Isoflurane Decreases Self-renewal Capacity of Rat Cultured Neural Stem Cells

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ABSTRACT

Background: In models, isoflurane produces neural and behavioral deficits *in vitro* and *in vivo*. This study tested the hypothesis that neural stem cells are adversely affected by isoflurane such that it inhibits proliferation and kills these cells.

Methods: Sprague-Dawley rat embryonic neural stem cells were plated onto 96-well plates and treated with isoflurane, 0.7, 1.4, or 2.8%, in 21% oxygen for 6 h and fixed either at the end of treatment or 6 or 24 h later. Control plates received 21% oxygen under identical conditions. Cell proliferation was assessed immunocytochemically using 5-ethynyl-2′-deoxyuridine incorporation and death by propidium iodide staining, lactate dehydrogenase release, and nuclear expression of cleaved caspase 3. Data were analyzed at each concentration using an ANOVA; $P < 0.05$ was considered significant.

Results: Isoflurane did not kill neural stem cells by any measure at any time. Isoflurane, 1.4 and 2.8%, reduced cell proliferation based upon 5-ethynyl-2′-deoxyuridine incorporation, whereas isoflurane, 0.7%, had no effect. At 24 h after treatment, the net effect was a 20–30% decrease in the number of cells in culture.

Conclusions: Isoflurane does not kill neural stem cells *in vitro*. At concentrations at and above the minimum alveolar concentrations required for general anesthesia (1.4 and 2.8%), isoflurane inhibits proliferation of these cells but has no such effect at a subminimum alveolar concentration (0.7%). These data imply that dosages of isoflurane at and above minimum alveolar concentrations may reduce the pool of neural stem cells *in vivo* but that lower dosages may be devoid of such effects.

What We Already Know about This Topic

- Certain general anesthetics have been shown to have adverse effects on neuronal survival and development
- Isoflurane reduced neuronal stem cell proliferation at high doses, but its effects at clinically relevant concentrations are unclear

What This Article Tells Us That Is New

- Isoflurane reduced proliferation of cultured rat embryonic neuronal stem cells in a dose-dependent manner but did not produce evidence of enhanced cell death
- Reduced stem cell proliferation could underlie impaired neurologic development and function if validated *in vivo*

A GROWING body of data suggest that exposure to anesthetics during certain periods of development has long-term deleterious effects. At the cellular level, there is evidence that anesthetic agents induce cell death, cause synaptic remodeling, and alter morphology of the developing brain.[1–5] Moreover, in humans, children exposed to anesthesia in early life have a higher incidence of learning deficits in adolescence.[6] It is possible that anesthetic effects on neural stem cells (NSCs) may mediate some of these morphologic and behavioral phenotypes. NSCs are pluripotent cells in the central nervous system that maintain the capacity for self-renewal and ultimately differentiate into astrocytes, oligodendrocytes, and neurons. Proliferation, differentiation,

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and migration of cells derived from embryonic NSCs are critical processes for normal brain development.[7] These processes are highly regulated and tightly choreographed, especially by γ-aminobutyric acid (GABA).[8,9] GABA is a major inhibitory neurotransmitter in the adult brain but depolarizes NSCs and immature neurons, essentially acting in the developing brain as a trophic factor that regulates NSC proliferation, differentiation, and migration.[8,9] Not surprisingly, excessive or prolonged γ-aminobutyric acid–mediated (GABAergic) stimulation during a critical period of neurodevelopment can derail neurogenesis and alter neural connectivity and behavior.[8,10–12]

It is relevant that many general anesthetic agents, including isoflurane, are γ-aminobutyric acid receptor type A ($GABA_A$) modulators.[13] However, few studies have investigated the effect of these agents on the capacity of NSCs to self-renew—the two main determinants of which are proliferation and death. There is evidence that the commonly used volatile anesthetic isoflurane affects the former but not the latter. Thus, isoflurane, 3.4%, impaired proliferation of hippocampal NSCs *in vitro*, and administration of an ED_{50} concentration to postnatal day 7 rats decreased hippocampal neurogenesis for at least 5 days.[2,14] Cell death was also studied in the *in vitro* experiment, but there was no evidence that isoflurane caused NSCs to die, despite apoptosis being a prominent histologic feature of the rodent brain exposed to isoflurane during the early postnatal period.[14] Interpretation of these results is difficult because the *in vitro* study used an isoflurane concentration (3.4%) significantly greater than the ED_{50} concentration required to maintain anesthesia, and the *in vivo* study varied the concentration as required to maintain a constant ED_{50} for movement (*i.e.*, minimum alveolar concentration), conditions that were associated with abnormal systemic physiology and a 25% death rate.[2,14,15] As such, it is unclear if the effects span the clinically relevant range. The current experiment was designed to test the hypothesis that isoflurane impairs proliferation and increases death of NSCs at high but not low concentrations of isoflurane.

Materials and Methods

The experimental protocol was approved by the Harvard Medical Area Standing Committee on Animals (Boston, MA) and consisted of treating NSCs cultured from embryonic day 18 Sprague-Dawley rats to 21% oxygen, with or without isoflurane (0.7, 1.4, or 2.8%), for 6 h. At the conclusion of exposure or 6 or 24 h later, cell viability and proliferation were evaluated by colorimetric assay or immunocytochemistry and high throughput, unbiased fluorescence microscopy, as appropriate.

Media

The media used were phosphate buffered saline (PBS)+: 500 ml sterile Dulbecco's phosphate-buffered saline (Invitrogen, Carlsbad, CA), 5 ml penicillin-streptomycin (Invitrogen), 5 ml Fungizone® antimycotic (Invitrogen); and B27 media: 500 ml Dulbecco's Modified Eagle Medium:F12 high glucose (Invitrogen), 2.5 ml 200 mM glutamine (Invitrogen), 5 ml Fungizone® antimycotic, 5 ml penicillin-streptomycin, and 10 ml B27 supplement without vitamin A (Invitrogen).

