ことにつながり，その結果として神経変性疾患に対する治療的な効果につながることを示している．この現象はそれぞれの疾患の発症機序とは無関係に観察されることから，NAD·Sir2 を介した軸索保護機構の臨床応用が可能になれば，数多くの神経疾患に共通した治療法となることが期待される．

その一方でこれまでに NAD・Sir2 を介した軸索変性遅延効果が認められない可能性が示唆されている疾患がある．運動神経変性疾患として非常に重要な筋萎縮性側索硬化症（ALS）の動物モデルとされている変異型スーパーオキシドジスムターゼ 1（SOD1）を過剰発現するトランスジェニックマウスに関しては，*wld*^{s} マウスとのかけ合わせによる寿命の延長や症状の軽減などの治療的な効果は非常に弱いかほとんど認められないことが報告されている[10]．また，運動神経とその標的となる筋細胞との神経連絡形成は，上述のようにいったん形成された神経突起がなくなる過程であるという意味において軸索変性過程の一つであると考えることが可能であるが，*wld*^{s} マウスにおいては成熟型の神経筋連絡の形成に明らかな異常を認めないことが既に報告されている[11]．

これらの結果の解釈としてはいくつかの可能性がある．その一つは観察上軸索変性に類似した過程であると考えられる過程は分子メカニズムとしては単一ではなく，そのため NAD・Sir2 を介したメカニズムで遅延させることができる過程とできない過程がある，という可能性である．可能性の第二としては，軸索変性に類似した過程は分子レベルでも単一であるが，*wld*^{s} マウスにおいて軸索変性遅延表現型の原因となっている Wld^s 変異タンパク質の発現が安定的でないため，*wld*^{s} マウスを用いて行われてきたこれまでの実験結果は分子レベルでの神経軸索変性様過程の多様性を反映するものではない，という考え方が可能である．（*wld*^{s} マウスにおける Wld^s 変異タンパク質の発現は UFD2a 遺伝子のプロモーターが制御しているが，このタンパク質の発現は成熟型の運動神経連絡が形成される発生過程や ALS の SOD1モデルにおいて神経症状の悪化を認める生後 7～8 カ月齢以降では発現レベルが低下しており，このために十分な軸索変性遅延効果が得られなかった可能性がある．）これらの可能性のうちの，いずれが正しいのか，もしくは第三の可能性があるのかに関しては，今後の課題となっている．

11-4 • おわりに

神経軸索変性過程が，アポトーシス apoptosis（プログラム細胞死）に匹敵するような一連のタンパク質新生と酵素反応を介した細胞内反応系である可能性が示唆されたのは比較的最近であり，軸索変性過程に関して知られていることはアポトーシスに比べても非常に少なく，研究はまだその端緒についたばかりであるといってよい．軸索変性に関する研究は本章で述べたように，神経変性疾患の治療，神経系の生理的な老化現象の理解とその予防につながるものである．今後のこの分野の研究の一層の進展が期待される．

●文 献

1）Raff MC, Whitmore AV, Finn JT：Axonal self-destruction and neurodegeneration. Science, 296：868–871, 2002.

2）Lunn, ER, Perry VH, Brown MC, Rosen H, Gordon S.：Absence of Wallerian Degeneration does not Hinder Regeneration in Peripheral Nerve. Eur. J. Neurosci., 1：27–33, 1989.

3) Conforti L, Tarlton A, Mack TG, Mi W, Buckmaster EA, Wagner D, Perry VH, Coleman MP: A Ufd2/D4Cole1e chimeric protein and overexpression of Rbp7 in the slow Wallerian degeneration (WldS) mouse. Proc. Natl. Acad. Sci. U. S. A., 97: 11377–11382, 2000.

4) Mack TG, Reiner M, Beirowski B, Mi W, Emanuelli M, Wagner D, Thomson D, Gillingwater T, Court F, Conforti L, Fernando FS, Tarlton A, Andressen C, Addicks K, Magni G, Ribchester RR, Perry VH, Coleman MP: Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. Nat. Neurosci., 4: 1199–1206, 2001.

5) Araki T, Sasaki Y, Milbrandt J: Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. Science, 305: 1010–1013, 2004.

6) Imai S, Armstrong CM, Kaeberlein M, Guarente L: Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature, 403: 795–800, 2000.

7) Kaeberlein M, McVey M, Guarente L: The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev., 13: 2570–2580, 1999.

8) Tissenbaum HA, Guarente L: Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. Nature, 410: 227–230, 2001.

9) Guarente L, Picard F: Calorie Restriction- the SIR2 Connection. Cell, 120: 473–482, 2005.

10) Coleman M: Axon degeneration mechanisms: commonality amid diversity. Nat. Rev. Neurosci., 6: 889–898, 2005.

11) Hoopfer ED, McLaughlin T, Watts RJ, Schuldiner O, O'Leary DD, Luo L: Wlds protection distinguishes axon degeneration following injury from naturally occurring developmental pruning. Neuron, 50: 883–895, 2006.

Development/Plasticity/Repair

Stimulation of Nicotinamide Adenine Dinucleotide Biosynthetic Pathways Delays Axonal Degeneration after Axotomy

Yo Sasaki,[1] Toshiyuki Araki,[2] and Jeffrey Milbrandt[1]

[1]Departments of Pathology and Neurology, Hope Center for Neurological Disorders, Washington University School of Medicine, St. Louis, Missouri 63110, and [2]Department of Peripheral Nervous System Research, National Institute of Neuroscience, National Centre of Neurology and Psychiatry, Kodaira, Tokyo 187-8502, Japan

Axonal degeneration occurs in many neurodegenerative diseases and after traumatic injury and is a self-destructive program independent from programmed cell death. Previous studies demonstrated that overexpression of nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1) or exogenous application of nicotinamide adenine dinucleotide (NAD) can protect axons of cultured dorsal root ganglion (DRG) neurons from degeneration caused by mechanical or neurotoxic injury. In mammalian cells, NAD can be synthesized from multiple precursors, including tryptophan, nicotinic acid, nicotinamide, and nicotinamide riboside (NmR), via multiple enzymatic steps. To determine whether other components of these NAD biosynthetic pathways are capable of delaying axonal degeneration, we overexpressed each of the enzymes involved in each pathway and/or exogenously administered their respective substrates in DRG cultures and assessed their capacity to protect axons after axotomy. Among the enzymes tested, Nmnat1 had the strongest protective effects, whereas nicotinamide phosphoribosyl transferase and nicotinic acid phosphoribosyl transferase showed moderate protective activity in the presence of their substrates. Strong axonal protection was also provided by Nmnat3, which is predominantly located in mitochondria, and an Nmnat1 mutant localized to the cytoplasm, indicating that the subcellular location of NAD production is not crucial for protective activity. In addition, we showed that exogenous application of the NAD precursors that are the substrates of these enzymes, including nicotinic acid mononucleotide, nicotinamide mononucleotide, and NmR, can also delay axonal degeneration. These results indicate that stimulation of NAD biosynthetic pathways via a variety of interventions may be useful in preventing or delaying axonal degeneration.

Key words: axotomy; neuropathology; neuroprotection; dorsal root ganglion; nucleus; GFP

Introduction

Axonal degeneration is observed after physical damage to axons and in a variety of neuropathological conditions, including diabetic neuropathies, demyelinating diseases, and neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinson's disease. In these diseases, the degeneration of axons often precedes the death of neuronal cell bodies and likely contributes significantly to the development of clinical symptoms (Raff et al., 2002). Axonal degeneration appears to be a unique self-destructive process that is linked to, but distinct from, apoptosis (Coleman, 2005). These new insights suggest that axonal degeneration may act upstream of neuronal cell death and thereby represents a potential therapeutic target for prevention of certain neurodegenerative conditions.

The active nature of axonal degeneration was originally brought to light by the discovery of a spontaneous dominant mutation in mice called Wallerian degeneration slow (*wld*^{S}). *wld*^{S} mice have significantly delayed axonal degeneration in both central and peripheral axons after nerve transection (Lunn et al., 1989; Perry et al., 1991). The *wld*^{S} mutation comprises an 85 kb tandem triplication that results in overexpression of a chimeric nuclear protein called Wld^S (Conforti et al., 2000). This protein consists of the N-terminal 70 amino acids of a ubiquitin ligase, Ufd2a (ubiquitin fusion degradation protein 2a), fused to full-length nicotinamide mononucleotide adenylyltransferase 1 (Nmnat1), an enzyme in the nicotinamide adenine dinucleotide (NAD) salvage pathway (Mack et al., 2001; Wang et al., 2001). Using an *in vitro* Wallerian degeneration assay, we and others have shown previously that the Nmnat1 portion of the Wld^S protein is sufficient to protect axons after mechanical or chemical injury (Araki et al., 2004; Wang et al., 2005). Mutations in the Wld^S protein or Nmnat1 that render them enzymatically inactive also abolish their ability to delay axonal degeneration (Araki et

Received June 1, 2006; revised June 27, 2006; accepted July 3, 2006.

This work was supported by the Hope Center for Neurological Disorders, National Institutes of Health Grants AG13730, NS39358, and NS049087, and Muscular Dystrophy Association Grant 3972 (J.M.). We thank Kelli Simburger and Tim Fahrner for experimental assistance, members of the Milbrandt laboratory for their comments on this manuscript, and E. Johnson and S. Imai for helpful discussions. We and Washington University may derive benefit from a licensing agreement with Sirtris Pharmaceuticals, which did not provide any support for this work.

Correspondence should be addressed to Jeffrey Milbrandt, Departments of Pathology and Neurology, Hope Center for Neurological Disorders, Washington University School of Medicine, St. Louis, MO 63110. E-mail: jmilbrandt@wustl.edu.

DOI:10.1523/JNEUROSCI.2320-06.2006

 0270-6474/06/268484-08$15.00/0

al., 2004). Furthermore, the *wld*^{S} phenotype can be mimicked by treating neurons with NAD itself, suggesting that it is the biosynthesis of NAD by Nmnat1 that leads to axonal protection.

Despite the fact that expression of Nmnat1 is sufficient to save axons *in vitro* (Araki et al., 2004; Wang et al., 2005), there remains a question as to the exact mechanism of this effect. Others have suggested that the N-terminal Ufd2a portion of the Wld^S protein is critical, and that the NAD biosynthetic function is not relevant to axonal protection (Conforti et al., 2006). In this study, we manipulated each of the steps in the NAD biosynthetic pathway and found that overexpression of nuclear or non-nuclear forms of Nmnat provide strong protection against axonal degeneration. Nicotinamide phosphoribosyl transferase (NmPRT) and nicotinic acid (Na) phosphoribosyl transferase (NaPRT), which convert the precursor molecules nicotinamide and nicotinic acid, to the respective mononucleotides, also provide axonal protection. We also found that exogenous application of nicotinic acid mononucleotide (NaMN), nicotinamide mononucleotide (NMN), and nicotinamide riboside (NmR) all promoted axonal protection to a similar extent as NAD itself. These results show that stimulating the activity of the NAD biosynthetic pathway at multiple steps provides protection against axonal degeneration after axotomy. Furthermore, they supply evidence that the Wld^S effect is mediated via alteration of the NAD biosynthetic pathway and present new avenues for potential therapeutic agents to slow axonal degeneration in diseases of the nervous system.

Materials and Methods

Construction of expression plasmids and mutagenesis. The coding region of each NAD biosynthetic enzyme was amplified by PCR using Herculase (Stratagene, Garden Grove, CA) from expressed sequence tagged (EST) clones [GenBank accession numbers BC011191 for murine quinolinate phosphoribosyl transferase (QPRT), BQ938564 for murine NaPRT, BC004059 for murine NmPRT, BC038133 for murine Nmnat1, BC005737 for murine Nmnat3, BC016495 for murine nicotinamide riboside kinase 1 (Nrk1), and AI239871 for human Nrk2]. A hexahistidine tag was added at the C terminus of each enzyme. Human glutamine-dependant NAD synthetase (QNS) hexahistidine-tagged cDNA was kindly provided by Nobumasa Hara (Shimane University, Shimane, Japan) (Hara et al., 2003).

