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 (Wld<sup>S</sup>) protects axons dose dependently, but its mechanism is still elusive. We recently showed that Wld<sup>S</sup> 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 Wld<sup>S</sup> (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 Wld<sup>S</sup>, 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 Wld<sup>S</sup> (Wallerian degeneration slow) (Lunn et al., 1989) established axonal degeneration as a tightly regulated process. In this strain, the chimeric Wld<sup>S</sup> protein delays the progressive degeneration of experimentally transected nerves (Wallerian degeneration) (Waller, 1850), preserving axons and synapses in a nonlinear, dose-dependent fashion (Mack et al., 2001). Importantly, Wld<sup>S</sup> 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 Wld<sup>S</sup> protection remains largely elusive at the molecular level. Wld<sup>S</sup> arose from a de novo triplication, which links the N-terminal 70 aa of the ubiquitination factor Ube4b (N70) to the nuclear NAD<sup>+</sup>-synthesizing enzyme nicotinamide mononucleotide adenyltransferase (NMNAT1), via a unique 18 aa sequence (Wld18) (Conforti et al., 2000). Despite its abundance in the nucleus, we recently detected Wld<sup>S</sup> in axons, where it accumulates after nerve conduction (Beirowski et al., 2009). Since axonally transported proteins typically accumulate at constriction sites (Cavalli et al., 2005), Wld<sup>S</sup> may be transported, either directly or by associating with vesicles or organelles. Moreover, redistribution of Wld<sup>S</sup> to cytoplasm strikingly enhanced axonal and synaptic protection (Beirowski et al., 2009), possibly by increasing the delivery to axons and synapses where Wld<sup>S</sup> may function locally.

Further support for this model comes from studies of the NMNAT1 portion of Wld<sup>S</sup>. The intrinsic catalytic activity of Wld<sup>S</sup> 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 Wld<sup>S</sup> (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 Wld<sup>S</sup> (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 Wld³ 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 Wld³ construct, carrying the R213A and R215A mutations (Beirowski et al., 2009). The FLAG reverse primer (BamHI site underlined, mutated stop codon in bold, FLAG tag italicized: 5′-CGGCGATCCATAGTCGTTCAATGTCGTTATGATTCACAGG-TGGATGTTGTTGTTGCTGTTGC-3′) (Wilbrey et al., 2008) was used together with the following forward primers (5′ HindIII cloning tag underlined, start codon in bold, axonal targeting sequence italicized):

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

For Ax-NMNAT1-MEM-FLAG: 5′-ATCCCAAGCTTTAGTTCCTCGTCGTCCTTCATCTCGTAATGATGAGGACCAAAAGATC-3′ (Mack et al., 2001). Ax-NMNAT1-mCherry was produced by PCR amplification using BamHI- and HindIII-tagged primers and described by Mack et al. (2001). Ax-NMNAT1-mCherry was produced from the previously reported 13292 • J. Neurosci., October 6, 2010 • 30(40):13291–13304 Babetto et al.

GAP43 MEM-FLAG: 5′-ATCCCAAGCTTTAGTTCCTCGTCGTCCTTCATCTCGTAATGATGAGGACCAAAAGATC-3′.

For GAP43 MEM-ΔNLS NMNAT1-FLAG: 5′-ATCCCAAGCTTTAGTTCCTCGTCGTCCTTCATCTCGTAATGATGAGGACCAAAAGATC-AAG-3′.

For AICD-ΔNLS NMNAT1-FLAG (subsequently named Ax-NMNAT1): 5′-ATCCCAAGCTTTAGTTCCTCGTCGTCCTTCATCTCGTAATGATGAGGACCAAAAGATC-AAG-3′.

For N70-FLAG: 5′-CGGCGATCCATAGTCGTTCAATGTCGTTATGATTCACAGG-TGGATGTTGTTGTTGCTGTTGC-3′.

For N70-Wld³-FLAG: 5′-CGGCGATCCATAGTCGTTCAATGTCGTTATGATTCACAGG-TGGATGTTGTTGTTGCTGTTGC-3′.

The FLAG-tagged construct expressing the 18 unique as followed by the full-length NMNAT1 cDNA (18-ΔNLS NMNAT1-FLAG) was amplified from the same template using the following forward primer (5′ HindIII cloning tag underlined, stop codon in bold, FLAG tag italicized):

For N70-FLAG: 5′-CGGCGATCCATAGTCGTTCAATGTCGTTATGATTCACAGG-TGGATGTTGTTGTTGCTGTTGC-3′.

For N70-Wld³-FLAG: 5′-CGGCGATCCATAGTCGTTCAATGTCGTTATGATTCACAGG-TGGATGTTGTTGTTGCTGTTGC-3′.

