Supporting Information

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SI Materials and Methods

Neonatal Hypoxic-Ischemic Brain Injury. Neonatal hypoxic-ischemic (H-I) brain injury was performed on postnatal (P) day 7 mouse pups as described (1). The pups were anesthetized on a heating pad to maintain body temperature with 5% isoflurane for induction and 1.5% isoflurane for maintenance (balance, room air). The left common carotid artery was exposed surgically and ligated permanently. The pups were returned to their cages with their mother to recover for 2 h before being placed in an hypoxia chamber maintained at 37 °C through which 8% oxygen (balance, nitrogen) flowed for 45 min. After hypoxia the pups were returned to the cages with their mother until they were killed.

Cortical Culture and Excitotoxicity Assay. The cortex was dissected from embryonic (E) day 14.5 C57BL6 embryos and dissociated with 0.5% trypsin for 15 min at 37 °C. Dissociated cortical neurons were washed with 10% serum containing DMEM and then were washed three times with complete medium [Neurobasal (21103; Gibco), 2% B27 (Invitrogen), 1 μM 5-fluoro-2′-deoxyuridine, and 1 μM uridine]. Cortical neurons were seeded in a 96-well culture dish coated with poly-D-lysine and laminin (Invitrogen) at a density of 1.2×10^5 cells per well. Neurons were infected with lentivirus (10^5 infectious particles) expressing EGFP, cytoplasmic nicotinamide mononucleotide adenylyl transferase 1 (cytNmnat1), or B cell lymphoma-extra large (Bcl-XL) protein (2) on day in vitro (DIV) 3, and 50% of the medium was replaced with fresh medium every 4 d. For the axonal degeneration assays, neurons at DIV10 were treated with NMDA, fixed with 4% paraformaldehyde containing PBS, stained with antibody against α-tubulin (1:2,000; Sigma), and visualized with Cy3-conjugated secondary antibody. MK801 (5 μM; Sigma), and the pan-caspase inhibitor N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VAD-FMK) (50 μM; Calbiochem) were added ∼5 min before the addition of NMDA. Axonal degeneration was quantified by ImageJ-based software (National Institutes of Health) with slight modifications (3). The neurite degeneration index was expressed as the total area of fragmented neurites recognized as particles over the total area covered by all neurites from binarized images from α-tubulin staining.

Histology and Determination of Tissue Loss. At day P14 pups were anesthetized with pentobarbital (200 mg/kg) and perfused transcardially with 3 U/mL heparin in PBS, pH 7.4.

The brains were removed rapidly and were immersion-fixed in 4% paraformaldehyde at 4 °C for 24 h. They subsequently were maintained in 30% sucrose until frozen in powdered dry ice and cut into 50-μm coronal sections with a freezing sliding microtome. Every sixth section (giving an approximate distance of 300 μm between slides) from the genu of corpus callosum through the caudal end of the hippocampus was mounted and stained with cresyl violet as described (1). The stained tissues were scanned using a NanoZoomer digital pathology system (Hamamatsu Photonics). Tissue loss was calculated by comparing the volume of distinct regions of the brain in the injured (left) hemisphere and the noninjured (right) hemisphere using the NanoZoomer quantitation software.

Caspase-3 Asp-Glu-Val-Asp (DEVD) Cleavage Activity. Twenty-four hours after H-I, pups were anesthetized with pentobarbital (200 mg/kg) and perfused transcardially with 3 U/mL heparin in PBS, pH 7.4. The right and left hippocampal and cortical regions were dissected on ice and then frozen on dry ice. Brain samples were homogenized and centrifuged at 19,000 × *g* for 15 min at 4 °C. Protein concentrations in the lysates were determined using a BCA kit (Pierce), and DEVD cleavage activity after the procedure was measured as previously described (1). DEVD cleavage activity was normalized to total protein concentration for each of the samples, and the data were reported as pmol amino-4-methyl coumarin (AMC) generated per mg of protein per minute.