To produce the Nmnat1 cytoplasmic mutant (cytNmnat1), the residues corresponding to the nuclear localization site (PGRKRKW located at residues 211–217 to PGAAAAW) were mutated using PCR-mediated site-directed mutagenesis (Ausubel, 2001). A mutant of Nmnat3 (nucNmnat3) localized to the nucleus was generated by adding a nuclear localization signal (KPKKIKTED) derived from human topoisomerase to the C-terminal end of Nmnat3 as described previously (Araki et al., 2004). All of the modified cDNAs encoding these enzymes were cloned into the FCIV lentiviral shuttle vector as described previously (Araki et al., 2004). The integrity of each clone was verified by nucleotide sequence analysis.

NAD biosynthesis substrates. All substrates for NAD biosynthetic enzymes were purchased from Sigma (Na, N0761; nicotinamide, N0636; NMN, N3501; NaMN, N7764; nicotinic acid adenine dinucleotide (NaAD), N4256; and NAD, N1636). NmR was synthesized from NMN using a phosphatase reaction as described previously (Bieganowski and Brenner, 2004). The conversion of NMN to NmR was monitored using an HPLC reverse-phase column LC-18T (Supelco, Bellefonte, PA) with a buffer containing 50 mM K_2HPO_4 and 50 mM KH_2PO_4, pH 7.0. Using a flow rate of 1 ml/min, NmR eluted at 260 ± 10 s and NMN eluted at 150 ± 10 s. The biological activity of NmR was confirmed using yeast strains BY165 and BY278 kindly provided by Charles Brenner (Dartmouth Medical School, Hanover, NH) (Bieganowski and Brenner, 2004).

Quantitation of NAD biosynthetic enzyme expression. Male Sprague Dawley rats (200–300 g; Harlan Bioproducts, Indianapolis, IN) were anesthetized, and the right sciatic nerve was transected at the hip level using surgical scissors. Dorsal root ganglia (DRGs) from L4 and L5 were collected from three animals at the indicated time points and pooled, and RNA was prepared via Trizol method (Invitrogen, Carlsbad, CA). First-strand cDNA templates were prepared from two independent cDNA syntheses for each sample using 1 μg of RNA template using standard methods. Quantitative reverse transcription (RT)-PCR was performed by monitoring in real time the increase in fluorescence of the SYBR-green dye on a TaqMan 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). The expression levels of glyceraldehye-3-phosphate dehydrogenase was used for normalization of variations between the levels of total cDNA template across different samples. Each RT-PCR quantitation experiment was performed twice using duplicate samples from two independently generated cDNA templates (Araki et al., 2001). All surgical procedures were performed according to National Institute of Health guidelines for care and use of laboratory animals at Washington University.

Cell culture, in vitro axotomy, and quantification of axonal degeneration. Mouse DRGs dissected from embryonic day 12.5 (E12.5) to E13.5 embryos were cultured as explants on collagen-coated 24-well dishes in DMEM containing 10% FCS and 25 ng/ml nerve growth factor (2.5S; Harlan Bioproducts) as described previously (Araki et al., 2004). Non-neuronal cells were removed from the cultures by adding a mixture of 1 μM 5-fluoro-2′-deoxyuridine and 1 μM uridine to the culture media. Transection of axons was performed at 14–28 d *in vitro* using an 18 gauge needle to remove the neuronal cell bodies. Lentiviruses expressing NAD biosynthetic enzymes were generated as described previously (Araki et al., 2004). Lentiviruses (10^4 to 10^5 total infectious units) were added to each DRG explant, and protein expression from the lentivirus was allowed to accumulate for 4–7 d before axonal transection. Expression of the transgene was confirmed by expression of an enhanced green fluorescent protein (EGFP) reporter that is located downstream of an internal ribosome entry site within the lentiviral vector. For each condition, two DRG explants were used for each experiment, and two to three independent experiments were performed for each condition. Quantitative analysis of axonal degeneration was performed as described previously (Araki et al., 2004). Statistical analysis was performed by Student's *t* test.

Monitoring protein expression and localization. The expression of each NAD biosynthetic enzyme from the respective lentivirus was verified using infected HEK293T cells. Five days after infection, lysates were prepared using buffer containing 20 mM Tris-HCl, pH7.5, 300 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, and protease inhibitor cocktail (Roche, Indianapolis, IN). The expression levels of the hexahistidine-tagged enzymes were analyzed by immunoblotting using anti-hexahistidine tag monoclonal antibody (R & D Systems, Minneapolis, MN). The subcellular localization of each protein was analyzed using HEK293T cells expressing each of the enzymes. The cells were fixed with 4% paraformaldehyde and permeabilized with PBS containing 0.1% Tween 20 (PBS-T). They were then incubated with PBS-T containing 5% BSA for 1 h. Cells were incubated with 1:1000 diluted anti-hexahistidine tag antibody in PBS-T containing 5% BSA for 16 h at 4°C. After incubation with Alexa Fluor 594-conjugated secondary antibody (Invitrogen) in TBS containing 0.1% Tween 20 for 1 h, the proteins were visualized by fluorescence microscopy (Nikon, Melville, NY).

Results

Overexpression of multiple NAD biosynthetic enzymes delays axonal degeneration

The expression of Wld^S protein or Nmnat1 itself is sufficient to protect axons in an *in vitro* Wallerian degeneration assay, suggesting that increased NAD production is crucial for axonal protection (Araki et al., 2004). Overexpression of NmPRT, which converts nicotinamide to NMN, increases intracellular NAD concentrations in fibroblasts (Revollo et al., 2004), stimulating us to search for additional manipulations of the NAD biosynthetic pathway that can promote axonal protection.

NAD is recognized as a key molecule for cellular metabolism

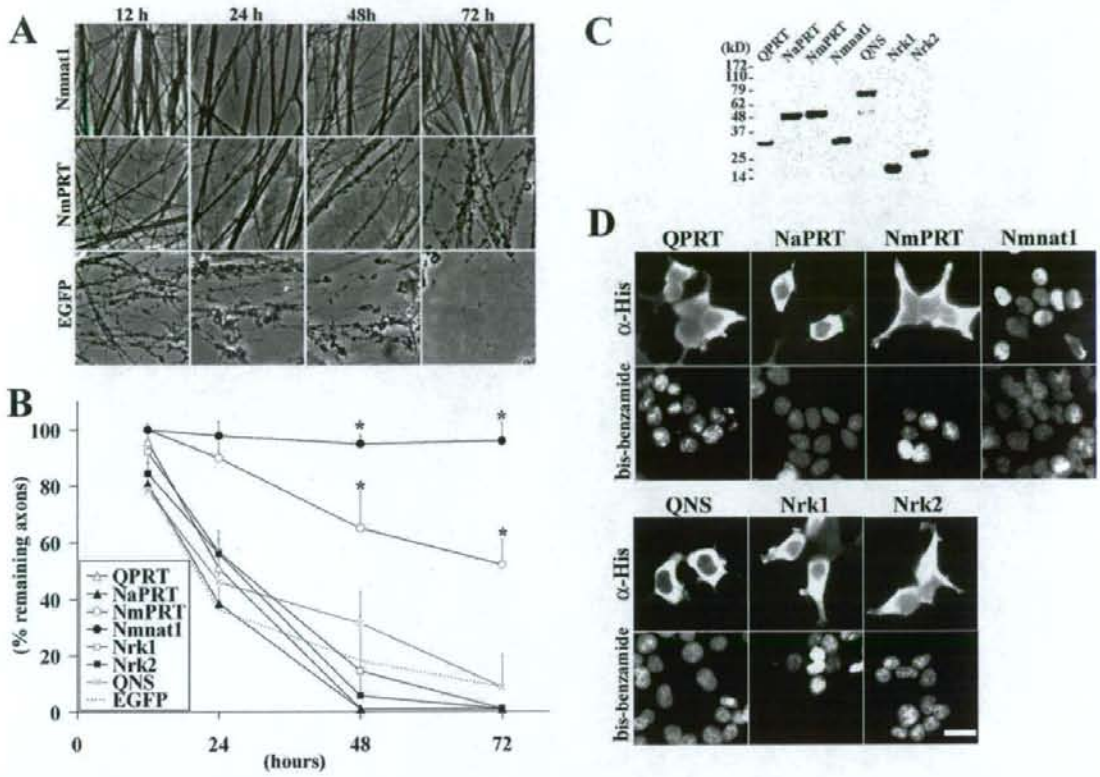


Figure 1. Axonal protection is mediated by enforced expression of NAD biosynthetic enzymes. *In vitro* Wallerian degeneration assays were performed using DRG neuronal explant cultures. ***A***, DRG neurons were infected with lentivirus expressing the indicated enzyme (Nmnat1, NmPRT, or EGFP control) 5 d before axotomy. Representative pictures taken at 12, 24, 48, and 72 h after transection are shown. ***B***, *In vitro* Wallerian degeneration assays were performed and axonal degeneration was quantified as outlined in Materials and Methods. The percentage ± SD of remaining axons relative to pretransection at 12, 24, 48, and 72 h after transection is displayed. $*p < 0.0001$ indicates a significant difference ($n = 6$) with EGFP-expressing cells. ***C***, Protein expression of QPRT, NaPRT, NmPRT, Nmnat1, QNS, Nrk1, and Nrk2 by lentivirus was confirmed by immunoblot analysis of HEK293T cells infected with each virus. The enzymatic activity of each clone was confirmed as described in the supplemental data (available at www.jneurosci.org as supplemental material). ***D***, The subcellular localization of the indicated NAD biosynthetic enzymes was examined using immunocytochemistry with an anti-hexahistidine tag antibody of transfected HEK293T cells. The cells were also stained with bis-benzimide to highlight the nucleus to determine the nuclear versus cytoplasmic distribution of each protein. Scale bar, 10 μm.

in organisms ranging from archebacteria to humans. It serves as a cofactor for most dehydrogenases, is important for aerobic and anaerobic ATP generation, and acts as a substrate for proteins such as PARP [poly(ADP-ribose) polymerase] or Sir2 (silent information regulator 2). NAD is synthesized via three major pathways in both prokaryotes and eukaryotes: the *de novo*, salvage, and Preiss-Handler independent. In the *de novo* pathway, NAD is synthesized from tryptophan (supplemental Fig. S1, available at www.jneurosci.org as supplemental material), whereas in the salvage pathway, NAD is generated from vitamins, including nicotinic acid and nicotinamide, and, in a recently discovered third route, NAD is derived from NmR. The mammalian homologs for most of the yeast NAD biosynthetic enzymes have been cloned and characterized (supplemental Fig. S1, available at www.jneurosci.org as supplemental material), including NmPRT, QNS, Nrk1/2, and Nmnat1–Nmnat3. For this work, we also identified mammalian homologs of NaPRT, which was present in the database as an EST (BQ938564) having NaPRT conserved domain [National Center for Biotechnology Information (NCBI) conserved domain cd01570.2], and QPRT, which was present in the database as an EST (GenBank accession number BC011191) having a QPRT conserved domain (NCBI clone cd01572.2). All of these enzymes were epitope tagged and cloned into the FCIV lentivirus vector (Araki et al., 2004). HEK293 cells were infected with these viruses, and cell lysates were used to confirm the expression (Fig. 1*C*). The catalytic activity of each enzyme was confirmed by HPLC (supplemental Fig. S2, available at www.jneurosci.org as supplemental material).

