

REVIEW

# Wld<sup>S</sup>, Nmnats and axon degeneration—progress in the past two decades

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## ABSTRACT

A chimeric protein called Wallerian degeneration slow (Wld<sup>S</sup>) was first discovered in a spontaneous mutant strain of mice that exhibited delayed Wallerian degeneration. This provides a useful tool in elucidating the mechanisms of axon degeneration. Over-expression of Wld<sup>S</sup> attenuates the axon degeneration that is associated with several neurodegenerative disease models, suggesting a new logic for developing a potential protective strategy. At molecular level, although Wld<sup>S</sup> is a fusion protein, the nicotinamide mononucleotide adenylyl transferase 1 (Nmnat1) is required and sufficient for the protective effects of Wld<sup>S</sup>, indicating a critical role of NAD biosynthesis and perhaps energy metabolism in axon degeneration. These findings challenge the proposed model in which axon degeneration is operated by an active programmed process and thus may have important implication in understanding the mechanisms of neurodegeneration. In this review, we will summarize these recent findings and discuss their relevance to the mechanisms of axon degeneration.

**KEYWORDS** axon degeneration, Wallerian degeneration, Wld<sup>S</sup>, NAD, neurodegenerative diseases

## Wld<sup>S</sup> AS A MODEL TO STUDY THE MECHANISMS OF AXON DEGENERATION

Axon degeneration is a common pathological event that is associated with different types of pathological conditions such as acute trauma, toxic insults and chronic neurodegenerative diseases (Raff et al., 2002; Coleman, 2005; Hilliard, 2008).

The degeneration of an axon separates the neuronal soma from its synaptic targets, which disrupts neuronal function. Thus, understanding the underlying mechanisms of axon degeneration may facilitate the development of novel protective strategies.

A simple model in studying the mechanisms of axon degeneration is the Wallerian degeneration, a self-destructive process observed at the distal portion of a transected axon (Waller, 1850). In vertebrates, the distal portion of a severed axon remains viable and conducts action potentials *in vivo* for up to a few days, after which they undergo a rapid structural destruction where the axolemma and axonal cytoskeleton are dismantled (Raff et al., 2002; Coleman, 2005; Luo and O'Leary, 2005). Morphologically, such a degenerative process is characterized by a beading appearance followed by granular disintegration of the axons. In addition, initial studies suggested that the axons undergoing Wallerian degeneration do not seem to possess detectable activation of the caspase 3 protease (Finn et al., 2000), suggesting that Wallerian degeneration and apoptosis may represent two distinct self-destruction programs. However, this concept has been challenged by recent observations that suggest the involvement of other types of caspases in axon degeneration (Nikolaev et al., 2009).

An important landmark is the discovery of a spontaneous mutant mouse strain, C57BL/Wld<sup>S</sup>, whose axons survived for as long as weeks after transection (Lunn et al., 1989; Glass et al., 1993). Genetic analysis suggests that the slow Wallerian degeneration phenotype can be attributed to a dominant mutation that acts intrinsically in neurons (Perry et al., 1990; Deckwerth and Johnson, 1994; Ferri et al., 2003). By *in vivo* imaging methods, Kerschensteiner et al. also reported that after axotomy, the proximal ends of severed

axons undergo a rapid retrograde (dying-back) degeneration, which could be efficiently protected in *Wld<sup>S</sup>* mice (Kerschens- steiner et al., 2005). *In vivo* protective effect of the *Wld<sup>S</sup>* protein has also been demonstrated in different species. For example, it was also found to protect physically injured axons in transgenic rats (Adalbert et al., 2005), as well as surgically severed *Drosophila* olfactory receptor neurons (ORN) (Hoop- fer et al., 2006; MacDonald et al., 2006). In addition, the *Wld<sup>S</sup>* is able to protect axon degeneration in other disease models (reviewed by Coleman et al., 2005, also see below). These studies suggest that these different types of axon degenera- tion may share similar mechanisms that might be relevant to *Wld<sup>S</sup>*.

