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NMNAT: It's an NAD⁺ Synthase... It's a Chaperone... It's a Neuroprotector

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Abstract

Nicotinamide mononucleotide adenylyl transferases (NMNATs) are a family of highly conserved proteins indispensable for cellular homeostasis. NMNATs are classically known for their enzymatic function of catalyzing NAD⁺ synthesis, but also have gained a reputation as essential neuronal maintenance factors. NMNAT deficiency has been associated with various human diseases with pronounced consequences on neural tissues, underscoring the importance of the neuronal maintenance and protective roles of these proteins. New mechanistic studies have challenged the role of NMNAT-catalyzed NAD⁺ production in delaying Wallerian degeneration and specified new mechanisms of NMNAT's chaperone function critical for neuronal health. Progress in understanding the regulation of NMNAT has uncovered a neuronal stress response with great therapeutic promise for treating various neurodegenerative conditions.

Introduction

Humans have three *NMNAT* genes that produce three NMNAT protein isoforms with distinct tissue expression patterns and subcellular localizations [1]. NMNAT1 is ubiquitously expressed and is enriched in the nucleus. NMNAT2 is predominantly expressed in the brain and is localized to the cytosol and enriched in membrane compartments. NMNAT3 is also widely expressed but is highest in liver, heart, skeletal muscles, and red blood cells [2]. NMNAT3 is reported to have two splice variants, encoding mitochondrial localized FKSG76 and cytosolic NMNAT3v1, though the expression and function of these endogenous protein variants are still under question [3]. Given the essential role for NAD⁺ in cellular metabolism, it is not surprising that the enzyme is required for the survival of all living organisms, from archaeobacteria to humans. The discovery of the remarkable neuroprotective function of NMNAT proteins sparked a burst of investigations on NMNAT in the nervous system. More recently, NMNAT mutations have been identified to cause a severe form of retinal degeneration and NMNAT deficiency has been associated with complex neurological diseases. In this review, we focus on the function of NMNAT in the nervous system and

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discuss the recent advances in understanding the regulatory mechanisms of neuronal maintenance that are relevant for neuroprotective therapies against neurodegenerative conditions. For previously published reviews that focus on the neuroprotective effects of NMNAT particularly in axon degeneration and injury, we refer readers to [1,4–6].

Genetic links between NMNAT and diseases of the nervous system

The only known monogenetic disease associated with NMNAT proteins is Leber congenital amaurosis (LCA), one of the most common forms of inherited blindness in children. Compound heterozygous or homozygous mutations in *NMNAT1* cause LCA9 (OMIM 608553), an autosomal recessive condition characterized by severe early-onset and rapid progression of vision loss and retinal degeneration [7]. To date more than 30 mutations spread across the *NMNAT1* gene have been reported, including missense, nonsense, and splicing mutations (Figure 1) [8–11]. Importantly, most LCA9 patients have reported normal physical and mental health, suggesting a specific requirement of NMNAT1 for maintenance of the neural tissue in the retina [9].

So far no human diseases have been shown to be directly caused by mutations in *NMNAT2* or *NMNAT3*, though several studies have signified a putative contribution of NMNAT deficiency to the progression of complex neurological diseases. Microarray studies have shown that *NMNAT2* mRNA levels are reduced in various neurodegenerative diseases, including Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease [12–14]. In AD, *NMNAT2* transcript levels negatively correlate with cognitive dysfunction and AD pathology [15]. Genome-wide association studies have indicated a SNP (*rs952797*) located 126 kb downstream of the *NMNAT3* gene that is associated with late-onset AD [16]. Although these association studies do not demonstrate a causal relationship, they do recommend NMNAT as an important contributor to neuronal health.

Molecular functions of NMNAT necessary for neuronal maintenance and protection

LCA9 and other neurological disease phenotypes associated with NMNAT deficiency are consistent with its neural maintenance function, yet the neuroprotective capacity of NMNAT had already emerged with the serendipitous discovery of the slow Wallerian degeneration mutant (*Wld^Δ*) mouse [17,18]. In the *Wld^Δ* mouse, degeneration of the distal axon following axotomy (Wallerian degeneration) is remarkably delayed by a dominant mutation causing overexpression and redistribution of Nmnat1 to the cytoplasmic compartment of the axon [4]. The neuroprotective role of NMNAT is efficacious against not only axonal injury, but also models of various neurodegenerative conditions such as toxic neuropathy, glaucoma, spinocerebellar ataxia, tauopathy, and Huntington's disease [1,5,19–25]. Furthermore, NMNAT homologues across species, including archaeobacteria, yeast, fly, mouse, and human, exhibit a conserved cytoprotective function, even when expressed in different model organisms [1,19]. While these studies chronicle the potent and conserved protection NMNAT proteins confer against a variety of neuronal insults, the precise mechanism by which NMNAT engenders this protection has been hard to pinpoint.