To test whether expression of any of these NAD biosynthetic enzymes could promote axonal protection, we infected primary cultured DRG neurons with lentiviruses expressing each enzyme and performed *in vitro* Wallerian degeneration experiments. Consistent with previous reports, axonal degeneration from DRG neurons expressing Nmnat1 was delayed for >72 h after axotomy, whereas axons from DRG neurons expressing EGFP degenerated within 24 h after axotomy (Fig. 1*A*). Next, we compared the extent of axonal degeneration in DRGs expressing each of the NAD biosynthetic enzymes with those expressing Nmnat1 or EGFP. We found that expression of NmPRT delayed axonal degeneration (Fig. 1*A*), whereas QPRT, NaPRT, Nrk1, Nrk2, and QNS did not protect against axonal degeneration. We quantified the extent of axonal degeneration by calculating the percentage of

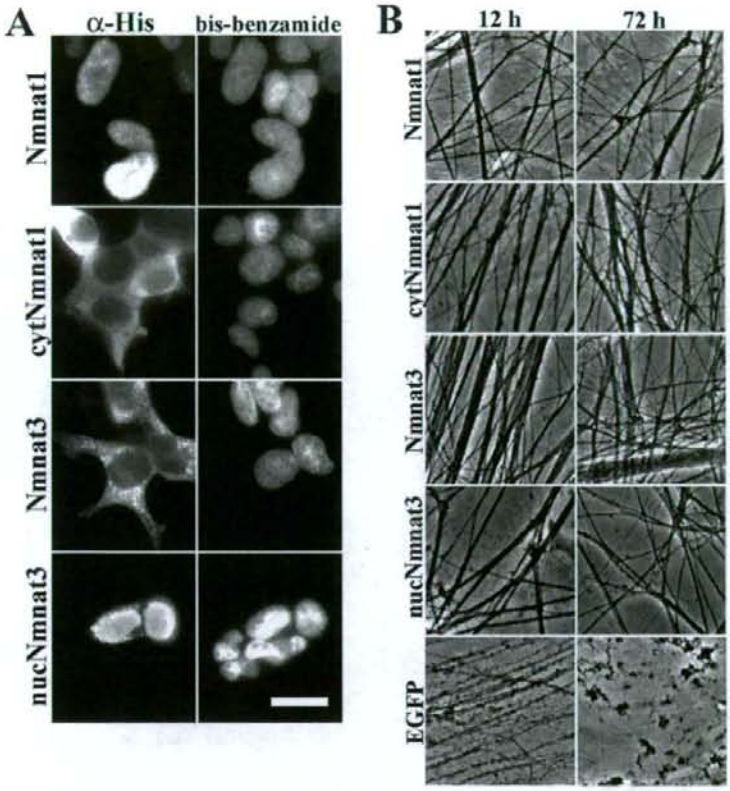


Figure 2. Nmnat enzymes located in the nucleus, cytoplasm, or mitochondria all promote axonal protection. ***A***, Subcellular localization of Nmnat1, cytNmnat1, Nmnat3, or nucNmnat3 in HEK293T cells. Immunohistochemistry using antibody against the hexahistidine epitope tag was used to detect each protein. The cells were also stained with bis-benzimide. As expected, the cytNmnat1 mutant is located in the cytoplasm, and the nucNmnat3 mutant is located in the nucleus. Scale bar, 10 μm. ***B***, *In vitro* Wallerian degeneration assay using lentivirus-infected DRG neuronal explant cultures expressing Nmnat1, cytNmnat1, Nmnat3, nucNmnat3, or EGFP control. Representative pictures taken at 12 and 72 h after transection show robust protection against axonal degeneration regardless of the subcellular distribution. ***C***, Quantitative analysis of axonal degeneration in DRG explant cultures expressing Nmnat1, cytNmnat1, Nmnat3, or nucNmnat3 at 12, 24, 48, and 72 h after transection. Nmnat1-, cytNmnat1-, Nmnat3-, and nucNmnat3-expressing cells have a significant difference ($p < 0.0001$; $n = 6$) with EGFP-expressing cells at 24, 48, and 72 h after axotomy.

remaining axons at the indicated times after transection. This analysis revealed that Nmnat1 and NmPRT resulted in a 10-fold and fivefold increase in the number of axons remaining 48 h after axotomy, respectively (Fig. 1*B*). The protection mediated by NmPRT was observed only when neurons were cultured in medium containing nicotinamide; thus, these results suggest that increased activity of the NAD biosynthetic pathway stemming from nicotinamide promotes axonal protection in this *in vitro* Wallerian degeneration assay.

Nmnat expression in multiple cellular compartments can promote axonal protection

In *wld*^{S} mice, the Wld^S protein is located within the nucleus and is absent from sciatic nerve and motor neuron terminals (Mack et al., 2001). Nmnat1 is also a nuclear protein; however, the localization of the upstream enzymes in these pathways, including NmPRT, which also promotes axonal protection, has not been well described. We therefore used immunocytochemistry to examine the subcellular localization of each NAD biosynthetic enzyme. HEK293T cells expressing each NAD biosynthetic enzyme were stained with anti-hexahistidine tag antibodies, and the proteins were visualized with the Texas Red-conjugated secondary antibody. As reported previously, Nmnat1 is present in the nucleus. However, QPRT, NaPRT, NmPRT, Nrk1, and Nrk2 are diffusely distributed throughout the cell, and QNS is localized primarily to the cytoplasm (Fig. 1*D*).

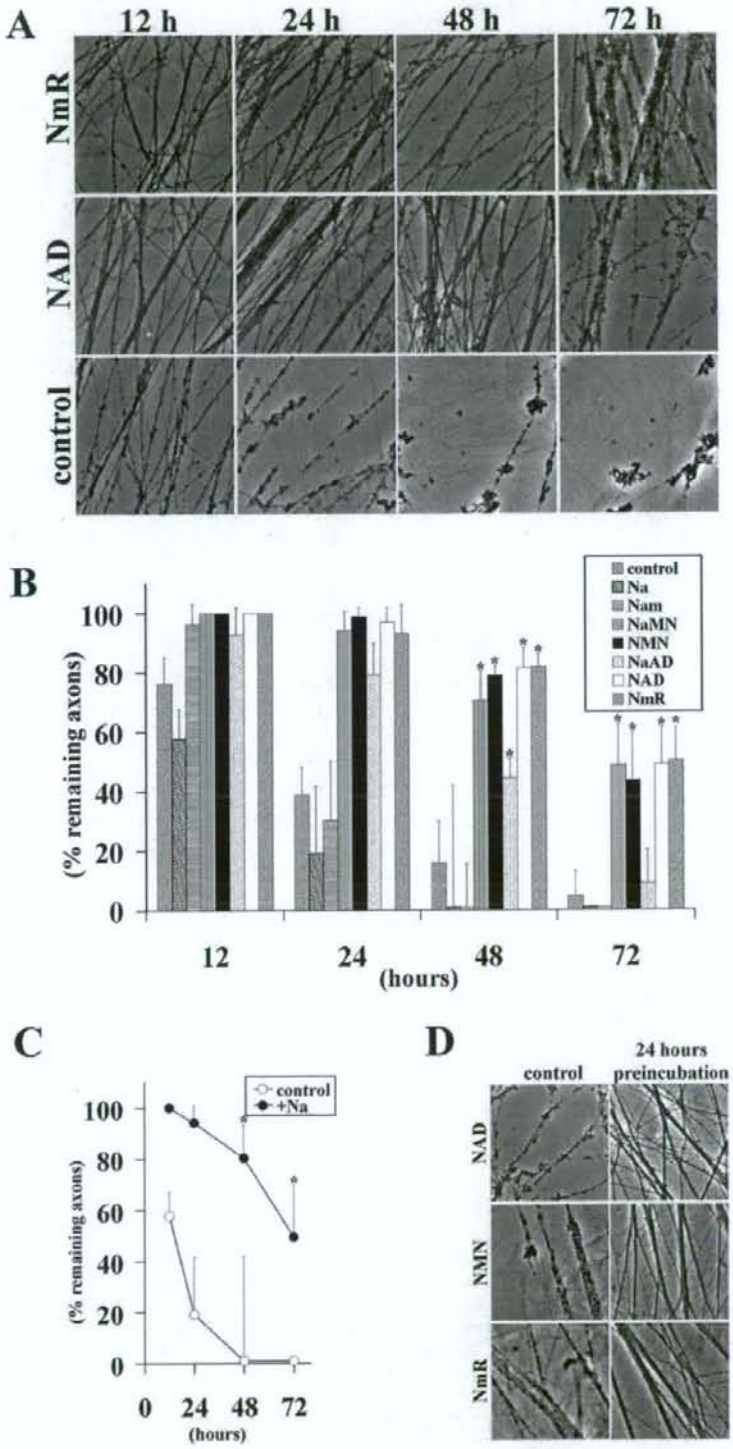
Of the two enzymes that promoted axonal protection, Nmnat1 is predominantly nuclear, and NmPRT is found distributed throughout the cell. These data coupled with the nuclear localization of the Wld^S fusion protein suggested that the nuclear production of NAD might be important for the protective function. To test the association between subcellular distribution of the Nmnat enzyme and axonal protection, we mutated the putative Nmnat1 nuclear localization signal (PGRKRKW) located at residues 211–217 to PGAAAAW to generate a cytoplasmic Nmnat1 (designated cytNmnat1). Wild-type and mutant Nmnat1 proteins were expressed in HEK293 cells, and the successful generation of a cytoplasmic Nmnat1 was demonstrated (Fig. 2*A*). The enzymatic activity of cytNmnat1 was compared with Nmnat1 using proteins affinity purified from transfected HEK293 cell lysates using His-select Nickel Affinity Gel as described in Materials and Methods. We found that the amount of NAD synthe-

sized by the cytNmnat1 was indistinguishable from that produced by wild-type Nmnat1 (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). To test the ability of cytNmnat1 to protect axons, we expressed it in DRG neurons and performed *in vitro* Wallerian degeneration assays. We found that cytNmnat1 promoted strong axonal protection that was indistinguishable from wild-type Nmnat1 (Fig. 2*B*,*C*).

We extended these results by using an Nmnat isoform, Nmnat3, which has comparable enzymatic activity with Nmnat1 (Raffaelli et al., 2002) and is localized within mitochondria (Zhang et al., 2003). We prepared hexahistidine-tagged versions of Nmnat3 and a Nmnat3 mutant in which we added a C-terminal nuclear localization signal (KPKKIKTED) from human topoisomerase I to generate nuclear Nmnat3 (designated nucNmnat3). We expressed wild-type Nmnat3 or nucNmnat3 in HEK293T cells and analyzed their subcellular localization and enzymatic activity. Nmnat3 was distributed outside of the nucleus with bright punctuate staining in the cytoplasm, consistent with a mitochondrial distribution as reported previously, whereas nucNmnat3 was predominantly found in the nucleus as expected (Fig. 2*A*). The enzymatic activity of Nmnat3 and nucNmnat3 were measured *in vitro*, and both proteins produced amounts of NAD that were comparable with that of Nmnat1 (supplemental Fig. S3, available at www.jneurosci.org as supplemental material). When these two Nmnat3 proteins were tested in the axonal degeneration assay, we found that both of them afforded protection to a similar extent as Nmnat1 (Fig. 2*B*,*C*). Overall, these experiments confirmed the potent axonal protection afforded by Nmnat enzymes and demonstrated that the subcellular site of increased Nmnat enzymatic activity is not important for mediating the delay in axonal degeneration. This would be consistent with the observation that addition of NAD to the culture medium also delays axonal degeneration, suggesting that NAD can diffuse to multiple cellular compartments.

Treatment with NAD precursors can delay axonal degeneration in DRG neurons

Previous work has shown that the addition of NAD to cultured neurons delays axonal degeneration (Araki et al., 2004; Wang et al., 2005). The expression of Nmnat enzymes as well as NmPRT, which ultimately convert NAD precursor substrates to



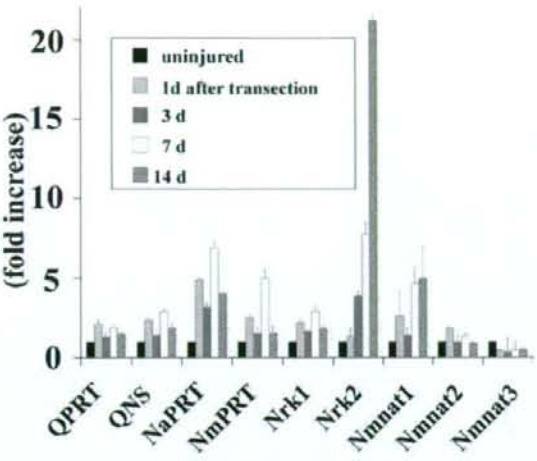


Figure 4. Expression of NAD biosynthetic enzymes is regulated by injury in DRG neurons. Quantitative RT-PCR analysis of indicated NAD biosynthesis enzyme mRNA levels in rat DRGs 0, 1, 3, 7, and 14 d after sciatic nerve transection. The expression level was normalized to glyceraldehyde-3-phosphate dehydrogenase and is indicated relative to expression in uninjured DRGs.

