Sphingosine Kinase 2 Mediates Cerebral Preconditioning and Protects the Mouse Brain Against Ischemic Injury

Lai Ming Yung, PhD; Ying Wei, MD; Tao Qin; Yumei Wang, MD; Charles D. Smith, PhD; Christian Waeder, PhD

Background and Purpose—Cerebral preconditioning provides insights into endogenous mechanisms that protect the brain from ischemic injury. Hypoxia and the anesthetic isoflurane are powerful preconditioning agents. Recent data show that sphingosine 1-phosphate receptor stimulation improves outcome in rodent models of stroke. Endogenous sphingosine 1-phosphate levels are controlled by the expression and activity of sphingosine kinases (SPK). We hypothesize that SPK upregulation mediates preconditioning induced by isoflurane and hypoxia and reduces ischemic injury.

Methods—Male wild-type C57BL/J, SPK1−/− and SPK2−/− mice were exposed to isoflurane or hypoxia preconditioning before transient middle cerebral artery occlusion. Infarct volume and neurological outcome were measured 24 hours later. SPK inhibitors (SKI-II and ABC294640) were used to test the involvement of SPK2. Expressions of SPK1, SPK2, and hypoxia-inducible factor 1α were determined. Primary cultures of mouse cortical neurons were exposed to isoflurane before glutamate- or hydrogen peroxide-induced cell death.

Results—Isoflurane preconditioning and hypoxia preconditioning significantly reduced infarct volume and improved neurological outcome in wild-type and SPK1−/− mice but not in SPK2−/− mice. Pretreatment with SKI-II or ABC294640 abolished the isoflurane preconditioning-induced tolerance. Western blot showed a rapid and sustained increase in SPK2 level, whereas SPK1 level was similar between preconditioned mice and controls. Hypoxia-inducible factor 1α was upregulated in wild-type isoflurane-preconditioned mice but not in SPK2−/−. Isoflurane preconditioning protected primary neurons against cell death, which was abolished in ABC294640-treated cells.

Conclusions—Applying genetic and pharmacological approaches, we demonstrate that neuronal SPK2 isoform plays an important role in cerebral preconditioning. (Stroke. 2012;43:199-204.)

Key Words: cell death ■ cerebral ischemia ■ hypoxia ■ isoflurane ■ neurons ■ preconditioning ■ sphingosine kinase 2

Cerebral preconditioning is a procedure by which a noxious stimulus is applied to a tissue or organ below the threshold of damage. After a recovery period, organs such as the brain develop a tolerance to the same or even different noxious stimuli given above the threshold of damage.2,12 Studying cerebral preconditioning may provide insight into endogenous protective mechanisms that could be exploited therapeutically. Known preconditioning stimuli include isoflurane,3-5 hypoxia,6-8 cortical spreading depression,9,10 and proinflammatory agents (such as lipopolysaccharide).11,12 Isoflurane, an inhalational anesthetic used widely and safely in surgical procedures, induces tolerance to ischemia in many organs, including the brain,5,13,14 heart,15 and kidney.16,17

Stroke is the leading cause of death and disability in developed countries. Despite the accumulating knowledge on the cellular and molecular mechanisms underlying ischemia/reperfusion injury, there is still a lack of effective treatment for stroke.18 The sphingosine 1-phosphate (S1P) receptor agonist Fingolimod (FTY720) has been shown to be protective in several animal models of cerebral ischemia.19-21 FTY720 is phosphorylated by sphingosine kinase (SPK) into the active compound phospho-FTY720, which then acts on 4 of the 5 known S1P receptor subtypes.22 In the central nervous system, S1P regulates multiple cellular processes, including proliferation, survival, and migration of neurons.23 Intracellular S1P is tightly regulated by the expression and activity of SPK. Previous reports suggested that SPK plays a role in heart,24,25 kidney,16,17 and brain preconditioning.8 We previously showed that SPK2 is the predominant isoform in brain.26 The aim of this study was to test the hypothesis that SPK2 is a universal mediator of both isoflurane- and hypoxia-induced preconditioning. To test the hypothesis that neu-
ronal SPK2 accounted for preconditioning, we used primary cultures of mouse cortical neurons to examine whether pretreatment with a specific SPK2 inhibitor could block isoflurane preconditioning (IsoPC)-induced protection against cell death in vitro.