NSC Harvest and Culture

Neural stem cells were harvested from timed pregnancy embryonic day 14 Sprague-Dawley rats (Harlan Sprague-Dawley, Indianapolis, IN). Pregnant rats were killed by carbon dioxide intoxication and embryo cortices harvested and placed in ice-cold PBS+ in a 50-ml centrifuge tube. Cortices were washed three times by centrifuging at 3,000 rpm, aspirating the supernatant, and adding fresh PBS+. The cortices were incubated for 5 min in 5 ml high-glucose Dulbecco's Modified Eagle Medium (Invitrogen) to which 10 μl papain, 4% (Worthington Biochemical Corporation, Lakewood, NJ), 50 μl Dispase II (neutral protease, Roche Diagnostics, Indianapolis, IN), and 50 μl Dnase 1 (recombinant Rnase-free Dnase 1, Roche Diagnostics) were added. Cortices were triturated 10 times with 1,000-μl pipette, filtered through a 40-μm filter, washed three times in PBS+, suspended in 5 ml B27 media and the number of cells counted with a hemocytometer. Five-million cells were plated in 75 ml tissue culture flasks (BD Biosciences, Bedford, MA) containing 25 ml B27 media, and placed in a humidified cell culture incubator at 37°C with 5% CO_2. This tissue harvesting always occurred on a Tuesday, and the remaining steps were completed over 8 days. The flasks were fed with 5 ml fresh media on Thursday; cells were passaged the first time on Saturday and returned to 75-ml tissue culture flasks; cells were fed with 5 ml fresh media on Monday; finally, the cells were passaged onto 96-well plates on Tuesday. Cells were treated with isoflurane or oxygen alone the day after plating onto the 96-well plates. For passaging, both media and cells were removed and placed into a 50-ml centrifuge tube and centrifuged at 3,000 rpm for 3 min; the media was removed, and Accutase (5 ml, Millipore Corporation, Temecula, CA) was added and incubated at 37°C for 5 min, at which time the cells were triturated 10 times using a 1,000-μl plastic pipette tip. PBS+ was added to the cells, and the cells were washed three times and plated at density 5×10^6 cells in 25 ml B27 media in 75-ml tissue culture flasks. All experiments were performed at the second passage. On the second passage, 10^4 cells in 100 μl media was added to each well of matched (control and isoflurane) 96-well poly-L-ornithine–laminin-coated microplates (BD BioCoat, BD Biosciences, San Jose, CA) using a multichannel pipette (Eppendorf, Westbury, NY). While in flasks, cells were primarily in the form of spheres (fig. 1 A & B), whereas they grew as individual undifferentiated cells when on plates, as reflected by more than 98% of the cells being stained positive for both nestin and SOX2 (fig. 1C), including in the 24-h control plates.

Isoflurane Administration. Identical airtight chambers (Billups-Rothenberg, Del Mar, CA) and gas-content–certified

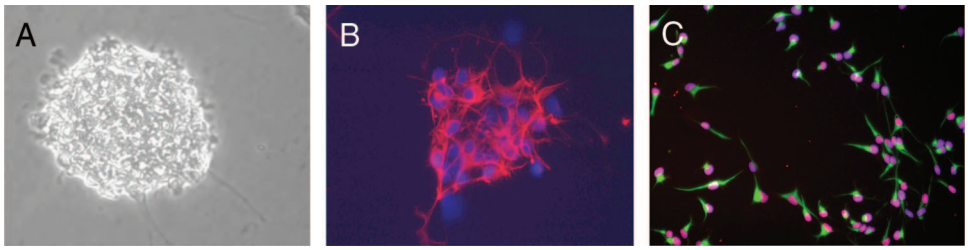

Fig. 1. Photomicrographs of neural stem cells (NSCs) in culture. Cells were isolated from embryonic rat brain at day 14 and cultured in proliferation media containing growth factors (see Methods). Phase contrast photograph taken before fixation of a neurosphere grown in 75-ml flasks *(A)*. Immunofluorescence image of a neurosphere that was fixed and stained with nestin (*pink*) and then counterstained with 4′6-diamidino-2-phenylindole, dilactate (*blue*), as the nuclear marker *(B)*. Immunofluorescence image of NSCs when grown on 96-well poly-D-lysine laminin-coated plates stained with both nestin (*green*) and SOX2 (*pink*) at passage 2. More than 98% of the plated cells stained positive for both nestin and SOX2 at the end of anesthesia and 24 h later, demonstrating they were NSCs *(C)*.

canisters containing 21% oxygen, 5% CO_2, and nitrogen (Airgas, Hingham, MA) were equilibrated to 37°C overnight in a heated room. Plates were randomly placed in either control or isoflurane-humidified chambers flushed with gas alone (control) or gas containing isoflurane at a rate of 2 l/min for 15 min followed by 100 ml/min for a total of 6 h at 37°C. Isoflurane, oxygen, and carbon dioxide concentrations were measured every 30 min with an agent analyzer (RGM 5250; Ohmeda, Louisville, CO). Removing the plates from the chambers terminated treatment. Experiments with isoflurane, 0.7, 1.4, and 2.8%, along with corresponding controls, were performed as distinct experiments on different days with cells from multiple harvests because of the availability of a single vaporizer.

Lactate Dehydrogenase Assay. Cell cytotoxicity was estimated by measuring the release of lactate dehydrogenase (LDH) into the culture medium. LDH was quantified using a commercially available colorimetric cytotoxicity detection kit (Roche Applied Science, Mannheim, Germany). Briefly, 100 μl supernatant was removed from 12 wells per plate (number based on previous experience) at the end of treatment or at 6 or 24 h after treatment and placed into a 96-well plate. One hundred microliters reaction mixture was added to each well and incubated (protected from light) for 30–60 min at room temperature. The absorbance of the samples was measured using a plate reader (SpectraMax M2; Molecular Devices, Sunnyvale, CA) and the absorbance of the reference wavelength (690 nm) subtracted from the absorbance at 492 nm. All values are expressed as a percent of the corresponding control for each dose at each time point.

EdU Incorporation Assay. Cell proliferation was determined based on EdU incorporation assay using a commercially available kit according to the manufacturer's instructions (Click-iT™ EdU Alexa Fluor® High-throughput Imaging (HCS) Assay; Invitrogen). EdU, like BrdU, is a thymidine analog that is incorporated into cells only during S-phase of cell division and is used to assess cell proliferation.[16] Briefly, 100 μl proliferation media containing 20 μM EdU (final concentration 10 μM) was added to 12 wells of a 96-well plate (number based on previous experience with the assay), containing cells in proliferation media either immediately before treatment with isoflurane, at end of treatment, or 18 h after treatment, for a total of 6 h. Cells were then fixed with paraformaldehyde, 4%, in PBS for 15 min. The fixative was removed, and the cells were washed twice with 100 μl bovine serum albumin (BSA), 3%, in PBS, and then incubated in 100 μl Triton® X-100 (Sigma, St. Louis, MO), 0.5%, in PBS for 20 min at room temperature. The cells were then washed twice and incubated with 100 μl Click-iT™ reaction cocktail for 30 min at room temperature. The reaction cocktail was removed, and the cells were washed twice and then blocked with 100 μl/well BSA, 3%, in PBS and incubated with an antinestin (Millipore, Billerica, MA; 1:500 dilution) primary antibody overnight at 4°C. The wells were then washed three times with 100 μl/well BSA, 3%, in PBS and the secondary antibody applied and incubated for 1 h at room temperature. The wells were then washed three times with 100 μl/well BSA, 3%, in PBS and incubated with 100 μl, 5 μg/ml Hoechst 33342 in PBS for 30 min at room temperature and then washed three times and stored in the dark at 4°C until image acquisition and analysis. Hoechst 33342 is a nuclear stain used to determine total cell counts.

