NMNAT3 is protective against the effects of neonatal cerebral hypoxia-ischemia

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Abstract

Objective: To determine whether the NAD⁺ biosynthetic protein, nicotinamide mononucleotide adenylyltransferase-3 (NMNAT3), is a neuroprotective inducible enzyme capable of decreasing cerebral injury after neonatal hypoxia-ischemia (H-I) and reducing glutamate receptor-mediated excitotoxic neurodegeneration of immature neurons. Methods: Using NMNAT3-overexpressing mice we investigated whether increases in brain NMNAT3 reduced cerebral tissue loss following H-I. We then employed biochemical methods from injured neonatal brains to examine the inducibility of NMNAT3 and the mechanism of NMNAT3-dependent neuroprotection. Using AAV8-mediated vectors for in vitro neuronal NMNAT3 knockdown, we then examine the endogenous role of this protein on immature neuronal survival prior and following NMDA receptor-mediated excitotoxicity. Results: NMNAT3 mRNA and protein levels increased after neonatal H-I. In addition, NMNAT3 overexpression decreased cortical and hippocampal tissue loss 7 days following injury. We further show that the NMNAT3 neuroprotective mechanism involves a decrease in calpastatin degradation, and a decrease in caspase-3 activity and calpain-mediated cleavage. Conversely, NMNAT3 knockdown of cortical and hippocampal neurons in vitro caused neuronal degeneration and increased excitotoxic cell death. The neurodegenerative effects of NMNAT3 knockdown were counteracted by exogenous upregulation of NMNAT3. Conclusions: Our observations provide new insights into the neuroprotective mechanisms of NMNATs in the injured developing brain, adding NMNAT3 as an important neuroprotective enzyme in neonatal H-I via inhibition of apoptotic and necrotic neurodegeneration. Interestingly, we find that endogenous NMNAT3 is an inducible protein important for maintaining the survival of immature neurons. Future studies aimed at uncovering the mechanisms of NMNAT3 upregulation and neuroprotection may offer new therapies against the effects of hypoxic-ischemic encephalopathy.

Introduction

Neonatal cerebral hypoxia-ischemia (H-I) is an often devastating condition constituting a significant cause of newborn death, and chronic neurological and neurodevelopmental disability in children.¹ Despite its relatively high incidence among newborns with cerebral injury, limited therapies are available for its prevention and treatment.²,³ Furthermore, unlike the aging adult brain, neuronal death in the immature CNS is not only a pathological cellular event of brain injury but also a biological process required for early normal brain development.⁴ Therefore, understanding the cellular mechanisms that actively modulate neuronal survival in the neonatal brain may help better understand the cellular pathways that regulate the cell fate of developing neurons as well as provide

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potential new approaches for the treatment of H-I in newborns.

The mammalian family of NAD\(^+\) metabolizing enzymes, nicotinamide mononucleotide adenylyltransferases (NMNATs), may play an important role in the regulation of neuronal survival during brain development, and their neuroprotective actions could offer new targets against the effects of neonatal H-I. NMNATs are potent inhibitors of axonal degeneration in the peripheral nervous system, they consist of three structurally distinct isoforms differentially expressed in all tissues, including brain, and each localized to different cellular compartments.\(^5\)\(^-\)\(^8\) Although the neuroprotective actions of NMNATs in the mammalian brain remain largely unexplored, recent evidence suggests that NMNATs are also strongly protective against axonal and neuronal CNS degeneration. For example, increases in the expression of NMNAT1 and NMNAT3 decrease mouse retinal ganglion cell degeneration following ischemia.\(^9\)\(^,\)\(^10\) and overexpression of cytoplasmic NMNAT1 protects the term-equivalent neonatal mouse brain against the degenerative effects of cerebral H-I via inhibition of glutamate-dependent excitotoxic cell injury.\(^11\) In contrast, depletion of NMNAT2, the NMNAT isoform most abundant in the brain, restricts the outgrowth of retinal and cortical fibers in vitro.\(^12\) Interestingly, mutations in NMNAT1 have been recently identified as a cause of Leber congenital amaurosis, a degenerative disease of the young human retina,\(^13\) and genome-wide and linkage analysis have demonstrated an association between NMNAT3 and late-onset Alzheimer's disease.\(^14\)

The above observations suggest that all three NMNAT isoforms are likely important for the survival of both, peripheral axons and CNS neurons and their processes. Nevertheless, the neuroprotective role of each of these proteins following neonatal H-I remains poorly understood. In this study, we used NMNAT3-overexpressing mice and AAV8 vectors to manipulate NMNAT3 endogenous and exogenous expression in order to examine whether NMNAT3 protects the immature brain from the degenerative effects of neonatal cerebral injury. We find that alterations in the expression of neuronal NMNAT3 in vivo and in vitro significantly modify the survival of healthy and injured immature neurons possibly via inhibition of glutamate-dependent excitotoxic degeneration involving the calpastatin/caspase/calpain-mediated death pathway. These data suggest that NMNAT3 is important for the survival of developing healthy and injured neurons following neonatal H-I. Investigating the neuroprotective actions of NMNAT3 in the ischemic immature brain may offer new putative cellular targets for the prevention and treatment of perinatal brain injury, and may also provide new insights into the cellular mechanisms that modulate cell death in the healthy and injured developing brain.

Methods

Animals and surgical procedures

Mice overexpressing the mouse NMNAT3 gene (B6.J.B6D2-Tg; CAG-Nmnat3; line #819) under the control of the chicken \(\beta\)-actin promoter on a C57BL/6 background were derived from sperm obtained from RIKEN (Saitama, Japan) as previously described.\(^15\) To produce NMNAT3 and wild-type littermate pups for the H-I experiments, NMNAT3 mice on a C57BL/6 background were bred to C57BL/6 nontransgenic, wild-type, mice (Charles Rivers Labs, New York). Genotyping was performed by PCR on genomic DNA from the toes of animals using the following primers: NMNAT3-Tg, 5'-GACCGGGCGGCTCTAGAGCCTCTGCTAA-3' and 5'-GCTCAAAGGCTCTCAGATGTGCCAATA-3'. Animal care and use in our laboratory were in strict accordance with the National Institute of Health guidelines on the use of laboratory animals. All procedures were also approved by the animal studies committee at Washington University.