### AXON DEGENERATION, *Wld<sup>S</sup>* AND NEURODEGENERATIVE DISEASE

It has become clear that many types of neurodegenerative diseases involve major synapse and axon losses before the appearance of symptoms, and even before the loss of neurons (Raff et al., 2002; Bjartmar et al., 2003; Saxena and Caroni, 2007). A variety of neurological disorders, including amyotrophic lateral sclerosis (ALS), spinal muscular atrophy, spinocerebellar disorders, peripheral neuropathies, nutritional neurological disorders, various intoxications and AIDS, are characterized by initial degeneration of the distal regions of long axons (Luo and O'Leary, 2005). Preventing the axon degeneration but not the apoptosis of the motoneuron cell body was able to rescue the symptoms and extends life span in *p<sub>mn</sub>* mice, a mouse model of motoneuron disease (Sagot et al., 1995; Sagot et al., 1998; Ferri et al., 2003). Clinical symptoms in the SOD1 G93A model of ALS result specifically from damage to the distal motor axon and not from activation of the death pathway (Gould et al., 2006). Therefore, it is intriguing to prevent the loss of axon and dendrites for therapeutic treatment of these neurodegenerative diseases.

Does *Wld<sup>S</sup>* protect these different forms of axon degenera- tion? *In vitro*, *Wld<sup>S</sup>* protein can be neuroprotective against neuropathy that is induced by vincristine or Taxol (Wang et al., 2001a, b, 2002). In disease models, *Wld<sup>S</sup>* mice show resistance to the 6-hydroxydopamine-induced dopamine fiber loss in Parkinson disease (Sajadi et al., 2004) or the MPTP-induced nigrostriatal axon degeneration (Hasbani and O'Malley, 2006). In addition, the protective roles of *Wld<sup>S</sup>* have been observed in mouse models of different neurodegenera- tive diseases, including progressive motor neuropathy (*p<sub>mn</sub>* mice) (Ferri et al., 2003), multiple sclerosis (experimental autoimmune encephalomyelitis) (Kaneko et al., 2006), myelin-related axonopathy (myelin protein zero null mutants) (Samsam et al., 2003), gracile axonal dystrophy (*gad* mutant mice) (Mi et al., 2005) and glaucoma (Beirowski et al., 2008). However, *Wld<sup>S</sup>* fails to protect axon degeneration in other degenerative disease models, such as the SOD1 transgenic

mouse model of ALS (Vande Velde et al., 2004; Fischer et al., 2005), the proteolipid protein (Plp) null animal model of hereditary spastic paraplegia, an spinal muscular atrophy model (Kariya et al., 2009) and prion infected models (Gultner et al., 2009). On the other hand, *Wld<sup>S</sup>* also cannot prevent developmentally regulated axonal pruning in both *Drosophila* and mice (Hoopfer et al., 2006), suggesting the existence of *Wld<sup>S</sup>*-independent pathways (Hilliard, 2008).

### MOLECULAR MECHANISMS OF *Wld<sup>S</sup>*-MEDIATED PROTECTION

Genetic studies showed that the slow Wallerian degeneration phenotype in the *Wld<sup>S</sup>* mice is due to the over-expression of an fusion protein *Wld<sup>S</sup>* that consists of the amino-terminal 70 amino acids of UFD2, which is an evolutionally conserved protein involved in protein poly-ubiquitination (Koepl et al., 1999), and the nicotinamide mononucleotide adenylyltrans- ferase (*Nm<sub>nat</sub>1*) (Conforti et al., 2000). Initially, there was some controversy regarding whether individual fragments of the *Wld<sup>S</sup>* gene (UFD2 or *Nm<sub>nat</sub>1*), or the entire chimeric gene, are responsible for the phenotypes (Araki et al., 2004; Wang et al., 2005; Zhai et al., 2006; Conforti et al., 2007). In cultured neurons, we and others found that while inhibiting the ubiquitin proteasome system activity can slow down Wallerian degeneration (Zhai et al., 2003; MacInnis and Campenot, 2005), over-expressing *Nm<sub>nat</sub>1* alone could mimic the protective effects of *Wld<sup>S</sup>* (Araki et al., 2004; Wang et al., 2005; Sasaki et al., 2006). Such protective effects could be mimicked by exogenously provided NAD or its biosynthetic precursors such as nicotinamide or nicotinamide riboside (Araki et al., 2004; Wang et al., 2005; Sasaki et al., 2006), suggesting that *Wld<sup>S</sup>/Nm<sub>nat</sub>1* may act, at least partially, through NAD biosynthesis. Similarly, independent studies also showed that *Nm<sub>nat</sub>1* could protect axon degeneration in *Drosophila*-based *in vivo* models (MacDonald et al., 2006; Zhai et al., 2006).

