Nicotinamide Phosphoribosyltransferase Protects Against Ischemic Stroke Through SIRT1-Dependent Adenosine Monophosphate–Activated Kinase Pathway

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Objective: Stroke is a leading cause of mortality and disability. Nicotinamide phosphoribosyltransferase (Nampt) is the rate-limiting enzyme in mammalian nicotinamide adenine dinucleotide (NAD)⁺ biosynthesis and contributes to cell fate decisions. However, the role of Nampt in brain and stroke remains to be investigated.

Methods: We used lentivirus-mediated Nampt overexpression and knockdown to manipulate Nampt expression and explore the effects of Nampt in neuronal survival on ischemic stress both in vivo and in vitro. We also used adenosine monophosphate (AMP)-activated kinase-α2 (AMPKα2) and silent mating type information regulation 2 homolog 1 (SIRT1) knockout mice to investigate the underlying mechanisms of Nampt neuroprotection.

Results: Nampt inhibition by a highly-specific Nampt inhibitor, FK866, aggravated brain infarction in experimentally cerebral ischemia rats, whereas Nampt overexpression in local brain and Nampt enzymatic product nicotinamide mononucleotide (NMN) reduced ischemia-induced cerebral injuries. Nampt overexpression and knockdown regulated neuron survival via the AMPK pathway. Neuroprotection of Nampt was abolished in AMPKα2⁻/⁻ neurons. In neurons, Nampt positively modulated NAD⁺ levels and thereby controlled SIRT1 activity. SIRT1 coprecipitated with serine/threonine kinase 11 (LKB1), an upstream kinase of AMPK, and promoted LKB1 deacetylation in neurons. Nampt-induced LKB1 deacetylation and AMPK activation disappeared in SIRT1⁻/⁻ neurons. In contrast, Ca²⁺/calmodulin-dependent protein kinase kinase-β (CaMKK-β), another upstream kinase of AMPK, was not involved in the neuroprotection of Nampt. More important, Nampt overexpression-induced neuroprotection was abolished in SIRT1⁻/⁻ and AMPKα2⁻/⁻ mice.

Interpretation: Our findings reveal that Nampt protects against ischemic stroke through rescuing neurons from death via the SIRT1-dependent AMPK pathway and indicate that Nampt is a new therapeutic target for stroke.

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.22236

Received Jun 4, 2010, and in revised form Aug 9, 2010. Accepted for publication Aug 20, 2010.

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Additional Supporting Information can be found in the online version of this article.

Stroke is the second most common cause of death and major cause of disability worldwide.1,2 Ischemic stroke is the most common type of stroke and occurs when there is an acute blockage of arterial blood flow to the brain. The defense to energy exhaustion and metabolic stress contributes to the survival of cells under cerebral ischemia, and it also determines the outcome of acute brain injury caused by ischemic stroke.3
Elucidation of the defense mechanisms against stroke injury is considered key to the development of new therapies for stroke prevention and treatment.

Nicotinamide phosphoribosyltransferase (Nampt) converts nicotinamide into nicotinamide mononucleotide (NMN), which is subsequently converted to nicotinamide adenine dinucleotide (NAD$^+$). Nampt is the rate-limiting enzyme in mammalian NAD$^+$ biosynthesis. This protein drew much more interest after it was reported as a visceral fat-derived adipokine and renamed visfatin. We and other groups have demonstrated that both intracellular and extracellular forms of the protein have Nampt enzymatic activity. Genetic deletion of Nampt is lethal in mice, indicating the importance of Nampt for life. Nampt positively regulates the activity of silent mating type information regulation 2 homolog 1 (SIRT1), a putative longevity protein, and exerts an antiapoptotic action in many cell types. These indicate that Nampt is an emerging endogenous protector, defending the organism against damage.

However, the role of Nampt in the brain and neural cells has been poorly investigated. Here we show that Nampt plays a defensive role against stroke damage. Nampt is neuroprotective both in vivo and in vitro. The neuroprotection of Nampt is mediated by the SIRT1-dependent AMP-activated kinase (AMPK) pathway.

Materials and Methods

Animals

Male Sprague-Dawley rats were purchased from Sino-British SIPPR/BK Lab Animal Ltd. (Shanghai, China). AMPK$\times$2 knockout mice, SIRT1 knockout mice, and their corresponding wild-type (WT) mice were generated as described. All animals were used in accordance with our institutional guidelines for animal care.

