Anesthetic Protection of Neurons Injured by Hypothermia and Rewarming

Roles of Intracellular Ca\(^{2+}\) and Excitotoxicity

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ABSTRACT

Background: Mild hypothermia is neuroprotective after cerebral ischemia but surgery involving profound hypothermia (PH, temperature less than 18°C) is associated with neurologic complications. Rewarming (RW) from PH injures hippocampal neurons by glutamate excitotoxicity, N-methyl-D-aspartate receptors, and intracellular calcium. Because neurons are protected from hypoxia-ischemia by anesthetic agents that inhibit N-methyl-D-aspartic acid receptors, we tested whether anesthetics protect neurons from damage caused by PH/RW.

Methods: Organotypic cultures of rat hippocampus were used to model PH/RW injury, with hypothermia at 4°C followed by RW to 37°C and assessment of cell death 1 or 24 h later. Cell death and intracellular Ca\(^{2+}\) were assessed with fluorescent dye imaging and histology. Anesthetic agents were present in the culture media during PH and RW or only RW.

Results: Injury to hippocampal CA1, CA3, and dentate neurons after PH and RW involved cell swelling, cell rupture, and adenosine triphosphate (ATP) loss; this injury was similar for 4 through 10 h of PH. Isoflurane (1% and 2%), sevoflurane (3%) and xenon (60%) reduced cell loss but propofol (3 μM) and pentobarbital (100 μM) did not. Isoflurane protection involved reduction in N-methyl-D-aspartate receptor-mediated Ca\(^{2+}\) influx during RW but did not involve γ-amino butyric acid receptors or K\(_{ATP}\) channels. However, cell death increased over the next day.

Conclusion: Anesthetic protection of neurons rewarmed from 4°C involves suppression of N-methyl-D-aspartate receptor-mediated Ca\(^{2+}\) overload in neurons undergoing ATP loss and excitotoxicity. Unlike during hypoxia/ischemia, anesthetic agents acting predominantly on γ-aminobutyric acid receptors do not protect against PH/RW. The durability of anesthetic protection against cold injury may be limited.

CONTROLLED mild hypothermia (core temperature 32–34°C) improves neurologic outcomes after neonatal asphyxia\(^{1,2}\) and adult cardiac arrest.\(^3\) However, profound hypothermia (PH), defined here as temperatures less than 18°C, is associated with neurologic injury. Concerns about the deleterious effects of hypothermia date from the early days of cardiac surgery,\(^1–6\) with deeper levels of PH (less than 18°C) associated with frequent neurologic complications.\(^7,8\) The causes of neurologic injuries caused by hypothermia have been studied sparingly compared with hypoxic or ischemic injury.
Experimental studies examining the effects of PH on the central nervous system often have not separated injury caused by hypothermia with that caused by experimental ischemia or the cardiopulmonary bypass techniques. However, a number of laboratory studies suggest that PH/rewarming (RW) injures neurons separately from injury caused by cerebral blood flow insufficiency. For example, in dogs cooled to 12°C during normal blood flow cardiopulmonary bypass, DeLeon et al. documented extensive neurologic damage in the cerebrocortex. Similarly, Watanabe et al. found widespread and persistent neural injury, including substantial loss of hippocampal neurons, in dogs after normal flow cardiopulmonary bypass in which the dogs were merely cooled to 20°C and not subjected to any ischemic stress. Alam et al. found that PH, independent of cerebral ischemia or other factors, causes central nervous system injury in swine. Damage and loss of hippocampal neurons are prominent findings after PH with or without circulatory arrest, similar to hippocampal damage caused by global ischemia.

The mechanisms by which PH and/or RW injures neurons remain unclear and little studied. In a recent study, we found that an increase in intracellular Ca\(^{2+}\) is a key pathologic event in hippocampal neurons during PH and/or RW. We found that the increase in Ca\(^{2+}\) was solely due to N-methyl-D-aspartate receptors (NMDARs), with essentially no contribution from voltage gated Ca\(^{2+}\) channels, 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl)propanoic acid-type glutamate receptors, or metabotropic glutamate receptors. This is distinct from excitotoxicity after hypoxia or ischemia, in which multiple Ca\(^{2+}\) entry processes are involved and can be targets for experimental neuroprotection.

Anesthetic agents are neuroprotective in focal or global brain ischemia. Numerous mechanisms of anesthetic protection have been examined in experimental models of ischemia, including attenuation of glutamate excitotoxicity by block of NMDARs or reduction of glutamate release, opening of K\(_{ATP}\) channels, augmentation of \(\gamma\)-aminobutyric acid (GABA) receptor currents, and activation of neuroprotective intracellular signaling pathways and prosurvival gene expression. Because anesthetic agents such as isoflurane blunt increases in intracellular Ca\(^{2+}\) mediated by glutamate excitotoxicity during and after ischemic insults and hypothermia involves excitotoxicity, we hypothesized that isoflurane and other anesthetic agents would also protect against injury caused by PH/RW. Although desflurane protects the brain during deep hypothermic cardiac arrest, the presumption is that desflurane targets the ischemic and not the hypothermic component of the injury. There has not been, to our knowledge, any study of how anesthetic agents affect the survival of neurons during and after PH/RW. The purpose of this study was therefore to determine whether anesthetics improve the cold tolerance of neurons, independent of ischemia-like conditions, and by what mechanisms.

