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Isoflurane Reduces Ischemia-induced Glutamate Release in Rats Subjected to Forebrain Ischemia

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Background: **The release of excitatory neurotransmitters during ischemia is thought to contribute to ischemic neuronal injury. Volatile anesthetics have been shown to reduce excitatory neurotransmission *in vitro,* and it is conceivable that they reduce ischemia-induced neurotransmitter release. The current investigation was conducted to evaluate the effect of isoflurane and N_2O–fentanyl anesthesia on ischemia-induced glutamate release in the rat and to compare it with that of mild hypothermia, an intervention known to reduce glutamate release significantly.**

Methods: **Microdialysis probes were implanted into the parietal cortex and dorsal hippocampus of four groups of anesthetized rats (n = 5 per group). The hypothermic group was anesthetized with 1.2% halothane. The two isoflurane groups were anesthetized with 0.5 minimum alveolar concentration or electroencephalographic burst-suppression doses of isoflurane (≈2 minimum alveolar concentration). The control group was anesthetized with 70% N_2O–30% O_2 and fentanyl. The pericranial temperature was maintained at 34°C in the hypothermic group and at 38°C in the remaining groups. Ischemia was induced by bilateral carotid artery occlusion with simultaneous hypotension to 35 mmHg for 10 min, followed by a reperfusion period of 70 min. Dialysate was collected before, during, and after ischemia. The concentrations of glutamate and glycine in the dialysate were measured by high-performance liquid chromatography.**

Results: **Preischemic glutamate and glycine concentrations in the dialysate were similar among the groups. Ischemia resulted in a significant increase in glutamate and glycine concentrations in the N_2O–fentanyl groups in the parietal cortex and in the hippocampus. This increase in neurotransmitter concentrations did not occur in the hypothermic group in either structure. Isoflurane reduced glutamate concentrations in both structures and glycine concentrations in the hippocampus. In the parietal cortex, glycine concentrations did not increase in either isoflurane group.**

Conclusions: **Hypothermia inhibits ischemia-induced excitatory neurotransmitter release in the rat. Isoflurane, in comparison with a N_2O–fentanyl–anesthetized state, significantly attenuates excitatory neurotransmitter release in the hippocampus. This effect of isoflurane is comparable to that of mild hypothermia. (Key words: Anesthetics, volatile: isoflurane. Animals: rat. Brain, ischemia: forebrain. Neurotransmitters, excitatory: glutamate; glycine. Temperature: hypothermia.)**

A large body of evidence supports the concept that an important mechanism of ischemic neuronal death is the result of exposure of neurons to toxic concentrations of excitatory neurotransmitters, particularly glutamate. Ischemia results in the release of substantial quantities of glutamate.[1,2] The released glutamate induces ion fluxes across cell membranes, including uptake of Ca^{2+}, and can lead to excitotoxic neuronal death.[3,4] In addition, ischemia results in a substantial release of glycine.[5] Glycine is a positive modulator of the N-methyl-D-aspartate subtype of glutamate receptor and is thought to contribute to ischemic neuronal injury by enhancing excitotoxicity.[6] Isoflurane has been shown to reduce excitatory neurotransmission *in vitro,* and it has been suggested that part of this effect may be mediated by an inhibition of the release of excitatory neurotransmitters.[7,8] It is therefore conceivable that protective effects attributed to isoflurane may also be mediated by the inhibition of ischemia-induced glutamate and glycine release.

The current study was conducted to evaluate this possibility. The effect of isoflurane, in 0.5 minimum alveolar concentration (MAC) and electroencephalographic (EEG) burst-suppression doses, on ischemia-

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induced glutamate and glycine release was assessed in rats subjected to incomplete forebrain ischemia. The effect of isoflurane was compared with that of mild hypothermia (an intervention that has been shown to reduce significantly ischemia-induced glutamate release) and to a N_2O–fentanyl–anesthetized control state.