Propidium Iodide (PI) Staining. Propidium iodide (Invitrogen) was added to each well and allowed to incubate for 5 min before fixation. PI is a fluorescent molecule that binds DNA; because it is membrane impermeant, PI labels only nonviable cells and immunocytochemical detection of nestin, as described.

Immunocytochemistry. Cells were fixed with paraformaldehyde, 4%, in PBS for 15 min either at the end of treatment or 6 or 24 h after treatment. The fixative was removed, and the cells were washed three times with 100 μl BSA, 3%, in PBS and blocked with 100 μl/well BSA, 3%, in PBS for 20 min at room temperature, and then incubated with the primary antibodies nestin (Millipore; 1:500 dilution), SOX2 (Invitrogen,1:200 dilution), or activated caspase 3 (Abcam Inc., Cambridge, MA; 1:500 dilution) overnight at 4°C. The wells were then washed three times and the secondary antibody (Alexa Fluor® 488 or Alexa Fluor® 555, Invitrogen) applied and incubated for 1 h at room temperature.

The wells were then washed 3 times and incubated with 100 μl, 5 μg/ml Hoechst 33342 in PBS for 30 min at room temperature and then washed three times with PBS and stored in the dark at 4°C until image acquisition and analysis. Cells treated with staurosporine (3 μM), a caspase 3 activator, served as a positive control for PI staining and cleaved caspase immunocytochemistry.

Image Acquisition and Analysis

Eight to 12 images (number based on previous experience with this system) were acquired per well using an IN Cell Analyzer 1000 (GE Healthcare, Piscataway, NJ) in an automated unbiased fashion. This compact bench-top instrument includes an automated Nikon™ (Nikon Inc., Melville, NY) microscope, high-resolution charge-coupled device camera, xenon lamp-based illumination, filter wheel-based wavelength control, and laser-based autofocus and associated image acquisition and analysis software (GE Healthcare). Because image acquisition is automated and large numbers of images can be acquired, cell selection bias is eliminated, and the impact of experimental and biologic variation is reduced.[17] With the use of a multitarget analysis, cells were first identified based upon having a nuclear area greater than 25 μm^2 using top-hat segmentation. This threshold setting excludes noncellular debris from the analysis. Cells were then outlined based on nestin staining overlapping a nucleus, as defined by staining with Hoechst, using multiscale top-hat segmentation with the characteristic area set at 100 μm^2 and a sensitivity of 50. For identification and analysis of EdU-, PI-, and cleaved caspase 3-positive cells, we used a two-step filtering process. In the first step, nestin-negative cells were excluded; in the second, threshold setting was used to determine the number of EdU-, PI-, and cleaved caspase 3-positive cells. Thus, only nestin-positive cells were analyzed and included in the final analysis. Imaging parameters were set based on a control plate stained with the fluorophore or antibody of interest and the parameters used to image an entire set of matched control and isoflurane-treated plates. Once the parameters were set, images were acquired automatically, meaning there was no cell selection bias. In these experiments, eight images were acquired per well using at 20× objective from 8 to 12 wells per treatment condition per assay per time (N = 8–12) in matched control and isoflurane-treated plates. Based on control experiments with staurosporine, a PI- or cleaved caspase 3-positive cell was defined as one with a nuclear/cellular intensity ratio greater than 2 or

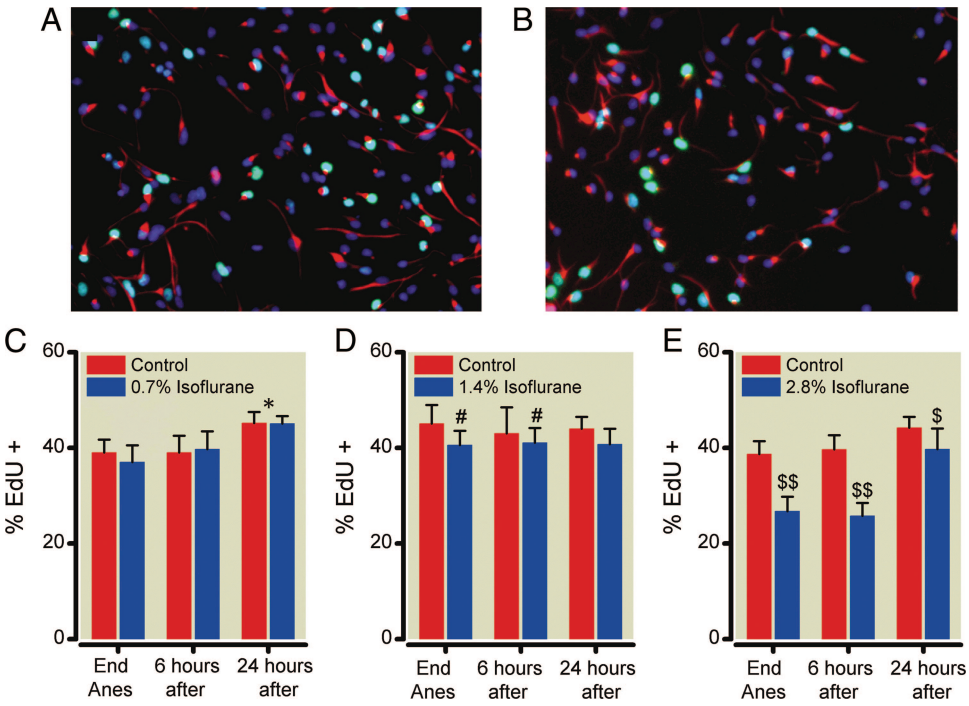

Fig. 2. Isoflurane and stem cell proliferation. With the use of matched plates, neural stem cells were exposed to control conditions or isoflurane, 0.7, 1.4, or 2.8%, for 6 h. Dividing cells were labeled at the end of treatment or 6 or 24 h later by incorporation of EdU. EdU staining in a control plate *(A)*. EdU staining in a matched plate treated with isoflurane, 2.8%, for 6 h. Hoechst (33342) nuclear staining (blue); EdU staining (Alexa Fluor 488) (*green*); nestin staining (Alexa Fluor 555) (*red*) *(B)*. Isoflurane, 0.7%, had no effect at any time point ($P = 0.60$) *(C)*. In contrast, isoflurane, 1.4 ($P = 0.004$) *(D)* and 2.8% ($P = <0.001$) *(E),* decreased the number of EdU-positive cells; the change lasted at least 24 h with isoflurane, 2.8%. Data at each concentration were analyzed by two-way ANOVA, using treatment condition and time posttreatment as the variables. Data are expressed as mean ± SD for N = 8 wells per treatment condition (eight images per well using a 20× objective) at each time point. * $P < 0.001$, 24 h after isoflurane *versus* end of treatment and 6 h after treatment; # $P < 0.01$, $ $P < 0.001$ end of isoflurane and 6 h after treatment *versus* control; $ $P < 0.01$ 24 h after isoflurane treatment *versus* control.