However, a study reported that over-expression of *Nm<sub>nat</sub>1* in transgenic mice model failed to protect Wallerian degeneration of sciatic nerves *in vivo* (Conforti et al., 2007). The exact mechanisms for these different results are unclear. It is possible that the differences in both the expression levels and protein localizations of the over-expressed *Nm<sub>nat</sub>1* vs. *Wld<sup>S</sup>* contribute to these discrepancies due to the nature of these gain-of-function studies. For example, while endogen- ous *Nm<sub>nat</sub>1* is a nuclear protein (Emanuelli et al., 2003), some of the over-expressed *Wld<sup>S</sup>/Nm<sub>nat</sub>1* protein can be detected in the axons of cultured neurons (Wang et al., 2005; Zhai et al., 2006). Perhaps the *Nm<sub>nat</sub>1* expressed in transgenic mice generated by Conforti (Conforti et al., 2007) did not reach a high level and still primarily localized in the nucleus; therefore, insufficient amounts of *Nm<sub>nat</sub>1* were available in the axons for the protective effects. In addition, although many reports suggest that *Wld<sup>S</sup>* is localized to the

nucleus of neurons in the Wld<sup>S</sup> mice (Coleman, 2005), all of these studies relied on an antibody prepared against an 18-amino acid peptide in Wld<sup>S</sup> (between UFD2 and Nmnat1) (Conforti et al., 2000; Fang et al., 2005). UFD2 itself localizes to both the nucleus and axons (Fang et al., 2005), and thus the fusion protein Wld<sup>S</sup> might be brought to axons by the UFD2 fragment. In support of this hypothesis, recent genetic studies demonstrated that a mutant form of Wld<sup>S</sup> with mutations at its nuclear localization signal (thus leading to increased cytoplasmic localization of the protein) has dramatically increased protective effects on Wallerian degeneration (Beirowski et al., 2009), which substantiates our finding that non-nuclear Wld<sup>S</sup> determines its neuroprotective efficacy for axons (Wang et al., 2005). Furthermore, more recent studies revealed the existence of the Wld<sup>S</sup> protein in axon in Wld<sup>S</sup> mice *in vivo*, likely in the mitochondria (Yahata

et al., 2009). It appears that such cytoplasmically localized Wld<sup>S</sup> shows protective effects on axon degeneration. Various mutants of Wld<sup>S</sup> in axon degeneration are summarized in Table 1.

Recent structure-function studies suggested that N70 and/or W18 portion of Wld<sup>S</sup> is likely to contribute for axonal protection (Watanabe et al., 2007). Wld<sup>S</sup> directly binds valosin-containing protein (VCP)/TER94 and is partially relocated it in the nucleus through N70 (Laser et al., 2006; Avery et al., 2009). N70 binds VCP through a VCP-binding motif in its N-terminal 16 amino acids (Laser et al., 2006). Knocking down TER94 by RNAi reduced the axon-protective effects of Wld<sup>S</sup> to levels indistinguishable from Nmnat1 and Wld<sup>S-ΔN16</sup>. Removing the N-terminal 16 amino acids in N70 portion of Wld<sup>S</sup> (Wld<sup>S-ΔN16</sup>) decreased axon protection in transgenic mice and also in *Drosophila* (Avery et al., 2009;

