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Isoflurane Delays but Does Not Prevent Cerebral Infarction in Rats Subjected to Focal Ischemia

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Background: Several investigations have shown that volatile anesthetics can reduce ischemic cerebral injury. In these studies, however, neurologic injury was evaluated only after a short recovery period. Recent data suggest that injury caused by ischemia is a dynamic process characterized by continual neuronal loss for a prolonged period. Whether isoflurane-mediated neuroprotection is sustained after a longer recovery period is not known. The current study was conducted to compare the effect of isoflurane on brain injury after short (2-day) and long (14-day) recovery periods in rats subjected to focal ischemia.

Methods: Fasted Wistar-Kyoto rats were anesthetized with isoflurane and randomly allocated to an awake (n = 36) or an isoflurane (n = 34) group. Animals in both groups were subjected to focal ischemia by filament occlusion of the middle cerebral artery. Pericranial temperature was servocontrolled at 37°C throughout the experiment. In the awake group, isoflu-

rane was discontinued and the animals were allowed to awaken. In the isoflurane group, isoflurane anesthesia was maintained at 1.5 times the minimum alveolar concentration. After 70 min of focal ischemia, the filament was removed. Animals were killed 2 days (awake, n = 18; isoflurane, n = 17) and 14 days (awake, n = 18; isoflurane, n = 17) after ischemia. The volumes of cerebral infarction and selective neuronal necrosis in the animals were determined by image analysis of hematoxylin and eosin-stained coronal brain sections.

Results: Cortical and subcortical volumes of infarction were significantly less in the isoflurane 2-day group (26 ± 23 mm³ and 17 ± 6 mm³, respectively) than in the awake 2-day group (58 ± 35 mm³, $P < 0.01$; and 28 ± 12 mm³, $P < 0.01$, respectively). By contrast, cortical and subcortical volumes of infarction in the awake (41 ± 31 mm³ and 28 ± 16 mm³, respectively) and isoflurane (41 ± 35 mm³ and 19 ± 8 mm³, respectively) 14-day groups were not different (cortex, $P = 0.99$; subcortex, $P = 0.08$). The volume of cortical tissue in which selective neuronal necrosis was observed, however, was significantly less in the isoflurane 14-day group (5 ± 4 mm³) than in the awake 14-day group (17 ± 9 mm³, $P < 0.01$). The total number of necrotic neurons in the region of selective neuronal necrosis was significantly smaller in the isoflurane 14-day group than in the awake 14-day group ($P < 0.01$).

Conclusion: Compared with the awake state, isoflurane reduced the extent of infarction assessed 2 days after focal ischemia in rats. At 14 days, however, only selective neuronal necrosis, but not infarction, was reduced by isoflurane. These results suggest that isoflurane delays but does not prevent cerebral infarction caused by focal ischemia. Isoflurane may attenuate the delayed development of selective neuronal necrosis in periinfarct areas in this animal model. (Key words: Infarction; volume; middle cerebral artery occlusion; volatile anesthetics.)

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A NUMBER of investigations have shown that volatile anesthetics can reduce the ischemic cerebral injury.¹⁻³ Warner *et al.*^{1,2} demonstrated that halothane and sevoflurane both substantially reduced the volume of infarction after focal ischemia compared with the awake state. Baughman *et al.*^{3,4} showed that isoflurane reduced ischemic cerebral injury in a model of hemispheric ischemia compared with a nitrous oxide-fentanyl anesthetized state. Miura *et al.*⁵ demonstrated that hippocampal CA1 and cortical injury after near-complete global ischemia were less in rats anesthetized with isoflurane com-

pared with ketamine or 70% nitrous oxide and fentanyl. Soonthon-Brant *et al.*⁶ also showed that infarct volume after focal cerebral ischemia in the rat anesthetized with isoflurane was significantly less than in either awake or fentanyl-sedated animals. In these studies, however, volatile anesthetic-mediated neuroprotection was shown after a survival period of 16 h to 7 days after ischemia.

Recent data suggest that injury caused by ischemia is a dynamic process characterized by ongoing neuronal loss for at least 14 days after ischemia.⁸⁻¹¹ For example, Due *et al.*⁸ showed that, in rats subjected to 30 min of focal ischemia, cerebral injury was not apparent 24 h after ischemia. After a 14-day recovery period, however, substantial cerebral infarction was evident. It is therefore conceivable that volatile anesthetic-mediated neuroprotection, which has been shown repeatedly, may be a result of short recovery periods. The current study therefore was conducted to evaluate the effect of isoflurane on brain injury after short (2-day) and long (14-day) recovery periods in rats subjected to focal ischemia and to determine, by comparison with nonanesthetized ischemic control animals, whether any neuroprotective effect of isoflurane is evident after 14 days of survival.