3, respectively. The nuclear intensity threshold for an EdU-positive cell was defined by the intensity of EdU-positive staining in matched control cells. A similar two-step filtering process was used to determine the percentage of cells staining positive for nestin and SOX2; cellular intensity of nestin was used as the first screen and nuclear intensity of SOX2 as the second.

Statistical Analysis

With the exception of PI staining, which failed normality testing, the remainder of the data from each assay at each time point from matched control and isoflurane-treated cells (plates) were analyzed using SigmaStat (Systat Software, Chicago, IL) software using two-way ANOVA with treatment condition and time as the between-group factors and Bonferroni corrections for multiple comparisons. Data for PI staining were analyzed using Kruskal-Wallis one-way ANOVA followed by Dunn's testing for multiple comparisons. Staurosporine experiments used as a positive control for apoptosis (cleaved caspase 3) and cell death (PI) were analyzed using an unpaired two-tailed Student *t* test. Cell number and SOX2 intensity 24 h after treatment were analyzed using unpaired two-tailed Student *t* test with a Bonferroni correction for multiple comparisons. Data are expressed as mean ± SD; $P < 0.05$ was considered statistically significant.

Results

In our culture system, more than 98% of control cells plated on poly-D-lysine laminin- coated plates stained positive for both nestin and SOX2 at the end of treatment and 6 and 24 h later, confirming the cells studied were NSCs (fig. 1). As mentioned, cells that were not nestin-positive were excluded from subsequent immunocytochemical analysis.

Cell Proliferation

Isoflurane, 0.7%, had no effect on proliferation of NSCs ($P = 0.60$; fig. 2), but there was an effect of time posttreatment, with increased proliferation 24 h after treatment in both the control and isoflurane-treated groups ($P < 0.001$) but no interaction between treatment condition and time posttreatment ($P = 0.42$; fig. 2C). With isoflurane, 1.4%, there was an effect of treatment condition, with impaired proliferation in isoflurane-treated cells relative to controls ($P = 0.004$) but no effect of time posttreatment ($P = 0.82$) and no interactions between treatment condition and time posttreatment ($P = 0.63$; fig. 2D). With isoflurane, 2.8%, there was an effect of treatment condition ($P < 0.001$) and time posttreatment ($P < 0.001$) and an interaction between isoflurane treatment and time posttreatment (end of treatment, control 39 ± 3%, isoflurane 27 ± 3%; 6 h after treatment, control 40 ± 3%, isoflurane 26 ± 3%; 24 h after treatment, control 44 ± 2%, isoflurane 40 ± 4%; $P < 0.001$, fig. 2E). This resulted in there being more EdU-positive cells 24 h after the experiment began under both control and isoflurane conditions, but the plates treated with isoflurane, 2.8%, had fewer EdU-positive cells than did time-matched control plates. These data suggest that isoflurane has no effect on NSC proliferation at low concentrations but sustained effects at higher concentrations.

Staurosporine and Cell Death

Staurosporine (6 h), as expected, increased the percentage of cleaved caspase 3-positive cells from 0.6 ± 0.1 under control conditions to 11.7 ± 1.6 and the percentage of PI-positive

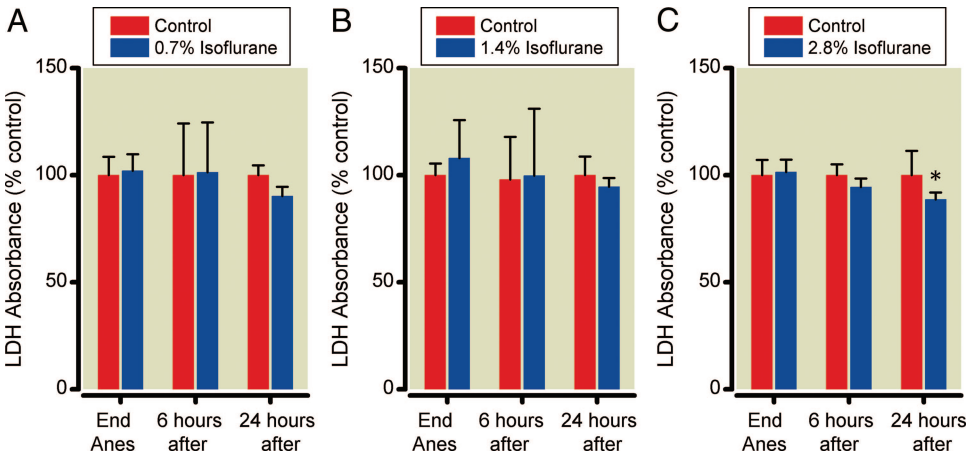

Fig. 3. Effect of isoflurane on lactate dehydrogenase (LDH) release. With the use of matched plates, neural stem cells were exposed to control conditions or isoflurane, 0.7, 1.4, or 2.8%, for 6 h. LDH was assayed in the media by colorimetric assay at the end of treatment or 6 or 24 h later. There was no effect of 0.7% isoflurane on LDH release ($P = 0.55$) *(A)*. Similarly, there was no effect of isoflurane, 1.4%, on LDH release ($P = 0.72$) *(B)*. In contrast, 24 h after treatment with isoflurane, 2.8%, LDH was reduced ($P = 0.006$) *(C)*. Data at each concentration were analyzed by two-way ANOVA, using treatment condition and time after treatment as the variables. Data are expressed as a percentage of matched control (mean ± SD) for N = 12 wells per treatment condition per time; * $P < 0.01$.

cells from 0.90 ± 0.40 under control conditions to 6.0 ± 0.40 ($P < 0.001$). There was clear differentiation between positive and negative cells when the nuclear/cellular intensity of cleaved caspase 3 and PI were more than 3 and 2, respectively. Accordingly, we used these parameters to set thresholds for defining caspase 3- and PI-positive cells in subsequent experiments.

Effects of Isoflurane on NSC Cytotoxicity and Cell Death

The concentration of LDH in the culture media was analyzed with a colorimetric assay (fig. 3). At isoflurane, 0.7%, there was no effect of treatment condition ($P = 0.55$) or time posttreatment ($P = 0.31$) and no interaction between the two ($P = 0.31$; fig. 3A). Likewise, there was no effect of treatment condition ($P = 0.72$) or time posttreatment ($P = 0.39$) and no interaction between the two ($P = 0.42$) with isoflurane, 1.4% (fig. 3B). In contrast, with isoflurane, 2.8%, there was an effect of treatment condition ($P = 0.002$) and time posttreatment ($P = 0.006$) and an interaction between the two ($P = 0.006$), with a decrease in LDH to 88 ± 3% of control 24 h after isoflurane was withdrawn (fig. 3C), perhaps because there are fewer cells at that time.