 Neonatal hypoxic-ischemic brain injury was performed postnatal day 7 mice pups as previously described.\(^16\) Only pups with a body weight greater than 3.0 g at time of surgery (P7) were used in this study. Briefly, pups were anesthetized using 3% halothane for induction and 2.5% halothane for maintenance (balance room air). Under anesthesia, blood flow through the left carotid artery was permanently interrupted by carotid artery cautery through an incision in the neck. The pups were returned to the dam and allowed to recover for a minimum of 3 h before being placed at 36.5°C in a thermoregulated hypoxia chamber containing 8% oxygen (balanced with nitrogen) for a period of 15 or 45 min. After completion of H-I, pups were returned to the dam until the day they were euthanized.

Histology and determination of tissue loss

At 7 days following HI, P14 mice pups were deeply anesthetized with pentobarbital (150 mg/kg) and perfused transcardially with ice-cold PBS containing 3 U/mL heparin. Brains were removed and immersion fixed in 4% (w/v) paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at 4°C for 24 h, and then cryoprotected in 30% (w/v) sucrose in 0.1 mol/L phosphate buffer until freezing in powdered dry ice and sectioned into 50 \(\mu\)m coronal sections with a freezing sliding microtome. The uninjured, contralateral hemisphere was noted before sectioning by nicking that hemisphere with a razor blade. A set
of coronal brain sections (spaced 50 μmol/L apart) beginning at the genu of the corpus extending to the mid body of the dorsal hippocampus were mounted in DAPI mounting medium (Vector Labs) and imaged via Nanozoomer (Hamamatsu Photonics). Percent tissue loss was calculated by comparing areas of distinct brain regions (cortex, striatum, and hippocampus) in the injured hemisphere (left) and uninjured hemisphere (right) using the Nanozoomer imaging software as previously described. Three coronal sections per quantification area per animal were used for each of the brain regions studied. Striatal volume was calculated using those sections containing the striatum at the level of the genu of the corpus callosum. Hippocampal injury was determined by analyzing sections containing all layers of the dorsal hippocampal formation as seen in Figure 3C. Cortical areas were analyzed on the sections that contained the dorsal hippocampus. The investigators were blinded to the genotype during area analysis.

**Immunohistochemistry and immunofluorescence**

To evaluate the localization of NMNAT3 in the brain, pup brain tissue was sectioned as previously described. Brain sections were processed for DAB- or fluorescent-based immunohistochemistry utilizing the free-floating method. Primary antibodies used were NMNAT3 (1:200, Santa Cruz), NF200 (1:500, Millipore), and NeuN Alexa Fluor® 488 conjugate (1:100, Millipore). Antigen retrieval was undertaken in 1 mg/mL pepsin in 0.2 mol/L HCl at 37°C for 10 min. Endogenous peroxidases were blocked with 0.3% H2O2 diluted in TBS (DAB-IHC only), and proteins were blocked using 3% dried milk in Tris-buffered saline and 0.25% (vol/vol) Triton-X (TBS-X). Brain sections were incubated with primary antibodies in diluting buffer (1% dried milk in TBS-X) overnight at 4°C, followed by incubation in fluorescent-tagged secondary antibodies Alexa Fluor® 488 donkey anti-mouse or Alexa Fluor® 568 donkey anti-goat (1:500, Life Technologies) or biotinylated donkey anti-goat secondary antibody (1:500, Jackson Immunoresearch Lab) followed by a final incubation in avidin-biotin-horseradish peroxidase solution (Vectastain Elite ABC kit; Vector Laboratories). Detection of the bright-field immunohistochemical sections was achieved using 3,3′-diaminobenzidine (DAB) chromogen (Sigma).

**Caspase-3 activity assay**

At 24 h following the end of H-I, mice pups were sacrificed by rapid decapitation. Brains were extracted and the left and right hippocampi dissected and frozen using dry ice. Each hippocampus was homogenized in lysis buffer (50 mmol/L HEPES, 5 mmol/L DTT, 0.1 mmol/L EDTA, 0.1% CHAPS, pH 7.4) and centrifuged at 10,000 x g for 15 min at 4°C. Tissue lysates incubated in a 96-well plate with 40 μL of assay buffer (100 mmol/L NaCl, 50 mmol/L HEPES, 10 mmol/L DTT, 1 mmol/L EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4) containing 30 μmol/L of the substrate Ac-DEVD-AMC using a Caspase-3 Activity Assay Kit according to the manufacturer’s instructions (Calbiochem/Millipore). The emitted fluorescence was measured every 10 min for 120 min at 37°C at an excitation wavelength of 360 nm and an emission wavelength of 460 nm using a fluorescent microplate reader (Bio-Tek Instruments). Protein concentration of each hippocampal lysate was determined by Bradford Protein Assay (Bio-Rad). DEVD-AMC cleavage activity was obtained from the slope by plotting fluorescence units against time. The enzyme activity was calculated as pico moles of AMC generated per milligram of protein per minute.

**Western blotting**

Hippocampal lysates prepared as described above for the caspase assay were subjected to Western blotting after the addition of protease inhibitors (Roche). For high-molecular-weight proteins, samples were resolved on 8–16% Tris-Glycine gels; for low-molecular-weight proteins, samples were resolved on 4–12% Bis-Tris NuPage gels (Invitrogen). Proteins (20 μg/lane) were separated by SDS-PAGE and transferred to nitrocellulose membranes using an iBlot (Life Technologies). Blots were blocked with 5% dried milk in TBS containing 0.05% Tween-20 overnight at 4°C. Blots were then incubated for 3 h at room temperature in primary antibody: NMNAT3 (1:200, Santa Cruz), Calpastatin (1:1000, CST), Alpha-spectrin (1:500, Millipore), or Beta-actin (1:50,000, Sigma). Membranes were then incubated with the appropriate HRP-labeled secondary antibody (1:2000, Santa Cruz), developed using either SuperSignal West Pico (Pierce) or ECL Ultra (Luminogen), and visualized on an ImageStation (Syngene). The resulting blot images were quantified using ImageJ software.