Materials and Methods

The study was approved by our institutional animal care and use committee. All experimental procedures were performed in accordance with the guidelines established in the PHS Guide for the Care and Use of Laboratory Animals.

Wistar-Kyoto rats (Simonson Laboratories, San Diego, CA) weighing 275–325 g fasted overnight. Access to water was provided. The rats were anesthetized with an inspired concentration of 5% isoflurane (Ohmeda, Liberty Corner, NJ). Animal tracheas were intubated, and lungs were ventilated mechanically with a gas mixture of 30% oxygen and 70% nitrogen. The end-tidal concentration of isoflurane was reduced to 2.5%. A needle thermistor (Mon-a-Therm; Mallinckrodt, St. Louis, MO) was inserted between the temporalis muscle and the skull, and the pericranial temperature was servocontrolled to $37.0 \pm 0.2^\circ\text{C}$ by surface heating or cooling. A cannula was inserted in the tail artery using PE-50 tubing. The mean arterial pressure was monitored continuously. A cannula was inserted in the right external jugular vein using PE-50 tubing.

The animals were prepared surgically for the occlusion of the middle cerebral artery according to the technique

of Zea-Longa *et al.*¹² The right common carotid artery was exposed via a midline pretracheal incision. The vagus and sympathetic nerves were separated carefully from the artery. The external carotid artery was ligated 2 mm distal to the bifurcation of the common carotid artery. The internal carotid artery was dissected distally to expose the origin of the pterygopalatine artery. The common carotid artery then was ligated permanently 5–10 mm proximal to its bifurcation. Baseline values for arterial oxygen (Pa_{O_2}) and carbon dioxide (Pa_{CO_2}) tensions and pH, plasma glucose concentration, hematocrit, mean arterial pressure, and heart rate were measured. Via a small arteriotomy, a 0.25-mm-diameter nylon monofilament previously coated with silicone was inserted into the proximal common carotid artery and was advanced into the internal carotid artery to a distance of 18–20 mm from the carotid artery bifurcation until slight resistance was felt.

With the use of randomization tables, the animals were allocated to one of two experimental groups. In the awake group ($n = 36$), isoflurane administration was discontinued. At resumption of spontaneous ventilatory effort, mechanical ventilation was discontinued, and the endotracheal tube was removed. The animals were transferred to a heated and humidified incubator, through which oxygen was flushed continuously. The animals were anesthetized briefly with isoflurane 6 min before the end of the 70-min ischemic interval. The pretracheal incision was reopened, and the monofilament was removed from the common carotid artery at the end of the 70-min ischemic interval. The tail artery and jugular vein catheters were removed and the wound was resutured. Then the animals were allowed to awaken.

In the isoflurane group ($n = 34$), the end-tidal concentration of isoflurane was reduced to 1.8% (approximately 1.5 times the minimum alveolar concentration [MAC])¹³ after middle cerebral artery occlusion. At the end of the 70-min ischemic interval, the monofilament was removed. The tail artery and jugular vein catheters were removed and the wound was resutured. All wounds were infiltrated with 0.25% bupivacaine (total dose 0.5 mg). Then, isoflurane was discontinued. At resumption of spontaneous ventilatory effort, mechanical ventilation was discontinued, and the endotracheal tube was removed. The animals were transferred to the incubator, as described previously. During the recovery period, the pericranial temperature was recorded at 1-h intervals for 3 h. Thereafter, the temperature probe was removed.

The animals were divided further into four groups with respect to the reperfusion period (2 days or 14

days); the awake 2-day group (n = 18), the awake 14-day group (n = 17), the isoflurane 2-day group (n = 18), and the isoflurane 14-day group (n = 17). Neurologic evaluations were performed at 2 or 14 days after ischemia. Each rat was assigned a score according to an eight-point behavioral rating scale¹⁴: 0 = no neurologic deficit; 1 = failure to extend left forepaw fully; 2 = decreased grip of the left forelimb; 3 = spontaneous movement in all direction, contralateral circling only if pulled by the tail; 4 = circling or walking to the left (or right); 5 = walking only if stimulated; 6 = unresponsiveness to stimulation, with a depressed level of consciousness; and 7 = dead. Neurologic testing was performed by a single observer blinded to group assignment.