Immunohistochemistry of Brain Tissue. To determine tissue loss pups were killed 24 h after H-I, and the brain tissue was sectioned as described previously. A set of free-floating sections, consisting of approximately every sixth section from the genu of the corpus callosum through the caudal end of the hippocampus, was processed for immunohistochemistry as described (1). Briefly, the tissue was blocked with 5% goat serum in Tris-buffered saline (TBS) and 0.25% (vol/vol) Triton-X. Brain sections were incubated with rabbit antibody against active caspase-3 (1:200; Cell Signaling) at 4 °C overnight, followed by incubation in secondary antibody (1:500 goat anti-rabbit; Alexa Fluor 488). The fluorescent tissues were scanned using the NanoZoomer digital pathology system. To visualize microglia, brain tissue blocked with 5% goat serum in TBS with 0.25% (vol/vol) Triton-X was incubated in rabbit antibody against ionized calcium-binding adaptor molecule 1 (IBA1) (1:5,000; Wako) at 4 °C overnight and then in biotinylated goat anti-rabbit secondary antibody (1:1,000). To assess the level of astrocytic gliosis qualitatively, brain tissue first was blocked with 5% donkey serum in TBS with 0.25% (vol/vol) Triton-X. The tissue then was incubated in rabbit antibody against GFAP (1:1,000; DAKO) and subsequently was incubated in biotinylated donkey anti-rabbit secondary antibody (1:1,000). All sections were scanned using the NanoZoomer digital pathology system capturing images in both bright-field mode (for GFAP and IBA1 immunostaining) and fluorescent mode (for caspase-3 staining). The resulting scanned images were quantified using Image J software.

Cytokine Measurement. After H-I, pups were anesthetized with pentobarbital (200 mg/kg), and the right and left hippocampal regions were dissected and then frozen on dry ice. Brain samples were homogenized and centrifuged at 19,000 × *g* for 5 min at 4 °C. Protein concentrations in the lysates were determined using a BCA kit (Pierce), and the levels of 28 cytokines were evaluated by Rules Based Medicine, Inc., using Rodent CytokineMAP A v. 1.0, Rodent CytokineMAP B v. 1.0, and Rodent CytokineMAP C v. 1.0 (4). IL-1β, IL-12 subunit p70, IL-17α, IL-3, IL-6, growth-regulated alpha protein, and the T-cell–specific protein RANTES were below the detection limit in our injured and noninjured hippocampal hemispheres of P9 (48 h after H-I) wild-type and cytNmnat1-Tg animal samples.

Nucleotide Measurements. The cortex and hippocampus were frozen in liquid nitrogen immediately after dissection. Tissues were homogenized by sonication in five volumes of 1 M $HClO_4$ and were centrifuged at 19,000 × *g* for 15 min. The cleared lysates were neutralized with 3 M KCO_3 and recentrifuged at 19,000 × *g* for 15 min. One volume of supernatant was mixed with 50 volumes of phosphate buffer (pH 7.5) and injected into an HPLC (Shimazu) equipped with a C18 column (Speclo). Each sample (50 mL) was injected, and the nucleotides were separated with MeOH gradient as described previously (Sasaki et al., 2009). The elution peaks of NAD, ATP, ADP, and AMP were

compared with standards for identification, and the levels were quantified with a series of standard concentrations. The nucleotide levels were normalized to tissue weight of the brain regions analyzed. The steady-state levels of ATP in the hippocampus of wild-type and cytNmnat1-Tg mice were determined by using a bioluminescence assay kit (A22066; Invitrogen) and were normalized to the tissue weight of the brain regions analyzed (Fig. S2).

Lactate Dehydrogenase Release. The conditioned media of cortical neuron cultures were collected and assayed for lactate dehydrogenase (LDH) activity using a cytotoxicity detection kit (Roche Applied Science). Briefly, LDH catalyzes the conversion of lactate to pyruvate upon reduction of NAD^+ to $NADH/H^+$; and then the added tetrazolium salt is reduced to formazan. The amount of formazan formed correlates with LDH activity. The formazan product was measured with a microtiter plate reader at 490 nm. The basal levels of LDH released by cortical neurons were different in cells transfected and/or treated with EGFP, cytNmnat1, Bcl-XL, MK801, and the pan-caspase inhibitor ZVAD. To calculate the LDH released in various treatment conditions, we subtracted the basal level before treatment from the LDH in the conditioned media after treatment.

