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Isoflurane Differentially Affects Neurogenesis and Long-term Neurocognitive Function in 60-day-old and 7-day-old Rats

Greg Stratmann, M.D., Ph.D., Jeffrey W. Sall, M.D., Ph.D.,† Laura D. V. May, M.A.,‡ Joseph S. Bell, B.A.,‡ Kathy R. Magnusson, D.V.M., Ph.D.,§ Vinuta Rau, Ph.D.,‖ Kavel H. Visrodia, B.A., B.S.,‡ Rehan S. Alvi, M.D.,‡ Ban Ku, B.A.,‡ Michael T. Lee, M.A.,‡ Ran Dai, B.A.‡*

Background: **Anesthetic agents cause cell death in the developing rodent brain and long-term, mostly hippocampal-dependent, neurocognitive dysfunction. However, a causal link between these findings has not been shown. Postnatal hippocampal neurogenesis affects hippocampal function into adulthood; therefore, the authors tested the hypothesis that isoflurane affects long-term neurocognitive function *via* an effect on dentate gyrus neurogenesis.**

Methods: **The S-phase marker 5-bromodeoxyuridine was administered at various times before, during, and after 4 h of isoflurane given to postnatal day (P)60 and P7 rats to assess dentate gyrus progenitor proliferation, early neuronal lineage selection, and long-term survival of new granule cell neurons. Fear conditioning and spatial reference memory was tested at various intervals from 2 weeks until 8 months after anesthesia.**

Results: **In P60 rats, isoflurane increased early neuronal differentiation as assessed by BrdU/NeuroD costaining, decreased progenitor proliferation for 1 day, and subsequently increased progenitor proliferation 5–10 days after anesthesia. In P7 rats, isoflurane did not induce neuronal lineage selection but decreased progenitor proliferation until at least 5 days after anesthesia. Isoflurane improved spatial reference memory of P60 rats long-term, but it caused a delayed-onset, progressive, persistent hippocampal deficit in P7 rats in fear conditioning and spatial reference memory tasks.**

Conclusion: **The authors conclude that isoflurane differentially affects both neurogenesis and long-term neurocognitive function in P60 and P7 rats. Neurogenesis might mediate the long-term neurocognitive outcome after isoflurane at different ages.**

ANESTHESIA kills neonatal brain cells of several animal species, including primates.[1] A combination of γ-aminobutyric-acid (GABA)–ergic and *N*-methyl-D-aspartate antagonist agents is particularly neurotoxic; however propofol, isoflurane, ketamine, and midazolam have all caused apoptosis individually.[1] We have shown that 4 h of hypercapnia, which caused a similar degree and distribution of cell death as 4 h of isoflurane, did not cause a neurocognitive deficit.[2] Likewise, 2 h of isoflurane caused cell death in many areas of the brain relevant to the behavioral outcome tested, but no neurocognitive deficit was observed.[2] The only intervention that caused both cell death and neurocognitive dysfunction was 4 h of isoflurane. This raises suspicion about whether anesthesia-related brain cell death in postnatal day (P)7 rats can really account for the cognitive deficit observed after anesthesia.[2–5]

The developing brain has a high degree of plasticity. In P7 rats, cell birth is a very common event. Additional division(s) of progenitors in the P7 rat brain should be able to compensate for the loss of cells due to anesthesia-induced cell death unless anesthesia also impairs neurogenesis. Neurogenesis occurs during development and persists in the subventricular zone and the hippocampal dentate gyrus (DG) in the adult brain, where it is important for learning and memory.[6–10] Neurogenesis in the subventricular zone, and the DG has been shown to increase when the brain loses neurons, such as in stroke (for review see Sharp *et al.*[11]). This begs the question of whether isoflurane-induced brain cell death might also increase neurogenesis. Neurogenesis is the composite outcome of progenitor proliferation, neuronal differentiation, migration, and survival through activity-dependent integration into existing neural networks. We hypothesized that isoflurane, in addition to causing cell death in the developing rat brain, impairs one or more events required for DG neurogenesis such as progenitor proliferation, neuronal differentiation, and new neuronal survival.

Curiously, young adult rats do not develop neurocognitive decline after anesthesia. In fact, Culley *et al.*[12] have shown an unexplained improvement in neurocognitive function of young adult rats after anesthesia. Neurogenesis occurs both during development and in the adult brain and can by itself affect cognitive outcome[6–10]; we therefore hypothesized that anesthesia enhances some aspect of neurogenesis and improves neurocognitive outcome in young adult rats.