After neurologic evaluation, the animals were anesthetized with chloral hydrate. They were killed by transcardiac perfusion with 200 ml of heparinized saline followed by 200 ml phosphate-buffered paraformaldehyde. The animals were decapitated. Their brains were removed carefully, immersed in fixative, and refrigerated at a temperature of approximately 4°C for 24–48 h. The brains then were prepared for histologic analysis. After dehydration in graded concentration of ethanol and butanol, the brains were embedded in paraffin. Eight-micron-thick coronal sections were obtained at 0.75-mm intervals and stained with hematoxylin and eosin.

Selective neuronal necrosis (SNN) and infarction were assessed using light microscopy, and within each section the areas of infarction and SNN were traced. Infarction area was defined as pannecrosis consisting of the loss of affinity for hematoxylin that affects simultaneously all cell types (neuronal, glial, and vascular) except infiltrated inflammatory cells or the areas in which more than 90% of neurons are necrotic. Necrotic neurons were identified as exhibiting one or more of pyknosis, karyorrhexis, and karyolysis, and cytoplasmic eosinophilia or loss of affinity for hematoxylin. The area of SNN was defined as the area in which more than 20% of neurons were necrotic with preserved neuropil. The areas of infarction and SNN were evaluated by image analysis using National Institutes of Health Image 1.60 software and an Apple Power Macintosh 8500 computer (Apple Computer, Cupertino, CA). The total volume of injury was determined by integration of the area of injury in each section (between 9 to 12 sections of the brain, spanning the entire region of ischemic injury, were analyzed) according to the technique of Swanson *et al.*¹⁵ In the animals in the awake 14-day and isoflurane 14-day groups, the total number of necrotic neurons in the area of SNN was counted in a coronal plane 3.0–3.5 mm

posterior to the bregma, as identified by reference to the atlas of the rat brain created by Palkovits and Brownstein.¹⁶ The brain section that manifested the greatest number of selectively necrotic neurons was chosen for analysis. The analysis was performed by two observers who had no previous knowledge of the experimental groups.

The physiologic values and volume of tissue injury were compared among groups by two-factor analysis of variance (Statview 4.0; Abacus Concepts, Berkeley, CA). If the analysis of variance identified significant differences, unpaired *t* tests with Bonferroni corrections were used for intergroup comparisons. Neurologic scores between groups were analyzed by Mann-Whitney U tests. *P* < 0.05 was considered to be statistically significant. All data except for neurologic score are presented as the mean ± SD. Neurologic scores are reported as the median (quartile deviation).

Results

The physiologic variables are presented in table 1. There were no significant differences in weight, mean arterial pressure, heart rate, pH, P_{CO₂}, and Pa_{O₂}, glucose concentration, hematocrit, and pericranial temperature among the four experimental groups.

Of a total of 70 animals studied, 5 (including 3 in the awake groups and 2 in the isoflurane groups) died before histologic analysis. These animals were considered to have experienced neurologic deaths. The results of behavioral testing are shown in table 2. Two days after ischemia, the neurologic scores were significantly lower in the isoflurane group than in the awake group. After 14-day recovery period, however, there were no significant differences in neurologic scores between the awake and isoflurane groups.

Histologic Analysis, 2-day Recovery Period

Cortical and subcortical infarct volumes were significantly smaller in the isoflurane group (26 ± 23 mm³ and 17 ± 6 mm³, respectively) than in the awake group (58 ± 35 mm³, *P* < 0.01; and 28 ± 12 mm³, *P* < 0.01, respectively) (fig. 1). Volumes of SNN in the cortex and subcortex were similar between animals in the awake and isoflurane groups (cortex, *P* = 0.25; subcortex, *P* = 0.25) (fig. 2).

