Anesthesiology 2009; 110:826–33

Isoflurane Inhibits Growth but Does Not Cause Cell Death in Hippocampal Neural Precursor Cells Grown in Culture

Jeffrey W. Sall, M.D., Ph.D., Greg Stratmann, M.D., Ph.D.,† Jason Leong,‡ William McKleroy, B.A.,‡ Daniel Mason, B.S.,§ Shanti Shenoy, B.A.,‖ Samuel J. Pleasure, M.D., Ph.D.,# Phillip E. Bickler, M.D., Ph.D.***

Background: **Isoflurane causes long-term hippocampal-dependent learning deficits in rats despite limited isoflurane-induced hippocampal cell death, raising questions about the causality between isoflurane-induced cell death and isoflurane-induced cognitive function. Neurogenesis in the dentate gyrus is required for hippocampal-dependent learning and thus constitutes a potential alternative mechanism by which cognition can be altered after neonatal anesthesia. The authors tested the hypothesis that isoflurane alters proliferation and differentiation of hippocampal neural progenitor cells.**

Methods: **Multipotent neural progenitor cells were isolated from pooled rat hippocampi (postnatal day 2) and grown in culture. These cells were exposed to isoflurane and evaluated for cell death using lactate dehydrogenase release, caspase activity, and immunocytochemistry for nuclear localization of cleaved caspase 3. Growth was assessed by cell counting and BrdU incorporation. Expression of markers of stemness (Sox2) and cell division (Ki67) were determined by quantitative polymerase chain reaction. Cell fate selection was assessed using immunocytochemistry to stain for neuronal and glial markers.**

Results: **Isoflurane did not change lactate dehydrogenase release, activity of caspase 3/7, or the amount of nuclear cleaved caspase 3. Isoflurane decreased caspase 9 activity, inhibited proliferation, and decreased the proportion of cells in s-phase. messenger ribonucleic acid expression of Sox2 (stem cells) and Ki67 (proliferation) were decreased. Differentiating neural progenitor cells more often select a neuronal fate after isoflurane exposure.**

Conclusions: **The authors conclude that isoflurane does not cause cell death, but it does act directly on neural progenitor cells independently of effects on the surrounding brain to decrease proliferation and increase neuronal fate selection. These changes could adversely affect cognition after isoflurane anesthesia.**

OVER the past 5 yr, a wide variety of anesthetic agents used alone or in combination in neonatal rodents have been reported to cause cell death and cognitive dysfunction (reviewed in Loepke *et al.*[1]). Isoflurane alone has been shown to produce both cell death and long-term cognitive deficits when given to neonatal rodents.[2–5] The cognitive deficits that have been reported after anesthetic exposure are largely hippocampal in origin despite modest or no cell death in the hippocampus compared to other brain structures.[3,4,6,7]

Neurogenesis in the hippocampal dentate gyrus (DG) is required for certain types of learning and memory; inhibiting it by genetic manipulation or radiation leads to specific hippocampal cognitive deficits[8–12] similar to those seen in postnatal day 7 rats after exposure to isoflurane.[4] We have recently reported a decrease in proliferation of precursors in the DG of both adult and postnatal day 7 rats after exposure to isoflurane[4]; however, it is unknown if this effect is mediated by isoflurane acting directly on precursor cells or by its action on the surrounding brain, where it inhibits neural signaling in neonates and adults and causes cell death in neonates.

γ-aminobutyric acid type A ($GABA_A$) and *N*-methyl-D-aspartate receptors provide important cues for proliferation and differentiation of neural progenitor or stem cells in the developing and adult brain,[13–16] and isoflurane acts at both of these receptors. Besides acting to decrease signaling of nearby established neurons, isoflurane could act directly on precursor cells to alter their growth and differentiation. We propose that a direct effect of isoflurane on precursor cells could be an alternative or additional explanation for the cognitive deficits observed in rodents.

In this study, we hypothesize that isoflurane acts on neural progenitor cells (NPCs) to decrease proliferation independent of its effects (cell death and decreased neural transmission) on the surrounding brain. To test this hypothesis, NPCs isolated from rat postnatal day 2 hippocampus were grown in culture and exposed to isoflurane. In the following experiments, we demonstrate that isoflurane is not toxic to NPCs and acts independently of surrounding brain to induce cell cycle exit and increase neuronal fate selection.

Materials and Methods

Hippocampal Precursor Cell Isolation and Culture

All animals were cared for according to procedures approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. NPCs were isolated by following methods previously described with slight modification.[17–20] Unanesthetized postnatal day 2 Sprague-Dawley rats were separated from the dam and decapitated using a guillotine. Hip-

* Assistant Professor, † Associate Professor, ‡ Staff Research Associate, ** Professor, Department of Anesthesia and Perioperative Care, University of California, San Francisco; § Medical Student, Eastern Virginia Medical School, Norfolk, Virginia; ‖ Medical Student, University of Chicago, Chicago, Illinois; # Associate Professor, Department of Neurology, University of California, San Francisco.

Received from the Department of Anesthesia and Perioperative Care, University of California, San Francisco. Submitted for publication November 12, 2008. Accepted for publication December 19, 2008. Supported by Foundation for Anesthesia Education and Research , Rochester, Minnesota, and the Anesthesia Patient Safety Foundation, Indianapolis, Indiana. Presented in part at the 34th Annual Meeting of the Society of Neuro-Surgical Anesthesia and Critical Care, Atlanta, Georgia, October 13, 2006; the 36th Annual Meeting of the Society for Neuroscience, Chicago, Illinois, October 14–18, 2006; Western Anesthesia Residents Conference, Sacramento, California, May 4–6, 2007; the Annual Meeting of the American Society of Anesthesiologists, Orlando, Florida, October 18–22, 2008; and the 38th Annual Meeting of the Society for Neuroscience, Washington, DC, November 15–19, 2008.