Materials and Methods

Animals
Male C57BL/6J mice (23–25 g; Charles River, Wilmington, MA) and age-matched wild-type, SPK1−/− and SPK2−/− mice were maintained on a 12/12-hour light/dark cycle and fed ad libitum. Experiments were conducted according to protocols approved by the Animal Research Committee of Massachusetts General Hospital and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were randomly allocated; after preconditioning or drug treatments, their identity and genotype were coded with tail marks to blind the investigators to the treatment groups; cerebral ischemia, infarct volumes measurement, and neurological deficit evaluations were performed in a blinded fashion. Total number of mice included and mortality during surgery are summarized in Supplemental Tables I and II (http://stroke.ahajournals.org).

Cerebral Preconditioning
For IsoPC, mice were exposed to 1% isoflurane (in 70% nitrogen and 30% oxygen) for 3 hours in an airtight chamber. Mice were allowed to recover in an incubator (at 28°C) for approximately 30 minutes and then for 24 hours in their original cages. For hypoxic preconditioning (HPC), mice were kept in an airtight chamber flushed with 8% O2 for 4 hours. Mice were allowed to recover for 72 hours. Naïve mice were placed in the airtight chamber flushed with air for the same duration of time.

Treatment With SPK Inhibitors
Fifteen minutes before IsoPC, mice were administered a specific SPK inhibitor (SKI-II; Chembridge) or an isoform-selective SPK2 inhibitor (ABC294640; Apogee Biotechnology Corporation) at 100 mg/kg through oral gavage (dissolved in 100 µL of polyethylene glycol 400). Dosage, solvent, and route of administration were based on the published pharmacokinetics.

Transverse Middle Cerebral Artery Occlusion Model
The middle cerebral artery was occluded for 90 minutes using a commercially available coated monofilament (Docoll Corporation) as reported previously.

Expression Studies
Mice were exposed to isoflurane (1% in 70% N2 and 30% oxygen) for 3 hours and euthanized at the following time points: immediately after exposure (t=0) or 1, 6, 24, or 48 hours after exposure. Mice were perfused transcardially with cold saline as described before.

Primary Culture of Neurons and IsoPC
Primary cultures of neurons were exposed to IsoPC. The extent of glutamate- and hydrogen peroxide-induced cell death in control and preconditioned neurons was compared (Supplement).

Statistical Analysis
Data are expressed as mean±SD. For infarct volumes and cell viability assay, statistical difference between groups was calculated by analysis of variance. Neurological deficit score was compared using Mann-Whitney U test. Gene and protein expression levels were compared with controls by 1-way analysis of variance. P<0.05 was considered statistically significant.

Results

IsoPC Reduced Infarct Volumes and Improved Neurological Outcomes
IsoPC significantly protected the brain from transient middle cerebral artery occlusion (MCAO) as shown in a representative 2,3,5-triphenyltetrazolium chloride staining coronal brain slides (1 mm thick each) from naïve and preconditioned (IsoPC) mice. Representative pictures of 2,3,5-triphenyltetrazolium chloride (TTG)-stained coronal brain slides (1 mm thick each) from naïve and preconditioned (IsoPC) mice. B, Infarct areas in consecutive coronal slices. C, Cortical and subcortical infarct volumes in naïve and preconditioned mice were measured and compared. Data are mean±SD (n=8). Probability values for cortical, subcortical, and total infarct volumes were 0.063, 0.041, and 0.026, respectively. D, Neurological deficit was evaluated and scored based on 5 categories: Grade 0, no observable neurological deficit (normal); Grade 1, failure to extend forepaw fully on lifting the whole body by the tail (mild); Grade 2, circling to the contralateral side (moderate); Grade 3, falling to 1 side (severe); Grade 4, no spontaneous walking, depressed level of consciousness (very severe).