**Table 1** Mutants of Wld<sup>S</sup> in axon degeneration

| gene                         | species | localization                                                                                            | models                       | type of injury | effectiveness                                                     | literature               |
|------------------------------|---------|---------------------------------------------------------------------------------------------------------|------------------------------|----------------|-------------------------------------------------------------------|--------------------------|
| Wld <sup>S</sup> (W258A)     | mice    | NT <sup>a</sup>                                                                                         | DRG                          | transection    | N <sup>b</sup>                                                    | (Araki et al., 2004)     |
|                              | mice    | as Wld <sup>S</sup> mice, nucleus, mitochondria, other fractions as well                                | transgenic mice              | transection    | N                                                                 | (Yahata et al., 2009)    |
|                              | mice    | nucleus                                                                                                 | transgenic mice              | transection    | N                                                                 | (Conforti et al., 2009)  |
| Wld <sup>S</sup> (F28A)      | mice    | nucleus                                                                                                 | SCG from transgenic mice     | vincristine    | N                                                                 | (Conforti et al., 2009)  |
|                              | mice    | nucleus                                                                                                 | SCG                          | transection    | N                                                                 | (Jia et al., 2007)       |
| Wld <sup>S-dead</sup> (H24A) | mice    | nucleus                                                                                                 | transgenic <i>Drosophila</i> | transection    | W, dramatically reduced                                           | (Avery et al., 2009)     |
| ΔNLS-Wld <sup>S</sup>        | mice    | cytoplasm                                                                                               | transgenic mice              | transection    | Y, 14 days after injury, stronger than wild-type Wld <sup>S</sup> | (Beirowski et al., 2009) |
| ΔN16-Wld <sup>S</sup>        | mice    | without amino acids 2–16, cytoplasm                                                                     | transgenic mice              | transection    | N                                                                 | (Conforti et al., 2009)  |
|                              | mice    | cytoplasm                                                                                               | SCG from transgenic mice     | vincristine    | N                                                                 | (Conforti et al., 2009)  |
| Wld <sup>S-ΔN16</sup>        | mice    | Wld <sup>S</sup> with a deletion of the first 16 amino acids, nucleus                                   | transgenic <i>Drosophila</i> | transection    | W, significantly reduced                                          | (Avery et al., 2009)     |
| ATX3-Wld <sup>S</sup>        | mice    | replaced the Wld <sup>S</sup> VCP-binding domain with an ataxin-3-derived VCP-binding sequence, nucleus | transgenic mice              | transection    | Y, similar to Wld <sup>S</sup>                                    | (Conforti et al., 2009)  |
|                              | mice    | nucleus                                                                                                 | SCG from transgenic mice     | vincristine    | Y, similar to Wld <sup>S</sup>                                    | (Conforti et al., 2009)  |

<sup>a</sup> NT, Not tested; <sup>b</sup> Y, Yes, have protective function; W, Weak protection; N, No protective function.

Conforti et al., 2009). Replacing the *Wld<sup>S</sup>* VCP-binding domain with an alternative ataxin-3-derived VCP-binding sequence restores its protective function (Conforti et al., 2009). N16-Nmnat1, a fusion of N16 to full-length Nmnat1, robustly increased the ability of Nmnat1 to protect severed *Drosophila* ORN axons from degeneration *in vivo* (Avery et al., 2009).

How N16-VCP/TRE94 interaction modulates axon degeneration is still an open question. A possible mechanism is related to the ubiquitin-proteasome system (UPS). It has been reported that the UPS is intrinsically required for both developmental and injury-induced axon degeneration (Zhai et al., 2003; Hoopfer et al., 2006). In cultured neurons, inhibiting the activity of the UPS can slow down Wallerian degeneration (Zhai et al., 2003). It was reported that VCP binds Npl4 and Ufd2 to target proteins for proteasomal degradation, or bind Ufd1 and Plap to release refolded proteins into the cytoplasm (Halawani and Latterich, 2006). Knocking down the *Drosophila* orthologue of above genes failed to suppress the ability of *Wld<sup>S</sup>* to protect degenerated axons (Avery et al., 2009). Another possible model is that N16-VCP interaction allows the Nmnat1 protein to relocate to cytoplasmic compartment.