Statistics. All data are presented as mean ± SEM, and different conditions were compared using one-way ANOVA followed by Dunnett's post test to compare control with treatments. Student's *t* test was used for comparing conditions with only two groups. Statistical significance was set at $P < 0.05$. Statistics were performed using GraphPad Prism (GraphPad Software Inc.).

1. West T, Atzeva M, Holtzman DM (2006) Caspase-3 deficiency during development increases vulnerability to hypoxic-ischemic injury through caspase-3-independent pathways. *Neurobiol Dis* 22(3):523–537.
2. Vohra BP, et al. (2010) Amyloid precursor protein cleavage-dependent and -independent axonal degeneration programs share a common nicotinamide mononucleotide adenylyltransferase 1-sensitive pathway. *J Neurosci* 30:13729–13738.
3. Sasaki Y, Vohra BP, Lund FE, Milbrandt J (2009) Nicotinamide mononucleotide adenylyl transferase-mediated axonal protection requires enzymatic activity but not increased levels of neuronal nicotinamide adenine dinucleotide. *J Neurosci* 29: 5525–5535.
4. Craig-Schapiro R, et al. (2011) Multiplexed immunoassay panel identifies novel CSF biomarkers for Alzheimer's disease diagnosis and prognosis. *PLoS ONE* 6:e18850.

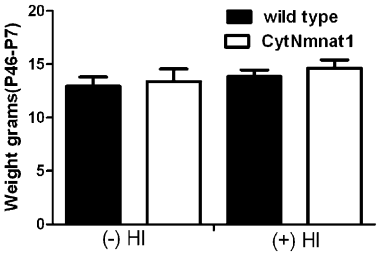

Fig. S1. Growth rate of wild-type and cytNmnat1-Tg animals. (*A*) The growth rates of wild-type ($n = 10$) and cytNmnat1-Tg ($n = 8$) mice that had (+) or had not (–) undergone H-I were calculated from the difference in their weight on days P7 and P46 (P46 – P7).

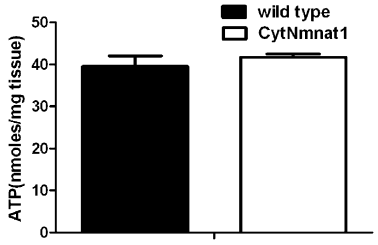

Fig. S2. Steady-state levels of ATP are similar in wild-type and cytNmnat1-Tg mice. ATP levels in hippocampal tissue lysates of wild-type ($n = 5$) and cytNmnat1-Tg ($n = 5$) P7 mice were assayed using a bioluminescence assay.

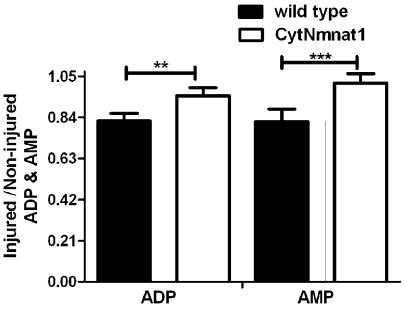

Fig. S3. H-I–induced changes in metabolism are blocked in cytNmnat1-Tg mice. ADP and AMP levels in hippocampus tissue lysates of injured and noninjured of wild-type ($n = 5$) and cytNmnat1-Tg ($n = 5$) mice were assayed 24 h after H-I using HPLC. Error bars show SE; $^{**}P < 0.001$ and $^{***}P < 0.0001$.