NMNAT proteins have two distinct functions that can bestow neuronal maintenance and protection: NAD synthase activity and chaperone activity [26]. NMNAT catalyzes the reversible conversion of NMN (nicotinamide mononucleotide) to NAD⁺ in the final step of both the *de novo* biosynthesis and salvage pathways. NAD⁺ is a vital coenzyme important for metabolism and redox biology, as well as a substrate in multiple signaling processes [27]. Thus, NMNAT enzymes play a straightforward role in neuronal maintenance via balancing NAD⁺ consumption with production. A molecular chaperone function has also been demonstrated by a number of NMNAT proteins tested, including *Drosophila* Nmnat, mouse Nmnat2, and human NMNAT3 [15,19,28]. *In vitro* these NMNAT proteins bind to client proteins and prevent thermal-stress induced unfolding [15,19]. Thus, NMNAT chaperones can contribute to neuronal protein homeostasis.

There is still considerable controversy surrounding the mechanism of NMNAT-mediated neuroprotection. The prevailing hypothesis is that NMNAT overexpression provides continuous enzyme activity in injured neurons, thus preventing the consequent decrease in NAD⁺ and the accumulation of the precursor NMN [6]. However, recent work revealed that NAD⁺ depletion following axon injury is due to a dramatic increase in consumption by the pro-degenerative SARM1 (sterile alpha and TIR motif-containing 1), but that overexpression of cytoplasmic Nmnat1 blocks SARM1-dependent NAD⁺ consumption without increasing NAD⁺ synthesis [29]. Thus, NMNAT-mediated axon protection hinges on its ability to block pro-degenerative SARM1 signaling, but this does not rely on enzymatic conversion of NMN to NAD⁺. One alternative is that NMNAT provides neuroprotection via an enzyme-independent function, leading to the hypothesis that NMNAT mediated protection is chaperone-dependent. Recently, it has been shown that Nmnat2's enzyme activity is dispensable for relieving the toxic phosphorylated tau burden in a model of fronto-temporal dementia and parkinsonism 17 (FTDP-17), and that Nmnat2 complexes with the classical HSP90 chaperone, possibly to promote refolding of toxic tau [15]. In another study, overexpression of cytoplasmic Nmnat1 partially preserved neuronal function in a model of early-onset FTDP-17 by decreasing insoluble tau aggregates, without altering phosphorylated tau [30]. Similarly, overexpression of yeast homologs of NMNAT, NMA1 and NMA2, suppresses proteotoxicity in yeast models of polyglutamine- and α -synuclein-induced neurodegeneration also by enhancing clearance of misfolded proteins [22]. Though the precise mechanism may vary by disease model, it is clear that NMNAT overexpression alleviates proteotoxic stress in neurodegenerative conditions associated with protein misfolding, consistent with a chaperone function. Further supporting the chaperone function of NMNAT is the identification of its endogenous 'client' protein in *Drosophila* synapses, where Nmnat maintains active zone structure by directly interacting with the active zone protein, Bruchpilot (BRP) in an activity-dependent manner [28]. Perhaps a source of resistance to this hypothesis is that although enzyme-inactive mouse Nmnat proteins fail to protect axons [31–33], enzyme-inactive *Drosophila* Nmnat is sufficient to protect against activity-induced retinal degeneration, Wallerian degeneration, and axonal degeneration induced by loss of JNK (c-Jun N-terminal kinase); in these conditions NMNAT chaperone-dependent protection is perhaps less implicit since here the client for such a chaperone role is not apparent [34–36]. Continued advances in NMNAT overexpression-mediated protection in neurodegenerative models will be invaluable for identifying not only the

underlying mechanisms of NMNAT's protection, but also by extension, the primary triggers of neurodegeneration.