## Nmnats AND AXON DEGENERATION

The requirement of Nmnat1 for the protective effect of *Wld<sup>S</sup>* prompts further analysis of this NAD synthetic enzyme. The Nmnat enzymes catalyze the last step in three major NAD biosynthesis pathways in both prokaryotes and eukaryotes: the *de novo*, salvage, and Preiss-Handler-independent pathway (Sasaki et al., 2006, 2009b). Three Nmnat isoforms have been identified in mammals and they differ in subcellular localization and tissue distribution (Emanuelli et al., 2001; Zhang et al., 2003; Yalowitz et al., 2004). While endogenous Nmnat1 is localized exclusively to nucleus (Schweiger et al., 2001; Zhang et al., 2003), Nmnat2 is mainly expressed in Golgi complex and Nmnat3 is in mitochondria (Berger et al., 2005). In terms of tissue distribution, Nmnat1 appears to be almost ubiquitously expressed while Nmnat2 and Nmnat3 exhibit rather specific tissue distributions. For example, Nmnat2 is predominantly expressed in brain and weakly expressed in heart and skeletal muscle (Emanuelli et al., 2001; Raffaelli et al., 2002; Berger et al., 2005). Specifically, Nmnat2 is highly expressed in cerebral cortex and detectable in midbrain, cerebellum, olfactory bulb, striatum and hippocampus (Yan et al., 2009). On the other hand, mitochondrial Nmnat3 is strongly expressed in kidney, lung and spleen, where Nmnat2 expression is low (Lau et al., 2009).

The protective effects of different Nmnat isoforms on axon degeneration was intensively analyzed (Table 2). Over-expression of Nmnat1 delayed axon degeneration after axotomy in primary dorsal root ganglia (DRG) cultures (Araki et al., 2004; Wang et al., 2005). Nmnat1 also prevented axon degeneration caused by exposure to vincristine in

cultured DRG (Araki et al., 2004; Press and Milbrandt, 2008) and differentiated Neuro2A cells (Watanabe et al., 2007). Axon damage induced by rotenone, a mitochondria complex I inhibitor, was inhibited by Nmnat1 as well (Press and Milbrandt, 2008). It is important to note that although the endogenous Nmnat1 is a nuclear protein, over-expressed Nmnat1 is detected in the cytoplasmic compartments and axons. Because NAD or nicotinamide, a NAD synthetic precursor, could protect axon degeneration in the axonal preparation without cell bodies (Wang et al., 2005), it is likely that such axonal and perhaps mitochondrial localized Nmnat1 functions in protecting axon degeneration.

Our recent research revealed that Nmnat2 mRNA level was remarkably decreased in hippocampus of aged APPswe/PS1dE9 transgenic mice (Yan et al., 2009), a model for Alzheimer's disease (Morrissette et al., 2009). Over-expression of human Nmnat2 was reported to protect the axons from degeneration in transected cultured SCGs (Yan et al., 2009), while over-expression of mouse Nmnat2 failed to suppress axon degeneration in transected *Drosophila* ORN (Avery et al., 2009).

Similar to Nmnat1, Nmnat3 was also reported to protect axon against mitochondrial dysfunction induced by a variety of insults like injury or chemicals in culture DRG (Sasaki et al., 2006; Press and Milbrandt, 2008) (Table 2). Nmnat3 had increased protection compared to Nmnat1 in DRG (Sasaki et al., 2006) and transgenic *Drosophila* (Avery et al., 2009). Interestingly, Nmnat3 transgenic mice showed robust protection as *Wld<sup>S</sup>* (Yahata et al., 2009), again supporting the protective effects of non-nuclear Nmnat and *Wld<sup>S</sup>* proteins.

Furthermore, Nmnat-mediated axonal protection is evolutionarily conserved. Nmnat enzymes with diverse sequences and structures from various species, including *Drosophila*, yeast, and archaeobacterium (Lau et al., 2009), all mediate robust axonal protection in cultured mouse DRG (Sasaki et al., 2009b).