Materials and Methods

The experimental protocol was approved by the institutional Animal Use Subcommittee. Animals were prepared as described previously.[9,10] Fasted male Wistar-Kyoto rats (Harlan Sprague-Dawley, Indianapolis, IN) of the same age and weight (275–350 g) were anesthetized with 3% halothane in O_2. After orotracheal intubation, mechanical ventilation of the lungs was initiated with a gas mixture of 2% halothane in 30% O_2–balance N_2. The end-tidal concentration of the expired gases, sampled at the level of the carina, was measured intermittently with an infrared anesthetic agent analyzer (RGM 5250 Infrared Gas Analyzer, Ohmeda, Englewood, CO). Mechanical ventilation was adjusted to maintain normocapnia (arterial CO_2 tension 35–40 mmHg). Pericranial temperature was measured with a thermistor (Mon-a-Therm temperature sensor, Mallinckrodt Anesthesia Products, St. Louis, MO) that was inserted between the temporalis muscle and the skull. The pericranial temperature was controlled by servomechanism to 38°C with an overhead heat lamp that was directed at the animal's body. The tail artery and the right external jugular vein were cannulated. *Via* a midline pretracheal incision, both carotid arteries were exposed and were encircled loosely with silk sutures. Platinum needle EEG electrodes (Grass Instruments, Quincy, MA) were inserted into the scalp in a frontooccipital configuration, and the EEG was recorded continuously (Accutrace 200A, Beckman, Fullerton, CA).

The animal's head was secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Burr holes 4 mm in diameter were drilled caudal to the bregma bilaterally. Under a stereoscopic microscope, the dura was carefully incised with a 30-G needle without injuring the underlying cortex. Two microdialysis probes with 2-mm membrane tips (CMA-12, Bioanalytical Systems, West Lafayette, IN) were used in each experiment. The probes were mounted on micromanipulators and inserted into the parietal cortex (2.8 mm caudal and 4.6 mm lateral to the bregma; depth 4.5 mm from the surface of the skull) and the dorsal hippocampus (2.8 mm caudal and 2.0 mm lateral to the bregma; depth 5.3 mm from the surface of the skull). The coordinates for probe insertion were derived from a stereotaxic atlas of the rat brain.[11] At the conclusion of the surgical preparation, all wounds were infiltrated with 0.25% bupivacaine (approximate dose 0.4–0.5 mg) Sensorcaine, Astra Pharmaceutical Products, Westborough, MA) and the halothane was adjusted to achieve an end-tidal concentration of 1.2%. Pancuronium was administered in 0.2-mg boluses as necessary to maintain muscle relaxation.

After probe insertion, the animals were left undisturbed for 60 min. The animals were then allocated randomly to one of four groups (n = 5 rats per group). In the hypothermic group, the pericranial temperature was allowed to decrease spontaneously and it was then servo-controlled at 34°C. In the N_2O–fentanyl group, N_2 administration was discontinued and the inspired gas mixture was changed to 70% N_2O–30% O_2. Simultaneously, a fentanyl infusion was initiated (bolus of 25 μg/kg and infusion rate of 25 $\mu g \cdot kg^{-1} \cdot h^{-1}$) and the administration of halothane was discontinued. In the two isoflurane groups, administration of halothane was discontinued and simultaneously, isoflurane was introduced. In the 0.5 MAC isoflurane group, the end-tidal concentration of isoflurane was maintained at 0.6% (0.5 MAC). In the large-dose isoflurane group, the end-tidal concentration of isoflurane was increased until EEG burst-suppression (4 or 5 bursts/min) was achieved (2.1–2.3% end-tidal). The animals were then left undisturbed for an equilibration period of 70 min. Serum glucose, hematocrit, and mean arterial pressure were recorded during this period. Arterial CO_2 and O_2 tensions and arterial *p*H were measured with a blood gas analyzer (IL-1306, Instrumentation Laboratories, Lexington, MA).

The animals in all groups were then subjected to 10 min of forebrain ischemia.[12] Heparin, 30 U intravenous, was injected through the right external jugular vein catheter. Hypotension was induced by intravenous injection of 3 mg trimethaphan (Arfonad, Roche Laboratories, Nutley, NJ) followed by adjustment of intravascular volume to achieve a mean arterial pressure of 35 mmHg (approximately 2.5–3.0 ml of blood was withdrawn). Both carotid arteries were then occluded with vascular clamps for 10 min. At the conclusion of the ischemic interval, the vascular clamps were removed and blood pressure was restored by rapid reinfusion of the withdrawn blood. Protamine, 0.3 mg

(QUAD Pharmaceuticals, Indianapolis, IN), and $NaHCO_3$, 0.25 mEq, were administered to reverse the heparinization and metabolic acidosis, respectively. During the reperfusion period, mean arterial pressure was maintained above 70 mmHg by infusion of up to 2.5 ml/kg of normal saline. Seventy minutes after reperfusion was established, 1 ml/kg 3% Evans' blue dye was administered intravenously. The animals were sacrificed 5 min afterward and their brains harvested and sliced coronally. The location of the probes within the cortex and hippocampus was identified macroscopically.