The FLAG-tagged construct expressing the 18 unique as followed by the full-length NMNAT1 cDNA (ΔNLS NMNAT1-FLAG) was amplified from the same template using the following forward primer (5′ HindIII cloning tag underlined, start codon in bold) and Wld³-FLAG reverse (Wilbrey et al., 2008): 5′-CGGGAACCTTTAAGAAGCCATGAGCAACATCGTGC-3′. The Wld³-FLAG construct was reported previously (Wilbrey et al., 2008).

All the PCR products were double digested with BamHI and HindIII restriction enzymes and cloned into pHAP1-1 vector downstream of the β-actin promoter for expression in neurons and other cell types as described by Mack et al. (2001). Ax-NMNAT1-mCherry was produced by PCR amplification using BamHI- and HindIII-tagged primers and subcloned in-frame to the mCherry sequence into pcDNA3 vector. The sequence of each construct was then verified. For transfection, plasmids were prepared using the EndoFree Plasmid Maxi-kit (Qiagen).

The Ax-NMNAT1 construct within pHAP1-1 was digested with EcoRI/Ndel and the resulting 5.7 kb linear DNA was used for pronuclear microinjection into an F1 C57BL/6J CBA strain by the in-house Gene Targeting Facility of the Babraham Institute. Founders were crossed to homozygous YFP-H mice (Feng et al., 2000) and their progeny genotyped by Southern blotting. For this study, we also used double-heterozygous native Wld³/YFP-H mice, triple-heterozygous tg-Wld³/Wld³/YFP-H mice, homozygous natural Wld³ mice, NMNAT1-overexpressing/YFP-H mice (line 7104) (Conforti et al., 2007), double-hemizygous ΔNLS Wld³/YFP-H mice (line 2 and 3) (Beirouz et al., 2009), and double-hemizygous ΔN16 Wld³/YFP-H mice (line 1) (Conforti et al., 2009). Triple-heterozygous tg-Wld³/Wld³/YFP-H mice express levels of Wld³ protein similar to that in homozygous natural Wld³ mice and display a similarly retarded time course of axon degeneration (Beirouz 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 0.5 M NaOH with two changes at 30 min intervals. The gel was Southern blotted overnight onto Hybond XL (GE Healthcare) in 0.4 M NaOH, and hybridized with a probe of radioactive Wld³ cDNA. Posthybridization washes were performed at a stringency of 0.1X 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 5–6. Neurons were cut with a scalpel 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). Aphidicolin (2 μM) was added to dissociated and explant DRG cultures to reduce the number of non-neuronal cells. Neurons were fixated with 4% paraformaldehyde (PFA) in 0.1 M PBS for 20–48 h later for immunofluorescence and high-resolution confocal imaging. 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. Vincuristine (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; Beirouz et al., 2004; Beirouz et al., 2005; Conforti et al., 2009). Mice were anesthetized with isoflurane (Abbot Animal Health) and subcutaneous injection of 2.5 μg of buprenorphine (Vetergesic, Alstoe Lt Animal Health) and unilateral sciatic nerve lesion was performed as described by Beirouz 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 0.1 M PBS and processed for imaging on a Zeiss LSM 510 Meta confocal system as previously described (Beirouz et al., 2004). Confocal z-stack series from longitudinally embedded nerve were taken using a 20X magnification objective, and z-projections were generated for final presentation using algorithms from Zeiss LSM Software Release 5.2. We quantified unfragmented YFP-positive axons in the distal tibial nerve as a percentage of the mean number of YFP-positive axons in uninjured preparation.

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

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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/PI). DRG explant cultures in presence of microtubule destabilizing agents (see Fig. 7) were cut and imaged in the same way. Axonal preservation 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 variocities or fragmentation 48 h after cut was quantified (±SD), 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-protease EDTA-free, Roche)]. 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 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 previous section of the sciatic nerve was verified by reexposing the wound in the thigh. Tibial nerve–muscle preparations were killed by cervical dislocation and subsequent section of the sciatic nerve was verified by reexposing the wound in the thigh. Tibial nerve–muscle preparations were killed by cervical dislocation and previous section of the sciatic nerve at the ankle. NMAT activity was obtained by normalization with protein content calculated by Bradford assay (Bio-Rad).

For immunofluorescence of transfected hippocampal neurons, cells were fixed in 4% PFA, 0.1% PBS for 10 min, permeabilized with 1% Triton X-100 for 10 min, blocked [5% NGS (Sigma) in PBS, 1 h], and immunostained with mouse anti-FLAG antibody (1:2000) (Sigma F3165) overnight followed by 1 h incubation with secondary Alexa647-goat-antibody-rabbit antibody (1:200) (Invitrogen), both diluted in 5% NGS in PBS. In hippocampal cultures, dendrites were counterstained with chicken anti-MAP-2 antibody (1:30,000) (Abcam) and secondary Alexa647-goat-antibody-rabbit antibody (Invitrogen). Proximal axons were stained with rabbit anti-Ankyrin G antibody (1:400) (Santa Cruz Biotechnology) and secondary Alexa488-goat-antibody-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-antibody-rabbit antibody (1:200) (Invitrogen), both diluted in 5% NGS in PBS. In hippocampal cultures, dendrites were counterstained with chicken anti-MAP-2 antibody (1:30,000) (Abcam) and secondary Alexa647-goat-antibody-rabbit antibody (Invitrogen). Proximal axons were stained with rabbit anti-Ankyrin G antibody (1:400) (Santa Cruz Biotechnology) and secondary Alexa488-goat-antibody-rabbit antibody (Invitrogen).

