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

ANDREW A. PIEPER^{1,2,3} AND STEVEN L. MCKNIGHT⁴

¹*Harrington Discovery Institute, University Hospitals Cleveland Medical Center, Cleveland, Ohio 44106, USA*

²*Department of Psychiatry, Case Western Reserve University, Cleveland, Ohio 44106, USA*

³*Geriatric Research Education and Clinical Centers, Louis Stokes Cleveland VAMC, Cleveland, Ohio 44106, USA*

⁴*Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75390, USA*

Correspondence: steven.mcknight@utsouthwestern.edu; andrew.pieper@harringtondiscovery.org

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 re-

sulted 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 (*Wld^S*) 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) made their serendipitous discovery of a line of mice characterized by abnormally delayed axon degeneration after sciatic nerve transection. Known as the C57BL/Ola 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

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

	<i>Wld^S</i>	SARM1	P7C3
Peripheral nerve disease			
Po mouse	Protective	Not tested	Not tested
Pmp22 rat	Protective	Not tested	Not tested
Peripheral nerve toxicity			
Paclitaxel-induced peripheral neuropathy	Protective	Protective	Protective
Vincristine-induced peripheral neuropathy	Not tested	Protective	Not tested
High fat diet/hyperglycemia-induced peripheral neuropathy	Not tested	Protective	Not tested
Peripheral nerve injury			
Sciatic nerve transection	Not tested	Protective	Not tested
Sciatic nerve crush	Not tested	Not tested	Protective
Spinal cord disease			
<i>pnm</i> 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

Wld^S, SARM1, and P7C3 pathways converge on augmentation of neuronal NAD⁺ levels and offer protection across common preclinical models of nervous system damage and disease.

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 C57BL6/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. 1990b). Three years later, the responsible gene was mapped to the distal end of mouse chromosome 4 at a locus designated *Wld*, which was syntenic to human chromosomal region 1p34–1p36 (Lyon et al. 1993). The symbol *Wld^S* (Wallerian degeneration slow) was assigned to the mutant allele, and C57/Ola mice were renamed *Wld^S* mice. Over the next few years, the Perry laboratory progressively eliminated candidate genes in this region (Coleman et al. 1996) and ultimately identified an 85-kb tandem triplication of the distal region of chromosome 4 that was unique to C57BL/*Wld^S* among 36 strains tested and thus was a strong candidate for the protective mutation (Coleman et al. 1998). This triplication was later found to be

stably inherited across divergent breeding colonies of *Wld^S* 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 *Wld^S* 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 *Wld^S* 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 *Wld^S* (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-

phology. Furthermore, the protective effect was dose-dependently 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 biosynthetic activity and was named NMNAT2. The original NMNAT that had been identified as the human protein homologous to *D4Cole1e* 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), optimization of mitochondrial function (Avery et al. 2012; Fang et al. 2012), control of expression of axonal receptors for glial-mediated engulfment of degenerating axons (Fainzilber and Twiss 2006; Hoopfer 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; Coleman and Freeman 2010). The work by Araki et al. (2004) showed that NMNAT1 activity alone was sufficient to protect axons of explanted dorsal root ganglion neurons subjected 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 recently, 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 receptors that facilitate uptake of extracellular NAD⁺ into nerve cells (Bruzzone 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/lysosomes, 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* somehow inhibits SARM1 after 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 prominent feature of neuropathies and other neurodegenerative diseases (Saxena and Caroni 2007), this discovery has prompted exploration of the potential benefits of augmenting 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 deficient in the peripheral myelin component P0, a model of human peripheral neuropathy, and observed delayed motor 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 paclitaxel-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). Furthermore, the protection of neuronal cell bodies without preserving axons may be insufficient to prevent neurologic 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 application of *Wld^S* mice to the central nervous system was by Perry et al. (1991) through optic nerve transection experiments in which *Wld^S* mice showed delayed degeneration of retinal ganglion cells and their axons. Later work, however, showed that *Wld^S* rats were protected only from axon degeneration with no effect on retinal ganglion cell body deterioration after both optic nerve transection and a photocoagulation 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 degeneration ~8–9 mo of age, followed by retinal ganglion cell death. Crossing these mice with *Wld^S* mice yielded animals 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 (*pnn*) 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 *pnn* 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 SOD^{G37R} and SOD^{G85R} mouse models, *Wld^S* conferred no protection in disease onset, axon degeneration, synaptic integrity, or motor neuron death (Velde et al. 2004). In the SOD1^{G93A} 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 α /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 (Gerdtts 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 (Gerdtts et al. 2016). Around this same time, others showed that SARM1 ablation in NMNAT2-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 carbonyl 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 (Gerdtts 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 (Gerdtts 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 (Gerdtts et al. 2013; Summers et al. 2016). Indeed, dimerization of the TIR domain alone has been shown to induce rapid axon degeneration (Gerdtts 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 (Gerdtts 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; Verstaappen 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; Lavoie 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.

