Sevoflurane Preconditioning Attenuates the Fall in Adenosine Triphosphate Levels, but Does Not Alter the Changes in Sodium and Potassium Levels during Hypoxia in Rat Hippocampal Slices

Brandon R. Esenther, M.D.,* Zhijun Ge, M.D.,† Fanli Meng, M.S.,‡ James E. Cottrell, M.D.,§ Ira S. Kass, Ph.D.||

ABSTRACT

Background: Sevoflurane preconditioning improves recovery after hypoxia. Sevoflurane administered before and during hypoxia improved recovery and attenuated the changes in intracellular sodium, potassium, and adenosine triphosphate (ATP) levels during hypoxia. In this study, the authors examine the effects of sevoflurane applied only before hypoxia on sodium, potassium, and ATP.

Methods: Hippocampal slices from adult male Sprague–Dawley rats were pretreated with 4% sevoflurane, washed, and then subjected to hypoxia (n ≥ 8 animals/group). The cornus ammonis 1 regions of the hippocampal slices were micro-dissected and sodium, potassium, and ATP concentrations measured.

Results: Pretreatment with sevoflurane for 15 or 60 min did not attenuate the increase in intracellular sodium or the decrease in intracellular potassium during hypoxia. After 60 min of preconditioning and 5 min of hypoxia, sodium increased 57% (*vs.* nonpreconditioned hypoxia 54% increase) and potassium decreased 31% (*vs.* 26%). These changes were not statistically significant *versus* untreated hypoxia. The 60-min sevoflurane preconditioning group had statistically significant higher ATP levels at 5 min of hypoxia (3.8 nmol/mg dry wt.) when compared to untreated hypoxic tissue (2.1 nmol/mg). There was no significant difference in ATP levels between the sevoflurane preconditioned and the untreated tissue before hypoxia (8.9 *vs.* 8.5 nmoles/mg, respectively).

Conclusion: Preconditioning with sevoflurane for 60 min before hypoxia does not alter changes in intracellular sodium and potassium during hypoxia but does attenuate the fall in intracellular ATP levels during hypoxia. Thus, there are differences between anesthetic preconditioning and when anesthetics are present before and during hypoxia.

What We Already Know about This Topic

- Sodium influx, potassium efflux, and a decrease in adenosine triphosphate content represent early phenomena that trigger the ischemic/hypoxic cascade
- Sevoflurane administered before and during hypoxia attenuates changes in sodium, potassium, and adenosine triphosphate during hypoxia

What This Article Tells Us That Is New

- Using the acute hippocampal slice, the authors provide evidence that sevoflurane-induced preconditioning (4%, 60 min given before a 5-min period of hypoxia) attenuates the decrease in adenosine triphosphate stores, but not the changes in sodium and potassium levels
- These results suggest that improved adenosine triphosphate content, but not Na^+ or K^+ levels, correlates with improved physiological recovery induced by sevoflurane preconditioning

CEREBRAL ischemia and hypoxia are significant causes of disability and neuropsychological deficits in patients undergoing major vascular, cardiac, and neurosurgical procedures.[1–6] A significant body of work has provided evidence

* Medical Student, SUNY Downstate Medical Center, Brooklyn, New York. Current position: Resident Physician, Department of Anesthesiology, Columbia University Medical Center, New York, New York. † Research Assistant Professor, Department of Anesthesiology, SUNY Downstate Medical Center. Current position: Vice President, Yixing People's Hospital, Yixing City, Jiangsu Province, China. ‡ Graduate Student, Program in Neural and Behavioral Sciences, SUNY Downstate Medical Center. Current position: Associate Researcher II, Department of Genetics and Genomic Sciences, Mount Sinai School of Medicine, New York, New York. § Dean for Clinical Practice, Distinguished Service Professor, Chair, Department of Anesthesiology, SUNY Downstate Medical Center. || Professor, Department of Anesthesiology, Department of Physiology and Pharmacology, Robert F. Furchgott Center for Neural and Behavioral Science, SUNY Downstate Medical Center.

Received from the Department of Anesthesiology, SUNY Downstate Medical Center, Brooklyn, New York. Submitted for publication September 6, 2012. Accepted for publication February 6, 2013. The authors gratefully acknowledge the generous funds to carry out this research that were provided by the Anesthesiology Department through the Brooklyn Anesthesia Research division of University Physicians of Brooklyn, Brooklyn, New York. The authors are also grateful to the Foundation for Anesthesia Education and Research, Rochester, Minnesota, for the Medical Student Anesthesia Research Fellowship in 2008 that Brandon Esenther received to begin this research.

Address correspondence to Dr. Kass: Department of Anesthesiology, Box 6, SUNY Downstate Medical Center, 450 Clarkson Ave, Brooklyn, New York 11203–2098. ira.kass@downstate.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

 Anesthesiology 2013; 119:119-28

for improvements in neurologic outcomes with volatile anesthetics administered prior to ischemic injury.[7–14] Volatile anesthetic agents have been shown to improve neuronal recovery and decrease total infarct size when administered before hypoxia/ischemia.[15–17] This improvement is gained not only when sevoflurane is administered before and during hypoxia but also with pretreatment alone, sevoflurane preconditioning.[18,19] These findings may impact the anesthetic management for patients with a higher risk of having an intraoperative stroke or poor cerebral perfusion while exposing patients to no additional risks.

The hippocampal cornus ammonis 1 (CA1) region was studied because tissue enriched with CA1 pyramidal neurons is easily isolated and these neurons demonstrate a high susceptibility to ischemic injury after short periods of ischemia.[20–22] To examine the mechanism of sevoflurane preconditioning protection, intracellular potassium (K^+), sodium (Na^+), and adenosine triphosphate (ATP) levels were measured in the CA1 region. Maintaining the intracellular concentrations of these two ions requires a major expenditure of neuronal ATP stores due to the activity of the Na^+/K^+-ATPase pump, which is required to pump the Na^+ and K^+ that have leaked down their electrochemical gradients. Intracellular ion concentrations have profound consequences on cellular volume and metabolic pathways, both of which contribute to the overall homeostasis of the cell. Sodium influx, potassium efflux, and consumption of ATP stores represent components of the ischemic cascade.

The experiments in this article will determine whether the maintenance of intracellular ATP, sodium, and potassium concentrations during hypoxia contributes to the mechanism by which sevoflurane-induced preconditioning reduces neuronal injury. Previous studies with sevoflurane administration before and during hypoxia found reduced changes in Na^+, K^+, and ATP levels during hypoxia. This study will determine whether this is also the case for sevoflurane-induced preconditioning and explicitly test the hypothesis that sevoflurane-induced preconditioning leads to improved Na^+, K^+, and/or ATP levels during hypoxia. We will also test whether sevoflurane-induced preconditioning alters the levels of Na^+, K^+, and/or ATP before hypoxia.

Material and Methods

Slice Preparation

All procedures involving animals were in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the State University of New York Downstate Medical Center, Brooklyn, New York. The minimum number of animals needed to achieve statistical significance was used.

