cut, allowing time for prior synthesis and axonal targeting of some Ax-NMNAT1 protein (Fig. 7A–C). Only 5% and 19% of axons remained free of extensive varicosities or did not fragment 48 h after cut when microtubules were destabilized early by nocodazole and vincristine, respectively (Fig. 7C,III,CIV), compared to 69% of axons preserved in absence of drugs. If microtubules were disrupted at the time of axotomy, 24 h after Ax-NMNAT1 expression and axonal delivery, axotomized neurites were preserved as strongly as in absence of drugs (Fig. 7C,III,CIII). Thus, preventing axonal NMNAT1 entry abolishes the protective capacity.

Although the drug treatment induces some axonal varicosities (supplemental Fig. 8, available at www.jneurosci.org as supplemental material), a general neuronal intoxication is not responsible for the neurite degeneration above because Ax-NMNAT1 can protect in presence of vincristine and nocodazole, if drugs are applied at the time of axotomy. As a further control for this, we applied nocodazole at a lower concentration (5 μg/ml), obtaining similar results, with a reduction of axonal survival to 29% when nocodazole was applied at the time of transfection (Fig. 7C). The slightly higher survival compared to the result obtained with 4 times more concentrated nocodazole (20 μg/ml) may reflect the lower effectiveness of microtubule destabilization, and thus the lower efficacy of preventing Ax-NMNAT1 from entering axons.

Wild^5^ explant cultures are known to be resistant to vincristine toxicity (Conforti et al., 2009), and explants from Ax-NMNAT1 transgenic embryos can be cultured for several days before axotomy, allowing longer time for expression and axonal delivery of axonally targeted NMNAT1 stably. Thus, we axotomized DRG explants from Ax-NMNAT1 line 1 homozygotes 6 d after plating (Fig. 7D). To mimic the conditions of our previous transected DRGs, we pretreated the explants with high concentrations of vincristine or nocodazole for 24 h before neurite transection. Six days after cut, neurites of Ax-NMNAT1 cultures were still intact.

**Figure 5.** Cytoplasmic redistribution of axonally targeted NMNAT1. Representative Western blot of nuclear and postnuclear (cytoplasmic) fractions from brains of Ax-NMNAT1 mice in comparison to native Wild^5^ mice, mice expressing the nonprotective variant of Wild^5^ lacking the first 16 N-terminal amino acids (ΔN16 Wild^5^ hemi), and mice expressing the enhanced protective extranuclear variant (ΔNLS Wild^5^ L3 hemi) (n = 4). Nuclear and cytoplasmic factions, blotted with anti-NMNAT1 antibody 183 (top left and right), are shown at different exposure times to optimize the visualization of the faint bands (Ax-NMNAT1, Wild^5^, and ΔN16Wild^5^). Sp1 is the loading control for the nuclear fraction. The densitometry (bottom) shows the intensity of the cytoplasmic bands, normalized to β-actin, and is expressed as percentage of ΔNLS Wild^5^ levels for comparative analysis. The axonally targeted NMNAT1 protein shows the lowest levels if compared to Wild^5^ and variants in the other strains. *Nonspecific band.
Axonally targeted NMNAT1 localizes to vesicular fractions and it is cotransported with mitochondria

The potency of Ax-NMNAT1 in protecting axons and synapses suggests that axonally targeted NMNAT1 is delivered with high efficiency to the location where Wld^3 functions. To investigate the specific subcellular localization of Ax-NMNAT1 protein, we fractionated brains of Ax-NMNAT1 mice and (variant) Wld^3 mice by sequential centrifugation at increasing speeds. We obtained fractions differently enriched in organelles as validated by nuclear, mitochondrial, endoplasmic reticulum (ER), Golgi, and synaptic vesicle markers (Fig. 8A). The distribution of Ax-NMNAT1 and (variant) Wld^3 mostly resembled the one of synaptic vesicles, with particular abundance in vesicle fractions V0 and V1, where also ER and Golgi markers were present. These proteins, and particularly Ax-NMNAT1, were abundant also in the mitochondrial fraction (M) (Fig. 8B). Thus, we find here that both Ax-NMNAT1 and Wld^3 are mostly concentrated in a small membranous compartment in vivo, extending our previous finding of high levels of (variant) Wld^3 in mitochondria and microsome fractions and fine granular staining of extranuclear Wld^3 in sciatic nerve axoplasm (Beirowska et al., 2009).