Materials and Methods

The studies were approved by the University of California San Francisco Committee on Animal Research and conform to relevant National Institutes of Health guidelines for the use of animals in research.

Preparation of Hippocampal Slice Cultures

Organotypic hippocampal slice cultures (HSCs) were prepared by standard methods. Eight- or 9-day-old Sprague-Dawley rats (Charles River Laboratories, Hollister, CA) were anesthetized with 3% isoflurane until they did not move in response to a vigorous tail pinch, and then were decapitated. Seven-day-old animals were not anesthetized before decapitation, per University of California San Francisco animal care guidelines. After decapitation, the hippocampi were quickly removed and placed in 4°C Gey balanced salt solution with 20 mM glucose. Further preparation and culture were as described by Bickler et al. Approximately 16 slices are harvested from each rat pup, and a total of approximately 250 pups were used in the entire study.

Study Design: Cold Injury in HSCs and Treatment with Anesthetic Agents

Slice cultures were exposed to hypothermia by placing them in a Billups-Rothenberg modular incubator chamber (Del Mar, CA) filled with humidified 95% air/5% carbon dioxide and placed in a 4 ± 1°C cold room for varying amounts of time. In a mock study, a thermocouple probe was placed in the culture media to measure temperature changes and it was found that the media cools to 4°C in approximately 1 h and that RW is complete in a similar period of time. Survival measurements and histology studies were done 1 h after completion of RW to 37°C. In some studies, cell death was measured 24 h after RW. To determine whether the rate of RW is related to damage caused by hypothermia, we also measured cell death after 6 h of PH followed by a 2.5-h RW period. For this RW profile, slices were first rewarmed to 25°C over the course of 1 h and then warmed to 37°C over the next 1.5 h, achieving an approximately linear rate of warming over the 2.5-h period.

Exposures to anesthetic agents and study drugs were handled as follows. For the anesthetic agents isoflurane and sevoflurane, slice culture trays were placed open in Billups-Rothenberg chambers through which the anesthetic agent was flowed via a calibrated vaporizer with the carrier gas (air/5% CO\(_2\)) for 5–8 min at 3 l/min flow to ensure that the desired anesthetic concentration was achieved within the chamber. Isoflurane was studied at 1% and 2% and sevoflurane at 3% and was measured with a calibrated clinical infrared anesthetic analyzer. Isoflurane concentration in slice culture media was also measured in several mock experiments by withdrawing media through a polyethylene tube into a glass syringe. Samples in the syringe were mixed with nitrogen to extract anesthetic vapor and the isoflurane concentration in the nitrogen bubble was measured with a gas chromatograph.
When xenon was studied, we mixed this gas with 5% CO2/air in 1 l precision spirometer calibration syringes and flushed through the chamber several times. Xenon concentration was not measured directly. Propofol (3 µM), pentobarbital (100 µM), or other study drugs (e.g., NMDAR antagonists and altered concentrations of K+) were present in the culture media 30 min before the start of the hypothermia.

Assessment of Cell Death in HSCs
Cell death was measured with propidium iodide (PI) or Sytox® fluorescence (Molecular Probes, Invitrogen, Eugene, OR), which provide essentially the same assessment of cell death. Each fluorescent dye penetrates damaged plasma membranes and binds to DNA. Confocal microscopy showed that PI and Sytox® both penetrate about 40 µm into the slice from both sides, labeling most dead neurons (slice cultures typically thin to approximately 100 µm). Sytox® (0.5 µM) was added to the wells of the culture trays 1 h after the slices were transferred to the 37°C incubator. After 15 min, the Sytox® was washed out and digital images of fluorescence were acquired. The Sytox® excitation light wavelength was 504 nm and the emission was 523 nm. PI (2.3 µM) was added to the wells of the culture trays 30 min after the slices were transferred to the 37°C incubator. After 30 min, the HSCs were rinsed in fresh media. The excitation light wavelength was 535 nm and emission was 620 nm for PI. For both Sytox® and PI, digital images were taken using a SPOT Jr. Digital Camera (Diagnostic Instruments, Sterling Heights, MI) and an inverted microscope. The fluorescence intensity of these dyes in slice cultures is a linear function of cell death25,26 and was analyzed in different regions of the cultures (CA1, CA3, and dentate) with Image J software by a blinded observer.

Histologic Analysis: Cresyl Violet and Fluorojade
HSCs for histologic examinations were grown on membrane “confetti” as described by Lacour et al.27 Briefly, slices were cultured on discs of permeable membrane (Millipore, Billerica, MA; FHLC01300) on top of the usual slice culture inserts for 3 days before study. After experiments, the cultures et al. were fixed in 4% paraformaldehyde in phosphate buffered saline for 1–2 h at 4°C. The cultures were then horizontally “resliced” as follows. Using a Z-axis controlled vibratome (Campden Instruments smz7000, Lafayette, IN), a flat surface was cut on a block of 3% agar. The confetti containing HSC was removed from the culture insert and glued to the flat agar bed with cyanoacrylate cement, providing an absolutely horizontal tissue for slicing. From the approximately 100-µm thick HSC, one 30-µm horizontal slice was obtained and mounted on a gelatin slide to dry. The dried and fixed slices were stained with cresyl violet to assess cell morphology or fluorojade to identify degenerating neurons. Confocal microscopy was used to image fluorojade-labeled neurons.