Male Sprague–Dawley rats (100–120 days old) were preoxygenated in a Plexiglass chamber for 3 min and anesthetized for 1 min with 1% isoflurane, followed by 2 min of 2% isoflurane. When confirmed to be unconscious, the rat was decapitated, and its brain was quickly removed and placed into chilled (2–4°C) artificial cerebrospinal fluid (aCSF) saturated with 95% O_2–5% CO_2.[23] Hippocampal slices of 500 μm thickness were sectioned perpendicular to the long axis of the hippocampus in chilled aCSF (4–6°C) using a tissue slicer with a vernier micrometer (Stoelting; Wood Dale, IL). The slices were held in a beaker containing ice-cold aCSF saturated with 95% O_2–5% CO_2, and quickly transferred to grids in 20-ml beakers containing 10 ml of oxygenated aCSF. The composition of the aCSF was, in mm, NaCl, 126; KCl, 3; KH_2PO_4, 1.4; $NaHCO_3$, 26; $MgSO_4$, 1.3; $CaCl_2$, 1.4; glucose, 4; at pH, 7.4, and was equilibrated with 95% O_2–5% CO_2.[18] The gridded hippocampal slices were maintained in oxygenated (95% O_2–5% CO_2) aCSF at room temperature for 30 min and then slowly heated to 37°C. The slices were pretreated for either 15 or 60 min with 4% sevoflurane delivered from a calibrated sevoflurane vaporizer, the anesthetic was washed out for 5 min with 95% O_2–5% CO_2, and then the slices were subjected to either 5 or 10 min of hypoxia (95% N_2–5% CO_2) (fig. 1). Normoxic untreated control tissue was maintained at 37°C in the oxygenated aCSF for the duration of the experiment. A control group in each trial was maintained in oxygenated aCSF and exposed to 4% sevoflurane in the absence of a hypoxic event. The time in which the control group was exposed to sevoflurane matched the corresponding sevoflurane time in the preconditioned trial group. The experiments are carried out with hippocampal slices from the same animal in four different beakers, each of which is subject to one of four different protocols: control no hypoxia, sevoflurane no hypoxia, hypoxia alone, and hypoxia after sevoflurane preconditioning. The values of Na^+, K^+, or ATP of the CA1 regions from the three or four slices in each beaker are treated as one data point for that beaker and are normalized for dry weight; the CA1 tissue from the different slices is pooled before analysis. The procedures used in the current experiments are carried out identically to our previous physiology experiments, but the differences between the physiology and biochemistry chambers may have some effect on the slices.[18] The physiology experimental chamber does not allow different experimental protocols to be carried out at the same time on tissue from the same animals; this would reduce the statistical power of the biochemical analyses.

Sodium and Potassium Measurements

Following the experiment, slices were placed in agitated ice-cold (4°C) isotonic sucrose for 10 min in order to wash extracellular ions from the tissue (fig. 1). A washout period of 10 min was used because this duration of time primarily washes out the extracellular space with a minimal effect on the intracellular concentrations.[24,25] The CA1 regions were micro-dissected and the tissue was dried at 85°C for 48 h and weighed. A minimum tissue weight of 450 μg was used in order to guarantee sufficient tissue for ion measurements. Ions were extracted in 0.1 N nitric acid, and the sodium

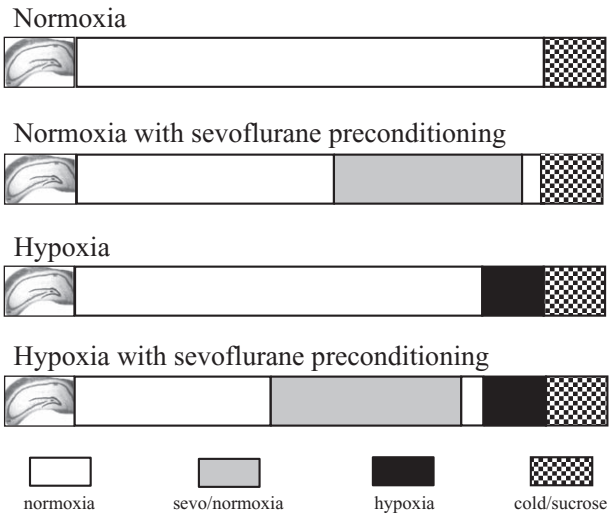

Fig. 1. Experimental paradigm to examine the effects of sevoflurane-induced preconditioning. Hippocampal slices were maintained in oxygenated (95% O_2–5% CO_2; normoxia) artificial cerebrospinal fluid (aCSF) at room temperature for 30 min and then slowly heated to 37°C. The slices were pretreated for either 15 or 60 min with 4% sevoflurane in 95% O_2–5% CO_2 (sevo/normoxia); only the 60-min treatment is shown. The anesthetic was washed out for 5 min with 95% O_2–5% CO_2 and then subjected to either 5 or 10 min of hypoxia (95% N_2–5% CO_2). The sevoflurane was administered using a calibrated sevoflurane vaporizer in line with the 95% O_2–5% CO_2 gas mixture before the aerator oxygenating the aCSF. Normoxic untreated control tissue was maintained at 37°C in oxygenated aCSF following the initial 30-min period at room temperature. A preconditioned normoxic control group was maintained under oxygenated aCSF and exposed to 4% sevoflurane in the absence of an ischemic event. The time in which the control group was exposed to sevoflurane matched the corresponding sevoflurane time in the preconditioned trial group. At the end of the sodium and potassium experiments, the slices were placed in ice-cold (2–4°C) isotonic sucrose for 10 min to wash out the extracellular ions. In the adenosine triphosphate (ATP) experiments, the slices were immediately frozen in liquid nitrogen at the end of the experiment and were not washed in sucrose.

and potassium concentrations were assayed using a flame photometer.[24,25] Percentage values are referenced to values in tissue from the same animal not treated with either drugs or hypoxia; each animal has a concurrent control. Each group was composed of eight different rats; a total of eight animals were used in each experiment as each animal contributed slices to each group in a particular experiment.

ATP Measurement

ATP levels were measured within the CA1 regions. The experimental treatment of the slices was as described ealier, except that there was no sucrose washout period. At the end of the hypoxia, or normoxia in the controls, the slices were rapidly frozen in liquid nitrogen, maintained in liquid nitrogen for up to a week, and then lyophilized. The CA1 regions were micro-dissected from the lyophilized tissue slices and weighted. ATP was extracted following tissue homogenization in 3 N ice-cold perchloric acid. ATP levels were then measured using a firefly luciferin–luciferase assay as described in previous publications.[25–27] Percentage values are referenced to values in tissue from the same animal not treated with either drugs or hypoxia; each animal has a concurrent control. Each group contained 10 different animals for each preconditioning time period; each animal contributed slices to each group of one experimental run.