**Hippocampal and cortical neuronal cell culture and excitotoxicity assay**

The cortex and hippocampus were microdissected from embryonic day 18 C57BL/6 embryos incubated in Hank’s balanced salt solution with 2.5% (10x) trypsin for 7 min at 37°C. Cortices and hippocampi were gently triturated, diluted in neurobasal media seeded in 24-well plates pre-coated with poly-L-Lysine. To avoid plating neurons at different densities in different wells and to allow for the
quantification of LDH activity and MAP2 immunofluorescence in the same well, cells were plated at the same time from a common dissociated neuronal preparation at a calculated equal neuronal concentration of 100,000 cells/well; all neurons were treated equally to all the conditions except the dependent variable of viral exposure status. Cells were maintained in neurobasal media plus 1X B-27 supplement, 0.5 mmol/L L-glutamine with penicillin-streptomycin, and 50% of the media was replaced every 3 days. At DIV12 cultured neurons were washed with a controlled salt solution (CSS) containing in mmol/L: 120 NaCl, 5.4 KCl, 1.2 CaCl2, 15 glucose, and 25 Tris-HCl, pH = 7.40, at room temperature. Magnesium was omitted to avoid blockade of the NMDA receptor channel. Cells were then exposed to 50 µmol/L L-ibotenic acid with 100 µmol/L glycine in CSS or CSS alone for 5 min then washed an incubated in neurobasal media for 24 h prior to cytological assessment by immunofluorescence and LDH quantification as previously described.19

In vitro immunofluorescence quantification

Quantification of neurites was achieved by staining PFA-fixed cortical and hippocampal neurons with a mouse MAP2 antibody (1:1000, Millipore). Sample digital images of MAP2 stained neurons of a fixed area were obtained from the same location in the 24-well plate of control or experimental cultures blindly and the amount of immunofluorescence was quantified utilizing ImageJ analysis software.18 Data were expressed as the amount of MAP2 stain per unit area. A single N value represents average stain quantified per area per a single culture well.

Lactate dehydrogenase assay

At 24 h postexcitotoxic injury with L-ibotenic acid or prior to paraformaldehyde fixation, the media of cortical and hippocampal neuron cultures were collected and assayed for lactate dehydrogenase (LDH) using a cytotoxicity detection kit (Roche) as previously described.11 Briefly, 100 µL of culture medium per well/sample in duplicate was incubated with gentle shaking with the kit reagents for 30 min in the dark, after which the product was measured at 490 nm utilizing a standard 96-well microplate reader (Bio-Tek Instruments). Following collection of conditioned media for LDH, cells were fixed with 4% paraformaldehyde in phosphate buffer for 30 min at room temperature. After washing with PBS, cells were permeabilized with PBS-X, blocked in normal serum, and probed for immunocytochemistry.

Real-time qPCR analysis

Real-time qPCR was used for mRNA quantitation of dissociated culture neurons or the microdissected hippocampus and cerebral cortex. At 1 day, 3 days, and 7 days post-H-I pups were sacrificed by lethal injection of pentobarbital (150 mg/kg) and decapitated. The right and left hemispheres were separated, and hippocampal and cortical tissue were dissected out. RNA was extracted from frozen hippocampal and cortical tissue using the RNaseasy kit (Qiagen). Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription kit (Life Technologies). Real-time qPCR was conducted with TaqMan primers (Life Technologies) and the TaqMan Universal PCR Master Mix (Life Technologies) using an ABI Prizm 7500 Thermocycler. Relative gene expression levels in NMNAT3 Tg and NMNAT3 WT mice in different brain regions were compared using the ΔΔCt method with 18S mRNA as a control reference.

NMNAT3 knockdown and NMNAT3 overexpression using adeno-associated viral vectors

Five candidate mouse shNMNAT3 clones were initially obtained (McDonnell Genome Institute, Washington University, St. Louis, MO) and introduced into HeLa cells utilizing lipofectamine-mediated transfection. We then identified a shNMNAT3 clone capable of reducing endogenous NMNAT3 mRNA levels in these cells by approximately 90% (88 ± 9%). This shRNA was also able to decrease the NMNAT3 mRNA levels of wild-type non-infected and AAV8-mediated NMNAT3 overexpressing neurons by approximately 40% (Fig. 6A and B). The candidate shNMNAT3 construct (NM_144533.1-870s1c1) was then engineered into a serotype 8 adeno-associated viral vector (AAV8) under the control of a U6 promoter also containing a CMV-GFP reporter (Washington University Viral Core, St. Louis, MO). AAV serotype 8 vector was chosen due to its high tropism toward neurons. For all viral experiments, cells were exposed to 7.1E+9 vg/µL of AAV8-shNMNAT3 virus or AAV8-shScramble control (unless otherwise specified) until the cells and cultured media were examined or the cells subjected to the excitotoxic death assay as described above. For exogenous NMNAT3 overexpression of dissociated cortical and hippocampal neurons, an AAV8 virus was produced containing the human sequence of NMNAT3 (MIM:608702; Location 3q23) under the regulation of the neuron-specific synapsin-1 promoter. This vector when used at a concentration of 8.3E10 + 9 vg/100 K cells was found to increase neuronal NMNAT3 mRNA by 98 ± 8.8-fold 6 days postinfection (Fig. 6A).

Neurons

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were infected with the AAV8-syn1-NMNAT3-overexpressing vector at a concentration of 8.3E10 + 9 vg/µL unless otherwise specified.

Statistics

Data are presented as mean ± SEM and were compared using one-way ANOVA followed by Bonferroni test for multiple comparisons, one-sample t-test for hypothetical value comparisons, and one- or two-tailed Student’s t-test for two-group comparisons. Statistical significance was set at 0.05, where *P < 0.05, **P < 0.01, and ***P < 0.001. Statistics were performed using GraphPad Prism (GraphPad Software).

