

Isoflurane Prevents Delayed Cell Death in an Organotypic Slice Culture Model of Cerebral Ischemia

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Background: General anesthetics reduce neuronal death caused by focal cerebral ischemia in rodents and by *in vitro* ischemia in cultured neurons and brain slices. However, in intact animals, the protective effect may enhance neuronal survival for only several days after an ischemic injury, possibly because anesthetics prevent acute but not delayed cell death. To further understand the mechanisms and limitations of volatile anesthetic neuroprotection, the authors developed a rat hippocampal slice culture model of cerebral ischemia that permits assessment of death and survival of neurons for at least 2 weeks after simulated ischemia.

Methods: Survival of CA1, CA3, and dentate gyrus neurons in cultured hippocampal slices (organotypic slice culture) was examined 2–14 days after 45 min of combined oxygen–glucose deprivation at 37°C (OGD). Delayed cell death was serially measured in each slice by quantifying the binding of propidium iodide to DNA with fluorescence microscopy.

Results: Neuronal death was greatest in the CA1 region, with maximal death occurring 3–5 days after OGD. In CA1, cell death was $80 \pm 18\%$ (mean \pm SD) 3 days after OGD and was 80–100% after 1 week. Death of $70 \pm 16\%$ of CA3 neurons and $48 \pm 28\%$ of dentate gyrus neurons occurred by the third day after OGD. Both isoflurane (1%) and the *N*-methyl-D-aspartate antagonist MK-801 (10 μ M) reduced cell death to levels similar to controls (no OGD) for 14 days after the injury. Isoflurane also reduced cell death in CA1 and CA3 caused by application of 100 but not 500 μ M glutamate. Cellular viability (calcein fluorescence) and morphology were preserved in isoflurane-protected neurons.

Conclusions: In an *in vitro* model of simulated ischemia, 1% isoflurane is of similar potency to 10 μ M MK-801 in preventing delayed cell death. Modulation of glutamate excitotoxicity may contribute to the protective mechanism.

THE neuroprotective qualities of isoflurane remain controversial. In several *in vivo* studies involving rodents, isoflurane was found to reduce infarct volume after cerebral ischemia.^{1,2} However, a recent study reported that isoflurane's protection may be short-lived: 2 weeks after temporary occlusion of the middle cerebral artery in rats, cortical infarction volume was not different between isoflurane and control animals.³ It was suggested

that isoflurane may prevent acute (necrotic) but not delayed (apoptotic) cell death. Earlier studies, limited to examination of cell injury after less than a week of recovery from ischemia, may thus have underestimated the final extent of injury (reviewed by Warner⁴). The neuroprotective qualities of isoflurane have also been tested in *in vitro* models of cerebral ischemia. Isoflurane protects brain slices from acute injury and death, an effect possibly related to isoflurane's action as an *N*-methyl-D-aspartate (NMDA) receptor antagonist.^{5,6}

In vitro models of ischemia have some advantages over *in vivo* models, including the ability to control or eliminate such variables as blood flow, temperature, ionic environment, nutrient availability, and the ability to serially assess injury or injury surrogates, such as increases in intracellular calcium concentration. However, all the *in vitro* models used for examining anesthetic neuroprotection have limitations that may restrict their ability to predict neuroprotective efficacy in intact animals. For example, in dissociated cell cultures, the anatomic relations of neurons, glia, and synapses are lost and sensitivity to anoxia or ischemia is much reduced compared with intact animals. In acutely prepared and studied brain slices, anatomic integrity is preserved, but delayed cell loss, an important feature of ischemic injury,⁷ cannot be assessed. Accordingly, we developed an organotypic slice culture model of cerebral ischemia that retains cellular integrity and sensitivity to anoxia and allows serial measurement of cell survival over many days. Using this model, we asked whether isoflurane prevents delayed death of ischemia-sensitive hippocampal neurons after simulated ischemia, whether the protection is comparable with an NMDA receptor antagonist, and whether suppression of glutamate excitotoxicity contributes to protection.