Statistical Methods

As described in the previous section, the slices were distributed and the data were obtained such that a single animal contributed tissue to each of the groups in an experiment. While some slices were lost in some groups during the experiments and before any analysis, all groups contained at least two slices from each animal even after this loss. Each data point in the analysis consists of the sodium, potassium, or ATP levels normalized by dry weight for all the CA1 regions from slices in a particular beaker. All four beakers in a single experiment contained tissue from the same animal; this allows for a paired comparison between beakers. CA1 tissue per beaker and not slices are compared in a paired manner. A preplanned paired *t* test examined hypoxia *versus* sevoflurane-hypoxia and normoxia *versus* sevoflurane-normoxia for both 15- and 60-min preconditioning. The 15- and 60-min preconditioning experiments were completely separate; they were performed at different times and compared to their own separate controls. In the sodium and potassium experiments, each group contained eight animals and the analyses were performed with eight pairings; for the ATP experiments, each group contained 10 animals and the analyses were performed with 10 pairings.

In addition to this preplanned paired *t* test, all data were subjected to an ANOVA followed by the Newman–Keuls multiple comparison test. Data were tested for normality using the D'Agostino and Pearson omnibus normality test and only these data were tested with parametric statistics. For data comparisons that passed one test but not another for significance, the results of both tests are explicitly stated in the results section. This occurred only for the ATP data with 15-min preconditioning and 5-min hypoxia. All statistics were carried out using Graphpad Prism 4 software (Graphpad Software Inc., San Diego, CA). In the text when we state a result is significant, we mean that it is statistically significant with $P < 0.05$ using a two-tailed test; all values in the text are given as the mean ± SD.

Results

Sodium and Potassium

Fifteen Minutes Sevoflurane Preconditioning Followed by 5 min Hypoxia. Sodium and potassium measurements were made at the end of 5-min of hypoxia. In normoxic tissue, the

mean (± SD) sodium concentration was 89 ± 14 nmol/mg and the mean potassium concentration was 151 ± 17 nmol/mg; all percentages in this section are referenced to these values. Sodium levels increased by 70% (to 152 ± 29 nmol/mg), while potassium levels decreased by 26% (to 112 ± 26 nmol/mg) after 5 min of hypoxia without preconditioning (fig. 2A). Sodium levels increased by 71% (to 153 ± 22 nmol/mg) and potassium levels decreased by 29% (to 108 ± 15 nmol/mg) in the 5-min hypoxic tissue preconditioned for 15 min with 4% sevoflurane. There was no significant difference in intracellular sodium and potassium concentrations between the CA1 regions exposed to hypoxic conditions and those exposed to hypoxic conditions following sevoflurane preconditioning ($P = 0.90$ for sodium and $P = 0.64$ for potassium; paired t test; n = 8 pairs).

In the control group treated with sevoflurane alone (no hypoxia), sodium increased by 4% (to 93 ± 12 nmol/mg) and potassium decreased by 4% (to 108 ± 15 nmol/mg), these changes were not significant when compared to normoxic tissue. Thus there was no difference in intracellular sodium and potassium levels between the untreated oxygenated CA1 regions and the oxygenated CA1 regions exposed to 4% sevoflurane for 15 min ($P = 0.49$ for sodium and $P = 0.46$ for potassium; paired t test; n = 8 pairs).

Fifteen Minutes Sevoflurane Preconditioning at 10-min Hypoxia. The impact of 15 min of sevoflurane preconditioning on hippocampal slices subjected to 10 min of hypoxia was examined (fig. 2B). The mean sodium concentration in normoxic untreated tissue was 119 ± 20 nmol/mg and the