## NAD SYNTHESIS PATHWAY AND AXON DEGENERATION

Despite a lot of efforts, how increased Nmnat activity influences axon degeneration is not entirely known. The total content of NAD was not significantly altered in *Wld<sup>S</sup>* transgenic mice (Mack et al., 2001) and the steady state NAD levels did not change in the PrP-cytNmnat1 transgenic mice as well (Sasaki et al., 2009a). *In vitro* cultured DRG study showed that neither *Wld<sup>S</sup>* nor Nmnat1 significantly increased neuronal NAD levels in intact neurons (Araki et al., 2004; Wang et al., 2005; Sasaki et al., 2006). However, by measuring NAD levels in degenerating axons, Wang et al. found a rapid NAD depletion in degenerating axons and this occurs prior to the development of degeneration signs, suggesting that NAD depletion is an early event in the process of axon degeneration (Wang et al., 2005). Importantly, both

**Table 2** Nmnats and axon degeneration

| gene          | species | localization                                   | models                 | type of injury                                                             | effectiveness                                   | citation                                    |
|---------------|---------|------------------------------------------------|------------------------|----------------------------------------------------------------------------|-------------------------------------------------|---------------------------------------------|
| Nmnat1        | mice    | nucleus                                        | DRG                    | transection                                                                | Y <sup>a</sup> , comparable to Wld <sup>S</sup> | (Araki et al., 2004)                        |
|               | mice    | nucleus                                        | DRG                    | vincristine                                                                | Y, up to 9 days                                 | (Araki et al., 2004)                        |
|               | mice    | NT <sup>b</sup>                                | DRG                    | transection                                                                | Y, up to 5 days                                 | (Wang et al., 2001b)                        |
|               | mice    | nucleus                                        | DRG                    | transection                                                                | Y, > 72 h                                       | (Sasaki et al., 2006; Sasaki et al., 2009a) |
|               | mice    | NT                                             | Neuro2A cell           | vincristine                                                                | Y, but weaker than Wld <sup>S</sup>             | (Watanabe et al., 2007)                     |
|               | mice    | nucleus                                        | transgenic mice        | transection                                                                | N                                               | (Conforti et al., 2007)                     |
|               | mice    | nucleus                                        | transgenic primary DRG | transection                                                                | N                                               | (Conforti et al., 2007)                     |
|               | mice    | nucleus                                        | DRG                    | vincristine                                                                | W                                               | (Conforti et al., 2007)                     |
|               | mice    | nucleus                                        | transgenic mice        | transection                                                                | N                                               | (Yahata et al., 2009)                       |
|               | mice    | NT                                             | DRG                    | rotenone                                                                   | Y, > 72 h                                       | (Press and Milbrandt, 2008)                 |
|               | mice    | NT                                             | DRG                    | transection                                                                | Y                                               | (MacDonald et al., 2006)                    |
| Nmnat3        | mice    | nucleus                                        | transgenic Drosophila  | transection                                                                | Y, less effective than Wld <sup>S</sup>         | (Avery et al., 2009)                        |
|               | mice    | mitochondria                                   | DRG                    | axotomy                                                                    | Y, > 72 h                                       | (Sasaki et al., 2006)                       |
|               | mice    | NT                                             | DRG                    | rotenone, vincristine, complex I inhibitors, h <sub>2</sub> O <sub>2</sub> | Y                                               | (Press and Milbrandt, 2008)                 |
|               | mice    | mitochondria                                   | transgenic mice        | transection                                                                | Y, similar to Wld <sup>S</sup>                  | (Yahata et al., 2009)                       |
| Nmnat2        | mice    | cytoplasm                                      | transgenic Drosophila  | transection                                                                | N                                               | (Avery et al., 2009)                        |
| Nmnat2        | human   | Golgi apparatus                                | SCG                    | transection                                                                | Y                                               | (Yan et al., 2009)                          |
| Nmnat1(W170A) | mice    | NT                                             | DRG                    | transection                                                                | N                                               | (Araki et al., 2004)                        |
|               | mice    | NT                                             | DRG                    | transection                                                                | N                                               | (Sasaki et al., 2009b)                      |
| Nmnat1(H24A)  | mice    | nucleus                                        | transgenic Drosophila  | transection                                                                | N                                               | (Avery et al., 2009)                        |
|               | mice    | NT                                             | DRG                    | transection                                                                | N                                               | (Sasaki et al., 2009b)                      |
| Nmnat2(H24D)  | human   | Golgi apparatus                                | SCG                    | transection                                                                | N                                               | (Yan et al., 2009)                          |
| N16-Nmnat1    | mice    | a fusion of N16 to full-length Nmnat1, nucleus | transgenic Drosophila  | transection                                                                | Y, increased protection relative to Nmnat1      | (Avery et al., 2009)                        |

