Enhancing NAD<sup>+</sup> Salvage Pathway Reverts the Toxicity of Primary Astrocytes Expressing Amyotrophic Lateral Sclerosis-linked Mutant Superoxide Dismutase 1 (SOD1)*

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Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) participates in redox reactions and NAD<sup>+</sup>-dependent signaling pathways. Although the redox reactions are critical for efficient mitochondrial metabolism, they are not accompanied by any net consumption of the nucleotide. On the contrary, NAD<sup>+</sup>-dependent signaling processes lead to its degradation. Three distinct families of enzymes consume NAD<sup>+</sup> as substrate: poly(ADP-ribose) polymerases, ADP-ribosyl cyclases (CD38 and CD157), and sirtuins (SIRT1–7). Because all of the above enzymes generate nicotinamide as a byproduct, mammalian cells have evolved the NAD<sup>+</sup> salvage pathway capable of resynthesizing NAD<sup>+</sup> from nicotinamide. Overexpression of the rate-limiting enzyme in this pathway, nicotinamide phosphoribosyltransferase, increases total and mitochondrial NAD<sup>+</sup> levels in astrocytes. Moreover, targeting nicotinamide phosphoribosyltransferase to the mitochondria also enhances NAD<sup>+</sup> salvage pathway in astrocytes. Supplementation with the NAD<sup>+</sup> precursors nicotinamide mononucleotide and nicotinamide riboside also increases NAD<sup>+</sup> levels in astrocytes. Amyotrophic lateral sclerosis (ALS) is caused by the progressive degeneration of motor neurons in the spinal cord, brain stem, and motor cortex. Superoxide dismutase 1 (SOD1) mutations account for up to 20% of familial ALS and 1–2% of apparently sporadic ALS cases. Primary astrocytes isolated from mutant human superoxide dismutase 1-overexpressing mice as well as human post-mortem ALS spinal cord-derived astrocytes induce motor neuron death in co-culture. Increasing total and mitochondrial NAD<sup>+</sup> content in ALS astrocytes increases oxidative stress resistance and reverts their toxicity toward co-cultured motor neurons. Taken together, our results suggest that enhancing the NAD<sup>+</sup> salvage pathway in astrocytes could be a potential therapeutic target to prevent astrocyte-mediated motor neuron death in ALS.

Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) is an essential redox molecule and a key player in several signaling pathways that govern fundamental biological processes (1, 2). In the redox reactions, a hydride equivalent is reversibly transferred at the nicotinamide moiety, resulting in a switch between oxidized (NAD<sup>+</sup>) and reduced (NADH) forms of the nucleotide. Although the redox reactions are critical for efficient mitochondrial metabolism, they are not accompanied by any net consumption of the nucleotide. On the contrary, NAD<sup>+</sup>-dependent signaling processes lead to its degradation.

Three distinct families of enzymes consume NAD<sup>+</sup> as substrate: poly(ADP-ribose) polymerases (PARPs); ADP-ribosyl cyclases (CD38 and CD157), and sirtuins (SIRT1–7) (3–5). PARPs hydrolyze NAD<sup>+</sup> and transfer the ADP-ribose moiety of NAD<sup>+</sup> to a receptor amino acid, building poly(ADP-ribose) polymers. PARPs regulate DNA damage repair, tumorigenesis, cell differentiation, and metabolism (3, 6, 7). CD38 is a ubiquitously expressed multifunctional enzyme that catalyzes the production of second messengers (like cyclic ADP-ribose) (8), which act as potent intracellular calcium-mobilizing agents to control cell cycle, insulin signaling, and microglial activation (8–10). Sirtuins are a highly conserved family of proteins capable of catalyzing NAD<sup>+</sup>-dependent deacetylation and monodeoxy (ADP-ribose)ylation reactions (11). Sirtuin activation has been shown to modulate mitochondrial biogenesis and all major mitochondrial processes, including the tricarboxylic acid cycle, fatty acid metabolism, oxidative phosphorylation, and antioxidant response (4, 12–15). Because all of the above NAD<sup>+</sup>-consuming enzymes generate nicotinamide (NAM) as a byproduct, mammalian cells have evolved an NAD<sup>+</sup> salvage pathway capable of resynthesizing NAD<sup>+</sup> from NAM (16).

Although NAD<sup>+</sup> synthesis can occur from l-tryptophan (kynurenine pathway), nicotinic acid (Preiss-Handler pathway), or nicotinamide riboside (NR) (17–19), the salvage pathway appears to account for the majority of NAD<sup>+</sup> synthesis in mammalian cells. The enzyme nicotinamide phosphoribosyltransferase (NAMPT) catalyzes the conversion of NAM and 5'-phosphoribosyl 1-pyrophosphate to nicotinamide mononucleotide (NMN); subsequently nicotinamide mononucleotide

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adényltransferases (NMNATs) transfer adenine from ATP to NMN to generate NAD⁺ (20, 21). NAMPT is the rate-limiting enzyme in this pathway. Accordingly, overexpression of NAMPT, but not NMNATs, increases NAD levels (22–24).