Here we show that 4 h of isoflurane does not cause cell death in P60 rats and increases neuronal differentiation,

* Associate Professor, † Assistant Professor, ‡ Research Assistant, ‖ Assistant Researcher, Department of Anesthesia, University of California, San Francisco; § Professor, Department of Biomedical Sciences, College of Veterinary Medicine, Oregon State University, Corvallis, Oregon.

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Address correspondence to Dr. Stratmann: Department of Anesthesia and Perioperative Care, University of California San Francisco, Box 0464, Room U286, 513 Parnassus Ave, San Francisco, California 94143. stratman@anesthesia.ucsf.edu.

causing a temporary decrease and subsequent compensatory increase in progenitor proliferation followed by an improvement in neurocognitive function 8 weeks later. In P7 rats on the other hand, isoflurane decreases progenitor proliferation without increasing neuronal differentiation and causes a neurocognitive deficit that is progressive and persistent.

Materials and Methods

Rat Anesthesia

All experiments were approved by the Institutional Animal Care and Use Committee of the University California, San Francisco, California. Male P60 rats (n = 40) and P7 rats (n = 141) were anesthetized in groups of 10–20 at 1 minimum alveolar concentration (MAC) as determined by tail clamping. MAC was initially assumed to be 3.5% for P7 and 2.4% for P60 rats. A supramaximal pain stimulus was generated by application of an alligator clamp to each rat's tail for 30 s or until the rat moved. Movement was defined as any movement except breathing. Tail clamping was repeated every 15 min, starting 15 min after induction of general anesthesia. The anesthetic concentration estimated as MAC and alterations in the delivered concentration were obtained from the determined concentration and the empirically derived algorithm in table 1 (see legend to table 1). The algorithm takes into account the tendency for anesthetic requirements to decrease over time in P7 rats. Custom-made temperature probes were inserted subcutaneously over the skull to facilitate control of temperature at 36.5 ± 1°C using computer-controlled Peltier heater/cooler plates integrated into the floor of the anesthetizing chamber. Hemoglobin oxygen saturation and heart rate were measured by application of a rodent transreflectance sensor (Nonin 2000T; Nonin Medical, Minneapolis, MN) to the ventral thoracic chest wall. The probe was coupled to a Nonin V8600 pulse oximeter (Nonin Medical). At various intervals throughout the anesthetic, blood (0.25 ml) was withdrawn either blindly through a 30-gauge needle from the heart of designated homeostatic P7 rats or from a P10 polyethylene catheter inserted into the tail artery of P60 cardiorespiratory control rats (n = 6). PH, arterial oxygen, and carbon dioxide tensions, base excess, blood hemoglobin, and blood glucose was analyzed by a blood gas analyzer (ABL 520; Radiometer, Copenhagen, Denmark). Although a total of 4 blood draws were performed in P60 rats, blood was withdrawn from only 1 rat per timepoint in P7 rats.

Table 1. Protocol for MAC Determination and Adjustment of Isoflurane Concentration in Response to Tail Clamping Every 15 Minutes for 4 Hours

Percent of Rats Moving in Response to Tail Clamping	Subsequent Percent Adjustment of Inspired Isoflurane Concentration		
	P7	P60, 15–60 min	P60, 75–240 min
0%	–1.0%	–0.6	–0.6
10%	–0.8%	–0.4	–0.4
20%	–0.6%	–0.3	–0.3
30%	–0.4%	–0.2	–0.2
40%	–0.2%	–0.1	–0.1
50%	No change	No change	No change
60%	No change	No change	+0.1
70%	+0.1%	+0.1	+0.2
80%	+0.2%	+0.2	+0.3
90%	+0.3%	+0.3	+0.4
100%	+0.5%	+0.5	+0.6

The anesthetic concentration was initially set to 3.5–4% isoflurane (see fig. 1A). Tail clamping commenced at 15 minutes and was repeated every 15 minutes until the end of anesthesia. The anesthetic concentration was adjusted according to the rats' responses to tail clamping as depicted in this table. The fact that the algorithm changes after 75 minutes in postnatal day (P)60 rats reflects the fact that the minimum alveolar concentration (MAC) reaches a plateau at this time in P60 rats; in P7 rats, MAC changes with increasing duration of anesthesia (fig. 1). The above algorithm resulted in the MAC values depicted in figure 1A.

