Benefits of Enhancing Nicotinamide Adenine Dinucleotide Levels in Damaged or Diseased Nerve Cells

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Three unbiased lines of research have commonly pointed to the benefits of enhanced levels of nicotinamide adenine dinucleotide (NAD+) to diseased or damaged neurons. Mice carrying a triplication of the gene encoding the culminating enzyme in NAD+ salvage from nicotinamide, NMNAT, are protected from a variety of insults to axons. Protection from Wallerian degeneration of axons is also observed in flies and mice bearing inactivating mutations in the SARM1 gene. Functional studies of the SARM1 gene product have revealed the presence of an enzymatic activity directed toward the hydrolysis of NAD+. Finally, an unbiased drug screen performed in living mice led to the discovery of a neuroprotective chemical designated P7C3. Biochemical studies of the P7C3 chemical show that it can enhance recovery of NAD+ from nicotinamide by activating NAMPT, the first enzyme in the salvage pathway. In combination, these three unrelated research endeavors offer evidence of the benefits of enhanced NAD+ levels to damaged neurons.

This review covers three independent lines of investigation commonly concluding that the loss of nicotinamide adenine dinucleotide (NAD+) accompanies the demise of damaged or diseased axons. It is likewise argued that the same three lines of investigation predict that pathways facilitating either the preservation of NAD+ or its enhanced biosynthesis may be protective of damaged or diseased axons. The first of these discoveries evolved from studies of a strain of mice that was found to be protected from Wallerian degeneration. The mutational event causative of this protective activity was traced to overexpression of a chimeric protein that includes the entire open reading frame encoding one of the three mammalian isoforms of the nicotinamide mononucleotide adenylyltransferase (NMNAT) enzyme. Enhanced expression of this enzyme facilitates preservation of NAD+ levels in damaged or diseased axons (see Table 1). The second discovery relevant to the relationship between NAD+ and axon health involved description of the fly gene designated dSarm. Inactivation of this gene in flies, or of the mouse ortholog Sarm1, blocks NAD+ decline in injured or diseased axons, thereby helping preserve axon integrity (see Table 1). The third discovery pertinent to these concepts entailed characterization of a synthetic chemical, designated P7C3, that modulates activity of the nicotinamide phosphoribosyltransferase (NAMPT) enzyme. Administration of the P7C3 chemical to mice or rats elicits a protective effect on neuron integrity in several models of axon injury or disease (see Table 1). Each of these three discoveries resulted from serendipitous, unbiased research. That all three lines of investigation commonly identified preservation of NAD+ levels as a means of protecting damaged or diseased axons offers credence to the concept that patients suffering deficits resulting from axon injury might benefit from agents that either preserve existing NAD+ or facilitate enhanced NAD+ synthesis.

DISCOVERY OF WALLERIAN DEGENERATION SLOW (WldS) MICE

Wallerian degeneration is the process by which the portion of an axon degenerates distal to its site of injury. Early events in Wallerian degeneration include breakdown of the axon membrane and cytoskeleton, followed by myelin degeneration and macrophage infiltration (Beuche and Friede 1984). This process is named after Augustus Volney Waller, who discovered in 1850 that degenerating distal axons of transected frog glossopharyngeal nerves coalesced into droplets that could be visualized by cytological staining (Waller 1850). It was by use of this technique of visualizing nerve fiber degeneration that Lunn et al. (1989) discovered a line of mice characterized by delayed axon degeneration after sciatic nerve transection. Known as the C57BL/6J strain and derived from a spontaneous mutation at Harlan-Olac in the United Kingdom, these mice were identified by their unusual ability to continue transmitting nerve impulses in...
The distal portion of severed axons for 2 wk after transection, in contrast to only 2–3 d in control mice (Lunn et al. 1989; Perry et al. 1992). Otherwise, C57BL/Ola mice were indistinguishable from C57BL/6J in appearance, behavior, and histocompatibility.

The resistance to Wallerian degeneration in C57BL/Ola mice was subsequently identified as intrinsic to nerve cells, and not related to Schwann cells (Glass et al. 1993) or circulating monocytes (Perry et al. 1990a). C57BL6/Ola mice also showed a protective effect in the central nervous system, with delayed retinal ganglion cell degeneration following optic nerve transection (Perry et al. 1991). A later observation that motor neuron cell death was similarly delayed in C57BL6/Ola mice after sciatic nerve axotomy confirmed a functional link between axon degeneration and neuronal cell death (Lapper et al. 1994).

