Supplementary Data

Supplementary Materials and Methods

Creation of peroxiredoxin 2 transgenic mice

The chimeric transgene used to create the transgenic mice contains human peroxiredoxin 2 (PRX2) cDNA under the control of neuron-specific synapsin I promoter. A hemagglutinin (HA) tag was added before the PRX2 coding region. The final plasmids were verified by transfection in HEK 293 cells followed by immunoblotting to detect the protein expression of HA-PRX2. The plasmids were digested with restriction enzymes, purified, and then microinjected into fertilized eggs of C57BL/6J×SJL/J mice. Transgenes were detected by polymerase chain reaction of mouse-tail DNA. Founders were used to establish independent transgenic lines by breeding to wild-type F1 hybrid mice. All lines of mice were backbred to the C57BL/6J background for at least seven generations to minimize the potential influence of genetic heterogeneity on the susceptibility to stroke. The offspring of matings between heterozygous Tg-PRX2 ($PRX2^{+/-}$) mice showed genotypes in a ratio consistent with mendelian transmission without disproportionate pre- or perinatal lethality. Intersibling matings were used to generate experimental subjects. The current study employed homozygous Tg-PRX2 ($PRX2^{+/+}$) mice and wild-type littermates from the same transgenic colonies.

Murine model of focal cerebral ischemia

Focal cerebral ischemia/reperfusion was produced by intraluminal occlusion of the left middle cerebral artery (MCA) as described previously (1). Briefly, male 8- to 12-week-old C57/B6 mice (25–30 g) were anesthetized with 1.5% isoflurane in a 30% O_2/68.5% N_2O mixture under spontaneous breathing. Rectal temperature was continually monitored and kept at 37°C to 37.5°C using a heating pad. A silicon-coated 8.0 monofilament surgical suture was introduced into the internal carotid artery through the external carotid artery stump, and wedged into the circle of Willis to obstruct the origin of the MCA. To ensure the induction of ischemia by MCA occlusion (MCAO), regional cerebral blood flow was monitored in each animal by laser Doppler flowmetry (LDF, PeriFlux System 5000; Perimed). In selected animals, cerebral blood flow was also measured using the PeriCam high-resolution laser speckle imaging system (Perimed AB). Cortical ischemic areas were calculated according to the manufacturer's instructions. Blood pressure, blood gases, and blood glucose concentration were monitored and maintained in the normal range throughout the experiments. In all experiments, the surgeon was blinded to the genotypes of mice.

Neurobehavioral tests

Behavioral tests were performed to assess sensorimotor deficits before surgery and on days 3–21 after 60 min of MCAO in PRX2 transgenic mice and wild-type littermates. All behavioral assessments were made during the light phase of the circadian cycle starting ~4 h after the light phase onset, and were made by an observer who was blinded to the experimental conditions. The assessment for sensorimotor deficits consisted of three different tests: The rotarod test was performed by placing mice on a rotating drum with a speed accelerating from 4 to 40 rpm during a 5-min period. The time at which the animal fell off the drum was evaluated. On each testing day, mice underwent three trials on the rotarod with intervals of 15 min, and the data are expressed as the mean duration per day. The corner test was performed as described previously (6), in which the ischemic mouse turns preferentially toward the nonimpaired (left) side. The direction of each turn was recorded from 10 trials for each test. The cylinder test was adapted for use in mouse to assess forepaw use and rotation asymmetry. The mouse was placed in a transparent cylinder 9 cm in diameter and 15 cm in height, and videotaped for 5 min. Videotapes were analyzed in slow motion, and forepaw (left/right/both) use of the first contact against the cylinder wall after rearing and during lateral exploration was recorded. Nonimpaired forepaw (left) preference is expressed as the relative proportion of left forepaw contacts, which was calculated as: (left − right)/(right + left + both) × 100 (5). Noninjured animals will show no preference for either forepaw initiation, but in injured animals preferences will increase toward a left forepaw preference depending on the severity of the insult.

