

Effects of Isoflurane Versus Fentanyl–Nitrous Oxide Anesthesia on Long-term Outcome from Severe Forebrain Ischemia in the Rat

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Background: This study examined long-term outcome from severe forebrain ischemia in the rat, as a function of anesthetic given during the ischemic injury.

Methods: Rats were subjected to 10 min of near-complete forebrain ischemia while anesthetized with either 1.4% isoflurane or 70% nitrous oxide–fentanyl. Neurologic and histologic outcomes were measured at 5 days, 3 weeks, or 3 months after ischemia.

Results: At 5 days, isoflurane-anesthetized rats had less damage than did fentanyl–nitrous oxide–anesthetized rats (mean \pm SD, percent alive hippocampal CA1 neurons = 58 ± 29 vs. 20 ± 16 , respectively; $P = 0.011$). This was accompanied by improved motor function in the isoflurane group ($P = 0.002$). At 3 weeks, there was no difference between groups for either outcome variable (percent alive CA1 neurons = 35 ± 26 and 36 ± 28 for isoflurane and fentanyl–nitrous oxide, respectively). Similarly, at 3 months, there was no difference between groups (percent alive CA1 neurons = 56 ± 27 and 60 ± 27 for isoflurane and fentanyl–nitrous oxide, respectively). Morris water maze performance at 3 months was similar between anesthetic groups and was also similar to sham performance. The percent alive CA1 neurons in the fentanyl–nitrous oxide group increased with duration of recovery ($P = 0.004$). There were no differences among isoflurane groups over time (5 days vs. 3 weeks, $P = 0.26$; 5 days vs. 3 months, $P = 0.99$; 3 week vs. 3 months, $P = 0.32$).

Conclusions: This study found no change in the percent alive CA1 hippocampal neurons as a function of duration of recovery from severe forebrain ischemia in isoflurane anesthetized rats. In contrast, the percent alive CA1 neurons in fentanyl–nitrous oxide–anesthetized rats tripled over 3 months of recovery. The natural history of long-term responses to forebrain ischemia requires further study before conclusions can be drawn with respect to the permanence of isoflurane neuroprotection.

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BECAUSE of the potentially devastating consequences of cerebral ischemia and the risks of such injuries during anesthesia and surgery, efforts have been made for at least 40 yr to pharmacologically increase the tolerance of the brain to ischemia.¹ Volatile anesthetics make good candidates as neuroprotectants for several reasons. It is intuitively attractive to consider drugs that depress cerebral energy requirements. More important, volatile anesthetics simultaneously interact with multiple receptor systems, potentially providing superior benefit over drugs that have singular receptor interactions within the complex milieu of ischemia–reperfusion. Indeed, well-controlled laboratory studies have shown substantially improved outcome in animals subjected to an ischemic injury while anesthetized with various volatile anesthetics.^{2–6} Most of these positive studies have accounted for potentially confounding side effects of anesthetics that might alter outcome independent of direct effects on neural substrate (e.g., perfusion pressure, temperature, and blood glucose).

Isoflurane is a neuroprotective anesthetic candidate. It delays onset of terminal depolarization,⁷ reduces quantity of tissue at risk for infarction (as defined by cerebral blood flow thresholds),⁸ antagonizes glutamate at the *N*-methyl-D-aspartate receptor and blocks *in vivo* glutamatergic excitotoxicity,^{9–11} inhibits penumbral spontaneous depolarizations,¹² and potentiates γ -amino butyric acid type A-mediated neurotransmission.^{13,14} Isoflurane has been shown protective against focal or global cerebral ischemia in the mouse,¹⁵ rat,^{2,4,6} and dog.⁵ All such studies, however, assessed outcome at intervals of 1–5 days after ischemia. Concern was recently raised by a report that the protective effect of isoflurane against focal cerebral ischemia, apparent at 48 h after ischemia, had completely dissipated when outcome was assessed at 2 weeks after ischemia.¹⁶ This is consistent with the failure of several other interventions to provide persistent protection, including brief (but not prolonged¹⁷) postischemic moderate hypothermia,¹⁸ dizocilpine,^{19,20} 1,2,3,4-tetrahydro-6-nitro-2,3-dioxo-benzo(f)quinolone-7-sulfonamide (NBQX),²¹ and ziconitide.²¹ We have reported substantial neuroprotective benefit from isoflurane in rat and mouse models of severe forebrain ischemia with outcome assessed at 1–5 days after ischemia.^{6,15,22} The pathophysiologic responses to focal stroke *versus* severe forebrain ischemia (such as could occur during cardiac arrest) are inherently different (i.e., pancellular necrosis *vs.* selective neuronal necrosis, re-

spectively). We hypothesized that isoflurane protection against a severe forebrain ischemic injury would persist over prolonged durations of recovery.