These foundational observations prompted interest in discovering the genetic basis for resistance of C57BL/Ola mice to Wallerian degeneration. Crossing C57BL6/Ola mice with BALB/c mice revealed that this property was inherited in single autosomal dominant fashion (Perry et al. 1992). Otherwise, C57BL/Ola mice were stably inherited across divergent breeding colonies of WldS mice (Mi et al. 2003).

In a relatively short time, Conforti et al. (2000) identified a chimeric gene within the 85-kb tandem triplication region that was abundantly expressed in the nervous system and appeared responsible for resistance to Wallerian degeneration in WldS mice. This gene encoded an in-frame 42-kDa fusion protein consisting of the amino-terminal 70 amino acids of the 1173 amino acid-long E4 ubiquitin ligase Ube4b protein, which was joined by an aspartic acid to the protein encoded by D4Cole1e. Given that only a very short region of Ube4b was included, it was of no surprise that the fusion protein lacked ubiquitin ligase activity. In contrast, the entire coding region of D4Cole1e was fully included and quickly identified as being nearly identical to the recently cloned gene for human NMNAT (Emanuelli et al. 2001; Fernando et al. 2002), a metabolic enzyme that catalyzes NAD+ synthesis from nicotinamide mononucleotide (NMN) and adenosine triphosphate (ATP) (Magni et al. 1999). Indeed, sequence alignment with human NMNAT showed that nucleotides 282–1140 of WldS contained the entire NMNAT open reading frame. Although a third gene, retinol binding protein 7 (Rbp7), was also positioned within the 85-kb repeat unit, this gene was expressed predominantly in white adipose and mammary gland tissue, and found to be unrelated to the protective effect of WldS (Conforti et al. 2000).

Within the next year, Mack et al. (2001) confirmed the critical role of NMNAT in the 85-kb region by expressing the Ube4b/Nmat chimeric gene in transgenic mice. They observed NMNAT enzymatic activity and neuroprotective efficacy specified by the fusion protein in sensory and motor axons with respect to nerve conduction, synaptic transmission, vesicle recycling, and nerve terminal mor-

### Table 1. Efficacy of nicotinamide adenine dinucleotide–based therapies in preclinical models of the damaged or diseased nervous system

| Peripheral nerve disease | WldS | SARM1 | P7C3 |
|--------------------------|------|-------|------|
| Po mouse                 | Protective | Not tested | Not tested |
| Pmp22 rat                | Protective | Not tested | Not tested |
| Peripheral nerve toxicity| Protective | Protective | Protective |
| Paclitaxel-induced peripheral neuropathy | Not tested | Protective | Not tested |
| Vincristine-induced peripheral neuropathy | Not tested | Protective | Not tested |
| High fat diet/hyperglycemia-induced peripheral neuropathy | Not tested | Protective | Not tested |
| Peripheral nerve injury  | Not tested | Protective | Not tested |
| Sciatic nerve transaction| Not tested | Protective | Not tested |
| Sciatic nerve crush      | Not tested | Not tested | Protective |
| Spinal cord disease      | pmn mouse | Protective | Not tested | Not tested |
| Traumatic brain injury   | Controlled cortical impact | Protective | Not tested | Protective |
|                          | Weight drop | Not tested | Protective | Not tested |
|                          | Blast-mediated injury | Protective | Not tested | Protective |
| Stroke                   | Cerebral ischemia/reperfusion | Not tested | Not tested | Protective |
| Parkinson’s disease      | 6-hydroxydopamine toxicity | Protective | Not tested | Protective |
|                          | MPTP toxicity | Protective | Not tested | Protective |
| Alzheimer’s disease      | TgF344-AD rat | Not tested | Not tested | Protective |

WldS, SARM1, and P7C3 pathways converge on augmentation of neuronal NAD+ levels and offer protection across common preclinical models of nervous system damage and disease.
phology. Furthermore, the protective effect was dose-de-
pendently related to protein expression levels. They also
showed that \( Wld^S \) mice display a fourfold increase in
NMNAT enzyme activity in the brain compared to
C57BL/6j mice (Mack et al. 2001). The following year,
a novel human cDNA encoding a 34.4-kDa protein with
significant homology with the 31.9-kDa NMNAT protein
was also discovered. The new protein also had NAD bio-
synthetic activity and was named NMNAT2. The original
NMNAT that had been identified as the human protein
homologous to \( D_{4}C_0l_{e} \) was then renamed NMNAT1 (Raffaelli et al. 2002).