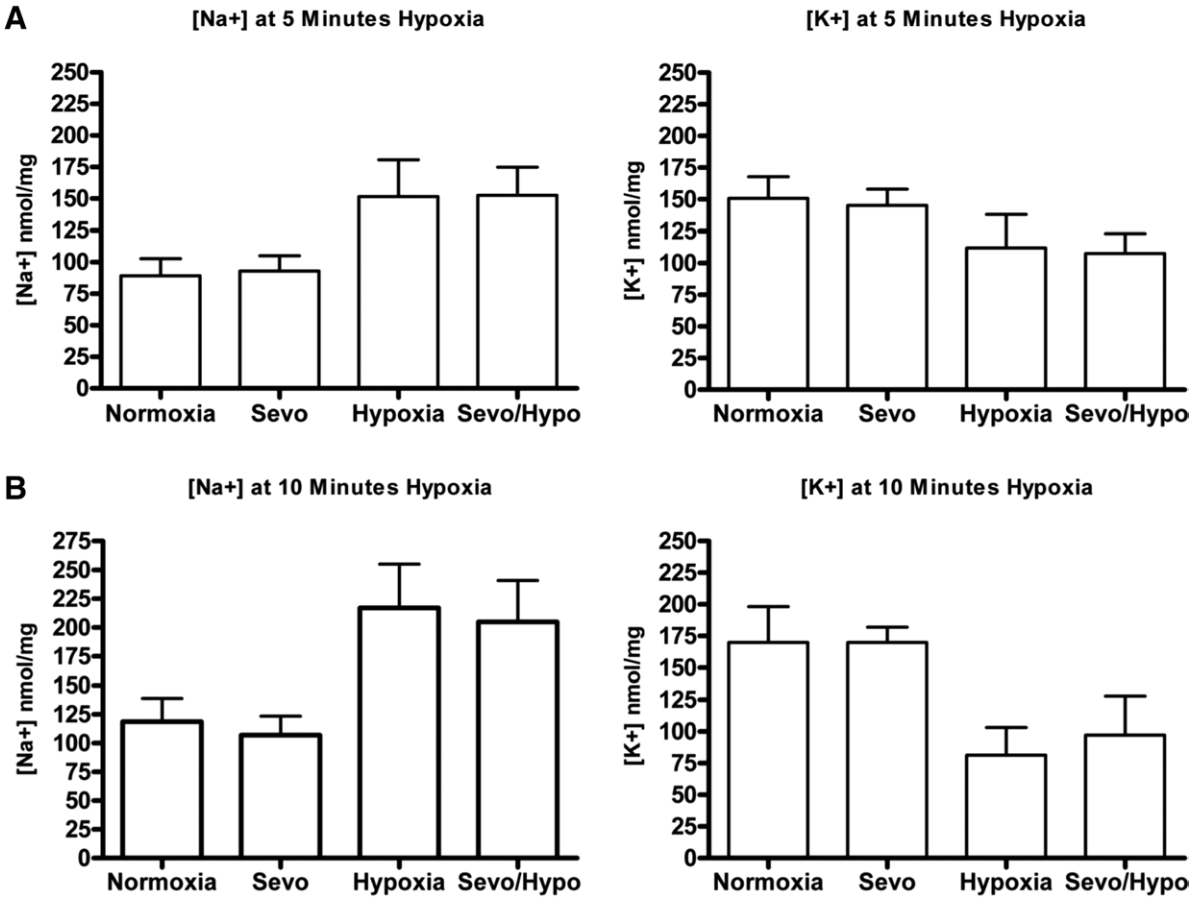

Fig. 2. Effect of 15-min sevoflurane preconditioning on sodium and potassium concentrations during hypoxia. Sodium (Na^+) and potassium (K^+) concentrations were measured in the cornus ammonis 1 (CA1) region of rat hippocampal slices at 5 (*A*) and 10 (*B*) min of hypoxia (Hypo). The tissue was treated with 4% sevoflurane (Sevo) in 95% O_2–5% CO_2 for 15 min and then the anesthetic was washed out with 95% O_2–5% CO_2 for 5 min (Sevo or Sevo/Hypo). The tissue was then subjected to either 5 min (*A*) or 10 min (*B*) of hypoxia by aerating the tissue with 95% N_2–5% CO_2; the Normoxia and Sevo groups were not subjected to hypoxia. At the end of the experiment, the tissue from each group was placed in ice-cold isotonic sucrose for 10 min to wash out the extracellular ions. The CA1 regions were then dissected, dried, weighed, and analyzed in a flame photometer. Values are the mean ± SD (n = 8 animals per group) and are expressed as nmol per mg dry weight. The normoxia groups (Normoxia and Sevo) are significantly different from the hypoxia groups (Hypoxia and Sevo/Hypo); $P < 0.05$ (Newman–Keuls multiple comparison test; n = 8/group); however, the Hypoxia and Sevo/Hypo groups are not significantly different from each other (Newman–Keuls or paired t test).

potassium concentration was 170 ± 28 nmol/mg; all percentages in this section are referenced to these values. Intracellular sodium increased by 83% (to 217 ± 38 nmol/mg), while potassium levels decreased by 52% (to 81 ± 22 nmol/mg) in hypoxic tissue compared to the normoxic tissue. In ischemic tissue preconditioned for 15 min with 4% sevoflurane, intracellular sodium increased by 73% (to 205 ± 36 nmol/mg) and intracellular potassium levels decreased by 43% (to 97 ± 31 nmol/mg) compared to normoxic tissue concentrations (fig. 2B). The change due to sevoflurane preconditioning was not significant ($P = 0.39$ for sodium and $P = 0.21$ for potassium; paired t test; n = 8 pairs).

In the normoxic group treated with sevoflurane alone, the intracellular sodium concentration decreased by 10% (not significant) and the potassium mean concentrations were identical to those of the normoxic untreated CA1 regions ($P = 0.30$ for sodium and $P = 1.00$ for potassium; paired t test; n = 8 pairs).

Thus there was a significant increase in intracellular sodium and a significant decrease in potassium concentrations in both groups exposed to hypoxic conditions when compared to the normoxic group ($P < 0.001$; Newman–Keuls test; n = 8/group). No statistically significant difference in intracellular sodium and potassium concentrations was seen between the CA1 regions exposed to hypoxic conditions and those exposed to hypoxic conditions following 15 min of sevoflurane preconditioning (Na^+ $P = 0.90$; K^+ $P = 0.64$; paired t test; n = 8 pairs).

Sevoflurane 60-min Preconditioning at 5 min of Hypoxia. The effect of a more prolonged 60-min sevoflurane preconditioning period was next examined. In normoxic tissue, the mean sodium concentration was 92 ± 14 nmol/mg and the mean potassium concentration was 170 ± 25 nmol/mg; all percentages in this section are referenced to these values. In untreated tissue, sodium increased by 54% (to 142 ± 11 nmol/mg) and potassium decreased by 26% (to 125 ± 29 nmol/mg) after 5 min of hypoxia in the hippocampal CA1 region when compared to the normoxic tissue. In tissue preconditioned with sevoflurane for 60 min prior to the 5-min period of ischemia, intracellular sodium was found to increase by 57% (to 144 ± 17 nmol/mg) and intracellular potassium decreased by 31% (to 118 ± 28 nmol/mg) compared to the normoxic tissue concentrations (fig. 3A).

While both preconditioned and nonpreconditioned groups demonstrated a significant difference when hypoxic and normoxic tissue are compared for sodium and potassium ($P < 0.001$; Newman–Keuls test; n = 8/group), there was no significant difference in intracellular sodium and potassium concentrations when the hypoxic and the sevoflurane preconditioned hypoxic tissue were compared ($P = 0.60$ for sodium and $P = 0.25$ for potassium; paired t test; n = 8 pairs). There was also no difference between the normoxic and sevoflurane-normoxic tissue ($P = 0.91$ for sodium and $P = 0.87$ for potassium; paired t test; n = 8 pairs).

Sevoflurane 60-min Preconditioning at 10-min Hypoxia. In normoxic tissue, the mean sodium concentration was 79 ± 8 nmol/mg and the mean potassium concentration was 175 ± 9 nmol/mg; all percentages in this section are referenced to these values. In the untreated group after 10 min of hypoxia, the sodium levels increased by 118% (to 172 ± 22 nmol/mg) and potassium levels decreased by 50% (to 88 ± 11 nmol/mg) when compared to normoxic tissue. Sodium concentrations in preconditioned tissue after 10 min of hypoxia increased by 114% (to 169 ± 17 nmol/mg) and potassium concentrations decreased by 60% (to 78 ± 14nmol/mg) compared to the normoxic tissue (fig. 3B). At the end of 10-min hypoxia, the concentrations of sodium and potassium in the sevoflurane preconditioned and untreated hypoxic groups were not significantly different ($P = 0.79$ for sodium and $P = 0.12$ for potassium; paired t test; n = 8 pairs). However, both the sevoflurane-hypoxia and the untreated hypoxia groups were significantly different from the normoxic group for both sodium and potassium ($P < 0.001$; Newman–Keuls test; n = 8/group).

In the normoxia group treated with sevoflurane alone, the mean sodium concentration was 5% lower (to 75 ± 13 nmol/mg) and the mean potassium concentration was 4% lower (to 169 ± 15 nmol/mg) when compared to untreated normoxia. There was no significant difference in the sodium or potassium concentrations of the normoxic tissue and the normoxic-sevoflurane treated tissue ($P = 0.45$ for sodium and $P = 0.30$ for potassium; paired t test; n = 8 pairs).

ATP Concentrations following 15-min Preconditioning at 5-min Ischemia. ATP levels were measured after 5 min of hypoxia because this is the time when most of the damaging cellular changes are initiated and also the time at which we found the largest differences between groups in previous studies.[27] All percentages in this section are referenced to the normoxic unpretreated ATP level (6.4 ± 1.5 nmol/mg). In slices not subjected to hypoxia, the ATP level was not altered by 15 min of sevoflurane; ATP measured in the 15-min sevoflurane group was 3% higher (to 6.6 ± 1.8 nmol/mg) than its level in the nonpreconditioned group; this difference was not significant ($P = 0.41$, paired t test; n = 10 pairs; fig. 4A). In trials where the hippocampal slices were not preconditioned prior to 5 min of hypoxia, the fall in ATP levels in the CA1 region of the hypoxia group was to 19% of its normoxic levels (it fell to 1.2 ± 0.4 nmol/mg at 5 min of hypoxia); in slices that were preconditioned with 4% sevoflurane prior to the hypoxic period, the ATP fell to 27% of its normoxic level (it fell to 1.7 ± 0.6 nmol/mg at 5 min of hypoxia; fig. 4A). A paired t test demonstrated that the sevoflurane-hypoxic and the untreated hypoxic groups were significantly different ($P = 0.0008$; n = 10 pairs); however, a Newman–Keuls multiple comparison test that did not use pairing did not show significance (n = 10/group).