Materials and Methods

The following study was approved by the Duke University Animal Care and Use committee (Durham, North Carolina). Male Sprague-Dawley rats (age, 8–10 weeks; Harlan Sprague-Dawley, Inc., Indianapolis, IN) were fasted in regard to food but were allowed free access to water for 12–16 h before the experiments. The animals were anesthetized with 5% isoflurane in oxygen. After orotracheal intubation, the lungs were mechanically ventilated (30% O₂–balance N₂) to maintain normocapnia. The inspired isoflurane concentration was reduced to 1–2%. Surgery was performed with an aseptic technique. The tail artery was cannulated and used for blood pressure monitoring and blood sampling. *Via* a ventral neck incision, the right jugular vein was cannulated with a silicone catheter for drug infusion and blood withdrawal. The common carotid arteries were encircled with suture. The vagus nerves and cervical sympathetic plexus were left intact. Muscle paralysis was provided by a 1-mg intravenous bolus of succinylcholine, repeated as necessary to allow control of ventilation during ischemia. Pilot studies had been performed to assure that rats would not exhibit an escape response in the absence of succinylcholine given the respective anesthetic regimens. A bilateral cortical electroencephalogram was continuously monitored during the experiment from active subdermal electrodes positioned over the parietal cortex bilaterally, a reference electrode placed on the nasion, and a ground lead positioned in the tail.

A 22-gauge needle thermistor (model 524; YSI Co., Yellow Springs, OH) was percutaneously placed adjacent to the skull beneath the temporalis muscle, and pericranial temperature was servoregulated (model 73ATA Indicating Controller; YSI Co.) at $37.5 \pm 0.1^\circ\text{C}$ by surface heating or cooling. Heparin (50 U) was given intravenously. Exposure to isoflurane during anesthesia induction and surgical preparation lasted approximately 1 h.

Rats were then randomly assigned to one of two anesthetic conditions. In one group, delivered isoflurane was adjusted to 1.4% in 30% O₂–balance N₂. In the other group, isoflurane was discontinued, and the inspiratory gas mixture was changed to 70% N₂O in 30% O₂. Fentanyl was given as a 10- $\mu\text{g}/\text{kg}$ intravenous bolus followed by 25 $\mu\text{g} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$. The anesthetic doses in the two groups were chosen on the basis of previous studies that showed short-term (*i.e.*, 3–5 days) outcome differences, as a function of these anesthetic conditions, in rats and mice subjected to 10 min of severe forebrain ischemia.^{6,15,22} Anesthetic conditions were established 30 min before ischemia, during which time rats in both

groups were allowed to stabilize physiologically. Arterial blood gases and hematocrit were measured 10 min before ischemia and at 10 and 60 min after ischemia. Blood glucose was measured at 10 min before ischemia.

Ischemia was then induced by withdrawal of 6–10 ml blood from the jugular catheter so as to reduce mean arterial blood pressure to 25–30 mmHg.^{23,24} The carotids were then occluded with aneurysm clips, and a timer was started. Ischemia persisted for 10 min and was confirmed by the presence of an isoelectric electroencephalogram.

To terminate ischemia, shed blood was reinfused, and the aneurysm clips were removed. NaHCO₃ (0.3 mEq intravenous) was given to counteract systemic acidosis. The catheters were removed. The wounds infiltrated with 1% lidocaine and closed with suture. Anesthetic agents were continued after ischemia for variable periods of time (isoflurane, 110 min; fentanyl, 80 min; nitrous oxide, 110 min) to ensure that animals in both groups would recover the righting reflex at approximately 2 h after onset of reperfusion. On recovery of spontaneous ventilation, the trachea was extubated, and the rats were allowed to recover in an oxygen-enriched environment (fraction of inspired oxygen = 0.4). Temperature regulation was continued until recovery of the righting reflex. Animals were then returned to their cages with free access to food and water.

For each recovery interval, a set of sham rats was generated ($n = 6$). These animals were exposed to all aspects of isoflurane anesthesia and surgical preparation for ischemia. The carotids were not occluded, and systemic hypotension was not used.

After completion of the ischemia protocol, rats were allowed to recover for 5 days, 3 weeks, or 3 months ($n = 10$ –13 rats per anesthetic condition per recovery interval). On the final day of recovery, with the observer blinded to group assignment, motor function tests were performed according to an established protocol, including assays of prehensile traction and balance beam performance.^{25,26} The motor score was graded on a 0–9 scale (best score = 9).

In the 3-month recovery group, Morris water maze (place set) testing was performed over 5 days before euthanasia.²⁷ Spatial learning was tested in a black circular pool (having fixed visual cues) filled with water (27°C) so that the surface was 3 cm higher than a submerged invisible platform. The position of the platform was kept unchanged throughout testing. Rats were tested for 5 consecutive days, with four trials per day, beginning 86 days after ischemia. The rat was introduced in the pool at one of four equally spaced locations (randomly assigned with the provision that on each day, all four locations were tested) and was allowed to swim for a maximum of 90 s. If the platform was not found, the rat was placed by hand on the platform and left there for 30 s in the first trial and for 15 s on any

subsequent trial. Four trials were performed each day (intertrial interval = 10 min). The latency to escape times were captured using a computerized tracking system (Ethovision 2.2.14; Noldus Information Technology, Leesburg, VA).