Methods

Preparation of Cultured Brain Slices

All studies were approved by the University of California San Francisco Committee on Animal Research and conform to relevant National Institutes of Health guidelines.

Organotypic cultures of the hippocampus were made as described by Stoppini *et al.*⁸ and Laake *et al.*⁹ with several modifications. Sprague Dawley Rats (6–28 days old; Simonsen Laboratories, Gilroy, CA) were anesthetized with halothane (2%) for 2–3 min or until quiescent. The rats were then given an intraperitoneal injection of ketamine (10 mg/kg) and diazepam (0.2 mg/kg) and

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returned to halothane administration until they did not respond to a toe pinch. The rats were decapitated, and the hippocampi were removed and placed in 4°C Gey's Balanced Salt Solution (University of California San Francisco Cell Culture Facility). Next, the hippocampi were transversely sliced (400- μ m thick) with a tissue slicer (Stoelting, Wood Dale, IL) and stored in Gey's Balanced Salt Solution containing 0.038 mg/ml ketamine at 4°C for 1 h.¹⁰ The slices were then transferred onto 30-mm-diameter membrane inserts (Millicell-CM; Millipore, Bedford, MA) and put into six-well culture trays with 1.5 ml slice culture medium per well. The slice culture medium consisted of 50% Minimal Essential Medium (Eagles with Earles balanced salt solution; University of California San Francisco Cell Culture Facility), 25% Earles balanced salt solution (University of California San Francisco Cell Culture Facility), and 25% heat-inactivated horse serum (Hyclone Laboratories, South San Francisco, CA) with 6.5 mg/ml glucose and 5 mM KCl. During the first several days *in vitro*, the medium contained 100 μ M adenosine. The culture trays were stored at 37°C in a 5% CO₂-enriched-atmosphere, 100%-humidity incubator. The slice culture medium was changed twice a week. No antibiotics were used. Slices were kept in culture for 7–14 days before study.

Assessment of Cultured Slice Viability

The viability of the cultures was assessed in several ways. Field potentials at CA1 neuron cell bodies were elicited as described by Taylor *et al.*¹¹ The presence of field potentials after 7 days in culture indicated the health of the cells and the persistence of synaptic connections in the slices. Propidium iodide exclusion was used to establish that a high percentage of neurons in the CA1, CA3, and dentate cell fields were living. In addition, slices were exposed to 2 μ M calcein-AM (Molecular Probes, Eugene, OR), a vital dye that is cleaved by intracellular esterases to the fluorescent compound calcein (excitation 390 nm, emission 420 nm), to further show that neurons were intact. Finally, cultures were fixed in paraformaldehyde, dehydrated, embedded in paraffin, sectioned and stained with hematoxylin-eosin, and examined microscopically to assess cell morphology.

Simulation of Ischemia with in vitro Oxygen-Glucose Deprivation and Assessment of Cell Death

In vitro ischemia was simulated by anoxia combined with glucose-free media (oxygen-glucose deprivation [OGD]). Before hypoxia, the slices were washed three times with glucose-free Hank's balanced salt solution. The cultures were then placed into a 2-l air-tight Billups-Rothenberg Modular Incubator chamber (Del Mar, CA) through which 95% N₂-5% CO₂ gas, preheated to 37°C, was passed at 5–10 l/min. The temperature of the chamber was kept at 37°C by both passing preheated gas

through the chamber and by placing a heat lamp over the chamber. The temperature inside the chamber was monitored with a thermocouple thermometer. After 10 min of gas flow, the chamber was sealed and placed in a 37°C incubator. The partial pressure of oxygen was less than 0.2 mmHg, measured with a Clark-type oxygen electrode. For studies involving isoflurane, a calibrated isoflurane vaporizer was used to deliver 1% isoflurane in the anoxic gas. The isoflurane remained in the chamber during the injury. A Datex OSCAR multigas analyzer (Helsinki, Finland) was used to measure isoflurane concentration in the inflow gas. For MK-801 treatment, slices were rinsed with glucose-free Hank's balanced salt solution containing 10 μ M MK-801; the MK-801 remained for the duration of the 45-min injury. The 10- μ M concentration of MK-801 was chosen based on previous studies involving different MK-801 concentrations in brain slices.⁵ After the injury, the culture tray was removed from the chamber, the anoxic glucose-free Hank's balanced salt solution was aspirated from the wells, and standard (oxygenated) slice culture media was added.