Results

Cerebral protein expression and localization in NMNAT3 transgenic mice

In order to examine the neuroprotective properties of NMNAT3 following neonatal brain injury, we utilized NMNAT3-overexpressing transgenic mice (mouse line #819) previously described to have decreased axonal degeneration following sciatic nerve injury. We first aimed at characterizing the cerebral localization of NMNAT3 in the young postnatal mouse brain utilizing conventional immunohistochemical and immunoblotting methods. Compared to wild-type animals, NMNAT3 immunoreactivity (IR) of NMNAT3-overexpressing transgenic mice (NMNAT3-Tg) is highly abundant being predominantly found in the hippocampus and cerebral cortex (Fig. 1A). NMNAT3-IR was mainly neuronal as noted by its primary presence in NeuN+ neurons (Fig. 1B). NMNAT3-IR was also found to a much lesser extent in the hippocampal regions of high neurite density indicating that NMNAT3 is partially and differentially distributed across the neuronal axis that include the cytoplasm of the cell body and its projections (Fig. 1C and D). Furthermore, NMNAT3 immunofluorescence in the NMNAT3-Tg mouse brain appeared in a punctate-like manner in the cell body and its processes (Fig. 1B and D) consistent with the prior observation demonstrating that NMNAT3 is mainly localized to the mitochondrial compartment in these mice.

Endogenous cerebral NMNAT3 is induced by neonatal cerebral hypoxia-ischemia

Previous research and our own aforementioned observations suggest that endogenous NMNAT3 is expressed at relatively low baseline levels in the wild-type rodent brain, although recent evidence supports the notion that its CNS abundance may be region specific given that it has been substantially localized to the cytoplasm of mouse retinal neurons and their optic nerve fibers. Furthermore, endogenous NMNAT levels are known to increase in response to cellular stress including hypoxia indicating that NMNAT proteins are potentially adaptive molecules that may provide neuroprotective actions in response to brain injury. Therefore, we investigated whether endogenous NMNAT3 levels in the immature mouse brain are affected by in vivo cerebral hypoxia and/or ischemia. To examine this question, we measured endogenous NMNAT3 expression in hippocampal and cortical tissue from C57BL/6 neonatal naïve and injured mouse brains 24 h, 72 h, and 7 days after neonatal H-I (Fig. 2) utilizing the modified Levine method of neonatal brain injury at postnatal day 7 (unilateral carotid ligation followed by 45 min of 8% oxygen hypoxia). NMNAT3 mRNA and relative protein expression was then examined by conventional quantitative PCR and immunoblotting techniques. Mouse cortical and hippocampal NMNAT3 mRNA levels increased during the first 72 h following injury in both, the hypoxic and hypoxic-ischemic side of the brain when compared to age-matched naïve control tissue (Fig. 2A). NMNAT3 mRNA returned to baseline levels when examined 7 days after neonatal H-I suggesting that NMNAT3 is upregulated in response to cerebral hypoxia and/or ischemia in a postinjury time-dependent manner. Similarly, while NMNAT3 immunoreactivity by Western blot was consistently absent in the naïve uninjured neonatal mouse, we observed a measurable increase in NMNAT3 protein levels in the neonatal hypoxic and ischemic hippocampus when examined 24 h after neonatal H-I (Fig. 2B). Taken together, the above observations suggest that endogenous NMNAT3 is induced in vivo during the acute injury phase of neonatal cerebral hypoxia and ischemia.

NMNAT3 protects the immature hippocampus from the effects of neonatal cerebral hypoxia-ischemia

Having established that NMNAT3 Tg mice demonstrate high levels of NMNAT3 in brain regions commonly affected following newborn cerebral hypoxia-ischemia, we then examined whether NMNAT3 overexpression decreases total cerebral tissue injury following neonatal cerebral H-I. We first tested this hypothesis by subjecting human term-equivalent (P7) NMNAT3 Tg mice and WT littermates to the modified Levine brain injury technique utilizing 45 min of 8% hypoxia and then examining hippocampal, striatal, and cortical tissue injury 7 days after neonatal H-I. Utilizing this length of hypoxia, we first observed that total NMNAT3 Tg pup mortality during or
Figure 1. Immunohistochemical localization of NMNAT3 in the young NMNAT3-overexpressing postnatal brain. (A) NMNAT3 DAB immunohistochemistry of representative brain coronal sections from a NMNAT3 wild-type (NMNAT3-WT) and NMNAT3-overexpressing transgenic (NMNAT3-Tg) mouse brain at postnatal day 14. (B–D) Composite digital micrographs of NMNAT3 fluorescence immunoreactivity (NMNAT3-IR) in the postnatal NMNAT3-Tg hippocampus. NMNAT3-IR is primarily found colocalized to neurons and regions of enriched neuronal processes as demonstrated by double immunofluorescence of NMNAT3 with neuronal antigen (B; NeuN) and neurofilament 200 (C and D; NF-200). Scale bar = 110 μm (B), 440 μm (C), and 100 μm (D).
right after hypoxic-chamber exposure was reduced by half compared to that of WT littermates (Fig. 3A). Examination of the surviving mice brain tissue 7 days after H-I demonstrated that animals with NMNAT3 overexpression had decreased cortical tissue injury and a trend toward a decrease in hippocampal neurodegeneration (Fig. 3A). Given that total mortality was higher in WT compared to NMNAT3 Tg mice prior to tissue injury quantification, we reasoned that the lack of statistical significance in the hippocampal measurements might be attributed to a selection bias toward less severely injured WT mice brains. To eliminate this confounder, we repeated the experiment while lowering the period of 8% hypoxia to 15 min. Utilizing this approach, pup mortality during or immediately after hypoxia was absent in the WT and NMNAT3 Tg group. Furthermore, NMNAT3 overexpression decreased hippocampal tissue injury by 56% when compared to WT mice littermates when examined 7 days post-H-I (Fig. 3B). These observations suggest that NMNAT3 reduces the acute effects of cerebral hypoxia and ischemia, while it also protects the neonatal cerebral cortex and hippocampus against the neurodegenerative effects of neonatal H-I.