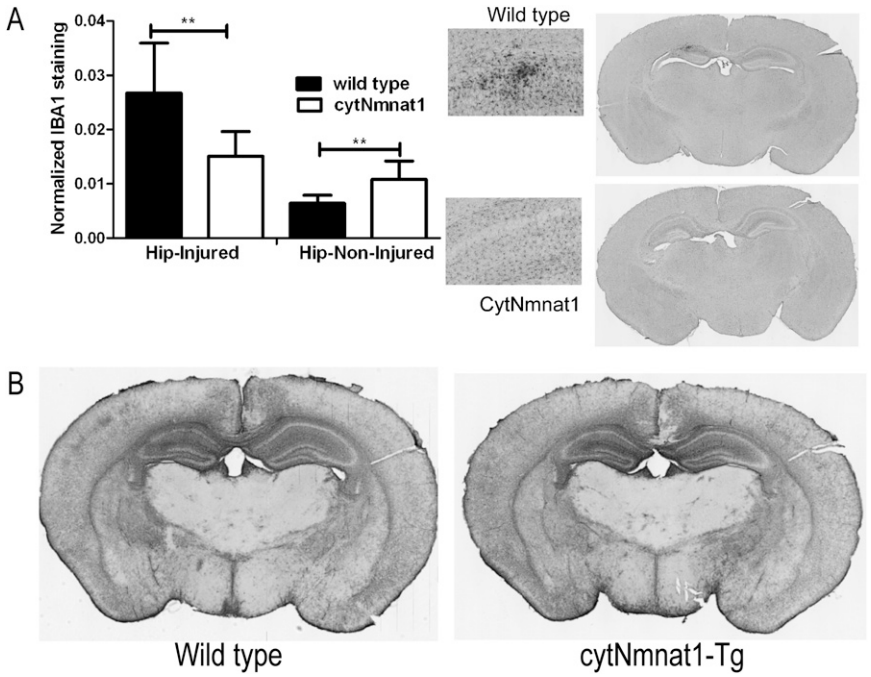

Fig. S4. Microglial and astrocyte activation after H-I in wild-type and cytNmnat1-Tg mice. (*A*) At P14, 7 d after neonatal H-I. the levels of IBA1, a marker for microglia, were quantified in the hippocampus of wild-type (n = 10) and cytNmnat1-Tg (n = 10) mice by immunostaining using an antibody against IBA1. The IBA1$^+$ stains in wild-type and cytNmnat1-Tg animals were normalized by calculating the ratio of positive stain present in the injured and uninjured hippocampus over the total hippocampal area. (*B*) The presence of GFAP, the marker of astroglyosis, was assessed in the hippocampus of wild-type (n = 10) and cytNmnat1-Tg (n = 10) mice 7 d after neonatal HI by immunostaining using an antibody against GFAP. Error bars show SE; $^{**}P < 0.001$.

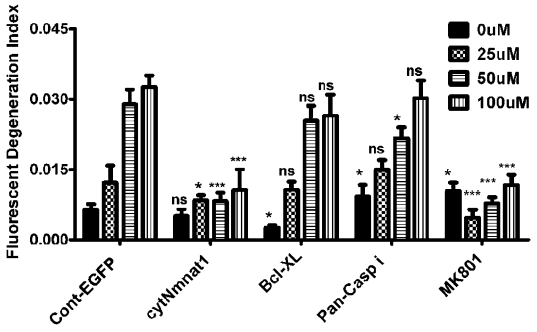

Fig. S5. CytNmnat1 protects neuronal processes against NMDA-mediated excitotoxicity. Primary cortical neurons infected with EGFP (control, Cont), cytNmnat1, or Bcl-XL lentiviruses were treated with 0, 25, 50, or 100 μM NMDA for 12 h on DIV10. In cells treated with the pan-caspase inhibitor Z-VAD-FMK (Pan-casp i) or the NMDA antagonist MK801, primary cortical neurons were incubated with these molecules on DIV10 before the 12-h treatment with 0, 25, 50, and 100 μM NMDA. The cells then were stained with α-tubulin (green, neuronal process) and with bizbenzimide (blue, cellular DNA). The fluorescent axonal degeneration index was quantified 12 h after NMDA treatment as described in *SI Materials and Methods*. *P* values were calculated by one-way ANOVA comparing EGFP (control) with other treatments. Error bars show SE; $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$; ns, not significant.