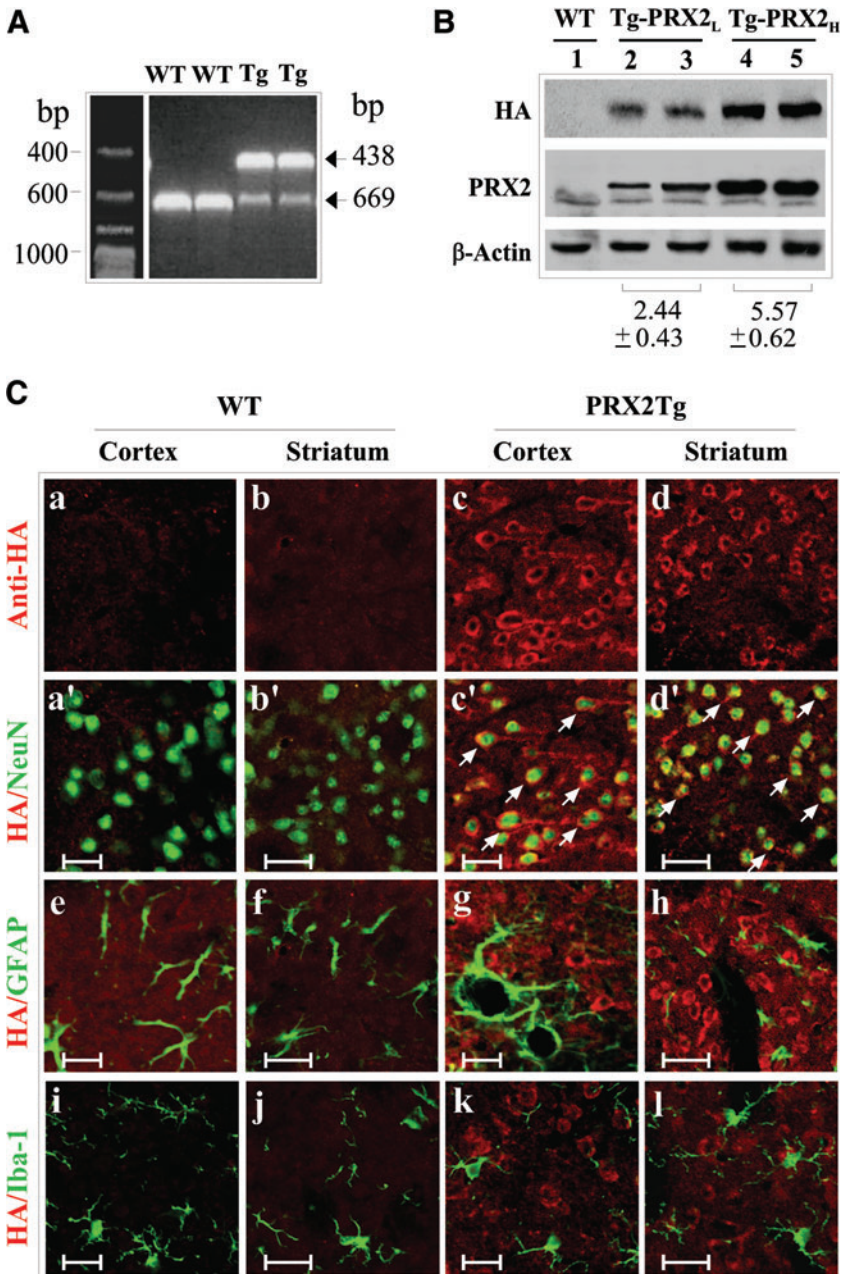
Determination of infarct volume

At 48 h after MCAO, brains were removed and the forebrain was sliced into coronal sections 1 mm thick. Sections were stained with 2% 2,3,5-triphenyltetrazolium chloride

Supplementary Table S1. Evaluation of Surface Cerebral Blood Vessels in Middle Cerebral Artery Territory and PcomA Plasticity in Mice

	MCA distance from the midline[a] *at the specified coronal plane of*[b]			*Number of hemispheres with PcomA scores of*[c]			
	2 mm	*4 mm*	*6 mm*	*0*	*1*	*2*	*3*
Wild-Type	2.29 (0.06)	2.18 (0.04)	2.41 (0.07)	2	6	2	0
$Tg\text{-}PRX2_H$	2.33 (0.05)	2.27 (0.06)	2.44 (0.05)	1	6	3	0

[a]Values shown are mean (S.E.) in millimeters ($n = 5$/group).
[b]A coronal plane is specified based on its distance from the frontal pole in millimeters.
[c]PcomA (posterior communicating artery) plasticity scoring criteria: 0, no anastomoses between PCA and SCA; 1, anastomoses between PCA and SCA in capillary phase; 2, small truncal anastomoses between PCA and SCA; 3, truncal anastomoses between PCA and SCA.
MCA, middle cerebral artery; $Tg\text{-}PRX2_H$, high-expressing PRX2 transgenic line; PCA, posterior cerebral artery; SCA, superior cerebral artery.