In another treatment group containing 46 slices, the effect of 1% isoflurane on glutamate-induced delayed cell death was evaluated. Slice cultures were exposed to 100 or 500 μ M L-glutamate with or without 1% isoflurane for 30 min at 37°C. Cell survival was evaluated at 2, 3, and 7 days after injury.

Assessment of Cell Death

Cell viability was assessed with propidium iodide fluorescence (Molecular Probes). Propidium iodide (PI), a highly polar fluorescent dye, penetrates damaged plasma membranes and binds to DNA. Before imaging, slice culture media containing 2.3 μ M PI was added to the wells of the culture trays. After 15 min, the slices were examined with a Nikon Diaphot 200 inverted microscope (Nikon Corp., Tokyo, Japan), and fluorescent digital images were taken using a SPOT Jr. Digital Camera (Diagnostic Instruments Inc., Sterling Heights, MI). Excitation light wave length was 490 nm, and emission was 590 nm. The sensitivity of the camera and intensity of the excitation light was standardized so as to be identical from day to day. PI fluorescence was measured in the dentate gyrus, CA1, and CA3 regions of the hippocampal slices. Slices were discarded if they showed more than slight PI fluorescence in these regions after 7–10 days in culture. Slices were imaged before OGD (signal assumed to represent 0% cell death) and after 2–7 days (group A) or 2–14 days following OGD. In preliminary studies, we observed that maximum post-OGD death occurred at approximately day 3, was similar at day 7, and did not increase further by days 10–14. Serial measurements of PI fluorescence intensity were made in a predefined area (manually outlining CA1, CA3, and dentate separately) for each slice using NIH Image software (developed at

the US National Institutes of Health, Bethesda, MD, and available on the Internet[#]). Thus, cell death was followed in the same regions of each slice after simulated ischemia. After the measurement of PI fluorescence on the 7th or 14th post-OGD day, all the neurons in the slice were killed to produce a fluorescence signal equal to 100% neuron death in the regions of interest. This was done by adding 100 μM KCN and 2 μM iodoacetate to the cultures for at least 20 min. One day later, final images of PI fluorescence (equated to 100% cell death) were acquired. Percentage of dead cells at 2–14 days after ischemia were then calculated based on these values. A linear relation exists between cell death and PI fluorescence intensity.^{9,10}

Study Design

The effect of isoflurane on post-OGD cell death was evaluated in two groups of cultured slices. In the first group (group A), cell death was followed for 1 week at time points of 0, 2, 3, and 7 days after OGD. The 36 slices comprising group A were obtained from eight rats (mean age at sacrifice, 16 days), with slices randomly assigned to the various treatment groups. These slices were maintained in culture 11–14 days before OGD. In the second group (group B), cell death was measured at 0, 2, 7–10, and 14–16 days after OGD. Group B contained 28 slices (mean age of pups, 12 days) kept in culture for 7 days before the simulated ischemia stress. These two groups were studied separately because we wished to examine both the evolution of cell death during the first week after OGD, when cell death has been reported to peak, and also during a longer (2-week) post-OGD period. Risk of culture contamination precluded making all the observations in the same slices.

Statistical Analysis

Data were analyzed using one-way or two-way analysis of variance. For comparison with a control value, we used the Dunnett multiple comparison procedure (InStat; GraphPad Software, Inc., San Diego, CA). Differences were considered significant for $P < 0.05$.