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Immunohistochemistry and immunochemistry. For immunofluorescence detection of axonally targeted NMNAT1 in brains, Ax-NMNAT1 mice were intracardially perfused with 4% PFA in 0.1% PBS and brains were postfixed for 24 h, cryopreserved for 3 d, embedded in OCT medium, and subsequently sectioned using a Leica cryostat. Perfused brains of transgenic mice expressing FLAG-tagged SOD1 (kind gift from Prof. Yasuhiro Watanebe, Tottori University, Tottori, Japan) were cryosectioned and processed as positive controls. Sections (20 μm) were mounted onto SuperFrost Plus glass slides (VWR) and incubated overnight in citrate buffer, pH 6.0, at 50°C for antigen retrieval. After permeabilization with 0.1% Triton X-100 plus 0.05 mM NH₄Cl in 0.05 mM TBS for 10 min, the sections were rinsed in fresh TBS, immunoblotted with 5% bovine serum albumin (Sigma) in TBS for 1 h, and incubated overnight at 4°C in primary antibody solution (Sigma F1804 mouse anti-FLAG antibody, 1:500 in 0.8% bovine serum albumin in TBS). After extensive washes, the secondary antibody solution (Alexa568-goat-antibody, 1:2000) in TBS was applied for 1 h at room temperature and slices were rinsed in TBS and diH₂O.
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

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**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. In the N-terminal axonal targeting sequence, evolutionarily conserved amino acids are highlighted in yellow.
axons and synapses in WldS 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 WldS (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 GFPQNQKEGRCS from exon 7 of the survival motor protein (SMN) (Zhang et al., 2003), the cytoplasmic targeting sequence (MEM) MLCCMRRTKQVEKNDEDQKI from growth cone-associated protein (GAP-43) (Zuber et al., 1989), and GYENPTYKFEEQMQN 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 immunostaining 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 dendritic staining 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 (Beiroukh 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 approximately 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 WldS in WldS 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 WldS 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; Beiroukh 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 WldS 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).

**Figure 2.** Axonally targeted NMNAT1 levels in brains of Ax-NMNAT1 mice from line 1 and 2. A, Western blotting of Ax-NMNAT1 line 1 mouse brain homogenates showing approximately twofold higher expression level of Ax-NMNAT1 protein in homozygotes than in hemizygotes, by anti-FLAG antibody detection (top) and anti-NMNAT1 antibody detection (bottom). B, Representative anti-NMNAT1 (antibody 183) Western blot of total brain homogenates of Ax-NMNAT1 mice. Anti-NMNAT1 antibody 183 detects both NMNAT1 and WldS. Axonally targeted NMNAT1 is 23 aa longer than murine native NMNAT1 and therefore migrates more slowly on SDS-polyacrylamide gel. Transfected HEK lysates indicate the molecular size of NMNAT1 and Ax-NMNAT1. Ax-NMNAT1-transfected HEK lyzates is 10-fold diluted; thus, its loading control band (β-actin) is not visible at this exposure time. In A and B, # indicates nonspecific band. C, Confirmation that axonally targeted NMNAT1 is enzymatically active in brain homogenates and that activity levels correlate with protein levels. Levels of NMNAT specific enzyme activity are higher in Ax-NMNAT1 mice from line 2 than in negative control littermates (WT). NMNAT enzyme activity of Ax-NMNAT1 mice from line 1 is similar to the WT one. Both Ax-NMNAT1 lines show levels lower than the ones from all the other strains. **p = 0.0037.
Robust axon protection \textit{in vivo} and \textit{in vitro}.