**NMNAT3 decreases neuronal degeneration in the injured neonatal brain via a caspase/calpain-mediated pathway**

In order to understand the cellular mechanisms involved in NMNAT3-dependent neuroprotection, we examined whether NMNAT3 overexpression affects the activity of calpain and caspase-3 in the neonatal hippocampus following H-I utilizing immunoblotting and caspase-3 enzymatic assays. Calpain and caspase-3 are cysteine proteases essential for the activation of necrotic and apoptotic neurodegeneration as a result of neonatal H-I.1,16,22,23 The cytoskeletal protein, spectrin, is cleaved by both caspase-3 and calpain resulting in 120 KDa fragment reflecting caspase cleavage and a 145 and 150 KDa fragment representing calpain cleavage when assessed using SDS-PAGE.23
Caspase-3 activity is marker of neuronal apoptosis, while calpain cleavage of spectrin after neonatal H-I is caspase-3 independent and thus serves as a measure of nonapoptotic cell death. Neonatal hippocampal lysates from control, WT littermates evaluated 24 h post-H-I (carotid ligation followed by 45 min of hypoxia) revealed markedly increased levels of both caspase- and calpain-specific spectrin cleavage products in the ipsilateral ligated, injured, side when compared to the contralateral, uninjured, hippocampus (Fig. 4A–D; WT). In contrast, the calpain and caspase cleavage products of NMNAT3-overexpressing mice were markedly decreased in the injured hippocampus (Fig. 4A–D; N3). Furthermore, NMNAT3-overexpressing mice showed no statistical differences in calpain and caspase cleavage products between their injured and uninjured hemispheres (Fig. 4A–D). To further corroborate the above findings, we examined the enzymatic activity of capase-3 in injured hippocampal lysates of NMNAT3 Tg and WT mice 24 h post-H-I. Congruent with our spectrin results, NMNAT3 overexpression resulted in a decrease in hippocampal caspase-3 activation (Fig. 4E). These results suggest that NMNAT3 protects the neonatal hippocampus against the neurodegenerative effects of H-I via a mechanism involving the inhibition of key proteins associated with both neuronal necrosis and apoptosis.

The neuroprotective effects of NMNAT3 after neonatal cerebral injury are associated with a decrease in the calpain-specific protease, calpastatin

In order to further explore the mechanism of NMNAT3-mediated calpain/caspase inhibition, we examined the contribution of calpastatin degradation on NMNAT3-dependent neuroprotection following neonatal H-I. Calpastatin (CASTN) is a calcium-activated cysteine protease responsible for the proteolytic inhibition of calpains.Increases in intracellular calcium after neonatal cerebral ischemia lead to the activation of calpains and subsequent degradation of CASTN resulting in unopposed activation of calpain-mediated neuronal cell death. Caspase-3 also promotes the degradation of CASTN in early apoptotic neurodegeneration. This effect allows further compromise of the neuronal cell membrane causing further increases in cytoplasmic calcium and calpain activation. Indeed, CASTN appears to be a key suicide substrate for the degeneration of neurons as a result of ischemic cerebral injury. Furthermore, CASTN has been recently implicated in the inhibition of axonal degeneration by NMNATs as overexpression of cytoplasmic NMNAT1 from injured axons of dorsal root ganglia (DRG) prevented injury-induced CASTN degradation in vitro.
Therefore, we investigated whether NMNAT3 overexpression results in the inhibition of CASTN degradation after neonatal H-I. Relative CASTN levels were measured by SDS-PAGE followed by Western blotting from neonatal P7 hippocampi of NMNAT3-overexpressing and WT mice 24 h after H-I. Consistent with our calpain and caspase findings, CASTN levels were significantly reduced in the ipsilateral, injured hippocampus of WT animals compared to the contralateral, injured, side (Fig. 5A and B). In contrast, NMNAT3 overexpression decreased injury-mediated CASTN degradation of the neonatal hippocampus (Fig. 5A and B). These data further implicate CASTN as a likely important mechanistic target involved in

Figure 4. NMNAT3 overexpression reduced the activation of calpain and caspase-3 following neonatal H-I in neonatal hippocampus. Quantitation (A, B, and D) and representative blot (C) of the relative immunoreactive band density by the corresponding spectrin breakdown product (SBPD; 145 and 150 kDa cleaved by calpain, 120 kDa cleaved by caspase) from wild-type (WT) and NMNAT3-overexpressing transgenic (N3) mice in the right (R) uninjured, nonischemic, versus left (L) injured, ischemic, hippocampus 24 h after H-I. (E). Quantitation of caspase-3 activity in WT and N3 transgenic mice of the injured and uninjured side measured 24 h after neonatal H-I. * p < 0.05; ** p < 0.01; *** p < 0.001

Figure 5. NMNAT3 overexpression decreases the degradation of calpastatin following H-I. Quantitation (A) and representative blot (B) of the relative calpastatin (CASTN) total band immunoreactive density by Western blot from wild-type (WT) and NMNAT3-overexpressing transgenic (N3) mice in the right (R) uninjured, nonischemic, versus left (L) injured, ischemic, hippocampus 24 h after H-I (N = 8/group). ** p < 0.01; *** p < 0.001
NMNAT3-mediated neuroprotection from the effects of neonatal H-I.

**Alterations in NMNAT3 expression in vitro affect baseline neuronal survival and glutamate-dependent excitotoxicity**

Lastly, we wanted to investigate whether alterations in endogenous and exogenous NMNAT3 expression in vitro affect the survival of nontransgenic mouse hippocampal and cortical neurons under basal and injurious conditions. To examine the effect of endogenous NMNAT3, we infected E18 embryonic dissociated cortical and hippocampal neurons on DIV3 with an AAV8 mouse shNMNAT3 knockdown viral vector containing a Green Fluorescent Protein (GFP) reporter. A shRNA scrambled AAV8 vector was used as a control for all viral experiments and in vitro neuronal injury assays. Neuronal infection as assessed by GFP reporter immunofluorescence cytochemistry was present in nearly all neurons by 3 days following viral exposure (Fig. 6C). Utilizing LDH levels as a relative quantitative measure of neuronal viability, we observed a significant increase in LDH activity as early as 6 days post-AAV8-shNMNAT3 exposure in both cortical (Fig. 7A; shN3) and hippocampal neurons (Fig. 8A; shN3). This increase was associated with visual qualitative fragmentation of GFP-positive neurites, and a quantitative decrease in MAP2 immunofluorescence in these two neuronal populations indicating that in vitro shNMNAT3 exposure increases the degeneration of neuronal processes (Fig. 7C and D and 8C and D; shN3). Five-minute exposure to the glutamate-agonist, ibotenic acid (50 \( \mu \)mol/L), resulted in further increases in cell death in neurons infected with the shNMNAT3 viral vector as compared to shScramble control when examined 24 h following transient excitotoxic agent administration (gray bars Figs. 7A and 8A; shN3 + IBO). Furthermore, shNMNAT3 vector exposure decreased endogenous mouse NMNAT3 mRNA levels by 40% without affecting the levels of NMNAT1 and NMNAT2 indicating that this effect is not caused by a decrease in the expression of the other, more abundant, NMNAT isoforms (Fig. 6B). The above observations suggest that endogenous NMNAT3 mRNA downregulation decreases baseline neuronal survival, and that this effect is further potentiated by glutamate-mediated excitotoxicity.