Measurement of Intracellular Ca2+
Intracellular Ca2+ in HSCs was measured with the fluorescent indicator calcium green 1-AM (Molecular Probes, Eugene, OR), because this dye loads into neurons in HSCs somewhat better than fura-2, which we have used previously. Cultures were loaded with 5–6 µM of the indicator during the 1-h RW period at 37°C. The cultures were rinsed and the fluorescence was quantified (excitation 488 nm, emission 520 nm) using an inverted microscope with the Spot Jr. camera. The background fluorescent signal from nonslice regions of the images was subtracted from the total fluorescent signal in the slice region. Fluorescence intensity was analyzed with Image J software.

ATP Measurements
The ENLITEN® Luciferase/Luciferin reagent (Promega, FF2021, San Diego, CA) and a luminometer was used to measure ATP in HSCs. Slices were removed from culture membranes in cold 5% trichloroacetic acid and frozen in liquid nitrogen to inactivate ATP-degrading enzymes and preserve ATP levels during storage. Before assay, the pH in the samples was neutralized using 100 mM Tris-acetate buffer. ENLITEN® reagent was added to samples and ATP reference standards and the resulting luminescence was measured with a MicroLumat Plus LB96V (EG&G Berthold Technologies, Bad Wildbad, Germany) luminometer. Because individual slice cultures are uniform in size and weight, [ATP] in experimentally treated slices was simply expressed relative to [ATP] in control slices.

Measurement of Glutamate Release from Cultures
Hippocampal slice cultures were grown for 7 days as described previously and randomly assigned to these experimental groups: (1) control; (2) 6 h PH/1 h RW; (3) 6 h PH/1 h RW with 2% isoflurane present during PH and RW; and (4) control plus 80 mM KCl to cause depolarization and full glutamate release. One ml of slice culture media was placed in each of the wells immediately before the experiment. The media was removed at each of the sampling points and snap frozen in an ethanol/dry ice bath before being analyzed for glutamate with a commercially available enzyme-linked immunosorbent assay kit (BA E-2300; Rocky Mountain Diagnostics, Colorado Springs, CO). Glutamate concentration in the culture media (very low; the media contained no added glutamate) was also measured and subtracted from that in the experiment groups.

Data Analysis
Neuron survival/injury experiments were designed to produce a normally distributed pattern of cell death as assayed by PI or Sytox® fluorescence. Therefore, analysis of variance (ANOVA) was used to compare the means of these data, and corrections were made for multiple comparisons with the Tukey-Kramer multiple comparison correction procedure; all statistical comparisons involve a two-tailed hypothesis of

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either an increase or decrease in a measured variable as a result of treatment. Differences were considered significant for $P < 0.05$. Other statistical comparisons involving multigroup design were also made with ANOVA and the Tukey-Kramer procedure. The GraphPad Prism software package was used (GraphPad, Inc., La Jolla, CA).

**Results**

**Effects of Anesthetic Agents on Hypothermia and RW Injury in Hippocampal Neurons**

Hippocampal slice cultures exposed to PH (4 ± 1°C) followed by 1 h RW to 37°C developed cell injury almost exclusively in the neuron cell body regions (fig. 1A). The duration of hypothermia was not significantly related to the amount of cell death (fig. 1B), suggesting that the RW period is the critical period in injury. Isoflurane (2%, approximately 1.3 minimum alveolar concentration), when present in the gas phase during hypothermia and RW, protected hippocampal neurons from cell death (one-way ANOVA with Tukey multiple comparison test, $P < 0.001$ for 4, 6, 8, and 10-h periods of hypothermia), with similar degrees of protection observed after the different durations of PH (fig. 1B). We also examined the effects of 1% isoflurane on cell death after PH/RW and found similar protection as with 2% iso-

