Effects of Volatile Anesthetics on N-methyl-D-aspartate
Excitotoxicity in Primary Rat Neuronal–Glial Cultures

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Background: Volatile anesthetics are known to ameliorate experimental ischemic brain injury. A possible mechanism is inhibition of excitotoxic cascade induced by excessive glutamate stimulation. This study examined interactions between volatile anesthetics and excitotoxic stress.

Methods: Primary cortical neuronal–glial cultures were exposed to N-methyl-D-aspartate (NMDA) or glutamate and isoflurane (0.1–3.3 μM), sevoflurane (0.1–2.9 μM), halothane (0.1–2.9 μM), or 10 μM (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate (MK-801). Lactate dehydrogenase release was measured 24 h later. In other cultures, effects of volatile anesthetics on Ca2+ uptake and mitochondrial membrane potential were determined in the presence or absence of NMDA (0–200 μM).

Results: Volatile anesthetics reduced excitotoxin-induced lactate dehydrogenase release by up to 52% in a dose-dependent manner. At higher concentrations, this protection was reversed. When corrected for olive oil solubility, the three anesthetics offered equivalent protection. MK-801 provided near-complete protection. Ca2+ uptake was proportionally reduced with increasing concentrations of anesthetic but did not account for reversal of protection at higher anesthetic concentrations. Given equivalent NMDA-induced Ca2+ loads, cells treated with volatile anesthetic had greater lactate dehydrogenase release than those left untreated. At protective concentrations, volatile anesthetics partially inhibited NMDA-induced mitochondrial membrane depolarization. At higher concentrations, volatile anesthetics alone were sufficient to induce mitochondrial depolarization.

Conclusions: Volatile anesthetics offer similar protection against excitotoxicity, but this protection is substantially less than that provided by selective NMDA receptor antagonism. Peak effects of NMDA receptor antagonism were observed at volatile anesthetic concentrations substantially greater than those used clinically.

VOLATILE anesthetics are known to ameliorate experimental brain injury when administered during, but not after, an ischemic insult.1–6 It has been suggested that interactions between volatile anesthetics and the N-methyl-D-aspartate (NMDA) receptor play an important role in this process.7 Volatile anesthetics are known to interact with the glutamatergic NMDA receptor.8–11 In some forms of ischemic brain injury, either preischemic or early postischemic administration of NMDA receptor antagonists provides potent neuroprotection,12–14 largely attributable to reduction of calcium transients associated with energy failure.15,16 Although the therapeutic window allowed by this mechanism of action is likely to be brief,17 it could be of relevance to anesthetized patients because volatile anesthetics are often present during intraoperative ischemic insults.

There is in vivo information regarding effects of the volatile anesthetics on glutamatergic neurotoxicity. Rats subjected to intracerebrocortical microinjections of NMDA or α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) had smaller lesion sizes if isoflurane anesthesia was maintained for 5 h after injection.7,18 In vitro, 2% isoflurane has been shown to reduce excitotoxicity resulting from incubation of hippocampal slices with 1 mM glutamate.19 This is consistent with an observed isoflurane-mediated reduction of Ca2+ uptake in neocortical slices incubated in 1 mM glutamate or 50 μM NMDA.10

The evidence just mentioned implicates an antagonistic role for volatile anesthetics in glutamatergic excitotoxic processes. However, several matters remain unknown. First, although efficacy of isoflurane has been defined, there is little information regarding other volatile anesthetics. As a result, whether this property is peculiar to isoflurane or is a property of this class of anesthetics is unclear. Second, there has been little information provided regarding relative potency of volatile anesthetics, defined as a function of dose–response relations, relative to known selective NMDA receptor antagonists. The goal of the current project was to examine these issues over a range of excitotoxic stresses in a primary mixed neuronal–glial cell culture system.

Materials and Methods

All animal procedures were approved by the Duke University Animal Care and Use Committee (Durham, North Carolina).