Sham Anesthesia

Control rats were placed in the anesthesia glove box for 4 h without exposure to anesthetic agent but otherwise identical conditions as animals in the isoflurane group.

BrdU Injections

Bromodeoxyuridine (BrdU; Sigma, St. Louis, MO; 15 mg/ml) was injected intraperitoneally as listed in table 2 to separately target the processes of progenitor proliferation, neuronal differentiation, and new neuronal survival. We were specifically interested in how isoflurane affects proliferation of progenitors that are in the S-phase of the cell cycle during the time of the anesthetic (labeling during anesthesia), how it affects proliferation and early lineage selection of progenitors that were labeled just before 4 h of anesthesia, how isoflurane affects progenitor proliferation 4 days after anesthesia, and if survival of these progenitor populations differently labeled with respect to the anesthetic is affected.

Tissue Preparation and Immonocytochemistry

Animals were deeply anesthetized with isoflurane and then transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4. The brains were removed, postfixed overnight in 4% paraformaldehyde/PBS, and placed in 20% sucrose until sunk. Coronal sections (40 μm) were cut on a microtome and stored in PBS. For immunocytochemical detection of BrdU-labeled nuclei, DNA was denatured to expose the antigen. Incubation with 50% formamide in PBS for 2 h at 65°C preceded 2-normal hydrochloric acid incubation for 30 min at 37°C and neutralization with 0.5 M boric acid pH 8.5 for 10 min at room temperature (RT) and 3 washes with PBS for 10 min in between each of these steps. Blocking of nonspecific epitopes with 3% serum and 0.1% bovine serum

Table 2. Experimental Protocol

	BrdU Injections: Timing/Dose (mg/kg)		Interval to Perfusion		ICC Stain	
Targeted Process	P7	P60	P7	P60	P7	P60
Proliferation	–4 h/300	–4 h/300	8 h	8 h	BrdU	BrdU
	–0 h/300	+0 h/300	12 h	12 h		
	+4 d × 2/50	+4 d × 2/50	12 h	12 h		
Differentiation	–4 h/300	–4 h/300	8 h	8 h	BrdU/NeuroD	BrdU/NeuroD
	–0 h/300	–0 h/300	8 h	8 h		
Survival	–4 h/300	–4 h/300	28 d	28 d	BrdU/NeuN	BrdU/NeuN
	+4 d × 8/50	+4d × 8/50	28 d	28 d		

Times of BrdU injections are given as hours or days before (−) or after (+) a 4-h isoflurane anesthetic. If multiple injections were made, the interval between injections was 12 hours. For example, a timing of −0 h and dose of 300 mg/kg denotes injection of 300 mg/kg right before the induction of a 4-hour isoflurane anesthetic; timing of +0 h and dose of 300 mg/kg indicates that 300 mg/kg BrdU was injected at the conclusion of a 4-hour isoflurane anesthetic; timing of +4 d × 8 and dose of 50 mg/kg denotes that the first of 8 injections of 50 mg/kg each was made 4 days after the end of a 4-hour isoflurane anesthetic with 12-hour intervals between injections. The interval to perfusion refers to the time from the last BrdU injection to transcardiac perfusion. Survival refers to survival of BrdU-labeled cells to 4 weeks after labeling. BrdU = 5-bromodeoxyuridine; ICC = immunocytochemical; NeuN = neuon-specific nuclear protein; P = postnatal day.

albumin in PBS with 0.3% Triton-X (Fisher Scientific, Pittsburg, PA) for 30 min at RT preceded incubation overnight at 4°C with the primary antibody listed in table 3 in PBS and bovine serum albumin 0.2%.

On day 2 of the single BrdU stain, incubation with a biotinylated antibody (sheep anti-mouse IgG, 1:200; Amersham [GE Healthcare], Piscattaway, NJ) for 2 h at RT was performed. Streptavidin-biotin treatment for 2 h at RT (Vectastain, ABC kit, Catalogue # PK 6100; Vector Laboratories, Burlingame, CA) was followed by three thorough washes to eliminate residual peroxidase activity. This was followed by incubation with diaminobenzidine + urea (Fast DAB tablets, Sigma) with nickel chloride augmentation (7.5 μl of 8% stock in 25 ml) for 5 min followed by mounting and coverslipping with Depex mounting medium (Electronmicroscopy Sciences, Fort Washington, PA).