Results

Viability of Cultured Slices

Hippocampal slices were maintained in culture for 3–4 weeks. Cultured slices retained their laminar appearance and became thinner and larger in area with time. At the end of several weeks in culture, the CA1 region had thinned to 10–15 neurons in depth (approximately 150 μm). Under phase contrast microscopy, neurons in CA1, CA3, and dentate were observed to retain their initial anatomic appearance, and sprouting of

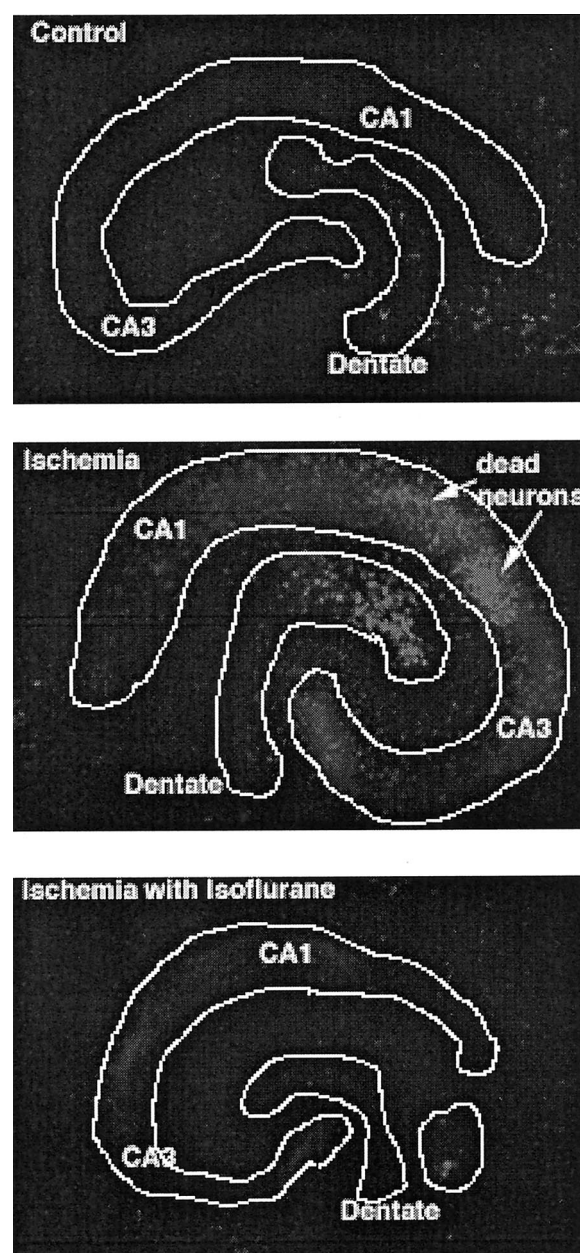


Fig. 1. Examples of propidium iodide fluorescence images from control, oxygen–glucose deprivation (“ischemia”), and oxygen–glucose deprivation plus isoflurane-treated hippocampal slice cultures. Images were acquired 3 days after the simulated ischemia. Bright areas indicate propidium iodide fluorescence (dead neurons). Outlines show CA1, CA3, and dentate cell body regions identified by standard and fluorescent microscopy used for serial analysis of cell death.

new neurites was seen at the margins of the slices. After 1 week in culture, the function of CA1 synapses was measured by placing a stimulating electrode in the Schaffer collateral pathway and placing a glass pipette recording electrode in stratum pyramidale (CA1). CA1 population spikes and excitatory postsynaptic potentials were present in 80–100% of the slices. In addition, after 7–10 days in culture, neurons in CA1, CA3, and dentate did not fluoresce after incubation in propidium iodide (fig.

[#] US National Institutes of Health: NIH Image software. Available at: <http://rsb.info.nih.gov/ni-image>. Accessed October 29, 2001.

1, top). These same cells exhibited fluorescence after incubation in calcein-AM, indicating viability. Microscopy of cross sections of fixed slices showed intact neuron cell bodies.

Effects of Isoflurane on 1- or 2-Week Survival after OGD

The percentage of dead neurons in CA1, CA3, and dentate lamina were assessed before injury and at 2, 3, and 7 days after OGD in group A slices. The degree of cell death varied with cell region, but death in all regions was maximal by 3 days after OGD. The greatest amount of cell death at the 3-day observation time was seen in CA1, with 80% death, followed by CA3 and dentate, with 70 and 55%, respectively (fig. 2).