Histologic Analysis, 14-day Recovery Period

Infarct volumes in the cortex and subcortex in the awake and isoflurane groups were similar (cortex *P* = 0.99; sub-

Table 1. Physiologic Variables in the Four Experimental Groups

	Awake-2 d	Iso-2 d	Awake-14 d	Iso-14 d
Number	18	17	18	17
Weight (g)	296 ± 12	295 ± 15	295 ± 11	296 ± 10
Mean arterial pressure (mmHg)				
Before MCAO	91 ± 8	96 ± 11	94 ± 13	97 ± 10
At reperfusion	85 ± 9	89 ± 6	85 ± 9	85 ± 5
Heart rate (beats/min)				
Before MCAO	431 ± 23	422 ± 21	421 ± 27	429 ± 19
At reperfusion	440 ± 12	437 ± 27	437 ± 24	441 ± 24
pH				
Before MCAO	7.38 ± 0.03	7.39 ± 0.05	7.40 ± 0.04	7.40 ± 0.04
At reperfusion	7.40 ± 0.03	7.38 ± 0.04	7.40 ± 0.04	7.39 ± 0.04
P _{CO₂} (mmHg)				
Before MCAO	37 ± 2	36 ± 2	36 ± 2	37 ± 2
At reperfusion	35 ± 2	37 ± 2	35 ± 3	36 ± 2
P _{O₂} (mmHg)				
Before MCAO	171 ± 17	183 ± 17	180 ± 21	166 ± 27
At reperfusion	182 ± 12	174 ± 19	182 ± 13	173 ± 14
Hematocrit (%)				
Before MCAO	41 ± 2	40 ± 2	41 ± 3	41 ± 3
At reperfusion	39 ± 1	39 ± 2	39 ± 3	40 ± 2
Glucose (mg/dl)				
Before MCAO	100 ± 18	97 ± 18	101 ± 17	96 ± 21
Temperature				
Before MCAO	37.1 ± 0.2	37.1 ± 0.1	37.0 ± 0.1	37.0 ± 0.1
I-35	37.0 ± 0.2	37.0 ± 0.1	36.9 ± 0.2	37.0 ± 0.1
At reperfusion	37.1 ± 0.1	37.0 ± 0.1	37.1 ± 0.1	37.0 ± 0.1
R-30	36.6 ± 0.4	36.5 ± 0.4	36.6 ± 0.5	36.5 ± 0.5
R-60	36.9 ± 0.4	36.8 ± 0.5	36.7 ± 0.5	36.8 ± 0.6
R-120	37.2 ± 0.5	37.0 ± 0.5	36.9 ± 0.5	37.0 ± 0.6
R-180	37.3 ± 0.3	37.1 ± 0.5	37.1 ± 0.4	37.0 ± 0.4

Data are mean ± SD.

MCAO = middle cerebral artery occlusion; At reperfusion = 5 min after reperfusion; 2 d, 14 d = 2 days and 14 days of reperfusion periods after MCAO respectively; I-35 = 35 min after MCAO; R-30, 60, 120, 180 = 30, 60, 120, 180 min after reperfusion, respectively.

Table 2. Neurologic Outcome in the Four Experimental Groups

	Awake-2 d	Iso-2 d	Awake-14 d	Iso-14 d
Number	18	17	18	17
Neurologic death	1	1	2	1
Behavioral rating scale	3 (2-4)	2 (2-3)*	2 (2-3)	2 (1-3)

Data are expressed as median (25-75 percentile). 2 d, 14 d; 2 days and 14 days of reperfusion periods after middle cerebral artery occlusion, respectively. Eight-points behavioral rating scale (Stroke 1997;28:2060-5); 0, no neurological deficit; 1, failure to extend light forepaw fully; 2, decreased grip of the left forelimb; 3, spontaneous movement in all directions, circling only if pulled by the tail; 4, circling; 5, walk only when stimulated; 6, unresponsive to stimulation with a depressed level of consciousness; 7, dead.

* $P < 0.05$ versus the awake group.

cortex $P = 0.08$; fig. 1). Within the cortex, the volumes of SNN were significantly less in the isoflurane group (5 ± 4 mm³) than in the awake group (17 ± 9 mm³; $P < 0.01$) (fig. 2). SNN volumes in subcortex were not different

between the groups ($P = 0.15$). Total numbers of necrotic cells in the area of SNN were significantly smaller in the isoflurane group than in the awake group (awake, 669 ± 344; isoflurane 160 ± 153; $P < 0.01$).