All the different types of NAD⁺-consuming reactions have been described in the mitochondria, but in general NAMPT appears to be absent from the mitochondrial compartment (22, 24–26), and the origin of the mitochondrial NAD⁺ pool in mammalian cells is not yet fully characterized. Treatment with intermediate precursors like NMN and NR has been shown to increase mitochondrial NAD⁺ levels in culture (24, 27). NR is likely converted to NMN in the cytosol, and it has been proposed that NMN is transported into the mitochondria for NAD⁺ synthesis (24, 28). Although the identity of the mitochondrial NAMPT remains to be established, the existence of a mitochondrial enzymatic activity capable of synthesizing NAD⁺ starting from NMN and ATP is widely accepted (2, 28–30).

Amyotrophic lateral sclerosis (ALS) is caused by the progressive degeneration of motor neurons in the spinal cord, brain stem, and motor cortex. Motor neuron death leads to muscle weakness and paralysis, causing death in 1–5 years from the time of symptom onset. Most ALS cases are sporadic (SALS), and exposure to yet unidentified environmental toxicants might be responsible for SALS (31). About 5–10% of the cases are inherited (familial ALS (FALS)), and the first ALS-linked gene identified was superoxide dismutase 1 (SOD1) (32). Mutations in SOD1 account for up to 20% of FALS and 1–2% of apparently SALS cases. Mutations in several other genes have now been identified in many FALS pedigrees (31, 33, 34). Each mutated gene has its own genetic and molecular signature, but FALS and SALS are phenotypically indistinguishable, and a significant share of our understanding comes from the study of rodent overexpressing ALS-linked mutant SOD1 that develop an ALS-like phenotype (35). The molecular mechanism underlying the toxic gain of function of mutant hSOD1s remains uncertain. However, several lines of evidence suggest that toxicity to motor neurons requires dysfunction of non-neuronal cells (36–38). In line with this observation, primary astrocytes isolated from mutant hSOD1-overexpressing mice induce motor neuron death in co-culture (39–41). The non-cell-autonomous component for other ALS-linked mutations remains under investigation, but it has been demonstrated that astrocytes differentiated from human post-mortem ALS spinal cord-derived progenitor cells and astrocytes obtained from the transdifferentiation of fibroblasts from FALS and SALS patients are also toxic for motor neurons in co-culture (42, 43).

Here, we determined the effect of NAD⁺ precursors and NAMPT overexpression on total and mitochondrial NAD⁺ content in non-transgenic and mutant hSOD1-expressing astrocytes. In addition, we investigated the effect of a mitochondrially targeted NAMPT on mitochondrial NAD⁺ recycling in astrocytes. Increasing total and mitochondrial NAD⁺ content in ALS astrocytes increases oxidative stress resistance and reverts their toxicity toward co-cultured motor neurons. Taken together, our results suggest that enhancing the NAD⁺ salvage pathway in astrocytes could be a potential therapeutic target for ALS.

### Enhancing NAD⁺ Salvage Pathway in ALS Astrocytes

#### Experimental Procedures

**Reagents**—All chemical and reagents were from Sigma-Aldrich unless otherwise specified. Culture media and serum were obtained from Life Technologies. Primers and siRNAs were obtained from Integrated DNA Technologies. NR was obtained from BOC Sciences.

**Animals and Primary Cultures**—B6.Cg-Tg(SOD1*G93A)1Gur/J (35) mice were obtained from The Jackson Laboratory. hSOD1H46R/H48Q mice were provided by Dr. David Borchelt (44). Primary astrocyte cultures were prepared from cortex and spinal cord of 1-day-old mice as described previously (39). Cortical astrocyte cultures have a significantly higher yield than spinal cord astrocyte cultures. Thus, to minimize the number of animals needed, some experiments were performed with cortical astrocytes, whereas the spinal cord astrocytes were reserved for critical experiments (e.g. the effect of mitochondrially targeted NAMPT, mitochondrial function, and co-cultures). Motor neuron cultures were prepared from 12.5-embryonic-day mouse spinal cords as described previously (45). For co-culture experiments, motor neurons were plated on mouse astrocyte monolayers at a density of 300 cells/cm² and maintained in supplemented L15 medium (39). Motor neurons were identified by immunostaining with anti-neurofilament (Sigma-Aldrich), and survival was determined by counting all cells displaying intact neurites longer than four cells in diameter. Counts were performed over an area of 0.90 cm² in 24-well plates.