Microdialysis

Before the use of the probes, the efficiency of the probe membrane was analyzed by immersing the probes in a 10 mM glucose solution at 38°C.[13] The probes were perfused with Ringer's solution at a rate of 2 μl/min and the *in vitro* rate of glucose recovery measured. The average recovery of glucose in new probes is approximately 20%.§ Probes with an *in vitro* recovery rate of less than 15% were discarded. The probes were perfused with Ringer's solution at a rate of 2 μl/min. The dialysate was collected in two 20-min epochs during the preischemic period, for four 5-min epochs during ischemia and for the first 10 min of reperfusion, and for three 20-min epochs for the remainder of the reperfusion period. The samples were frozen at −20°C for later analysis. The amino acids in the dialysate were derivatized with phenylisothiocyanate. The concentrations of the derived amino acids were measured with high-performance liquid chromatography with a reverse phase column. The derivatives were detected fluorometrically with an ultraviolet detector and the peaks were integrated and quantified by calibration with standards of known concentrations.

Statistical Analysis

The physiologic data were analyzed by factorial analysis of variance. Where analysis of variance identified differences, *post hoc* Fisher protected least significant difference tests were used for intergroup comparisons. The amino acid concentrations were log transformed and the transformed data were analyzed by a two factor repeated-measures analysis of variance. To preserve statistical power, only baseline and peak concentrations were compared *post hoc*. A paired two-tailed *t* test was used to compare peak with baseline concentrations (within group) and Fisher protected least significant difference tests were used to compare baseline and peak concentrations (among groups). A $P < 0.05$ was considered to be statistically significant. All data are presented as means ± SEM.

Results

A total of 22 animals were studied. The dialysates from 1 animal in the isoflurane burst-suppression group and 1 animal in the N_2O–fentanyl group were discarded because the volume of dialysate recovered was significantly less than the volume of Ringer's solution that was infused. Examination of the probes revealed a membrane leak. These animals were replaced to provide a uniform group size of 5 animals per group. Correct location of the probes in the hippocampus and cortex was confirmed in all animals.

In the hypothermic group, the end-tidal halothane concentration was 1.2 ± 0.1%. In the two isoflurane groups, the end-tidal concentration of isoflurane was 0.6 ± 0.1% and 2.1 ± 0.2%. With the exception of the hypothermic group, halothane was not detected in the exhaled gas that was sampled immediately before the induction of ischemia.

The physiologic data are presented in table 1. The weights of the animals in the four groups were not different. The mean arterial pressure in the N_2O–fentanyl group was slightly greater than in the other groups before ischemia. After ischemia, the mean arterial pressure was similar in all groups. There were no differences in the heart rate. Arterial blood gas tensions and *p*H were similar among the groups. The hematocrit was slightly less in the hypothermic group than in the other normothermic groups.

Glutamate

In the parietal cortex, the preischemic baseline glutamate concentrations were similar in all experimental groups (fig. 1). In the hypothermic group, glutamate concentrations did not increase during ischemia. In contrast, in the N_2O–fentanyl and both isoflurane groups, glutamate concentrations increased during ischemia and returned to baseline after 10 min of reperfusion. The increase was the greatest in the N_2O–fentanyl group and the peak concentration was significantly greater than that observed in the two isoflurane groups. Although the mean glutamate concentration in

§ Unpublished obervations.