We then asked whether AAV8-shNMNAT3-mediated neuronal degeneration can be rescued by NMNAT3 overexpression. To answer this question, we examined neuronal viability and neurite density in neurons exposed to a NMNAT3-overexpressing vector capable of increasing NMNAT3 by 100-fold in the presence or absence of the shNMNAT3 vector (Fig. 6A; Viral N3). The AAV8-NMNAT3-overexpressing vector contained a neuron-specific synapsin-1 promoter to investigate these effects exclusively in neurons. Exposure to the NMNAT3-overexpressing vector was divided into four groups and all exposures began at DIV5 and were assessed at DIV13: (1) neurons exposed to NMNAT3-overexpressing vector only (N3 only; 8.3E +9 vg/well); (2) neurons exposed simultaneously to nearly equal concentrations of shNMNAT3 (shN3; 7.1E +9 vg/well) and NMNAT3-overexpressing vector (N3; 8.3E + 9 vg/well); (3) neurons exposed to shN3 3 days PRIOR to
Figure 7. NMNAT3 mRNA depletion increases markers of neuronal cell death and enhances glutamate-dependent excitotoxic degeneration of cortical neurons in vitro. The prodegenerative effect of NMNAT3 mRNA knockdown is reduced by NMNAT3 overexpression. (A) Effect of endogenous NMNAT3 knockdown on LDH activity in cortical neuronal cultures exposed to AAV8-shNMNAT3-GFP (shN3) virus for 6 days prior ($N = 6$) and 24 h ($N = 4$) after 50 μmol/L ibotenic acid exposure (shN3 + IBO), or exposed to a human NMNAT3-overexpressing vector alone (N3; $N = 6$). The percent change in LDH activity was calculated as the change in LDH activity from that of experimentally paired neurons exposed to control AAV8-shScramble vector for 6 days prior and 24 h after IBO exposure. Notice the significant elevation in LDH activity in shN3-exposed neurons above shScramble control at baseline and following IBO exposure. LDH activity in N3-exposed neuronal cultures appeared to be mildly reduced compared to shScramble controls. (B and C) Percent change in baseline LDH activity compared to shScramble controls (B) and amount of MAP2 immunoreactivity per unit area (C) 9 days postinfection with the mouse NMNAT3 knockdown AAV8 (shN3) in the presence ($N = 4/group$) or absence ($N = 12/group$) of the human NMNAT3-overexpressing vector at the time (shN3 + N3), 3 days prior (N3 then shN3), or 3 days after (shN3 then N3) shN3 viral vector exposure. (D) Representative micrographs of shNMNAT3 GFP-positive neurons alone (high-power; bar = 110 μm) or stained with MAP2 (low-power; bar = 1100 μm) and infected with the shN3 vector with or without N3-overexpressing virus. GFP fragmentation, increased visualization of morphologically appearing astrocytes (see GFP-shN3 panel), and a decrease in MAP2 + neurite staining was found primarily in shN3-exposed neurons. The presence of the N3-overexpressing vector in shN3-treated neurons did not decrease, but rather qualitatively increase, shNMNAT3 GFP-positive fluorescence.* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$
N3-vector exposure; and (4) neurons infected with shN3 3 days AFTER N3-vector exposure. Findings were then simultaneously compared to neurons only exposed to the AAV8-shScramble control virus (shScramble; $7.1 \times 10^9$ vg/well). Concomitant exposure of the N3-overexpressing vector did not appear to negatively affect shNMNAT3 knockdown virus infection as the degree of GFP fluorescence generated by the shNMNAT3 vector did not appear to decrease in its presence (Fig. 7D). Cortical neurons exposed to the shN3 vector concomitantly with the N3 vector or infected with shN3 3 days after N3 vector exposure demonstrated significant improvement in shN3-mediated neuronal viability as assessed by decreases in LDH activity 8 days postviral infection (Fig. 7B and D). N3 vector exposure alone did not negatively affect neuronal survival or neurite density when compared to shScramble control (Fig. 7B and C). In fact, N3-only exposed cortical and hippocampal neurons had significantly lower baseline LDH activity levels when compared to the viral vector control-infected neurons suggesting a healthier baseline status (Figs. 7A and 8A; N3). Furthermore, shN3-mediated neurite degeneration as assessed by the amount of MAP2 immunofluorescence was significantly decreased in the presence of N3-overexpressing vector irrespective of the timing of shN3 exposure (Fig. 7C and D). A similar effect was also observed in hippocampal neurons (Fig. 8C and D). Taken together, the above data suggest that upregulation of NMNAT3 expression protects neurons against the prodegenerative effects of NMNAT3 mRNA depletion.

**Discussion**

In this study, we asked whether NMNAT3 protects the immature mouse brain from the effects of cerebral hypoxia-ischemia. We find that increases in cerebral NMNAT3 decrease total hippocampal and cortical injury following neonatal H-I. This effect is associated with a decrease in calpain and caspase-3 activity and a decrease in the injury-mediated degradation of calpastatin. In addition, decreases in baseline endogenous NMNAT3 mRNA in vitro increased neuronal degeneration and enhanced glutamate-dependent excitotoxicity, an injury mechanism thought to be important in the pathogenesis of perinatal cerebral injury. The neurotoxic effect observed from NMNAT3 knockdown in vitro and its near complete reversal by exogenous upregulation of NMNAT3 further supports the notion that NMNAT3 is important for maintaining the viability of developing neurons. To our knowledge, this is the first report demonstrating the neuroprotective actions of a natively found non-nuclear NMNAT in the neonatal injured brain. These observations also strengthen the increasing body of literature demonstrating the importance of NMNATs in promoting neuronal and axonal survival not only of the peripheral but also of the central nervous system.