Address correspondence to Dr. Sall: 513 Parnassus Ave, S257, Box 0542, San Francisco, California 94143-0542. sallj@anesthesia.ucsf.edu. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

pocampi were immediately dissected out and placed in 10-ml ice-cold Hanks Basic Salt Solution without calcium. Whole hippocampi pooled from 10 animals were collected by centrifugation for 2 min at 300 relative centrifugal force. Supernatant was removed, and hippocampi were minced with a razor blade then triturated 5 times using a 200-μl pipette. Cells were then resuspended in 10 ml of Hanks Basic Salt Solution for 2 min. After settling for 2 min, the supernatant was transferred to a new tube. Cells were then collected by centrifugation at 300 relative centrifugal force for 5 min. Supernatant was discarded, and cells were resuspended in proliferation medium consisting of 3:1 Dulbecco's Modified Eagle's Medium: Ham's F12 (University of California, San Francisco cell culture facility, San Francisco, CA), 1% penicillin and streptomycin, 1× B-27 supplement (Invitrogen, Grand Isle, NY), 20 ng/ml basic fibroblast growth factor (Chemicon, Temecula, CA), 0.75 units/ml heparin (Abraxis, Schaumburg, IL). Hippocampal precursor cells were then grown in 5% carbon dioxide in air at 37°C and with media changed thrice weekly and cells transferred to new flasks every 5 days so that adherent differentiated cells were left behind and nonadherent proliferating NPCs were moved to the new flask. NPCs had been grown in culture for 2 to 12 weeks at the time of use. To differentiate NPCs, basic fibroblast growth factor containing medium was replaced with differentiation medium consisting of Neurobasal-A (Invitrogen, Grand Isle NY), B27 supplement, 1% penicillin-streptomycin (University of California, San Francisco cell culture facility), L-glutamine (Invitrogen, Carlsbad CA), and 5% fetal bovine serum (University of California, San Francisco cell culture facility) on day one. The next day, medium was replaced with differentiation medium lacking serum.

Immunocytochemistry

Cells were plated at a density of 20,000 per well on 8-chamber microscope slides that were precoated with poly-L-ornithine (Sigma-Aldrich, St. Louis, MO) in water overnight and then with laminin (Sigma-Aldrich) in phosphate-buffered saline (PBS), pH7.4 overnight. At the end of the experiment, the slides were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 15 min at room temperature and blocked with 10% goat serum and 0.03% Triton X-100 (Sigma-Aldrich, St. Louis, MO) in PBS for 2 h at room temperature. Primary antibodies were diluted in PBS and added for overnight incubation at 4°C. Secondary antibodies were diluted 1:1000 in PBS and added for 2 h at room temperature. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI) for 10 min at room temperature. Slides were washed with PBS, and coverslips were applied with Aquapolymount (Polysciences, Warring, PA). Table 1 gives a summary of the antibodies used.

Table 1. List of Antibody Sources and Dilutions

Antibody	Name	Company	Dilution
BrdU	SC56258	Santa Cruz (Santa Cruz, CA)	1/75
Tuj-1	MMS-435P	Covance (Billerica, MA)	1/200
GFAP	AB5804	Chemicon (Emeryville, CA)	1/1,500
Map2	MAB3418	Chemicon	1/200
Sox2	2003600	Chemicon	1/500
Nestin	MAB353	Chemicon	1/200
Goat anti mouse IgG	Alexa 488	Invitrogen (Grand Isle, NY)	1/1,000
Goat anti rabbit IgG	Alexa 594	Invitrogen	1/1,000

Isoflurane Exposure

NPCs in slides or flasks were placed in a Billups-Rothenburg chamber (Billups-Rothenburg, Del Mar, CA). The chamber was flushed with isoflurane in humidified air with 5% carbon dioxied at 37°C for 15 min while monitoring the temperature and concentration of gases inside the chamber. The chamber was then sealed and placed inside a 37°C incubator. The concentrations of oxygen, isofluorane, and carbon dioxide in the chamber were monitored using a Datex Capnomac Ultima gas analyzer (Datex Ohmeda, Helsinki Finland) at 2-h intervals, and the chamber was reflushed if there was any change. Control experiments were performed in the same manner, except no isoflurane was added when flushing the chamber. We chose 3.4% isoflurane for these experiments on the basis of our experience anesthetizing postnatal day 7 animals. Using the method of Merkel and Eger,[21] we found the inspired minimum alveolar concentration of isoflurane to be 3.6– 4% initially, with a slow decline thereafter.[4]

Cell Death/Necrosis

NPCs were grown as neurospheres in suspension as described above and plated in 96-well plates in proliferation medium. The next day, the cells were exposed to 3.4% isoflurane for 4 h as described above (Isoflurane Exposure) and returned to standard incubator growth conditions for 18 h. NPCs were then analyzed for release of lactate dehydrogenase (LDH) using the Cytox-96 assay kit (Promega, Madison, WI) and following the manufacturers protocol. Treatment with Triton X-100 (0.9% volume/volume, provided by manufacturer) for 45 min at 37°C was used as a positive control.

Caspase Activation

NPCs (1×10^4) were plated in proliferation medium and grown as neurospheres in suspension in opaque 96-well plates. The next day, plates were exposed to 3.4% isoflurane, 500 ng/ml fentanyl citrate (0.95 μM), or control for 4 h, followed by standard incubator conditions for 2 h for caspase 3/7 or as indicated in the figure for caspase 9. Fentanyl has previously been shown to activate opioid receptors on cultured differentiated neu-

rons at concentrations of 0.1 to 2.0 μM[22–24] and did not induce caspase activation in the brain when used as a sole anesthetic.[25] Fentanyl was used as a control treatment because it does not interact with $GABA_A$ or *N*-methyl-D-aspartate receptors and does not cause cell death *in vivo* but is commonly used for clinical anesthesia. Cells were then lysed and analyzed for caspase 3/7 or caspase 9 activity (Promega, Caspase-Glo 3/7 or Glo 9 kit, Promega). Nuclear cleaved caspase 3 was quantified using immunocytochemistry methods described above and performed on NPCs plated in 8-chamber glass slides coated with poly-L-ornithine and laminin. The total (DAPI-labeled) and cleaved caspase 3 (immunofluorescence-labeled) nuclei were counted in multiple fields of view to determine the fraction of nuclei positive for cleaved caspase 3.