There were few dead or dying cells, as determined by PI staining, at any time under control conditions (less than 2% on average; fig. 4), indicating the cultures were healthy. There was no effect of treatment condition or time posttreatment with isoflurane, 0.7%, on the percentage of cells that stained positive for PI ($P = 0.49$, fig. 4C). At isoflurane, 1.4%, there was no effect of treatment condition, but there was an effect of time posttreatment, with a larger percentage of cells staining positive for PI 6 and 24 h after isoflurane treatment compared with those fixed at the end of treatment ($P < 0.001$; fig. 4D). The effect of isoflurane, 2.8%, was similar; there was no effect of treatment condition, but a larger percentage of cells stained positive for PI 6 and 24 h after isoflurane compared with those that were fixed at the end of treatment ($P < 0.01$, fig. 4E).

Similarly, few cells stained positive for cleaved caspase 3 under control conditions (less than 2% on average; fig. 5). With isoflurane, 0.7 and 1.4%, there was no effect of treatment condition ($P = 0.73$ and 0.87, respectively) or time posttreatment ($P = 0.82$ and 0.68, respectively), and there was no interaction between the two ($P = 0.99$ and 0.06, respectively) (figs. 5C and 5D). For isoflurane, 2.8%, there also was no effect of treatment condition ($P = 0.97$), but there was an effect of time posttreatment ($P < 0.001$) and an

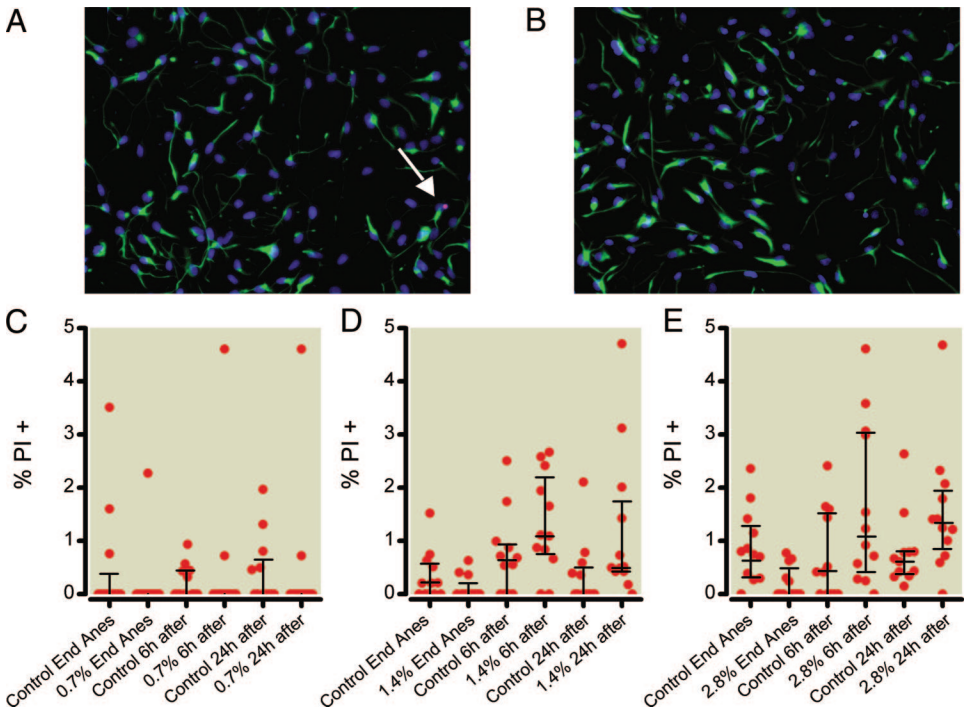

Fig. 4. Effect of isoflurane on cell death. With the use of matched plates, neural stem cells were exposed to control conditions or isoflurane, 0.7, 1.4, or 2.8%, for 6 h. The percentage of dead cells was determined by propidium iodide (PI) staining at the end of treatment or 6 or 24 h later. Image of PI-stained cells in a control plate *(A)*. Image of PI staining in a matched plate 24 h after isoflurane, 2.8% *(B)*. Hoechst (33342) nuclear staining (*blue*); nestin staining (Alexa Fluor 488) (*green*); PI (*pink*). PI-positive cells (*arrows*). Compared with the corresponding control, isoflurane, 0.7 *(C)*, 1.4 *(D)*, and 2.8% *(E)*, had no effect on the percentage of cells that stained positive for PI, but there was an effect of time after treatment, with a larger percentage of cells staining positive for PI 6 and 24 h after treatment with isoflurane, 1.4 and 2.8%, compared with the end of treatment time point. Because the data failed normality testing, results were analyzed using Kruskal-Wallis one-way ANOVA followed by Dunn's testing for multiple comparisons. Data are expressed as median (interquartile range) for N = 12 wells per treatment condition (eight images per well using a 20× objective) at each time point.