After neurologic evaluation, the rats were anesthetized with isoflurane, and the brains were fixed *in situ* by intraaortic infusion of buffered 10% formalin. After 24 h, the brains were removed and stored in 10% formalin. Paraffin-embedded brain sections were serially cut (5 μ m thick) and stained with acid fuchsin-celestine blue. With the investigator blinded to group assignment, injury to the hippocampal CA1 sector was evaluated by light microscopy. CA1 neuronal necrosis peaks at 3 days after ischemia, followed by progressive phagocytosis.²⁸ Therefore, the following procedure was used to assess the fraction of CA1 neurons surviving ischemia. First, the total number of hemispheric CA1 neurons at bregma -4.0 mm was counted and averaged for each sham group to account for any effect of aging on CA1 cell counts during the recovery interval. This value was taken as the common denominator for each respective recovery interval. Then, the number of viable CA1 neurons was counted in each hemisphere of the rats exposed to ischemia. Viable neurons were considered to be those with a blue hue and also an intact plasma membrane and visible nucleus. For each animal, the value from the hemisphere with the least number of surviving CA1 neurons was taken for further analysis. This value was divided by the respective common denominator, and the fraction was multiplied by 100 to obtain the percent alive CA1 neurons. In the 5-day animals, the cells were counted a second time. In this case, both viable and nonviable neurons were counted. Nonviable neurons were considered to be those being pyknotic and having an acidophilic reddish hue.²⁹ The total number of neurons (viable plus nonviable) was calculated for each animal. The percent alive CA1 neurons was calculated as viable neurons/total neurons \times 100. This process resulted in values almost identical to those obtained by use of the sham values as the denominator for the 5-day recovery group (linear regression $R^2 = 0.96$), validating concordance between the counting techniques. To provide consistency in counting technique across all recovery intervals, statistical analysis was performed using percent surviving neurons values calculated by use of respective sham common denominator values for each recovery interval.

Given the results of the above histologic analysis, the remaining tissue blocks from rats allowed to survive 3 months were again sectioned. The goal of this assessment was to determine whether the cells counted as survivors using acid fuchsin-celestine blue were in fact neurons by use of NeuN, a neuron-specific immunohistochemical marker.³⁰ Because this was a *post hoc* analysis, some tissue was lost (approximately 400 μ m) be-

tween the sections obtained for acid fuchsin-celestine blue *versus* NeuN staining. Coronal sections (5 μ m thick) were deparaffinized and hydrated in distilled water, then placed in a Coplin jar containing 0.01 M citrate buffer (pH 6.0) for antigen retrieval using a microwave set at 80% power for 5 min \times 2. Slides were kept in buffer for an additional 30 min until buffer reached room temperature in preparation for immunohistochemistry as follows. Slides were treated with 0.3% H_2O_2 -0.1% NaN_3 for 10 min, twice washed in phosphate buffered saline, incubated in 2% horse serum for 5 min, incubated in 10% horse serum for 30 min, and then incubated overnight in mouse antineuronal nuclei (NeuN) antibody (Fisher Chemical, Tustin, CA), 1:1,000 dilution, at 4°C. Slides were then washed in phosphate-buffered saline for 10 min \times 3, incubated in fluorescein isothiocyanate conjugated horse anti-mouse immunoglobulin G antibody, 1:200 dilution (Vector Labs, Burlingame, CA), for 3 h, and washed in phosphate-buffered saline for 10 min \times 3. A vectashield cover slip with 4',6-diamidino-2-phenylindole (Vector Labs) was applied and the slides stored at 4°C until analyzed.

NeuN-positive cells in hippocampal CA1 were counted using fluorescence microscopy. As was done for the acid fuchsin-celestine blue study, CA1 cell counts were made in both hemispheres. The hemisphere with the smallest number of NeuN-positive cells was used for further analysis. To define the percent alive neurons, the number of CA1 NeuN-positive cells in each animal was divided by the mean number of hemispheric CA1 NeuN-positive cells in the sham group (\times 100).

All experimental procedures with the exception of ischemia were performed by an experimenter blinded to group assignment.

Statistical Analysis

Physiologic values were compared qualitatively to conserve statistical power. The primary dependent variable was percent alive CA1 neurons as assessed with acid fuchsin-celestine blue staining. A two-way analysis of variance was used to compare the two anesthetic groups and the three times to recovery. The Tukey-Kramer adjustment for multiple comparisons was applied to pairwise comparisons. NeuN-positive cell counts were compared by the Student *t* test. Total motor scores were compared between anesthetic groups at each recovery interval by the Mann-Whitney U statistic. Morris water maze latencies were compared by repeated-measures analysis of variance with time as the repeated measure. Values are presented as mean \pm SD.

Results

Physiologic values are reported in table 1. There were no important differences among anesthetic groups.