**NAD⁺/NADH Quantification**—NAD⁺ and NADH levels were determined based on the differential sensitivity of the nucleotides to pH and temperature (46). Samples were divided in two: one part was homogenized in 0.5 m perchloric acid and maintained on ice (NAD⁺ fraction), and the other part was homogenized in 50 mM NaOH plus 1 mM EDTA and incubated at 60 °C for 30 min (NADH faction). Following neutralization, NAD⁺ and NADH levels were determined by an enzymatic cycling assay (47) and corrected by protein concentration determined with a BCA protein assay (Thermo Scientific). NADP⁺/NADPH quantification was performed with a fluorometric NADP⁺/NADPH Ratio Assay kit (AAT Bioquest).

**Expression Vectors and Adenovirus Packaging**—A mouse Myc-DDK-tagged NAMPT cDNA clone was obtained from OriGene Technologies (MR207867). To generate a mitochondrially targeted NAMPT, the mitochondrial targeting sequence from the human cytochrome c oxidase subunit VIII (48) was inserted in front of the NAMPT ORF. Subcloning into an adenoiral plasmid and adenoviral packaging were performed by Vector Biolabs. The pDsRed2-Mito plasmid was obtained from Clontech. To provide in situ evidence of increased mitochondrial NAD⁺ levels, we used the formation of immunodetectable poly(ADP-ribose) polymers mediated by a mitochondrially targeted fusion protein consisting of EGFP and the catalytic domain of PARP1. The mitochondrially targeted PARP plasmid, the control mitochondrially targeted EGFP plasmid, and the assay have been described previously (49, 50). SIRT1-FLAG- (ID 13812 (51)) and SIRT3-FLAG (ID 33309 (52))-coding plasmids were obtained from Addgene.
Cell Treatment, Transfections, and Mitochondrial Isolation—Confluent astrocyte monolayers were treated with 5 mM NMN, 5 mM NR, or vehicle control 24 h before motor neuron plating or subsequent analysis. Plasmid transfections were performed as described previously (53). Adenovirus-mediated transfections were performed at 50 multiplicity of infection, and astrocytes were used 48 h post-transfection. H2O2 was diluted in Dulbecco’s PBS and applied to astrocyte monolayers at the indicated final concentrations. Survival was assessed 24 h later by determining the release of lactate dehydrogenase using the CytoTox Non-Radioactive Cytotoxicity Assay kit (Promega). siRNA transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Astrocytes were transfected with 25 nM Sirt1 siRNA (5’-CAA-CAGACAUUCUUGCCUAUGUUGAA-3’), Sirt3 siRNA (5’-GGGAACGGUGCCAAGUGAAGGACG), or negative control (NC1) siRNA 24 h before motor neuron plating or 48 h before sample collection for analysis. Mitochondrial isolation was performed by differential centrifugation in a buffer containing 10 mM Tris, 1 mM EDTA, 0.32 mM sucrose, and 0.2 mg/ml digitonin. This preparation routinely yields mitochondrial fractions with less than 1.5% of the total cellular lactate dehydrogenase activity.

Mitochondrial Reactive Oxygen Species (ROS)—Confluent astrocyte monolayers were treated for 2 h with 300 μM H2O2 or vehicle. Following treatments, duplicate sets of cells were incubated for 30 min in Hanks’ balanced salt solution with 4 μM MitoSox (Life Technologies) or 0.2 μM MitoTracker Green (Life Technologies). Mitochondrial reactive oxygen species production (MitoSox; excitation/emission, 530/590 nm) was corrected by mitochondrial content (MitoTracker; excitation/emission, 485/530 nm).