Mouse models of NMNAT1-dependent LCA recapitulate several aspects of human disease and further delineate disease progression; importantly, degeneration occurs after retinal development, is observed first within the photoreceptors, and is only observed in inner retinal neurons and RPE (retinal pigment epithelia) cells in advanced stages [37]. The sensitivity of the retina, therefore, may reflect a twofold requirement of NMNAT: biosynthesis of NAD⁺ and chaperone activity for meeting the high metabolic and protein turnover demands of photoreceptors, respectively. Although LCA mutations are widely distributed across the *NMNAT1* gene, most NMNAT1 mutant proteins characterized exhibit several common features. NAD⁺ synthase activity is reduced by most mutations *in vitro*, yet patient fibroblasts exhibit normal basal NAD⁺ levels, suggesting that enzyme deficiency is not a primary cause of disease [10,38]. Importantly, ectopic expression of several LCA9 NMNAT1 mutants is sufficient to protect cultured neurons from Wallerian degeneration, indicating that LCA9-causing mutations do not disrupt the qualitative neuroprotective properties of NMNAT proteins [38]. However *in vitro*, NMNAT1 mutant proteins are more susceptible to stress-induced unfolding compared to wild type protein, which may amplified *in vivo* to cause disease [38]. Interestingly, *Drosophila* photoreceptors are also particularly vulnerable to loss of Nmnat, but Nmnat's enzyme activity is completely dispensable for maintenance of retinal health and function in this model [34]. So while LCA9 may be primarily attributed to a loss of NMNAT1 protein stability, more studies are needed to determine whether retinal degeneration arises from a consequential loss of NAD synthesis and/or loss of the molecular chaperone function (Figure 1).

Regulation of NMNAT during stress and disease

Due to the essential neuronal maintenance role of NMNAT and its capacity for neuroprotection, understanding the regulation of NMNAT levels becomes key to unlocking its therapeutic potential. It is critical that neurons maintain sufficient levels of NMNAT2; NMNAT2 loss is considered an initiating event in Wallerian degeneration and also appears to be consistent in progression of neurodegenerative conditions [39]. At the protein level, NMNAT2 is constitutively degraded by the ubiquitin-proteasome system, the exact players of which are beginning to emerge. Several ubiquitin ligases, including Phr1 (Highwire in *Drosophila*), Skp1a, and Fbxo45, have been identified, though direct evidence of NMNAT ubiquitination is lacking [40–42]. Genetic interference of these ligases increases NMNAT levels and subsequently enhances neuronal protection. In addition to protein regulation, two functional cAMP-response elements (CREs) have been identified in the *Nmnat2* promoter region that influence expression at the transcriptional level. In an FTDP-17 tauopathy model, a pathological reduction of phospho-CREB (CRE binding protein) levels and binding with the *Nmnat2* promoter resulted in decreased Nmnat2 transcript and protein [24]. Slowing or preventing NMNAT protein turnover and transcriptional repression can be a useful strategy for treating a variety of neurological diseases (Figure 2).

In contrast to mammals that use three *NMNAT* genes to produce three different NMNAT protein isoforms, *Drosophila* has only one *Nmnat* gene that is alternatively spliced to

generate two variants: *Nmnat-RA* mRNA variant encodes the nuclear Nmnat-PC protein isoform and *Nmnat-RB* variant encodes the cytosolic Nmnat-PD isoform. In a *Drosophila* model of spinocerebellar ataxia 1 (SCA1), overexpression of Nmnat-PD significantly improved behavioral and morphological defects by reducing neuronal mutant human Ataxin-1 (hAtx1-[82Q]) aggregates, yet surprisingly, overexpression of Nmnat-PC increased aggregate size and exacerbated neurodegeneration [43]. Importantly, cytoplasmic targeted Nmnat-PC is also ineffective in relieving hAtx-[82Q] proteotoxicity. This study revealed a functional divergence underlying *Drosophila* Nmnat isoform-specific neuroprotection. Taken together with the previous finding that the transcription of *Drosophila Nmnat* pre-mRNA is increased under various stress conditions[44], the observation that stress also drives post-transcriptional alternative splicing to preferentially generate *Nmnat-RB* sheds light on the complex neuronal stress response to achieve self-protection [43]. Therefore, alternative splicing in *Drosophila* uses one gene to produce two functionally exclusive mRNA variants: one housekeeping variant, *Nmnat-RA*, that is stably expressed under normal conditions, and one stress response variant, *Nmnat-RB*, that can be quickly induced under stress conditions for neuronal protection (Figure 3).