Multiple mechanisms have been proposed for how \( Wld^S \)
mice are protected from Wallerian degeneration (Wishart et
al. 2007; Wang and Barres 2012), including modification
of cell cycle pathways (Wishart et al. 2008), optimi-
zation of mitochondrial function (Avery et al. 2012; Fang
et al. 2012), control of expression of axonal receptors for
glial-mediated engulfment of degenerating axons (Fain-
zilber and Twiss 2006; Hooper et al. 2006; MacDonald
et al. 2006), and a hypothetical role of the Ube4b/NMNAT
fusion protein as a molecular chaperone (Zhai et al. 2008).
The most simple and likely correct explanation, however,
is that \( Wld^S \) mice benefit from the maintenance of steady-
state levels of NAD\(^+\) through increased NAD\(^+\) synthesis in
damaged or diseased nerve cells (Wang et al. 2005; Cole-
man and Freeman 2010). The work by Araki et al. (2004)
showed that NMNAT1 activity alone was sufficient to pro-
tect axons of explanted dorsal root ganglion neurons sub-
jected to either traumatic transection or toxic exposure to
vincristine, a chemotherapeutic drug that blocks tubulin
assembly into microtubules. Subsequent generation of an
additional \( Wld^S \) transgenic mammalian model—the \( Wld^S \)
transgenic rat—further bolstered confidence in the utility
of augmenting NAD\(^+\) synthesis for neuroprotection, as
these animals were resistant to Wallerian degeneration after
sciatic nerve transection (Adalbert et al. 2005). More re-
cently, in vitro studies have shown that extracellular NAD\(^+\)
recapitulates the axonal protection seen in \( Wld^S \) neurons
(Wang et al. 2015), presumably because of transport recep-
tors that facilitate uptake of extracellular NAD\(^+\) into nerve
cells (Bruzzzone et al. 2001).

Although initially found to be most highly abundant in
the nucleus, the \( Wld^S \) fusion protein was later noted to also
be enriched in mitochondria, cytosol, peroxisomes/lyso-
somes, endoplasmic reticulum, and axons (Yahata et al.
2009; Avery et al. 2012; Wang et al. 2015). Indeed, axonal
localization of \( Wld^S \) appears to enable normally nuclear
NMNAT1 to substitute for its axonal paralog NMNAT2,
which is impaired in maintaining NAD\(^+\) synthesis under
conditions of axonal injury or stress. This insufficiency of
NMNAT2 activity has been attributed to its short half-life
and dependence of distal neurites on constant delivery of
NMNAT2 from the soma (Berger et al. 2005; Gilley and
Coleman 2010; Neukomm and Freeman 2014). Sasaki et
al. (2016) have also reported that NMNAT2 inhibits the
NAD hydrolase activity of SARM1 (described below),
and that the NMNAT1 enzymatic domain of \( Wld^S \) some-
how inhibits SARM1 activity during damage-induced loss of axonal
NMNAT2.

In conclusion, although nuances of \( Wld^S \) function will
undoubtedly require further study, it is clear that NAD\(^+\)
synthesis plays a vital role in the resistance of mice to
Wallerian degeneration. Because axonal loss is a promi-
inent feature of neuropathies and other neurodegenerative
diseases (Saxena and Caroni 2007), this discovery has
prompted exploration of the potential benefits of augment-
ing NAD\(^+\) synthesis in neurodegeneration. Indeed, \( Wld^S \)
mice have been used extensively to study the physiology
of reinnervation and peripheral nerve damage, and the
applicability of \( Wld^S \) to peripheral and central nervous
system degeneration has also been explored.