![Fig. 1. Effects of inhaled and intravenous anesthetic agents on total death in neuron cell body regions (average of CA1, CA3, and dentate) in hippocampal slice cultures after profound hypothermia (4°C, PH) and rewarming (RW). (A) Images of propidium iodide (PI) fluorescence overlaid on bright-field images of cultures at baseline, after 6 h PH/1 h RW and after PH/RW with 2% isoflurane present. Red fluorescence indicates dead cells. (B) Cell death assessed with PI fluorescence, in hippocampal slice cultures following 4, 6, 8, or 10 h at 4°C and 1 h of RW to 37°C. There was 2% isoflurane in the gas phase present in the “Iso” groups during both PH and RW. (C) Isoflurane is protective when present throughout hypothermia and rewarming (“10 h PH/RW+Iso” group) or just during rewarming (“Iso only in RW” group). Data represent means ± SEM of PI fluorescence in CA1, CA3, and dentate regions. (D) Figure showing 3% sevoflurane (“Sevofl”) and 60% xenon protect hippocampal slice cultures from 6 h of PH (4°C) and RW. (E) Propofol (3 μM) and sodium pentobarbital (100 μM) present during 6 h of PH (4°C) and RW do not reduce cell death. (F) Isoflurane concentrations in slice culture media (in atmospheres, ATM) during a mock hypothermia and RW experiment. In this experiment only, 5% isoflurane was used in the gas phase of the Billups-Rothenberg modular incubator chamber. Samples of media were withdrawn at hourly intervals during hypothermia and after 1 h of RW. The chamber was flushed with air at 8 h. The number of cultures in each treatment group are indicated with numbers above the bars. The lines and asterisks above the error bars describe statistical differences between different treatment groups as follows: **$P$ less than 0.01, ***$P$ less than 0.001 and ****$P$ less than 0.00001 as measured with analysis of variance and a Tukey multiple comparison posttest.
flurane (data not shown). Isoflurane reduced PH/RW injury even if it was present only during the RW phase of the injury (fig. 1C, one-way ANOVA with Tukey test, \( P < 0.001 \)). The anesthetic agents sevoflurane (3%, approximately 1.3 minimum alveolar concentration) and xenon (60% of an atmosphere, approximately 0.4 minimum alveolar concentration for rats) were also protective against PH/RW injury (fig. 1D, \( P < 0.001 \), one-way ANOVA with Tukey test). Nitrogen substituted for air, as a control in the xenon experiments, did not increase the PH/RW injury, even though it diluted the carbon dioxide in the chamber to 2%. Experiments examining the effects of reducing the carbon dioxide in the atmosphere in the chamber during hypothermia showed that varying carbon dioxide level between 2% and 10% had no effect on hypothermia and RW injury (data not shown). We also examined propofol and pentobarbital and found that these agents, at concentrations commonly used in in vitro neuroprotection studies, had no effect on cold and RW injury (fig. 1E).

Histologic examination of resliced hippocampal cultures with cresyl violet after 6 h of PH (4°C) and a 1 h period of RW to 37°C revealed clear effects on the histologic appearance of neurons in the cell body regions. Rupture and loss of neurons was observed in cultures fixed immediately after RW (compare fig. 2A and B). Isoflurane prevented apparent cell loss in the cultures (fig. 2C). Acute neurodegeneration caused by NMDA application (fig. 2D) caused cell disruption and nuclear condensation similar in histologic appearance to cold and RW. In cultures fixed 24 h after RW, greater numbers of condensed nuclei were seen, although total cell death, based on histologic appearance, did not appear to be much greater at 24 h after RW compared with 1 h after RW (fig. 2E, compare to fig. 2B). Isoflurane also improved the histologic appearance of cultures examined 24 h after RW (fig. 2F). These findings are consistent with the central role of NMDAR-mediated excitotoxicity in PH/RW injury and observations that NMDAR antagonists uniquely prevent PH/RW injury.

Fluorojade, a neuron-specific dye method for assessing cell death, was also used in the PH/RW studies. We examined the clearest cell body region in each culture, which was typically the CA1 or CA3 region. As with PI, fluorojade staining revealed that both isoflurane and sevoflurane reduced neuron injury caused by hypothermia and RW (fig. 3A). Regional analysis of cell death in the cultures demonstrated isoflurane protection in all the neuronal areas (fig. 3B, one-way ANOVA with Tukey multiple comparison test).

In experimental models of neural injury after PH and cardiopulmonary bypass, the rate of RW may influence the degree of neurologic damage. Therefore, we compared our standard RW protocol (RW from 4°C to 37°C in approximately 1 h) with a slower RW protocol (4°C to 37°C over 2.5 h). We found that slower RW had no effect on neuronal death (fig. 4).

### Isoflurane and ATP during PH

ATP levels and cell death in HSCs after 6 h at 4°C followed by RW to 37°C are shown in figure 5. ATP measured in cultures at the end of 8 h at 4°C (frozen for ATP analysis before RW) was reduced by more than 80% compared with

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**Footnotes:**

1. Bickler et al.

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**Fig. 2.** Cresyl violet-stained resliced sections of hippocampal slice cultures, showing the CA1 or CA1-CA3 border region. Horizontal bar is 20 μm. (A) Control or baseline conditions. (B) Fixation after 1 h of rewarming (RW) to 37°C after 6 h at 4°C (profound hypothermia, PH). (C) Same as B but with isoflurane present through the hypothermia and RW. (D) Fixation 1 h after addition of N-methyl-D-aspartate cocktail (100 μM N-methyl-D-aspartate, Mg²⁺ -free media). (E) Fixation 1 h after addition of N-methyl-D-aspartate cocktail (100 μM N-methyl-D-aspartate, Mg²⁺ -free media). (F) Fixation 24 h after rewarming in a culture exposed to isoflurane during PH and the first hour of RW.
control cultures. ATP measured immediately after RW rebounded moderately from this nadir (one-way ANOVA with Tukey test, \( P < 0.01 \)), but remained low. Isoflurane present during hypothermia reduced ATP loss significantly only during the period of hypothermia (\( P < 0.05 \)) and did not preserve ATP after RW compared to the non-anesthetic treated groups. The moderately higher levels of ATP during hypothermia were apparently critical to cell survival because cell death in “sister” cultures from the same experiments in which the ATP measurements were made indicated with numbers above the bars. The lines and asterisks above the error bars describe statistical differences between different treatment groups as follows: * \( P < 0.05 \), ** \( P < 0.01 \), and *** \( P < 0.001 \), measured with analysis of variance and a Tukey multiple comparison posttest.