Preparation of Mixed Neuronal–Glial Cell Cultures

Mixed neuronal–glial cell cultures were prepared from fetal Sprague-Dawley rat brains at 18 days of gestation. Brains were harvested from 10–15 pups and dissected to separate cortex from meninges and subcortical structures using anatomic landmarks. Cortices were pooled and minced into 2-mm3 pieces in a buffered salt solution.
Preparation of Volatile Anesthetic Solutions

Stock solutions of volatile anesthetics dissolved in culture medium were prepared using a modification of the method of Blanck and Thompson. A 10-ml volatile anesthetic solution was made by injecting liquid anesthetic agent (100 μl halothane; Fluothane; Ayerst Laboratories Inc., Philadelphia, PA), 130 μl isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether; Abbott Laboratories Inc., Chicago IL), or 140 μl sevoflurane (Ultane; Abbott Laboratories Inc.) into 103 ml Mg²⁺-free BSS with 1.8 m CaCl₂ in a 100-ml volumetric flask. The flask was sealed with a glass stopper to exclude all air from the neck of the flask. The flask was wrapped in aluminum foil, and the solution was stirred for 24 h to solubilize the anesthetic agent. Immediately before use, 30 ml concentrated stock solution was poured into a 50-ml polycarbonate centrifuge tube and vortexed for 5–10 s to produce the working stock. An aliquot of the working stock was assayed by gas chromatography as described later in the current report to determine the concentration of dissolved volatile anesthetic. Then the working stock was diluted with Mg²⁺-free BSS containing 1.8 m CaCl₂ to produce the desired anesthetic concentration for treating cultures. In preliminary studies, the stability of the volatile anesthetic agent in solution was determined by incubating samples of the working stock under identical conditions used in treating cell cultures for a period of 30 min. Gas chromatography was used to measure the concentration before and after the incubation period. Values presented for anesthetic concentrations were corrected for percentage loss by averaging the concentrations at the start and end of the incubation.

Effects of Anesthetic Agents on NMDA or Glutamate Toxicity

Mature cultures (13–16 days in vitro) were washed with Mg²⁺-free BSS containing 20 m HEPES buffer, 1.8 m CaCl₂, pH 7.4, and dissolved anesthetic (0–3.2 m halothane, 0–3.3 m isoflurane, or 0–2.9 m sevoflurane). Unless otherwise indicated, cultures were maintained for 30 min in the presence of volatile anesthetics before addition of NMDA or glutamate. To determine the effect of anesthetic treatment on NMDA or glutamate toxicity, the cultures were returned to the incubator and maintained at 37°C for 30 min. Then the medium containing the dissolved anesthetic and NMDA was removed and replaced with MEM supplemented with 20 m glucose (with no dissolved anesthetic). The activity of lactate dehydrogenase (LDH) released (LDH release) into the media was measured 24 h after exposure as described later in the current study. The noncompetitive NMDA receptor antagonist, (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]-cyclohepten-5,10-imine hydrogen maleate (MK-801) (Tocris Cookson Inc., St. Louis, MO), 10 μM, was used as a positive control.

Lactate Dehydrogenase Release

Cellular injury was assessed 24 h after excitotoxic stress by measuring the amount of LDH released by damaged cells into overlying medium. LDH activity was determined by a modification of the method described by Amador et al. In brief, a 200-μl sample of culture medium was added to a polystyrene cuvette containing 10 ml lactate and 5 μmol nicotinamide adenine dinucleotide (NAD) in 2.75 ml 50 mm glycine buffer, pH 9.2, at 24°C. LDH activity was determined from the initial rate of reduction of NAD as calculated using a linear least square curve fit of the temporal changes in fluorescence signal from the cuvette (340 nm excitation, 450 nm emission) and expressed in units of enzymatic activity (nanomoles of lactate converted to pyruvate per minute). Analysis was performed on a fluorescence spectrophotometer (Perkin Elmer Model LS50B; Bodenseewerk GmbH, Uberlingen, Germany).

Effects of Anesthetic Agents on Cellular Calcium Uptake

Cellular calcium uptake from extracellular space was assessed using ⁴⁰CaCl₂ (American Radiolabeled Chemicals, St. Louis, MO). Cultures were washed with Mg²⁺-
free BSS containing 20 mM HEPES buffer and 1.8 mM CaCl₂, and dissolved anesthetic. The ³⁵ClCl₂ was added to each well (0.28 μCi/ml, 0.9 μCi/well), and NMDA was added to a final concentration of 0, 30, 50, 100, or 200 μM. The cultures were returned to the incubator and maintained at 37°C. Twenty minutes later, the exposure medium containing dissolved anesthetics was removed and each well was washed three times with ice-cold Mg²⁺-free BSS containing 2 mM HEPES buffer. Afterward, the cells were lysed by addition of 0.2% sodium dodecyl sulfate. An aliquot from each well was added to a liquid scintillation vial containing 10 ml Cytoscint (ICN Biochemicals, Costa Mesa, CA), and radioactivity was determined by scintillation counting.