Growth

After triturating to a single cell suspension, equal numbers of NPCs were plated into 6- or 12-well plates in proliferation medium and grown floating in culture. The next day, cultures were exposed to 3.4% isoflurane or control for 6 h before returning to the incubator for growth under standard conditions. Forty-eight hours later, the contents of each well were collected by centrifugation, triturated to a single cell suspension, and then resuspended in 500 μl of medium before counting the cells by trypan blue exclusion using a hemocytometer (VWR, Batavia, IL).

Quantitative Polymerase Chain Reaction

NPCs ($0.5–1 \times 10^5$) grown as neurospheres in suspension in T-25 flasks (Corning supplied by Fisher, Pittsburg, PA) were exposed to isoflurane for 4 h. At specified times, cells were collected by centrifugation, and RNA was isolated using Trizol (Invitrogen, Carlsbad, CA) by following the manufacturer's directions 6, 12, and 24 h after treatment. RNA (1 μg) was reverse-transcribed to make copy DNA using the Quigen Omniscript kit (Quiagen, Valencia, CA) by following manufactures instructions and using a mix of poly D-thymidine and random hexamers. Quantitative polymerase chain reaction was performed using Quiagen Quantitect SYBR Green kit (Quiagen) on an Stratagene MX 3000P thermocycler (Agilent Technologies, La Jolla, CA). Changes in gene expression were determined using the delta-delta cT method.[26] α-Actin was used as a reference gene for loading control, and expression of the gene of interest was defined relative to untreated cultures, which were arbitrarily assigned a value of 100%. Primer sequences for target genes are listed in table 2.

BrdU Incorporation

NPCs were plated in coated eight-chamber microscope slides in proliferation medium. The next morning, NPCs were exposed to isoflurane as described above (isoflurane exposure). 5-Bromodeoxyuridine (BrdU), a thymidine analog, was added to the medium for 2 h at the end of the isoflurane exposure or 12 h later. Slides were fixed and stained as described above and then photographed with 40× or 100× objective lenses on an E400 (Nikon, Melville, NY) fluorescence microscope equipped with filters at 385, 490, and 570 nM. Six to twelve sets of images were acquired at different locations and were subsequently merged using National Institutes of Health Image J software (Bethesda, MD), and the total number of cells (DAPI) as well as the number of BrdU-positive cells was determined.

Table 2. Primers Used for Quantitative Polymerase Chain Reaction

Gene	Primer Sequence
Sox2	CTCTGCACATGAAGGAGCAC
	CTCCGGGAAGCGTGTACTTA
Ki-67	CGATCGCAGAAACCTAGGAG
	GGCTCTGTGCAGGAGAAGAC
Actin	ACAGCTGAGAGGGAAATCGT
	TTCTCCAGGGAGGAAAGAGG

Differentiation

NPCs growing in suspension in proliferation medium were collected, switched to differentiation medium, and plated on coated 8-chamber microscope slides. The slides were immediately exposed to isoflurane as described above under isoflurane exposure and then returned to standard incubator conditions for 4 days. Media was replaced on day 2 with differentiation medium lacking serum. Slides were fixed and stained as described above (immunocytochemistry) and then photographed with 40× or 100× objective lenses on a Nikon E400 fluorescence microscope with filters at 385, 490, and 570 nM. Six to twelve sets of images were acquired at random locations and were subsequently merged using NIH Image J software. The total number of cells (DAPI) and the ratio of cells positive for neuron-specific beta 3 tubulin (Tuj1), to mark neurons, or glial fibrillary acidic protein (GFAP), to mark astrocytes, was determined.

Statistical Analysis

All statistical analyses were performed and all graphs were produced using Prism 4 (GraphPad Software, San Diego, CA). Data are expressed as mean ± SE and analyzed using parametric test except in cases for which assumptions required for parametric analyses (homogeneity of variance, normality) were violated, when data were expressed as medians ± interquartile ranges and analyzed using nonparametric tests. Caspase 3/7 and 9 enzyme activity was evaluated by analysis of variance (ANOVA) and Bonferroni post test to correct for multiple comparisons. BrdU incorporation immediately after isoflurane exposure was analyzed by Kruskal-Wallis with Dunn's post test. Nuclear localization of cleaved caspase 3, BrdU incorporation at 12 h, and expression of Tuj1 or

GFAP by differentiated NPCs were analyzed by Mann–Whitney U test. Growth experiments were analyzed by Wilcoxon signed rank test, with fold increases from control values being compared to the theoretical value of 1, assigned to the control group. Subsequent comparisons of fold increase in untreated *versus* isofluorane-treated cultures were done with Wilcoxon matched pairs test. Changes in gene expression in quantitative polymerase chain reaction experiments were analyzed by ANOVA with Dunnett post test to compare all time points to control untreated cultures.

Results

Hippocampal Neural Progenitor Cells Grown in Culture

Two to four days after isolation, NPCs in proliferation medium began to grow in characteristic neurospheres as has been described previously (fig. 1A).[17–20,27,28] The same NPCs plated on slides with poly-L-ornithine and laminin become adherent and put out processes (fig. 1B); however, they remain largely undifferentiated as evidenced by expression of stem cell markers nestin and Sox2 (fig. 1C) but little expression of neuronal marker Tuj1 or the astrocyte marker GFAP (fig. 1D). After 4 days in differentiation medium, cells positive for GFAP (fig. 1E, red) and Tuj1 (fig. 1E, green) were observed, confirming that the precursor cells had been multipotent before differentiation.

Isoflurane Does Not Cause Caspase Activation or LDH Release

The activity of the apoptotic proteins caspase 3/7 and caspase 9 was determined (fig. 2). Although 1 μM staurosporine increased caspase 3/7 activity, neither 4 h of 3.4% isoflurane nor 4 h of 500 ng/ml fentanyl citrate changed the activity of caspase 3/7 2 h after exposure (n = 3; ANOVA $P = 0.0005$; Bonferroni post test $P > 0.05$ for control *vs.* isoflurane and control *vs.* fentany; fig. 2A). Similarly, when cells were grown adherently on glass slides, no increase was seen in nuclear-cleaved caspase 3 staining 2 h after a 4-h isoflurane exposure (control n = 3; isoflurane n = 4; staurosporine n = 2; Mann–Whitney U $P > 0.05$; fig. 2C). Staurosporine (1 μM) did induce nuclear localization of cleaved caspase 3 (fig. 2, C and D). Caspase 9 activity was increased in the presence of 1 μM staurosporine but was unchanged by fentanyl and decreased after 2 or 4 h of isoflurane or 4 h of isoflurane + 2 h standard incubator conditions (n = 3; except staurosporine n = 5; ANOVA $P < 0.0001$; Bonferroni post test $P < 0.001$ control *vs.* any isoflurane group, $P < 0.05$ control *vs.* staurosporine, and $P > 0.05$ control *vs.* fentanyl; fig. 2B).