**Is Isoflurane Protection Durable?**

The durability of volatile anesthetic neuroprotection was a major concern in previous studies of neuroprotection after global or regional hypoxia or ischemia. Indeed, from figure 3A, it appears that both isoflurane and sevoflurane improve survival measured within 1 h after RW, but not 24 h later. We thought that this might be due to continuing excitotoxicity during the 24 h after RW. To test this, we exposed cultures to 1% isoflurane during PH/RW and for 24 h after RW. Figure 6 confirms that isoflurane protection from PH/RW injury fades significantly when assessed at 24 h after RW (protection significantly less in CA3 and dentate at 24 h, ANOVA with Tukey test, \( P < 0.001 \) and \( P < 0.05 \), respectively), and in addition shows that the continuous presence of 1% isoflurane during the 24-h period after RW was of no benefit in protecting neurons (\( P > 0.05 \) for all comparisons, one-way ANOVA with Tukey multiple comparison test). This pattern was seen in CA1, CA3, and to a lesser degree, in dentate neurons.

**NMDA Receptors and \( Ca^{2+} \) Are Involved in Isoflurane Protection against Cold and RW Injury, but Not GABA Receptors**

PH/RW injury is caused by glutamate excitotoxicity involving NMDA receptors. Because isoflurane attenuates NMDAR currents and NMDA-based excitotoxicity both

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**Fig. 3.** Isoflurane protection from profound hypothermia/rewarming (PH/RW) injury is neuron specific. (A) Fluorojade staining intensity of neurons in the CA1-CA3 rich regions in hippocampal slice cultures following hypothermia and rewarming with isoflurane (ISO). Images at right were obtained with confocal microscopy of fixed and resliced cultures. Bright fluorescence identifies dead cells. (B) Region-specific cell death (propidium iodide, PI, fluorescence) in hippocampal slice cultures in which PH was present for 4, 6, or 8 h followed by RW to 37°C for 1 h. DG = dentate gyrus. Bars represent means ± SEM. The number of cultures in each treatment group is indicated with numbers above the bars. The lines and asterisks above the error bars describe statistical differences between different treatment groups as follows: * \( P < 0.05 \), ** \( P < 0.01 \), and *** \( P < 0.001 \), measured with analysis of variance and a Tukey multiple comparison posttest.
in the context of hypoxia/ischemia and exogenously applied neurotoxic concentrations of glutamate or NMDA, we reasoned that isoflurane protection of neurons exposed to PH/RW would also involve this mechanism. We found that the selective NMDA receptor antagonist AP5 reduces PH/RW injury and prevents Ca$^{2+}$ entry (fig. 7 A-C). Isoflurane follows this pattern as well, suggesting that isoflurane is acting to antagonize NMDAR-mediated neuronal death in PH/RW injury.

Isoflurane’s protective effect in hypoxic-ischemic neuronal injury is partly mediated by GABAA receptor activation or augmentation. GABAA receptors are probably not related to protection against PH/RW injury because isoflurane remained protective when the GABAA antagonists bicuculline and picrotoxin were present in concentrations sufficient to block all GABAA receptor activity (fig. 7D, one-way ANOVA with Tukey test, $P < 0.05$ compared with the PH/RW group).

To further define the role of isoflurane in modulating glutamate excitotoxicity in PH/RW injury, we used media with 30 mM KCl during hypothermia to block glutamate release during the subsequent period of RW. This approach was based on the work of Hogins et al., who showed that 30 mM K$^+$ conferred a preconditioning effect against a stress of oxygen/glucose deprivation that was clearly related to presynaptic silencing of glutamate release. Thirty mM KCl was markedly protective against PH/RW injury, consistent with the importance of glutamate excitotoxicity in PH/RW injury (fig. 8, $P < 0.01$ one-way ANOVA with Tukey multiple comparison test). Isoflurane combined with 30 mM KCl produced additional protection, as did the NMDAR antagonist AP5. Because 30 mM K$^+$ depolarizes and clamps hippocampal neurons to approximately $13$ mV, membrane potential per se must not be the parameter that is responsible for isoflurane protection in PH/RW injury. We also investigated whether isoflurane decreases the release of glutamate during PH/RW injury. We found that isoflurane significantly reduced the release of glutamate into the culture media during the period of hypothermia (fig. 9), from a mean of approximately 0.35 μg glutamate per slice to 0.16 μg per slice ($P < 0.05$, one-way ANOVA with Tukey multiple comparison test).

K$_{ATP}$ Channels Are Not Involved in Isoflurane Protection against Cold and RW Injury

ATP-sensitive K channels are proposed to mediate part of isoflurane’s protection of hypoxic or ischemic myocardium and brain tissue. To determine whether this mechanism accounts for isoflurane protection after PH/RW, slice cultures were preincubated in the K$_{ATP}$ channel blocker glibenclamide for 30 min before cooling them to 4°C for 6 h. Glibenclamide did not prevent isoflurane from protecting neurons when they were rewarmed from this period of hypothermia (fig. 10, ANOVA with Tukey multiple comparison test, $P > 0.05$).