Table 1. Physiologic Values

	Isoflurane			Fentanyl-Nitrous Oxide		
	5 Days	3 Weeks	3 Months	5 Days	3 Weeks	3 Months
No.	11	12	13	12	10	13
Pre-BW, g	294 ± 15	295 ± 14	292 ± 11	298 ± 10	291 ± 19	285 ± 17
Final BW, g	305 ± 20	385 ± 16	463 ± 25	274 ± 16	379 ± 35	465 ± 25
10 min before ischemia						
MAP, mmHg	112 ± 6	105 ± 7	112 ± 12	134 ± 9	121 ± 15	126 ± 10
Arterial pH	7.38 ± .04	7.36 ± .03	7.36 ± .04	7.37 ± .03	7.34 ± .03	7.35 ± .03
Paco ₂ , mmHg	40 ± 3	39 ± 3	40 ± 3	39 ± 2	39 ± 2	40 ± 2
Pao ₂ , mmHg	120 ± 13	121 ± 15	119 ± 14	114 ± 12	122 ± 15	123 ± 11
Blood glucose, mg/dl	82 ± 6	80 ± 7	83 ± 8	87 ± 8	80 ± 7	85 ± 11
Hematocrit, %	43 ± 1	42 ± 2	43 ± 1	43 ± 1	42 ± 2	41 ± 2
10 min after ischemia						
MAP, mmHg	115 ± 4	108 ± 9	113 ± 9	117 ± 5	115 ± 7	112 ± 9
Arterial pH	7.35 ± .03	7.36 ± .03	7.36 ± .04	7.36 ± .04	7.35 ± .04	7.35 ± .03
Paco ₂ , mmHg	39 ± 1	39 ± 2	40 ± 2	40 ± 5	39 ± 2	40 ± 3
Pao ₂ , mmHg	120 ± 15	123 ± 17	123 ± 12	119 ± 15	122 ± 16	127 ± 17
Hematocrit, %	42 ± 1	41 ± 2	41 ± 1	42 ± 1	41 ± 2	41 ± 1
60 min after ischemia						
MAP, mmHg	98 ± 5	90 ± 5	94 ± 5	99 ± 5	94 ± 7	94 ± 9
Arterial pH	7.37 ± .03	7.36 ± .03	7.37 ± .03	7.38 ± .02	7.35 ± .04	7.38 ± .04
Paco ₂ , mmHg	40 ± 3	39 ± 3	39 ± 3	40 ± 3	39 ± 2	39 ± 3
Pao ₂ , mmHg	120 ± 17	118 ± 19	123 ± 12	116 ± 8	120 ± 10	127 ± 17
Hematocrit, %	42 ± 1	41 ± 2	41 ± 1	42 ± 1	40 ± 2	41 ± 2

Values are presented as mean ± SD.

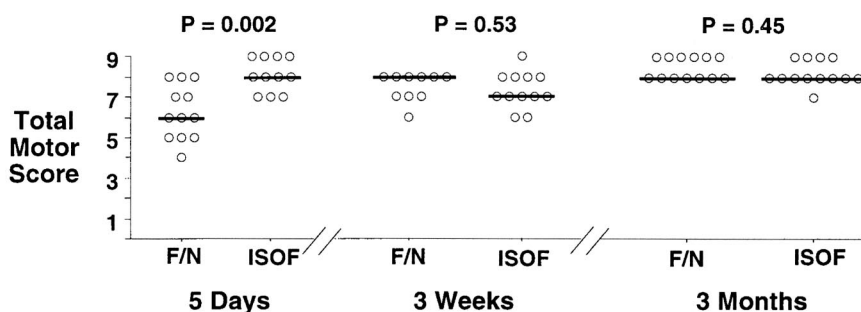
BW = body weight; MAP = mean arterial pressure; Paco₂ = arterial carbon dioxide tension; Pao₂ = arterial oxygen tension.

Total motor scores are reported in figure 1. All sham rats received a score of 9 (data not shown). Scores were better in the isoflurane *versus* fentanyl-nitrous oxide group at 5 days ($P = 0.002$). In contrast, at both 3 weeks ($P = 0.53$) and 3 months ($P = 0.45$) after ischemia, there was no difference between the anesthetic groups. There was no difference between groups for Morris water maze latency at 3 months after ischemia ($P = 0.29$; fig. 2), although there was a main effect for time over the testing interval ($P < 0.001$).

Figure 3 depicts the percent alive hippocampal CA1 neurons as defined by acid fuchsin-celestine blue staining. The distribution of the percent alive CA1 neurons was not severely nonnormal, and no outliers were present. The variances of the groups were similar. The overall analysis of variance model, considering both anesthetic groups and all recovery intervals, was significant ($P = 0.0011$), with $R^2 = 0.264$ (i.e., approximately 26% of the variability of the percent alive CA1 neurons was accounted for by the factors in the model). Although the

anesthetic effect was marginal ($P = 0.089$) and the duration of recovery effect was strong ($P = 0.008$), these effects were modified by their significant interaction ($P = 0.011$). That is, the effect of anesthetic depended on time to recovery, and *vice versa*. The anesthetic groups differed significantly at 5 days after ischemia, when the percent alive CA1 neurons was higher in the isoflurane group ($P = 0.011$). In contrast, there was no difference between anesthetic groups at 3 weeks ($P = 1.0$) or 3 months ($P = 0.99$). In the fentanyl-nitrous oxide groups, the percent alive CA1 neurons varied with duration of recovery, where at 3 months, the percent alive CA1 neurons was greater than at 5 days ($P = 0.004$). There were no differences among isoflurane groups over time (5 days *vs.* 3 weeks, $P = 0.26$; 5 days *vs.* 3 months, $P = 0.99$; 3 weeks *vs.* 3 months, $P = 0.32$). At 3 months, the percent alive CA1 neurons in both anesthetic groups remained less than that observed in the sham group ($P \leq 0.01$).