SUPPLEMENTARY FIG. S1. Verification of neuron-specific overexpression of Peroxiredoxin 2 (PRX2) in transgenic mice. HA-tagged cDNA containing the entire open-reading frame of human PRX2 was cloned downstream of the synapsin-I promoter bearing the first intron and was subsequently used to generate the Tg-PRX2 mice. **(A)** Wild-type (WT) and Prx2 transgenic (Tg) mice were identified by polymerase chain reaction genotyping. Primer pairs were designed to allow the detection of human PRX2 transgene and endogenous PRX2 of 438 and 609 bp, respectively. **(B)** Western blot verification of PRX2 overexpression in the brains from both the low-expressing PRX2 transgenic line (Tg-PRX2$_L$) and the high-expressing PRX2 transgenic line (Tg-PRX2$_H$). Immunoblotting was performed using either the anti-HA antibody (for the HA-tagged transgenic PRX2) or the anti-PRX2 antibody (for both endogenous and transgenic PRX2). Semi-quantification for Western blot results shows the Tg-PRX2$_L$ mice yielded 2.44 ± 0.43-fold increase (mean ± SEM, $n = 5$) in PRX2 protein expression, whereas the Tg-PRX2$_H$ mice yielded about 5.57 ± 0.62-fold increase (mean ± SEM, $n = 5$) in the brain. **(C)** Double-label immunofluoroscent staining for hemagglutinin (HA)-tagged transgenic PRX2 (red) with neuronal marker NeuN (green, **a–d**, **a′–d′**), astrocyte marker GFAP (green, **e–h**), or microglia marker Iba-1 (green, **i–l**) in the cortex and striatum of WT and Tg-PRX2 mice. Arrows indicate that transgenic PRX2 shows mainly a cytosolic localization and is expressed exclusively in neurons. Scale bars, 30 μm.

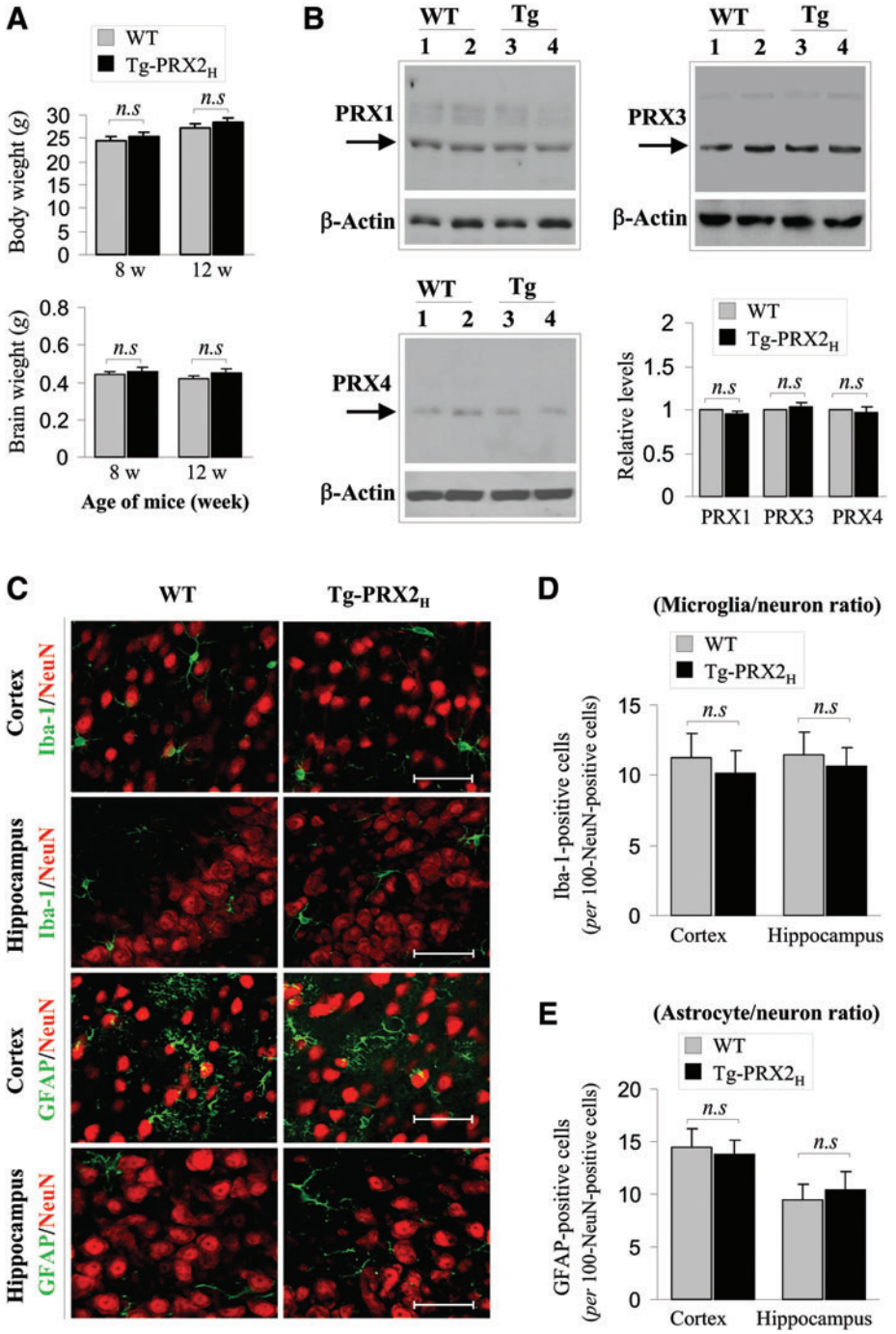
(TTC). For long-term measurement of infarct volume, animals were sacrificed at 21 days after MCAO. Coronal sections were processed for MAP-2 immunostaining (3). Infarct volume was determined with correction for brain edema using MCID image (3) by an observer blinded to experimental group assignment.