Discussion

The results of the current study indicate that the extent of cerebral injury caused by focal ischemia is influenced by the time point at which injury is measured. Isoflurane reduced cerebral infarction 2 days after focal ischemia in comparison to the awake state. If cerebral injury was evaluated 2 weeks after focal ischemia, this difference in infarct volumes was no longer apparent. These data suggest that isoflurane delays the development of cerebral infarction but does not prevent it. Neuronal injury caused by focal ischemia, however, is not limited to the area of completed infarction. A vari-

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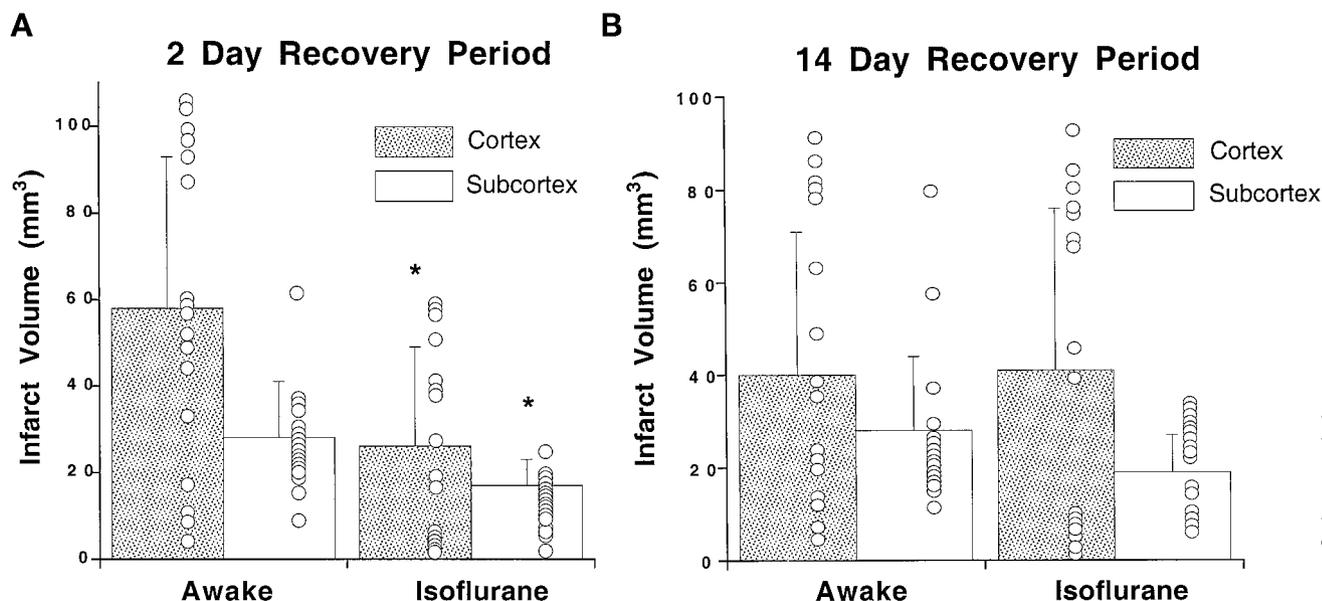


Fig. 1. Cortical and subcortical volumes of infarction 2 days (A) and 14 days (B) after 70 min of middle cerebral artery occlusion in rats of awake and isoflurane-anesthetized groups. Two days after ischemia, cortical and subcortical volumes of infarction were significantly less in the isoflurane group than in the awake group ($P < 0.01$). Data are presented as the mean \pm SD. * $P < 0.01$ versus the awake group.

able region of the cortex surrounding the infarct manifests SNN (neuronal death with preserved neuropil). Within this part of the cortex, isoflurane substantially reduced neuronal injury 2 weeks postischemia.

The effects of isoflurane on neuronal injury observed in the current study are consistent with previous reports in which isoflurane-mediated neuroprotection has been described. Isoflurane reduced neuronal injury in animal models of hemispheric severe cerebral ischemia⁴ and near complete ischemia.⁵ Previous data from our laboratory also have shown that isoflurane can reduce cerebral infarction caused by focal ischemia.⁶ These effects of isoflurane are similar to those of other volatile anesthetics. Both halothane and sevoflurane reduced infarct volume after focal ischemia.¹ Sevoflurane also has been shown to reduce neuronal injury caused by hemispheric ischemia.¹⁷ It is important to note that, in these studies, neuronal outcome was determined between 1 and 7 days postischemia.