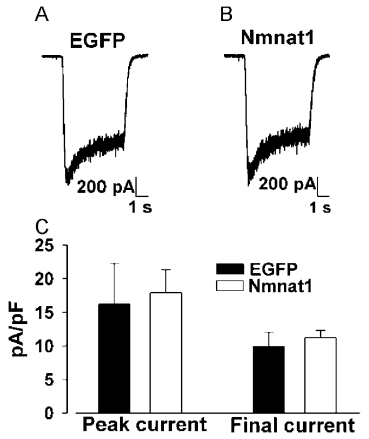

Fig. S6. CytNmnat1 overexpression does not alter NMDA receptor function. Mixed astrocyte/neuron hippocampal cultures were prepared from rat pups on day P1 and were plated on collagen substrate as described (1). Cells were plated in serum-containing medium and fed with a Neurobasal/B27 exchange 1 d after plating. Cultures were infected with EGFP or Nmnat1 viral vectors at day 3 after plating, and cells were whole-cell patch clamped at DIV10. The extracellular perfusate for recording included 138 mM NaCl, 4 mM KCl, 10 mM Hepes, 2 mM $CaCl_2$, no added Mg^{2+}, 10 mM glucose, 10 μM glycine, 1 μM 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), 0.5 μM TTX, and 25 μM bicuculline (pH 7.25). The pipette solution contained 140 mM potassium gluconate, 4 mM NaCl, 10 mM Hepes, 5 mM EGTA, and 0.5 mM $CaCl_2$ (pH 7.25). (*A*) Current induced by application of 10 μM NMDA in a control cell exhibiting EGFP fluorescence. (*B*) The protocol in *A* showing current induced in an $EGFP^+$ neuron in a sibling culture infected with cytNmnat1 virus. (*C*) Summary of the NMDA current density in eight control cells and eight cells overexpressing Nmnat1. There was no difference between groups in the peak current or in the amount of receptor desensitization.

1. Mennerick S, Que J, Benz A, Zorumski CF (1995) Passive and synaptic properties of hippocampal neurons grown in microcultures and in mass cultures. *J Neurophysiol* 73(1):320–332.