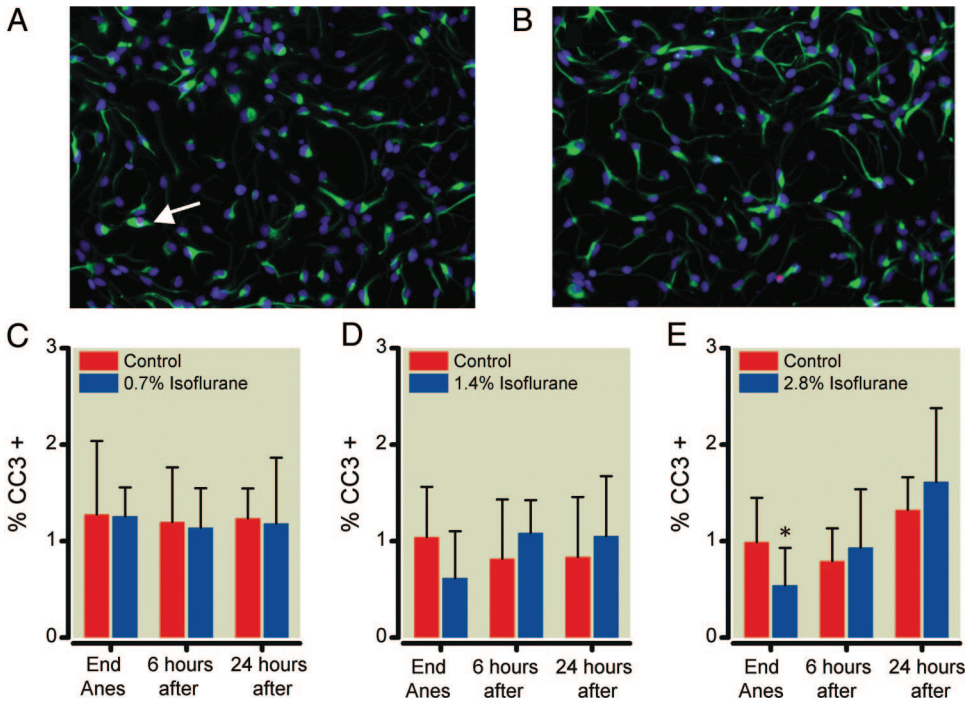

Fig. 5. Effect of isoflurane on cleaved caspase 3 (CC3). With the use of matched plates, neural stem cells were exposed to control conditions or isoflurane, 0.7, 1.4, or 2.8%, for 6 h. The percentage of CC3-positive cells was determined at the end of treatment or 6 or 24 h later. CC3-stained cells in a control plate 24 h after treatment *(A)*. CC3 staining in a matched plate 24 h after isoflurane, 2.8% *(B)*. Hoechst (33342) nuclear staining (*blue*); nestin (Alexa Fluor 488) (*green*); cleaved caspase 3 (*red*). CC3-positive cells (*arrows*). Isoflurane, 0.7 ($P = 0.73$) *(C)* and 1.4% ($P = 0.87$) *(D)* had no effect at any time. Isoflurane, 2.8% *(E)*, decreased the percentage of CC3-positive cells at the end of treatment ($P = 0.03$). Data were analyzed using a two-way ANOVA, with treatment condition and time after treatment as the variables. Data are expressed as mean ± SD for N = 12 wells per treatment condition (eight images per well using a 20× objective) at each time point. * $P < 0.05$ for isoflurane, 2.8%, *versus* control at the end of treatment.

interaction between the two ($P = 0.03$), indicating high-dose isoflurane reduces natural cell death in NSCs during the time of exposure but not subsequently (fig. 5E).

Effect of isoflurane on SOX2 expression. The percentage of cells expressing SOX2 did not change with isoflurane regardless of concentration, but the intensity of expression did (fig. 6). Thus, at 24 h after treatment with isoflurane, 0.7%, the nuclear intensity of SOX2 was no different from that in control cells (107 ± 9% of control; $P = 0.13$), but it was 7% (93 ± 5% of control; $P = 0.024$) and 14% (86 ± 7% of control; $P = 0.012$) lower in cells treated with isoflurane, 1.4 and 2.8%, respectively.

Ultimately, as determined by Hoechst staining and comparison with matched controls, these effects on self-renewal translated into there being 20–30% fewer cells in culture 24 h after treatment with isoflurane, 1.4 and 2.8% (fig. 7).

Discussion

The salient results of our study are that isoflurane decreases NSC proliferation but does not kill cultured embryonic NSCs. Importantly, we demonstrate these effects occur at concentrations of isoflurane that span the clinical range but not at a subminimum alveolar concentration. In fact, at the highest concentration, isoflurane reduced natural cell death as measured by PI staining and caspase activation, which confirms the previously identified ability of this agent to reduce or delay apoptosis in both *in vitro* and *in vivo* models.[18,19] Isoflurane's antiproliferative effect occurred only at 1.4 and 2.8% and persisted for at least 24 h only at the high concentration, but compared with matched controls, the net effect was a 20–30% decrease in the number of NSCs in culture 24 h after treatment. In addition, NSCs treated with isoflurane express less SOX2, a transcription factor important for maintaining an undifferentiated phenotype, which supports previous observations that this agent promotes early differentiation and neuronal fate selection.[14] Therefore, our results demonstrate that isoflurane impairs embryonic NSC proliferation at dosages at and above minimum alveolar concentrations, which implies it may impair neurogenesis and deplete the NSC pool *in vivo* at clinically relevant but not subminimum alveolar concentrations.

This is the first study to examine the effects of isoflurane at concentrations that span the clinical range on neural stem–progenitor cells, but it is not the first to investigate the impact of isoflurane on the death or proliferation of NSCs. A recent study using isoflurane, 3.4%, and NSCs harvested from postnatal day 2 rats reported a decrease in activated caspase 9 but not caspase 3/7 during and 2 h after exposure to isoflu-

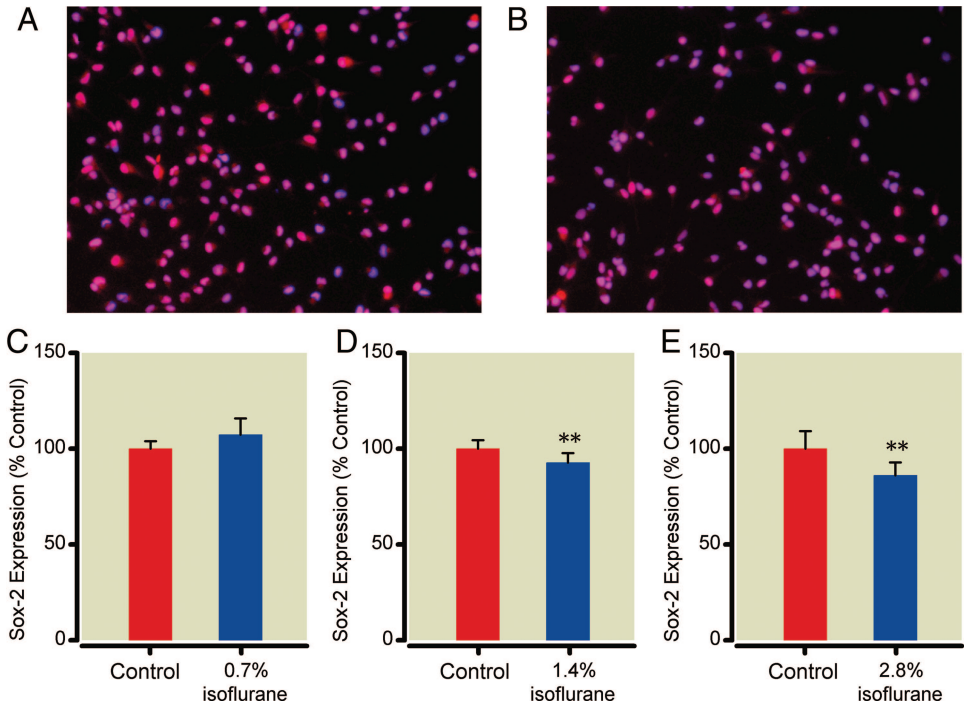

Fig. 6. Isoflurane decreases SOX2 expression. With the use of matched plates, neural stem cells were exposed to control conditions or isoflurane, 0.7, 1.4, or 2.8%, for 6 h. Cells were fixed and stained 24 h later with SOX2 (Alexa Fluor 555; *red*) and Hoechst 33342 (*blue*). SOX2-stained cells in a control plate 24 h after treatment *(A)*. SOX2 staining in a matched plate 24 h after isoflurane, 2.8%. Hoechst (33342) nuclear staining (*blue*); SOX2 staining (*red*) *(B)*. There was no effect of isoflurane on the percentage of cells expressing SOX2 *(C)*, but isoflurane 1.4 ($P = 0.024$) *(D)* and 2.8% ($P = 0.012$) *(E)* produced a 7% and 14% decrease, respectively, in nuclear expression of SOX2. Data are expressed as mean ± SD for N = 8 wells per treatment condition (eight images per well using a 20× objective and appropriate filters) at each time point. ** $P \leq 0.01$ by Student *t* test.