ATP Concentrations following 60-min Preconditioning at 5-min Ischemia. The effect of a more prolonged sevoflurane preconditioning period on ATP levels was next examined

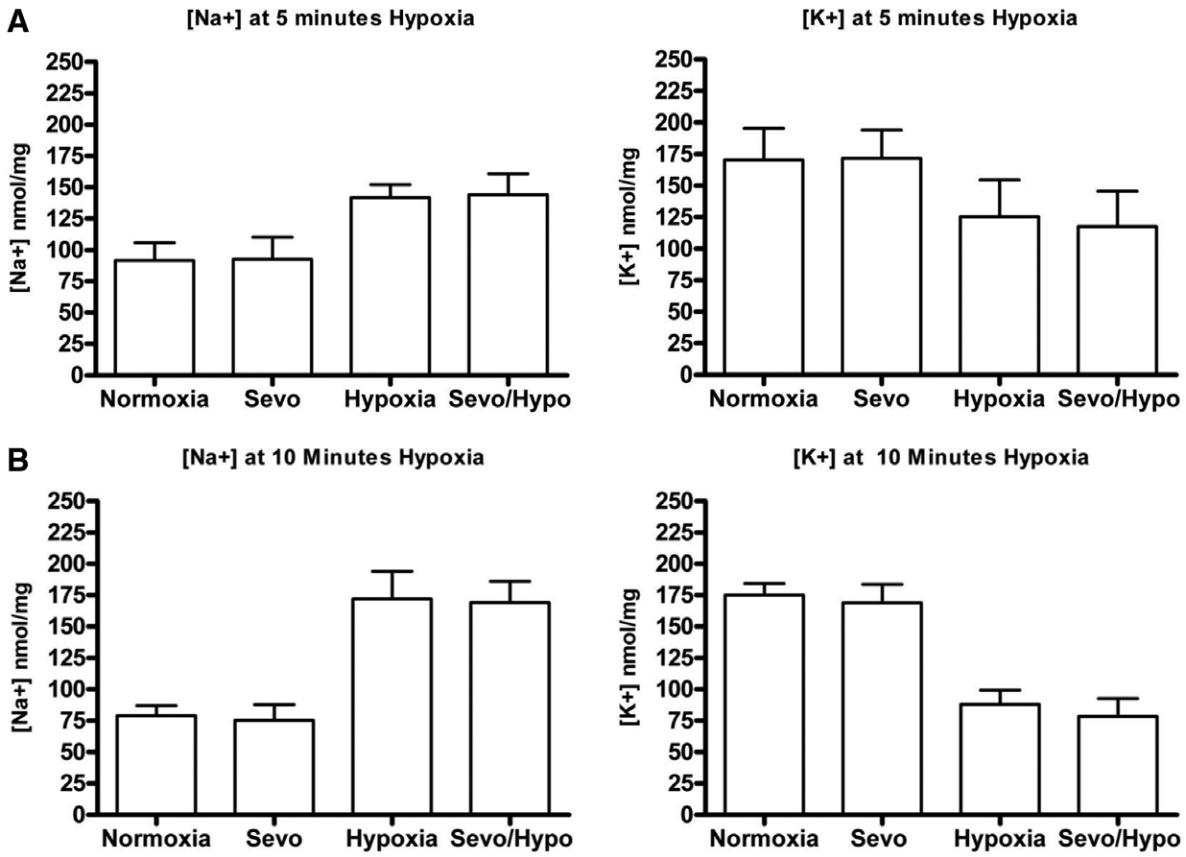

Fig. 3. Effect of 60-min sevoflurane preconditioning on sodium and potassium concentrations during hypoxia. Sodium (Na^+) and potassium (K^+) concentrations were measured in the cornus ammonis 1 (CA1) region of rat hippocampal slices at 5 (*A*) and 10 (*B*) min of hypoxia. The tissue was treated with 4% sevoflurane in 95% O_2–5% CO_2 for 60 min and then the anesthetic was washed out with 95% O_2–5% CO_2 for 5 min (Sevo or Sevo/Hypo). The tissue was then subjected to either 5 min (*A*) or 10 min (*B*) of hypoxia by aerating the tissue with 95% N_2–5% CO_2; the Normoxia and Sevo groups were not subjected to hypoxia. At the end of the experiment, the tissue from each group was placed in ice-cold isotonic sucrose for 10 min to wash out the extracellular ions. The CA1 regions were then dissected, dried, weighed, and analyzed in a flame photometer. Values are the mean ± SD (n = 8 animals per group) and are expressed as nmol per mg dry weight. The normoxia groups (Normoxia and Sevo) are significantly different from the hypoxia groups (Hypoxia and Sevo/Hypo); $P < 0.05$ (Newman–Keuls multiple comparison test; n = 8/group); however, the Hypoxia and Sevo/Hypo groups are not significantly different from each other (Newman–Keuls or paired *t* test).

(fig. 4B). All percentages in this section are referenced to the normoxic unpretreated ATP level (8.5 ± 0.9 nmol/mg). The ATP level in normoxic tissue with 60 min of sevoflurane preconditioning was increased by 5% (to 8.9 ± 1.3 nmol/mg); this difference was not significant compared to untreated normoxic tissue ($P = 0.53$, paired *t* test; n = 10 pairs). In tissue preconditioned for 60 min with 4% sevoflurane and then subjected to 5-min hypoxia, the fall in ATP levels in the CA1 region was significantly attenuated compared to that in the untreated hypoxic slices. The mean ATP level measured from the sevoflurane preconditioned group fell to 45% of its normoxic level (3.8 ± 0.8 nmol/mg), while in the untreated hypoxic group, it fell to 25% of its normoxic level (2.1 ± 0.6 nmol/mg); these differences were significant ($P = 0.0001$, paired *t* test, n = 10 pairs; $P = 0.001$ Newman–Keuls test n = 10/group). Thus, 60 min of 4% sevoflurane preconditioning significantly attenuated the fall in ATP levels during hypoxia; this may be one of the mechanisms by which sevoflurane preconditioning improves the recovery of neurons after hypoxia and ischemia.