Middle Cerebral Artery Occlusion

Middle cerebral artery occlusion (MCAO) surgery in rats and mice was performed as described. Briefly, the core temperature (rectum) was maintained at 36.5°C to 37.5°C by use of a homeothermic heating pad (CWE Inc., Andmore, PA) throughout the surgery. Cerebral focal ischemia was produced by intraluminal occlusion of the left MCA using a silicone rubber–coated nylon monofilament. Cortical blood flow was measured with a laser Doppler flow meter (VMS-LDF1; Moor Instruments, Axminster, UK). The laser Doppler probe was placed over the cortical area supplied by the MCA to ensure that cerebral blood flow was reduced by more than 85%. Two hours after MCAO, the occluding filament was withdrawn to allow reperfusion. The physiological parameters, including systolic blood pressure, diastolic blood pressure, arterial pH, arterial partial pressure of carbon dioxide (pCO$_2$), and arterial partial pressure of oxygen (pO$_2$), were monitored in mice before MCAO, during MCAO, and after reperfusion using a blood pressure instrument (ALCBIO, Shanghai, China) and a blood gas analyzer (Instrumentation Laboratory, Lexington, MA). At 24 or 48 hours after MCAO, rats and mice were killed for various examinations.

Neuron Culture and Oxygen-Glucose Deprivation Model

Primary rat and mouse neuronal cells were prepared from the cerebral cortex of neonatal animals within 6 hours after birth and genotyping, as described. One day after isolation, the cultures were replenished with Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with 2% B27 (Invitrogen). Glial growth was suppressed by addition of 5-fluoro-2-deoxyuridine and uridine (10µM), yielding cultured cells with >90% neurons, confirmed by staining for NeuN (neuron marker) and glial fibrillary acidic protein (GFAP) (astrocyte marker). After 7 days in vitro (DIV), we began to transfect the neurons with lentivirus for overexpression or knockdown of Nampt. The incubation time for transfection was 2 days. To establish oxygen-glucose deprivation (OGD) conditions, the cultured neurons were washed 3 times and incubated with glucose-free Earle’s balanced salt solution (EBSS) and placed for different times within a hypoxic chamber (Forma Scientific, Marietta, OH) that was continuously flushed with 95% N$_2$ and 5% CO$_2$ at 37°C to obtain <0.2% O$_2$. Control neuron cultures were placed in EBSS containing glucose (25 mmol/l) and incubated under normal culture conditions for the same period.

Preparation of Plasmids Encoding Complementary DNA of Nampt and Short Hairpin RNA and Lentivirus Production and Transfection

For plasmid encoding Nampt, we used pRNAT-U6.2-green fluorescent protein (GFP)/Lenti vector (Genescript, Piscataway, NJ). The rat Nampt (GenBank accession number NM_177928) complementary DNA (cDNA) was polymerase chain reaction (PCR) amplified, sequenced, and subcloned into a pRNA-U6.2/Lenti vector with Xma1/Kpn1 restriction sites to construct a pRNA-U6.2-GFP/Lenti-Nampt vector. The short hairpin RNA (sh-RNA)-mediated knockdown (sh-Nampt) vectors were constructed by subcloning the H1 promoter-sh-Nampt cassette into the Cclal-Cclal sites of the pLVTH vector, which was obtained from AddGene (http://www.addgene.org). Plasmids for lentiviral packaging, pMDLg/pRRe, pRSV-REV, and pMD2G were also obtained from AddGene. The infectious lentivirus was produced by transfecting lentivector and packaging vectors into 293T cells (Invitrogen).

Drug and Lentivirus Administration In Vivo

Rats were intraperitoneally injected with FK866 (4mg/kg) or vehicle (propylene glycol) twice a day for 3 days to inhibit Nampt before cerebral ischemia induced by MCAO. For NMN administration, 30 minutes after MCAO, rats were fixed in a stereotactic frame (ASI Instruments, Houston, TX) and were injected with 2µl of NMN (10mg/ml) or vehicle (saline) into
the left lateral cerebroventricle for 15 minutes. During the 15 minutes of intracerebroventricular (i.c.v.) infusion, we used a homeothermic heating blanket to maintain the core temperature (rectum) at 37°C, as we did during the MCAO operation. For lentivirus infusion, lentiviral vectors encoding Nampt (2μl per site for rats and 0.5μl per site for mice; 1 × 10⁹ transduction units [TU]/ml) or empty lentiviral vectors or saline control were stereotaxically injected into the cortex and hippocampus of 5-week-old rats and mice at 4 sites. Three weeks later, MCAO was performed in these animals.