Primary culture and in vitro *model of ischemia*

Primary cortical neurons were prepared from embryonic day 17 Sprague-Dawley rat embryos as previously described (4). Experiments were conducted at 10 to 12 days *in vitro* when cultures consisted primarily of neurons (> 97%) as determined using cell phenotype-specific immunocytochemistry. To model ischemia-like conditions *in vitro*, cultures were exposed to transient oxygen and glucose deprivation (OGD) for 60 min (4).

Construction of viral vectors

Lentiviral (Ln) vectors carrying either the human full-length (LnPRX2) or catalytically inactive mutant (LnPRX2$_{C/A}$) PRX2 cDNA were constructed as described previously (7). The HA-tagged cDNA was inserted into the Ln transfer vector FSW under the control of the neuron-specific synapsin I promoter. Ln vectors were also made expressing short hairpin interfering RNA (shRNA) against rat thioredoxin (Trx) and apoptosis signal–regulating kinase 1 (ASK1). The gene-specific targeting sequence (TRXt: 5′-GTCAAATGCATG CCGACCTAG-3′ and 5′-GCTCGAAGCCACTATTACG-3′; ASK1t: 5′-CCAACAACATCATCCTCTA-3′ and 5′-TGAACA CCATTACCGAAGA-3′) or its counterpart scramble sequence (Trxsc or ASK1sc) was inserted into the transfer vector FSW under the control of the U6 promoter. The constructed