Table 1. Physiologic Variables in the Experimental Groups

	Nitrous Oxide–Narcotic	Isoflurane, 0.5 MAC	Isoflurane, EEG B-S	Hypothermia
Number	5	5	5	5
Weight (g)	295 ± 3	304 ± 5	318 ± 13	293 ± 19
Preischemic MAP (mmHg)	120 ± 5*	107 ± 7	92 ± 6	87 ± 6
Postischemic MAP (mmHg)	99 ± 6	115 ± 7	102 ± 9	94 ± 10
Preischemic HR (beats/min)	394 ± 9	414 ± 23	380 ± 16	356 ± 22
Postischemic HR (beats/min)	396 ± 12	444 ± 33	360 ± 11	308 ± 10
Pa_{O_2}	169 ± 11	166 ± 18	200 ± 11	183 ± 26
Pa_{CO_2}	36.8 ± 0.3	36.3 ± 1.9	37.8 ± 1.0	38.7 ± 1.5
*p*H	7.38 ± 0.02	7.44 ± 0.02	7.41 ± 0.02	7.38 ± 0.02
Blood glucose (mmol/dl)	4.6 ± 0.3	4.7 ± 0.1	4.7 ± 0.1	4.4 ± 0.2
Hematocrit (%)	44 ± 1	45 ± 1	46 ± 1	42 ± 1†

Values are mean ± SEM.

EEG B-S = electroencephalogram burst–suppression; MAP = mean arterial pressure;; HR = heart rate.

* $P < 0.05$, nitrous oxide–fentanyl group *versus* 0.5 MAC isoflurane and hypothermic groups.

† $P < 0.05$, hypothermic group *versus* the two isoflurane groups.

the 0.5 MAC isoflurane group was greater than that in the EEG burst-suppression isoflurane group, the difference did not attain statistical significance.

In the dorsal hippocampus, glutamate concentrations before ischemia were similar in all groups (fig. 1). In the isoflurane EEG burst-suppression and hypothermia groups, glutamate concentrations did not increase during ischemia. In the N_2O–fentanyl and 0.5 MAC isoflurane groups, glutamate concentrations increased significantly during ischemia. The glutamate concentrations returned to baseline after 10–30 min of reperfusion. Glutamate concentrations were greater in the N_2O–fentanyl group than in the other three groups. The concentrations in the 0.5 MAC isoflurane group were also greater than in the EEG burst-suppression isoflurane group and in the hypothermic group.

Glycine

In the parietal cortex, there were small differences in the baseline glycine concentrations among the groups before ischemia (fig. 2). Glycine concentrations were statistically slightly greater in the N_2O–fentanyl and 0.5 MAC isoflurane groups than in the EEG burst-suppression isoflurane and hypothermic groups. During ischemia, glycine concentrations appeared to increase in all four groups. However, the increase reached statistical significance in only the N_2O–fentanyl group. The peak glycine concentrations in the N_2O–fentanyl group were significantly greater than in the other three groups. During reperfusion, glycine concentrations returned to baseline values.

In the dorsal hippocampus, glycine concentrations immediately before ischemia were similar in all groups (fig. 2). During ischemia, there were no changes in glycine concentrations in the hypothermia and isoflurane EEG burst-suppression groups. By contrast, glycine concentrations increased significantly from preischemic baseline values during ischemia in the N_2O–fentanyl and 0.5 MAC isoflurane groups. Glycine concentrations returned to preischemic baseline values during reperfusion. Because of a wide interanimal variability in glycine concentrations in this structure, the peak concentrations in the N_2O–fentanyl and in the 0.5 MAC isoflurane groups were not different from the hypothermic and EEG burst-suppression isoflurane groups.

Discussion

The results of the current investigation demonstrate that the anesthetic state influences ischemia-induced release of the neurotransmitters glutamate and glycine. Specifically, isoflurane, in comparison with N_2O–fentanyl, reduced concentrations of glutamate in the dialysates from the parietal cortex and from the dorsal hippocampus during ischemia. Furthermore, isoflurane reduced ischemia-induced glycine release in a manner that was qualitatively similar to that of glutamate release in both structures. In fact, the ability of isoflurane to suppress excitatory neurotransmitter release in this model of incomplete forebrain ischemia was compa-