Western Blotting, Immunoprecipitation, Real Time PCR, and Immunofluorescence—Western blotting and immunoprecipitation were performed as described previously (45, 54). Membranes were incubated overnight with one of the following antibodies: anti-NAMPT (Abcam, ab45890, lot GR71423–1), anti-ACT (Sigma, A5411, lot 061M4808), anti-VDAC1 (Cell Signaling Technology, 4661S, lot 4), anti-poly(ADP-ribose) (PAR) polymers (Abcam, ab119484, lot GR84202–2), anti-SIRT1 (Cell Signaling Technology, 9475S, lot 1), anti-SIRT3 (Cell Signaling Technology, 5490S, lot 3), anti-iso-citrate dehydrogenase 2 (IDH2) (Cell Signaling Technology, 12652S, lot 1), and anti-acetylated lysine (Cell Signaling Technology, 9814S, lot 5). Image acquisition was performed in a chemiluminescence Western blot scanner (LI-COR Biosciences) or exposed on Eastman Kodak Co. BioMax Light film. Quantifications were performed using Image Studio Software (LI-COR Biosciences) or Image software (National Institutes of Health). RNA extraction, RNA retrotranscription, and real time PCR were performed as described previously (45). Specific primers were as follows: Nampt/5’-GCCCACCTTTACCTTAGATCTCAGTC-3’; Nampt/3’, 5’-ATGGATGGAGGATGAGGGTCTTACT-TC-3’; Namat1/5’, 5’-GACGCTGGCCAGAGCTATTATG-3’; Namat1/3’, 5’-GGATGAGCCCTTTCTTCTTGT-3’; Namat2/5’, 5’-GGGAGGTGGGGAACGACTTTAGC-3’; Namat2/3’, 5’-CAGAGTACACCCATCCTTACCT-3’; Namat3/5’, 5’-CACAGGGTTTCCCATATCC-3’; Namat3/3’, 5’-CAA-ACAAGCAGGCATCTAAA-3’; Parp1/5’, 5’-GGAGACC- CGATTGGCTTAAT-3’; Parp1/3’, 5’-CCCTTGGTACT- TGCTGATA-3’; Cd38/5’, 5’-GTGCAGGATAGAGGT- ATGG-3’; Cd38/3’, 5’-TGAAGGCTGTTATGGGAATGTAG- G-3’; Sirt3/5’, 5’-ATCCCGTACCCCTGGAAGCCTCCTTT-3’; Sirt3/3’, 5’-TCAAGCCCCCTGATGTTCTGTGTA-3’; Sirt1/5’, 5’-AGCAACATCTCATGTTGGCCAAGG-3’; Sirt1/3’, 5’-TCTGGCACAGCGTATACATCCA-3’; Actin/5’, 5’-TTGTAATGTTGGGAATGGTGCTAAA-3’; and Actin/3’, 5’-TGTGGTGGCAGATCTTTCCATGT-3’. Immunofluorescence was performed as described previously (39) using an anti-FLAG antibody (Sigma, F1804, lot SLBK1346V).

Respirometry Assay, Mitochondrial Biogenesis, and ATP Measurement—Mitochondrial oxygen consumption rate measurements were performed using a Seahorse Bioscience XF-96 instrument (Seahorse Bioscience) as described previously (55). Briefly, average basal rates are the averages of the third and fourth basal rate measurements. At the end of the experiment, cells were fixed with 4% paraformaldehyde and stained with DAPI. The number of cells per well was determined in an IN Cell analyzer (GE Healthcare) and used to normalize the raw oxygen consumption rate data. DNA extraction from astrocytes was performed with a QIAamp DNA Mini kit (Qiagen). Relative mitochondrial and nuclear DNA copy determinations were performed by real time PCR as described previously (56). ATP determination was performed using a commercial kit (BioVision) and corrected by protein concentration.

Statistical Analysis—Each experiment was repeated at least in three independent primary culture preparations. Multiple group comparison was performed by one-way analysis of variance with Tukey’s post-test. When comparing the effect of genotype and treatments, two-way analysis of variance was used followed by Tukey’s post-test. Differences were declared statistically significant if p was <0.05. All statistical computations were performed using Prism 6.0 (GraphPad Software).

Results

NAD+ Precursors Increase Total and Mitochondrial NAD+ Content in Astrocytes—NMN and NR supplementation significantly increase total NAD+ in primary cortical astrocytes obtained from non-transgenic and hSOD1<sup>G93A</sup>-overexpressing mice (Fig. 1). Because total NAD+ increases more than total NADH levels (about 100 and 35% change, respectively), treatments also modify the NAD+/NADH ratio (Fig. 1, A and B). Both precursors also significantly increase mitochondrial NAD+ content, but no significant change was observed in mitochondrial NADH (Fig. 1, C and D). In spinal cord astrocytes, treatments induce a similar -fold change in total NAD+ and NADH as in cortical astrocytes (about 100 and 30% change, respectively; Fig. 1, E and F), indicating that astrocytes from these different regions of the CNS respond in a similar way to the treatments. Untreated astrocytes from both genotypes do not display significant differences in NAD+ or NADH content.

A Mitochondrially Targeted NAMPT Increases Total and Mitochondrial NAD+ Content in Astrocytes—To investigate the effect of enhancing NAD+ salvage in astrocytes, we overexpressed NAMPT or a mitochondrial targeted version of NAMPT (mNAMPT). Overexpression was confirmed by
Western blotting of total cell lysates using antibodies against NAMPT (Fig. 2A). The presence of the Myc-DDK tag increases the size of the exogenous proteins and allows the distinction from the endogenous NAMPT. NAMPT is absent from astrocytic mitochondrial fractions even when overexpressed (Fig. 2B). However, when fused to the leading peptide of human cytochrome c oxidase subunit VIII, NAMPT is directed to the mitochondria as evidenced by subcellular fractionation and immunofluorescence (Fig. 2, B and C). Overexpression of NAMPT and mNAMPT in spinal cord astrocytes increases total NAD⁺ about 90 and 55%, respectively. Mitochondrial NAD⁺ content increases about 30% with both approaches (Fig. 2, D and E). Because mitochondrial NAD⁺ synthesis is not yet fully characterized and to demonstrate that the effect of the mNAMPT is not only restricted to astrocytes, we used the formation of immuno-detectable PAR polymers in the mitochondrial fraction to assess the PARP activity. 