**Effects of Anesthetics on Mitochondrial Membrane Potential**

Mitochondrial membrane potential was determined with 5,5',6,6'-tetrachloro-1',3',3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Molecular Probes, Eugene, OR). In brief, cells were loaded by changing of culture medium to Mg²⁺-free BSS containing 10 μM JC-1 and incubation at 37°C for 30 min. Thereafter, cells were washed twice with Mg²⁺-free BSS containing 20 mM HEPES buffer and 1.8 mM CaCl₂, and dissolved anesthetics were added. Fluorescence (485/530 nm and 530/590 nm [excitation/emission]) was measured in a BIO-TEK FL600 fluorescence plate reader (BIO-TEK Instruments, Winoosk, VT) at the following time points: 1 and 5 min after addition of NMDA plus anesthetic or 1 h after NMDA exposure. An additional study was performed when fluorescence was measured 1 h after NMDA-anesthetic exposure (i.e., 30 min after washing out NMDA and volatile anesthetic from medium). The ratio of measured intensities at both wavelengths was calculated and used as an indicator of mitochondrial membrane potential.²³,²⁴ At the end of the experiments, 100 μM 2,4-dinitrophenol, a well-known uncoupler of oxidative phosphorylation, was added to each well and fluorescence was measured 1 and 5 min later.

**Measurement of Dissolved Volatile Anesthetic Concentration**

A 200-μl sample of medium containing dissolved anesthetic agent was transferred to a sealed 4.4-ml vial. The vial was vortexed for 1 min to equilibrate the volatile anesthetic in the gas and liquid phases. A gas-tight Hamilton syringe was used to collect a 500-μl sample of the air space within the vial. The sample was injected onto a 6-ft Supelcoport 100/120 gas chromatography column coated with 3% SP2340. The anesthetic agent was detected by flame ionization. The detector was calibrated using gas samples taken from an anesthetic vaporizer against values obtained with an infrared agent monitor for halothane and isoflurane (Model 5330; Ohmeda Inc., Louisville CO) and for sevoflurane (Capnomac Ultima; Datex Engstrom, Helsinki, Finland).

**Statistical Analysis**

Parametric data were compared by one-way analysis of variance. When indicated by a significant F ratio, post hoc analysis was performed using Scheffé test. Values are reported as mean ± SD. Significance was assumed when P < 0.05.

**Results**

**Prevention of NMDA-induced Cell Lysis and Calcium Uptake by Anesthetic Agents**

Exposure of the neuronal–glial cultures to 100 μM NMDA increased LDH release and increased Ca²⁺ uptake from the extracellular space. Treatment of cultures with media containing dissolved volatile anesthetic during NMDA exposure produced a dose-dependent change in NMDA-induced cell lysis (fig. 1). All three volatile anesthetics partially protected cells at lower concentrations. However, at increasingly higher concentrations, this protection began to reverse. MK-801 (10 μM) provided near-complete protection against 100 μM NMDA-induced cell lysis.

Studies were performed in sister cultures to directly compare the lipophilic phase potency of halothane, isoflurane, and sevoflurane. The aqueous phase concentrations (0.5 mM halothane, 1.5 mM isoflurane, or 2.0 mM sevoflurane) were chosen to attain equal concentrations in the hydrophobic membrane space by compensating for calculated differences in saline–olive oil partition coefficients.²⁵–²⁷ All three anesthetics similarly decreased LDH release induced by 100 μM NMDA (no NMDA–no anesthetic = 0.74 ± 0.28; NMDA-no anesthetic = 1.53 ± 0.43; NMDA-0.8 mM halothane = 1.03 ± 0.22; NMDA 1.5 mM isoflurane = 1.01 ± 0.23; NMDA-3.0 mM sevoflurane = 1.06 ± 0.26 nmol lactate converted to pyruvate per minute).