To determine if nonapoptotic cell death occurred, NPCs were exposed to isoflurane for 4 h, and the amount of LDH released into the media was determined.

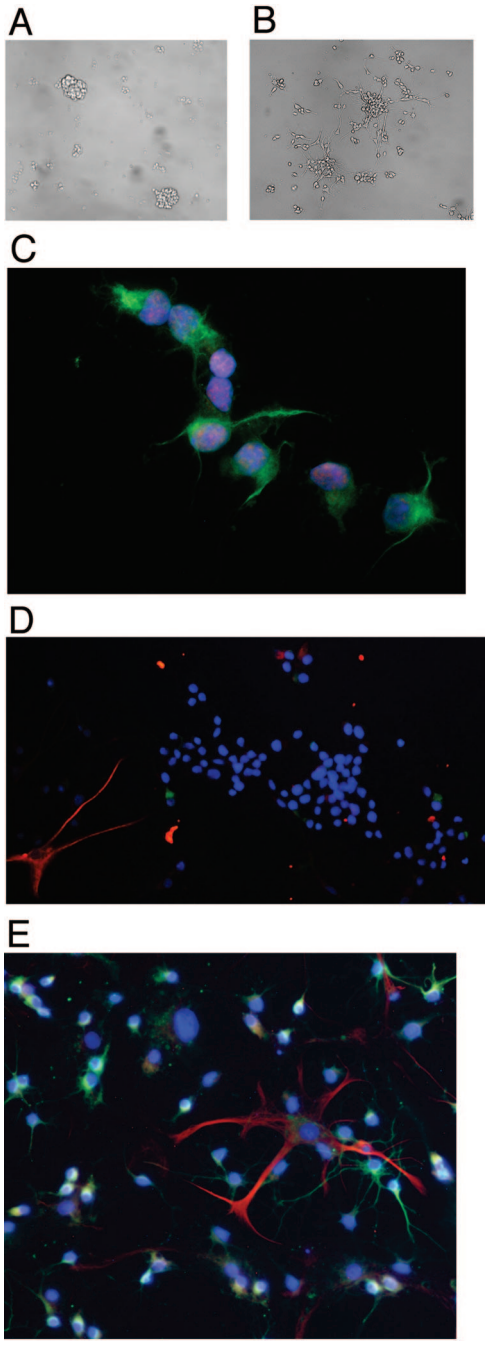

Fig. 1. Hippocampal neural progenitor cells (NPCs) grown in culture are multi-potent. (*A*) NPCs grown in culture flasks in proliferation media form floating neurospheres but become adherent when grown in poly-L-ornithine and laminin-coated glass slides (*B*). (*C*) NPCs grown in 8-chamber slides in proliferation media express Sox2 (*red*) and nestin (*green*). 4′,6-diamidino-2-phenylindole (DAPI) marks the DNA or nuclei (*blue*). (*D*) NPCs grown on coated slides in proliferation media for 24 h express very little glial fibrillary acidic protein (GFAP, *red*) or neuron-specific class III beta tubulin (Tuj1, *green*). (*E*) NPCs grown on coated slides in differentiation media for 4 days after basic fibroblast growth factor withdrawal and addition of serum show Tuj1 (*green*) staining neurons and GFAP (*red*) positive astrocytes. DAPI stains all nuclei (*blue*).