NAD, also promote axonal protection, suggesting that NAD precursors may also be effective in protecting axons. Because some of these precursors may be more stable or enter neurons more efficiently than NAD itself, we tested the ability of many of these substrates to promote axonal protection. These included nicotinamide (the substrate of NmPRT), NMN (the substrate of Nmnat), and nicotinic acid, NmR, NaMN, and NaAD. Each of these substrates was added to cultured DRG neurons 24 h before axotomy at a concentration of 1 mM. We found that NMN, NaMN, and NmR delayed axonal degeneration comparably with NAD, whereas NaAD showed only slight activity and nicotinic acid and nicotinamide had no activity (Fig. 3*A*,*B*). Interestingly, the protective effect of NaMN was equivalent to NMN, suggesting that the reaction converting NaAD to NAD catalyzed by QNS is sufficient to protect axons in the presence of an increased supply of NaAD. However, exogenous application of NaAD was only moderately effective in protecting axons, suggesting that this substrate is unstable or does not enter neurons efficiently (Fig. 3*B*).

We found it surprising that NaPRT was ineffective in protecting axons, whereas NmPRT mediated axonal protection because both NaMN and NMN, the respective products of these two enzymes, were able to delay axonal degeneration. The Wallerian degeneration assays are conducted in culture medium containing nicotinamide but lacking nicotinic acid. This suggested to us that the failure of NaPRT to protect axons could be attributable to insufficient amounts of its substrate, nicotinic acid. We therefore performed experiments in which both substrate and enzyme were increased. For this experiment, DRG neurons were infected with NaPRT lentivirus and cultured in the presence (or absence) of 1 mM nicotinic acid 24 h before axotomy. We found that the combination of increased NaPRT along with 1 mM nicotinic acid effectively delayed axonal degeneration, whereas neither increased enzyme or substrate alone delayed axonal degeneration (Figs. 1*B*, 3*C*).

We showed previously that neurons must be treated with NAD for ~24 h before axotomy to demonstrate axonal protection (Araki et al., 2004), whereas others have reported that NAD can protect axons when added contemporaneously with the injury (Wang et al., 2005). Because we have now identified multiple substrates capable of delaying axonal degeneration, we tested the time dependency of these substrates. NAD, NMN, or NmR (1 mM each) was added either simultaneously or 24 h before axotomy. At 24 h after transection, there was a significant decrease in the number of degenerating axons when the substrates were added before the injury (Fig. 3*D*), but no protection was afforded if they were added at the time of injury. Thus, like NAD, both NMN and NmR require a pretreatment period to provide axonal protection. These results strengthen the conclusion that increased NAD synthesis is the crucial step in promoting axonal protection, because a variety of manipulations of NAD biosynthetic pathways, including increased substrate availability, increased enzyme levels, or combinations thereof, delay axonal degeneration.

Expression of enzymes involved in NAD biosynthesis is induced in response to neuronal injury

The impact of NAD biosynthetic activity on axonal protection stimulated us to investigate the expression of the enzymes involved in this pathway in the nervous system during development and after neuronal injury. We surveyed their expression in brain, retina, spinal cord, and DRGs at ages E14, postnatal day 0 (P0), P7, P14, and P21 using quantitative RT-PCR. All enzymes were expressed ubiquitously in the nervous system throughout development and in adulthood, with the exception of Nrk2, whose expression is very low in all tissues examined (data not shown). To determine whether neuronal stress leads to increased expression of these NAD-synthesizing enzymes, we compared their RNA levels in DRGs before injury and at 1, 3, 7, and 14 d after sciatic nerve transection. The expression of most of the enzymes increased twofold to eightfold after injury, whereas Nrk2, which converts NmR to NMN, was induced >20-fold at 14 d after axotomy (Fig. 4). These results suggest that neurons express all NAD biosynthetic enzymes at a basal level and that the pathways that synthesize NAD are activated after neuronal injury to enable increased NAD production for downstream functions associated with the neuronal response to injury.

←

Figure 3. Treatment of cultured DRG neurons with NAD precursors delays axonal degeneration. ***A***, *In vitro* Wallerian degeneration assay using DRG explant cultures after exogenous application of NAD or NmR. Representative pictures at 12, 24, 48, and 72 h after transection are shown. ***B***, *In vitro* Wallerian degeneration assay using DRG neuronal explant cultures after exogenous application of Na, nicotinamide (Nam), NaMN, NMN, NaAD, NAD, and NmR. Quantitative analysis of axonal protection at 12, 24, 48, and 72 h after axotomy is shown. $*p < 0.002$ indicates a significant difference ($n = 6$) compared with control. ***C***, Axonal degeneration in DRG explants infected with NaPRT-expressing lentivirus is delayed only when 1 mM nicotinic acid is added to the medium 24 h before axotomy. Percentage of remaining axons at 12, 24, 48, and 72 h after transection is shown. $*p < 0.01$ indicates a significant difference ($n = 6$) compared with control. ***D***, NAD, NMN, or NmR (1 mM) was added either at the time of transection (control) or 24 h before axonal transection (24 h before incubation). Representative pictures taken 24 h after transection show axonal protection only in cultures in which substrates were added before injury.

Discussion

The mutation in the *wld*^{S} mutant mouse responsible for the delay in Wallerian degeneration results in the production of a fusion protein composed of a portion of the E3 ubiquitin ligase Ufd2a fused in-frame to the full-length Nmnat1 coding sequence (Conforti et al., 2000). Using an *in vitro* neuronal culture system, we demonstrated that axonal protection was mediated via the NAD biosynthetic activity of

the Nmnat1 portion of the Wld^S fusion protein (Araki et al., 2004). Indeed, mutant Wld^S or Nmnat1 protein, which harbors a mutation that destroys their NAD-synthesizing activity, fail to save axons, suggesting that an increased supply of NAD is responsible for preventing axonal degeneration. In addition, exogenous application of NAD to cultured neurons before axotomy also delays axonal degeneration, suggesting that NAD may modify a genetic program that results in altered axonal physiology. These studies led us to postulate that manipulation of other enzymatic reactions in the NAD biosynthetic pathway, either through increased expression of the pertinent enzymes or addition of their cognate substrates, might also provide axonal protection. We found that expression of Nmnat1 or Nmnat3 alone could delay axonal degeneration, whereas NmPRT and NaPRT could delay axonal degeneration when high levels of their requisite substrates, nicotinamide or nicotinic acid, were present in the culture medium. The enforced expression of other NAD synthetic enzymes, including QPRT, QNS, Nrk1, and Nrk2, did not promote axonal protection. The addition of multiple NAD precursors, including NaMN, NMN, NaAD, and NmR, gave protection similar to that provided by NAD itself, whereas nicotinic acid and nicotinamide did not. We added NMN or NmR at various times relative to axotomy and found that, like NAD, they afforded protection only if they were administered before axonal injury, suggesting that they protect axons via a similar mechanism. These studies have demonstrated that multiple manipulations that increase activity of the NAD pathway can promote axonal protection, supporting the hypothesis that increased NAD availability is a likely mechanism responsible for the delayed axonal degeneration mediated by the Wld^S protein.

The Nmnat enzymes catalyze the final step in NaAD and NAD synthesis by conversion of the mononucleotides NaMN and NMN to NaAD and NAD, respectively. These two mononucleotide compounds can be synthesized from three different pathways: the *de novo* pathway, the salvage pathway, and a Preiss-Handler independent pathway. NaMN can be synthesized from nicotinic acid via NaPRT (salvage steps) or quinolinic acid (*de novo* pathway). Our culture medium does not contain nicotinic acid, and production of quinolinic acid through the *de novo* pathway must be limited because expression of QPRT, which converts quinolinic acid to NaMN, cannot delay axonal degeneration; thus, it appears that NaMN synthesis is relatively low in our assay conditions. Furthermore, although NaAD can promote axonal protection, QNS expression, which converts NaAD to NAD, was not effective, presumably because of low intracellular levels of NaAD secondary to low concentrations of NaMN. These results indicate that the activity of the *de novo* pathway is insufficient to increase NAD levels high enough to promote axonal protection. Similarly, the intracellular levels of NmR must be low because enforced expression of the Nrk enzymes that convert NmR to NMN do not delay axonal degeneration, whereas addition of NmR itself protects equivalently to NAD administration. It thus appears that the predominant pathway involved in promoting axonal protection under our assay conditions is the salvage pathway that converts nicotinamide, which is present as a major vitamin in the culture medium, to NAD. Consistent with this hypothesis, if nicotinic acid is added to the medium, then enforced expression of NaPRT can also effectively delay axonal degeneration attributable to the increased production of NaMN. The pathways that are most active *in vivo* under normal versus pathological conditions will dictate which manipulations of NAD production will most effectively alter axonal degeneration dynamics under disease conditions.

Both the Wld^S and Nmnat1 proteins are localized to the nucleus and provide robust axonal protection, whereas NmPRT and NaPRT, which are distributed throughout multiple cellular compartments, mediate moderate protection. These results raise the possibility that NAD production in the nucleus is more effective in mediating axonal protection. Others have also suggested that the nuclear localization may be important for Wld^S-mediated axonal protection, possibly by the interaction of valosin-containing-protein (VCP/p97) with the Ufd2a portion of the Wld^S fusion protein (Conforti et al., 2006; Laser et al., 2006). However, we found that an Nmnat1 cytoplasmic mutant and the closely related Nmnat3, which is localized to mitochondria, also mediated robust axonal protection. These results suggest that neither Nmnat location nor interaction with VCP is crucial for axonal protection. Instead, it is likely that axonal protection is provided by NAD that is rapidly distributed throughout multiple cellular compartments. Furthermore, the more robust axonal protection mediated by Nmnat compared with NaPRT and NmPRT could result if NAD synthesis in neurons is limited by Nmnat activity.

A recent study from Wang et al. (2005) confirmed the ability of Nmnat1 and NAD to delay axonal degeneration; however, much higher levels of NAD (5 vs 1 mM) were required for protection in those experiments. They also noted that axonal degeneration could be delayed in their system when NAD was added at the time of injury or even slightly after the injury (i.e., no preincubation step was required). In contrast and in accord with our previous study, we find that neurons must be preincubated with NAD or other protective compounds such as NMN or NmR to protect against axonal degeneration. Our observations suggest that the mechanism of axonal protection mediated by preincubation with NAD, NMN, or NmR is different from that observed using postaxotomy treatment. In addition, another recent study claims that both Nmnat1 and NAD are ineffective in preventing axonal degeneration, whereas the Wld^S fusion protein mediated protection (Conforti et al., 2006). However, in this report, it was shown that an enzymatically inactive Wld^S mutant did not provide axonal protection as well as Wld^S, again indicating that NAD production is an important feature of Wld^S-mediated axonal protection. These discrepancies may be derived from differences in the neuronal culture conditions such as components in the culture medium, substrates for cell adhesion, or the age of the neurons. All of these parameters, in particular the number of days *in vitro*, affect intracellular calcium concentrations and requirements for trophic factors (Tong et al., 1996), and it is likely that aspects of Wallerian degeneration are also affected by these factors. In addition, *in vivo* studies with transgenic mice suggest that Nmnat1 overexpression provides weak axonal protection compared with Wld^S (Conforti et al., 2006), whereas dNmnat overexpression in *Drosophila* olfactory receptor neurons provided strong axonal protection (MacDonald et al., 2006). A detailed characterization of NAD-dependent delay in axonal degeneration *in vitro*, and eventually *in vivo*, will help reveal the precise molecular mechanisms of this protective phenomenon.

The expression of many NAD biosynthetic enzymes is altered after axotomy. In particular, the levels of Nrk2 were dramatically increased. This result suggests that Nrk2-dependent NmR conversion to NMN is an important aspect of the neuronal response to stress. The NAD biosynthetic pathway that uses NmR as a substrate is independent from the *de novo* and salvage pathways, because NmR is found in milk and has been proposed as a vitamin (Bieganowski and Brenner, 2004). In contrast to precursors such as nicotinamide and nicotinic acid, the exogenous applica-

tion of NmR protects axons from degeneration as well as NAD. This observation suggests that Nrk activity is high enough to convert exogenously applied NmR to NMN in cultured DRG neurons. This would imply that NmR levels in the culture medium are normally very low and would explain why enforced expression of Nrk1 or Nrk2 was ineffective in mediating axonal protection. Because NmR is the only dietary nutrient in the NAD biosynthetic pathway in our study that promotes axonal protection and the enzyme Nrk2, which converts it to NMN, is dramatically induced after axotomy, it may be useful in the treatment of diseases in which axonopathy is a major component of the pathophysiology.