Discussion

Deep hypothermia is currently used to facilitate complex surgical procedures, including those that involve cardiac arrest. Such procedures are frequently complicated by adverse neurologic outcomes, but it is unclear whether ischemia, hypothermia, or both cause the injury. The results of this study, and another from our laboratory, show that hypothermia (4°C) and RW damages rat hippocampal neurons. The experimental conditions for both these studies are such that we can exclude ischemia-like damage from the injury to the neurons. Studies in dogs suggest that PF, independent of other factors, causes neuronal injury. The rate of RW from hypothermia may influence the severity of injury.
caused by hypothermia or ischemia during the period of hypothermia, which is an important issue that is debated in the clinical use of hypothermia for cardiopulmonary bypass. However, when we slowed the RW period after hypothermia from 1.5 h to 2.5 h, no difference in neuron death was seen (fig. 4). Whether RW rate matters for less severe hypothermia and RW injury is currently under investigation in our laboratory.

We found that the volatile anesthetic agents isoflurane, sevoflurane, and xenon, but not the intravenous anesthetic agents propofol and pentobarbital, protect neurons from the injury caused by PF (4°C) and RW. The mechanism of isoflurane protection appears to involve limiting NMDA receptor-dependent Ca2+ overload that may be related to suppressing the release of glutamate caused by the stress of hypothermia and RW or by antagonizing NMDA receptors. Isoflurane also reduced the loss of ATP during hypothermia itself, but not during the entire hypothermia and RW period. We excluded several other targets that were thought to be involved in isoflurane protection; including ATP-sensitive K+ channels and GABA receptors. Because protection from cold injury was achieved when isoflurane was present only during RW, it appears that the predominant target for isoflurane is NMDA receptors during the RW phase when energy depletion is severe and the potential for excitotoxicity may be greatest.

A major finding was that isoflurane protection of neurons injured by PH/RW was not completely durable; that is, we observed protection after 1 h of RW, but 24 h later, the injury increased. This decrease in protection was also seen when the isoflurane was continued during the entire 24-h postrewarming period. There are several possibilities for these observations. The first is that PH/RW injury continues to evolve long after completion of RW, even though injury measured by PI and Sytox does not suggest this. The second is that isoflurane is relatively weak in preventing PH/RW injury and that excitotoxicity still kills many neurons if excitotoxicity persists for long enough of time. This possibility could be tested in experiments in which NMDA receptor antagonists remain in the culture media after RW, although NMDA antagonist toxicity was a problem when this was attempted.

Blocking NMDA receptors was effective in preventing PH/RW injury in hippocampal neurons (figs. 6 and 7), as was reducing extracellular [Ca2+] with the chelating agent EGTA. Similar to Ca2+-related neuron injury after hyp-
oxia or ischemia, hypothermia-RW injury involves glutamatergic excitotoxicity, where uncontrolled Ca\(^{2+}\) influx through NMDA receptors is caused by release of glutamate from depolarizing neurons. In contrast with hypoxic-ischemic injury, T-type Ca\(^{2+}\) channels and L-type Ca\(^{2+}\) channels and the reverse mode Na\(^{+}\)/Ca\(^{2+}\) exchanger are not involved in PH/RW injury.\(^1\)\(^2\) Volatile anesthetics are known to modulate excitotoxicity by several processes, including augmentation of glutamate reuptake transporters, inhibition of voltage-gated Ca\(^{2+}\) channels and activation of K\(_{ATP}\) channels, and suppression of glutamate release. Although we only examined the mechanisms of protection in the case of isoflurane, it is reasonable to suggest that similar processes apply in the case of sevoflurane and xenon. Neither propofol nor pentobarbital have significant effects on NMDA receptors and this is probably why they were ineffective in preventing PH/RW injury.

We observed that increasing extracellular K\(^+\) to 30 mM protects neurons from cold injury (fig. 7). This effect is observed in a variety of preparations, including brain slices and dissociated neurons\(^3\)\(^8\)\(^9\) and is most likely due to suppression of presynaptic glutamate release.\(^3\)\(^3\) These findings support our belief that glutamate neurotransmission is important to the excitotoxicity involved in PH/RW injury. As seen in figure 9, PH/RW involves glutamate release from the cultures. At much higher K\(^+\) concentrations (80–120 mM), neurons are killed by Ca\(^{2+}\) accumulation from activation of voltage-dependent Ca\(^{2+}\) channels.\(^3\)\(^9\) The data in figure 8 also make it unlikely that maintenance of a range of membrane potential is the key to surviving PH/RW; based on previous patch-clamp studies of isolated hippocampal neurons we showed that 30 mM K\(^+\) drives the membrane potential to approximately \(-13\) mV. Thirty mM K\(^+\) was markedly neuroprotective, making it unlikely that maintenance of normal or hyperpolarized membrane potential is uniquely necessary for cold survival. Furthermore, because isoflurane added to the neuroprotection afforded by 30 mM K\(^+\), it is unlikely that K channels (i.e., background or tandem-pore K channels) exclusively confer isoflurane’s protection against PH/RW injury because the membrane potential of neurons is effectively clamped to depolarized potentials by the high extracellular K\(^+\) concentration.