In slices exposed to 1% isoflurane during the 45-min period of simulated ischemia, no cell death in CA1, CA3, or dentate was observed (figs. 1 and 2). Cell morphology was maintained. Similar protection was also seen in slices with 10 μ M MK-801 present during OGD (fig. 2). In both isoflurane and MK-801-treated cultures, calcein fluorescence (indicating viable cells) in CA1, CA3, and dentate remained unchanged compared with control for 1 week after OGD.

In a second group of slices (group B), cellular viability was assessed for 14–16 days after OGD. These slices, which varied from those in group A in that they were mainly prepared from younger animals (mean age at sacrifice, 12 vs. 16 days) and were maintained in culture for only 7 days before OGD, showed 100% cell death in CA1–CA3 and dentate regions a week after OGD. Isoflurane was found to reduce cell death significantly, to levels similar to non-OGD controls, during 14 days of observation (fig. 3). In group B control slices (no OGD), up to 30% cell death was observed by the end of the third week in culture.

Effects of Isoflurane and MK-801 on Glutamate-induced Cell Death

We also tested whether isoflurane prevents cell death caused by application of 100 or 500 μ M glutamate. Glutamate, applied at a concentration of 500 μ M for 30 min, caused the death of more than 90% of CA1, CA3, and dentate neurons by the third to fifth day after exposure (fig. 4). MK-801, 10 μ M, prevented this injury, but 1% isoflurane present during the glutamate exposure did not reduce cell death. However, isoflurane did significantly attenuate the loss of CA1 and CA3 neurons caused by a similar application of 100 μ M glutamate (fig. 4).

Discussion

We found that the volatile anesthetic isoflurane in an *in vitro* model of simulated cerebral ischemia and recovery prevents delayed cell death for at least 2 weeks. In