Regulation of *Drosophila* Nmnat under neuronal stress may provide insight into regulation of NMNAT2 in mammals in neurodegenerative conditions. Notably, the transcript level of the neuroprotective *Drosophila* variant *Nmnat-RB* is reduced in late stages of models of tauopathy and SCA1, however it is increased in the early stages, suggesting an upregulation to combat pathogenic protein aggregation during disease progression that is perhaps overwhelmed at more advanced stages of neuronal degeneration and cell death [43]. Similarly, although many studies show a decrease of *NMNAT2* transcript postmortem in patients with neurodegenerative diseases, there is also evidence of increased levels at the early stages of disease. For example, in mild cognitive impairment and early stages of AD, *NMNAT2* mRNA levels are higher in the frontal cortex of patients exhibiting high levels of oxidative stress-induced neuronal DNA damage, compared to those with lower levels of DNA damage [45]; at this stage the nervous system is mounting a stress response as HSP90 is also upregulated in patient brains with high oxidative damage. In a genome-wide gene-expression study of another mouse model of FTDP-17, *Nmnat2* transcript is significantly upregulated in the hippocampus during early disease progression, but is downregulated at end stages of disease when neurofibrillary tangle burden is heavy [46]. Here the early rise of Nmnat2 is likely a proteotoxic stress response, while the late decrease is consistent with observed downregulation of synaptic genes corresponding to synaptic loss and upregulation of inflammatory and apoptotic genes indicative of neuronal cell death. Since endogenous upregulation of NMNAT under stress conditions seems to be insufficient to maintain neural integrity in the long term, NMNAT and its transcriptional and post-transcriptional regulation can serve as a therapeutic target for pharmacological intervention to slow the progression of neurodegenerative diseases.

Concluding remarks

The long life of neurons, up to 100 years in humans, makes neuronal maintenance an important challenge. It is conceivable that compromised maintenance would result in degeneration in an age-dependent manner. The emergence of the chaperone function of

NMNAT, an NAD⁺ synthase, specifically in the nervous system for neuronal maintenance exemplifies an evolutionarily conserved strategy of ‘repurposing’ (or ‘moonlighting’) housekeeping enzymes. The transcriptional and post-transcriptional regulation of NMNAT expression under stress conditions reveals the intricate balance of metabolic activity and stress response in neurons. Dissecting such regulatory mechanisms has huge implications for neuroprotective therapeutics as increasing NMNAT protein levels is sufficient to augment neuronal maintenance. Identifying druggable targets to enhance expression and/or reduce protein degradation would be a pharmacologically approachable way to achieve higher NMNAT protein levels and therefore confer neuroprotection.

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*Special interest

**Outstanding interest

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Highlights

- NMNATs are highly conserved multifunctional neuronal maintenance proteins.
- NMNAT deficiency is genetically linked with neurodegenerative diseases.
- NMNAT-mediated neuroprotection is contributed by NAD⁺-independent mechanisms.
- Advances in understanding NMNAT regulation have great therapeutic potential.

Figure 1. Genetic and transcriptional alterations of *NMNATs* in human diseases

Mutations across human *NMNAT1* gene cause LCA9, characterized by early-onset and rapid progression of vision loss and retinal degeneration. Decreased mRNA levels of human *NMNAT2* and *NMNAT3* are correlated with cognitive dysfunction and neurodegeneration. In both conditions, the NMNAT protein stability or levels are decreased affecting their enzyme activity and/or molecular chaperone capacity and comprising the neuroprotective function.

Figure 2. Regulation of NMNAT in neurons

Under stress conditions, binding of transcription factors to the *NMNAT* promoter region induces transcription as a neuronal self-protective mechanism. Under pathological conditions, binding of transcription factors to the promoter region is reduced, which leads to decreased NMNAT protein levels and exacerbates neurodegeneration. Constitutive proteasomal degradation of neuroprotective NMNAT is regulated by an atypical ligase complex comprising Skp1a, Phr1, and Fbxo45. Potential therapeutic targets include maintaining or enhancing *NMNAT* transcription (1) and suppressing NMNAT degradation (2) to combat neurodegeneration in a wide variety of settings.

NMNATs and Human Diseases

Figure 3. Transcriptional regulation of *Drosophila Nmnat* under stress

Under heat shock or hypoxic stress, *Drosophila Nmnat* transcription is upregulated directly through heat shock factor (HSF) or indirectly through hypoxia-inducible factor-1 α (HIF-1 α). Proteotoxic stress (in models of proteinopathy, such as tauopathy and spinocerebellar ataxia) also induces transcriptional upregulation of *Drosophila Nmnat*, although the mechanism remains to be determined. In neurons, the splicing of *Nmnat* pre-mRNA is switched to *Nmnat-RB* under stress conditions to promote the production of Nmnat-PD, therefore achieving neuronal protection (Figure modified from [43]).