An amount of Wld$^5$ protein comparable to this level of axonally targeted NMNAT1 would be unlikely to grant axonal protection in Wld$^5$ 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$^5$ 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$^5$ (ΔNLS Wld$^5$) (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$^5$ lines with similar transgene expression levels indicated a stronger axon sparing activity of Ax-NMNAT1. Mice from line 1 of the ΔNLS Wld$^5$ 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$^5$, even when expressed at substantially lower levels. Moreover, these data indicate that Ax-NMNAT1 confers higher axoprotective potency than extranuclear Wld$^5 \textit{in vivo}$. 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/)

**Figure 3.** Comparison of delay of Wallerian degeneration in Ax-NMNAT1 mice and in mice with the indicated genotypes expressing YFP in a representative subset of neurons. Longitudinal imaging (confocal z-projections) of sciatic and tibial nerves 7, 14, and 35 d after transection, and quantification of percentages of intact axons in distal tibial nerves 35 d after lesion. Nerves from NMNAT1-overexpressing mice (TgNMNAT1) fragment with a time course similar to WT nerves, whereas 35 d lesioned nerves from hemizygous Ax-NMNAT1 mice still show intact axons. Thirty-five days after axotomy, the number of unfragmented axons in Ax-NMNAT1 samples is significantly higher than the one in Wld$^5$ samples and similar to the one in ΔNLS Wld$^5$ samples. (**p = 0.003, one-sample t test; NS: p = 0.399).
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 ΔNLS WldS 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 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 WldS in vivo and in vitro, despite the greatly reduced expression level.

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Axonally targeted NMNAT1 localizes outside the nucleus in vivo at low levels
Because the protective action is determined by extranuclear WldS (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 WldS (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 ΔN16 WldS, 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 WldS, and ~96.5% lower than ΔNLS WldS in the respective strains. Nevertheless, axonal preservation in Ax-NMNAT1 line 2 mice is greater than in WldS and similar to ΔNLS WldS 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 WldS mice, and is absent in WldS heterozygotes 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 vesicle recycling activity in 100% of their area 6 d after axotomy. In contrast, in WldS 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 Wld<sup>3</sup> mice, mice expressing the nonprotective variant of Wld<sup>3</sup> lacking the first 16 N-terminal amino acids (ΔN16 Wld<sup>3</sup> hemi), and mice expressing the enhanced protective extranuclear variant (ΔNLS Wld<sup>3</sup> L3 hemi) (n = 4). Nuclear and cytoplasmic fractions, 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, Wld<sup>3</sup>, and ΔN16Wld<sup>3</sup>). 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 Wld<sup>3</sup> levels for comparative analysis. The axonally targeted NMNAT1 protein shows the lowest levels if compared to Wld<sup>3</sup> and variants in the other strains. *Nonspecific band.

**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 Wld<sup>3</sup> mice, mice expressing the nonprotective variant of Wld<sup>3</sup> lacking the first 16 N-terminal amino acids (ΔN16 Wld<sup>3</sup> hemi), and mice expressing the enhanced protective extranuclear variant (ΔNLS Wld<sup>3</sup> L3 hemi) (n = 4). Nuclear and cytoplasmic fractions, 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, Wld<sup>3</sup>, and ΔN16Wld<sup>3</sup>). 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 Wld<sup>3</sup> levels for comparative analysis. The axonally targeted NMNAT1 protein shows the lowest levels if compared to Wld<sup>3</sup> and variants in the other strains. *Nonspecific band.

which full-length Wld<sup>3</sup> 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<sup>+</sup> channels—FETQHHLHHCLEKTT (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 hemizygotests), 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 neuronts 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. 7Ai,Aii,Aiv,Bii,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. 7Ci,Ciii), 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. 7Ci,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.

Wld<sup>3</sup> 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 hemizygotests 6 d after plating (Fig. 7D). To mimic the conditions of our previous transfected 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.
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 WldS functions. To investigate the specific subcellular localization of Ax-NMNAT1 protein, we fractionated brains of Ax-NMNAT1 mice and (variant) WldS 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) WldS 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 WldS are mostly concentrated in a small membranous compartment in vivo, extending our previous finding of high levels of (variant) WldS in mitochondria and microsome fractions and fine granular staining of extranuclear WldS in sciatic nerve axoplasm (Beirowski 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). Ax-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 WldS by cotransfection with ΔNLS Wld3-EGFP (Beirowski et al., 2009). ANLS Wld3-EGFP 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
(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 Wld⁶ 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 Wld⁶ 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 Wld⁶ 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 Wld⁶ in mouse distal stumps 1 week after lesion (Beirousski 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 Wld⁶. 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 Wld⁶ 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 Wld⁶ protein in young mice nor a homozygous dose in older mice is sufficient to delay NMJ deter-
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, \( \Delta NLS \) 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 (Lubińska, 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...
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/X11, via the NPTY domain (Rusu et al., 1998). Mint/X11 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 neurixin, another protein interacting with Mint1. The exit of neurxin 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 calyxenin-1 (Ludwig et al., 2009). Calyxenin-1 is a neuronal transmembrane protein transported to axons in vesicular carriers in a kinesin 1-dependent manner (Koneca 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 calyxenin-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 calyxenin-1, and p38 SNPH, we cannot exclude a possible colocalization with other 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 (Devil 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 ∆NLS WldS cytoplasmic puncta colocalize with mitochondria (Beirovski 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