Mean calcium uptake in cultures exposed to 100 μM NMDA in the absence of isoflurane was 440 counts per minute per well. Isoflurane inhibited NMDA-induced calcium uptake in a dose-dependent manner (0.6 mM = 30 ± 9%, 1.2 mM = 40 ± 6%, and 3.1 mM = 60 ± 12% inhibition relative to cultures untreated with isoflurane, 11 culture wells per condition [P < 0.01]).

Studies were also performed to examine the effect of increasing NMDA concentrations (0-200 μM) on the magnitude of isoflurane inhibition of cell lysis. NMDA given in the absence of isoflurane caused a dose-dependent increase in LDH release. At all NMDA concentrations, 0.6 mM (approximately 2 minimum alveolar concentration [MAC]) isoflurane caused a reduction in LDH release defined as percentage protection relative to isoflurane untreated sister cultures (50 μM NMDA = 45 ± 20%; 50 μM
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Prevention of Glutamate-induced Cell Lysis by Isoflurane

Because glutamate, unlike NMDA, is an endogenous excitatory neurotransmitter in the mammalian nervous system, a more biologically relevant glutamate (0–1000 μM) dose–response relationship was also established. Assays performed 24 h after a 30-min exposure to glutamate demonstrated a glutamate concentration-dependent increase in LDH release. Cotreatment with isoflurane (either 0.6 or 1.2 mM) caused a modest but significant reduction in LDH release caused by up to 75–100 μM glutamate (fig. 4). No isoflurane protection was observed at higher glutamate concentrations (300 μM, 1 mM).

Volatile Anesthetic Effects on Mitochondrial Membrane Depolarization

JC-1 fluorescence was used to detect changes in mitochondrial inner membrane polarization. For both isoflurane and halothane (in the absence of NMDA), mitochondrial depolarization was observed only at the highest concentration tested (3.1 mM isoflurane, 2.7 mM halothane; figs. 5A and B). These concentrations were also found to be directly toxic to the cultures, causing increased LDH release in the absence of NMDA (figs. 5C and D). In fact, the magnitude of the LDH release in-

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duced by a 30-min exposure to 2.7 mM halothane was greater than that caused by 100 µM NMDA alone, suggesting recruitment of glial injury. Lower concentrations of isoflurane and halothane had no effect on mitochondrial depolarization. One hundred micromolar concentration of 2,4-dinitrophenol caused abrupt membrane depolarization in all cultures, regardless of isoflurane concentration (data not shown).

Excitotoxic stress itself induced a significant depolarization of the mitochondrial membrane potential measured at 5 min after application of 100 µM NMDA (fig. 6A). This depolarization was partially inhibited by 0.3 mM but not 0.6

Fig. 2. The effect of volatile anesthetics on Ca$$^{2+}$$ uptake and lactate dehydrogenase release depends on the severity of excitotoxic stress. (A) Cultures were exposed to isoflurane or sevoflurane during a 30-min exposure to N-methyl-D-aspartate (NMDA). Cultures were loaded with $^{45}$Ca$$^{2+}$$ before NMDA exposure. Twenty minutes later, $^{45}$Ca$$^{2+}$$ was measured (n = 8 culture wells per condition). CPM = counts per minute. (B) Values from figure 2A recalculated as percentage inhibition of NMDA-induced Ca$$^{2+}$$ uptake relative to cultures exposed to respective NMDA concentrations in absence of volatile anesthetic. For either anesthetic there was no difference in percentage inhibition of NMDA-induced Ca$$^{2+}$$ uptake between different doses of NMDA. (C) Same treatment paradigm as described for figure 2A (n = 12 culture wells per condition). In these experiments, no $^{45}$Ca$$^{2+}$$ was used. Cultures were exposed to volatile anesthetic and NMDA for 30 min. Twenty-four hours later, lactate dehydrogenase activity was assessed. Values are presented as percentage protection against NMDA-induced Ca$$^{2+}$$ uptake between different doses of NMDA. Values are mean ± SD. *P < 0.01 compared with respective controls. Isoflurane and sevoflurane 100% lactate dehydrogenase values were 1.64 ± 0.07 and 1.89 ± 0.18 nmol lactate converted to pyruvate per minute, respectively.