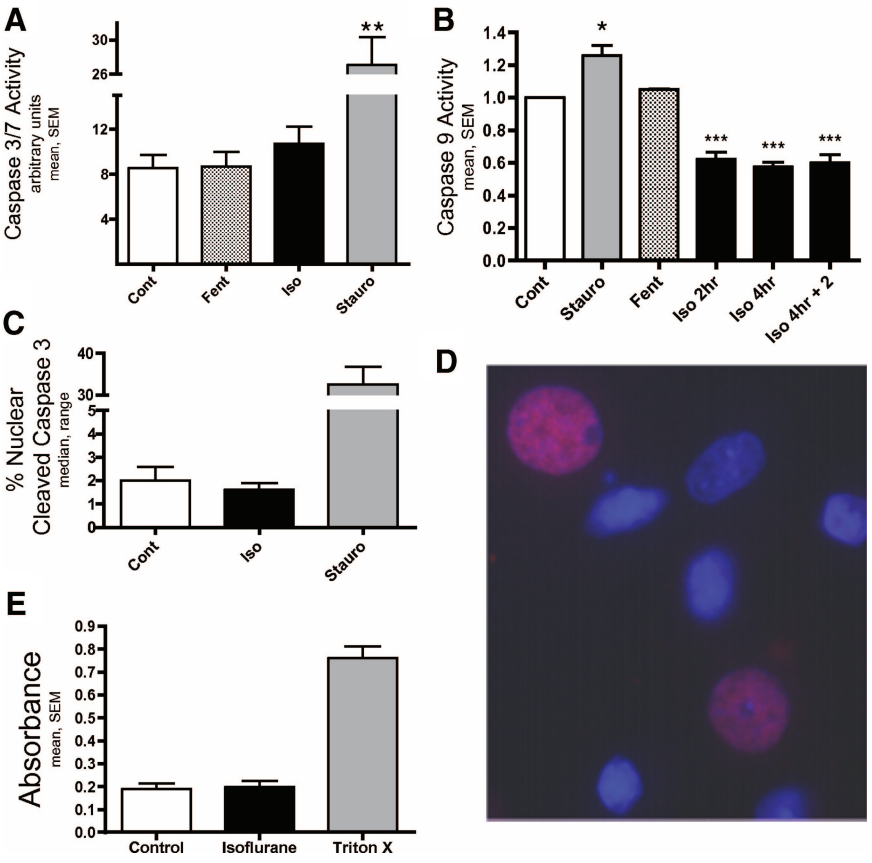

Fig. 2. No caspase activation or lactate dehydrogenase release after isoflurane treatment. Neural progenitor cells (NPCs) grown in proliferative conditions were exposed to isoflurane (Iso) and then lysed to determine the activity of caspase enzymes, fixed to identify nuclear translocation of cleaved caspase 3, or media-tested for lactate dehydrogenase release. (*A*) Staurosporine (1 μM; positive control) increased activity of caspase 3/7 more than threefold, but 4 h of 3.4% isoflurane or 6 h of 500 ng/ml fentanyl citrate had no effect (Bonferroni post test ** $P < 0.01$). (*B*) Immediately after 2 or 4 h of exposure to 3.4% isoflurane (Iso 2 h and Iso 4 h, respectively) the baseline activity of caspase 9 decreased. Caspase 9 activity remained decreased 2 h after removal from isoflurane after a 4-h exposure (Iso 4 h + 2). Fentanyl citrate (500 ng/ml) had no effect on activity of caspase 9 (Bonferroni post test * $P < 0.05$, *** $P < 0.001$). (*C*) Staurosporine (1 μM) dramatically increased the number of cells that were positive for nuclear-cleaved caspase 3, but isoflurane did not. (*D*) After addition of 1 μM staurosporine, all nuclei stained with 4, 6-diamidino-2-phenylindole (DAPI) are *blue* and cleaved caspase 3–positive nuclei are *red*. (*E*) To determine if nonapoptotic cell death occurred, NPCs were exposed to isoflurane for 4 h and the media assayed for lactate dehydrogenase activity. No difference was seen between control and isofluorane-exposed groups (*t* test $P = 0.80$). Triton X-100 (0.9% v/v) for 45 min at 37°C was used as a positive control.

No difference was seen between control and isofluorane-exposed groups, but addition of detergent (0.9% Triton X-100) to the medium did increase LDH release (n = 8; isoflurane *vs.* control *t* test $P = 0.80$; fig. 2E).

Isoflurane Inhibits Proliferation

NPCs were plated in 6- or 12-well plates, grown in nonadherent proliferative conditions, and exposed to 3.4% isoflurane for 6 h. Both untreated and isoflurane-treated cultures proliferated relative to time 0 (Wilcoxon signed rank test relative to theoretical value of 1 at time 0; control $P < 0.01$; isoflurane $P < 0.05$,). However, the fold increase in the number of cells 48 h later was smaller in isoflurane-treated than in untreated cultures (n = 9; Wilcoxon matched pairs *t* test $P < 0.05$; fig. 3).

Isoflurane Decreases Expression of Ki67 and Sox2 mRNA

NPCs grown in proliferative conditions were exposed to 3.4% isoflurane for 4 h, and expression of the cell cycle regulator Ki-67 and the stem cell marker Sox2 was evaluated 6, 12, and 24 h later by using quantitative polymerase chain reaction. Both Ki-67 and Sox2 messenger RNA were decreased maximally at 12 h (34 and 45% lower) and began to return to baseline by 24 h (Ki-67: n = 6, ANOVA $P > 0.05$ Dunnett post test 12 h $P < 0.05$, fig. 4A; Sox2: n = 5, ANOVA $P < 0.05$, Dunnett post test 12 h $P < 0.05$, fig. 4B).

Isoflurane Decreased the Number of Cells in S-Phase

To determine if the isoflurane-mediated decrease in proliferation was the result of a change in cell cycle, progenitors were exposed to 4 h of 3.4% isoflurane followed immediately by 2 h of BrdU. Control cultures showed a median of 21.5% of cells incorporated BrdU compared to only 13.1% of isoflurane-exposed cells. Fentanyl citrate (500 ng/ml) given for 6 h did not change BrdU incorporation (Kruskal-Wallis $P = 0.029$; Dunn post test $P < 0.05$, control *vs.* isofluorane; fig. 5A). To

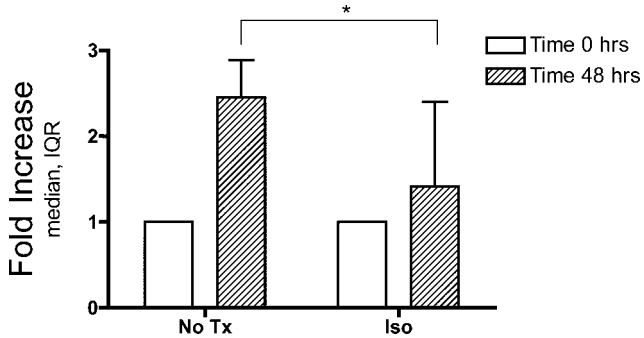

Fig. 3. Isoflurane (Iso) inhibits proliferation. Neural progenitor cells were plated in equal numbers and exposed to 3.4% isoflurane for 6 h. Fold increase relative to control cultures was determined 48 h later. All cultures showed growth over 48 h, but isoflurane exposure inhibited proliferation by 42% relative to untreated cultures. Wilcoxon matched pairs *t* test * $P < 0.05$; IQR = interquartile range; No Tx = no treatment.