In summary, we found that multiple manipulations that increase activity of the NAD pathway can promote axonal protection, supporting the hypothesis that increased NAD availability is a likely mechanism responsible for the delayed axonal degeneration mediated by the Wld^S protein. Additional investigation to apply these findings *in vivo* will be critical to assess the potential utility of this pathway as a therapeutic target.

References

Araki T, Nagarajan R, Milbrandt J (2001) Identification of genes induced in peripheral nerve after injury. Expression profiling and novel gene discovery. J Biol Chem 276:34131–34141.

Araki T, Sasaki Y, Milbrandt J (2004) Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. Science 305:1010–1013.

Ausubel FM (2001) Current protocols in molecular biology, Vol 1, Chap 8. New York: Wiley.

Bieganowski P, Brenner C (2004) Discoveries of nicotinamide riboside as a nutrient and conserved NRK genes establish a Preiss-Handler independent route to NAD+ in fungi and humans. Cell 117:495–502.

Coleman M (2005) Axon degeneration mechanisms: commonality amid diversity. Nat Rev Neurosci 6:889–898.

Conforti L, Tarlton A, Mack TG, Mi W, Buckmaster EA, Wagner D, Perry VH, Coleman MP (2000) A Ufd2/D4Cole1e chimeric protein and overexpression of Rbp7 in the slow Wallerian degeneration (Wld^S) mouse. Proc Natl Acad Sci USA 97:11377–11382.

Conforti L, Fang G, Beirowski B, Wang MS, Sorci L, Asress S, Adalbert R, Silva A, Bridge K, Huang XP, Magni G, Glass JD, Coleman MP (2006) NAD(+) and axon degeneration revisited: Nmnat1 cannot substitute for Wld(S) to delay Wallerian degeneration. Cell Death Differ, in press.

Hara N, Yamada K, Terashima M, Osago H, Shimoyama M, Tsuchiya M (2003) Molecular identification of human glutamine- and ammonia-dependent NAD synthetases. Carbon-nitrogen hydrolase domain confers glutamine dependency. J Biol Chem 278:10914–10921.

Laser H, Conforti L, Morreale G, Mack TG, Heyer M, Haley JE, Wishart TM, Beirowski B, Walker SA, Haase G, Celik A, Adalbert R, Wagner D, Grumme D, Ribchester RR, Plomann M, Coleman MP (2006) The slow Wallerian degeneration protein, WldS, binds directly to VCP/p97 and partially redistributes it within the nucleus. Mol Biol Cell 17:1075–1084.

Lunn ER, Perry VH, Brown MC, Rosen H, Gordon S (1989) Absence of Wallerian degeneration does not hinder regeneration in peripheral nerve. Eur J Neurosci 1:27–33.

MacDonald JM, Beach MG, Porpiglia E, Sheehan AE, Watts RJ, Freeman MR (2006) The *Drosophila* cell corpse engulfment receptor draper mediates glial clearance of severed axons. Neuron 15:869–881.

Mack TG, Reiner M, Beirowski B, Mi W, Emanuelli M, Wagner D, Thomson D, Gillingwater T, Court F, Conforti L, Fernando FS, Tarlton A, Andressen C, Addicks K, Magni G, Ribchester RR, Perry VH, Coleman MP (2001) Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. Nat Neurosci 4:1199–1206.

Perry VH, Brown MC, Lunn ER (1991) Very slow retrograde and Wallerian degeneration in the CNS of C57BL/Ola Mice. Eur J Neurosci 3:102–105.

Raff MC, Whitmore AV, Finn JT (2002) Axonal self-destruction and neurodegeneration. Science 296:868–871.

Raffaelli N, Sorci L, Amici A, Emanuelli M, Mazzola F, Magni G (2002) Identification of a novel human nicotinamide mononucleotide adenylyltransferase. Biochem Biophys Res Commun 297:835–840.

Revollo JR, Grimm AA, Imai S (2004) The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. J Biol Chem 279:50754–50763.

Tong JX, Eichler ME, Rich KM (1996) Intracellular calcium levels influence apoptosis in mature sensory neurons after trophic factor deprivation. Exp Neurol 138:45–52.

Wang J, Zhai Q, Chen Y, Lin E, Gu W, McBurney MW, He Z (2005) A local mechanism mediates NAD-dependent protection of axon degeneration. J Cell Biol 170:349–355.

Wang MS, Fang G, Culver DG, Davis AA, Rich MM, Glass JD (2001) The Wld^S protein protects against axonal degeneration: a model of gene therapy for peripheral neuropathy. Ann Neurol 50:773–779.

Zhang X, Kurnasov OV, Karthikeyan S, Grishin NV, Osterman AL, Zhang H (2003) Structural characterization of a human cytosolic NMN/NaMN adenylyltransferase and implication in human NAD biosynthesis. J Biol Chem 278:13503–13511.

特集 神経変性疾患の神経再生・神経移植

神経変性疾患の神経再生

Strategies for nerve regeneration in the adult central nervous system

国立精神・神経センター神経研究所疾病研究第5部部長
Toshiyuki Araki 荒木敏之

Summary

中枢神経が末梢神経と異なる最大の特徴の1つは，傷害された神経突起の再生の有無である。中枢神経軸索の再生が非常に難しい理由として，古くから①末梢神経に存在する再生促進因子の中枢における欠如，②末梢神経に存在しない再生阻害因子の中枢神経系における存在，が想定されていたが，それらはいずれも正しく，近年それぞれの分子基盤が徐々に明らかになりつつあり，それに伴って再生促進因子の補塡，阻害因子の除去を行えば，少なくとも限定的な中枢神経再生の実現が可能であることが，主として脊髄損傷などの中枢神経傷害モデル動物における活発な研究によって明らかになりつつある。本稿ではこれまでに報告されている方法について概説し，いくつかの問題点についても述べたい。

Key words

- spinal cord injury
- axonal regeneration
- 再生促進因子
- 再生阻害因子

I はじめに

神経変性疾患は，神経細胞の中でも特定の一群(たとえば運動神経細胞など)が変性，死滅する疾患であり，その治療は，パーキンソン病のL-ドーパ療法やアルツハイマー病(Alzheimer's disease；AD)のアセチルコリン療法などのように，疾患によって脱落した神経細胞の機能を補う補充療法が主流で，失われた神経を回復させる根本的な治療はいまだ研究段階にとどまっている。

いったん発達を終えた哺乳類の中枢神経系に損傷あるいは変性が起こると，再生・修復は不可能であるというのが従来の一般的常識であったが，このような考え方を覆す多くの実験結果が示されるようになった。1969年に，Raismanは，成体ラットにおいて，中隔核への求心線維のうち海馬采を切断すると，海馬采に由来するシナプスは変性するが，これらのシナプスが失われた部位に，他の求心線維である内側前脳束に由来する神経終末が発芽して，新しいシナプスが形成されることを示し，脳の可塑性(plasticity)という概念を確立した[1]。また，1982年に，Bjorklundは，損傷を受けた哺乳類成体の脳内に胎生神経組織を移植すると，移植組織は発達・分化して，宿主神経細胞とシナプスを形成し，神経回路の再構築が起りうることを，黒質－線条体投射系ならびに海馬－中隔核投射系で明らかにした[2]。これらの実験結果は，成体脳組織にも，損傷後，軸索の発芽・再生，シナプスの

再形成のような再生・修復能力が発現しうることをはじめて明確に示したものである。

本特集は神経変性疾患における再生・移植に関するものであるが，他稿で幹細胞の移植などによる治療に関しては扱われることになるので，本稿では「神経再生」ということで，神経軸索の再生機転の活性化に関して述べる。

中枢神経系における神経軸索の再生が困難な要因として，末梢神経系において存在する再生促進因子の中枢神経における欠如と，中枢神経における再生阻害因子の存在がある。

再生阻害因子の抑制による神経再生

中枢神経系に存在する神経突起伸展阻害因子に関する研究も近年成果を上げている。阻害因子として主として現在までに想定されているのは，中枢神経系の髄鞘構成成分と，傷害部位に集積するアストロサイトなどによって形成される瘢痕である。

1．髄鞘構成成分

Schwabらは，中枢神経系の髄鞘構成成分に対するモノクローナル抗体IN-1を作成し，この抗体が中枢神経の髄鞘がもつ神経突起伸展阻害効果を抑制する作用をもつことを，脊髄損傷の動物モデルにおいて示した[1]。この抗体が認識する物質は10年近い年月を経て同定され，Nogoと名づけられた[2]。その後，Nogoを介したシグナリングの詳細について研究が進んでいる[3]。またNogoに加えて，myelin-associated glycoprotein(MAG)，oligodendrocyte myelin glycoprotein(OMgp)がこのシステムに関連した神経突起伸展阻害因子としてさらに同定された[4]（図1）。

IN-1投与による再生促進効果に加えて，Nogoシステムの抑制に関しては他に，Nogo受容体に対する抗体の投与，Nogo様ペプチド(Nogo受容体阻害作用を有する)の投与などが動物の中枢神経傷害モデルにおいて試みられている[5]。

しかし，その一方で，いくつかのグループは中枢神経の軸索は白質領域を越えて伸展可能であるという実験結果を基に，中枢神経の髄鞘成分は神経突起伸展に対して抑制的ではないと考えている[6]。この考えによると，髄鞘が神経突起に対してrepulsiveに作用するのは突起の成長を髄鞘に沿った方向に導き，それ以外の方向への成長を妨げるためで，髄鞘成分は髄鞘と平行な方向への成長には促進的であるとされている。ただ，神経傷害時に

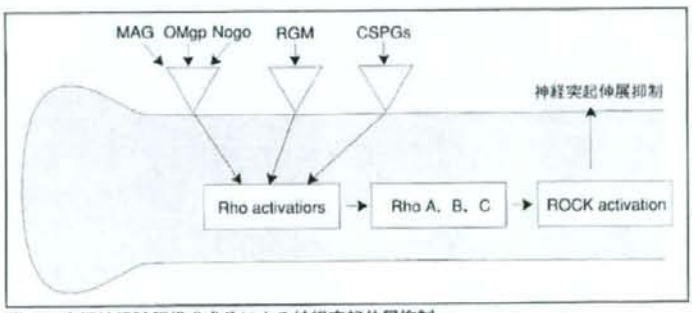


図1　中枢神経髄鞘構成成分による神経突起伸展抑制
神経突起の細胞表面受容体が髄鞘構成成分に由来するシグナルを受容し，RhoA ROCK系を活性化する。
MAG：myelin-associated glycoprotein，OMgp：oligodendrocyte myelin glycoprotein，RGM：repulsive guidance molecule

は正常時には方向性をもっているはずの髄鞘配列は乱れていて本来の作用をもつことができない(図2)。

2．瘢　痕

傷害部位にアストロサイト(反応性アストロサイト)が集積することは古くから知られていたが，この周辺では傷害部位に浸潤してくるマクロファージとアストロサイトの相互作用などによって細胞外マトリクスの改変が起こり，chondroitin sulphate proteoglican(CSPG)，keratin sulphate proteoglican(KSPG)などの蓄積が起こり，硬い瘢痕組織を形成する。この瘢痕組織は長期間にわたって傷害部位を占拠し，神経突起再生の妨げとなる(図

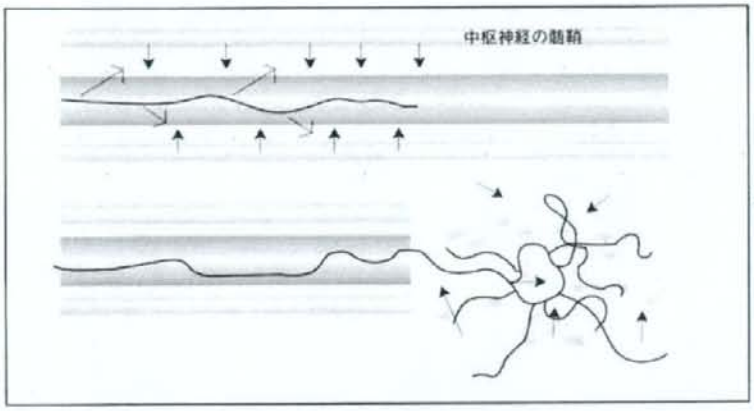


図2　中枢神経髄鞘構成成分による神経突起誘導仮説
矢印は髄鞘由来の抑制物質を示す。正常神経では髄鞘の存在が神経突起の伸展を正しい方向に誘導する(上図)が，傷害部位では髄鞘構成成分由来の突起伸展抑制は方向性がなくなり，神経突起の再生が阻害される(下図)。