Discussion

In the current study, the effects of sevoflurane-induced preconditioning on intracellular sodium, potassium, and ATP levels are examined during hypoxia using an *in vitro* model. Our study examines sevoflurane-induced immediate preconditioning. In this study, there is only a minimal time between sevoflurane application and washout and the onset of the hypoxia. This type of protection would be useful for surgical patients under general anesthesia who are at risk of ischemic brain injury. Sevoflurane is a commonly used anesthetic that allows for rapid awakening, and any clinically used anesthetic will not expose the patient to additional risks as anesthesia is required for the surgery. Previous studies have shown that volatile anesthetic agents, including sevoflurane, may protect against neuronal injury to CA1

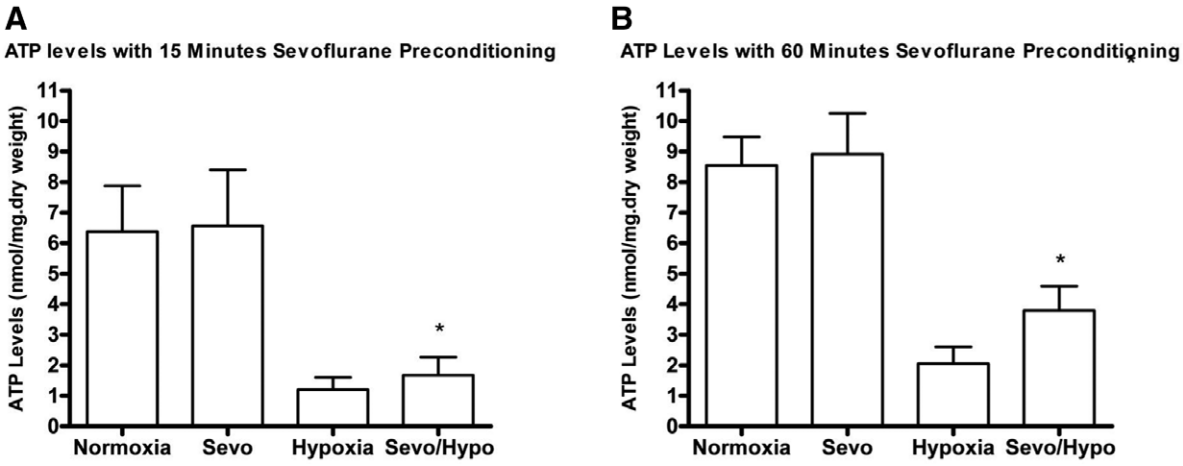

Fig. 4 . Effect of 15 or 60 min of sevoflurane preconditioning on adenosine triphosphate (ATP) concentrations during hypoxia. ATP was measured in the cornus ammonis 1 (CA1 region) of rat hippocampal slices at 5 min of hypoxia. The tissue was treated with 4% sevoflurane (Sevo) in 95% O_2–5% CO_2 for either 15 (*A*) or 60 (*B*) min and then the anesthetic was washed out with 95% O_2–5% CO_2 for 5 min (Sevo and Sevo/Hypo groups). At 5 min of hypoxia (Hypo) with 95% N_2–5% CO_2 (Hypoxia and Sevo/Hypo groups) or at an equivalent time period (Normoxia and Sevo groups), the tissue was frozen in liquid nitrogen. The hippocampal slices were lyophilized and the CA1 region was then dissected, weighed, and analyzed using a bioluminescent assay. Values are the mean ± SD (n = 10 rats per group) and are expressed as nmol per mg dry weight. A paired *t* test indicated that the Hypoxia *versus* the Sevo/Hypo groups were significantly different from each other as indicated by * ($P < 0.001$; n = 10 pairs). The normoxia groups (Normoxia *vs.* Sevo) were not significantly different from each other ($P > 0.4$; Paired *t* test; n = 10 pairs).

pyramidal cells when present during rewarming following hypothermia, a condition that may lead to excitotoxic injury.[28] Our previous *in vivo* studies have shown that sevoflurane preconditioning provided robust protection of CA1 hippocampal neurons 1 and 6 weeks after global ischemia.[18] The concentration of sevoflurane used in the current and previous studies was 4%, a level of anesthetic commonly used in the operating room with proper pharmacologic support of blood pressure.

We chose a dose of 4% sevoflurane for 15 min starting 20 min before a 10-min hypoxia as that is the concentration we found that improved recovery in a previous study.[29] This dose regimen increased the synthesis of a protein kinase that is necessary for preconditioning protection. In a previous study, we found that 2% sevoflurane for 15 min did not induce preconditioning protection of hippocampal slices and therefore we only examined preconditioning with 4% sevoflurane.[18] In the current study, we did examine a more prolonged application of sevoflurane and found increased preservation of ATP during hypoxia when 4% sevoflurane was present for 60 min before hypoxia.

Research examining the mechanisms of protection by anesthetic preconditioning have focused predominantly on minimizing neurotransmitter toxicity, modulation of apoptotic pathways, modulation of cerebral blood flow, modulation of reactive oxygen species, and maintenance of electrophysiological parameters and intracellular ion concentrations.[7,15,16,30–35] Current research suggests that sevoflurane preconditioning influences numerous pathways within the neurons; however, the identities of the pathways responsible for neuroprotection remain controversial.

Our *in vitro* experiments use procedures that have been found not to alter or obstruct the protective effects of anesthetics. We have found through past investigation that 2% isoflurane does not protect neuronal tissue from ischemic damage in the rat model even when present 10 min before and during a hypoxic event.[24,27,30,35–38] In the methods of the current study, 2% isoflurane is present for only 2 min, so it is unlikely to influence the results in the slices 2 h after its washout. In any case, the control and experimental animals were both subjected to 2 min of 2% isoflurane; therefore, any differences found represent the additional effects of 4% sevoflurane.

In our previous studies, when sevoflurane is present before and during hypoxia, direct neuroprotection with 4% sevoflurane significantly attenuated the increase in intracellular sodium and the fall in intracellular potassium.[27] In the current study with preconditioning, we did not find a statistically significant reduction of the effects of hypoxia on Na^+ and K^+ levels. These point to an important difference between preconditioning and application of anesthetic during the hypoxia. Anesthetics are known to have direct effects on glutamate and γ-aminobutyric acid transmission. This effect is thought to require the continuous application of anesthetic and would directly affect excitability and ion flux. This may be the cause of the reduced hypoxic changes in Na and K with application during hypoxia. Preconditioning is by definition after the anesthetics are washed off, so these direct effects on transmission and ion flux would be absent. Indeed we found no effect of anesthetic preconditioning on ion levels during hypoxia.

It remains unclear why the normoxic untreated ATP levels from the 15-min and 60-min sevoflurane-induced

preconditioning experiments are different (6.4 *vs.* 8.5) as the conditions of these experiments are identical. The two experiments were run 2 months apart and differences in the reagents, techniques, and/or animals may have caused altered baseline values. However, all experiments are compared back to a simultaneous control; therefore, within experiments the comparative values are valid. If there was a systematic error in values, the use of a simultaneous control as a comparison will correct for the different baseline values. The analysis of ATP was run in two large batches: one for all the 15-min experiments and the other for the 60-min experiments.

The fall in cellular ATP levels was statistically significantly attenuated with both sevoflurane preconditioning and sevoflurane direct neuroprotection. This may serve as one mechanism of protection during ischemic events. In our earlier study of sevoflurane direct neuroprotection, we attributed the attenuation of the fall in ATP to the reduced changes in intracellular Na^+ and K^+ concentrations and therefore reduced Na^+-K^+ ATPase pump activity. In our current study, we found that although changes in the Na^+ and K^+ concentrations are not significantly improved by sevoflurane preconditioning, the fall in ATP levels was statistically significantly attenuated. Therefore, reducing the net influx in Na^+ and the net efflux in K^+ during ischemia appears not to be the sole mechanism that leads to improved ATP levels during hypoxia.