**Neuroprotective role of NMNAT3 in injured developing neurons**

The result demonstrating that NMNAT3 overexpression decreased hippocampal and cortical degeneration...
following neonatal H-I is supported by previous observations showing similar neuroprotective effects in other neural tissues and species.\textsuperscript{9,12,29–31} In vivo increases in rodent NMNAT3 prevented the degeneration of injured axons of central olfactory receptor neurons in the Drosophila brain and of retinal ganglion neurons of the rat eye.\textsuperscript{9,29,30} Similarly, NMNAT3 overexpression conferred strong neuroprotection against axonal degeneration of the mouse injured sciatic nerve in vivo and of damaged DRG axons in vitro.\textsuperscript{15,30,31} Furthermore, our observation that NMNAT3 reduced cerebral tissue loss in the hippocampus and cerebral cortex suggests that the extent of the neuroprotective actions of NMNATs may be dependent on their anatomical and cellular expression as well as mode of injury. Indeed, immunohistochemical localization of cerebral NMNAT3 appeared to be primarily present in neuronal cell bodies of the cerebral cortex and hippocampus (Fig. 1), and previous research has shown that overexpression of cytoplasmic NMNAT1 following neonatal cerebral H-I resulted in a more global protective effect similar to the one found with NMNAT3 in this study.\textsuperscript{11} In addition, we find that endogenous NMNAT3 levels are increased during the first 72 h following neonatal H-I. This effect is congruent with previous observations demonstrating that NMNATs are inducible under conditions of anoxic cellular stress.\textsuperscript{21,32} Drosophila brain NMNAT levels are upregulated following hypoxia via a mechanism involving the activation of hypoxia-inducible factor alpha (HIFα) and heat shock factor.\textsuperscript{21} Interestingly, NMNAT1 and HIFα peripheral blood mRNA levels are also increased in people living at high altitudes suggesting that the expression of HIFα and NMNATs is also adaptive to low oxygen conditions in humans.\textsuperscript{33,34} Moreover, HIFα is a transcription factor importantly involved in the regulation of neuronal survival of injured neurons following neonatal H-I and it is also actively upregulated in response to repeated neonatal cerebral hypoxia.\textsuperscript{35} Therefore, increases in the endogenous expression of NMNAT3 in response to neonatal cerebral hypoxia and ischemia may represent an internal mechanism by which the immature neuron attempts to protect itself from the degenerative effects brought on by excessive metabolic stress. This hypothesis is supported by the observation that NMNAT3 upregulation in the fly brain is capable of enhancing the life span of flies in response to oxidative stress.\textsuperscript{21} Similarly, NMNAT3 overexpression reduced the degeneration of DRG axons after exposure to exogenous toxic oxidants such as rotenone and hydrogen peroxide,\textsuperscript{30} and increases in Drosophila NMNAT prevented hypoxia-induced degeneration of neuronal dendrites.\textsuperscript{20,36} In this study, we also find that NMNAT3-overexpressing mice have lower mortality during the acute phase of H-I compared to their littermate controls (Fig. 3A). The decreased mortality during and/or immediately following hypoxia-ischemia in the NMNAT3-overexpressing mice was an unexpected and interesting finding that likely needs future study. In addition to their aforementioned role in oxidative stress, NMNAT targeted to mitochondria have been shown to facilitate mitochondrial calcium buffering capacity.\textsuperscript{37} Mitochondrial dysfunction is well known to affect neuronal excitability through its role in calcium buffering, neurotransmitter biosynthesis, and redox homeostasis.\textsuperscript{38} It is thus possible that chronic increases in NMNAT3 expression, such as those seen in NMNAT3 transgenic mice, may contribute to an amelioration in neuronal excitability during excessive neuronal stress and may in turn decrease the risk for death during the acute phase of H-I.

Our results showing significant increases in cortical and hippocampal neurodegeneration after NMNAT3 mRNA downregulation and its subsequent rescue by exogenous human NMNAT3 (Figs. 7–8) further support the aforementioned increasing scientific evidence that endogenous NMNATs are likely important for maintaining neuronal survival. These findings are rather surprising given the relatively low expression of NMNAT3 in the immature brain. Our observation also contrasts previous research demonstrating that injection of NMNAT2 but not NMNAT3 siRNA to superior cervical ganglion neurons caused neurite degeneration when examined 72 h post-transfection.\textsuperscript{39} These differences may be attributed to the timing of observation, type of neuron, and duration of siRNA exposure, given that neuronal degeneration and processes fragmentation in our cultures were not observed until 6 days postinfection, and viral-mediated transduction of shRNA produces a more wide spread and long-lasting level of siRNA expression when compared to direct siRNA injection.\textsuperscript{40} Although we cannot conclusively exclude the possibility that neuronal exposure and infection with our shNMNAT3 viral vector negatively affect other survival genes, we did not observe changes in the endogenous expression of NMNAT1 and NMNAT2 (Fig. 6B). Additionally, exposure to the control shScramble vector did not affect neuronal survival (Figs. 7–8). Of note, NMNAT3 knockout mice appear viable,\textsuperscript{41} although it is not yet known whether these mice have abnormalities in neuronal development or survival under basal or injurious conditions. Lastly, increases in endogenous NMNAT3 24 h following H-I were primarily observed in the hypoxic side of the pup brain. Although NMNAT3 protein levels were relatively increased in the ischemic hippocampus, we did not observe a corresponding elevation in NMNAT3 mRNA in this region (Fig. 2). In our injury model, the hippocampus undergoes the most severe injury and it is possible that the mechanism of NMNAT3 induction is partially overwhelmed and/or
delayed by the extent of injury. This may be the reason why the highest increases in mRNA and NMNAT3 Western blot immunoreactivity were observed in the hypoxic-only tissues or the ischemic cerebral cortex, a brain region that undergoes a relatively lesser degree of injury. Future studies should aim at characterizing further the differential expression and cellular compartmentalization of NMNATs in the developing brain while further exploring the mechanisms responsible for NMNAT induction. The above likely will provide a better understanding of the regional, environmental, and internal cellular triggers that modulate neuronal NMNAT expression and NMNAT-mediated neuronal survival.

Taken together, our observations indicate that endogenous and exogenous NMNAT3 is neuroprotective against the effects of neonatal brain injury. Furthermore, studying the mechanisms and cellular triggers that modulate the endogenous expression of NMNATs is of translational importance as exploring biological strategies targeted at increasing the levels of these proteins in brain may prove beneficial against disease conditions of compromised neuronal health.