The observation that isoflurane was additive in protection to that conferred by 30 mM K\(^+\) (fig. 7) suggests that isoflurane’s effects on glutamate excitotoxicity are mediated by inhibition postsynaptic NMDARs rather than only by suppression of presynaptic glutamate release.\(^3\)\(^3\) This is because 30 mM K\(^+\) drives the membrane potential to approximately \(-13\) mV. Thirty mM K\(^+\) was markedly neuroprotective, making it unlikely that maintenance of normal or hyperpolarized membrane potential is uniquely necessary for cold survival. Furthermore, because isoflurane added to the neuroprotection afforded by 30 mM K\(^+\), it is unlikely that K channels (i.e., background or tandem-pore K channels) exclusively confer isoflurane’s protection against PH/RW injury because the membrane potential of neurons is effectively clamped to depolarized potentials by the high extracellular K\(^+\) concentration.

The observation that isoflurane was additive in protection to that conferred by 30 mM K\(^+\) (fig. 7) suggests that isoflurane’s effects on glutamate excitotoxicity are mediated by inhibition postsynaptic NMDARs rather than only by suppression of glutamate release. This is because 30 mM K\(^+\) acts to mute synaptic release of glutamate\(^3\)\(^3\); the fact that isoflurane produces additional protection in the presence of the high K\(^+\) means that it must have a separate protective effect. However, isoflurane did reduce the release of glutamate during hypothermia and RW. Taken together, we suggest that these data mean that isoflurane probably has both a presynaptic and postsynaptic effect on limiting glutamate excitotoxicity during PH/RW injury.

**Fig. 6.** Isoflurane protection of neurons in CA1, CA3, and dentate regions of slice cultures after profound hypothermia/re-warming (PH/RW) is time dependent. (A–C) Cell death was nominally measured 1 h after completion of RW from 6 h of PH. The PH/RW@24 h and PH/RW+IsoX24 h groups had cell death assessed 24 h after the RW. The PH/RW+IsoX24 h group had 1% isoflurane present during PH, RW, and during the 24-h period after RW. The IsoX24 h group was in 1% isoflurane for 24 h only. Note that isoflurane protection seen at 1 h after RW fades by 24 h, whether isoflurane (Iso) is present for the subsequent 24 h or not. The number of cultures in each treatment group is indicated with numbers above the bars. The lines and asterisks above the error bars describe statistical differences between different treatment groups as follows: *P less than 0.05, **P less than 0.01, and ***P less than 0.001, measured with analysis of variance and a Tukey multiple comparison posttest.
Study Limitations

The inference that isoflurane’s main target in protecting neurons from PH/RW injury is the NMDA receptor is limited by the fact that we did not directly measure inhibition of NMDARs in hypothermic or RW neurons. This was attempted, but was defeated by the difficulty of patch-clamping neurons in RW cultures (swelling makes the neurons rupture easily). The conclusion that isoflurane inhibits the NMDARs and that NMDARs are related to PH/RW injury and calcium overload is based on solid evidence, however. Isoflurane also decreases the release of glutamate, an effect that also would act to limit NMDAR-mediated Ca\(^{2+}\)/H\(^{1+}\) overload during PH/RW injury.

With the exception of isoflurane, we studied only single concentrations of each anesthetic agent and although the concentrations were similar to those used clinically, we cannot exclude the possibility that the protective effects of these compounds are dose-dependent. Another issue relates to anesthetic potency at different temperatures. What is the relevance of minimum alveolar concentration or anesthetic concentration at temperatures less than 28°C where low temperature itself produces immobility? It is important to consider that, when volatile anesthetic agents are used in patients with hypothermia, no specific temperature correction is used to adjust the delivered concentration. Further, we believe that the protective effect of isoflurane or the other anesthetic agents is exerted during the RW period when excitotoxicity must peak. This does not explain why isoflurane reduces ATP loss during the period of hypothermia but not during rewarming. It is possible that isoflurane decreases ATPase activity in hypothermic but not rewarming neurons.

Innate differences in the hypothermia tolerances of different species of animals means that extrapolation of our

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**Fig. 7.** Isoflurane protection of hippocampal neurons after profound hypothermia/rewarming (PH/RW) involves N-methyl-D-aspartate (NMDA) receptors but not γ-aminobutyric acid (GABA) receptors. (A) Isoflurane (Iso) and the competitive NMDA receptor antagonist AP5 both reduce PH/RW injury in slice cultures. (B) Isoflurane and AP5 prevent increases in intracellular Ca\(^{2+}\) in cultures exposed to 4 h PH and 1 h RW. (C) Typical images of calcium green fluorescence in slice cultures at baseline (37°C), after PH, after PH/RW with the competitive NMDA receptor antagonist AP5, and after PH/RW when isoflurane was present. (D) Lack of involvement of GABA\(_x\) receptors in isoflurane protection against PH and RW injury. The GABA\(_x\) antagonists bicuculline (Bicuc, 50 μM) and picrotoxin (Picro, 100 μM) were present during PH and RW. Bar graphs show means ± SEM. The number of cultures in each treatment group is indicated with numbers above the bars. The lines and asterisks above the error bars describe statistical differences between different treatment groups as follows: *P < 0.05, **P < 0.01, and ***P < 0.001, measured with analysis of variance and a Tukey multiple comparison posttest.
results to other species of animals is difficult. Rats are probably more hypothermia tolerant than humans and 7-day-old rats are extremely tolerant of hypothermia compared with adult rats. It is therefore likely that the neuronal damage after exposure of HSCs to 4°C would occur at higher temperatures or with shorter durations of hypothermia in less hypothermia tolerant species, including humans.