The precise mechanism by which isoflurane reduced the brain injury after a short recovery period is not defined clearly. Uncontrolled release of glutamate during ischemia and the consequent excessive stimulation of postsynaptic glutamate receptors (excitotoxicity) plays a major role in the initiation of neuronal injury.^{18,19} A number of investigations indicates that isoflurane can

reduce this excitotoxicity. Eilers *et al.*¹⁸ indicated that one minimum alveolar concentration of isoflurane significantly reduced glutamate release from brain slices during chemical anoxia. Patel *et al.*¹⁹ reported that isoflurane significantly attenuated glutamate release in a dose-dependent fashion in rats subjected to forebrain ischemia. In addition, inhibition of postsynaptic glutamate receptor-mediated responses has been shown. Isoflurane reduced neuronal depolarizing responses evoked by the application of glutamate to neocortical slices²⁰ and the frequency of *N*-methyl-D-aspartate (NMDA) receptor channel opening in response to stimulation by NMDA.²¹ Glutamate-mediated neuronal calcium influx in neocortical cells²² and hippocampal slices²³ is reduced by isoflurane. Data from our laboratory also indicate that isoflurane can reduce both NMDA- and α -amino-3-hydroxy-5-methyl-4-isoxazole-mediated cortical injury *in vivo*.^{24,25} Collectively, these data indicate that attenuation of excitotoxicity by isoflurane may have contributed to the observed reduction in ischemic injury in the isoflurane group 2 days postischemia.

The neuroprotective effect of isoflurane observed at 2 days was not apparent 14 days after ischemia, suggesting that isoflurane delays but does not prevent the development of infarction. This phenomenon also has been reported for other neuroprotective agents. The neuroprotective effect of NMDA receptor antagonists in ani-

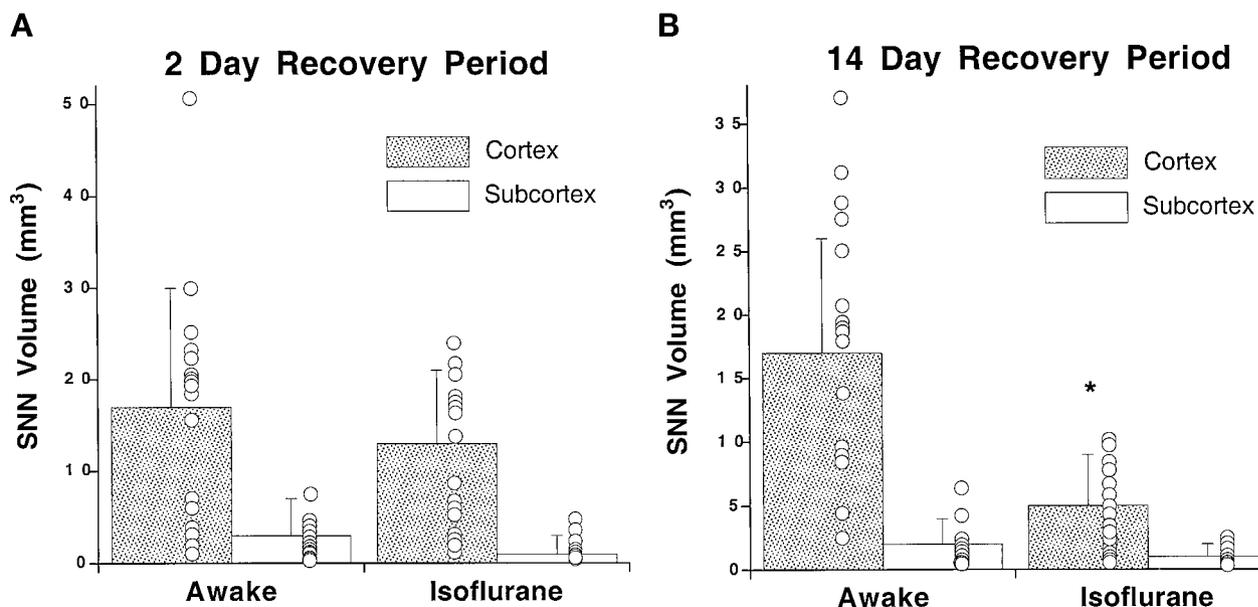


Fig. 2. Cortical and subcortical volumes of selective neuronal necrosis 2 days (A) and 14 days (B) after 70 min of middle cerebral artery occlusion in rats of the awake and isoflurane anesthetized groups. Cortical volume of selective neuronal necrosis was significantly less in the isoflurane group than in the awake group after a 14-day recovery period ($P < 0.01$). Data are presented as the mean \pm SD. * $P < 0.01$ versus the awake group.