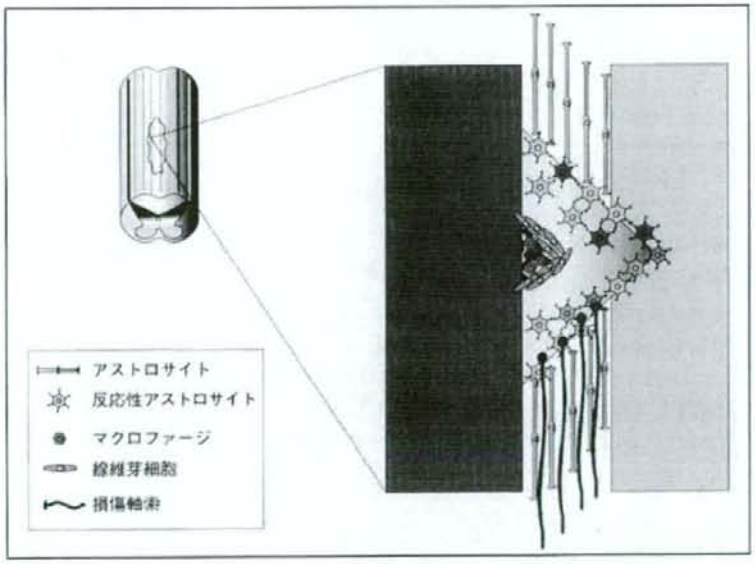


図3　中枢神経傷害部位での瘢痕形成
集積する活性化アストロサイトとマクロファージの作用によりCSPG(chondroitin sulphate proteoglican)，KSPG(keratin sulphate proteoglican)などからなる神経突起伸展阻害物質が蓄積する。

3)。

これに対して，chondroitinase を投与することによって細胞外マトリクスを酵素的に消化して除去する方法が試みられ，ラットの脊髄損傷モデルでは傷害された皮質脊髄路の神経線維の sprouting などの再生促進効果が認められている[36]。

3．再生阻害因子が刺激する細胞内シグナル

RhoGTPase は Ras スーパーファミリーの GTPase に属し，GTP，GDP との結合状態によってそれぞれ活性化・不活性型となって，数多くの細胞内シグナルを制御している。RhoGTPase は活性を制御する GTPase activating protein(GAP)，guanine nucleotide-exchange factor(GEF)，さらに guanine nucleotide-dissociation inhibitor(GDI)による制御を受けている。RhoGFPase によって制御を受ける effector の中では Rho-associated coiled-coil-containing protein kinase(ROCK)が最もよく知られている[37-41]。

Nogo，MAG，OMgp はいずれも Nogo 受容体(NgR 1)に結合し，さらにこの受容体と結合する p75，TROY などの co-receptor の作用により，RhoGTPase である RhoA を活性化する。ミエリン構成成分には最近報告された repulsive guidance molecule(RGM)を含め，他にも RhoA を活性化するものがあると考えられている。また傷害後瘢痕に存在する CSPG も Rho/ROCK 系を活性化することによって神経突起伸展を抑制していると考えられる(**図 1**)。このため，Rho キナーゼの阻害剤は神経再生促進作用をもつと考えられ，小分子物質による神経再生効果を得るための重要な創薬ターゲットとみなされている。Rho キナーゼ阻害剤は脊髄損傷モデルのほか，脳血管障害による脳損傷や *N*-methyl-D-aspartate 投与による傷害モデルにおいても再生促進刺激効果があるとする報告もなされている。

Rho キナーゼ阻害剤は神経変性疾患に対する有効性をもつ可能性も指摘されている。たとえば AD において，神経毒性の主要な原因物質と考えられる Aβ42の脳内レベルを非ステロイド性抗炎症薬(nonsteroidal antiinflammatory drug；NSAID)が低下させる作用が報告されているが，この作用は NSAID が RhoA/ROCK 系を抑制するためと考えられている。また，多発性硬化症においても，Rho キナーゼ阻害剤は白血球の中枢神経系への移行を阻害することによって疾患の進行を抑制する効果をもつ可能性が報告されている[42]。

Ⅲ 再生促進因子を利用した神経再生

再生促進因子を利用する神経軸索再生促進法としては，①神経栄養因子の投与，②突起伸展を促進するシグナル分子(cAMP など)の投与，③再生促進的環境を整える細胞の移植，などの方法が研究されている。

1．神経栄養因子

神経栄養因子は神経細胞の生存維持，突起伸展促進などの生理作用から中枢神経の再生促進作用を期待され，多くの因子に関して投与の量，時期，方法などの検討が行われ，因子ごとに異なるフェノタイプの神経細胞/突起に作用することが明らかになっている(**表 1**)。特に運動神経の再生促進に効果の期待される脳由来神経栄養因子(brain-derived neurotrophic factor；BDNF)，グリア細胞由来神経栄養因子(glial cell line-derived neurotrophic factor；GDNF)については直接的な投与のほかに，これらを発現するウイルスベクターの投与による強

表 1　神経栄養因子の神経突起伸展促進作用の有無に関する特異性

神経軸索	種　別	NGF	BDNF	GDNF	NT-3
感覚系	CGRP 陽性線維	＋	＋	＋	－
運動系	ChAT 陽性線維	＋	＋	＋	－
	TH 陽性線維	＋	＋	－	－
	皮質脊髄路	－	－	－	＋
	毛様体脊髄路	?	＋	?	?
	赤核脊髄路	?	＋	?	?

＋，－は神経突起伸展促進作用の有無をそれぞれ示す
NGF：nerve growth factor，BDNF：brain-derived neurotrophic factor，GDNF：glial cell line-derived neurotrophic factor，NT-3：neurotrophin-3，CGRP：calcitonin gene-related peptide，ChAT：choline acetyltransferase，TH：tyrosine hydroxylase
(文献13)より改変，引用)

制発現や，後に述べる細胞移植と組み合わせてこれらの因子を発現させるコンストラクトを組み込んだ細胞を移植する方法などが試みられている[13]。

2．突起伸展シグナル

2002年に複数のグループが脊髄運動神経細胞にcAMPを注入すると脊髄損傷後の神経突起の再生促進効果があることを示し，cAMPシグナルの増強による神経再生促進効果が注目された[14,15]。cAMPは成長円錐(growth cone)の先端において成長の方向性の決定に関与すること，上述のMAGなどの突起伸展抑制因子を超えて突起伸展を促す効果をもつことなどが，動物実験における中枢神経損傷後の突起伸展効果と関係しているものと考えられている。cAMPレベルの上昇を後に述べる細胞移植と組み合わせることによって一層の再生促進効果を得る方法も報告されている[16]。

3．細胞移植

1980年代に，Aguayoらは中枢神経損傷モデルにおける欠損部位を末梢神経の非神経組織で架橋することによって損傷された神経が再生することを示し，Schwann細胞が末梢神経のみならず中枢神経系の神経突起に対しても再生促進的な環境を提供することを明らかにした[17]。

Schwann細胞は通常末梢神経系における髄鞘を形成しているが，神経傷害後においては，傷害部位より末梢側において神経突起との接触を失うことが刺激となって細胞の性質を大きく変え，自律的に増殖しながら傷害された神経突起の伸展を促進すると考えられている。同様に，成体においても再生能を有していると考えられる嗅神経を取り巻くolfactory ensheathing cell(OEC)にもSchwann細胞と同様の神経突起伸展促進能があるものと考えられる。これらのほかにもradial glia，subventricular zone astrocyte，oligodendrocyte precursor cellなどが研究されているが，Schwann細胞とOECに関する報告が最も多い[18]。

Schwann細胞は外科的アクセスの比較的容易な末梢神経から採取でき，purificationや増殖も容易であるなどの点で優れているが，アストロサイトが存在する領域にSchwann細胞を侵入させるのが難しく，また中枢神経の突起をSchwann細胞のgraftを超えて伸展させるのも困難であるとされている。一方，OECは嗅球へのアクセス，OECのpurification，増殖のいずれをとってもSchwann細胞よりも困難であるが，アストロサイトと共存でき，軸索伸展効果も優れていることから移植に用いる細胞として有力視されている[19]。

Ⅳ さいごに

ここに述べた中枢神経の再生促進療法はいまだ動物実験段階のものであるが，複数の方法を組み合わせることによって一層有効性を高めるなどの工夫が行われている。今後，神経軸索再生の効率化とともに，再生した神経軸索の機能的なコネクションの回復をいかに実現するかが重要な課題となっている。

文　献

1) Raisman G：Neuronal plasticity in the septal nuclei of the adult rat. Brain Res **14**：25-48, 1969
2) Low WC, Lewis PR, Bunch ST, et al：Function recovery following neural transplantation of embryonic septal nuclei in adult rats with septohippocampal lesions. Nature **300**：260-262, 1982
3) Caroni P, Schwab ME：Antibody against myelin-associated inhibitor of neurite growth neutralizes nonpermissive substrate properties of CNS white matter. Neuron **1**：85-96, 1988
4) Chen MS, Huber AB, van der Haar ME, et al：Nogo-A is a myelin-associated neurite outgrowth inhibitor and an antigen for monoclonal antibody IN-1. Nature **403**：434-439, 2000
5) Schwab ME：Nogo and axon regeneration. Curr Opin Neurobiol **14**：118-124, 2004
6) Raisman G：Myelin inhibitors；Does NO mean GO？ Nat Rev Neurosci **5**：157-161, 2004
7) Silver J, Miller JH：Regeneration beyond the glial scar. Nat Rev Neurosci **5**：146-156, 2004
8) Barritt AW, Davies M, Marchand F, et al：Chondroitinase ABC promotes sprouting of intact and injured spinal systems after spinal cord injury. J Neurosci **26**：10856-10867, 2006

9) Van Aelst L, D'Souza-Schorey, C : Rho GTPases and signaling networks. Genes Dev **11** : 2295-2322, 1997
10) Burridge K, Wennerberg K : Rho and Rac take center stage. Cell **116** : 167-179, 2004
11) Bishop A, Hall A : Rho GTPases and their effector proteins. Biochem J **348**, 241-255, 2000
12) Mueller BK, Mack H, Teusch N : Rho kinase, a promising drug target for neurological disorders. Nat Rev Drug Discov **4** : 387-398, 2005
13) Jones LL, Oudega M, Bunge MB, et al : Neurotrophic factors, cellular bridges and gene therapy for spinal cord injury. J Physiol **533** : 83-89, 2001
14) Neumann S, Bradke F, Tessier-Lavigne M, et al : Regeneration of sensory axons within the injured spinal cord induced by intraganglionic cAMP elevation. Neuron **34** : 885-893, 2002
15) Qiu J, Cai D, Dai H, et al : Spinal axon regeneration induced by elevation of cyclic AMP. Neuron **34** : 895-903, 2002
16) Lu P, Yang H, Jones LL, et al : Combinatorial therapy with neurotrophins and cAMP promotes axonal regeneration beyond sites of spinal cord injury. J Neurosci **24** : 6402-6409, 2004
17) Aguayo AJ, David S, Bray GM : Influences of the glial environment on the elongation of axons after injury : Transplantation studies in adult rodents. J Exp Biol **95** : 231-240, 1981
18) Reier PJ : Cellular transplantation strategies for spinal cord injury and translational neurobiology. NeuroRx **1** : 424-451, 2004
19) Barnett SC, Riddell JS : Olfactory ensheathing cells (OECs) and the treatment of CNS injury : Advantages and possible caveats. J Anat **204** : 57-67, 2004

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 5, pp. 2957–2966, January 30, 2009

Roles of Meltrin-β/ADAM19 in Progression of Schwann Cell Differentiation and Myelination during Sciatic Nerve Regeneration*[S]