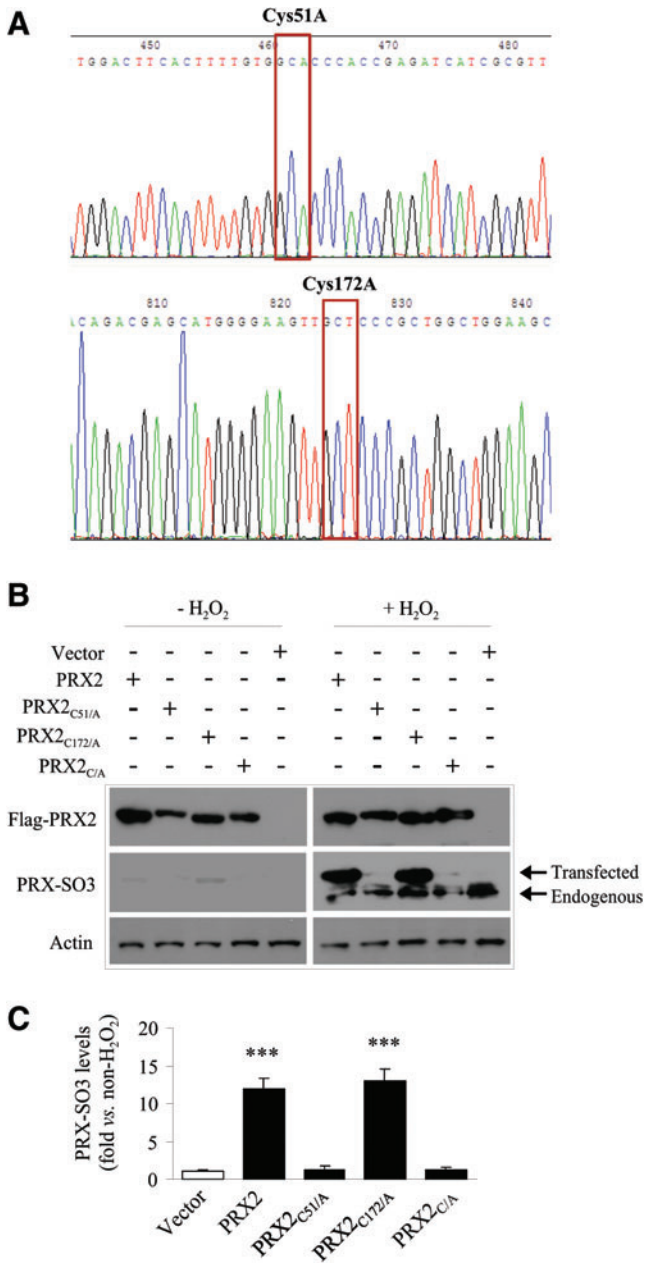
SUPPLEMENTARY FIG. S2. Characterization of PRX2 transgenic mice. Equal number of Tg-PRX2$_L$ mice and WT littermates were analyzed for body weight, brain weight, immunoblotting for PRX1, PRX3, and PRX4, and immunohistochemistry for GFAP and Iba-1 at either 8 or 12 weeks of age. **(A)** Comparison of body weight and brain weight between Tg-PRX2$_L$ and WT mice (mean ± SEM, $n=4$ per group). Note that there is no significant (*n.s.*) difference between Tg-PRX2$_L$ and WT mice in either age. **(B)** Representative Western blots showing compatible expression levels of PRX1, PRX3, and PRX4 in the brain of Tg-PRX2$_L$ and WT mice at 8 weeks of age. The forebrain cortical extracts were used for all blots. The bar graph illustrates the semi-quantitative data on the relative levels of PRX1, PRX3, and PRX4 (mean ± SEM, $n=4$ per group), where the average value of WT mice served as 1.0. **(C)** Double-label immunofluoroscent staining for the neuronal marker NeuN (red) with either the microglial marker Iba-1 (green) or the astrocyte marker GFAP (green) in the cortex and hippocampus of Tg-PRX2$_L$ and WT mice at 12 weeks of age. Scale bars, 50 μm. **(D, E)** Cell counting of Iba-1- or GFAP-positive cells, presented as the numbers *per* 100 NeuN-positive cells, in the cortex and hippocampus of Tg-PRX2$_L$ and WT mice (mean ± SEM, $n=4$ per group). Quantification was done on three consecutive coronal sections from each brain at the levels of dorsal hippocampus. Note that there is *n.s.* difference on the Iba-1/NeuN **(D)** or GFAP/NeuN **(E)** ratio between Tg-PRX2$_L$ and WT mice. Statistical analyses were done using Student's *t*-test comparing Tg-PRX2$_L$ *versus* WT mice.

transfer vectors were transformed into recombinase-free Stbl3 *Escherichia coli* (Invitrogen), and then isolated using the EndoFree Plasmid Maxi Kit (Qiagen). Large-scale production of the virus was performed using a protocol described previously (9). In brief, a plasmid mixture containing 435 μg pCMV ΔR8.9 (packaging construct), 237 μg pVSVG (envelope plasmid), and 675 μg FSW (transfer vector) was suspended in 34.2 ml $CaCl_2$ (250 m*M*) and added volume for volume into 2×BES (2 *N*,*N*-bishydroxyethyl-2-aminoethane-sufonic acid) buffer, pH 6.95. The DNA-$CaCl_2$ precipitate was added to human kidney 293 FT cells (1.125 ml to one 15-cm plate at a density of 1.1×10^7 cells/plate) and allowed to incubate for 12 h before switching to a fresh culture medium. The supernatant was collected 72 h after transfection, filtered through the 0.45-μm filter flask and centrifuged at 25,000 rpm for 1.5 h using an SW32 Ti rotor (Beckman Coulter). Viruses were further purified by sucrose gradient ultracentrifuge. The pellet was suspended in 9 ml of phosphate buffered saline (PBS), loaded on the top of 3 ml of 20% sucrose solution, and centrifuged at 25,000 rpm for 1.5 h using the SW41 Ti rotor (Beckman Coulter). The resulting pellet was resuspended in 300 μl of serum-free Dulbecco's modified

Eagle's medium, aliquoted, and stored at −70°C. The titer of the vector stock was determined using a lentivirus-associated p24 ELISA kit (Cell Biolabs) according to the manufacturer's instructions.