rane but no change in LDH release 18 h after withdrawal of the agent.[14] Our results generally agree with those of that study[14] inasmuch as we found no evidence for cytotoxicity in this model using either PI staining or cleaved caspase 3. This is interesting given that the concentrations of isoflurane (3.4% *vs.* 0.7, 1.4, and 2.8%), timing of assessment (during and 2 h after *vs.* during and 6 and 24 h), and age of the animals used for the stem cell harvest (postnatal rats *vs.* E14 animals) are different. This latter point is noteworthy because the expression of receptors and signaling systems relevant for the action of isoflurane (*e.g.*, GABA and *N*-methyl-D-aspartic acid receptors) are developmentally regulated[8,20,21] such that sensitivity to isoflurane might be expected to vary with developmental age. Thus, the fact that neither study found a cytotoxic effect is persuasive evidence that NSCs *in vitro*, compared with neurons, are resistant to the cytotoxic effects of isoflurane.

Isoflurane's effect on proliferation is a different story. Whereas isoflurane, 0.7%, had no effect at any time, both 1.4 and 2.8% reduced EdU incorporation significantly, with the latter change lasting at least 24 h. This decrease in NSC proliferation is broadly consistent with other *in vitro* and *in vivo* work with isoflurane. Studies report a 20–40% decrease in proliferation of NSCs in culture or in the hippocampus of postnatal day 7 animals exposed to isoflurane concentrations of 1.4–3.4%, and brief repeated exposure to 1.7% produces a 20% decrease in NSC proliferation acutely and a 42% decrease in the radial glia-like stem cell pool in P14 rats.[1,2,14,15] Although we found changes of a similar magnitude in our culture model, there are two important differences between the *in vitro* and *in vivo* data. First, whereas the antiproliferative effect of isoflurane is temporary at low dosages *in vitro* (recovering within 24 h at all but the two highest concentrations; fig. 2), it persists for days or even weeks *in vivo*.[1,2] This implies that although isoflurane initiates cell cycle arrest by a direct effect on NSCs, the sustained antiproliferative effect *in vivo* may be maintained by secondary effects of isoflurane on surrounding neural tissue. Importantly, our results indicate isoflurane, 0.7%, is without effect on NSC proliferation, implying low concentrations of isoflurane may have minimal or no adverse effects on developmental processes that depend on NSC self-renewal.

The mechanisms by which isoflurane impairs proliferation have not been investigated. Isoflurane is a pleiotropic agent that modulates GABA receptors and antagonizes *N*-methyl-D-aspartic acid receptors, but these are not the only targets of isoflurane that might be relevant to its effect on NSC proliferation.[13] Embryonic NSCs express GABA and *N*-methyl-D-aspartic acid receptors, but the latter are inactive at this stage of development,[8,20,21] leaving GABA receptors as a likely target in these cells. $GABA_A$ receptors are excitatory at this stage of neurodevelopment (in contrast to their

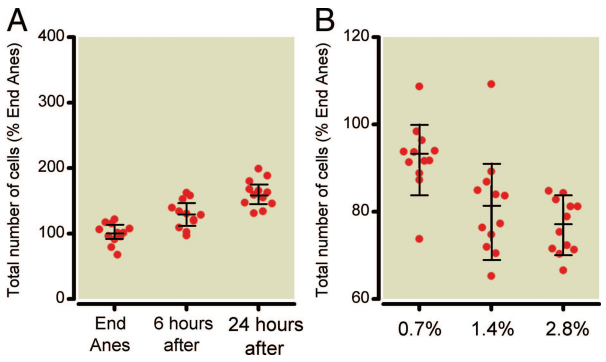

Fig. 7. Effect of isoflurane on number of neural stem cells (NSCs) in culture. With the use of matched plates, NSCs were exposed to control conditions or isoflurane, 0.7, 1.4, or 2.8%, for 6 h. The absolute number of cells was determined 24 h after anesthesia based on the number of Hoechst staining cell nuclei. NSCs in culture proliferate over time, leading to an increase in the number of control cells at 6 and 24 h after treatment ($P < 0.001$). Data expressed as a percentage of end of anesthesia controls and analyzed using a one-way ANOVA *(A)*. Consistent with its antiproliferative effect, isoflurane decreased the number of NSCs present 24 h after treatment relative to the number of cells in control plates ($P < 0.001$). Data expressed as a percentage of their time-matched controls and analyzed using a one-way ANOVA. Data are expressed as mean ± SD for N = 12 wells per treatment condition (eight images per well) *(B)*.

inhibitory effect in adulthood).[8,22] GABA is tonically released by NSCs and acts as a trophic factor regulating key developmental processes, including proliferation of NSCs, neuronal differentiation, and migration.[8,9] Thus, not surprisingly, excessive or prolonged GABAergic stimulation, such as with ethanol or valproic acid, which like isoflurane are GABA agonists/modulators, decreases NSC proliferation, increases differentiation, and alters neural connectivity.[11,12] As such, we theorize that the excessive GABA receptor-mediated excitation produced in NSCs during exposure to isoflurane may cause decreased proliferation of these cells. Indeed, although the percentage of cells expressing SOX2 was unchanged by isoflurane treatment, the intensity of SOX2 expression was reduced with isoflurane, 1.4 and 2.8%, which are the same concentrations that decreased proliferation. SOX2 is a key transcription factor for maintaining the self-renewal capacity of undifferentiated embryonic stem cells.[23] Therefore, our data suggest that isoflurane may reduce NSC proliferation and potentially promote early differentiation of NSCs *via* a SOX2-dependent mechanism.