**\( Wld^S \) IN NEUROPROTECTION**

Because Wallerian degeneration is a prominent feature
of injuries and disease in the peripheral nervous system,
the resistance of \( Wld^S \) mice to peripheral neuropathy has
been investigated in relevant animal models. For example,
Samsam et al. (2003) crossed \( Wld^S \) mice with mice defi-
cient in the peripheral myelin component P0, a model of
human peripheral neuropathy, and observed delayed mo-
tor and sensory axon degradation. Several years later,
Meyer zu Horste et al. (2011) crossed the \( Wld^S \) rat with the
Pmp22 rat, a transgenic model of Charcot–Marie–
Tooth (CMT) disease type 1A, and observed that \( Wld^S \)
reduced axon loss and behavioral deficits. With respect
to the toxicity of anticancer chemicals, Wang et al.
(2002) showed that \( Wld^S \) mice were resistant to paclitax-
el-mediated peripheral neuropathy.

In the central nervous system, axon loss occurs early in
many disorders, including spinal cord injury (Zhang et al.
1996), amyotrophic lateral sclerosis (ALS) (Dal Canto and
Gurney 1995; Fischer et al. 2004; Fischer and Glass
2007), Alzheimer’s and Parkinson’s diseases (Raff et al.
2002; Stokin et al. 2005; Kurowska et al. 2016), and
traumatic brain injury (TBI) (Yin et al. 2014, 2016). Fur-
thermore, the protection of neuronal cell bodies without
preserving axons may be insufficient to prevent neurolog-
ic disease (Sagot et al. 1995; Houseweart and Cleveland
1999). Thus, there is considerable interest in finding ways
to therapeutically protect axons in central nervous system
injury and disease. As described above, the first applica-
tion of \( Wld^S \) mice to the central nervous system was by
Perry et al. (1991) through optic nerve transection exper-
iments in which \( Wld^S \) mice showed delayed degeneration of
retinal ganglion cells and their axons. Later work, how-
ever, showed that \( Wld^S \) rats were protected only from axon
degeneration with no effect on retinal ganglion cell body
derioration after both optic nerve transection and a pho-
tocoagulation model of glaucoma (Beirowski et al. 2008).
More recently, Williams et al. (2017) re-addressed this
question in the DBA/2J (D2) mouse model of glaucoma,
in which ocular hypertension leads to optic nerve degene-
ratio \( \sim 8–9 \text{ mo of age} \), followed by retinal ganglion cell
dearth. Crossing these mice with \( Wld^S \) mice yielded ani-
mals showing increased retinal NAD\(^+\) levels. The optic
nerve axons and retinal ganglion cell bodies of these mice
were protected from ocular hypertension.
With respect to TBI, $Wld^S$ mice display improved performance over wild-type mice in cognitive and motor behavior after controlled cortical impact injury (Fox and Faden 1998). More recently, Yin et al. (2016) reported that $Wld^S$ mice are resistant to both retinal ganglion cell deterioration and axonal degeneration after blast-mediated TBI, and are protected from injury-induced cognitive and motor behavioral deficits. $Wld^S$ has additionally been evaluated in models of Parkinson’s disease because of the known degeneration of substantia nigra dopaminergic neurons. In 2004, Sajadi et al. tested susceptibility of $Wld^S$ mice to the catecholaminergic toxin 6-hydroxydopamine (6-OHDA) model of Parkinson’s disease. Following injection of the toxin into the median forebrain bundle, $Wld^S$ mice were partially protected from dopaminergic axon loss in the striatum. However, protection was restricted to portions of the axons distal to the site of toxin injection. As 6-OHDA is subject to retrograde transport from the site of injection back to the cell body, this selective regional protection presumably reflects unique neuronal processes governing primary and secondary injury after 6-OHDA exposure, with $Wld^S$ being protective of the latter. A possible role of $Wld^S$ for protection in Parkinson’s disease was further examined with another chemical toxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Specifically, Hasbani and O’Malley (2006) reported that $Wld^S$ mice were protected from nigrostriatal axon degeneration and striatal neurotransmitter loss in this model. However, as in the case of 6-OHDA (Sajadi et al. 2004), no protection from dopaminergic cell body death was observed in $Wld^S$ mice.