**Immunoblotting and Immunoprecipitation**

Tissues and cells were lysed and used for immunoprecipitation and immunoblotting as described. Blots were incubated with primary antibodies and IRDye800CW-conjugated secondary antibody. The image was captured by the Odyssey infrared imaging system (Li-Cor Bioscience, Lincoln, NE). All immunoblotting experiments were repeated at least 3 times. For details of the immunoprecipitation assay, see the Supporting Information.

**Immunohistochemistry and Immunofluorescence**

Immunohistochemistry and immunofluorescence were performed as described. Frozen 20-μm-thick brain sections and cultured neurons were fixed in 4% paraformaldehyde, blocked by 8% normal goat serum, and incubated in specific primary antibodies as follows: NeuN (1:200), GFAP (1:1000), Nampt (1:400), MAP-2 (1:400), GFP (1:400), and p-AMPK (1:200). After being washed 3 times with phosphate-buffered saline (PBS), the sections and cells were incubated with Alexa 488-conjugated or Cy3-conjugated secondary antibodies. An immunofluorescence terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed according to the manufacturer’s instructions (Roche, Penzberg, Germany). Images were obtained by fluorescence microscope (IX-71; Olympus, Tokyo, Japan) with a digital camera (Olympus). Digital images were recorded and analyzed using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD) and Adobe Photoshop software (Adobe, Mountain View, CA).

**Analysis of Cell Viability and Lactate Dehydrogenase Release**

Cell viability was evaluated by a nonradioactive cell counting kit (CCK-8) assay (Dojindo, Kumamoto, Japan). Lactate dehydrogenase (LDH) release analysis was performed with a colorimetric LDH cytotoxicity assay (Promega, Madison, WI).

**NAD⁺/NADH and SIRT1 Deacetylase Activity Assay**

NAD⁺ and NADH levels were determined with a NAD⁺/NADH quantification kit (BioVision, Mountain View, CA) according to the manufacturer’s instructions. To evaluate the SIRT1 activity, whole cell extracts were obtained using a mild lysis buffer plus protease inhibitor mix from cultured neurons in the presence of LV-GFP, LV-Nampt, NMN (300μM), or NAD (1mM). Generally, 100μg of extracts were used for the deacetylation assay using the Fluorimetric Drug Discovery Kit (Biomol, Plymouth Meeting, PA). SIRT activity was determined using a microplate fluorimeter (Tecan, Hillsborough, NC).

**Statistical Analysis**

Data are expressed as mean ± SEM. Differences were evaluated by 2-tailed Student t test (2 groups) or 1-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) post hoc test (3 or more groups). When experiments were designed to measure 2 different factors, or to test 2 factors at the same time, 2-way ANOVA followed by SNK post hoc analysis was performed. Statistical significance was set at p < 0.05.

**Results**

**Nampt Is Expressed in Brain and Neurons and Upregulated After Ischemia**

In initial experiments, we examined Nampt expression in rat brain and neural cells. Nampt was expressed in brain tissue (Fig 1A). It was also expressed in cultured neurons and astrocytes (see Fig 1B). However, the expression of Nampt in neurons was higher than in astrocytes. Furthermore, immunofluorescent staining demonstrated that Nampt was mainly colocalized with neurons indicated by a neuronal marker of NeuN (see Fig 1C) as opposed to astrocytes (see Fig 1D) in brain tissue. The coexistence of Nampt and neuronal markers of microtubule-associated protein-2 (MAP-2) in cultured neurons was also shown (see Fig 1E).

To explore whether Nampt is involved in ischemic stroke, we determined Nampt expression in a rat model of ischemic stroke experimentally produced by unilateral MCAO. In this focal cerebral infarction model, Nampt expression was markedly increased in both infarct core and peri-infarct penumbra, compared with the corresponding unaffected contralateral region (see Fig 1F). Additionally, Nampt was upregulated in an in vitro model of ischemic neuronal injury induced by OGD (see Fig 1G).