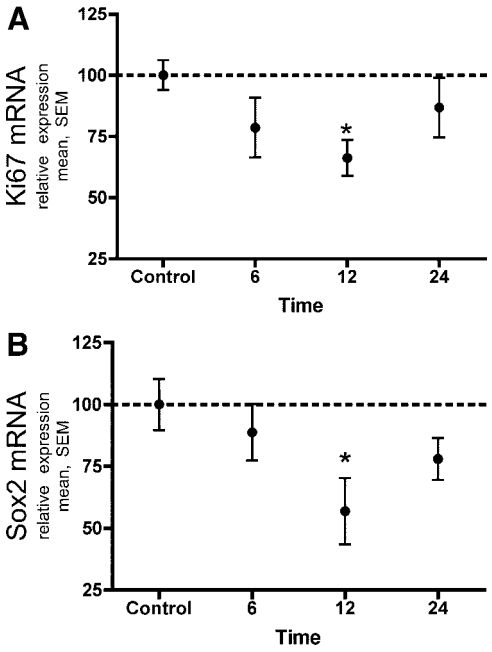

Fig. 4. Isoflurane decreases expression of Ki67 and Sox2 messenger ribonucleic acid (mRNA). Quantitative polymerase chain reaction 6, 12, and 24 h after exposure to 3.4% isoflurane for 4 h shows a decrease in the amount of the cell cycle gene Ki-67 and the stem cell gene Sox2 that is maximal at 12 h and begins to return to baseline by 24 h. The amount of mRNA for each timepoint is expressed relative to control cultures that were arbitrarily assigned a value of 100%. (*A*) Ki67 decreased by 34% from baseline at 12 h (Dunnett post test * $P < 0.05$). (*B*) Sox2 decreased by 43% from baseline at 12 h (Dunnett post test * $P < 0.05$).

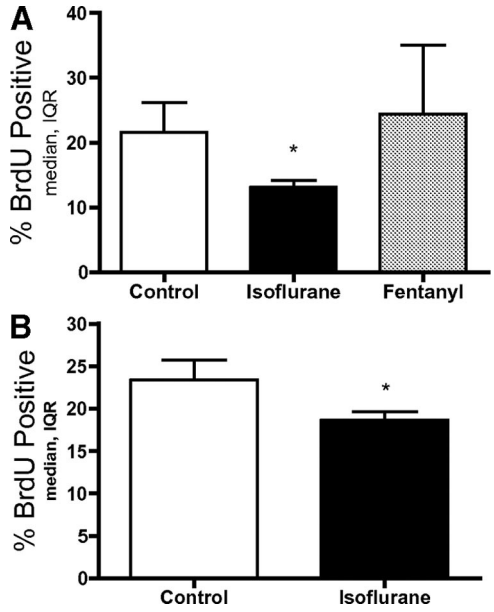

Fig. 5. Decreased number of cells in S-Phase. To determine whether isoflurane exposure changes the cell cycle, neural precursor cells were exposed to isoflurane for 4 h then removed before BrdU was added to the media for 2 h either immediately or 12 h later. (*A*) 3.4% isoflurane for 4 h, but not 500 ng/ml fentanyl citrate, decreased the number of cells that took up BrdU during the subsequent 2 h (Dunn post test * $P < 0.05$). (*B*) The inhibitory effect of isoflurane on cells taking up BrdU persists until at least 12 h after isoflurane exposure (Mann–Whitney * $P < 0.05$). IQR = interquartile range.

assess the duration of the effect BrdU was added 12 h after completion of the isoflurane treatment leading to a median 18.6% BrdU-positive cells in the isoflurane group compared to 23.3% in control (Mann–Whitney $P < 0.05$; fig. 5B).

Isoflurane Increased Neuronal Fate Selection

We next evaluated the effect of isoflurane on fate selection and differentiation by exposing NPCs to isoflurane for 4 h at the time of plating in differentiation medium. Cells were fixed 4 days later and stained for Tuj1 (neurons), GFAP (astrocytes), and DAPI (all nuclei). NPCs exposed to isoflurane were more likely to express a neuronal marker 4 days later (n = 6; $P < 0.01$, Mann–Whitney U; fig. 6). No difference was seen in the rate of expression of the astrocyte marker GFAP (n = 6; $P = 0.94$, Mann–Whitney U; fig. 6).

Discussion

Multiple anesthetics have previously been reported to lead to caspase activation and neuronal cell death throughout the brain of postnatal day 7 animals.[1] We report in an accompanying article in this issue that isoflurane decreases NPC proliferation (BrdU uptake) *in vivo* in adults and neonates.[4] This could be the result of isofluorane-mediated decreases in neuronal firing in neonatal and adult animals[29] or of direct toxicity in neonates where cell death has been observed.[3] Here we have attempted to determine whether this effect is independent of the actions of isoflurane on the surrounding brain by growing NPCs in an *in vitro* environment. Cultures of hippocampal precursor cells were isolated from postnatal day 2 rats to maximize the contribution of cells from the DG and minimize the contribution from the subventricular zone and other brain regions. Embryonic animals are often used for NPC cultures; however,

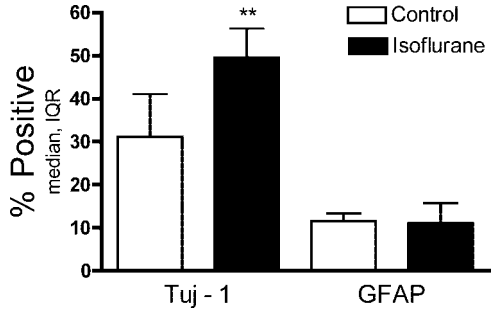

Fig. 6. Isoflurane increased neuronal fate selection. Proliferating neural precursor cells were plated on coated glass slides in differentiation media and immediately exposed to isoflurane for 4 h. The percentage of cells positive for neuron-specific class III beta tubulin (Tuj1) 4 days later was increased in isofluorane-exposed cultures, but no difference was seen in glial fibrillary acidic protein (GFAP)–positive cells (Mann–Whitney ** $P < 0.01$). IQR = interquartile range.

the hippocampus is poorly defined or absent until birth in rats, making isolation nearly impossible. We are most interested in NPCs specifically from the dentate to correlate with *in vivo* studies of this region; therefore, we obtained our cultures from postnatal day 2 animals.

Translating anesthetic studies from animals (let alone cell culture) to humans is at least flawed if not impossible. To minimize differences between *in vitro* and *in vivo* models of anesthesia, a comparable dose must be used. One can use an equal dose such as 1% isoflurane that is not clinically equivalent across species or one can choose to measure a clinical endpoint such as minimum alveolar concentration and use a dose that produces an equivalent endpoint between species. We have chosen to base our *in vivo* studies on minimum alveolar concentration and therefore use the same dose for the *in vitro* studies presented here. Fentanyl was used as a control in these studies because both $GABA_A$ and *N*-methyl-D-aspartate receptors mediate signals that are known to influence proliferation and differentiation of NPCs,[13–16] and most anesthetic drugs act at one or both of these receptors.[29] Opiates do not act at $GABA_A$ or *N*-methyl-D-aspartate receptors, and fentanyl is a commonly used opiate and was chosen as a control for that reason.