With respect to spinal cord disease, Ferri et al. (2003) evaluated efficacy of $Wld^S$ in progressive motor neuronopathy (pnm) mice, a model of human motor neuron disease. Here, $Wld^S$ blocked axon degeneration and preserved associated motor function. Notably, previous work allowing for the controlled inhibition of apoptosis to protect motor neuron cell bodies without preserving axons in pnm mice did not modify disease progression (Sagot et al. 1995). Last, efficacy of $Wld^S$ has been evaluated in animal models of ALS, based on a variety of mutations in the gene encoding Cu/Zn superoxide dismutase 1 (SOD1) that have been identified in subsets of families with ALS. $Wld^S$ has been evaluated in three of these models with marginal results. In SOD1G37R and SOD1G93R mouse models, $Wld^S$ conferred no protection in disease onset, axon degeneration, synaptic integrity, or motor neuron death (Velde et al. 2004). In the SOD1G93A mouse model, however, $Wld^S$ modestly prolonged survival and delayed denervation at the neuromuscular junction, but had no effect on motor axon loss (Fischer et al. 2005).

**DISCOVERY OF SARM1**

Tight conservation of the axonal protective efficacy of $Wld^S$ and NMNAT activity across species, including *Drosophila melanogaster*, prompted Marc Freeman to conduct a loss-of-function genetic screen in the fly with the goal of identifying other genes involved in controlling Wallerian degeneration (Osterloh et al. 2012). This unbiased screen revealed that mutational inactivation of dSarm (*Drosophila* sterile a/armadillo/Toll-interleukin 1 receptor [TIR] homology domain protein) suppressed Wallerian degeneration in a cell-autonomous manner for weeks after axotomy. Elimination of SARM1, the mammalian homolog of dSarm, in mice produced comparable long-term survival of damaged axons in vitro and in vivo. Freeman’s discovery was later confirmed by Jeff Milbrandt, who identified SARM1 in a quantitative, image-based shRNA screen for genes required for axotomy-induced axon degeneration of explanted dorsal root ganglion cells (Gerds et al. 2013). Milbrandt reported similarly reduced axon degeneration following sciatic nerve transection in SARM1 mutant mice, and showed that artificial activation of SARM1 in axons is both necessary and sufficient for Wallerian degeneration (Gerds et al. 2016). Around this same time, others showed that SARM1 ablation in MNAT2-deficient mice completely blocked axon degeneration and perinatal lethality (Gilley et al. 2015), indicating that these two proteins may function in opposition with respect to the physiology of damaged axons.

**MECHANISMS OF SARM1 ACTIVITY**

Prior to the aforementioned discoveries, SARM1 had been thought to function as an adaptor protein in innate immunity (Mink and Csizsar 2005; O’Neill and Bowie 2007; Peng et al. 2010). Perplexingly, however, SARM1 was found to be unique among TIR-containing proteins in its selective enrichment in the nervous system (Kim et al. 2007). Indeed, before Freeman discovered the role of SARM1 in Wallerian degeneration, physiologic roles for SARM1 had been claimed in neural fate specification (Chuang and Bargmann 2005), dendritic arborization (Chen et al. 2011), and microglial activation (Szretter et al. 2009). To date, much of the mechanistic work to clarify the role of SARM1 in the nervous system has been conducted in the Milbrandt laboratory. They have shown that induced loss of mitochondrial membrane potential in cultured primary mouse sensory neurons induces a form of cell death pharmacologically distinct from apoptosis or necrosis. Without SARM1, the mitochondrial poison cyanide m-chlorophenyl hydrazone (CCCP), an inhibitor of oxidative phosphorylation, elicits ATP depletion, excessive calcium influx, and accumulation of reactive oxygen species, yet fails to lead to axon degeneration or cell death (Summers et al. 2014). These observations, coupled with the finding that SARM1 elimination also protects neurons from prolonged exposure to reactive oxygen species (ROS), suggest that SARM1 acts downstream from ROS generation to induce cell death in times of oxidative stress. Milbrandt has thus proposed a form of programmed cell death in the peripheral nervous system downstream from ROS termed sarmoptosis (Summers et al. 2014).

Architecturally, SARM1 consists of 4 identifiable protein domains, including its MLS (mitochondrial localization signal), ARM (armadillo/HEAT motifs), SAM (sterile α motif), and TIR domains (Gerds et al. 2016). A mutated variant of SARM1 lacking the amino-terminal ARM
domain constitutively triggers axon degeneration, demonstrating that SARM1 may be auto-inhibited by its ARM domain (Gerfts et al. 2013). The SAM domain is required for SARM1 oligomerization and axon degeneration, and the TIR domain is also required for SARM1 to execute its role in axon degeneration (Gerfts et al. 2013; Summers et al. 2016). Indeed, dimerization of the TIR domain alone has been shown to induce rapid axon degeneration (Gerfts et al. 2015).