(Continued)

| gene                 | species         | localization                    | models                                                 | type of injury       | effectiveness                   | citation                 |
|----------------------|-----------------|---------------------------------|--------------------------------------------------------|----------------------|---------------------------------|--------------------------|
| cytNmnat1            | mice            | cytoplasm                       | DRG                                                    | axotomy              | Y, similar as Nmnat1            | (Sasaki et al., 2006)    |
|                      | mice            | cytoplasm                       | DRG from Cd38 <sup>-/-</sup> Parp1 <sup>-/-</sup> mice | transection          | Y                               | (Sasaki et al., 2009b)   |
|                      | mice            | cytoplasm, neuronal projections | transgenic mice                                        | axotomy              | Y, 7 days after injury          | (Sasaki et al., 2009a)   |
|                      | mice            | cytoplasm, high in axon         | DRG                                                    | axotomy, vincristine | Y, more efficiently than Nmnat1 | (Sasaki et al., 2009a)   |
| nuc-Nmnat3           | mice            | nucleus                         | DRG                                                    | axotomy              | Y, similar as Nmnat1            | (Sasaki et al., 2006)    |
| dNmnat <sup>c</sup>  | Drosophila      | cytoplasm                       | transgenic Drosophila                                  | transection          | Y, weaker than Wld <sup>S</sup> | (MacDonald et al., 2006) |
|                      | Drosophila      | NT                              | DRG                                                    | transection          | Y                               | (Sasaki et al., 2009b)   |
| yNmnat <sup>d</sup>  | yeast           | NT                              | DRG                                                    | transection          | Y                               | (Sasaki et al., 2009b)   |
| mjNmnat <sup>e</sup> | archaebacterium | NT                              | DRG                                                    | transection          | Y                               | (Sasaki et al., 2009b)   |

<sup>a</sup> Y, Yes, have protective function, W, Weak protection, N, No protection; <sup>b</sup> NT, Not tested; <sup>c</sup> Drosophila melanogaster Nmnat;

<sup>d</sup> *Saccharomyces cerevisiae* Nmnat; <sup>e</sup> archaebacterium *Methanocaldococcus jannaschii* Nmnat.

Nmnat1 and Wld<sup>S</sup> prevent the decrease of NAD levels in transected axonal segments *in vitro* (Wang et al., 2005). However, a recent study showed that elevated neuronal NAD levels in cultured DRG neurons from Cd38<sup>-/-</sup>Parp1<sup>-/-</sup> mice do not provide axonal protection (Sasaki et al., 2009b). It is suggested that the Wld<sup>S</sup> or Nmnats protect the axon loss by maintaining the homeostasis of NAD level. As it is reported that NAD levels in mitochondria remain at physiological levels following genotoxic stress when nuclear and cytoplasmic pools of NAD are depleted (Yang et al., 2007), it is also possible that they changed the NAD levels in specific organelles, which are not reflected by the whole cell NAD levels.

Another line of evidence that supports the role of NAD in protecting axon degeneration is from the observations that over-expression of other NAD-synthesizing enzymes, in addition to Nmnats, also showed protective effects. These enzymes include nicotinamide phosphoribosyltransferase, nicotinamide phosphoribosyltransferase (Namp1) and nicotinic acid phosphoribosyl transferase (Sasaki et al., 2006). In addition, exogenous application the substrate or intermediates of the NAD synthesis pathway, including nicotinamide, NAD, nicotinic acid mononucleotide and nicotinamide mononucleotide all promoted axonal protection (Araki et al., 2004; Wang et al., 2005; Kaneko et al., 2006; Sasaki et al., 2006). Exogenously pyruvate, a critical intermediate product of glycolysis, could partially protect axon degeneration (Wang et al., 2005). Together with the observed localization of Wld<sup>S</sup> in mitochondria (Yahata et al., 2009), it is likely that NAD protects axon degeneration via impacting energy metabolism

in the axon. Although it remains to be determined whether maintaining energy balance is the sole or major protective mechanism of Wld<sup>S</sup>, these findings highly suggested a passive wasting-away mechanism involved in axon degeneration, which challenges the proposed active program process of axon degeneration (Raff et al., 2002).