Double immunofluorescent stains were performed using an identical protocol for day 1 as the above single stain except for incubation with the Serotec (Serotec, Raleigh, NC) anti-BrdU antibody (table 2). On day 2, incubation with the appropriate secondary fluorescent antibodies Alexafluor 488 and Alexafluor 594 (1:500; Invitrogen, Carlsbad, CA) occurred for 2 h at RT. Nuclear counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI, 1:1000; Sigma) followed by mounting and coverslipping with an acqueous mounting medium.

FluoroJade Stain

Slices of P60 rat brain tissue were mounted on glass slides and covered in 100% ethanol for 3 min, then 70% ethanol for 1 min, and then deionized water (dH_2O) for 1 min. Each slide contained one positive control slice of brain tissue from a P60 rat treated with 10 mg/kg kainic acid intraperioneally. Tissue was incubated with 0.06% potassium permanganate for 20 min on a shaker at RT before a 1-min wash with dH_2O. Slides were then incubated with FluoroJade staining solution (4 ml of 0.01% FluoroJade [in dH_2O] stock solution, 36 ml of 0.1% acetic acid in dH_2O, 40 μl DAPI) for 30 min at RT before 3 washes with dH_2O for 1 min each. After air-drying, slides were briefly rinsed with xylene and coverslipped with Depex mounting solution. All areas of the brain were inspected for FluoroJade staining in every twelfth section.

Microscopy

Single-stained BrdU+ cells were detected using brightfield microscopy with a 4×, 20×, and 100× objective lenses if required to distinguish cells within tightly packed cell conglomerates. The granule cell layer and the subgranular zone of each DG was traced using StereoInvestigator® software (MicroBrightField, Williston, VT). An observer blinded to group assignment determined the number of BrdU+ cells per DG in every twelfth slice for each animal.

Table 3. Primary Antibodies

Raised Against	Supplier (ID)	Raised in	Dilution
BrdU	Roche, Clone (BMC9318)	Mouse, monoclonal	1:400
BrdU	Serotec, (MCA2060)	Rat, monoclonal	1:200
NeuroD (N-19)	Santa Cruz, (sc-1084)	Goat, polyclonal	1:500
NeuN	Chemicon, (MAB377)	Mouse, monoclonal	1:200

List of primary antibodies, their suppliers, the animal used to raise the antibodies in, and the working dilution. BrdU = 5-bromodeoxyuridine; ID = identification label in catalogue; NeuN = neuron-specific nuclear protein.

Using double immunofluorescent staining, the proportion of BrdU+ cells that coexpressed the immature neuronal marker NeuroD or the mature neuronal marker NeuN was assessed by an observer blinded to group assignment. Colocalization was confirmed using image stacks on a laser scanning confocal microscope (Zeiss LSM 510, 63× NA 1.3; Carl Zeiss, Peabody, MA) or a grid confocal microscope (Optigrid; Improvision, Waltham, MA) using a Volocity® image acquisition suite (Improvision). The number of new neurons in the DG cell layer was assessed in every twelfth slice.

Fear Conditioning

Four rats, counterbalanced for group assignment, were trained at a time. The chambers (length, 32 cm; width, 25 cm; height, 25 cm) were constructed of clear acrylic. The grid floor used to deliver shock was composed of 19 stainless steel bars, each 4 mm in diameter and spaced 16 mm center to center. These floors were connected to a shock delivery system (Med Associates, St. Albans, VT). The chambers were wiped with a pine-scented cleaner (5% Pine Scented Disinfectant, Midland, Inc.; Sweetwater, TN) before and after each session. The room in which training took place was illuminated with overhead fluorescent bulbs, and a ventilation fan provided background noise (65 db). The appearance, odor, and texture of the chambers and room comprised the training context.

After a 3-min baseline exploratory period in the chambers, rats received 3 tone (2000 Hertz, 90 db)–shock (1 mA, 2 s) pairings separated by 1 min. Freezing, the absence of all movement except that necessary for respiration, is an innate defensive fear response in rodents and a reliable measure of learned fear.[13] Each animal's behavior was scored every 8 s during the observation period, and a percentage was calculated using the formula 100× f/n, where f is the number of freezing events per rat and n is the total number of observations per rat.