Next, we studied this site using an in vitro imaging approach. Because of the lack of commercially available, sensitive antibodies for detection of Ax-NMNAT1 protein, we fused Ax-NMNAT1 to mCherry to observe the fluorescence directly in transfected hippocampal (Fig. 9A–D) and dissociated DRG neurons (Fig. 9E–K).

Axonally targeted NMNAT1-mCherry was distributed in vitro in pleomorphic vesicle-tubular structures. Similar structures were also evident in transfected non-neuronal cells that are present at low numbers in our DRG cultures (supplemental Fig. 7, available at www.jneurosci.org as supplemental material). Vesicles entered the axons (Fig. 9B) and, particularly in DRG neurons, were concentrated at the growth cones (Fig. 9F), where the fluorescence appeared clearly punctate (Fig. 9G, arrowheads). Next, we assessed colocalization with extranuclear Wld^3 by cotransfection with ΔNLS Wld^3-EGFP (Beirowska et al., 2009). ANLS Wld^3-mCherry and Ax-NMNAT1-mCherry gave a similar vesicle-tubular distribution, and we noticed partial colocalization in soma (Fig. 9C,D,H) and particularly in puncta along axons (Fig. 9C,D,I).

Finally, we assessed the identity of these structures by testing for colocalization (supplemental Fig. 8, available at www.jneurosci.org as supplemental material) with the same organelle markers used in the subcellular fractionation experiment (Fig. 8), along with additional markers of trans-Golgi-network/ER membranous compartment.
(calsyntenin-1) and microtubules (β-tubulin). There was some heterogeneity, with Ax-NMNAT1-mCherry fluorescence occasionally overlapping with Golgi staining (γ-adaptin) in the soma, and more frequent colocalization with MitoTracker within axons (Fig. 9). Time-lapse imaging in both DRG (Fig. 9K and supplemental Movie 1, available at www.jneurosci.org as supplemental material) and hippocampal neurons (supplemental Movie 2, available at www.jneurosci.org as supplemental material) revealed a number of Ax-NMNAT1-mCherry particles undergoing bidirectional axonal transport coordinated with movements of mitochondria. This is important because it is highly unlikely to reflect coincidental or transient colocalization. Velocity of mitochondria movement was similar in transfected and untransfected neurons with pauses between continuous transport (supplemental Movie 2, available at www.jneurosci.org as supplemental material), as described previously (Takenaka et al., 1990; Kang et al., 2008). The colocalization was evident on both static and moving mitochondria (supplemental Movie 1, available at www.jneurosci.org as supplemental material).

Together, these in vivo and in vitro data indicate that the highly protective axonally targeted NMNAT1 concentrates to the same subcellular structures where extranuclear WldS is also trafficked. Ax-NMNAT1 transport is regulated and at least partially coordinated with the axonal movement of mitochondria, pointing to a contribution by mitochondria to the delivery of Ax-NMNAT1 and WldS into axons.

Discussion
These data show that local delivery of NMNAT1 is essential to delay axonal and synaptic degeneration, and that even a marginal increase in NMNAT activity in axons is robustly protective. We extend previous work showing that WldS protects axons against drugs affecting microtubule stability, demonstrating Ax-NMNAT1 protection also against nocodazole toxicity for the first time. Ax-NMNAT1 transport to axons was necessary to prevent degeneration, and its presence within axons was sufficient to overcome toxicity from high doses of axonal transport blockers. This indicates an axonal mechanism of protection, consistent with the presence of WldS in mouse distal stumps 1 week after lesion (Beirouksii et al., 2009).