Table S1. Decreased cytokine levels in cytNmnat1-Tg animals after H-I

Cytokines	Injured wild-type mice (pg/mg protein)	Injured cytNmnat1-Tg mice (pg/mg protein)	Noninjured wild-type mice (pg/mg protein)	Noninjured cytNmnat1-Tg mice (pg/mg protein)
IL-7	8.30 ± 1.28	5.36 ± 0.68*	3.04 ± 0.91†	2.99 ± 1.24 ns§§
IL-10	27.93 ± 5.83	13.71 ± 2.24§	19.74 ± 5.44†	12.74 ± 3.07**,§§
IL-11	6.08 ± 0.57	3.38 ± 0.36§	1.86 ± 0.88†	1.64 ± 0.68 ns§§
IL-1α	31.56 ± 0.82	18.02 ± 2.46*	14.99 ± 1.29†	11.63 ± 2.19**,§§
IL-2	1.93 ± 0.23	1.34 ± 0.45¶	1.91 ± 0.36 ns	1.44 ± 0.41ns ns
Oncostatin-M	12.14 ± 1.21	7.17 ± 1.54§	3.09 ± 0.83†	2.96 ± 1.32 ns§§
TNF-α	2.95 ± 0.77	1.73 ± 0.47¶	BD	BD
Lymphotactin	2.62 ± 0.42	1.41 ± 0.23§	1.60 ± 0.19‡	1.12 ± 0.15††,‡‡
MIP-2	0.59 ± 0.054	0.44 ± 0.013§	0.32 ± 0.034†	0.33 ± 0.047 ns§§
GM-CSF	0.71 ± 0.24	0.33 ± 0.13¶	BD	BD
MCP-3	8.53 ± 1.49	5.99 ± 0.49*	7.14 ± 0.78 ns	6.22 ± 0.55 ns ns
IP-10	3.48 ± 1.41	1.78 ± 0.65¶	0.70 ± 0.13‡	0.61 ± 0.13 ns§§
MIP-1β	15.81 ± 3.29	10.69 ± 0.95¶	3.61 ± 1.15‡	2.89 ± 0.90 ns¶¶
MCP-1	7.41 ± 1.61	4.09 ± 0.90*	2.01 ± 0.23†	1.97 ± 0.22 ns¶¶
MCP-3	8.53 ± 1.49	5.99 ± 0.49*	7.14 ± 0.78 ns	6.22 ± 0.55 ns ns
TIMP-1 mouse	105.82 ± 23.16	57.56 ± 12.31*	20.34 ± 4.35†	17.78 ± 2.85 ns¶¶
VEGF	47.66 ± 3.79	41.12 ± 2.69¶	38.99 ± 3.85‡	39.30 ± 3.72 ns ns
MCP-5	3.62 ± 0.34	3.28 ± 0.33 ns	1.98 ± 0.21†	2.42 ± 0.28**,§§
SCF	131.23 ± 6.64	139.82 ± 35.00 ns	140.20 ± 27.65 ns	148.67 ± 28.98 ns ns
FGF-9	978.69 ± 88.15	947.71 ± 82.71 ns	1192.42 ± 117.70‡	1099.07 ± 150.32 ns ns

Cytokine levels in injured and noninjured hippocampus tissue lysates of wild-type (n = 5) and cytNmnat1-Tg (n = 5) mice were assayed 48 h after H-I using multianalyte profiling. Values represent means ± SEM. BD, below detection; IP-10, IFN-γ–induced protein 10; MIP-1β, macrophage inflammatory protein 1β; MCP-1, monocyte chemotactic protein 1; MCP-3, monocyte chemotactic protein 3; MCP-5, monocyte chemotactic protein 5; MIP-2, macrophage inflammatory protein 2; ns, not significant; SCF, stem cell factor; TIMP-1, tissue inhibitor of metalloproteinases 1 mouse. Statistical analysis of the data were done between: (*i*) injured wild-type and injured cytNmnat1-Tg animals (significance shown in column 3), (*ii*) injured and noninjured wild type animals (significance shown in column 4), (*iii*) noninjured wild type and noninjured cytNmnat1 (significance shown in column 5, sign on the left), and (*iv*) injured cytNmnat1-Tg mice and Noninjured cytNmnat1-Tg mice (significance shown in column 5, sign on the right).

*Significant difference between injured hippocampal hemispheres of wild-type and cytNmnat1-Tg animals; $P < 0.001$.

†Significant difference between injured and noninjured hippocampal hemispheres of wild-type animals; $P < 0.0001$.

‡Significant difference between injured and noninjured hippocampal hemispheres of wild-type animals; $P < 0.001$.

§Significant difference between injured hippocampal hemispheres of wild-type and cytNmnat1-Tg animals; $P < 0.0001$.

¶Significant difference between injured hippocampal hemispheres of wild-type and cytNmnat1-Tg animals; $P < 0.05$.

**Significant difference between noninjured hippocampal hemispheres of wild-type and cytNmnat1-Tg animals; $P < 0.05$.

††Significant difference between noninjured hippocampal hemispheres of wild-type and cytNmnat1-Tg animals; $P < 0.001$.

‡‡Significant difference between injured and noninjured hippocampal hemispheres of cytNmnat1-Tg animals; $P < 0.05$.

§§Significant difference between injured and noninjured hippocampal hemispheres of cytNmnat1-Tg animals; $P < 0.001$.

¶¶Significant difference between injured and noninjured hippocampal hemispheres of cytNmnat1-Tg animals; $P < 0.0001$.