mal models of focal cerebral ischemia in which the recovery period was relatively short (up to 2 days) has been well documented.^{26,27} Valtysson *et al.*²⁸ investigated the effect of the noncompetitive NMDA antagonist dizocilpine (MK-801) on histopathologic outcome 28 days after focal cerebral ischemia in rats. They demonstrated that MK-801 did not reduce infarction after a prolonged survival period and suggested that this NMDA antagonist may only delay the progression of ischemic brain damage. By contrast, Sauer *et al.*²⁹ showed that the competitive NMDA antagonist CGP40116 substantially reduced ischemic injury both 2 days and 28 days after focal ischemia in rats. The discrepancy between the different studies may result from the different pharmacokinetic properties of MK-801 and CGP40116. Sauer *et al.*²⁹ suggested that competitive NMDA antagonists like CGP40116 have longer half-lives in rat brain compared with MK-801 and therefore might be able to block the receptor for a longer time period. Because isoflurane is a short-acting agent and its half-life is shorter than MK-801, administration of isoflurane for a much longer period might result in long-term neuroprotection comparable to that achieved with CGP40116.

Our data suggest that, after focal ischemia, there is a delayed progression of ischemic injury beyond 48 h postischemia. Du *et al.*⁸ similarly demonstrated that neu-

ronal injury after focal ischemia continues long after the immediate reperfusion period and suggested that this delayed neuronal death may result at least in part from the process of apoptosis (cell suicide). If apoptosis is major mechanism leading to delayed neuronal death, then the observation that isoflurane did not reduce infarct volume 2 weeks after ischemia suggests that isoflurane does not inhibit apoptosis (as opposed to excitotoxic neuronal death). This premise, however, is speculative, and the effect of isoflurane on ischemia-induced neuronal apoptosis remains to be determined.

Two distinct morphologic patterns of injury produced by focal cerebral ischemia have been described.^{11,30-32} One is tissue necrosis, in which all neurons, glial cells, and endothelial cells are involved. This rapid progressive damage is called *infarction* or *pannecrosis*. The other pattern is a nonacute type of injury that is characterized by SNN. Investigations of temporary focal cerebral ischemia have shown that SNN occurs in regions of the brain in which ischemia is not severe.³¹ Unlike infarction, which progresses rapidly, the evolution of SNN occurs over a relatively long period (a few months),¹¹ and the contribution of SNN to total brain injury is significant. It is precisely in the regions of the cortex that manifested SNN that isoflurane mediated neuroprotection was evident. Therefore, isoflurane appears to exert differential

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effects on tissue destined to undergo infarction (severe ischemia) than on tissue that undergoes SNN (mild to moderate ischemia).

The mechanisms by which isoflurane reduced the extent of SNN, but not infarct, 14 days after ischemia are unknown. One possible mechanism is the inhibition of spreading depression, such as ischemic depolarizations that occur during focal ischemia. These ischemic depolarizations have been shown to increase neuronal calcium influx during ischemia, thereby increasing brain injury.³⁶ Recent data, reported by Back and colleagues,³⁴ indicate that ischemic depolarizations during focal ischemia do not increase the infarct volume but contribute significantly to the development of SNN. Agents such as MK-801, which can prevent ischemic depolarizations, also can reduce SNN substantially.³⁵ Previous work in our laboratory has shown that isoflurane can reduce both the frequency of ischemic depolarizations during focal ischemia and infarct volume.³⁷ Together, these studies suggest that the reduction in SNN that was observed in the current study might be mediated in part by a reduction in the frequency of ischemic depolarizations during isoflurane anesthesia.

In summary, compared with the awake state, isoflurane reduced the extent of ischemic injury 2 days after focal ischemia. This infarction-sparing effect was not apparent after a 2-week recovery period, indicating that isoflurane delays but does not prevent cerebral infarction caused by focal ischemia. This observation is noteworthy because, by delaying the development of infarction, isoflurane might increase the therapeutic window for other putative neuroprotective agents. Isoflurane significantly reduced SNN 2 weeks after the ischemia. The results suggest that, in a model of focal ischemia, isoflurane can reduce injury in regions of the brain in which ischemia is not severe. Our results also confirm that, in experimental studies in which the neuroprotective effects of pharmacologic agents are evaluated, consideration should be given to the selection of the appropriate time point at which to measure the extent of the histopathologic injury.

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