In the experiments presented here, with isoflurane as the sole anesthetic, we were unable to induce cell death, caspase activation, or LDH release in cultured NPCs. This suggests that NPCs do not undergo apoptosis or necrosis after isoflurane exposure, consistent with limited cell death being reported in the DG of the hippocampus (the source of these cultured cells) compared with other regions of the brain.[3] Decreased proliferation 48 h after isoflurane exposure combined with a decrease in the number of cells in S-phase and loss of the cell cycle regulator Ki-67 suggests that the cells are either in growth phase arrest or have exited the cell cycle as would occur with differentiation. Cell cycle exit and differentiation is further suggested by the loss of the stem cell gene Sox2 and increased neuronal fate selection.

Culturing cells in different states can change the characteristics of those cells and how they respond to different signals or stresses and represents a potential limitation of this study. These experiments were performed on cells in two different states: floating as neurospheres or adherent to glass slides but undifferentiated. The results we observed, however, are similar in both cases. Growth inhibition, loss of Ki-67, and lack of caspase activation in floating neurospheres and decreased ratio of cells in S-phase and lack of nuclear-cleaved caspase 3 in adherent cells are all consistent with a lack of cell death and a decrease in proliferation. Similar effects on proliferation *in vivo* are also reported in this issue.[4]

Isoflurane facilitates opening of the $GABA_A$ receptor and has previously been shown to raise the intracellular calcium concentration in neurons and slice cultures by liberating it from endoplasmic reticulum.[30–33] $GABA_A$ receptor opening and increased intracellular calcium decrease proliferation of precursor cells in subventricular zone[13,34] and increase differentiation and selection of a neuronal fate in hippocampal progenitors of the DG.[14,16,35] Isoflurane may lead to cell cycle exit and differentiation of hippocampal NPCs by facilitating $GABA_A$ receptor opening and increasing intracellular calcium. Future studies are necessary to determine the exact mechanism of isofluorane-mediated growth inhibition and increased neuronal differentiation we have observed in NPCs, but $GABA_A$ receptor activation and changes in calcium concentration are likely targets.

Decreased proliferation and increased neuronal differentiation caused by isoflurane could lead to cognitive dysfunction in neonates by permanently disrupting the architecture of the hippocampus during a critical period of development or by depleting the pool of precursor cells present for the duration of the animals life. The same mechanism in an adult might not cause any cognitive dysfunction when the hippocampus is already fully developed and connected and can more easily accept an increased number of cells choosing to become neurons at any given time. The adult hippocampus routinely integrates new neurons into its circuitry with learning.[10,36]

Elsewhere in this issue, we report isoflurane-mediated changes in NPC proliferation *in vivo*,[4] and the current experiments demonstrate an effect of isoflurane on hippocampal NPCs grown in culture, demonstrating that this effect is on the cells themselves rather than being mediated by the surrounding tissue of the neonatal brain or specifically the DG. In addition to suggesting a possible mechanism for isoflurane-induced hippocampal dysfunction, the role of anesthesia in the field of neural stem cells and neural stem cell transplantation remains largely un-explored. The results reported here show that isoflurane may have a direct impact on the proliferation and fate selection of transplanted stem cells. Future studies on the effect of volatile and nonvolatile anesthetics on NPCs are important for our understanding of how these drugs effect the biology of NPCs and eventually for choosing the appropriate anesthetic for trials of stem cell transplantation in animals and someday humans.

This model system can be used to efficiently screen many anesthetics that have been previously reported to cause neurodegeneration to determine if they have an effect on NPC proliferation and differentiation. This system may also be used to address questions of mechanism that are difficult to answer with *in vivo* studies. Understanding how anesthetics interact with the complex system of neurogenesis in the neonatal and adult brain may provide some insight into normal development and will guide our choice of drugs for future studies related to both anesthetic-mediated cognitive dysfunction and transplantation of NPCs.