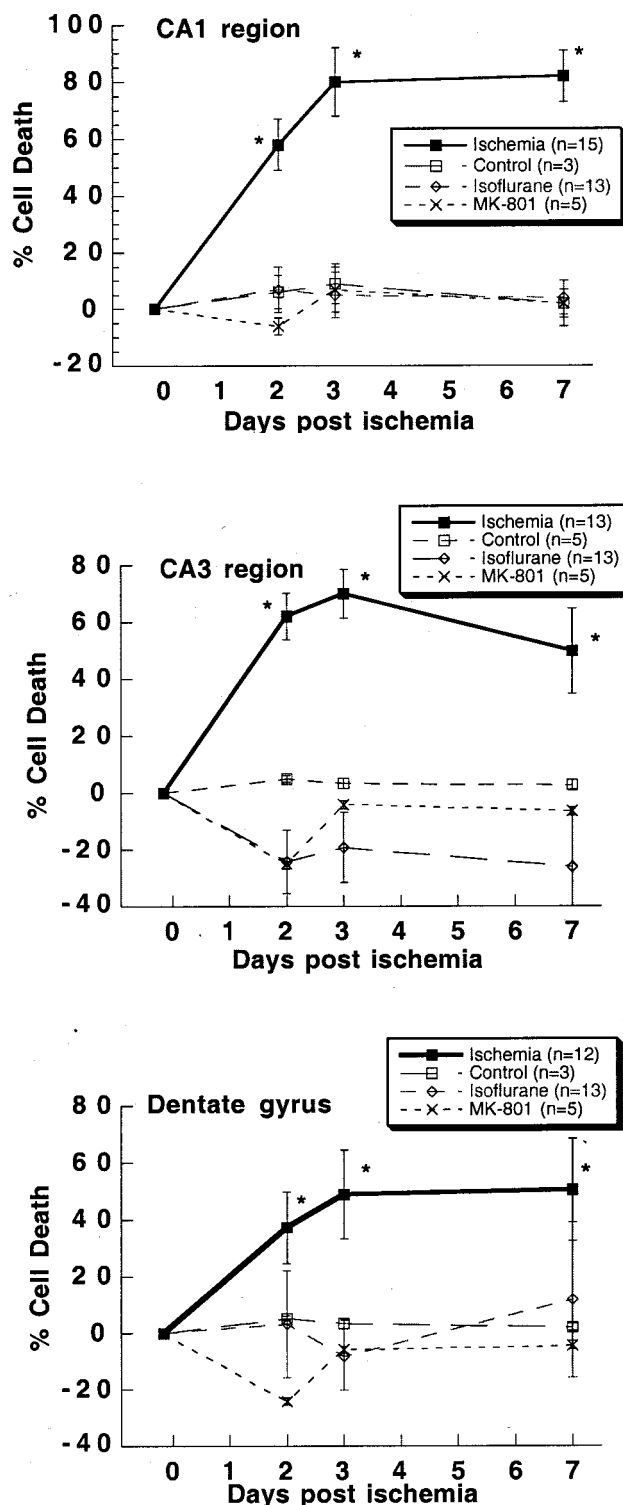


Fig. 2. Cell death in the CA1, CA3, and dentate region of cultured hippocampal slices before and after simulated ischemia (oxygen and glucose deprivation [OGD] for 45 min at 37°C). Error bars indicate SD of the mean. Ischemia = slices subjected to OGD without other agents present; control = slices never subjected to OGD but maintained in parallel culture for 7 days; MK-801 = slices subjected to OGD in the presence of 10 μ M MK-801; isoflurane = slices subjected to OGD with 1% isoflurane present in the anoxic gas. * P < 0.01 compared with the control (no OGD) group (two-way analysis of variance).

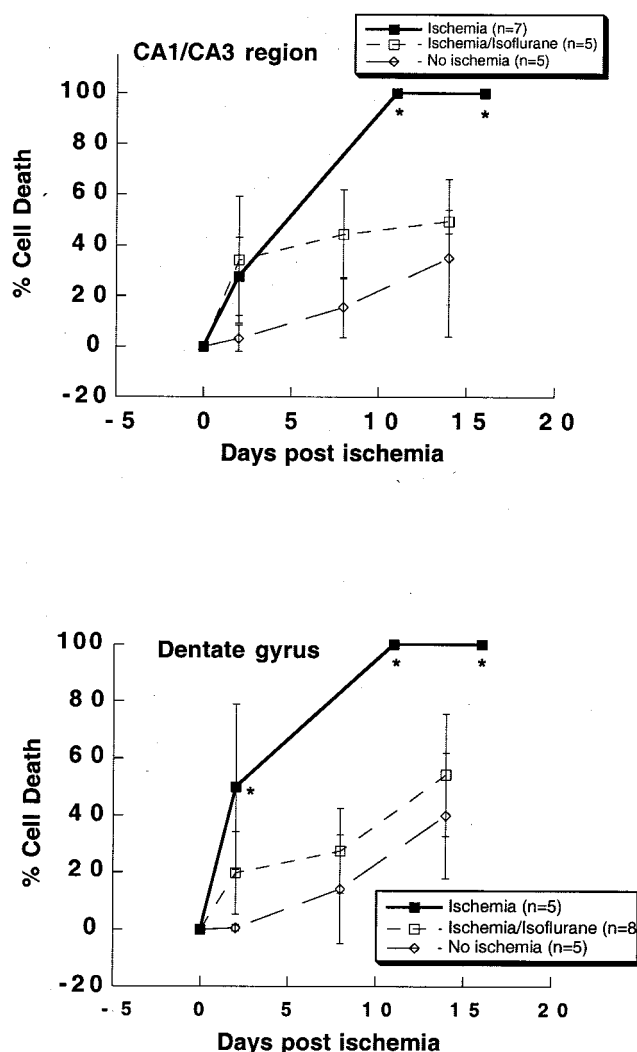


Fig. 3. Effects of isoflurane on cell death in hippocampal slice neurons for 2 weeks after *in vitro* ischemia (oxygen-glucose deprivation). * $P < 0.05$, ** $P < 0.01$, compared with the control (no oxygen-glucose deprivation) group (two-way analysis of variance).

addition, 1% isoflurane provided protection similar to the noncompetitive NMDA receptor antagonist MK-801. Furthermore, isoflurane was observed to reduce glutamate toxicity. The results thus suggest that the volatile anesthetic isoflurane provides neuroprotection at least in part by modulating glutamate excitotoxicity.