Next, to determine the function of Nampt in stroke, intervention studies were performed in vivo and in vitro using Nampt-specific inhibitor FK866, Nampt enzymatic product NMN, Nampt overexpression, and Nampt knockdown.

**Nampt Inhibition Aggravates Neuronal Injury**

To determine the effect of Nampt activity on ischemic stroke, experiments were performed using MCAO rats. FK866 (4mg/kg, intraperitoneally [i.p.]) twice a day for 3 days significantly inhibited Nampt activity, leading to a marked reduction of brain NMN level as well as resulting in a substantial enlargement of MCAO-induced infarction (Fig 2A–C). This effect of FK866 was
significantly prevented by supplementation with NMN (i.c.v.) via stereotactic infusion into lateral cerebroventricle 30 minutes after MCAO. TUNEL staining demonstrated that FK866 increased brain cell death in both infarct core and peri-infarct penumbra, which was totally prevented by supplementation with NMN (see Fig 2D). However, FK866 did not induce brain cell death in the unaffected contralateral region (see Fig 2D) and in normal rats without MCAO.

Next, we studied the effect of FK866 and NMN in cultured neurons. In normal conditions, inhibition of Nampt by FK866 had no significant effect on neuronal survival (see Fig 2E–G). However, under OGD, FK866 significantly aggravated neuronal injury, as evidenced by a reduction in MAP-2-positive neuronal cells and cell viability and by an increased release of LDH, which was prevented by supplementation with NMN. To further delineate the signal events involved in Nampt inhibition-induced neuron apoptosis, we studied the potential involvement of cleaved caspase 3 and mitochondrial bcl-2 family members. Under OGD, inhibition of Nampt increased expression of proapoptotic proteins (cleaved...
caspase 3, bax, and bad) and decreased expression of antiapoptotic proteins (bcl-2 and bcl-XL), which was prevented by supplementation with NMN (Supporting Fig 1A). In the same manner, cytochrome c released from mitochondria to the cytoplasm was increased by FK866 under OGD, which was prevented by supplementation with NMN (Supporting Fig 1B).

Nampt Enzymatic Product NMN Is Neuroprotective In Vivo and In Vitro
In addition to the prevention of NMN on neurotoxic effects of FK866 under ischemia, NMN itself had a neuroprotective effect in MCAO rats and OGD neurons. NMN injection significantly attenuated MCAO-induced cerebral infarction size (Fig 3A), neurological deficit (see Fig 3B), and neuronal cell death (see Fig 3C). Moreover, NMN protected cells against injury in OGD-treated neurons in vitro (see Fig 3D–F). NMN decreased proapoptotic protein levels and increased antiapoptotic protein levels in cultured neurons under OGD (Supporting Fig 1C). These results strongly suggest that Nampt promotes neuronal survival on ischemic stress.

Nampt Overexpression Protects Against Ischemic Stroke
The effect of Nampt on ischemic stroke was further investigated using local overexpression of Nampt in the brain. Lentiviral vectors encoding Nampt (LV-Nampt) or empty lentiviral vectors encoding green fluorescent protein (LV-GFP, vector control) were injected into rat cortex and hippocampus at 4 sites, MCAO was performed 3 weeks after injection, and animals were killed 24 hours after MCAO for various examinations (Fig 4A,B). Lentivirus-mediated Nampt expression in the brain and
neurons was confirmed by immunochemical analysis of GFP (see Fig 4C,D). Injection of LV-Nampt led to an approximately 2.0-fold increase of Nampt protein levels in targeted brain regions (see Fig 4E), and the neurological deficit score was ameliorated (see Fig 4G). Brain cell death in peri-infarct penumbra was found with a pronounced decrease in the LV-Nampt group (see Fig 4H). In both infarct core and peri-infarct penumbra of the LV-Nampt group, antiapoptotic proteins increased and proapoptotic proteins decreased, without alterations in unaffected contralateral regions (Supporting Fig 1D).