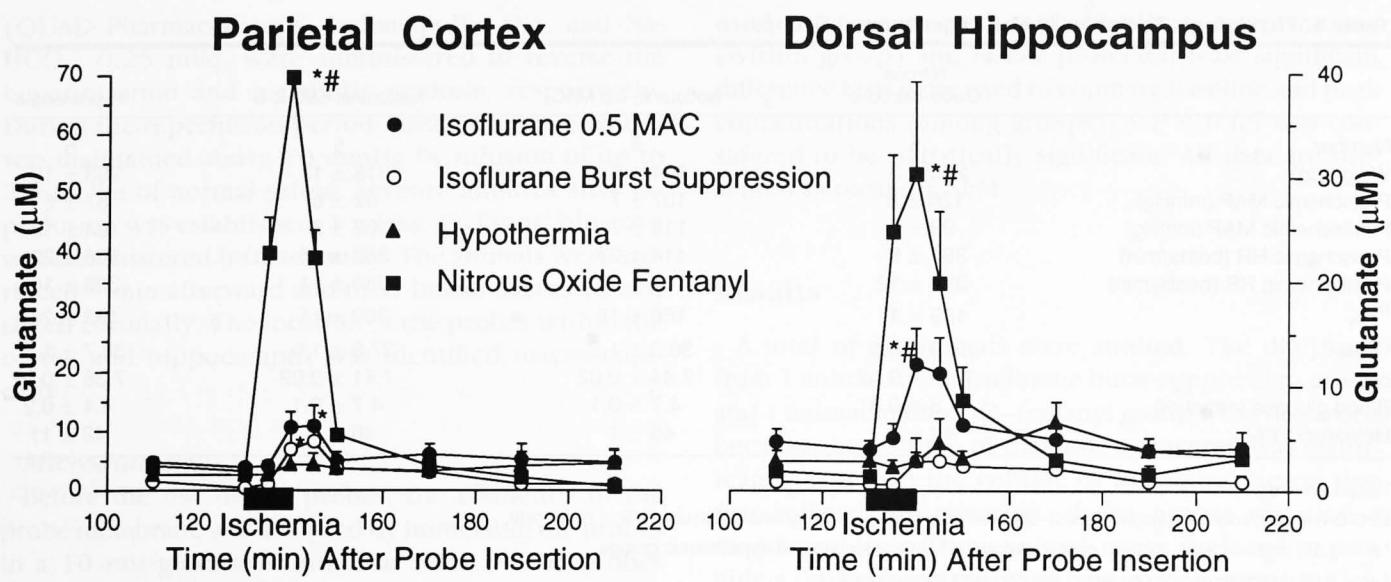

Fig. 1. Glutamate concentrations in the dialysate from the parietal cortex (*left*) and the dorsal hippocampus (*right*) in the four experimental groups. Incomplete forebrain ischemia (solid bar on the abscissa) was induced 130 min after the insertion of the probes. *$P < 0.05$, preischemic *versus* peak intraischemic concentrations within groups; #$P < 0.05$, N_2O–fentanyl group *versus* the other three groups (parietal cortex) and N_2O–fentanyl and isoflurane 0.5 minimum alveolar concentration group *versus* the hypothermic and isoflurane electroencephalographic burst-suppression groups (dorsal hippocampus). Data are means ± SEM.

rable to that of mild hypothermia, an intervention that has been shown previously to reduce ischemia-induced release of glutamate.[5,14]

Currently, the mechanism by which isoflurane reduces ischemia-induced glutamate release is not clear. The glutamate that is released during ischemia is derived from two potential sources: vesicular and nonvesicular cytoplasmic stores. Upon ischemia-induced depolarization, influx of Ca^{2+} into the presynaptic terminal normally leads to the exocytosis of vesicular glu-