Anesthetic Neuroprotection

Previous studies have found that isoflurane and other volatile anesthetics reduce infarct volume in *in vivo* focal ischemia in various rodent models,^{1,12-14} as well as protecting against excitotoxic (glutamate or NMDA) or anoxic injury in cultured neurons¹⁵ and brain slices.^{5,6} Recently, it has been suggested that neuroprotection with isoflurane is time limited, with reduction in infarct volume seen at 2 days but not after 2 weeks in a temporary middle cerebral artery occlusion model by Kawagu-

chi *et al.*³ In the study of Kawaguchi *et al.*,³ lasting protection of neurons in the periinfarct region of the cerebral cortex was seen. In contrast, no time-dependent limitation of isoflurane protection was seen in our model, at least for 14 days after the injury. In the organotypic slice culture model, post-oxygen-glucose deprivation death was maximal between days 2 and 7, consistent with other studies showing that delayed death peaks before or by 1 week after an injury in intact animal and *in vitro* models.⁷ Our results thus suggest that isoflurane confers lasting neuroprotection in cultured hippocampal slices.

Cell injury and death in an acute brain slice model of *in vitro* ischemia are significantly dependent on glutamate receptors,⁶ and the situation is similar in cultured slices.^{10,16} In this study, MK-801 (a specific NMDA receptor antagonist) prevented delayed cell death after *in vitro* ischemia and isoflurane significantly reduced glutamate toxicity. Isoflurane reduced cell death caused by 100 but not 500 μM glutamate, whereas MK-801 prevented death with both. These data suggest that part of isoflurane

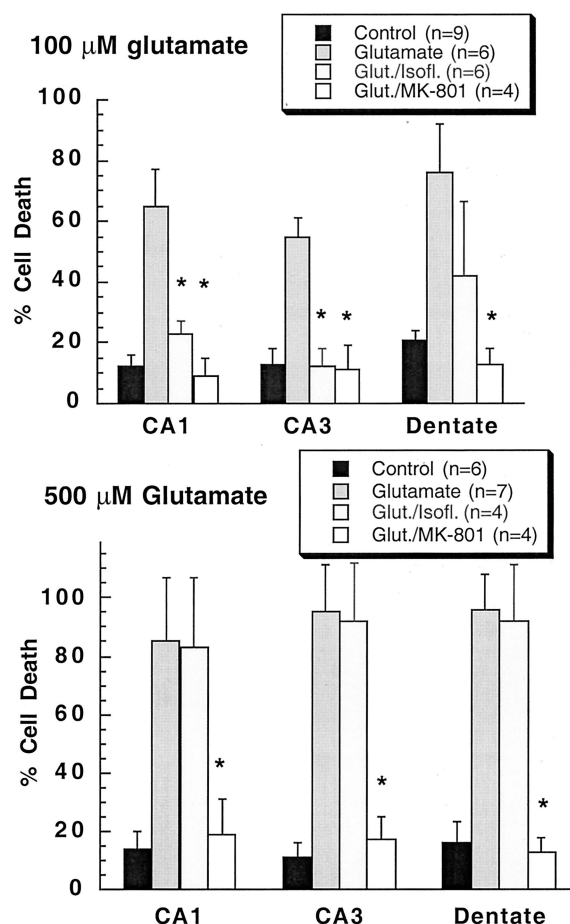


Fig. 4. Isoflurane, 1%, reduces cell death caused by 100 but not 500 μM glutamate in CA1 and CA3 neurons. Data indicate percent cell death in three different regions of the slices 1 week after 45 min of exposure to 100 μM glutamate at 37°C. *Significant difference compared with glutamate treatment group (analysis of variance, $P < 0.05$).