**Nampt Critically Regulates Neuron Survival**

We determined the role of Nampt in neuron survival using Nampt overexpression and knockdown in cultured neurons. Primary neurons were infected with lentiviral vectors encoding short hairpin RNA targeting Nampt (sh-Nampt) to prepare the corresponding neurons with lentivirus-mediated overexpression of Nampt (LV-Nampt) or lentivirus-mediated knockdown of Nampt (sh-Nampt). For all experiments, transfection efficiency was maintained at over 90% determined by flow cytometer, and no detectable cellular toxicity was observed (Supporting Fig 2). Expressions of Nampt in LV-Nampt and sh-Nampt neurons were 2.4-fold (overexpression) and 0.16-fold (knockdown), respectively, compared with their control. Overexpression of Nampt reduced OGD-induced neuronal injury, whereas knockdown of Nampt aggravated OGD-induced neuronal injury (Fig 5A,B). Using TUNEL analysis and mitochondrial membrane potential analysis with JC-1 staining to identify neuronal apoptosis, similar effects were observed (see Fig 5C,D). It was also noted that in normal conditions without OGD, both overexpression and knockdown of Nampt had no effect on the preceding parameters with respect to

**FIGURE 3: NMN, the enzymatic product of Nampt, is neuroprotective in MCAO rats and in OGD neurons.** (A, B) Intracerebroventricular injection (i.c.v.) of NMN (2μl, 10mg/ml) 30 minutes after MCAO reduces the infarction volume and neurological deficit score. \( p < 0.05, \ *p < 0.01 \) vs Vehicle; \( n = 8 \). (C) TUNEL staining showing NMN reduces brain cell death in peri-infarct penumbra after MCAO. \( \ast \ p < 0.01 \) vs Vehicle; \( n = 8 \). (D-F) NMN protects against neuronal injury under OGD. \( \ast \ p < 0.05, \ \ast \ast \ p < 0.01 \) vs Vehicle; \( n = 6 \). [Color figure can be viewed in the online issue, which is available at annalsofneurology.org.]
neuronal survival, except for a significant loss of mitochondrial membrane integrity in sh-Nampt neurons (see Fig 5D). Furthermore, annexin V and propidium iodide (PI) staining assay by flow cytometry showed that LV-Nampt neurons exhibited resistance not only to cell apoptosis (annexin V–positive) but also to cell necrosis (PI-positive) under OGD (see Fig 5E). In contrast, sh-Nampt neurons were susceptible to apoptosis and necrosis under OGD (see Fig 5F). Finally, the expression of apoptosis-related proteins in LV-Nampt and sh-Nampt neurons demonstrated that Nampt overexpression increased antiapoptotic proteins and attenuated proapoptotic proteins under OGD, whereas Nampt knockdown induced converse effects (Supporting Fig 3A,B).

Nampt-Evoked Neuron Survival Is Mediated by AMPK Pathway

AMPK acts as a metabolic master switch to maintain the local and systemic energy balance under energetic stress.22 We first investigated the function of AMPK in ischemic injury in cultured neurons. AMPK activator 5-aminimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR) exhibited a neuroprotective effect in OGD neurons (Supporting Fig 4). Next, we examined the effect of Nampt on the AMPK signaling pathway. AMPK was significantly activated by OGD (Fig 6A). Intriguingly, under OGD, Nampt overexpression further increased phosphorylation of AMPK and of its substrate acetylcoenzyme A (CoA)-carboxylase (ACC) (see Fig 6B),
whereas Nampt knockdown and inhibition induced converse effects (see Fig 6B). Moreover, the increased phosphorylation of AMPK and ACC by LV-Nampt under OGD could be blocked by Nampt inhibitor FK866, and decreased phosphorylation of AMPK and ACC induced by sh-Nampt and FK866 could be reversed by administration of NMN (Supporting Fig 5). It was also shown that AMPK phosphorylation and Nampt expression were enhanced and colocalized in the penumbra area of MCAO rat brain (see Fig 6C). In addition to the AMPK pathway, we excluded the possibility of 2 other important stress-responsive and survival signal pathways, PI3K-Akt and STAT3 (Supporting Fig 6A,B), which have been shown to be involved in the diverse functions of Nampt.11,23

To know whether Nampt-evoked neuron survival is mediated by the AMPK pathway, we further examined the effect of Compound C, a chemical inhibitor of AMPK. AMPK inhibition by Compound C aggravated OGD-induced neuronal injury (see Fig 6D–F). Moreover, Compound C significantly reduced LV-Nampt-induced neuroprotection.