Fig. 1. Rats were subjected to 10 min of severe forebrain ischemia while anesthetized with fentanyl-nitrous oxide (F/N) or isoflurane (ISOF) and were allowed to survive for 5 days, 3 weeks, or 3 months. The total motor score (9 = normal) is depicted for the respective recovery intervals. There was a difference between anesthetic groups at 5 days ($P = 0.002$) but not at 3 weeks or 3 months after ischemia. Horizontal bars = group median values; open circles = values for individual rats.



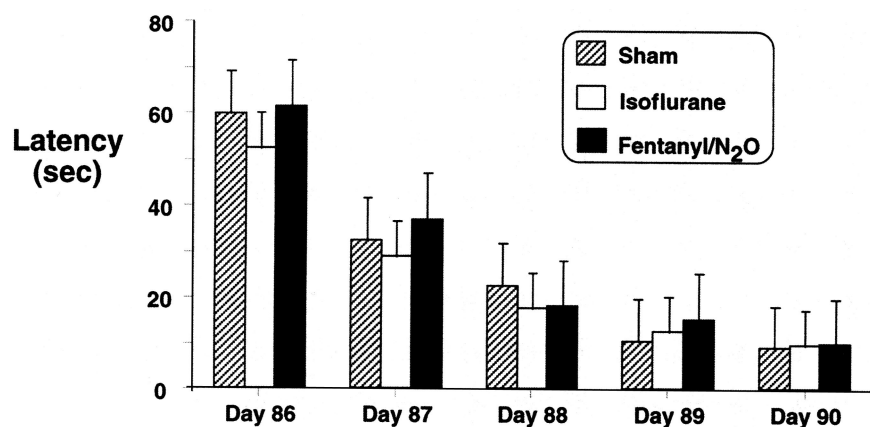


Fig. 2. Rats were allowed to survive for 3 months after 10 min of severe forebrain ischemia while anesthetized with fentanyl-nitrous oxide or isoflurane. Concurrent sham-operated animals were not subjected to ischemia. On days 86–90 after ischemia (or sham surgery), place-set Morris water maze performance was assessed. Although performance improved over the 5-day training intervals in all groups ($P < 0.001$), there were no differences among groups ($P = 0.29$). Values are presented as mean \pm SD.

Analysis of the NeuN results at 3 months after ischemia showed no effect of anesthetic group (number CA1 NeuN positive: fentanyl-nitrous oxide, 320 ± 135 ; isoflurane, 341 ± 124 , $P = 0.69$). The percentages of CA1 neurons alive (fentanyl-nitrous oxide, $70 \pm 27\%$; isoflurane, $74 \pm 27\%$) were numerically greater than values reported for acid fuchsin-celestine (fentanyl-nitrous oxide, $60 \pm 27\%$; isoflurane, $56 \pm 27\%$), perhaps attributable to the more caudal CA1 samples used for NeuN staining.

Discussion

There is a growing body of evidence showing that pharmacologic neuroprotection can have a transient nature, dissipating over the first few weeks of recovery after an ischemic injury.^{19–21} In the current study, both histologic and neurologic outcome from 10 min of severe forebrain ischemia were substantially better in rats anesthetized with isoflurane *versus* fentanyl-nitrous oxide when assessed at 5 days after ischemia. This is consistent with several previous reports.^{6,15,22} However, when recovery was extended to 3 weeks or 3 months, there was no differential effect of intraischemic anesthetic on histologic or behavioral outcome.

The intention of our study was to define the persistence of isoflurane neuroprotection against a severe forebrain ischemic injury. We speculated that findings in our study would be different from those of Kawaguchi *et*

al.,¹⁶ who observed only transient protection by isoflurane in a focal cerebral ischemia model. We hypothesized that absence of a hippocampal ischemic penumbra in the global injury might distinguish the persistence of isoflurane neuroprotection from that observed in a focal lesion.³¹ In support of our hypothesis, Sullivan *et al.*³² reported persistent protection (2 weeks) by isoflurane against oxygen-glucose deprivation in organotypic hippocampal slices, an *in vitro* model used to simulate global cerebral ischemia. Unfortunately, our data neither supports nor refutes the hypothesis that isoflurane neuroprotection is persistent, but it presents potentially important observations relevant to the study of long-term outcome responses to severe forebrain ischemia.