Fig. 3. The interaction between Ca$$^{2+}$$ uptake and lactate dehydrogenase (LDH) release depends on volatile anesthetic concentration. Cultures were exposed to 0, 10, 30, 50, 100, or 200 µM N-methyl-D-aspartate (NMDA) for 30 min in the presence of 0.6 (A), 1.2 (B), or 3.1 (C) mM isoflurane. Subset cultures from each condition were assessed for Ca$$^{2+}$$ uptake at 20 min after NMDA exposure or LDH release at 24 h after NMDA exposure. LDH release and Ca$$^{2+}$$ uptake (mean ± SD) are presented for the respective isoflurane concentrations as percentage values relative to control cultures unique to each anesthetic concentration that were untreated with isoflurane. Open circles represent cultures treated with respective isoflurane concentrations. Filled circles represent cultures untreated with isoflurane. Approximate minimum alveolar concentration (MAC) equivalent values are given.
or 1.2 mM isoflurane. When cultures were allowed to incubate with both 100 μM NMDA and 0.7 mM isoflurane (approximately 2 MAC), a 45% inhibition of NMDA-induced mitochondrial depolarization occurred. In contrast, 10 μM MK-801 completely inhibited mitochondrial depolarization (fig. 6B).

Discussion

The principal findings of this study are as follows. As reported in other systems,⁷¹⁰,28 isoflurane reduced excitotoxic cell death in a dose-dependent manner. This was associated with a reduction in Ca²⁺ uptake and preservation of mitochondrial membrane potential.

When corrected for lipid solubility, isoflurane, halothane, and sevoflurane provided similar magnitudes of protection. However, when cultures were treated with doses of volatile anesthetic substantially greater than those used in clinical anesthesia, the volatile anesthetic itself was sufficient to increase LDH release and cause reversal of the mitochondrial membrane potential. This was associated with enhanced sensitivity to a given Ca²⁺ load, suggesting that benefits from NMDA receptor antagonism become overshadowed by intrinsic volatile anesthetic toxicity at supraclinical concentrations.

To some extent, these data support the hypothesis that in vivo neuroprotection is mechanistically associated with direct effects on the NMDA, and possibly, AMPA, glutamate receptor subtypes. However, the maximal protective benefit of volatile anesthetics was appreciably less than that provided by MK-801. There are both advantages and disadvantages of use of isolated cell systems to study excitotoxicity as a possible component of ischemic brain injury. The system can be highly controlled with respect to manipulating independent stimuli such as NMDA receptor agonists or antagonists. Poorly defined vascular and humoral events can be eliminated from interactions under investigation. At the same time, cerebral ischemia is largely a vascular disease, and in vivo circumstances may not be appropriately represented. Our mixed neuronal–glial cell cultures were obtained from brains of embryonic rats in which ontogenetic events, particularly those pertaining to receptor expression, are not mature as would be expected in an in vivo model. In addition, the studies were conducted in Mg²⁺-free media to promote NMDA toxicity. In vivo, Mg²⁺ serves as a potential endogenous inhibitor of NMDA toxicity, again pointing to limitations of the in vitro system we used as a model for mechanisms of in vivo injury.

Our findings, at first appearance, would seem to be inconsistent with other reports using either whole animal or hippocampal slice preparations where protective effects of clinically relevant concentrations of volatile anesthetics were found to offer greater protection. As a result, we must speculate on contrasts between models and the interpretation of these findings in the context of in vivo events.

Popovic et al.¹⁹ studied rat hippocampal slices subjected to 10 or 20 min of oxygen-glucose deprivation as a model of cerebral ischemia. Both 0.7% and 2% “intraischemic” isoflurane provided equivalent neuroprotection in a magnitude similar to that provided by 10 μM MK-801 or reduction of media temperature to 33°C. Bickler et al.¹⁰ subjected rat neocortical slices to either 50 μM NMDA or 1.0 mM glutamate and observed increases in cytosolic Ca²⁺ concentrations. NMDA-stimulated cytosolic Ca²⁺ accumulation was reduced by up to 63% by coadministration of approximately 1.7% isoflurane. Subsequently, it was shown that similar concentrations of...
isoflurane inhibit increases in extracellular glutamate concentrations caused by 100 μM NaCN in a magnitude similar to that provided by moderate hypothermia. Harada et al. performed microinjection of 50 μM NMDA into rat cerebral cortex and found near-complete protection in rats anesthetized with 2.2% isoflurane, a dose sufficient to cause electroencephalographic burst suppression. This protection was of similar magnitude to that obtained by 10 mg/kg intravenous MK-801. Therefore, in these models, considerable evidence is provided that isoflurane serves as a potent antagonist of excitotoxicity mediated at the NMDA receptor.