The next day, rats were tested for fear to the training context and fear to tone. For the context test, each rat was once again placed in the chamber in which it was trained for a period of 8 min (in the absence of tone and shock). For the tone test, groups of rats were transported in separate plastic pots (height, 14 cm; diameter, 15.5 cm) to a distinct context in a different room. The test chambers were triangular in shape with an acrylic floor (length, 28 cm; width, 25 cm) and two acrylic sidewalls (length, 28 cm; width, 22 cm) at a 45-degree angle. The chambers were equipped with a speaker and were wiped down with acetic acid (1%; Fisher Scientific, St. Louis, MO) before and after each session. The room appeared dark to the rats, being lit by a single 30-Watt red bulb. A different kind of white noise (65 db) was used for background noise. Rats were given a 3-min exploratory period, followed by three 30-s tones (2000 Hertz, 90 db) separated by 60 s. Rats were removed from the chamber after an additional 30 s. The order of the context and tone tests was counterbalanced, such that half of each treatment group was tested to context first and tone second and *vice versa*. Freezing was scored by two observers blinded to group assignment during the 3-min exploratory period, the training, and both tests.

Spatial Reference Testing in the Morris Water Maze

Two cohorts of rats underwent a total of three tests of spatial reference memory in the Morris water maze tests. Initial testing was performed on 8-month-old animals (n = 25); after data analysis, a separate cohort (n = 9 controls, n = 8 isoflurane) was anesthetized and tested in the Morris water maze to identify the earliest timepoint at which a neurocognitive deficit is apparent. In the water maze test, the ability of rats to locate a hidden submerged platform in a circular pool (180 cm in diameter, 50 cm deep) filled with warm (24°C) opaque water was assessed. The rats were trained first to locate a visible platform (cued trials) and then to locate a hidden platform (place trials). In a subsequent trial, the hidden platform was removed (probe trial). Rats received two training sessions per day for five consecutive days. Each session consisted of three trials with a 60-s intertrial interval. The cohort was stratified to be spread evenly over one of four escape platforms located in the center of each quadrant. The escape location remained the same for each rats. The animals were placed in the tank pseudorandomly at one of six drop locations relative to their escape location. The various drop locations resulted in a short, medium, and long swim for each session. The interval between the two daily sessions was 5 h. When the rats located the platform, they were allowed to remain on it for 20 s. Time to reach the platform (latency), path length, and swimming speed were recorded with an EthoVision video tracking system (Noldus Instruments, Wageningen, Holland) set to analyze 10 samples per second. To assign credit for searching in the close proximity to the platform during the search, we chose as a primary outcome variable the time-integrated cumulative distance to the platform. During the 90-s probe trial, the proportion of time spent in the target quadrant *versus* each of the other quadrants was determined separately for each of three 30-s intervals.

Statistical Methods

Data were expressed as medians and interquartile ranges (IQR), except blood glucose concentrations (median, range) and survival (mean, 95% confidence interval). A two-tailed Mann-Witney U test was performed to determine between-group differences.

Water maze data were analyzed by mixed model regression using SAS version 9 (SAS Institute, Cary, NC) Proc MIXED. Within each set of sessions, a complicated

model was used first from which nonstatistically significant terms were sequentially dropped, usually starting with nonstatistically significant random effects and then dropping higher-order interactions. When all terms remaining in the model were considered statistically significant or required to be in for face validity (*e.g.*, session number) the model was declared a final model, which included group, session, and a quadratic term for session. *Post hoc* tests were used to compare the least squares means, and Bonferroni method was used to adjust for multiple comparisons. Probe trial data were analyzed by using the Mann–Whitney U test. $P < 0.05$ was considered significant.

Sample Size Calculation

For immunocytochemistry, a minimal group size of 6 animals was required to detect a difference between means of 40% with an 80% power at a significance level of 0.05.

For the initial water maze test, the sample size required to detect a 30% difference between groups at 80% power and a significance level of 0.05 was 12 animals per group. A group size of 14 was chosen to include a margin of error. For a second water maze experiment in the P60 group, the sample size required to demonstrate a mean difference in time-integrated distance to platform of 14 ms with an SE of 10 s, as observed in the first experiment, with 80% power at a significance level of 0.05 was eight animals per group. Nine animals were enrolled per group to include a margin of error.