Gene transfection in primary neurons by viral vectors

The primary neuronal cultures were infected with the LnPRX2, LnPRX2$_{C/A}$, LnTrxt, LnASK1t, or the control vector (LnGFP) for 6 h and then incubated in vector-free normal media for 72 h. For each of the shRNA-targeted genes, a scrambled sequence containing the same nucleotide composition in a randomized order was constructed and used as control. Overexpression of PRX2 in neurons was confirmed by immunocytochemistry and Western blot, respectively.



SUPPLEMENTARY FIG. S3. Verification of site-directed mutations of the PRX2 catalytic site cysteines. (A) Site-directed mutagenesis was performed to convert Cys^{51} and/or Cys^{172} to alanine, and the incorporated mutations were confirmed by sequencing. **(B)** Human *293* cells were transfected for 24 h with empty vectors or vectors carrying the Flag-tagged WT PRX2 cDNA or PRX2 cDNA containing the Cys→Ala mutation at Cys^{51} (PRX2$_{C51/A}$) or Cys^{172} (PRX2$_{C172/A}$), or both (PRX2$_{C/A}$). The cells were then challenged with H_2O_2 (0.5 mM) or treated with saline for 20 min; cell protein extracts were subjected to immunoblotting against Flag or PRX-SO_3. Note that H_2O_2 treatment induces PRX-SO_3 resulting from endogenous PRXs (the *lower bands*) in all transfected cells, whereas it induces PRX-SO_3 resulting from transfection of WT PRX2 or PRX2$_{C172/A}$ (the *upper bands*), but not PRX2$_{C51/A}$ or PRX2$_{C/A}$. **(C)** Semi-quantification of PRX-SO_3 resulting from gene transfection (the *upper bands*) as described in **(B)**. Data are expressed as fold increases over the saline treatment controls, mean ± SEM, ***$p < 0.001$ *versus* vector controls from three independent experiments; based on one-way analysis of variance (ANOVA), followed by Bonferroni's/Dunn's *post hoc* analysis.

Cell death/viability assessment in cultured neurons

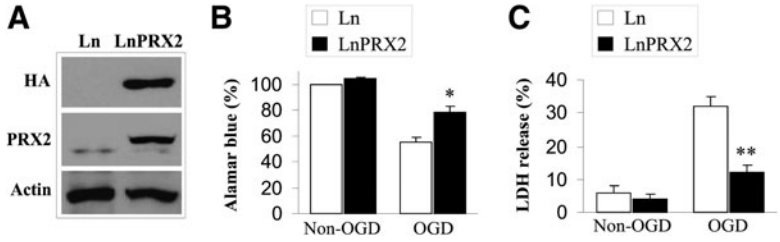
Fluorescence of Alamar blue (Accumed International), an indicator that changes from blue to red and fluoresces when reduced by cellular metabolic activity, was used to measure the viability of the cultured neurons at 24 h after OGD (4). One-half of the culture medium was replaced with MEM-Pak containing 10% (v/v) Alamar blue, and cultures were incubated for 1.5 h at 37°C in humidified 95% air and 5% CO_2. Fluorescence was determined using an automated plate-reading fluorometer (CytoFluor 2300; Millipore) with excitation at 530 nm and emission at 590 nm.

OGD-induced cell death was quantified by measuring lactate dehydrogenase (LDH) release from damaged cells into the culture medium (4). In brief, 10-μl aliquots of medium taken from the cell culture wells were added to 200 μl of LDH reagent (Sigma). Using a spectrophotometer plate reader (Bio-Rad Laboratories), the emission was measured at 340 nm, which is proportional to the amount of LDH in the medium. The data are expressed as the percentage of cell death that was calculated as described previously (4).

In selective experiments, DNA damage or fragment was evaluated by TUNEL staining (10). To quantify the MCAO-induced DNA damage in brain sections, TUNEL-positive cells were counted with stereology using the Bioquant Image Analysis program (Bioquant) as detailed previously (4). To quantify the OGD-induced DNA damage, the percentage of TUNEL-positive cells was calculated by counting at least 3000 cells under each experimental condition (three randomly selected fields per well, four to six wells per condition per experiment, and three independent experiments).