Our study is limited in several important ways. First, EdU is a thymidine analog that is incorporated into DNA only during the S-phase of cell division or during DNA repair. Accordingly, an alternative explanation for our results is that isoflurane damages DNA and the EdU incorporation reflects DNA repair, rather than cell division. However, this seems unlikely given that isoflurane decreased the absolute number of NSCs relative to the control state. Second, we can only speculate about the pharmacologic and molecular mechanisms responsible for the effects we have observed. Third, we do not know whether isoflurane has consequences for brain development beyond or in addition to affecting the self-renewal capacity of NSCs. For example, GABA plays a prominent role in neuronal migration, but that aspect of isoflurane's action has yet to be examined.[10] Fourth, the SOX2 results are based on changes in intensity of immunostaining, which is less reliable than cell counting. As such, conclusions about the effect of isoflurane on NSC differentiation must be made cautiously and will require independent confirmation.

The biologic significance of these results is unclear. Normal brain development depends upon a readily available pool of NSCs to generate, in the right location, the proper numbers of neurons and glia.[10] NSCs are also present in the mature brain, albeit in lower numbers, where they are implicated in processes ranging from learning and memory to mood and epilepsy and in brain maintenance and repair.[24] Thus, even a transient adverse effect of isoflurane on self-renewal of NSCs might have far-reaching consequences for brain development and function across the life-span. To date, such developmental consequences have not been demonstrated. However, a 15–20% decrease in neurogenesis *in vivo*, which resembles the effect observed here, is sufficient to impair hippocampal-dependent memory in rodents,[25] and even more striking, recall of remote spatial memory in adult animals depends on recruiting as few as 1–4% newly born neurons into hippocampal circuits.[26] Therefore, these results may have implications for the use of isoflurane at critical periods of brain development as well as in adulthood.

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References

1. Zhu C, Gao J, Karlsson N, Li Q, Zhang Y, Huang Z, Li H, Kuhn HG, Blomgren K: Isoflurane anesthesia induced persistent, progressive memory impairment, caused a loss of neural stem cells, and reduced neurogenesis in young, but not adult, rodents. J Cereb Blood Flow Metab 2010; 30:1017–30
2. Stratmann G, Sall JW, May LD, 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
3. 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
4. Loepke AW, Istaphanous GK, McAuliffe JJ 3rd, Miles L, Hughes EA, McCann JC, Harlow KE, Kurth CD, Williams MT, Vorhees CV, Danzer SC: The effects of neonatal isoflurane exposure in mice on brain cell viability, adult behavior, learning, and memory. Anesth Analg 2009; 108:90–104
5. Zhao Y, Liang G, Chen Q, Joseph DJ, Meng Q, Eckenhoff RG, Eckenhoff MF, Wei H: Anesthetic-induced neurodegeneration mediated *via* inositol 1,4,5-trisphosphate receptors. J Pharmacol Exp Ther 2010; 333:14–22

6. Wilder RT, Flick RP, Sprung J, Katusic SK, Barbaresi WJ, Mickelson C, Gleich SJ, Schroeder DR, Weaver AL, Warner DO: Early exposure to anesthesia and learning disabilities in a population-based birth cohort. Anesthesiology 2009; 110:796–804
7. Uylings HB: Development of the cerebral cortex in rodents and man. Eur J Morphol 2000; 38:309–12
8. Ge S, Pradhan DA, Ming GL, Song H: GABA sets the tempo for activity-dependent adult neurogenesis. Trends Neurosci 2007; 30:1–8
9. Ben-Ari Y: Excitatory actions of gaba during development: The nature of the nurture. Nat Rev Neurosci 2002; 3:728–39
10. Métin C, Vallee RB, Rakic P, Bhide PG: Modes and mishaps of neuronal migration in the mammalian brain. J Neurosci 2008; 28:11746–52
11. Ieraci A, Herrera DG: Single alcohol exposure in early life damages hippocampal stem/progenitor cells and reduces adult neurogenesis. Neurobiol Dis 2007; 26:597–605
12. Umka J, Mustafa S, ElBeltagy M, Thorpe A, Latif L, Bennett G, Wigmore PM: Valproic acid reduces spatial working memory and cell proliferation in the hippocampus. Neuroscience 2010; 166:15–22
13. Franks NP: General anaesthesia: From molecular targets to neuronal pathways of sleep and arousal. Nat Rev Neurosci 2008; 9:370–86
14. Sall JW, Stratmann G, Leong J, McKleroy W, Mason D, Shenoy S, Pleasure SJ, Bickler PE: Isoflurane inhibits growth but does not cause cell death in hippocampal neural precursor cells grown in culture. Anesthesiology 2009; 110:826–33
15. Stratmann G, Sall JW, Eger EI 2nd, Laster MJ, Bell JS, May LD, Eilers H, Krause M, Heusen F, Gonzalez HE: Increasing the duration of isoflurane anesthesia decreases the minimum alveolar anesthetic concentration in 7-day-old but not in 60-day-old rats. Anesth Analg 2009; 109:801–6
16. Chehrehasa F, Meedeniya AC, Dwyer P, Abrahamsen G, Mackay-Sim A: EdU, a new thymidine analogue for labelling proliferating cells in the nervous system. J Neurosci Methods 2009; 177:122–30
17. Dragunow M: High-content analysis in neuroscience. Nat Rev Neurosci 2008; 9:779–88
18. Sakai H, Sheng H, Yates RB, Ishida K, Pearlstein RD, Warner DS: Isoflurane provides long-term protection against focal cerebral ischemia in the rat. Anesthesiology 2007; 106:92–9; discussion 8–10
19. Kawaguchi M, Drummond JC, Cole DJ, Kelly PJ, Spurlock MP, Patel PM: Effect of isoflurane on neuronal apoptosis in rats subjected to focal cerebral ischemia. Anesth Analg 2004; 98:798–805
20. 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
21. Haberny KA, Paule MG, Scallet AC, Sistare FD, Lester DS, Hanig JP, Slikker W Jr: Ontogeny of the *N*-methyl-D-aspartate (NMDA) receptor system and susceptibility to neurotoxicity. Toxicol Sci 2002; 68:9–17
22. Maher BJ, LoTurco JJ: Stop and go GABA. Nat Neurosci 2009; 12:817–8
23. Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R: Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 2003; 17: 126–40
24. Zhao C, Deng W, Gage FH: Mechanisms and functional implications of adult neurogenesis. Cell 2008; 132:645–60
25. Imayoshi I, Sakamoto M, Ohtsuka T, Takao K, Miyakawa T, Yamaguchi M, Mori K, Ikeda T, Itohara S, Kageyama R: Roles of continuous neurogenesis in the structural and functional integrity of the adult forebrain. Nat Neurosci 2008; 11: 1153–61
26. Trouche S, Bontempi B, Roullet P, Rampon C: Recruitment of adult-generated neurons into functional hippocampal networks contributes to updating and strengthening of spatial memory. Proc Natl Acad Sci USA 2009; 106:5919–24