Our data also show that NMNAT1 can be transformed into a highly potent neuroprotective molecule by axonal targeting. We show here that when targeted to axons by a specific targeting motif NMNAT1 acquires an efficacy greater than WldS. We resolve the question of whether 15-fold overexpression of extranuclear NMNAT1 is necessary for protection in vivo (Sasaki et al., 2009b), demonstrating that very low levels preserve axons and their synaptic endings if NMNAT1 is axonally targeted. Western blotting with two different primary antibodies, immunohistochemistry and NMNAT enzyme assays unanimously indicated low to undetectable levels of expression of Ax-NMNAT1 in transgenic mice. Because WldS protective efficacy is dose dependent (Mack et al., 2001), axon protection would not be expected at such low levels, but low doses of axonally targeted NMNAT1 delay Wallerian degeneration for even 5 weeks. Moreover, neither a heterozygous dose of WldS protein in young mice nor a homozygous dose in older mice is sufficient to delay NMJ dener-

Figure 7. Blockage of axonal entry abolishes Ax-NMNAT1 protective capacity. A, Diagram illustrating vincristine and nocodazole application (gray bars) at the indicated times to DRG neurons coexpressing EGFP and Ax-NMNAT1-mCherry. Vincristine was applied at 0.04 μM (i and iii), and nocodazole at 5 or 20 μg/ml (iii and iv), at the time of transfection (i and iii), or 24 h later at the time of axotomy (i and iii). B, Representative EGFP epifluorescence images of DRGs expressing Ax-NMNAT1-mCherry, at the time of axotomy (i and iii) and 48 h later (iv), treated with 0.04 μM vincristine (i and iii), or with 20 μg/ml nocodazole (iii and iv), at the times indicated in A. Dashed line marks the cut location. C, Quantification of axonal preservation 48 h after axotomy (imaging iv) in DRGs cultured and treated as explained in A. The diagram shows the percentage of axons (±SD) that did not develop swelling or fragmentation between imaging i and imaging iv. D, Diagram illustrating 0.04 μM vincristine, and 20 μg/ml nocodazole treatments on DRG explant cultures from wild-type mice and Ax-NMNAT1 homozygotes from line 1. Treatments started 6 d after plating, 24 h before axotomy. E, Phase-contrast images (0.3 mm × 0.3 mm) of Ax-NMNAT1 or wild-type explants, as indicated, at the time of cut (i and 6 d later (iv), treated with vincristine as illustrated in D. F, Phase-contrast images (0.3 mm × 0.3 mm) of Ax-NMNAT1 or wild-type, as indicated, at the time of cut (i and 6 d later (iv), treated with nocodazole as illustrated in D. Scale bars: B, E, F, 0.1 mm.
vation (Gillingwater et al., 2002; Wong et al., 2009), whereas these very low doses of axonally targeted NMNAT1 preserve axotomized NMJs for 6 d and retain this ability even in older mice. Even though Ax-NMNAT1 has substantially higher potency than WldS, important similarities indicate that its mechanism of protection is the same as the WldS mechanism. Ax-NMNAT1 protection efficacy was also dose dependent, and axonal atrophy and fragmentation in Ax-NMNAT1 mice followed a proximo-distal gradient (Beirowski et al., 2005).

These data strongly suggest that WldS acts locally within axons. We cannot rule out the possibility that low somatic NMNAT1 activity increases the expression or transport of an axonal effector, but this seems unlikely in view of our previous data demonstrating that a truncated form of WldS, H9004N16 WldS, is inefficient in protecting axons despite its extranuclear localization and enzymatic activity (Conforti et al., 2009).

An axonal role for WldS and Ax-NMNAT1 has several interesting implications for the mechanism of Wallerian degeneration and synapse loss in disease. It has been postulated that depletion below a critical threshold of transported substances essential for axonal integrity triggers Wallerian degeneration (Lubinska, 1977). We recently identified NMNAT2 as a survival factor in wild-type axons (Gilley and Coleman, 2010). We proposed that in WldS nerves axonal delivery of this NMNAT1 fusion protein compensates for the postinjury depletion of NMNAT2. This study now confirms one key prediction of that proposal: that WldS itself acts in axons.