PRX activity assay

Peroxidase activity was determined by the consumption of NADPH mediated by Trx and mammalian Trx reductase (7). The peroxiredoxin activity assay kit (Redoxica) was used for the measurement according to the manufacturer's instructions. In brief, 250 μg of the precipitated proteins from



SUPPLEMENTARY FIG. S4. Lentiviral overexpression of PRX2 protects against oxygen and glucose deprivation (OGD)-induced neuronal injury in mouse cortical neurons. Primary mouse cortical neurons at 9 days *in vitro* were infected for 3 days with empty lentivirus or lentiviral (Ln) vector carrying HA-tagged human PRX2 (LnPRX2). **(A)** Lentivirus-mediated overexpression of PRX2 in neurons was confirmed by Western blotting against the HA tag and PRX2, respectively. The blots are representatives of two experiments with similar results. **(B, C)** Transfection of PRX2 protects against neuronal cell death at 24 h after 60 min of OGD. Cell viability was quantified using the Alamar blue assay **(B)**; cell death was assessed by measuring lactate dehydrogenase (LDH) release **(C)**. $n = 6$ from three independent experiments. All quantitative data are presented as mean ± SEM, $^*p < 0.05$; $^{**}p < 0.01$ *versus* Ln-infected cell cultures; based on one-way ANOVA, followed by Bonferroni's/Dunn's *post hoc* analysis.

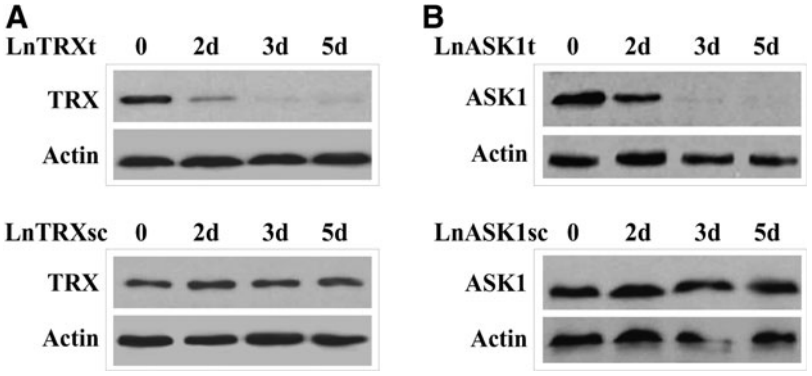
cultured neurons or brain tissues was incubated with 5 mm Trx, 1 mm Trx reductase, and 100 mm NADPH in HEPES, pH 7.5. The reaction was initiated by the addition of H_2O_2 at a final concentration of 0.2 mm, and incubated at 30°C for 1 h. The consumption of NADPH was measured at 340 nm by spectrophotometer, and the linear rate of decrease in absorbance in the first 120 s was used for calculation. The data are expressed as percentage changes in PRX activity over control noninjured cell cultures or animals.

Trx activity assay

Trx activity was determined by the insulin disulfide reduxing assay as previously described (8). Briefly, brain tissue or primary neuron lysates containing 50 μg of protein were incubated for 30 min at 37°C in reaction buffer (0.1 *M* Tris-Cl, pH 7.5; 2 m*M* EDTA) containing 1 m*M* NADPH, 0.15 U/ml Trx reductase, and 2.5 mg/ml insulin. The reaction was terminated by the addition of stop buffer containing 6 M guanidine-HCl, 1 m*M* DTNB (Sigma), and 0.2 *M* Tris-Cl (pH 8.0), and absorbance was measured at 405 nm. The data were expressed as percentage changes in PRX activity over control noninjured cell cultures or animals.

Immunohistochemistry

Animals were sacrificed in a carbon dioxide chamber at the indicated time points after induction of ischemia, and then perfused with 50 ml heparinized 0.9% saline followed by 50-ml 4% paraformaldehyde in 0.1 *M* phosphate-buffered saline (pH 7.4). Brains were removed, and after postfixation and cryoprotection, coronal sections were cut using a microtome. Standard protocols were used for immunohistochemistry. Briefly, sections were incubated with anti-NeuN (1:500; Millipore), anti-PRX-SO_3 (1:200; AbFrontier), or anti-p-c-Jun antibody (1:200; Cell Signaling Technology) at 4°C for 24 h. After being washed in PBST three times, sections were incubated with secondary antibodies conjugated with the fluorochrome Cy3 (1:1000; Jackson ImmunoResearch) for 2 h at room temperature. For double-label immunofluorescence staining, sections were first incubated with anti-HA (1:50; Cell Signaling Technology), anti-p-MKK4 (1:100; Cell Signaling Technology),