We cannot identify major methodologic issues that might weaken our findings. It is plausible that use of a learning-set rather than place-set Morris water maze would have provided a different result at 3 months.²⁶ However, the decreases in latency times over the 5-day observation interval were nearly identical between either anesthetic-ischemia group and shams, making it unlikely that major memory dysfunction went undetected. This is consistent with the relatively large percent alive CA1 neurons in both groups. *Post hoc*, we questioned the validity of the acid fuchsin-celestine blue histologic analysis because the percent alive CA1 neurons *increased* over the 3-month observation interval in the fentanyl-nitrous oxide group (which presumably was not confounded by any transient preservation of

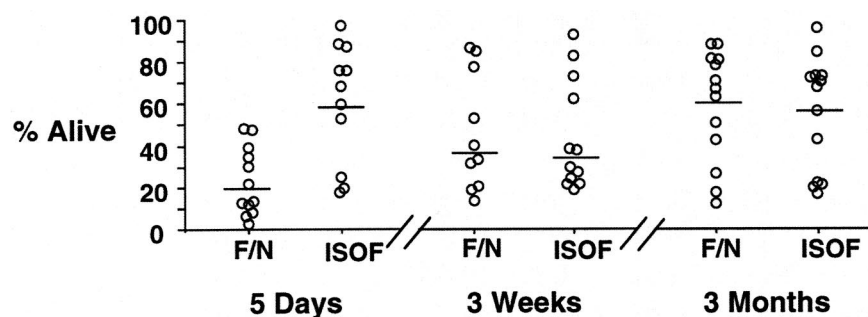


Fig. 3. Rats were subjected to 10 min of severe forebrain ischemia while anesthetized with fentanyl-nitrous oxide (F/N) or isoflurane (ISOF) and were allowed to survive for 5 days, 3 weeks, or 3 months. Percent alive hippocampal CA1 neurons, as assessed with acid fuchsin-celestine blue staining, are shown for the respective recovery intervals. A difference was present between anesthetic groups at 5 days after ischemia ($P = 0.011$). There was no difference between groups at 3 weeks or 3 months. Horizontal bars = group mean values; open circles = values for individual rats.

neuronal survival, as may have been the case for isoflurane). The validity of acid fuchsin staining of acidophilic neurons as a marker of hippocampal neuronal death has been validated by presence of concurrent electron microscopic markers of neuronal death,²⁹ but it remained possible that cells counted as living neurons at 3 months were in fact other cell types. To assure that the counted cells were neurons, the analysis was repeated using NeuN immunohistochemistry, and the results were validated. This is suggestive that neurogenesis occurred, an event others have recently reported in postischemic hippocampus.³³⁻³⁵ However, we cannot claim evidence for neurogenesis in the absence of proof of postischemic mitosis (commonly assessed by use of 5-bromo-2'-deoxyuridine mitotic labeling combined with NeuN immunohistochemistry).

The percent alive CA1 neurons in the fentanyl-nitrous oxide group increased dramatically over the 3-month observation interval (5 days, 20 ± 16 ; 3 months, 60 ± 27). This magnitude of change was not anticipated, despite reports of neurogenesis in the ischemia literature.³³⁻³⁵ However, we are not aware of any studies that have examined forebrain ischemia outcome intervals as long as 3 months. As a result, our study was confounded by a changing baseline in the comparator group against which isoflurane protection was assessed. This caused a probable reduction in statistical power that is suggested by the failure of this study to detect statistical significance for a 40% change in the percent alive CA1 neurons in the isoflurane group between 5 days (58 ± 29) and 3 weeks (35 ± 26 , $P = 0.26$) after ischemia. When P values were not corrected for multiple comparisons, this difference approached significance ($P = 0.053$). Therefore, the difference in the percent alive CA1 neurons between 5 days and 3 weeks after ischemia in the isoflurane group may warrant further investigation.

The curious observation in this study is that while the fentanyl group showed a statistically significant increase in the percent alive CA1 neurons over 3 months of recovery, the isoflurane group did not. We do not have data to explain this difference. Isoflurane did reduce acute neuronal necrosis when measured at 5 days after ischemia. The fraction of those "protected" neurons that remained alive at 3 weeks is unknown, particularly because it is also possible that the same mechanism that caused the percent alive CA1 neuron cells to increase in the fentanyl-nitrous oxide group may also have been simultaneously operational in the isoflurane group. As a result, it is plausible that the difference in the percent alive CA1 neurons in the isoflurane group at 3 weeks *versus* the percent alive CA1 neurons alive in the fentanyl-nitrous oxide group at 5 days reflects a mixture of mechanisms (e.g., neuroprotection and neurogenesis). Added to this is the possibility that some CA1 neurons in the isoflurane group were simultaneously undergoing apoptotic cell death.³⁶ Further work is clearly needed to

understand the response of hippocampal CA1 to ischemia over extended recovery intervals, and specific assay of the modulation of this effect by isoflurane is warranted.