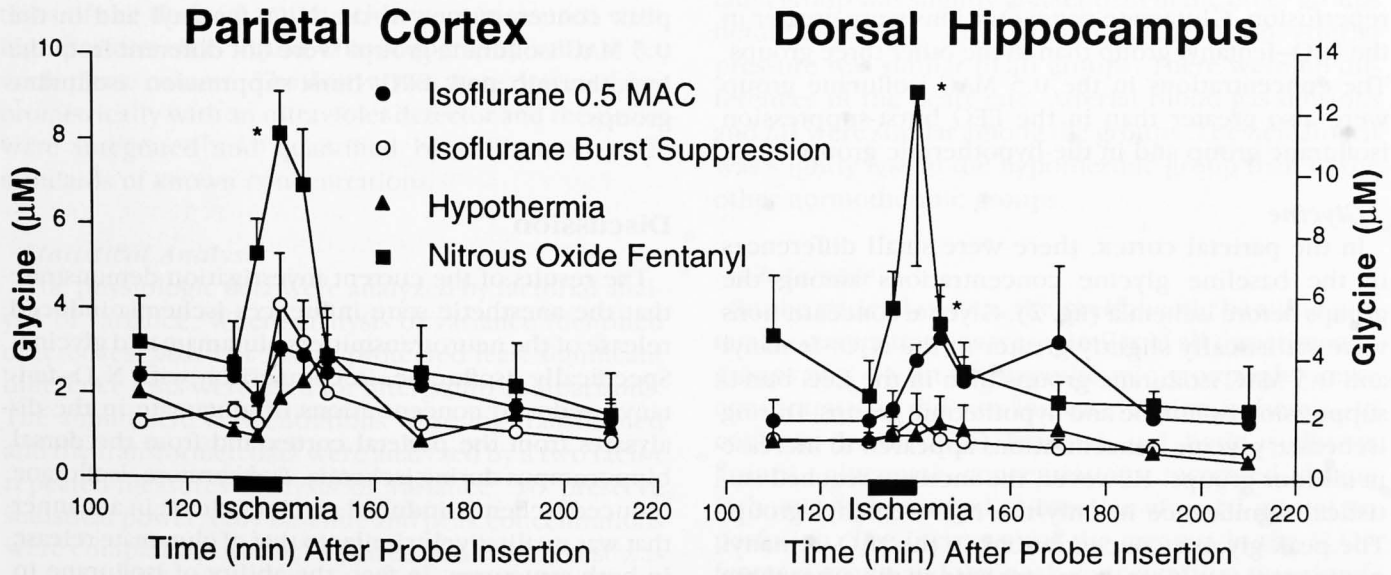

Fig. 2. Glycine concentrations in the dialysate from the parietal cortex (*left*) and the dorsal hippocampus (*right*) in the four experimental groups. Incomplete forebrain ischemia (solid bar on the abscissa) was induced 130 min after the insertion of the probes. *$P < 0.05$, preischemic *versus* peak intraischemic concentrations within groups. Data are means ± SEM.

tamate.[15] This Ca^{2+}-dependent release of vesicular glutamate is an energy requiring process and is inhibited once adenosine triphosphate is depleted.[16] Consequently, this mechanism is probably responsible for little of the glutamate release that occurs during ischemia. Depletion of adenosine triphosphate also leads to the collapse of the normal Na^+ gradient across the neuronal cell membrane. The subsequent reversal of the glutamate transporter[17] can lead to further glutamate efflux into the extracellular space. It is conceivable that isoflurane may have reduced glutamate release by inhibiting both the Ca^{2+}-dependent and Ca^{2+}-independent release processes. Although the effect of isoflurane on glutamate release has not been evaluated specifically, isoflurane has been shown to reduce the stimulated release of neurotransmitters in *in vitro* preparations.[18,19] The reduction of transmitter release has been attributed to an anesthetic mediated reduction in Ca^{2+} influx into the cells.[18,19] The ability of isoflurane to reduce Ca^{2+}-dependent transmitter release may have contributed to the reduction in glutamate release that was observed in the current investigation.

A second possibility is that isoflurane may have inhibited the release of glutamate from nonvesicular cytoplasmic sources by affecting the glutamate transporter. Arai *et al.* have shown that halothane does not interfere with the release or uptake of labeled aspartate (aspartate is also transported by the glutamate transporter).[20] If these results are also applicable to isoflurane, then the results of Arai *et al.*[20] suggest that isoflurane does not interfere with the function of the glutamate transporter *per se*. However, it is possible that isoflurane may have delayed Ca^{2+}-independent release of glutamate by delaying the onset of ischemia-induced anoxic depolarization. The glutamate release in large part results from reversal of the glutamate transporter, which occurs with the onset of anoxic depolarization and adenosine triphosphate depletion.[21–23] Isoflurane has been shown to delay the onset of anoxic depolarization in rats subjected to incomplete forebrain ischemia.[24] This may explain the lower glutamate concentrations in the two isoflurane groups. However, data in support of this supposition are lacking, and further experimental clarification, particularly of the effect of anesthetic agents on ischemia-induced glutamate release with identical intervals of anoxic depolarization, is needed.