SUPPLEMENTARY FIG. S5. Time course of short hairpin RNA (shRNA)-mediated gene knockdown in cortical neurons. (A) Knockdown of thioredoxin (Trx) in neurons. Primary rat cortical neurons were infected for 0, 2, 3, or 5 days with Ln vectors containing shRNA targeting the Trx sequence (LnTRXt, *the top panel*) or the scrambled sequence (LnTRXsc, *the bottom panel*). Immunoblotting results confirmed the knockdown of Trx expression. **(B)** Knockdown of apoptosis signal–regulating kinase 1 (ASK1) in neurons. Primary rat cortical neurons were infected for 0, 2, 3, or 5 days with Ln vectors containing shRNA targeting the ASK1 sequence (LnASK1t, *the top panel*) or the scrambled sequence (LnASK1sc, *the bottom panel*). Immunoblotting results confirmed the knockdown of ASK1 expression. The blots are representatives of two independent experiments with similar results.

or active caspase-3 antibody (1:1000; Cell Signaling Technology) at 4°C for 24 h, followed by incubation for 2 h at room temperature with secondary antibodies Cy3 immunoconjugate (1:1000; Jackson ImmunoResearch). The sections were rinsed with PBST and then incubated at 4°C overnight with anti-NeuN, anti-GFAP (1:500; Millipore), anti-Iba-1 (1:50; Abcam), or anti-NSE antibody (1:50; Millipore), followed by DyLight-488-conjugated secondary antibodies (1:1000; Jackson ImmunoResearch) at room temperature for 2 h. For the assessment of nonspecific staining, alternating sections from each experimental condition were incubated without the primary antibody. To quantify NeuN-positive cells after ischemia, cell counting was performed with stereology using the Bioquant Image Analysis program as described previously (4).

To confirm the virus-mediated overexpression of PRX2 or $PRX2_{C/A}$ in neurons, the primary neuronal cultures were infected with the LnPRX2, $LnPRX2_{C/A}$ or control vector. After 3 days, cells were fixed with 1% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS for 10 min at room temperature. Fixed cells were pretreated with 2% normal goat serum in PBS for 1 h and incubated with mouse anti-HA antibody and rabbit anti-MAP2 antibody (1:1000; Novus Biologicals) at 4°C overnight. Cells were washed three times in PBS and stained with Alexa Fluor-488 and Cy3-conjugated goat antibodies (1:2000; Invitrogen).

Western blot and immunoprecipitation

Protein isolation from brain tissues or neuronal cell cultures, based on total cell extracts or subcellular fractionation (cytosolic and mitochondrial), was performed as described previously (2). Western blot was performed using the standard method and enhanced chemiluminescence detection reagents (GE Healthcare Bio-Sciences). Immunoreactivity was semiquantified by gel densitometric scanning and analyzed with the MCID image analysis system (Imaging Research, Inc.). The following primary antibodies were used: rabbit monoclonal antibodies against p-JNK (Thr183/Tyr185) (clone 81E11, 1:1000), and p-c-Jun (Ser63) (clone 54B3, 1:1000), purchased from Cell Signaling Technology; rabbit polyclonal antibodies against cleaved caspase-9 (1:1000), COX IV (1:1000), p-MKK4 (Ser257/Thr261) (1:1000), p-MKK7 (Ser271/Thr275) (1:1000), p-ASK1 (Thr845) (1:1000), total JNK (1:1000), total MKK-4 (1:1000), and total MKK-7 (1:1000), from Cell Signaling Technology; rabbit polyclonal antibodies against ASK1 (1:1000), Bax (1:1000), β-actin (1:2000), cytochrome *c* (1:1000), AIF (1:1000), and HA (1:1000), from Santa Cruz; rabbit polyclonal antibodies against PRX2 (1:2000), and PRX-SO_3 (1:2000), from AbFrontier; goat anti-Trx antibody (1:1000), from American Diagnostica; and rabbit polyclonal anti-PUMA antibody, a gift from Dr. Jian Yu (Pittsburgh Cancer Institute, Pittsburgh, PA).

Immunoprecipitation was performed to examine protein–protein interactions between Trx and ASK1 after ischemia using a procedure described previously (2). Briefly, cytosolic protein was isolated from brain tissues or primary neurons at indicated time points after MCAO or OGD using RIPA A (150 m*M* NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 m*M* Tri-HCl pH 8.0) buffer. Equal amounts of protein from each experimental condition (300 μg per sample) were subjected to immunoprecipitation using the anti-Trx or anti-ASK1 antibody. The resulting immunoprecipitates were then analyzed by immunoblotting with anti-Trx and anti-ASK1 antibodies, respectively.

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