To more specifically confirm that Nampt-induced neuroprotection is mediated by the AMPK pathway, we tested this effect in cultured neurons from AMPKa2 catalytic subunit knockout mice (AMPKa2−/−).12 In our

FIGURE 5: Lentivirus-mediated overexpression and knockdown of Nampt regulate neuron survival under OGD. (A–D) Nampt overexpression by LV-Nampt prevents neuronal injury, whereas Nampt knockdown by sh-Nampt aggravates neuronal injury, in cultured neurons under OGD. Cell viability and LDH release, n = 8; TUNEL staining, n = 6; JC-1 staining flow cytometer analysis of mitochondrial membrane potential, n = 4. *p < 0.05 vs LV-GFP; #p < 0.05 vs sh-Scramble. (E, F) Flow cytometer analysis of cell apoptosis (Annexin V staining, R2+R3) and necrosis (PI staining, R3+R4), n = 3. *p < 0.05, **p < 0.01 vs LV-GFP or sh-Scramble. R1 = alive cells; R2 = early apoptotic cells (Annexin V–positive, PI–negative); R3 = late apoptotic or necrotic cells (Annexin V–positive, PI–positive); R4 = dead cells and cell fraction (Annexin V–negative, PI–positive). [Color figure can be viewed in the online issue, which is available at annalsofneurology.org.]
study, the AMPKα2 subunit was found to be expressed at much higher levels (>10-fold) than the AMPKα1 subunit in neurons (Supporting Fig 6), in line with a previous report. OGD-induced neuron injury was aggravated by ablation of AMPKα2 (Fig 7A,B). Moreover, neuroprotection of Nampt overexpression was abolished in AMPKα2-null neurons. These data indicate that the AMPK pathway plays an important role in Nampt-evoked neuron survival. Deletion of AMPKα2 or AMPK inhibition by Compound C did not hamper the upregulation of Nampt induced by OGD (see Fig 7C,D), indicating that Nampt upregulation by OGD is independent
of AMPK activation. All the previously mentioned results reveal that Nampt overexpression–induced AMPK activation is a new pathway for rescuing neurons from death.

**SIRT1 Is Essential for Nampt-Induced AMPK Activation and Neuroprotection**

In view of the observation that Nampt colocalizes with AMPK in the brain and regulates AMPK activation (see Fig 6C), we determined whether Nampt can directly interact with AMPK in cultured neurons using an immunoprecipitation assay. Our data did not show a direct interaction between these 2 proteins (Supporting Fig 7). As several biological effects of Nampt are mediated by SIRT1, we hypothesized that NAD$^+$-dependent SIRT1 activity upregulated by Nampt may affect AMPK activation. Although previously verified in other cell types, the theory that NAD$^+$ level and SIRT1 activity are regulated by Nampt has never been tested in mammalian neurons. In normal conditions, Nampt overexpression mildly enhanced SIRT1 activity, whereas Nampt knockdown and FK866 markedly reduced SIRT1 activity (Supporting Fig 8). OGD decreased NAD$^+$ and NAD$^+/NADH$ ratios significantly. Nampt overexpression attenuated the decrease of NAD$^+$ and NAD$^+/NADH$ ratios induced by OGD, which could be blocked by FK866 (Fig 8A). Consistent with these results, OGD-induced reduction of SIRT1 activity could be recovered by NMN, NAD$^+$, and Nampt overexpression (see Fig 8B). Next we compared AMPK activation on OGD in WT and SIRT1$^{-/-}$ neurons; AMPK activation was swift (3 hours) and strong in WT neurons but slow (24 hours) and weak in SIRT1$^{-/-}$ neurons (see Fig 8C), indicating that deletion of SIRT1 impairs the activation of AMPK in neurons. Moreover, the increase in AMPK phosphorylation by Nampt overexpression was also abolished in SIRT1$^{-/-}$ neurons (see Fig 8D).

**LKB1, But Not Ca$^{2+}$/Calmodulin-Dependent Protein Kinase Kinase-$\beta$, Is the Mediator of SIRT1 Regulation on AMPK**

To further determine how AMPK activation is impaired by deletion of SIRT1 and the potential link between these 2 proteins in neurons, we first targeted the LKB1, the major upstream kinase of AMPK. LKB1 is active after being deacetylated.$^{18}$ We found that endogenous SIRT1 coprecipitated with endogenous LKB1 in neurons (Fig 9A). Thus we naturally proposed that SIRT1 regulates the AMPK pathway via LKB1 in neurons. Supporting this speculation, deacetylation of LKB1 induced by OGD was apparently impaired in SIRT1$^{-/-}$ neurons (see Fig 9B). In addition, Nampt overexpression enhanced the deacetylation of LKB1 in both normal and OGD conditions, whereas FK866 induced converse effects (see Fig 9C,D; Supporting Fig 9). However, in SIRT1$^{-/-}$ neurons, the effects of Nampt overexpression and FK866 both disappeared (see Fig 9C,D).