The observation that isoflurane reduced ischemia-induced glutamate release in a model of severe incomplete forebrain ischemia suggests that isoflurane might also reduce ischemic neuronal injury. In models of forebrain ischemia, isoflurane does not reduce injury in comparison with a N_2O-anesthetized[25] or halothane-anesthetized[26] states. Such a lack of correlation between dialysate glutamate concentrations and neuronal outcome after ischemia has been reported before. Lekieffre and colleagues have reported that, in rats subjected to forebrain ischemia, kynurenic acid inhibited ischemia-induced glutamate release in the hippocampus but that it did not reduce injury.[27] The results of that study and of the current study question the relevance of ischemia-induced glutamate release to neuronal injury. The data supporting the pivotal role of excessive glutamate release during ischemia in neuronal death are particularly strong (reviewed recently by Diemer *et al.*[28]) and cannot be refuted easily. However, the efficacy of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole) antagonists[29,30] and mild hypothermia[31] in reducing ischemic neuronal injury even when applied in the postischemic phase (when extracellular glutamate concentrations have returned to baseline) suggests that processes other than intraischemic glutamate release are also important to postischemic neuronal outcome. A speculative explanation for the results of the current study (apparent dissociation between glutamate release, as assessed by microdialysis, and neuronal outcome) is that isoflurane, even when it reduces intraischemic glutamate release, does not modulate pathophysiologic processes in the postischemic phase to appreciably reduce neuronal injury. If so, then the data do suggest that inhibition of glutamate release *in isolation* may not be sufficient to reduce injury in the setting of forebrain ischemia.

Not all the data regarding the influence of volatile anesthetics on ischemia-induced glutamate release are consistent. Although our results are in keeping with those of Koorn *et al.*, who reported recently that isoflurane substantially reduced ischemia-induced dopamine release in the striatum of rats subjected to forebrain ischemia,[32] they differ from those of Illievich *et al.*[13] These investigators observed that, in a rabbit model of global ischemia, isoflurane in EEG burst-suppression doses did not reduce ischemia-induced glutamate release in the hippocampus when compared with a 1 MAC halothane anesthetized state.[13] In fact, in both the halothane- and the isoflurane-anesthetized rabbits, a three- to fourfold increase in glutamate concentrations was evident, whereas in hypothermic rabbits, glutamate release was attenuated substantially. The reason for these

apparently discrepant results is not clear. They may be related to interspecies differences (rat *vs.* rabbit) or perhaps to the severity of ischemia (forebrain *vs.* global ischemia). Although the results of the study of Illievich *et al.*[13] and those of the current investigation do not completely define the relative effect of volatile anesthetics and mild hypothermia on ischemia-induced glutamate release, our data do demonstrate unequivocally that isoflurane reduces glutamate release in comparison with N_2O–fentanyl control (15-fold increase occurred in this group).

Control group animals received fentanyl and the possibility that glutamate concentrations were influenced by this narcotic should be considered. The opioid receptor antagonist nalmefene has been shown to reduce ischemia-induced glutamate and glycine release in the hippocampus of rats subjected to global ischemia.[33] However, the ischemia-induced glutamate release inhibiting effect of nalmefene has been attributed primarily to its ability to antagonize κ opioid receptors and not μ receptors.[33] Fentanyl is a μ receptor agonist with insignificant activity at κ receptors[34] and therefore, would not be expected to modulate ischemia-induced glutamate release. Given these data, it seems unlikely that fentanyl influenced the glutamate concentrations in the dialysate to any great degree.

In summary, in a rat model of severe incomplete forebrain ischemia, isoflurane reduced ischemia-induced glutamate and glycine release in a dose-dependent manner. In the hippocampus, this reduction in neurotransmitter release was similar in magnitude to that associated with mild hypothermia. The data suggest that reduced ischemia-induced excitatory neurotransmitter release may contribute to the protective effects attributed to isoflurane in some experimental situations. However, because a protective effect of isoflurane has been difficult to demonstrate in many experimental situations[25,35–37] and because hypothermia appears to provide greater degrees of protection than volatile agents,[26] our data suggest that the reduction of excitatory neurotransmitter release may be a necessary but not sufficient condition for achieving cerebral protection. In fact, an evaluation of the effect of isoflurane on ischemia-induced glutamate release and on histopathologic injury in models of focal ischemia is necessary before it can be determined whether a relation between its effect on glutamate release and on neuronal outcome exists.

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