Expression of SPK1 and SPK2
In IsoPC mice, cortical spk2 mRNA was upregulated (peak level of approximately 2.4-fold increase at t=0 and 1 hour) in preconditioned mice (Figure 2A). SPK2 protein was rapidly upregulated (approximately 1.7-fold increase at t=0, ie, immediately after the 3-hour isoflurane exposure) and the peak SPK2 level was found at 1 hour after isoflurane exposure (2.7-fold increase). The upregulated SPK2 expression was still 2.2 times higher than the control at 24 hours (the time at which MCAO was induced; Figure 2B). In contrast, cerebral SPK1 mRNA (P=0.467) and protein (P=0.053)
expression remained unchanged at the different time points examined after IsoPC.

**Pharmacological Approaches**

We first established that infarct volumes were unaffected in naïve mice treated with a specific SPK inhibitor (SKI-II at 100 mg/kg, oral gavage) or vehicle (PEG400) 24 hours before cerebral ischemia (Figure 3A). SKI-II treatment (15 minutes before isoflurane exposure) abolished the protective effect of preconditioning, resulting in infarct volumes comparable to those seen in naïve mice (111.9±22.6 versus 107.2±12.8 mm³ in naïve; Figure 3A). SKI-II treatment also prevented IsoPC-induced improvement in neurological outcomes (Figure 3B).

ABC294640 is a novel isoform-selective inhibitor for SPK2. In a preliminary study, we investigated whether this compound was neuroprotective and found similar infarct volumes in mice treated with 100 mg/kg ABC294640 either 24 hours before MCAO or 30 minutes after reperfusion (Supplemental Figure I). Pretreatment with ABC294640 15 minutes before isoflurane exposure blocked the protective effect of IsoPC; infarct volumes were similar in IsoPC mice treated with ABC294640 either 24 hours before MCAO or 30 minutes after reperfusion (Figure 3C). ABC294640 also blocked the improvement in neurological score in preconditioned mice (Figure 3D).

**Genetic Tools**

The circle of Willis did not show obvious differences in the 3 mouse strains investigated and the naïve wild-type. The change in relative cerebral blood flow during MCAO and reperfusion was similar (Supplementary Table III), and SPK1−/− and SPK2−/− mice had similar infarct volumes (104.5±15.3, 98.5±18.2, and 91.9±15.5 mm³ respectively; Supplemental Figure II). IsoPC reduced infarct volumes in SPK1−/− mice (69.4±10.9 versus 98.5±18.2 mm³, P<0.005; Figure 4B) by 30%, comparable to that observed in wild-type mice. In contrast, infarct volumes in naïve and preconditioned SPK2−/− mice did not differ (91.9±15.5 versus 84.9±11.9 mm³; Figure 4B).

**In Vitro IsoPC**

The extent of cell death was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. Glutamate (Figure 5A) and H2O2 (Figure 5B) induced cell death in primary culture of mouse cortical neurons, which was significantly prevented by IsoPC. This protection was not observed when cells were pretreated (30 minutes before IsoPC) with 1 μmol/L SKI-II or 10 μmol/L ABC294640.

The degree of cell death was also quantified by Hoechst 33342 staining (Supplemental Figure III). Neurons with condensed nuclei (ie, undergoing apoptosis) were counted (Figure 5C), providing results similar to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide measurements.

**SPK2, a General Mediator for Cerebral Preconditioning**

Hypoxic preconditioning significantly reduced infarct volumes in wild-type mice (64.8±26.7 versus 104.5±15.3 mm³, P<0.01; Figure 6A), whereas infarct volumes were similar in naïve and preconditioned SPK2−/− mice (89.1±14.4 mm³; Figure 6A). Neurological outcome was significantly improved in hypoxia-preconditioned wild-type
mice but not in SPK2\(^{-/-}\) mice (Figure 6B). Cerebral spk2 mRNA level was upregulated (Figure 6C). Western blot revealed an elevated SPK2 protein expression starting at 2 hours and maintained up to 72 hours after HPC (Figure 6D).