References

1. Loepke AW, Soriano SG: An assessment of the effects of general anesthetics on developing brain structure and neurocognitive function. Anesth Analg 2008; 106:1681–707
2. Ma D, Williamson P, Januszewski A, Nogaro MC, Hossain M, Ong LP, Shu Y, Franks NP, Maze M: Xenon mitigates isoflurane-induced neuronal apoptosis in the developing rodent brain. ANESTHESIOLOGY 2007; 106:746–53
3. Stratmann G, May LDV, Sall JW, Alvi RS, Bell JS, Ormerod BK, Rau V, Hilton JF, Dai R, Lee MT, Visrodia KH, Ku B, Zusmer EJ, Guggenheim J, Firouzian A: Effect of hypercarbia and isoflurane on brain cell death and neurocognitive dysfunction in 7-day-old rats. ANESTHESIOLOGY 2009; 110:849–61
4. Stratmann G, Sall JW, May LDV, Bell JS, Magnusson KR, Rau V, Visrodia KH, Alvi RS, Ku B, Lee MT, Dai R: Isoflurane differentially affects neurogenesis and long-term neurocognitive function in 60-day-old and 7-day-old rats. ANESTHESIOLOGY 2009; 110:834–48
5. Johnson SA, Young C, Olney JW: Isoflurane-induced neuroapoptosis in the developing brain of nonhypoglycemic mice. J Neurosurg Anesthesiol 2008; 20:21–8
6. Jevtovic-Todorovic V, Hartman RE, Izumi Y, Benshoff ND, Dikranian K, Zorumski CF, Olney JW, Wozniak DF: Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. J Neurosci 2003; 23:876–82
7. Fredriksson A, Ponten E, Gordh T, Eriksson P: Neonatal exposure to a combination of N-methyl-D-aspartate and gamma-aminobutyric acid type A receptor anesthetic agents potentiates apoptotic neurodegeneration and persistent behavioral deficits. ANESTHESIOLOGY 2007; 107:427–36
8. Zhang CL, Zou Y, He W, Gage FH, Evans RM: A role for adult TLX-positive neural stem cells in learning and behaviour. Nature 2008; 451:1004–7
9. Zhao C, Deng W, Gage FH: Mechanisms and functional implications of adult neurogenesis. Cell 2008; 132:645–60
10. Dupret D, Revest JM, Koehl M, Ichas F, De Giorgi, F Costet P, Abrous DN, Piazza PV: Spatial relational memory requires hippocampal adult neurogenesis. PLoS ONE 2008; 3:e1959
11. Madsen TM, Kristjansen PE, Bolwig TG, Wortwein G: Arrested neuronal proliferation and impaired hippocampal function following fractionated brain irradiation in the adult rat. Neuroscience 2003; 119:635–42
12. Czurko A, Czeh B, Seress L, Nadel L, Bures J: Severe spatial navigation deficit in the Morris water maze after single high dose of neonatal x-ray irradiation in the rat. Proc Natl Acad Sci U S A 1997; 94:2766–71
13. LoTurco JJ, Owens DF, Heath MJ, Davis MB, Kriegstein AR: GABA and glutamate depolarize cortical progenitor cells and inhibit DNA synthesis. Neuron 1995; 15:1287–98
14. Ganguly K, Schinder AF, Wong ST, Poo M: GABA itself promotes the developmental switch of neuronal GABAergic responses from excitation to inhibition. Cell 2001; 105:521–32
15. Ge S, Goh EL, Sailor KA, Kitabatake Y, Ming GL, Song H: GABA regulates synaptic integration of newly generated neurons in the adult brain. Nature 2006; 439:589–93
16. Tozuka Y, Fukuda S, Namba T, Seki T, Hisatsune T: GABAergic excitation promotes neuronal differentiation in adult hippocampal progenitor cells. Neuron 2005; 47:803–15
17. Aberg MA, Aberg ND, Palmer TD, Alborn AM, Carlsson-Skwirut C, Bang P, Rosengren LE, Olsson T, Gage FH, Eriksson PS: IGF-I has a direct proliferative effect in adult hippocampal progenitor cells. Mol Cell Neurosci 2003; 24:23–40
18. Ray J, Raymon HK, Gage FH: Generation and culturing of precursor cells and neuroblasts from embryonic and adult central nervous system. Methods Enzymol 1995; 254:20–37
19. Palmer TD, Ray J, Gage FH: FGF-2-responsive neuronal progenitors reside in proliferative and quiescent regions of the adult rodent brain. Mol Cell Neurosci 1995; 6:474–86
20. Gage FH, Ray J, Fisher LJ: Isolation, characterization, and use of stem cells from the CNS. Annu Rev Neurosci 1995; 18:159–92
21. Merkel G, Eger EI 2nd: A comparative study of halothane and halopropane anesthesia including method for determining equipotency. ANESTHESIOLOGY 1963; 24:346–57
22. Vansteensel MJ, Magnone MC, van Oosterhout F, Baeriswyl S, Albrecht U, Albus H, Dahan A, Meijer JH: The opioid fentanyl affects light input, electrical activity and Per gene expression in the hamster suprachiasmatic nuclei. Eur J Neurosci 2005; 21:2958–66
23. McDowell TS: Exogenous nerve growth factor attenuates opioid-induced inhibition of voltage-activated Ba2+ currents in rat sensory neurons. Neuroscience 2004; 125:1029–37
24. Cahill CM, Morinville A, Lee MC, Vincent JP, Collier B, Beaudet A: Prolonged morphine treatment targets delta opioid receptors to neuronal plasma membranes and enhances delta-mediated antinociception. J Neurosci 2001; 21: 7598–607
25. Rizzi S, Carter LB, Ori C, Jevtovic-Todorovic V: Clinical anesthesia causes permanent damage to the fetal guinea pig brain. Brain Pathol 2008; 18:198–210
26. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402–8
27. Shetty AK, Turner DA: *In vitro* survival and differentiation of neurons derived from epidermal growth factor-responsive postnatal hippocampal stem cells: Inducing effects of brain-derived neurotrophic factor. J Neurobiol 1998; 35:395–425
28. Campos LS: Neurospheres: Insights into neural stem cell biology. J Neurosci Res 2004; 78:761–9
29. Sonner JM, Antognini JF, Dutton RC, Flood P, Gray AT, Harris RA, Homanics GE, Kendig J, Orser B, Raines DE, Trudell J, Vissel B, Eger E III : Inhaled anesthetics and immobility: Mechanisms, mysteries, and minimum alveolar anesthetic concentration. Anesth Analg 2003; 97:718–40
30. Kindler CH, Eilers H, Donohoe P, Ozer S, Bickler PE: Volatile anesthetics increase intracellular calcium in cerebrocortical and hippocampal neurons. ANESTHESIOLOGY 1999; 90:1137–45
31. Bickler PE, Fahlman CS: The inhaled anesthetic, isoflurane, enhances Ca2+-dependent survival signaling in cortical neurons and modulates MAP kinases, apoptosis proteins and transcription factors during hypoxia. Anesth Analg 2006; 103:419–29
32. Bickler PE, Zhan X, Fahlman CS: Isoflurane preconditions hippocampal neurons against oxygen-glucose deprivation: role of intracellular Ca2+ and mitogen-activated protein kinase signaling. ANESTHESIOLOGY 2005; 103:532–9
33. Gray JJ, Bickler PE, Fahlman CS, Zhan X, Schuyler JA: Isoflurane neuroprotection in hypoxic hippocampal slice cultures involves increases in intracellular Ca2+ and mitogen-activated protein kinases. ANESTHESIOLOGY 2005; 102: 606–15
34. Liu X, Wang Q, Haydar TF, Bordey A: Nonsynaptic GABA signaling in postnatal subventricular zone controls proliferation of GFAP-expressing progenitors. Nat Neurosci 2005; 8:1179–87
35. Deisseroth K, Singla S, Toda H, Monje M, Palmer TD, Malenka RC: Excitation-neurogenesis coupling in adult neural stem/progenitor cells. Neuron 2004; 42:535–52
36. Shors TJ, Miesegaes G, Beylin A, Zhao M, Rydel T, Gould E: Neurogenesis in the adult is involved in the formation of trace memories. Nature 2001; 410:372–6