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Mitochondrial NAD+ for mitochondrial antioxidant defenses. An increase in mitochondrial ROS generation in response to H2O2 (Fig. 3, A). To determine whether decreased mitochondrial oxidative stress contributes to the protection observed, the fluorescent probe MitoSox was used. MitoSox is selectively accumulated in mitochondria and oxidized as a function of ROS generation. MitoTracker Green was used in parallel to quantify total mitochondrial mass. Treatment of control astrocyte cultures with H2O2 induced a significant increase in mitochondrial ROS, which was prevented by NMN pretreatment (Fig. 3B). Likewise overexpression of NAMPT or mNAMPT also provides protection and reduces mitochondrial ROS production in response to H2O2 (Fig. 3, C and D). Moreover, NMN treatment and overexpression of NAMPT or mNAMPT also appear to decrease mitochondrial ROS levels under basal conditions (Fig. 3, B and D). The mitochondrial IDH2 plays a key role in mitochondrial antioxidant defenses through its ability to generate NADPH, an essential reducing equivalent for mitochondrial antioxidant defenses. An increase in mitochondrial NAD+ availability could lead to SIRT3-mediated deacetylation and consequent activation of IDH2 (57, 58). NMN treatment induces about a 15% increase in NADPH content in astrocytes (Fig. 3E). Fig. 3, F and G, show a decrease in IDH2 acetylation following NMN treatment. This suggests that, at least in part, an increase in IDH2 activity could explain the observed increased resistance against peroxide toxicity and decreased mitochondrial ROS generation.

Although an increase in NAD+ levels has been shown to promote oxidative metabolism (27), we found that NMN treatment significantly decreases the basal oxygen consumption rate in non-transgenic and hSOD1<sup>G93A</sup> astrocytes (Fig. 4A). However, we did not observe changes in ATP content (Fig. 4B); mitochondrial content (Fig. 4C); transcription of the mitochondrial genome-related genes Ppara<sub>1</sub>, Ppara<sub>1</sub>B, Nirf1, or Gabpa (Fig. 4D); or changes in mitochondrial membrane potential (data not shown).

Enhancing NAD+ Salvage Pathway Reverts the Toxicity of Primary Astrocytes Expressing ALS-linked Mutant SOD1—hSOD1<sup>G93A</sup> astrocytes do not display differences in NAD+ levels when compared with non-transgenic astrocytes (Fig. 1). In addition, we did not find evidence of decreased expression of NAD+ salvage enzymes or increased expression of NAD+ consuming enzymes, including SIRT1 and SIRT3, in hSOD1<sup>G93A</sup> astrocytes (Fig. 5). In contrast to the trophic support provided