These results are apparently inconsistent with findings made in whole animal cerebral ischemia preparations. Sano et al. exposed rats to incomplete forebrain ischemia while the animals were anesthetized with halothane or isoflurane. In the halothane group, a subset of animals was also made hypothermic during the insult. There was no difference in histologic score between normothermic halothane and normothermic isoflurane rats, but there was major reduction in damage when the halothane rats were made hypothermic. This suggests that hypothermia is markedly more neuroprotective than is isoflurane, in contrast to findings made in vitro. However, Sano et al. used trimethaphan to induce systemic hypotension. It has since been demonstrated that isoflurane neuroprotection is reversed by coadministration of trimethaphan. A repetition of the study by Sano et al., performed in the absence of trimethaphan, would be of interest. Sarraf-Yazdi et al. examined rats subjected to temporary middle cerebral artery occlusion. Rats that were given MK-801 in a dose sufficient to reduce isoflurane MAC by 50% showed a 44% reduction in cerebral infarct size relative to awake controls. In contrast, 0.5 MAC isoflurane alone did not have a significant effect on infarct size, although numerically, infarct size was reduced by 22% relative to awake controls. These data suggest that at equianalgesic doses, MK-801 is a substantially more potent neuroprotective agent than is isoflurane. These data are, at first appearance, consistent with the data derived from our cell culture studies. Although a dose–response analysis for MK-801–mediated reduction in NMDA-induced LDH release was not performed, it is clear from our results that no isoflurane dose could provide protection equivalent to that provided by 10 μM MK-801. These findings were also true for halothane and sevoflurane, which have similar efficacy against in vitro focal ischemic insults. However, the cell culture findings do not rule out that, should isoflurane be examined in vivo, at a peak neuroprotective...
In all cases in which isoflurane was found to exhibit protective concentration (presumably, > 0.5 MAC), reductions in cerebral infarct size could be similar to those observed for MK-801. However, other mechanisms in addition to NMDA receptor antagonism would be required to explain such protection.

For example, if the principal mechanism by which isoflurane causes neuroprotection is via interactions with glutamate receptors, it would be predicted that isoflurane would have neuroprotection similar to that of MK-801 in a model of global (or forebrain) cerebral ischemia. These two drugs have not been simultaneously compared. However, there is compelling evidence that isoflurane substantially reduces global ischemic brain damage whereas MK-801 does not under normothermic conditions of controlled pericranial (or brain) temperature.\(^5\)\(^{10}\)\(^{13}\)\(^{14}\) This discrepancy may serve to allow synthesis of several pieces of information alluded to earlier in this report and the findings from our cell culture system. In all cases in which isoflurane was found to exhibit potent neuroprotection against ischemia, excitotoxicity, or simulated ischemia, either an intact animal or brain slices were used. These systems would have intact presynaptic glutamatergic receptors and postsynaptic yamino- butyric acid (GABA) afferentation. Volatile anesthetics, when studied at clinically relevant concentrations, have been shown to interact with both of these systems in a manner consistent with providing neuroprotection.\(^34\)\(^{38}\) Anesthesia provided by volatile anesthetics is thought to be, at least in part, mechanistically attributable to agonism of postsynaptic GABA receptor systems causing hyperpolarization of the postsynaptic membrane, thereby reducing excitability.\(^38\)\(^{39}\) Volatile anesthetics have also been shown to diminish presynaptic NMDA receptor-mediated responses associated with excitation.\(^37\) The GABAergic property, in particular, has been suggested to account for the efficacy of halothane as a neuroprotectant against paradoxical cell death in the cingulate cortex of rats given parenteral MK-801, presumably caused by inactivating glutamate-activated inhibitory (i.e., GABAergic) neurons.\(^36\) Further, administration of benzodiazepine receptor agonists provides neuroprotection against global ischemia,\(^40\) in contrast to MK-801, which does not.\(^31\) Accordingly, we suggest that data obtained from our primary mixed neuronal-glial cell culture are informative with respect to defining the limits of direct volatile anesthetic protection against excitotoxicity and that these findings are consistent with a substantial body of literature which indicates that NMDA receptor antagonism is insufficient to account for the neuroprotective effect of this class of anesthetics against ischemic brain injury. It is tempting to speculate that either additive or synergistic interactions with both glutamatergic and GABAergic systems will be sufficient to afford by volatile anesthetics.