THE TIR DOMAIN OF SARM1 SPECIFIES AN NAD\(^+\) HYDROLASE ACTIVITY

Numerous observations giving evidence that NAD\(^+\) levels decline in damaged axons, complemented by the observation that forced expression of an activated form of SARM1 was capable of eliciting this same effect (Gerfts et al. 2015), prompted interest in how SARM1 might provide a link between NAD\(^+\) loss and axon degeneration. In a surprising and exciting series of experiments, the Milbrandt laboratory found that the purified TIR domain of SARM1 itself specifies an NAD\(^+\) hydrolase activity (Essuman et al. 2017). Through homology-based modeling of other proteins with related TIR domains, the Milbrandt group identified a glutamic acid residue essential for the TIR domain to hydrolyze NAD\(^+\) (Essuman et al. 2017). The variants of SARM1 mutated at this single-amino-acid position fully eliminate the ability of SARM1 to execute its role in triggering Wallerian degeneration of axons. Most recently, the Milbrandt laboratory has made reference to TIR domains from prokaryotic organisms that also display NAD\(^+\) hydrolase activity, suggesting a new class of TIR-containing proteins as evolutionarily ancient metabolic regulatory enzymes (Essuman et al. 2018).

SARM1 IN NEUROPROTECTION

In studies of the peripheral nervous system, Geisler et al. (2016) exposed SARM1-deficient mice to a model of vincristine toxicity that mimics the neuropathy experienced by up to 80% of patients treated with vincristine (Casey et al. 1973; Verstappen et al. 2005), and is the main dose-limiting side effect of this form of chemotherapy (DeAngelis et al. 1991; Haim et al. 1994; Reinders-Messelink et al. 2000; Lavioie Smith et al. 2015). SARM1-deficient mice were protected in all parameters of vincristine toxicity. More recently, Turkiew et al. (2017) reported that SARM1-deficient mice are also protected from peripheral neuropathy induced by either paclitaxel or a high-fat diet. More recently, Turkiew et al. (2017) reported that SARM1-deficient mice are also protected from peripheral neuropathy induced by either paclitaxel or a high-fat diet. In contrast, animals missing the paralogous NPAS3 transcription factor display impaired hippocampal neurogenesis. Experimental evidence from the laboratory of Rusty Gage had likewise shown that adult neurogenesis in mice can be influenced by environment (Kempermann et al. 1997). Mice availed access to siblings and an enriched environment, such as a running wheel, display substantively enhanced hippocampal neurogenesis relative to mice deprived of these conditions. Recognizing from these various observations that the process of adult neurogenesis might be under dynamic control, we performed an unbiased, in vivo screen in search of proneurogenic chemicals (Pieper et al. 2010).

With help from Patrick Harran, a synthetic organic chemist in the Department of Biochemistry at University of Texas Southwestern Medical Center, a compound file consisting of 200,000 synthetic chemicals was trimmed down to 1000 individual molecules best representative of the diversity of the entire collection. These chemicals were combined into 100 pools of 10 individual compounds and administered directly into the brains of living mice. Continuous infusion for a period of 1 wk was achieved via Alzet mini-pumps implanted beneath the skin of the backs of individual animals. To visualize neurogenesis, bromodeoxyuridine (BrdU) was coadministered along with each pool of compounds. After 1 wk animals were killed, allowing brain tissue to be sectioned and stained for BrdU incorporation into neurons localized to the subgranular zone of the dentate gyrus. The seventh of the 100 pools yielded a twofold enhanced level of hippocampal neurogenesis, and breakdown of this pool assigned proneurogenic activity to the third compound. This compound, designated pool seven compound three (P7C3) corresponded to an aminopropylcarbazole. Analysis of synthetic derivatives of the P7C3 chemical yielded the A20 variant, which showed reliably enhanced proneurogenic activity readily detected upon intraperitoneal or oral administration to either mice or rats (Pieper et al. 2010). It was subsequently shown that P7C3 achieved its proneurogenic effect by augmenting the survival of newborn hippocampal neurons without affecting their initial rate of proliferation.