**FIGURE 3.** Increasing NAD+ levels confers resistance against hydrogen peroxide toxicity and decreases mitochondrial reactive oxygen species. A, confluent cortical non-transgenic (NonTG) and hSOD1<sup>G93A</sup> (G93A) astrocyte monolayers were incubated with 5 mM NMN and 24 h later treated with the indicated concentrations of H2O2. Toxicity was assessed by lactate dehydrogenase release 24 h after peroxide treatment. Data are expressed as the percentage of the respective control. B, confluent non-transgenic and hSOD1<sup>G93A</sup> astrocyte monolayers were incubated with 5 mM NMN for 24 h. Following a change of medium, cultures were treated with H2O2 (300 μM) or vehicle, and 2 h later mitochondrial ROS (Mito ROS) production was determined. Mitochondrial reactive oxygen species was corrected by mitochondrial content (Mito mass). Data are expressed as the percentage of the non-transgenic control. C, confluent non-transgenic and hSOD1<sup>G93A</sup> astrocyte monolayers were transfected with adenovirus expressing GFP, NAMPT, or mNAMPT. 48 h post-transfection astrocytes were treated with vehicle or H2O2 (300 μM). Toxicity was assessed by lactate dehydrogenase release 24 h after peroxide treatment. Data are expressed as the percentage of their respective controls. D, confluent non-transgenic and hSOD1<sup>G93A</sup> astrocyte monolayers were transfected with adenovirus expressing GFP, NAMPT, or mNAMPT. 48 h post-transfection astrocytes were treated with H2O2 (300 μM) or vehicle. 2 h after treatment mitochondrial ROS production was determined as indicated above. Data are expressed as the percentage of non-transgenic GFP. For A, B, C, and D, each data point represents the mean ± S.D. (error bars) of at least three independent experiments. *, significantly different from vehicle-treated control/GFP for each respective genotype (p < 0.05). #, significantly different from H2O2-treated control/GFP for each respective genotype (p < 0.05). E, confluent non-transgenic and hSOD1<sup>G93A</sup> astrocytes were treated with 5 mM NMN, and 24 h later NADPH levels were determined as described under “Experimental Procedures” and corrected by protein (prot). content. Each data bar represents the mean ± S.D. (error bars) of at least three independent experiments. *, significantly different from its respective control (p < 0.05). F, confluent astrocytes were treated with 5 mM NMN for 24 h. Following protein extraction, IDH2 was immunoprecipitated and analyzed by Western blotting using an antibody against acetylated lysine (AcK). G, lysine-acetylated proteins were immunoprecipitated (IP) and then analyzed by Western blotting using an antibody against IDH2 (WB: IDH2). For both panels, as input control (INPUT), 20 μg of whole protein extracts were analyzed by Western blotting using an antibody against IDH2.
by non-transgenic astrocytes, astrocytes isolated from hSOD1<sup>G93A</sup> mice induce approximately a 40% decrease in the survival of co-cultured motor neurons (39, 40). Pretreatment with NMN or NR completely reverts the neurotoxic phenotype of hSOD1<sup>G93A</sup> astrocytes toward non-transgenic and hSOD1<sup>G93A</sup> motor neurons (Fig. 6, A and B). Accordingly, overexpression of NAMPT or mNAMPT in astrocytes also prevents motor neuron death in co-cultures (Fig. 6C). A similar protection was observed in co-cultures using astrocytes overexpressing the experimental hSOD1 mutation H46R/H48Q. Increased NAD<sup>+</sup>/H<sub>11001</sub> availability has been associated with increased sirtuin activity (4); thus we wanted to test whether the reversion of the toxic phenotype induced by the increase in NAD<sup>+</sup> levels was sirtuin-dependent. Because SIRT1 and SIRT3 have been previously implicated in models of ALS (59–61), we focused on these two enzymes. The ideal experimental approach would consist in decreasing the expression of the sirtuin before treatment with NMN to find whether the protection is lost. However, decreasing SIRT1 or SIRT3 expression in untreated non-transgenic astrocytes reduces <i>per se</i> the survival of co-cultured motor neurons to the levels observed in untreated hSOD1<sup>G93A</sup> astrocytes (Fig. 6, D, E, and F). Hence, because a mechanism different from the inherent toxicity of the hSOD1<sup>G93A</sup> astrocytes is also at play under these conditions, this experiment cannot provide a definite answer. Because a lack of a protective effect following an increase in NAD<sup>+</sup> levels in the absence of SIRT1 or SIRT3 could not be accurately attributed to a sirtuin-dependent pathway, we did not pursue this approach. To partially circumvent this issue, we overexpressed SIRT1 and SIRT3 in astrocytes before co-culturing with motor neurons (Fig. 6, G and H). Fig. 6I shows that overexpression of SIRT3, but not SIRT1, reverts the toxic phenotype of hSOD1<sup>G93A</sup> astrocytes.

**Discussion**

Although the mechanism responsible for the toxicity of ALS astrocytes toward motor neurons remains under investigation, we have shown before that decreasing oxidative stress in astrocytes prevents astrocyte-mediated toxicity (45, 55). Here we show that increasing NAD<sup>+</sup> levels leads to decreased mito-
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The protective effect was linked to an increase in NAD$^+$ availability above normal levels. hSOD1$^{G93A}$ astrocytes do not display decreased NAD$^+$ levels or altered expression of enzymes involved in NAD$^+$ degradation or the NAD$^+$ salvage pathway. Overexpression of a mitochondrially targeted NAMPT reverts the toxicity toward co-cultured motor neurons to the same extent as NAMPT overexpression. These data

FIGURE 6. Enhancing NAD$^+$ salvage pathway reverts the toxicity of astrocytes expressing ALS-linked mutant SOD1. A, confluent non-transgenic (NonTG) and hSOD1$^{G93A}$ (G93A) spinal cord astrocyte monolayers were treated with vehicle (Control), 5 mM NMN, or 5 mM NR. 24 h later purified motor neurons from non-transgenic E12.5 mice were plated on top of the astrocyte monolayer. B, the same experimental setup as in A was used, and purified motor neurons from hSOD1$^{G93A}$ E12.5 mice were plated on top of the astrocyte monolayer. For A and B, motor neuron survival was assessed 72 h later. *, significantly different from non-transgenic control ($p < 0.05$). C, confluent spinal cord astrocyte monolayers obtained from non-transgenic, hSOD1$^{G93A}$, and hSOD1$^{H46R/H48Q}$ (H46R/H48Q) mice were transfected with adenovirus expressing GFP, NAMPT, or mNAMPT. 24 h later purified motor neurons from non-transgenic E12.5 mice were plated on top of the astrocyte monolayer. Motor neuron survival was assessed 72 h later. *, significantly different from non-transgenic control ($p < 0.05$).