**Conclusion**

We conclude that the most important neuroprotective target for volatile anesthetics in neurons undergoing PH/RW is NMDA receptor-mediated Ca\(^{2+}\) influx caused by glutamate excitotoxicity, which may be mediated both by reduced glutamate concentration in the media during profound hypothermia/rewarming (PH/RW). Glutamate was assayed in control cultures and after 6 h at 4°C followed by 1 h of rewarming (PH/RW), and with 2% Iso in the gas phase during the entire PH/RW period. The KCl group was treated with 80 mM KCl at 37°C to cause maximal glutamate release. The number of cultures in each treatment group is indicated with a number above the bars. The lines and asterisks above the error bars describe statistical differences between different treatment groups as follows: **P** less than 0.01, measured with analysis of variance and a Tukey multiple comparison posttest. D-AP5 = D-2-amino-5-phosphonovaleric acid; MK-801 = Merck compound 801, dizocilpine, an NMDA receptor antagonist.

**Potassium-adenosine triphosphate channels are not involved in isoflurane protection against profound hypothermia and rewarming (PH/RW) injury in hippocampal slice cultures.** Glibenclamide (100 µM, Glib) or isoflurane (2%, Isof) was present during the entire PH and RW period in the PH/RW+Isof, PH/RW+glib, and PH/RW+Glib+Iso groups. Data represent means ± SEM. The number of cultures in each treatment group is indicated with numbers above the bars. PI = propidium iodide. The lines and asterisks above the error bars describe statistical differences between different treatment groups as follows: **P** less than 0.01 and ***P** less than 0.001, measured with analysis of variance and a Tukey multiple comparison posttest.

**Fig. 8.** Effects of 30 mM K\(^+\), isoflurane (Iso) or N-methyl-D-aspartate (NMDA) antagonists on neuron death after profound hypothermia/rewarming (PH/RW). (A) There was 30 mM K\(^+\) added to the media before hypothermia to prevent glutamate release at later times (i.e., during RW); 30 mM K\(^+\) will clamp the membrane potential to approximately −13 mV. Sytox\(^\text{®}\) fluorescence (Molecular Probes, Invitrogen, Eugene, OR) values are means ± SEM. (B) Typical images of Sytox\(^\text{®}\) fluorescence overlaid on the corresponding bright-field images of cultures from several treatment groups. The number of cultures in each treatment group is indicated with numbers above the bars. The lines and asterisks above the error bars describe statistical differences between different treatment groups as follows: **P** less than 0.01, measured with analysis of variance and a Tukey multiple comparison posttest.

**Fig. 9.** Isoflurane (Iso) decreases glutamate concentration in the media during profound hypothermia (PH) and rewarming (RW). Glutamate was assayed in control cultures and after 6 h at 4°C followed by 1 h of rewarming (PH/RW), and with 2% Iso in the gas phase during the entire PH/RW period. The KCl group was treated with 80 mM KCl at 37°C to cause maximal glutamate release. The number of cultures in each treatment group is indicated with a number above the bars. The lines and asterisks above the error bars describe statistical differences between different treatment groups (P less than 0.05), measured with analysis of variance and a Tukey multiple comparison posttest.

**Fig. 10.** Potassium-adenosine triphosphate channels are not involved in isoflurane protection against profound hypothermia and rewarming (PH/RW) injury in hippocampal slice cultures. Glibenclamide (100 µM, Glib) or isoflurane (2%, Isof) was present during the entire PH and RW period in the PH/RW+Isof, PH/RW+glib, and PH/RW+Glib+Iso groups. Data represent means ± SEM. The number of cultures in each treatment group is indicated with numbers above the bars. PI = propidium iodide. The lines and asterisks above the error bars describe statistical differences between different treatment groups as follows: **P** less than 0.01 and ***P** less than 0.001, measured with analysis of variance and a Tukey multiple comparison posttest.
Weigl M, Tenze G, Steinlechner B, Skhirtladze K, Reining G, measuring isoflurane concentration in media samples. The authors thank Ted Eger, M.D., Professor of Anesthesia, University of California, San Francisco, San Francisco, California, for help measuring isoflurane concentration in media samples.

References

15. Eilers H, Bickler PE: Hypothermia and isoflurane similarly inhibit glutamate release evoked by chemical anoxia in rat cortical brain slices. ANESTHESIOLOGY 1996; 85:600–7

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A Towering View of Charles T. Jackson’s Grave

Washington Tower (left) occupies the highest point within America’s premier landscaped cemetery, Mount Auburn in Cambridge, Massachusetts. As visitors climb the tower, they can peek over its side and spot a nearby pair of headstones (center) for Dr. Charles T. Jackson (1805–1880) and his wife, Susan Bridge Jackson (1816–1899). Recognized as the geologist-chemist-physician who suggested that W. T. G. Morton try sulphuric ether for anesthesia, Dr. Jackson suffered a cerebrovascular accident and was terminally nursed by the insane asylum to which he had provided medical consultations. Slightly shadowed by his wife’s marker (right), Charles Jackson’s tombstone reads, “Through his observations of the peculiar effects of sulphuric ether on the nerves of sensation, and his bold deduction therefrom, the benign discovery of painless surgery was made.” (Copyright © the American Society of Anesthesiologists, Inc.)

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