**Discussion**

Cerebral preconditioning elicited a global neuroprotective effect and reduced infarct volumes. We observed upregulated cerebral SPK2 but not SPK1 protein expression in preconditioned mice, suggesting that the former isoform may play a role in preconditioning. Indeed, we also show that the reduced infarct volumes and improved neurological outcomes are absent in mice treated with a selective SPK2 inhibitor or in mice lacking SPK2. Because SPK inhibition blocked IsoPC-induced tolerance in primary neurons, we conclude that upregulation and activation of neuronal SPK2 is essential in cerebral preconditioning and protects the brain against ischemic injury and cell death.

The 2 SPK isoforms share high sequence homology (80\% amino acid homology) yet differ in the central regions and N-termini.\(^{32}\) SPK1 and SPK2 show different subcellular localizations and enzymatic properties as well as different expression in various tissues.\(^{32}\) We have previously observed that the SPK2 isoform predominates in different regions and cell types in the mouse brain.\(^{26}\) However, SPK1 is the more abundant isoform in renal proximal tubules and cardiomyocytes, and it was shown to be upregulated and activated in preconditioning in the kidney and heart.\(^{16,17,25}\) In contrast, an effect of SPK2 activation in preconditioning has been reported in 1 study of brain ischemia, which suggests a role of cerebral microvessel SPK2 in hypoxia preconditioning.\(^{8}\) This study reported that SPK inhibition abolished the induced ischemic tolerance.\(^{5}\) However, the SPK inhibitor used in this study, dimethylsphingosine, is known to inhibit the SPK1 isoform\(^{31,33}\) and possibly other enzymes such as protein kinase C.\(^{25}\) Following up on this study, we used knockout mice lacking either 1 of the SPK isoforms and a new selective SPK2 inhibitor, ABC294640, which dose-dependently inhibits SPK2 with a concentration that produces 50\% inhibition of approximately 60 \(\mu\text{mol/L}\), without affecting the activity of SPK1 at concentrations up to at least 100 \(\mu\text{mol/L}\).\(^{31}\) Taken together, our study adds further support to the notion that SPK2 is a general mediator in cerebral preconditioning in vivo and in vitro.

The present data reveal a rapid and sustained upregulation of SPK2 protein expression in cortical samples in preconditioned mice (approximately 2.2-fold increase at 24 hours after
IsoPC and approximately 2.5-fold increase at 72 hours after HPC). Taking into account that the published therapeutic windows for IsoPC and HPC are 24 hours and approximately 2.5-fold increase at 72 hours, respectively, our data strongly suggest a functional role of SPK2 in mediating preconditioning. Interestingly, Wacker et al showed an elevated SPK2 protein expression (1.7-fold increase at 2 hours after HPC) in microvessel-enriched brain extracts, suggesting that the endothelium of cerebral microvessels is the major cellular source for SPK during HPC. However, this SPK2 upregulation was transient, because it declined back to baseline in 24 hours in HPC mice. We previously observed an elevated spk2 mRNA expression in neurons treated with oxygen–glucose deprivation. We now show that selective SPK2 inhibition (by ABC294640) suppresses the neuroprotective effect of IsoPC in these cells, suggesting an autocrine effect of neuronal SPK2 in response to preconditioning.

Cerebral preconditioning requires gene and protein synthesis. IsoPC upregulates antiapoptotic protein (Bcl-2) and vascular endothelium growth factor in the brain, whereas hypoxia-inducible factor 1α (HIF1α) has been shown to mediate HPC. As a master regulator of transcription, HIF binds to hypoxia responsive elements of hypoxia-inducible genes. Hypoxia upregulates sptk1 in cancer and endothelial cells. However, less is known about the transcription regulation of spk2. In attempt to explore the role of HIF1α in cerebral preconditioning, we find an upregulated HIF1α in the cerebral cortex after IsoPC in wild-type mice but not in SPK2−/− (Supplemental Figures IV and V). This supports the previous observations that SPK is activated by hypoxia and SPK stabilizes HIF1α expression. Taken together, our findings pinpoint the crucial role of neuronal SPK as a universal regulator that mediates preconditioning and protects the brain against ischemic injury.