D and E, confluent non-transgenic (N) and hSOD1$^{G93A}$ (G) astrocytes were transfected with a negative control (NC), Sirt1, or Sirt3 siRNA, and 48 h later protein levels were determined by Western blotting. Actin levels were used as a loading control. A representative image is presented, and the bottom panels show the quantification of at least three experiments for each siRNA. *, significantly different from non-transgenic negative control ($p < 0.05$).

F and G, confluent non-transgenic (N) and hSOD1$^{G93A}$ (G) astrocytes were transfected with plasmids coding for Sirt1, Sirt3, or an empty control plasmid, and 24 h later purified motor neurons from non-transgenic E12.5 mice were plated on top of the astrocyte monolayer. Motor neuron survival was assessed 72 h later. *, significantly different from non-transgenic negative control ($p < 0.05$).

H, I, and J, confluent non-transgenic and hSOD1$^{G93A}$ spinal cord astrocyte monolayers were treated as in D and E, and 24 h later purified motor neurons from non-transgenic E12.5 mice were plated on top of the astrocyte monolayer. Motor neuron survival was assessed 72 h later. *, significantly different from non-transgenic empty ($p < 0.05$). For all panels, each data bar represents the mean ± S.D. (error bars) of at least three independent experiments.
suggest that increasing the recycling of NAM to NAD⁺ in the mitochondria of ALS astrocytes is sufficient to revert the toxic phenotype and point to the critical role of the mitochondrial NAD⁺ pool.

Increasing cellular NAD⁺ availability can influence mitochondrial redox reactions as well as the activity of NAD⁺-consuming enzymes. Compartmentalization of NAD⁺ biosynthesis and utilization remains to be fully characterized, but it is known that the mitochondrial NAD⁺ pool can be relatively uncoupled from the cytosolic pool (26). Because NMN is the product of the rate-limiting enzyme in NAD⁺ salvage and can generate NAD⁺ following a single enzymatic reaction, NMN seems the most obvious choice for NAD⁺ precursor supplementation. However, an NMN transporter has not been identified in eukaryotic cells, and it appears that the nucleotide is degraded to the nucleoside (NR) to enter the cell (24). Once in the cytosol, NR can be phosphorylated by NR kinases to produce NMN, which is taken up by the mitochondria to serve as substrate for NAD⁺ synthesis. Regardless of the exact mechanism of transport and uptake, we show here that mitochondrial NAD⁺ levels parallel total NAD⁺ increases following NMN and NR treatments in astrocytes. In astrocytes, we observed that mitochondrial NAD⁺ accounts for ~70% of total NAD⁺ in control conditions or following treatments with NMN and NR. Although there was an increase in total NADH, we did not detect an increase in mitochondrial NADH. This observation could be explained, at least in part, by the intrinsic difficulty of accurately quantifying NADH following the mitochondrial isolation process. However, it could also reflect that NADH levels are regulated by other mechanisms in addition to NAD⁺ availability (62).

NAD⁺-consuming enzymes generate NAM as a by-product, and continuous resynthesis of NAD⁺ by NAMPT is essential for mammalian cells (16). Because all NAD⁺-consuming reactions have been described in the mitochondria, we investigated the effect of increasing NAM recycling in the mitochondria by targeting NAMPT to the organelle. Our results show that a mitochondrially targeted NAMPT effectively increases total and mitochondrial NAD⁺ in astrocytes. This observation has several important implications. First, mitochondrial NAD⁺ degradation accounts for a significant portion of the NAD⁺ turnover in the cell. Thus, it is possible to modulate NAD⁺ levels by enhancing NAD⁺ salvage in a compartment-specific manner. Second, overexpression of NAMPT causes a larger increase in total NAD⁺ than overexpression of the mitochondrially targeted NAMPT, which is likely due to the fact that the latter form of the enzyme will not have access to the NAM generated by NAD⁺-consuming enzymes in the nucleus and the cytoplasm. However, both forms of the enzyme increase mitochondrial NAD⁺ levels in a similar fraction, suggesting that cytoplasmic NAMPT activity limits mitochondrial NAD⁺ levels in astrocytes. Finally, the data provide additional evidence for the existence of a mitochondrial enzymatic activity capable of using NMN to synthesize NAD⁺. Although NMNAT3 was originally identified as the mitochondrial adenylyltransferase responsible for this activity, this notion has been recently challenged (30). However, isolated mitochondria can synthesize NAD⁺ from NMN (29), and although the NMN generated by the mitochondrially targeted NAMPT could be exported to the cytoplasm to be converted to NAD⁺ and then imported back to the mitochondria, this appears to be a less likely explanation. To the best of our knowledge, this is the first work to report the effect of a mitochondrially targeted NAMPT in the NAD⁺ salvage pathway.