An effect of sevoflurane that may explain the attenuation of the fall of ATP and the other actions of sevoflurane preconditioning, such as membrane hyperpolarization, may be enhanced protein kinase M zeta and ATP-dependent potassium channel (K_{ATP} channel) activity *via* the activation of the mammalian target of rapamycin pathway.[29,39] Both plasma membrane and mitochondrial K_{ATP} channels have been implicated in the protection due to ischemic and anesthetic preconditioning in cardiac and neuronal tissue.[31,40–42] In studies where mitochondrial K_{ATP} channels were blocked with 5-hydroxydecanoic acid, the preconditioning effects of sevoflurane were blocked.[31,34,43,44] Chelerythrine, a blocker of protein kinase C family of isoenzymes which includes protein kinase M zeta blocked the preconditioning effects of sevoflurane.[18,42] A recent *in vivo* study found that sevoflurane-induced preconditioning led to a mitochondrial K_{ATP} channel-dependent increase in protein kinase C epsilon, which is an enzyme similar to protein kinase M zeta that we found increased *in vitro*.[29,43] Protein kinase M zeta is a constitutively active protein kinase C family member that has a long half-life and remains active in the neurons for prolonged periods of time.[39] Additionally, it has been demonstrated that a mitochondrial K_{ATP} channel opener, diazoxide, mimics sevoflurane preconditioning by reducing infarct size and improving neurological recovery in a rat model of ischemia.[44] The role of K_{ATP} channels may be integral to the mechanism of sevoflurane preconditioning protection.[29] Our previous article proposed a pathway for sevoflurane preconditioning protection; the mammalian target of rapamycin pathway increases protein kinase M zeta and this leads to increased K_{ATP} channel activity.[29] This previous article used tolbutamide and glybenclamide to block the K_{ATP} channels and prevent preconditioning protection; preliminary experiments indicate that 5-hydroxydecanoic acid, a mitochondria-specific K_{ATP} channel blocker, is also effective in preventing preconditioning protection in our preparation.

Previous studies found an enhanced hypoxic hyperpolarization with sevoflurane preconditioning that was blocked by glibenclamide and tolbutamide; this implicated enhanced activity of K_{ATP} channels. The current study did not find a change in either Na or K levels during hypoxia with sevoflurane preconditioning; however, it did find improved ATP levels. The enhanced ATP is not likely due to reduced pumping; if anything, K pumping should be increased due to the increased activity of the membrane K_{ATP} channel. Therefore, the improved ATP is likely due to a direct effect on the mitochondria, perhaps *via* enhanced mitochondrial K_{ATP} channel activity preserving function.

There are many similarities between anesthetic preconditioning and anesthetic postconditioning: both affect cell signaling pathways and are not reliant on direct continuous anesthetic action on membrane ion channels such as γ-aminobutyric acid and glutamate receptor ion channels. Thus, it is likely that pre- and postconditioning share similar mechanisms. In this study, we found that sevoflurane-induced preconditioning did not directly affect sodium or potassium levels and, therefore, did not likely directly activate or inhibit membrane ion channels. Sevoflurane preconditioning causes metabotropic responses that alter cellular signaling pathways which may then alter ion channel activity and/or activate other pathways.[29,39] It seems likely that during postconditioning the same or similar metabotropic pathways are activated by sevoflurane.

In conclusion, we found that sevoflurane preconditioning does not attenuate changes in intracellular sodium and potassium following periods of ischemia, but did lead to better maintenance of intracellular ATP levels. The effect of sevoflurane preconditioning is different from the effect of sevoflurane before and during hypoxia with respect to Na^+ and K^+ levels, but the two anesthetic regimens have similar effects with respect to ATP preservation. Thus, the improved ATP but not Na^+ and K^+ levels correlate with the improved physiological recovery due to sevoflurane preconditioning.

References

1. Arrowsmith JE, Grocott HP, Reves JG, Newman MF: Central nervous system complications of cardiac surgery. Br J Anaesth 2000; 84:378–93
2. Boeken U, Litmathe J, Feindt P, Gams E: Neurological complications after cardiac surgery: Risk factors and correlation