There are other points that warrant consideration. The three volatile anesthetics were not equipotent against NMDA excitotoxicity in our cell culture system when aqueous phase concentrations were compared, but equipotency was observed when aqueous phase concentrations were adjusted to produce similar calculated lipid phase concentrations. This suggests that the predominant site of action of the volatile anesthetics was in the lipid phase of our cell culture system, consistent with action at the NMDA receptor as defined by other authors.\(^41\) There has been little work performed to determine relative potencies of volatile anesthetics as NMDA receptor antagonists. To our knowledge, sevoflurane has not been previously investigated as an NMDA receptor antagonist. Although we found the three anesthetics to be equipotent when correction for lipid phase solubility was made, we cannot be sure that other undefined mechanisms (e.g., AMPA receptor antagonism) were differentially recruited at the same concentrations. Nonetheless, it is clear from this work that definition of po-

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**Fig. 6. Effects of isoflurane on N-methyl-D-aspartate (NMDA)-induced mitochondrial membrane potential. (A) Cortical primary mixed neuronal-glial cell cultures were exposed to 100 μM NMDA in the presence or absence of different concentrations of isoflurane. Five minutes later, mitochondrial inner membrane depolarization (5,5',6,6'-tetracloro-1,1',3,3'-tetraceethylbenzimidazolylcarbocyanine iodide [JC-1] ratio) was measured. An increase in the JC-1 ratio resulted from NMDA application. This was inhibited by 0.3 mM isoflurane but not by 0.6 or 1.2 mM isoflurane. (B) The experimental conditions were similar except that mitochondrial depolarization was measured 1 h after onset of exposure to 100 μM NMDA and 0.7 mM (2 minimum alveolar concentration [MAC]) isoflurane. Isoflurane provided partial protection against depolarization. In contrast, protection was complete with MK-801. Values are mean ± SD; n = four to eight culture wells per condition. a = P < 0.01 compared with cultures exposed neither to NMDA nor to isoflurane; b = P < 0.01 compared with 100 μM NMDA-no isoflurane.**
tendencies of different volatile anesthetics as NMDA antagonists will be contingent upon partition coefficients used to correct for varying solubilities in aqueous and lipid compartments.

It was of interest that when cells were subjected to increasing NMDA concentrations, both isoflurane and sevoflurane provided a consistent proportional inhibition of Ca$^{2+}$ uptake. However, because absolute Ca$^{2+}$ uptake increased with increasing NMDA concentrations, it can be suggested that a only a subset of NMDA receptors was affected by isoflurane and that increasing NMDA concentrations resulted in longer channel-open times in the remaining receptors. This would be consistent with a previous demonstration of differential sensitivities of NMDA receptor subtypes to noncompetitive antagonists. As an alternative, if the NMDA concentration determined the frequency of channel opening and volatile anesthetics shortened channel-open times, that would also account for the proportional inhibition of Ca$^{2+}$ uptake across the range of NMDA doses and the increase in Ca$^{2+}$ uptake at higher NMDA concentrations. Support for this hypothesis is provided by Yang and Zorumski, who studied single NMDA receptor channel-open duration and frequency of opening, both of which were reduced by isoflurane in a dose-dependent manner. These effects cannot be differentiated in our cell culture system. However, it is tempting to speculate that different subpopulations of NMDA receptors exist with differing sensitivities to volatile anesthetics.

In conclusion, by use of primary mixed neuronal–glial cell cultures, we examined interactions between NMDA-induced excitotoxicity and volatile anesthetics. A dose-dependent reduction of LDH release, indicative of cell death, was observed for halothane, isoflurane, and sevoflurane. The three drugs appeared to be equipotent when corrections were made for lipid solubility. However, the magnitude of protection against NMDA excitotoxicity was small at clinically relevant concentrations, particularly when compared with effects of MK-801, a selective noncompetitive NMDA receptor antagonist. We conclude that the contribution of NMDA receptor antagonism to the overall ability of clinically relevant concentrations of volatile anesthetics to provide protection against ischemic brain injury is likely to be small and that other properties of volatile anesthetics account for the efficacy against excitotoxic stress observed in vivo.

References