Cerebral ischemia/reperfusion triggers acute cellular injury (for example, neuronal cell death took place within hours) and late-phase tissue damage (such as inflammatory responses, which progresses and peak in days after ischemic insult). Although numerous reports support the notion that preconditioning could protect brain cells (including neurons, endothelial cells, and astrocyte) from cell death, there is limited information regarding the role of SPK in mediating the acute neuroprotection by preconditioning. To this end, we evaluate the effects of cerebral preconditioning on stroke outcomes (infarct volumes and neurological deficit score) at 24 hours after transient MCAO. It will be interesting to follow-up on the potential roles of SPK in mediating long-term neuroprotection (such as angiogenesis and neurogenesis) that may lead to improved motor function and recovery.

A review of preconditioning literature finds that a large number of pathways seem to mediate this phenomenon. This suggests that these pathways might act through common mediators to induce tolerance. Interestingly, SPK is known to be activated by a wide array of stimuli, including cell depolarization, G protein receptor agonists (muscarnic receptor agonists, formyl peptide, nucleotides, bradykinin, cannabinoids, lysophosphatidic acid, and S1P), agonists at receptor tyrosine kinases (platelet-derived growth factor, endothelial growth factor, nerve growth factor, vascular endothelial growth factor), crosslinking of immunoglobulin receptors, tumor necrosis factor-α, transforming growth factor-α, interleukins, Ca2+ increasing agents, and phospholipids. Furthermore, sphingolipids stimulate many signaling pathways (including HIF signaling, see previously) and modulate most cellular functions. It is therefore tempting to speculate that sphingolipid signaling plays a central role in the many pathways involved in preconditioning.

In summary, the present data demonstrates that SPK2 is a universal mediator in isoflurane- and hypoxia-induced preconditioning. Further investigation of the crosstalk between the SPK/S1P axis and HIF is likely to provide insights into the endogenous signaling that could protect the brain against ischemia/reperfusion injury.

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We thank Drs Michael Moskowitz and Cenk Ayata for discussion and comments and Dr Zhongcong Xie for assistance in isoflurane preconditioning in vitro.

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Disclosures
C.D.S. is CEO and President of Apogee Biotechnology Corp.

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Supplemental Material

Sphingosine kinase 2 mediates cerebral preconditioning and protects brains against ischemic injury

Lai Ming Yung, Ph.D1#; Ying Wei, M.D.1; Tao Qin1; Yumei Wang1, M.D.; Charles Smith, Ph.D2; Christian Waeber, Ph.D1*

1Stroke and Neurovascular Regulation Laboratory, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Charlestown, MA 02129; 2Apogee Biotechnology Corporation, Hummelstown, PA 17036, USA

*Correspondence to Dr Christian Waeber, Building 149, Room 6403, MGH, Charlestown, MA 02129.
Email: waebert@helix.mgh.harvard.edu
Phone: (617) 726 0768
Fax: (617) 726 0765

#Present address: Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA 02115
Supplemental Methods

Middle cerebral artery occlusion (MCAo) – Mice were anesthetized with 2% isoflurane, and maintained on 1.5% isoflurane in 30% oxygen and 70% nitrous oxide. Mice were placed on a heating pad to maintain rectal temperature at 37°C and MCAo was performed. The left MCA was occluded for 90 min with a commercially available coated monofilament (Doccol Corporation). At 24 hours after reperfusion, neurological deficit were scored into 4 categories as described1. Mice were sacrificed with i.p. administration of chloral hydrate (1 g/kg). Brains were harvested and cut into 1 mm-sections. Infarct sizes were stained with 2,3,5-triphenyltetrazolium chloride (TTC) and measured using a computerized image analysis system.

Expression studies for SPK1 and SPK2 - Cortical samples were harvested and frozen immediately. Samples were homogenized and total RNA and protein were extracted. Real-time PCR and western blot analysis were performed to detect SPK1 and SPK2 expressions. Polyclonal antibodies sc-22702 and sc-22704 (1:500, Santa Cruz) were used to detect SPK1 and SPK2 protein levels respectively. Expression levels were normalized to β-actin (1:4000, Sigma) and compared to naïve mice.