Additionally as the peculiar degeneration of WldS axons proceeds proximo-distally independently from the type of injury (Beirowski et al., 2005), this could reflect a spatial gradient of WldS loss along the nerve. The weaker WldS protection at NMJs, compared to the axonal trunk (Gillingwater et al., 2002), could be explained by the longer distance that WldS needs to travel to reach the nerve endings and by dilution into the extensive terminal axonal branches. The age dependence of synapse withdrawal could be due to progressive decrement of axonal transport efficiency correlating with aging. At least in some cases, axonal transport decreases along the nerve with a proximo-distal gradient (Brunetti et al., 1987). Indeed NMNAT could have a local synaptic role since the single Drosophila NMNAT isoform is also localized at NMJs (Zhai et al., 2006).

The same model could explain why WldS retards anterograde degeneration more effectively than retrograde degeneration in some models of Parkinson’s disease (Cheng and Burke, 2010). Consistent with this, optimizing the delivery of extranuclear NMNAT1 in this present report enhances NMJ protection in older mice. This is particularly important for pathologies in which synapses are affected at early stages, such as amyotrophic lateral sclerosis (ALS). WldS has been proven protective for axons in many diseases but was relatively ineffective in SOD1 G93A transgenic mice, the most widely used model for familial ALS (Coleman, 2005; Fischer et al., 2005), and failed to reduce symptoms in others (Mi et al., 2005). Interestingly, although protection depends on transport of NMNAT1 to axons, degeneration caused by axonal trans-
port block is delayed if Ax-NMNAT1 has been delivered to axons before the damage occurred. This could explain the great protection WldS exerted in the pmn model (Ferri et al., 2003). Thus, enhanced targeting of WldS or NMNAT1 locally may help preserving synapses and counteract damage caused by axonal transport blockage.

Axonal WldS can be detected in the form of granular staining in vivo and in multisize particles in vitro. We show that Ax-NMNAT1 and extranuclear WldS share a common subcellular location in small membranous compartments, in particular subcellular fractions enriched in mitochondria, Golgi, and synaptic vesicles. In this context, we show for the first time coordinated movement of Ax-NMNAT1 with mitochondria in axons, in line with the previously demonstrated association between ΔNLS WldS and mitochondria (Beirowski et al., 2009).

In view of the employed axonal targeting motif, the mitochondrial association seems surprising. The targeting sequence is part of the C-terminal cytoplasmic domain of APP, which, after cleavage

Figure 9. Localization of Ax-NMNAT1 protein in transfected primary cultures. A, Confocal z-projection of the subcellular distribution of Ax-NMNAT1-mCherry in transfected hippocampal neuron. B, Single confocal plane of the axonal inset in A. C, Confocal z-projection of Ax-NMNAT1-mCherry and ΔNLS WldS-EGFP cotransfected hippocampal neuron. D, Single confocal plane of the soma and axon insets in C. E, F, Confocal z-projection of the subcellular distribution of Ax-NMNAT1-mCherry in transfected DRG and its growth cone respectively. G, Single confocal plane of the inset in F; arrowheads point to Ax-NMNAT1-mCherry puncta at the growth cone. H, I, Single confocal plane of soma and axon, respectively, of an Ax-NMNAT1-mCherry and ΔNLS WldS-EGFP cotransfected DRG. In D, H, and I, arrows indicate partial colocalization of Ax-NMNAT1 with ΔNLS WldS. Scale bars: A, C, E, F, 20 μm; B, D, G–I, 2 μm. In A–I, blue is DAPI staining, red is Ax-NMNAT1-mCherry fluorescence, and green is ΔNLS WldS-EGFP fluorescence. J, Single confocal plane showing colocalization (arrows) of Ax-NMNAT1-mCherry particles and mitochondria (MitoTracker Green) in a transfected DRG axon. Scale bar, 2 μm. K, Time-lapse imaging of the experiment in J, showing coordinated retrograde movement of an Ax-NMNAT1-mCherry particle (arrow, left) with a mitochondrion (arrow, right). Images were zoomed from supplemental Movie 1 (available at www.jneurosci.org as supplemental material).
from the full-length APP, is sorted to the axon in carrier vesicles (Muresan et al., 2009). The NPTY motif contained in the axonal targeting peptide is involved in the trafficking of synaptic vesicles. In Drosophila, it mediates axonal accumulation of synaptic markers upon APP overexpression (Rusu et al., 2007). Although APP interacts with a vast number of proteins, the 15 aa peptide we used is only likely to interact with Mint/X1, via the NPTY domain (Rusu et al., 1998). Mint/X1 are adaptors containing distinct domains for binding various proteins, one of which is the synaptic vesicle fusion protein Munc18-1 (Okamoto and Südhof, 1997). Mint deletion produces presynaptic functional deficit in KO mice (Ho et al., 2006), whereas overexpression ameliorates long-term potentiation deficits in Alzheimer’s disease mouse models (Mitchell et al., 2009).