Besides LKB1, we studied calmodulin-dependent protein kinase kinase-$\beta$ (CaMKK-$\beta$) another upstream
kinase of AMPK, which is also expressed at a high level in the brain. Inhibition of CaMKK-β by specific inhibitor STO-609 slightly aggravated neuronal injury at 12 hours after OGD but not at 24 hours after OGD (see Fig 9E). This agent did not affect the neuroprotective ability of Nampt at both 12 and 24 hours after OGD. Moreover, AMPK phosphorylation induced by OGD and Nampt overexpression was not affected by STO-609 (see Fig 9F), indicating that CaMKK-β is neuroprotective to some extent but not involved in the neuroprotection of the Nampt-AMPK cascade.

**Nampt-SIRT1-AMPK Cascade Is Neuroprotective In Vivo**

On the basis of the preceding in vitro data showing that Nampt regulates AMPK activation via SIRT1-dependent deacetylation of LKB1 in OGD-treated neurons, we investigated whether SIRT1 and AMPK are essential for the neuroprotection of Nampt in vivo. Because SIRT1−/− homozygous mice are perinatal lethal, we used SIRT1+/− heterozygous mice for in vivo study. As shown in Supporting Tables 1 and 2, the physiological parameters were not significantly changed in SIRT1+/− and AMPKα2−/− mice compared with their respective WT mice. Lentivirus-mediated Nampt overexpression in the local brain ameliorated the MCAO-induced infarction and neurological deficit in WT mice, whereas these effects were blunted in SIRT1+/− mice (Fig 10A,B) and AMPKα2−/− mice (see Fig 10C,D).

**Discussion**

The most important finding is that Nampt is a neuroprotector on ischemic stress. We gave the first demonstration for preferential expression of Nampt in neurons as opposed to astrocytes. Remarkably, Nampt was significantly upregulated in the peri-infarct area and infarct core of MCAO models. Inhibition of Nampt augmented infarction in MCAO rats, whereas overexpression of Nampt in local brain protected against stroke injury in MCAO rats, indicating that Nampt is a key protective factor in ischemic stroke. Furthermore, the neuroprotection of Nampt was confirmed in cultured neurons of the OGD model. Inhibition or knockdown of Nampt aggravated neuronal injury, whereas overexpression of Nampt
FIGURE 9: LKB1, but not CaMKK-β, is the mediator of SIRT1 regulation on AMPK in neurons. (A) Immunoprecipitation analysis shows a direct interaction between SIRT1 and LKB1 in WT neurons. SIRT1−/− neurons are used as a negative control. IP = immunoprecipitation; IB = immunoblotting. (B) OGD activates LKB1 deacetylation (active form) in WT neurons, which disappears in SIRT1−/− neurons. Anti-acetyl-lysine (Ac-Lys) antibody is used to determine acetylation status of LKB1 (inactive form). *p < 0.05 vs WT neurons under normal conditions; n = 3. (C, D) Nampt overexpression by LV-Nampt activates LKB1 deacetylation, while Nampt inhibition by FK866 reduces LKB1 deacetylation in WT neurons under OGD. Both effects disappear in SIRT1−/− neurons. *p < 0.05 vs LV-GFP in WT neurons; †p < 0.05 vs Vehicle in WT neurons; n = 3. (E) Treatment of CaMKK-β inhibitor STO-609 (3μM) promotes neuronal injury at 12 hours after OGD but not at 24 hours after OGD. STO-609 does not affect the neuroprotection of Nampt at 12 or 24 hours after OGD. *p < 0.05 vs Vehicle; †p < 0.05 vs LV-GFP. (F) STO-609 does not affect the AMPK activation induced by OGD in LV-GFP and LV-Nampt neurons. *p < 0.05 vs without OGD; ‡p < 0.05 vs LV-GFP.
reduced neuronal injury. All these in vivo and in vitro results identify Nampt as a chief dictator for neuron survival in ischemia and implicate Nampt as a crucial factor involved in energy balance regulation.