P7C3 COMPOUNDS FUNCTION AS NAMPT MODULATORS

Subsequent to completion of the target-agnostic in vivo screen that led to the P7C3 chemical, coordinated efforts were initiated with the medicinal chemistry laboratory of Joseph Ready to probe its mechanism of action. A bio-
logically active derivative of P7C3, known as P7C3-S326, was synthesized in the Ready laboratory to contain both a benzophenone for photo-cross-linking and an alkyne for CLICK chemistry (Wang et al. 2014). The observation that P7C3-A20, P7C3-S326, and other active derivatives of P7C3 (MacMillan et al. 2010; Naidoo et al. 2013, 2014), but not inactive variants, protected cultured U2OS cells from doxorubicin-induced toxicity provided a cell culture system for target discovery. The active enantiomer (−)-P7C3-S243 was protective from doxorubicin, whereas the inactive (−)-P7C3-S243 enantiomer was less protective. Incubation of P7C3-S326 in cultured U2OS cells showed that it could be photo-cross-linked to a 70-kDa protein. Cross-linking was competed by coinubcation with 30× excess of P7C3-A20. Roughly 150 P7C3 analogs were assayed for their ability to compete for photo-cross-linking of the 70-kDa protein by P7C3-S326, and comparison of the observed pattern of competition to efficacy of the same compounds in protecting U2OS cells from doxorubicin toxicity yielded a significant correlation (Wang et al. 2014). Through cell fractionation, 2D gel electrophoresis, and mass spectrometry, it was determined that the 70-kDa protein photo-cross-linked by P7C3-S326 was NAMPT, the rate-limiting enzyme in NAD⁺ salvage by which cells sequentially convert nicotinamide into NAD⁺. Administration of14C-labeled nicotinamide to U2OS cells pretreated with doxorubicin showed that toxin-mediated loss of NAD⁺ was dose-dependently restored by cotreatment with P7C3-A20. Correlation was noted between the ability of P7C3 variants to preserve NAD⁺ levels and their ability to protect U2OS cells from doxorubicin-mediated toxicity (Wang et al. 2014). Administration of14C-labeled nicotinamide to U2OS cells pretreated with doxorubicin, and subsequent analysis by thin layer chromatography, revealed enhanced conversion of radiolabeled nicotinamide into NAD⁺ upon exposure to P7C3-A20. A triple-coupled assay was then developed to test the activities of P7C3 variants on a set of recombinant enzymes. The enzymes included NAMPT (which converts nicotinamide to NMN), NMNAT1 (which converts NMN to NAD⁺), and alcohol dehydrogenase (which converts NAD⁺ to NADH). Flux of nicotinamide through this triply coupled enzyme assay was monitored by increase in fluorescence resulting from the production of NADH. By testing a large number of synthetic variants of P7C3 that had already been assayed for proneurogenic activity in mice, and for protection of U2OS cells from doxorubicin, a strong correlative relationship between this triply coupled enzyme assay and the two assays of P7C3 function in living animals or cells was revealed.

**P7C3 Compounds Are Neuroprotective**

Just as the role of NAD⁺ in WldS, SARM1, and P7C3. The mouse that ended up encoding a triplicated version of the fusion protein linking a small bit of a ubiquitin ligase to the full open reading frame of NMNAT1 did not come from a comprehensive genetic screen, but instead emerged from careful analysis of a random mouse strain. It is remarkable that this one strain carried the aforementioned mutation, and perhaps even more remarkable that the Oxford scientists happened to evaluate the properties of this strain in the context of Wallerian degeneration. Studies pinpointing the product of dSARM1 in the pathway leading to degeneration of damaged axons were carefully conceived and meticulously executed. In this case, serendipity came in the discovery of a protein endowed with an un-
anticipated NAD⁺ hydrolase activity essential for Wallerian degeneration. Finally, the drug screen leading to the discovery of the P7C3 chemical evaluated only 1000 synthetic chemicals. That this small collection included the neuroprotective, NAMPT-modulating aminopropylcarbazole chemical is undoubtedly serendipitous. Most remarkable of all is the apparent fact that all three approaches to the study of axon or neuron health commonly led to the same metabolite—NAD⁺. If these conclusions prove to be correct, they give evidence that the concept of NAD⁺ centrality to axon and neuron health is supported by a three-legged stool (see Fig. 1). It is hoped that the apparent stability of this foundation may properly point toward the utility of therapeutics that either enhance NAD⁺ biosynthesis or prevent SARM1-mediated NAD⁺ hydrolysis.

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