to the surgical procedure. Thorac Cardiovasc Surg 2005; 53:33–6

3. Bucerius J, Gummert JF, Borger MA, Walther T, Doll N, Onnasch JF, Metz S, Falk V, Mohr FW: Stroke after cardiac surgery: A risk factor analysis of 16,184 consecutive adult patients. Ann Thorac Surg 2003; 75:472–8
4. Lund C, Sundet K, Tennøe B, Hol PK, Rein KA, Fosse E, Russell D: Cerebral ischemic injury and cognitive impairment after off-pump and on-pump coronary artery bypass grafting surgery. Ann Thorac Surg 2005; 80:2126–31
5. Jensen BØ, Rasmussen LS, Steinbrüchel DA: Cognitive outcomes in elderly high-risk patients 1 year after off-pump *versus* on-pump coronary artery bypass grafting. A randomized trial. Eur J Cardiothorac Surg 2008; 34:1016–21
6. Norkienė I, Samalavičius R, Ivaškevičius J, Budrys V, Paulauskiene K: Asymptomatic carotid artery stenosis and cognitive outcomes after coronary artery bypass grafting. Scand Cardiovasc J 2011; 45:169–73
7. Kitano H, Kirsch JR, Hurn PD, Murphy SJ: Inhalational anesthetics as neuroprotectants or chemical preconditioning agents in ischemic brain. J Cereb Blood Flow Metab 2007; 27:1108–28
8. Clarkson AN: Anesthetic-mediated protection/preconditioning during cerebral ischemia. Life Sci 2007; 80:1157–75
9. Werner C, Möllenberg O, Kochs E, Schulte J am Esch: Sevoflurane improves neurological outcome after incomplete cerebral ischaemia in rats. Br J Anaesth 1995; 75:756–60
10. Xiong L, Zheng Y, Wu M, Hou L, Zhu Z, Zhang X, Lu Z: Preconditioning with isoflurane produces dose-dependent neuroprotection via activation of adenosine triphosphate-regulated potassium channels after focal cerebral ischemia in rats. Anesth Analg 2003; 96:233–7
11. Zheng S, Zuo Z: Isoflurane preconditioning induces neuroprotection against ischemia via activation of P38 mitogen-activated protein kinases. Mol Pharmacol 2004; 65:1172–80
12. Bendo AA, Kass IS, Cottrell JE: Anesthetic protection against anoxic damage in the rat hippocampal slice. Brain Res 1987; 403:136–41
13. Head BP, Patel P: Anesthetics and brain protection. Curr Opin Anaesthesiol 2007; 20:395–9
14. Warner DS: Anesthetics provide limited but real protection against acute brain injury. J Neurosurg Anesthesiol 2004; 16:303–7
15. Engelhard K, Werner C, Eberspächer E, Pape M, Blobner M, Hutzler P, Kochs E: Sevoflurane and propofol influence the expression of apoptosis-regulating proteins after cerebral ischaemia and reperfusion in rats. Eur J Anaesthesiol 2004; 21:530–7
16. Pape M, Engelhard K, Eberspächer E, Hollweck R, Kellermann K, Zintner S, Hutzler P, Werner C: The long-term effect of sevoflurane on neuronal cell damage and expression of apoptotic factors after cerebral ischemia and reperfusion in rats. Anesth Analg 2006; 103:173–9
17. Warner DS, McFarlane C, Todd MM, Ludwig P, McAllister AM: Sevoflurane and halothane reduce focal ischemic brain damage in the rat. Possible influence on thermoregulation. Anesthesiology 1993; 79:985–92
18. Wang J, Lei B, Popp S, Meng F, Cottrell JE, Kass IS: Sevoflurane immediate preconditioning alters hypoxic membrane potential changes in rat hippocampal slices and improves recovery of CA1 pyramidal cells after hypoxia and global cerebral ischemia. Neuroscience 2007; 145:1097–107
19. Payne RS, Akca O, Roewer N, Schurr A, Kehl F: Sevoflurane-induced preconditioning protects against cerebral ischemic neuronal damage in rats. Brain Res 2005; 1034:147–52
20. Araki T, Kato H, Kogure K: Selective neuronal vulnerability following transient cerebral ischemia in the gerbil: Distribution and time course. Acta Neurol Scand 1989; 80:548–53
21. Cronberg T, Jensen K, Rytter A, Wieloch T: Selective sparing of hippocampal CA3 cells following *in vitro* ischemia is due to selective inhibition by acidosis. Eur J Neurosci 2005; 22:310–6
22. Ordy JM, Wengenack TM, Bialobok P, Coleman PD, Rodier P, Baggs RB, Dunlap WP, Kates B: Selective vulnerability and early progression of hippocampal CA1 pyramidal cell degeneration and GFAP-positive astrocyte reactivity in the rat four-vessel occlusion model of transient global ischemia. Exp Neurol 1993; 119:128–39
23. Zhu H, Cottrell JE, Kass IS: The effect of thiopental and propofol on NMDA- and AMPA-mediated glutamate excitotoxicity. Anesthesiology 1997; 87:944–51
24. Amorim P, Cottrell JE, Kass IS: Effect of small changes in temperature on CA1 pyramidal cells from rat hippocampal slices during hypoxia: Implications about the mechanism of hypothermic protection against neuronal damage. Brain Res 1999; 844:143–9
25. Kass IS, Abramowicz AE, Cottrell JE, Chambers G: The barbiturate thiopental reduces ATP levels during anoxia but improves electrophysiological recovery and ionic homeostasis in the rat hippocampal slice. Neuroscience 1992; 49:537–43
26. Wang J, Chambers G, Cottrell JE, Kass IS: Differential fall in ATP accounts for effects of temperature on hypoxic damage in rat hippocampal slices. J Neurophysiol 2000; 83: 3462–72
27. Wang J, Meng F, Cottrell JE, Kass IS: The differential effects of volatile anesthetics on electrophysiological and biochemical changes during and recovery after hypoxia in rat hippocampal slice CA1 pyramidal cells. Neuroscience 2006; 140:957–67
28. Bickler PE, Warren DE, Clark JP, Gabatto P, Gregersen M, Brosnan H: Anesthetic protection of neurons injured by hypothermia and rewarming: Roles of intracellular Ca2+ and excitotoxicity. Anesthesiology 2012; 117:280–92
29. Wang J, Meng F, Cottrell JE, Sacktor TC, Kass IS: Metabotropic actions of the volatile anaesthetic sevoflurane increase protein kinase M zeta synthesis and induce immediate preconditioning protection of rat hippocampal slices. J Physiol (Lond) 2012; 590:4093–107
30. Frädorf J, Huhn R, Weber NC, Ebel D, Wingert N, Preckel B, Toma O, Schlack W, Hollmann MW: Sevoflurane-induced preconditioning: Impact of protocol and aprotinin administration on infarct size and endothelial nitric-oxide synthase phosphorylation in the rat heart *in vivo*. Anesthesiology 2010; 113:1289–98
31. Kehl F, Payne RS, Roewer N, Schurr A: Sevoflurane-induced preconditioning of rat brain *in vitro* and the role of KATP channels. Brain Res 2004; 1021:76–81
32. Yang Q, Dong H, Deng J, Wang Q, Ye R, Li X, Hu S, Dong H, Xiong L: Sevoflurane preconditioning induces neuroprotection through reactive oxygen species-mediated up-regulation of antioxidant enzymes in rats. Anesth Analg 2011; 112: 931–7
33. Hirota K, Roth SH: Sevoflurane modulates both GABAA and GABAB receptors in area CA1 of rat hippocampus. Br J Anaesth 1997; 78:60–5
34. Velly LJ, Canas PT, Guillet BA, Labrande CN, Masmejean FM, Nieoullon AL, Gouin FM, Bruder NJ, Pisano PS: Early anesthetic preconditioning in mixed cortical neuronal-glial cell cultures subjected to oxygen-glucose deprivation: The role of adenosine triphosphate dependent potassium channels and reactive oxygen species in sevoflurane-induced neuroprotection. Anesth Analg 2009; 108:955–63

35. Kass IS, Lipton P: Mechanisms involved in irreversible anoxic damage to the *in vitro* rat hippocampal slice. J Physiol (Lond) 1982; 332:459–72
36. Kass IS, Lipton P: Protection of hippocampal slices from young rats against anoxic transmission damage is due to better maintenance of ATP. J Physiol (Lond) 1989; 413:1–11
37. Wang T, Raley-Susman KM, Wang J, Chambers G, Cottrell JE, Kass IS: Thiopental attenuates hypoxic changes of electrophysiology, biochemistry, and morphology in rat hippocampal slice CA1 pyramidal cells. Stroke 1999; 30:2400–7
38. Bendo AA, Kass IS, Cottrell JE: Anesthetic protection against anoxic damage in the rat hippocampal slice. Brain Res 1987; 403:136–41
39. Sacktor TC: How does PKMζ maintain long-term memory? Nat Rev Neurosci 2011; 12:9–15
40. Ardehali H, O'Rourke B: Mitochondrial K(ATP) channels in cell survival and death. J Mol Cell Cardiol 2005; 39:7–16
41. Inoue I, Nagase H, Kishi K, Higuti T: ATP-sensitive K+ channel in the mitochondrial inner membrane. Nature 1991; 352:244–7
42. Zaugg M, Lucchinetti E, Spahn DR, Pasch T, Schaub MC: Volatile anesthetics mimic cardiac preconditioning by priming the activation of mitochondrial K(ATP) channels via multiple signaling pathways. Anesthesiology 2002; 97:4–14
43. Ye Z, Huang YM, Wang E, Zuo ZY, Guo QL: Sevoflurane-induced delayed neuroprotection involves mitoK(ATP) channel opening and PKC ε activation. Mol Biol Rep 2012; 39:5049–57
44. Adamczyk S, Robin E, Simerabet M, Kipnis E, Tavernier B, Vallet B, Bordet R, Lebuffe G: Sevoflurane pre- and post-conditioning protect the brain via the mitochondrial K ATP channel. Br J Anaesth 2010; 104:191–200