**Role of mitochondria in NMNAT3-mediated neuroprotection**

Numerous studies have demonstrated that NMNAT3 is likely the primary NMNAT isoform present in mitochondria,6,7,9,15,42–46 and our observations and those of previous reports suggest that NMNAT3 in NMNAT3-Tg mice is also primarily localized to this organelle.15,31 Mitochondria play an essential role in neuronal survival following cerebral injury and recent findings suggest that mitochondria are important cellular compartments involved in the neuroprotective actions of NMNATs.37,47 For example, and as mentioned above, NMNAT targeted to the mitochondria enhanced mitochondrial calcium buffering, and reduced axotomy-induced calcium elevation and Wallerian degeneration of mouse *Drosophila* axons.37 Furthermore, genetic elimination of axonal mitochondria in *Drosophila* prevented NMNAT’s ability to reduce Wallerian degeneration of injured neuronal fibers.47 In contrast, increases in NMNAT3 expression enhanced the ATP synthetic activity of mitochondria and reduced axonal degeneration following mouse sciatic nerve injury.15 Therefore, NMNAT3 is likely important in maintaining neuronal integrity during disease conditions that alter mitochondria-associated ATP metabolism and calcium homeostasis such as during and after neonatal H-I. If NMNAT3 protects neurons against neonatal hypoxic-ischemic neurodegeneration via a pathway that involves alterations in mitochondrial bioenergetics and mitochondrial-mediated calcium buffering, one is expected to find NMNAT3-mediated decreases in the function of death-promoting cellular substrates activated as a result of this organelle’s dysfunction. In support of this hypothesis, we find that NMNAT3 overexpression following neonatal H-I decreased the activity of calpain and caspase-3 (Fig. 4), two proteases that are well known to be triggered by the cellular events that lead to the permeabilization and altered function of mitochondria in ischemic neurons.48,49 Furthermore, we also observed that NMNAT3 upregulation was associated with a significant decrease in the degradation of calpastatin (Fig. 5), a calcium-sensitive protease known to inhibit the activity of both calpains and caspase-3.23–27 It is thus tempting to suggest that the biological mechanism by which NMNAT3 protects neurons against the effects of neonatal H-I involves an increase in the capacity of mitochondria to buffer the rapid increases in intracellular calcium as a result of ischemia-induced glutamate-dependent excitotoxicity, thereby preventing calpastatin degradation and the subsequent activation of calpain and caspase-3. This is supported by our in vitro observation showing a substantial increase in cortical and hippocampal neuronal degeneration upon NMNAT3 mRNA depletion 24 h following glutamate receptor agonist administration (Figs. 7A, 8A). Therefore, future research efforts may aim at characterizing the specific signaling mechanisms that link NMNATs and calcium-activated death-modulating proteins such as calpastatin. Notably, although our observations agree with previously published work indicating that NMNATs are important enzymes for the regulation of neuronal degeneration, our experiments do not directly assess the specific sequence of biochemical events that lead to NMNAT3-mediated neuroprotection following neonatal hypoxia-ischemia, an important subject that needs future study. Yet, published observations suggest that the calpastatin-calpain pathway is a likely common downstream cellular mechanism involved in NMNAT-mediated neurodegeneration. For example, injured axons of dorsal root ganglia neurons transfected with mutant cytoplasmic NMNAT1 prevented axotomy-induced calpastatin degradation, while knockdown of calpastatin accelerated the degeneration of injured neuronal fibers.15 More recent evidence indicates that NMNAT2 inhibits the depletion of ATP triggered by the activation of sterile alpha and Toll/interleukin-1 receptor motif-containing protein-1 (SARM1) following axotomy, a prodegenerative NADase involved in axonal degeneration.50,51 Interestingly, calpain inhibition or calpastatin overexpression did not prevent the energy depletion observed after axotomy.52 Taken together these observations suggest that NMNATs functions upstream to the cellular cascade that triggers the depletion of ATP and subsequent activation of the calpain-calpastatin degenerative cascade following axotomy. Although we cannot
conclude that this same mechanism occurs in injured immature brain neurons in response to increases in NMNAT3, the above findings and the observation that similar results have been noted in developing injured retinal ganglion neurons\(^{28}\) suggest that NMNATs may involve common death signaling pathways in both, the peripheral and central nervous system. Yet, the various NMNAT isoforms may individually differ in the cellular mechanisms that they employ to protect the injured brain. For example, in this work we found that NMNAT3 overexpression following H-I decreased caspase-3 activation, whereas this apoptotic enzyme appeared not to be affected following HI in neonatal mice expressing high levels of cytoplasmic NMNAT1.\(^{11}\) These differences further contribute to newer evidence showing that the neuroprotective effects of NMNATs are not exclusively related to their NAD\(^+\) synthetic activity.\(^{53}\) For example, recent studies show that NMNAT1 and NMNAT2 decreased axon degeneration by preventing the SARM1-dependent depletion of NAD\(^+\).\(^{50,54}\) Interestingly, increases in the expression of NMNAT prevented SARM1-dependent NAD\(^+\) depletion without increasing NAD\(^+\) production.\(^{54}\) In addition, NMNATs have been recently shown to have chaperon function in animal models of tauopathies.\(^{40,55}\) The above findings suggest that NMNATs have a broader and more complex neuroprotective function and therefore, targeting the mechanisms that affect the endogenous expression of these proteins may offer novel and more wide spread neurotherapeutic approaches.

**Conclusions**

NMNAT3 overexpression protects the term-equivalent neonatal mouse hippocampus and cortex from the degenerative effects of cerebral H-I. Cerebral hypoxia and/or ischemia increases the expression of endogenous NMNAT3. The neuroprotective effect of NMNAT3 involves a decrease in cerebral apoptotic and necrotic degeneration possibly via a decrease in injury-related calpstatatin degradation and resultant inhibition of calpain and caspase-3 activation. Endogenous NMNAT3 is important for the survival of cortical and hippocampal neurons in vitro under baseline and following glutamate receptor-mediated excitotoxic injury, an effect that is rescued by exogenous upregulation of NMNAT3. Our results suggest that NMNAT3 is an important prosurvival molecule in the injured developing brain.

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**Conflicts of Interest**

The authors declare no competing financial or nonfinancial interests.

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