Therefore, the results of the current investigation can be interpreted in one of two ways. Because there was both a behavioral and a histologic difference between the isoflurane and fentanyl-nitrous oxide groups at 5 days but no difference for either outcome assay at 3 weeks or 3 months, it could be concluded that the protective advantage of isoflurane *versus* fentanyl-nitrous oxide is transient in nature. This conclusion would limit clinical relevance of isoflurane neuroprotection (and potentially that of other similar anesthetics), should the same phenomena be present in humans. Global ischemic injuries are known to cause selective neuronal necrosis in humans, with functional deficits consistent with hippocampal CA1 damage,^{37,38} but see Grubb *et al.*³⁹ Conversely, because the fentanyl-nitrous oxide group showed a threefold increase in the percent alive CA1 neurons over the 3-month observation interval, the protective effect of isoflurane may have been obscured. Indeed, rats in the isoflurane group were better for at least part of the recovery interval. More important, the study indicates that the basic response of the hippocampal CA1 sector to severe forebrain ischemia is poorly understood and thus presents a current limitation of the model in defining long-term outcome effects of isoflurane. Further investigation into both the natural history of the brain's response to severe forebrain ischemia and how isoflurane interacts with that process will be required before firm conclusions can be drawn with respect to the persistence of isoflurane neuroprotection against selective neuronal necrosis.

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References

1. Wells BA, Keats AS, Cooley DA: Increased tolerance to cerebral ischemia produced by general anesthesia during temporary carotid occlusion. *Surgery* 1963; 54:216-23
2. Baughman VL, Hoffman WE: Neurologic outcome in rats following incomplete cerebral ischemia during halothane, isoflurane, or N₂O. *ANESTHESIOLOGY* 1988; 69:192-8
3. Werner C, Mollenberg O, Kochs E, Schulte am Esch J: Sevoflurane improves neurological outcome after incomplete cerebral ischaemia in rats. *Br J Anaesth* 1995; 75:756-60
4. Soonthon-Brant V, Patel P, Drummond JC, Cole DJ, Kelly PJ, Watson M: Fentanyl does not increase brain injury after focal cerebral ischemia in rats. *Anesth Analg* 1999; 88:49-55
5. Blanck TJ, Haile M, Xu F, Zhang J, Heerd P, Veselis RA, Beckman J, Kang R, Adamo A, Hemmings H: Isoflurane pretreatment ameliorates postischemic neurologic dysfunction and preserves hippocampal Ca²⁺/calmodulin-dependent protein kinase in a canine cardiac arrest model. *ANESTHESIOLOGY* 2000; 93:1285-93
6. Miura Y, Grocott H, Bart RD, Pearlstein RD, Warner DS: Differential effects of anesthetic agents on outcome from near-complete but not incomplete global ischemia in the rat. *ANESTHESIOLOGY* 1998; 89:391-400
7. Verhaegen M, Todd MM, Warner DS: Ischemic depolarization during halothane-nitrous oxide and isoflurane-nitrous oxide anesthesia: An examination of cerebral blood flow thresholds and times to depolarization. *ANESTHESIOLOGY* 1994; 81:965-73