Received for publication, April 25, 2008, and in revised form, November 25, 2008 Published, JBC Papers in Press, December 2, 2008, DOI 10.1074/jbc.M803191200

Shuji Wakatsuki[‡§1], **Norihiro Yumoto**[‡], **Koji Komatsu**[‡], **Toshiyuki Araki**[§], **and Atsuko Sehara-Fujisawa**[‡2]

From the [‡]Department of Growth Regulation, Institute for Frontier Medical Sciences, Kyoto University, Kawahara-cho 53, Shogo-in, Kyoto 606-8507, Japan and the [§]Department of Peripheral Nervous System Research, National Institute of Neuroscience, National Center for Neurology and Psychiatry, 4-1-1 Ogawahigashi-cho, Kodaira 187-8502, Japan

Remyelination is an important aspect of nerve regeneration after nerve injury, but the underlying mechanisms are not fully understood. Here, we show that meltrin-β (ADAM19), a member of the ADAM (a disintegrin and metalloprotease) family, plays crucial roles in nerve regeneration after a crush injury to the sciatic nerves. The expression of meltrin-β was up-regulated in neurons after the crush injury. Morphometrical analysis revealed a delay in remyelination in meltrin-β-deficient nerves, whereas no significant defects were observed in their axon elongation. The activation of Krox-20, an indispensable transcription factor for myelination, was delayed in meltrin-β-deficient nerves and was accompanied by the retarded expression of myelin-related proteins. Expression of Krox-20 in Schwann cells was mediated by Akt. Phosphorylation of Akt but not that of Erks was reduced in regenerating nerves of meltrin-β-deficient mice. The cell membrane fraction prepared from meltrin-β-deficient nerves showed a defective activation of Akt in the membrane-loaded Schwann cells. Meltrin-β-deficient mice exhibited delayed sciatic functional recovery after the nerve crush. Altogether, these results reveal a role of meltrin-β in Schwann cell differentiation and re-myelination in nerve regeneration. Moreover, this study suggests that meltrin-β functions as a modulator of juxtacrine signaling from axons that activate the Akt pathway and the Krox-20 expression, which is the prerequisite for Schwann cell differentiation.

The peripheral nervous system (PNS)[3] is mainly composed of neurons and glial cells. Differentiation of Schwann cells, a group of glial cells that sheathe axons of the peripheral nerves, including sensory and motoneurons, is a multistep process. Immature Schwann cells migrate along axons and then differentiate to promyelin-stage Schwann cells, which sheathe single axons, before differentiation into myelinating Schwann cells (1). Differentiation from immature to promyelin and from promyelin to myelinating Schwann cells requires the POU domain transcription factor Oct-6 (also called suppressed cAMP-inducible protein, SCIP) and the zinc-finger transcription factor Krox-20 (Egr-2), respectively (2–4).

Differentiation of Schwann cells is regulated by various growth factors, such as neuregulin-1 (NRG1), the major ErbB ligand acting as a glial growth factor (5). Receptor-tyrosine kinases, including ErbBs, transmit intracellular signals mainly through extracellular signal-regulated kinases (Erks) and phosphatidylinositol 3-kinase (PI3K)-Akt pathways (6), of which the latter pathway is crucial for initiation of myelination (7, 8). NRG1-ErbB signaling in promyelin and myelinating Schwann cells activates Erks and PI3K-Akt pathways, respectively. These studies reveal the importance of identifying modifiers that alter growth factor signaling, including NRG1, from the Erks- to PI3K-Akt-dependent pathway for the initiation of myelination.

Here, we show that meltrin-β (ADAM19) plays roles in Schwann cell differentiation during regeneration of the sciatic nerves after a crush injury. Meltrin-β is a member of the ADAM (a disintegrin and metalloprotease) family of proteins that contains the active metalloproteases domain. Evidence suggests modulatory roles of ADAM proteases in the ectodomain shedding of various membrane proteins. Kuzbanian/ADAM10 is involved in the ectodomain shedding of multiple substrates, including Notch ligands, ephrins, and cadherins (9–11). Tumor necrosis factor-α-converting enzyme/ADAM17 plays essential roles in the phorbol ester-stimulated ectodomain shedding of various membrane-anchored growth factors, receptors, or adhesion molecules (12). We and Inoue *et al.* identified mouse meltrin-β cDNA previously (13–15). Meltrin-β is highly expressed in the developing PNS and enhances the generation of soluble ligands from membrane-anchored type I NRG1 in cultured neurons (5, 14, 16–18). Although physiological substrates of meltrin-β remain elusive, the protease domain of meltrin-β could, thus, regulate the growth factor signaling required for PNS development. From the structural point of view, meltrin-β and several other ADAM proteins can be classified as subfamily proteins; these proteins contain well conserved dis-

* This work was funded in part by a grant-in-aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science, and Technology, a Research Grant for Nervous and Mental Disorders from the Ministry of Health, Labor, and Welfare, and a Research Grant from Core Research for Evolutional Science and Technology, the Japan Science and Technology Corporation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Data 1 and 2.

[1] A researcher with the 21st Century COE Program, Integration of Transplantation Therapy and Regenerative Medicine at Kyoto University.

[2] To whom correspondence should be addressed. Tel.: 81-75-751-3826; Fax: 81-75-751-4642; E-mail: asehara@frontier.kyoto-u.ac.jp.

[3] The abbreviations used are: PNS, peripheral nervous system; PBS, phosphate-buffered saline; siRNA, small interfering RNA; NRG1, neuregulin-1; Erk, extracellular signal-regulated kinases; PI3K, phosphatidylinositol 3-kinase; RT, reverse transcription; PLSD, protected least significant difference; DAC, days after crush; IGF-I, insulin-like growth factor I; DRG, dorsal root ganglia.

integrin and other cysteine-rich domains, conserved domains originally found in viper venoms, whereas these domains are less conserved in tumor necrosis factor-α-converting enzyme/ADAM17 and Kuzbanian/ADAM10 (13, 19, 20). The functions of disintegrin and cysteine-rich domains of meltrin-β have not been elucidated.

Most meltrin-β-deficient mice died soon after birth, probably due to developmental defects in the heart (19, 21, 22). However, some of meltrin-β-deficient mice carrying a mixed genetic background of 129/Sv and C57BL/6, but not those carrying a single C57BL/6 background, survived to adulthood despite these defects (22). These survivors enabled us to investigate whether meltrin-β participates in the development of the PNS after birth. In this study we found meltrin-β-deficient mice exhibited a delay in remyelination and a prolonged period of hind limb dysfunction after injury. Meltrin-β-deficient nerves showed delayed up-regulation of Krox-20, leading to retarded activation of the genes for myelin-related proteins. Moreover, Akt activation, an essential process for expression of Krox-20 in Schwann cells, is less efficient in lesioned meltrin-β-deficient nerves. These results revealed that meltrin-β functioned as one of the modulators of Schwann cell differentiation from promyelin to myelinating stages during regeneration through the activation of Akt signaling pathway.

EXPERIMENTAL PROCEDURES

Animals—Meltrin-β-deficient mice were generated as previously described (22). Heterozygous mice of the mixed background (129Sv × C57BL6) were crossed, and the offspring were genotyped by PCR. No gross abnormalities were observed in wild type and heterozygous littermates on overall development, growth characteristics, and histology (22); therefore, littermates with wild type genotypes were used as controls throughout this study. All animals were maintained in accordance with the guidelines of Kyoto University. The technical protocols for animal experiments in this study were approved by a review committee of the Institute for Frontier Medical Sciences, Kyoto University.

Antibodies—The antibodies used and their sources were as follows: rabbit polyclonal anti-C terminus of meltrin-β antibody (16), rabbit polyclonal anti-Akt and anti-phospho-Akt antibodies (9272, 9271, Cell Signaling Technology), mouse monoclonal anti-Erk1 and anti-Erk2 antibodies (610030, 610103, BD Biosciences), rabbit polyclonal anti-phospho-Erk1/2 antibody (9101S, Cell Signaling), rabbit polyclonal anti-Krox-20 antibody (PRB-236P, Covance), goat polyclonal anti-Oct-6 antibody (sc-11661, Santa Cruz), rat monoclonal anti-F4/80 antibody (clone Ab-3, Serotec), rabbit polyclonal anti-myelin basic protein antibody (A0623, Dako), rabbit polyclonal anti-GAP-43 antibody (AB5220, Chemicon), mouse monoclonal anti-neurofilament 160 antibody (clone NN18, Sigma), and horseradish peroxidase-conjugated secondary antibodies (Vector Laboratories).

Immunoblotting and Histology—Several days after surgery, distal portions of the sciatic nerve from the crush-lesioned or unlesioned side were homogenized in radioimmune precipitation assay buffer (1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5). Twenty micrograms of sciatic nerve lysates were analyzed by SDS-PAGE followed by immunoblotting. Immunoblots were developed with a horseradish peroxidase-conjugated secondary antibody and detected by enhanced chemiluminescence. The intensity was determined by laser densitometry of immunoblots using a densitometer and ImageQuant software (GE Healthcare). Scans at multiple exposures were obtained to ensure that the results fell within the linear range of the instrument.

For immunohistochemical analyses, sciatic nerves and lumbar DRG were dissected at various days after the nerve crush, flash-frozen in optimal cutting temperature compound, and cryosectioned at 8 μm. After preparation, sections were fixed with ice-cold acetone and immunostained using antibodies against NF160, GAP-43, and meltrin-β. For the staining with antibodies against Krox-20 and Oct-6, sections were fixed with 4% paraformaldehyde in PBS. For toluidine blue staining, sciatic nerves were postfixed overnight in PBS containing 2% glutaraldehyde and then dehydrated and embedded in plastic for microtome sectioning. For quantification of immunostained nerves, longitudinal cyrosections (10 μm) of uninjured or injured nerves at 7, 12, and 21 days after crush were prepared from wild type and mutant mice. Each section was immunostained with antibodies against transcription factors Oct-6 and Krox-20 and counterstained with 4′,6-diamidino-2-phenylindole. Photographs were taken from three wild type and three mutant nerves, and immunostained nuclei were counted with reference to the 4′,6-diamidino-2-phenylindole-stained signals.

Membrane Preparation and Analysis of Akt or Erks Activation in Schwann Cells—DRG neurons from 35-mm dish cultures were washed twice with ice-cold PBS and collected with fine forceps and homogenized with 20 strokes of a 0.2-ml micro tissue grinder (Wheaton, Millville, NJ) in 150 μl of ice-cold PBS. The volume was brought up to 1.5 ml with ice-cold PBS, and the homogenate was centrifuged (100 × *g*, 20 min, 4 °C) to remove debris. The supernatant, diluted up to 3 ml, was then centrifuged at 35,000 × *g* for 1 h at 4 °C. The supernatant was discarded, and the pellet was resuspended in culture media by vortexing. To analyze the activation levels of either Akt or Erks in Schwann cells, the membrane fraction prepared from either wild type or meltrin-β-deficient neurons was loaded onto Schwann cell cultures, and after 20 min cell lysates were prepared, blotted, and probed for phospho-Akt, total Akt, phospho-Erk, and total Erks as described above. Conditioned media were prepared as previously described (23). Briefly, conditioned media from ~12 cultures each of wild type and meltrin-β-deficient DRG were collected and concentrated 100-fold. Concentrated media were diluted 10-fold with Dulbecco's modified Eagle's medium and exposed to the Schwann cell for 20 min. Schwann cell lysates were prepared, blotted, and analyzed described above.

Quantitative Reverse Transcription (RT)-PCR—Total RNA was extracted from distal parts of sciatic nerves at various days after crush injury or cultured Schwann cells using the RNeasy MiniKit (Qiagen, Basel, Switzerland). RNA samples were further purified by DNase digestion and extraction with phenol and chloroform and subjected to RT-PCR. Briefly, 0.5 μg of

total RNA was converted to cDNA using Superscript II (Invitrogen) in the presence of random hexamer primers. The mRNAs for Krox-20, P0, and myelin basic protein were measured by real-time quantitative RT-PCR using the Applied Biosystems prism model 7700 sequence detection instrument. The mRNA levels were corrected for glyceraldehyde-3-phosphate dehydrogenase.