Ax-NMNAT1 and WldS are abundant in brain subcellular fractions enriched for Golgi apparatus and ER. Perhaps not coincidentally, a strikingly similar distribution has been observed in neurons transfected with neurexin, another protein interacting with Mint1. The exit of neurexin from the ER/Golgi and its trafficking to synapses in vesicles depends on its C-terminal sequence, where the domain for interaction with Mint1 resides (Fairless et al., 2008). Although a direct interaction between APP and kinesin is controversial (Kamal et al., 2001; Lazarov et al., 2005), the exit of vesicles containing APP from the trans-Golgi-network is mediated by calssytin-1 (Ludwig et al., 2009). Calssytin-1 is a neuronal transmembrane protein transported to axons in vesicular carriers in a kinesin 1-dependent manner (Konecna et al., 2006; Ludwig et al., 2009). Strikingly, by using the same subcellular fraction method that we use in the present study, Ludwig et al. (2009) showed that calssytin-1 concentrates in the subcellular vesicle fractions V0 and V1, where we found Ax-NMNAT1 and WldS mostly present. Although we show that Ax-NMNAT1 does not colocalize with calssytin-1, and p38 SNPH, we cannot exclude a possible colocalization with others of vesicles of similar size. In fact, Ax-NMNAT1-mCherry fluorescence was not confined to mitochondria. In this context, it is interesting to note evidence for physical connection between ER and mitochondria (de Brito and Scorrano, 2008).

Since the C-terminal fragment of APP is not reported to be targeted to mitochondria (Devi and Anandatheerthavarada, 2010), it is possible that the striking mitochondria localization of Ax-NMNAT1 is determined by NMNAT1 itself. When ectopically present in axons NMNAT1 may dynamically associate with mitochondria. Accordingly, we noticed a progressive increase in number of cytoplasmic particles of ANLS WldS EGFP with time after transfection (data not shown). Because ΔNLS WldS cytoplasmic puncta colocalize with mitochondria (Beirowski et al., 2009) their increase in number may underlie a progressive delivery of WldS and NMNAT1 to these organelles after transfection. A mitochondrial involvement in the mechanism of WldS and NMNAT1 protection would be in line with recent findings showing that mitochondrial NMNAT3 protects in vivo (Yahata et al., 2009). Further targeting studies may help to resolve whether WldS, NMNAT1, and other axon-protective molecules mechanistically act at a specific axonal location and whether this coincides with mitochondria.

In summary, our data provide the first evidence that NMNAT1 transport to axons and synapses is indispensable for protection. The unprotective nuclear NMNAT1 protein was transformed into a strong inhibitor of Wallerian degeneration by specific targeting to axons. We shed light on possible axonal sites of action facilitating future analysis of the molecular mechanism. Importantly, this could be therapeutically exploited, especially in disorders where synapse loss is a limiting factor, and the local mechanism of protection could pave the way for direct intervention in the distal stump of a nerve after injury.

References