8. Warner DS, Hansen TD, Vust LH, Todd MM: Distribution of cerebral blood flow during deep isoflurane vs. pentobarbital anesthesia in rats with middle cerebral artery occlusion. *J Neurosurg Anesth* 1989; 1:219-26
9. Yang J, Zorumski CF: Effects of isoflurane on *N*-methyl-D-aspartate gated ion channels in cultured rat hippocampal neurons. *Ann N Y Acad Sci* 1991; 625: 287-9
10. Kimbro JR, Kelly PJ, Drummond JC, Cole DJ, Patel PM: Isoflurane and pentobarbital reduce AMPA toxicity in vivo in the rat cerebral cortex. *ANESTHESIOLOGY* 2000; 92:806-12
11. Harada H, Kelly PJ, Cole DJ, Drummond JC, Patel PM: Isoflurane reduces *N*-methyl-D-aspartate toxicity in vivo in the rat cerebral cortex. *Anesth Analg* 1999; 89:1442-7
12. Patel P, Drummond JC, Cole DJ, Kelly PJ, Watson M: Isoflurane and pentobarbital reduce the frequency of transient ischemic depolarizations during focal ischemia in rats. *Anesth Analg* 1998; 86:773-80
13. Moody EJ, Suzdak PD, Paul SM, Skolnick P: Modulation of the benzodiazepine/ γ -aminobutyric acid receptor chloride channel complex by inhalation anesthetics. *J Neurochem* 1988; 51:1386-93
14. Bickler PE, Warner DS, Strattman G, Schuyler J: GABA receptors contribute to isoflurane neuroprotection in organotypic hippocampal cultures. *Anesth Analg* 2003; 97:564-71
15. Homi HM, Mixco JM, Sheng H, Pearlstein RD, Warner DS: Severe hypotension is not essential for isoflurane neuroprotection against forebrain ischemia in mice. *ANESTHESIOLOGY* 2003; 99:1145-51
16. Kawaguchi M, Kimbro JR, Drummond JC, Cole DJ, Kelly PJ, Patel PM: Isoflurane delays but does not prevent cerebral infarction in rats subjected to focal ischemia. *ANESTHESIOLOGY* 2000; 92:1335-42
17. The Hypothermia After Cardiac Arrest Study Group: Mild therapeutic hypothermia to improve the neurologic outcome after cardiac arrest. *N Engl J Med* 2002; 346:549-56
18. Dietrich WD, Busto R, Alonso O, Globus MYT, Ginsberg MD: Intracerebral but not postischemic brain hypothermia protects chronically following global forebrain ischemia in rats. *J Cereb Blood Flow Metab* 1993; 13:541-9
19. Block F, Bozdag I, Nolden-Koch M: Inflammation contributes to postponed neuronal damage following treatment with a glutamate antagonist in rats. *Neurosci Lett* 2001; 298:103-6
20. Valtysson J, Hillered L, Andine P, Hagberg H, Persson L: Neuropathologic endpoints in experimental pharmacotherapy: The importance of both early and late evaluation. *Acta Neurochir (Wien)* 1994; 129:58-63
21. Colbourne F, Li H, Buchan AM: Continuing postischemic neuronal death in CA1: Influence of ischemia duration and cytoprotective doses of NBQX and SNX-111 in rats. *Stroke* 1999; 30:662-7
22. Mackensen GB, Nellgård B, Miura Y, Dexter F, Pearlstein RD, Warner DS: Sympathetic blockade masks positive effect of isoflurane on histologic outcome from near-complete ischemia in the rat. *ANESTHESIOLOGY* 1999; 90:873-81
23. Smith M-L, Bendek G, Dahlgren N, Rosen I, Wieloch T, Siesjö BK: Models for studying long-term recovery following forebrain ischemia in the rat: II. A 2-vessel occlusion model. *Acta Neurol Scand* 1984; 69:385-401
24. Gionet TX, Warner DS, Verhaegen M, Thomas JD, Todd MM: Effects of intra-ischemic blood pressure on outcome from 2-vessel occlusion forebrain ischemia in the rat. *Brain Res* 1992; 586:188-94
25. Combs DJ, D'Alecy LG: Motor performance in rats exposed to severe forebrain ischemia: Effect of fasting and 1,3-butanediol. *Stroke* 1987; 18:503-11
26. Gionet TX, Thomas JD, Warner DS, Goodlett CR, Wasserman EA, West JR: Forebrain ischemia induces selective behavioral impairments associated with hippocampal injury in rats. *Stroke* 1991; 22:1040-7
27. Morris RGM: Developments of a water maze procedure for studying spatial learning in the rat. *J Neurosci Meth* 1984; 11:47-60
28. Smith M-L, Kalimo H, Warner DS, Siesjö BK: Morphologic lesions in the brain preceding the development of post-ischemic seizures. *Acta Neuropathol* 1988; 76:253-64
29. Auer RN, Kalimo H, Olsson Y, Siesjö BK: The temporal evolution of hypoglycemic brain damage: II. Light- and electron-microscopic findings in the hippocampal gyrus and subiculum of the rat. *Acta Neuropathol* 1985; 67:25-36
30. Wolf HK, Buslei R, Schmidt-Kastner R, Schmidt-Kastner PK, Pietsch T, Wiesler OD, Bluhmke I, Neun N: A useful marker for diagnostic histopathology. *J Histochem Cytochem* 1996; 44:1167-71
31. Mackensen GB, Nellgård B, Kudo M, Sheng H, Pearlstein RD, Warner DS: Perischemic cerebral blood flow does not explain beneficial effects of isoflurane on outcome from near-complete forebrain ischemia in rats. *ANESTHESIOLOGY* 2000; 93:1101-06
32. Sullivan BL, Leu D, Taylor DM, Fahlman CS, Bickler PE: Isoflurane prevents delayed cell death in an organotypic slice culture model of cerebral ischemia. *ANESTHESIOLOGY* 2002; 96:189-95
33. Schmidt W, Reymann KG: Proliferating cells differentiate into neurons in the hippocampal CA1 region of gerbils after global cerebral ischemia. *Neurosci Lett* 2002; 334:153-6
34. Kokaia Z, Lindvall O: Neurogenesis after ischaemic brain insults. *Curr Opin Neurobiol* 2003; 13:127-32
35. Felling RJ, Levison SW: Enhanced neurogenesis following stroke. *J Neurosci Res* 2003; 73:277-83
36. Inoue S, Drummond JC, Davis DP, Cole DJ, Patel PM: Isoflurane and caspase 8 inhibition reduce cerebral injury in rats subjected to focal cerebral ischemia (abstract). *ANESTHESIOLOGY* 2003; 99:A-886
37. Cummings JL, Tomiyasu S, Read SJ, Benson DF: Amnesia with hippocampal lesions after cardiopulmonary arrest. *Neurology* 1984; 34:679-81
38. Zola-Morgan S, Squire L, Amaral DG: Human amnesia and medial temporal region: Enduring memory deficit following a bilateral lesion limited to field CA1 of the hippocampus. *J Neurosci* 1986; 6:2950-67
39. Grubb NR, Fox KA, Smith K, Best J, Blane A, Ebmeier KP, Glabus MF, O'Carroll RE: Memory impairment in out-of-hospital cardiac arrest survivors is associated with global reduction in brain volume, not focal hippocampal injury. *Stroke* 2000; 31:1509-14