In the central nervous system, minimal protection of SARM1-deficient mice was noted after optic nerve crush, and subsequent retinal ganglion cell death still occurred, as was observed for *Wld^S* mice (Fernandes et al. 2018). The Freeman laboratory has shown that SARM1-deficient mice display reduced axon degeneration and preserved neuronal function after exposure to TBI (Henninger et al. 2016). With respect to spinal cord disease, elimination of SARM1 was not protective in the SOD1^{G93A} mouse model of ALS (Peters et al. 2018).

DISCOVERY OF THE P7C3 CLASS OF NEUROPROTECTIVE CHEMICALS

Studies of two paralogous transcription factors designated neuronal PAS domain protein 1 (NPAS1) and neuronal PAS domain protein 3 (NPAS3) have given evidence for their role in the control of adult hippocampal neurogenesis in mice (Pieper et al. 2005; Stanco et al. 2014; Michaelson et al. 2017). Animals deficient in the NPAS1 transcription factor display enhanced hippocampal neurogenesis. 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 coinubation 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 NMN and then NAD⁺ (Preiss and Handler 1958).

Metabolite analysis of U2OS cells exposed to 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 of ¹⁴C-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 *Wld^S* and SARM1 neuroprotection was discovered via unbiased genetic approaches, the in vivo discovery of the P7C3 family of

neuroprotective compounds was also unbiased. In combination, these three lines of evidence favor the concept of therapeutic utility of augmenting NAD⁺ synthesis in damaged or diseased neurons. The neuroprotective efficacy of P7C3 compounds has been reported for central and peripheral forms of nerve cell injury or disease.

P7C3 treatment of neonatal rats subjected to sciatic nerve crush revealed protection of both motor and sensory nerve degeneration (Kemp et al. 2015). The potentially protective activity of P7C3 in peripheral neurons has also been tested by the laboratory of William Clarke in a rat model of taxol-induced peripheral neuropathy. Robustly favorable measures of compound-mediated protection from both allodynia and nerve fiber degeneration were reported for this model of chemotherapy-induced peripheral neuropathy (LoCoco et al. 2017).

Beneficial effects of P7C3-mediated NAD-enhancement have been reported in the central nervous system for animal models of depression and anxiety (Walker et al. 2014; De Jesús-Cortés et al. 2016; Lee et al. 2016), TBI (Blaya et al. 2014), acute and chronic stroke (Loris et al. 2017a,b), and Alzheimer's disease (Choi et al. 2018; Voorhees et al. 2018). In addition to augmenting postnatal neurogenesis, P7C3 compounds also block axon degeneration and neuronal cell death in these models, with correlative protection from deficits in cognition and motor function. Also in TBI, Dutca et al. (2014) reported that (–)-P7C3-S243-mediated protection of retinal ganglion cell function subsequent to blast-mediated TBI. In both MPTP and 6-OHDA rodent models of Parkinson's disease, P7C3 compounds display a dose-dependent ability to preserve motor function and block degeneration of substantia nigra dopaminergic neurons and their axonal projections to the striatum (De Jesús-Cortés et al. 2012, 2015; Naidoo et al. 2014). Finally, P7C3-A20 has recently been found to promote the survival of young hippocampal neurons in nonhuman primates (Bauman et al. 2018). In the latter study, adult male rhesus monkeys receiving 38 wk of daily, orally administered P7C3-A20 displayed sustained plasma exposure and elevated levels of postnatally born hippocampal neurons (Bauman et al. 2018).

CONCLUSION

We close this review with emphasis on the serendipity involved in the discoveries of *Wld^S*, 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-

Figure 1. Three independent and unbiased discoveries have revealed neuroprotective efficacy of preservation or augmentation of NAD⁺ levels in nerve cells.

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.

ACKNOWLEDGMENTS

A.A.P. is supported by the Brockman Medical Research Foundation, the Elizabeth Ring Mather & William Gwinn Mather Fund, the S. Livingston Samuel Mather Trust, and the Department of Veterans Affairs Merit Review 11O1BX002444 to A.A.P. A.A.P. and S.L.M. thank Sandi Estil, Joseph Ready, Gelin Wang, Ting Han, and many other coworkers who have been instrumental in the discovery and characterization of P7C3. Work on the discovery and characterization of the P7C3 neuroprotective molecule was supported by a National Institutes of Health (NIH) Director's Pioneer Award and the provision of research funds to S.L.M. by an anonymous donor. The contents of this manuscript do not represent the views of the U.S. Department of Veterans Affairs or the U.S. Government.

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Cold Spring Harbor Symposia on Quantitative Biology

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Andrew A. Pieper and Steven L. McKnight

Cold Spring Harb Symp Quant Biol 2018 83: 207-217 originally published online February 20, 2019

Access the most recent version at doi:[10.1101/sqb.2018.83.037622](https://doi.org/10.1101/sqb.2018.83.037622)

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