protection involves reduction of glutamate excitotoxicity, but that this action may not explain all of the protective effect. Reduction of glutamate excitotoxicity is consistent with data showing that isoflurane decreases NMDA receptor activity.^{17,18} In addition, volatile anesthetics, such as isoflurane, may increase glutamate uptake¹⁹ and suppress glutamate release.^{20,21} Glutamate receptor antagonism has been shown to be effective in reducing ischemic brain injury in a variety of animal models. Compounds such as the NMDA receptor antagonist MK-801 protect against cerebral injury in focal^{13,22,23} and transient global ischemia²⁴ in intact rodent models. Isoflurane may have other protective effects on hippocampal neurons, including augmentation of γ -aminobutyric acid-mediated inhibition²⁵ or a variety of other actions.

Organotypic Slice Culture Models of Cerebral Ischemia

Organotypic culture of brain slices provides an *in vitro* model of simulated cerebral ischemia that is superior in some respects to other *in vitro* models. Because cultured slices retain synaptic connectivity, maintain *in vivo* anatomic relations, and undergo limited de-differentiation, they retain sensitivity to anoxia, glucose lack, and glutamate toxicity that more closely mimics the intact brain than do cultured neurons.^{10,16,26} For example, cultured cortical neurons in our laboratory are not injured by 1 h of anoxia, whereas acutely prepared brain slices or organotypic cultures show demonstrable injury after 5–15 min of oxygen or glucose deprivation or both.²⁷ Because of the preserved anatomic relations, cultured slices are expected to duplicate more closely the *in vivo* accumulation of glutamate and other excitatory neurotransmitters than do monolayers of cultured neurons bathed in culture medium. Cultured slices also retain the selective vulnerability of CA1 neurons seen in intact animals.⁹ Probably the greatest advantage of cultured slices over other *in vitro* or *in vivo* models is the ability to follow the development of cell death serially in the same regions for several days to several weeks in an intact multicellular neuronal system, while retaining direct control of the extracellular environment. Although delayed cell death can be followed in dissociated neuron cultures, the de-differentiated state of cultured neurons places this model further from the intact brain. Most previous studies of anesthetic neuroprotection using *in vitro* ischemia in brain slices have used measures such as increase in intracellular calcium concentration,¹⁷ glutamate release,²¹ or synaptic failure²⁸ to indicate cell damage, without direct evidence that cell death would necessarily follow. In acutely prepared brain slices, cell death can only be assessed for hours after isolation.

Slice culture cannot duplicate all the features of ischemia and recovery that are present in the intact brain. Some of these differences could limit the neuroprotec-

tive qualities of isoflurane in intact animals compared with brain slices. One example is the contribution of leukocytes to postischemic free radical injury and inflammation. It is possible that in intact animals, this or other processes might kill neurons initially protected by isoflurane. If so, then anesthetics may provide only a window of therapeutic opportunity to abort the injury cascade leading to delayed death.

Group B slices, which were studied after 7 days in culture, exhibited more ongoing cell death than did group A slices, which remained in culture 10–14 days before study (compare figs. 2 and 3). This difference most likely reflects the fact that up to 14 days is required for slice preparation-related cell death to wane. Even so, isoflurane substantially reduced cell death in group B slices for 2 weeks after the injury (total of 3 weeks in culture).

Delayed cell death is an important component of cerebral injury after ischemia. Cell death in hypoxic and ischemic brain injuries involves a cascade of events that may lead to early cell death by necrosis as well as delayed cell death.^{29–31} However, the nature of the delayed cell death remains unclear because although in some ways the death resembles apoptosis (programmed cell death), in other respects, it may be more properly termed delayed necrotic death.³² It is thus uncertain whether techniques designed to detect apoptotic cell death accurately define postischemic cell loss in the brain. The propidium iodide method used in this study has the advantage of identifying nonviable cells, regardless of the nature of the death process.⁹

In summary, 1% isoflurane was found to provide 2 weeks of protection after *in vitro* ischemia in hippocampal neurons in cultured slices. This protection, which was similar to that afforded by 10 μ M MK-801, may be related to suppression of glutamate toxicity during *in vitro* ischemic stress.

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