Furthermore, we demonstrate a novel "Nampt-SIRT1-AMPK" neuroprotective signaling pathway under cerebral ischemic stress (see Fig 10E). Utilizing both pharmacological and genetic manipulations, this study showed for the first time that upregulation of Nampt positively modulated NAD$^+$ levels and thereby controlled SIRT1 activity, leading to LKB1 deacetylation and then AMPK activation in vitro. More important, the in vivo study using SIRT1$^{1/2}$ and AMPK$^{2/2}$ mice confirmed that SIRT1 and AMPK are essential for the neuroprotection of Nampt overexpression in ischemic stroke. LKB1, an upstream kinase of AMPK, is a key link between the SIRT1 and AMPK in this cascade. SIRT1 directly interacted with LKB1 and promoted LKB1 deacetylation, and this process was controlled by Nampt. In contrast, CaMKK-$\beta$, another upstream kinase of AMPK, was found not to be involved in the neuroprotection of Nampt. In addition, we excluded the possibility of 2 other stress-responsive and survival signaling pathways (Akt and STAT3) involved in Nampt neuroprotective signaling.

Poly (ADP-ribose) polymerase (PARP)-1 activation plays a role in neuronal death on ischemic stress through NAD$^+$ depletion. It has been reported that NAD$^+$ depletion is necessary and sufficient for PARP-1-mediated neuronal death. Poly (ADP-ribose) polymerase (PARP)-1 activation plays a role in neuronal death on ischemic stress through NAD$^+$ depletion. Therefore, supplementation of NAD$^+$ is neuroprotective and prevents PARP-1-mediated cell death in vitro. According to our results, Nampt overexpression-induced neuroprotection was abolished in SIRT1 knockout mice, indicating that SIRT1 is a key player. Regarding SIRT1 and PARP-1, it has been reported that PARP-1-dependent cardiac myocyte cell death during heart failure is mediated by NAD$^+$ depletion and reduced SIRT1 activity. Thus it is possible that PARP-1-mediated neuronal death is through depleting NAD$^+$ and then reducing SIRT1 activity under ischemic stress. This remains to be investigated in the future.

The present signaling model is different from that previously proposed, in which AMPK lies upstream of Nampt and regulates Nampt expression and subsequently SIRT1 activity. Canto and colleagues also demonstrated another disparate model in which AMPK regulates SIRT1 activity through a non-Nampt link. These 2 studies were performed in myocytes, which are very different from the central nervous system. In our study,
Nampt upregulation by OGD in neurons was not affected by deletion of AMPKα2 or AMPK chemical inhibition, clearly indicating that Nampt upregulation during ischemic stress is not driven by AMPK in neurons.

As a therapeutic target for stroke, Nampt seems superior to SIRT1. Although both are neuroprotective, it should be emphasized that SIRT1 activity is controlled by NAD⁺ levels. In ischemic stroke, brain NAD⁺ levels decrease swiftly, which makes SIRT1 activators and SIRT1 overexpression inefficient. In this case, upregulation of Nampt may produce more NAD⁺ to ensure SIRT1 function but also reduce nicotinamide, a potent inhibitor of SIRT1.⁵¹ Therefore, Nampt activators or molecules that enhance Nampt expression might be an effective therapy for stroke.

In conclusion, this study demonstrates that upregulation of Nampt in ischemic stroke protects neurons against energy stress. Nampt promotes neuronal survival through SIRT1-dependent AMPK pathways (see Fig 10E). Our results provide new insights into endogenous defense mechanisms in ischemic stroke and suggest that Nampt is a new therapeutic target for stroke prevention and treatment.

Acknowledgments
This research was supported by grants from the National Natural Science Foundation of China for Distinguished Young Scholars (30525045 to C.-Y.M.), the National Basic Research Program of China (2009CB521902 to C.-Y.M.), the Program of Shanghai Subject Chief Scientist (10XD1405300 to C.-Y.M.), the Foundation for National Excellent Doctoral Thesis Author (200369 to C.-Y.M.), and the National Science and Technology Major Project (2009ZX09303-002 to C.-Y.M.).

We thank Apoxis SA, a Swiss corporation, for donating Nampt inhibitor FK866 (also known as APO866).

Potential Conflicts of Interest
Nothing to report.

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