In cell cultures, hydrogen peroxide toxicity is in part due to NAD⁺ depletion caused by excessive PARP1 activation that could lead to glycolytic inhibition and mitochondrial failure (63, 64). Thus, higher NAD⁺ levels result in protection against peroxide treatment in astrocytes, and in particular, increasing specifically the mitochondrial NAD⁺ salvage pathway seems to confer significant protection. NMN treatment and overexpression of both forms of NAMPT decrease basal and peroxide-induced ROS production in the mitochondria. In general, an increase in the activity of NAD⁺-dependent sirtuins enhances metabolic efficiency and oxidative stress resistance (4) and could be partially responsible for the protective effect and decrease in mitochondrial ROS levels observed in astrocyte cultures. For example, an increase in NAD⁺ levels could promote SIRT1-mediated mitochondrial biogenesis (65), whereas an increase in mitochondrial NAD⁺ levels could promote SIRT3-mediated deacetylation and activation of superoxide dismutase 2 and isocitrate dehydrogenase 2 (58, 66). However, we did not observe an increase in basal oxidative metabolism or mitochondrial biogenesis that could indicate a SIRT1-mediated response. We did observe a decrease in IDH2 acetylation that can be partially responsible for the increased antioxidant defenses. The key role of IDH2 activity regulating mitochondrial antioxidant defenses has been clearly linked to the protection conferred by SIRT3 activation (58, 67). However, at the level of total protein acetylation, we did not find a consistent correlation between NAD⁺ levels and changes in protein acetylation (data not shown), which could indicate that only a small number of targets are changing acetylation status. In agreement with our results, a recent study has found that redistributing cellular NAD⁺ from the cytosol to the mitochondria decreases oxidative phosphorylation without significantly affecting global protein acetylation patterns (68). Alternatively, the observed decrease in mitochondrial ROS production could also be explained by a direct effect of shifting the NAD⁺/NADH ratio. Superoxide production by complex I is favored when the concentration of NADH is significantly higher than the levels of NAD⁺, whereas superoxide production decreases as the relative NAD⁺ concentration increases (69, 70). In astrocytes, treatment with NAD⁺ precursors leads to a much larger increase in mitochondrial NAD⁺ levels than NADH and to a decrease in mitochondrial oxygen consumption, suggesting that this mechanism could be responsible for the decrease in ROS production.

In our co-culture model, only 40% of the motor neurons seem to be susceptible to the underlying toxic mechanism (39, 40), and decreasing SIRT1 or SIRT3 expression in hSOD1G93A does not cause further reduction in motor neuron survival. However, decreasing SIRT1 or SIRT3 expression in non-transgenic astrocytes decreases motor neuron survival to the levels observed in hSOD1G93A co-cultures. This prevented us from directly testing whether a sirtuin-dependent mechanism is
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responsible for the observed protection conferred by an increase in NAD\textsuperscript{+} availability. SIRT1 activation has been shown to be protective in models of ALS (59, 60). However, overexpression of SIRT1 does not revert the toxicity of hSOD1\textsuperscript{G93A} astrocytes and could suggest that SIRT1 protection is a cell-autonomous mechanism mediated by SIRT1 activation directly in the neurons. On the contrary, SIRT3 overexpression is able to revert the neurotoxic phenotype of hSOD1\textsuperscript{G93A}. However, the protection conferred by overexpression of SIRT3 does not directly address whether a sirtuin-dependent mechanism is responsible for the protection conferred by an increase in NAD\textsuperscript{+} availability. Moreover, the increase in NAD\textsuperscript{+} availability caused by the different treatments could lead to the activation of other members of the sirtuin family with potential beneficial effects. Nevertheless, the decrease in IDH2 acetylation following NMN treatment, the reversion of the toxic phenotype by only increasing mitochondrial NAD\textsuperscript{+} salvage (mNAMPT overexpression), and the protective effect of the mitochondrial SIRT3 overexpression point out to a critical role of the mitochondrial NAD\textsuperscript{+} pool in the protection observed. Regardless, our findings demonstrate that it is possible to modulate astrocyte-motor neuron interaction by enhancing NAD\textsuperscript{+} salvage pathway in the astrocytes. Interestingly, aminopropyl carbazole derivatives previously shown to preserve motor function in hSOD1\textsuperscript{G93A} mice have been recently identified as NAMPT activators (71, 72). In summary, because we have shown that therapeutic targets identified in this co-culture system may have a beneficial effect when translated into animal models of ALS (45), our data suggest that further investigation regarding the therapeutic potential of increasing NAD\textsuperscript{+} availability to prevent astrocyte-mediated motor neuron death in ALS is deserved.

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