However, the axonal protection provided by these chemical treatments is less potent than that provided by Nmnat over-expression. In addition, inhibition of Namp1 that catalyzes the rate-limiting step for NAD biosynthesis from nicotinamide, could strongly reduced NAD levels in Wld<sup>S</sup> SCG cultures, but only incompletely reversed the Wld<sup>S</sup> phenotype. Inhibition of Namp1 did not affect the protective activity of Nmnat1 as well (Sasaki et al., 2009b). These data suggest the potential NAD-independent functions of Nmnat1. In this aspect, other downstream metabolites should be considered. Alternatively, NAD synthesis activity may function at specific loci such as mitochondria, Golgi apparatus or endoplasmic reticulum, and could not be measured in the whole cell lysates.

In addition to the critical role in energy metabolism, NAD is also a substrate for the Sirtuin family of histone deacetylases. Enhancing the dosage of Namp1 could increase total NAD levels and regulate the transcriptional repressive activity of SIRT1 in mouse NIH3T3 fibroblasts (Revollo et al., 2004). It was shown that SIRT1 protein are effector of the axonal protection mediated by increased Nmnat activity (Araki et al., 2004), but this mechanism is not supported by subsequent studies. Genetic deletion of SIRT1 in mice and Drosophila does not affect the protective effects of Wld<sup>S</sup> on axon degeneration (Wang et al., 2005; Avery et al., 2009). However, there

are several cytoplasmic SIRT members, such as SIRT2, SIRT3 and SIRT4 (Haigis and Guarente, 2006), whose roles in NAD-dependent protection remain to be determined.

## OTHER MECHANISMS

Recent studies in *Drosophila* showed that Nmnat functions as a chaperone in protecting neuronal degeneration, which is independent of its NAD synthesis activity (Zhai et al., 2008). Another study in mice showed that chaperone activity in the mutant protein Wld<sup>S</sup> (W258A) was similar to that reported for enzyme-dead Nmnat, but the Wld<sup>S</sup> (W258A) transgenic mice show no axon protection (Conforti et al., 2009). Thus, the chaperone activity was unlikely to be sufficient in the axonal protection of Nmnat or Wld<sup>S</sup> (Conforti et al., 2009).

Other researches focus on the downstream effectors of Wld<sup>S</sup>. Pituitary tumour-transforming gene-1 (Pttg1) could be down-regulated by Wld<sup>S</sup>, but Pttg1<sup>-/-</sup> mice did not show neuroprotective phenotype (Gillingwater et al., 2006). A recent study suggested that Wld<sup>S</sup> could regulate cell cycle status, which possibly involve Pttg1/Ube1, NAD and VCP pathways (Wishart et al., 2008). In addition, previous observations showed decreased macrophage/microglial activation in transected nerves of Wld<sup>S</sup> mice as well as in the experimental autoimmune encephalomyelitis model mediated by elevated expression of CD200 (Chitnis et al., 2007). But how these pathways influence Wld<sup>S</sup>/Nmnat-related axonal protective function remains to be further investigated.

## SUMMARY AND PERSPECTIVES

As discussed above, great progress has been achieved for understanding axon degeneration during the past two decades. However, many challenges remain. Neither the exactly cell organelles nor the downstream pathway that Wld<sup>S</sup> or Nmnat works on has been elucidated. Furthermore, in addition to Wld<sup>S</sup>, the protective function of Nmnats in neurodegenerative diseases, and the correlation of Nmnats and neurodegenerative diseases have not been fully characterized. There are still a lot of puzzles to be solved.

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## ABBREVIATIONS

ALS, amyotrophic lateral sclerosis; DRG, dorsal root ganglia; NAD, nicotinamide adenine dinucleotide; Nampt, nicotinamide phosphoribosyltransferase; Nmnat, nicotinamide mononucleotide adenyl

transferase; ORN, olfactory receptor neuron; SCG, superior cervical ganglia; VCP, valosin containing protein; UPS, ubiquitin-proteasome system

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