Transfection of siRNA—For transfection of siRNA, control or Akt targeting siRNA were electroporated into primary Schwann cells using the nucleofector mouse embryo fibroblast mixture 1 (program T20; Amaxa Biosystems) and then plated onto 6-well culture plates (100,000 cells per well). The siRNA effect was analyzed by immunoblotting 48 h after transfection. All the siRNAs used in this study were purchased from Qiagen (negative control siRNA, catalog no. 1033076; Akt targeting siRNAs, catalog no. 1022427 and SI02652419). RNA samples were prepared from Schwann cells and analyzed as described above.

Sciatic Nerve Crush—Mice were anesthetized by intraperitoneal injection of 2.5% avertin. The sciatic nerve was exposed at the mid-thigh region and crushed for 30 s using Dumont no. 5 forceps. This was repeated at the same site to completely sever the axons.

Quantitation of Degenerating and Myelinated Axons—For the assessment of the extent of axonal degeneration and regeneration, we measured numbers of degenerating and myelinated axons. Toluidine blue-stained sections prepared from at least five control mice and five mutant mice were photographed and 10,000 μm^2 areas were chosen at random for analysis. Irregular and darkly stained myelin profiles were classified as degenerating myelin sheaths. Myelin sheaths that wrap axons to a greater or lesser extent were counted as myelinated.

Tracing of DRG Neurons and Motoneurons—Crystals of Fluorogold (Molecular Probes) were applied onto the cut nerve 7 mm distal to the crush lesion. After 48 h mice were deeply anesthetized with pentobarbital and perfused with 4% paraformaldehyde. Spinal cords and DRG were removed and processed for cryosectioning. For counting DRG neurons, all experiments were evaluated using cresyl violet staining for serial sections and Fluorogold-labeled large cells with abundant cytoplasm and prominent nucleolus in each section were counted. Thus, the number of neurons can be counted based on the number of nucleolus in Fluorogold-labeled cells. Numbers of motoneurons were counted similarly using these criteria.

Behavior Analysis—At various day points after unilateral crush injury, mice were tested for recovery of hind limb function on the crushed side by measuring toe spread and the ability to grip an inverted wire screen (24, 25). For the outer toe spread assay, the hind feet of the mice were painted with black pigment ink; the mice were then allowed to walk freely on a blank sheet of paper. The distance between the first and fifth digits (outer toe spread) was measured. For the grip assay mice were placed on a wire screen mesh, which was then turned over to test the ability of the mice to grip the screen correctly with their hind paws. The test was repeated 10 times for each mouse, and the number of failed trials was recorded.

Statistical Analysis and Ethical Considerations—Results were expressed as the mean ± S.E. Differences between groups were examined for statistical significance using Fischer's protected least significant difference (PLSD) test. $p < 0.05$ denoted the presence of a statistically significant difference.

RESULTS

Expression of Meltrin-β (ADAM19) in Peripheral Neurons after Crush Injury—To investigate whether meltrin-β participates in the development of the PNS after birth, heterozygous mice of the mixed background of 129/Sv and C57BL/6 were crossed, and the meltrin-β-deficient mice were generated. Approximately 10–15% of the meltrin-β-deficient mice survived to adulthood despite the heart defects (22). These survivors enabled us to investigate roles of meltrin-β in the development of the PNS after birth. Although the numbers of axons in the meltrin-β-deficient mice were slightly lower than those in the wild type mice (about 77% at L5 level), both myelinating and non-myelinating axons were normal in appearance. The *g*-ratio of myelinating axons in the meltrin-β-deficient mice was similar to that in the wild type mice. IB4-positive, calcitonin gene related peptide-positive, and parvalbumin-positive neurons of meltrin-β-deficient mice were more or less equally reduced in number compared with those of wild type mice, suggesting that the smaller number of axons in the sciatic nerves is not due to defects in the formation of certain types of neurons. Meltrin-β-deficient mice showed no abnormality in walking or in sensing heat or touch (data not shown).

We adopted another approach to explore the roles of meltrin-β in PNS, hypothesizing that the roles of meltrin-β might be elucidated by examining the regeneration processes after the sciatic nerve crush, which is a well established paradigm for nerve regeneration. We examined the expression of meltrin-β before and after the nerve crush by Western blotting and immunohistochemical analyses (Fig. 1). Meltrin-β protein was scarcely detectable in uninjured DRG neurons. Several days after the sciatic nerve crush at the mid-femoral level, meltrin-β expression increased strongly in L3-L5 DRG neurons (Fig. 1, *C* and *G*) and motor neurons (data not shown). GAP-43, which is a highly specific marker for regenerating peripheral neurons including DRG neurons (Fig. 1, *E* and *I*) (26), was induced similarly after the sciatic nerve crush. Thus, expression of meltrin-β is up-regulated after the sciatic nerve crush at the early phase of regeneration and persists at least for 2 weeks.

Decreased Myelination in the Early Stage of Regeneration after Sciatic Nerve Crush in Meltrin-β-deficient Mice—To understand the roles of meltrin-β in nerve regeneration processes, we first examined whether the absence of meltrin-β affected nerve regeneration at the morphological level. The sciatic nerves of wild type and meltrin-β-deficient animals were crush-lesioned at the mid-femoral level, and the extent of morphological degeneration and regeneration was assessed by serial sectioning and microscopic analyses of the lesioned nerves. After the nerve damage, self-destruction of axons or Wallerian degeneration (27) was observed in the nerve distal to the lesion, resulting in loss of axon-Schwann cell contact. Five days after the nerve injury, demyelinating features were prominent in the part distal to the lesion in both wild type and mutant mice nerves (Fig. 2*A*). There was no significant differ-

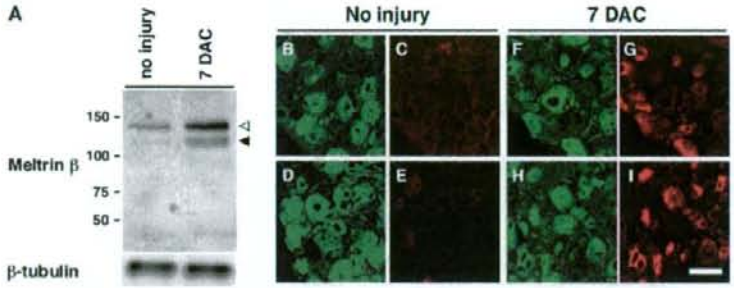


FIGURE 1. **Induction of meltrin-β in DRG by sciatic nerve crush.** *A*, an immunoblot for meltrin-β protein that indicates its up-regulation in adult L3-L5 DRG at 7 days after nerve crush. *Open* and *filled arrowheads* indicate ~120-kDa prodomain-bearing and ~100-kDa prodomain-lacking, active forms of meltrin-β, respectively. The active form of meltrin-β is scarcely detectable in uninjured samples. Molecular weight markers are shown at the *left* (*upper panel*). The expression of β-tubulin protein was used as a control for protein loading (*lower panel*). *B–I*, immunohistochemical analysis that demonstrates activation of meltrin-β in DRG neurons after sciatic nerve crush. DRG sections before (*B–E*) and 7 DAC (*F–I*) were double-stained with anti-neurofilament 160 (*green*) and anti-meltrin-β (*red*) antibodies (*B*, *C*, *F*, and *G*) or with anti-neurofilament 160 (*green*) and anti-GAP-43 (*red*) antibodies (*D*, *E*, *H*, and *I*). Meltrin-β is activated similarly to GAP-43, a specific marker for regenerating peripheral neurons. *Scale bar*, 50 μm.

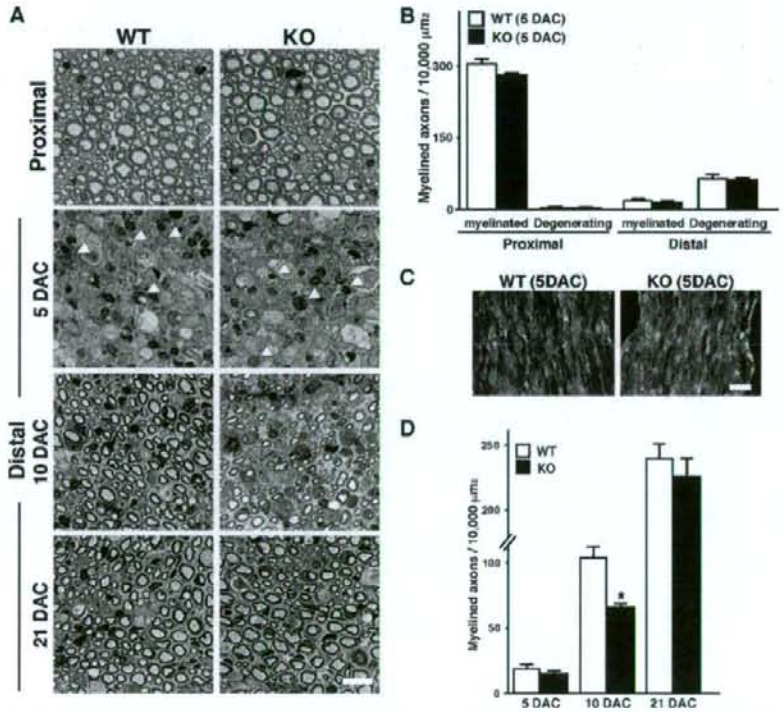


FIGURE 2. **Decreased myelination in the early stage of regeneration after sciatic nerve crush in meltrin-β-deficient mice.** *A*, semi-thin toluidine-blue-stained sections of sciatic nerves 3 mm proximal or distal to the lesion at 5, 10, and 21 DAC. Degenerating profiles from wild type (*WT*) and meltrin-β-deficient (*KO*) mice on panels of five DACs are indicated with *arrowheads*. The number of myelinating nerves in meltrin-β KO mice was less than that in wild type mice at 10 DAC, whereas similar numbers of myelinated nerves were found in wild type and meltrin-β KO mice at 21 DAC. *Scale bar*, 25 μm. *B*, the densities of myelinated fibers and degenerating axon profiles were measured in wild type (*open bars*) and meltrin-β-deficient nerves (*filled bars*) 3 mm proximal or distal to crushed sites at 5 days after the injury. Degeneration occurred similarly in the nerves of both genotypes. The mean ± S.E. obtained with five wild type and five mutant mice are represented. *C*, immunostaining of invaded macrophages using an antibody against F4/80 in the lesioned nerves. Macrophages invaded into injured nerves similarly in wild type and mutant-deficient mice. *Scale bar*, 100 μm. *D*, the densities of myelinated axon profiles were measured in wild type and mutant-deficient nerves 3 mm distal to crushed sites at 5, 10, and 21 days after crush injury. *Scale bar*, 25 μm.

ence between the numbers of myelinated (intact) or demyelinated (degenerating) axons in the sciatic nerve of the wild type and mutant mice groups 5 days after crush (DAC) (Fig. 2*B*). Immunochemical analyses of the markers for macrophages, F4/80 (Fig. 2*C*) and MOMA-2 (data not shown) showed similar profiles between each genotype, suggesting proper invasion of macrophages into the lesioned sites in meltrin-β-deficient mice. In addition, many myelin ovoids that had not been cleared by macrophages and dark-stained macrophages with phagocytosed myelin were prominent in most areas of the cross-sections prepared from the lesioned mice of both genotypes (Fig. 2*A*, *arrowhead*). These results suggest that meltrin-β is dispensable for nerve degeneration ahead of regeneration.

After demyelination, remyelination starts about a week after the crush (7 DAC) in wild type mice. Then, the progression of myelination during the second and third weeks results in the regeneration of nerves with thick myelin sheath. Fig. 2, *A* and *D*, show representative results in which wild type and meltrin-β-deficient nerves were compared on 10 DAC (the early myelinating stage) and 21 DAC (the late myelinating stage). Ten days after crush many myelinating nerve fibers were already found in wild type mice, indicating their active regeneration. In contrast, only a few myelinated fibers and various degrees of myelination were observed in the distal segments from mutant mice, indicating that myelination of regenerating fibers was much less advanced at the early stage of regeneration in meltrin-β-deficient mice. However, the number of myelinated axons gradually increased at the late stage of regeneration in these mice, and similar numbers of myelinated axons were found in wild type and meltrin-β-deficient mice 3 weeks after the injury (Fig. 2, *A* and *D*). Overall, these results indicated that nerve regeneration is indeed affected in