Primary culture of mouse cortical neurons – E14-16 embryos of CD1 mice were collected and their brains were harvested in sterile PBS. Cortices were dissected, freed from meninges and choroids plexus, minced and digested in trypsin-EDTA. Trypsin action was stopped with FBS and the tissue were homogenized by trituration with a pipette, passed through a cell strainer and spun down. The pellet were re-suspended in Neurobasal medium (Invitrogen)
with L-glutamine, B27 supplement and penicillin/streptomycin, centrifuged, re-suspended in Neurobasal medium, and plated onto polylysine-coated 24-well plate at a density of $2 \times 10^5$ cell/well.

In vitro IsoPC and cell death – After 8 days in culture, neurons were exposed to 2% isoflurane for 30 min in an air-tight chamber and allowed to recover for 24 hours as previously described\(^2\)\(^-\)\(^4\). This duration of exposure and concentration of isoflurane did not induce significant neuronal toxicity\(^5\),\(^6\). In order to test the involvement of SPK in IsoPC in vitro, cells were treated with SKI-II (0.3 and 1 µmol/L)\(^7\) and ABC294640 (3 and 10 µmol/L)\(^8\) 30 min before IsoPC.

After the 24-hour recovery period, glutamate or H\(_2\)O\(_2\) was added to induce cell death. Neurons were treated with 100 µmol/L glutamate for 5 min or 30 µmol/L H\(_2\)O\(_2\) (drugs prepared in NBM with no supplement) for 30 min, washed and replaced with fresh pre-warmed NBM\(^9\). Cell death was quantified with MTT 24 hours later. Neurons were incubated in 200 µg/ml Thiazoly1 Blue Tetrazolium Bromide (Sigma, St. Louis) at 37°C for 2 hours. Culture medium was aspirated and cells were lysed in 250 µl DMSO. Color intensity was measured at 570 nm using a Victor\(^3\)\(^\text{V}\) plate reader (Perkin Elmer, Waltham, MA). Results are expressed as percent absorbance of control wells.

A separate cohort of neurons was fixed with 4% PFA for 10 min and nuclei were stained with Hoechst 33342. Cells undergoing cell death, characterized by condensed nuclei, and the percentages of healthy-looking cells in three random fields were counted in a blinded fashion.
Table 1: Total number of C57BL/J mice subjected to middle cerebral artery occlusion (MCAo).

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<th>Mortality</th>
<th>Total number of mice</th>
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60min-MCAo (Supplementary figure 1)

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Table 2: total number of age-matched wild-type (WT), SPK1⁻/⁻ and SPK2⁻/⁻ mice subjected to MCAo.

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<th>Mortality</th>
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Figure S1. Effect of ABC294640 on infarct volumes. ABC294640 was given (100 mg/kg, oral gavage) either 24 hours before or 30 min after a 60 min-MCAo. There was no difference in infarct volume in naïve mice receiving ABC294640 treatment. PEG400 is the vehicle control. Data are mean±SD (n=6-8).
Figure S2. Infarct volumes of WT, SPK1⁻/⁻ and SPK2⁻/⁻ mice. Mice underwent a 90 min-MCAo and a 24 hr-reperfusion. The researcher performing MCAo and infarct volume measurements was blinded from the mouse genotypes. Data are mean±SD (n=7-8).
Figure S3. Extent of cell death as shown by nuclear staining. After exposure to glutamate (100 µmol/L for 5 min) or H₂O₂ (30 µmol/L for 30 min), neurons were fixed with 4% PFA and stained with Hoechst 33342. Filled and open arrows indicate dead and healthy neurons respectively. Scale bar (15 µm) applies to all panels.
**Figure S4.** Expression of hypoxia inducible factor 1alpha (HIF1α) in cerebral cortex harvested at different time points after isoflurane preconditioning (IsoPC). The primary antibody against HIF1α (Sigma, H6536) was used at 1:1000. HIF1α protein expression was normalized to loading control (β-actin) and expressed as fold compared to naïve control. Bars represent mean±SD (n=3). **p<0.01 compared to naïve control by one-way ANOVA.
Figure S5. Protein expression of HIF1α in control and preconditioned wild-type and SPK2⁻/⁻ mice. Cortical samples were collected 1 hr after isoflurane preconditioning. HIF1α level was determined and normalized to loading control (β-actin) and expressed as fold compared to naïve control. Bars represent mean±SD (n=3).
Supplemental References