SAS version 9 (SAS Institute, Cary, NC) proc power was used for all sample size calculations.

Results

Anesthetic Requirements and Physiologic Data

In P60 rats, the inspired MAC of isoflurane reached a stable plateau of approximately 1.6% after roughly 75 min. Inspired MAC of isoflurane in P7 rats, on the other hand, decreased progressively from 3.5% isoflurane at 15 min to 1.4% at 4 h (fig. 1A). No hypoxia (fig. 1B) or hypoglycemia (fig. 1C) occurred during anesthesia in either age group; however, profound hypercapnia (fig. 1D) and acidosis (fig. 1E) developed in P7 but not in P60 rats immediately after induction of general anesthesia, with a median peak arterial carbon dioxide tension of 137 mmHg (range, 92–186 mmHg) at 1 h and a steady decrease thereafter to roughly 80 mmHg at 4 h. No P60 rat died during the anesthetic, and the mortality in the P7 group was 21% (fig. 1F). Mortality was greatest during the fourth hour of anesthesia. The mechanism of death is neither hypoxia nor hypoglycemia because neither of these occurred during anesthesia. The mechanism is neither hypercapnia nor respiratory acidosis because a matched control group of unanesthetized P7 rats created by insufflation of carbon dioxide described elsewhere suffered no ill effects.[2] We were unable to prevent mortality by intraperitoneal injection of 1 mg/kg naloxone at 3 h (data not shown) or 0.5–1 ml of normal saline with or without 1 μg of epinephrine at 2 h of anesthesia (data not shown) or by withholding tail clamping until the fourth hour (data not shown).

Oxygen saturations remained stable at roughly 85% with a wide range throughout the anesthetic and did not correlate with arterial Po_2 measurements (data not shown). In P60 cardiorespiratory control animals, the blood hemoglobin concentration decreased to roughly 10 mg/dl by the fourth blood draw, near the conclusion of anesthesia. In P7 rats, the blood hemoglobin concentration progressively increased from a median of 8.3 (range, 6.9–9.5; n = 4) just after induction of general anesthesia to a median of 11.3 (range, 8.7–12.5; n = 5; $P < 0.05$, Mann–Whitney U) at 4 h of anesthesia. Due to the normal blood gas results in P60 rats, the respiratory rate of P60 rats was not recorded. The respiratory rate of P7 rats decreased from roughly 100 breaths/min to approximately 20 breaths/min from immediately after induction of general anesthesia and remained stable until 3 h of anesthesia, when it steadily increased to 35 breaths/min at 3.75 h of anesthesia.

Brain Cell Death

In P60 rats, there were hardly any FluoroJade+ cells in the brain of anesthetized (fig. 2) or unanesthetized animals. There was cell death in both anesthetized and unanesthetized P7 brains, the extent of which is described in detail in an accompanying article in this issue of ANESTHESIOLOGY.[2]

Neuronal Differentiation

When BrdU was injected 4 h before anesthesia in P60 rats, the proportion of BrdU+ cells coexpressing the early neuronal marker NeuroD (fig. 3A) at the end of the anesthetic was 41%, (IQR 32.9–50%) compared to a median of 27.8% (IQR 25.0–34.0%) in unanesthetized animals ($P < 0.01$, Mann–Whitney U; fig 3B). Using the same protocol in P7 rats, the proportion of BrdU+ cells expressing NeuroD was unchanged at the end of 4 h of isoflurane anesthesia (control median 21.5%, IQR 17.7–23.3% *vs.* isoflurane median 20.0, IQR 18.4–23.4%; $P > 0.05$; fig. 3B). The proportion of BrdU+ cells coexpressing NeuroD was significantly greater in sham anesthetized P60 than in sham anesthetized P7 rats ($P < 0.001$, Mann–Whitney U; fig. 3B).

Progenitor Proliferation

Neuronal differentiation of progenitor cells requires cell cycle exit, and isoflurane induced neuronal differentiation only in P60 rats; therefore, isoflurane should decrease progenitor proliferation in P60 but not in P7 rats. We found, however, that BrdU administration during anesthesia decreased the number of BrdU+ cells per DG (fig. 4A) in both age groups by 56.8% and

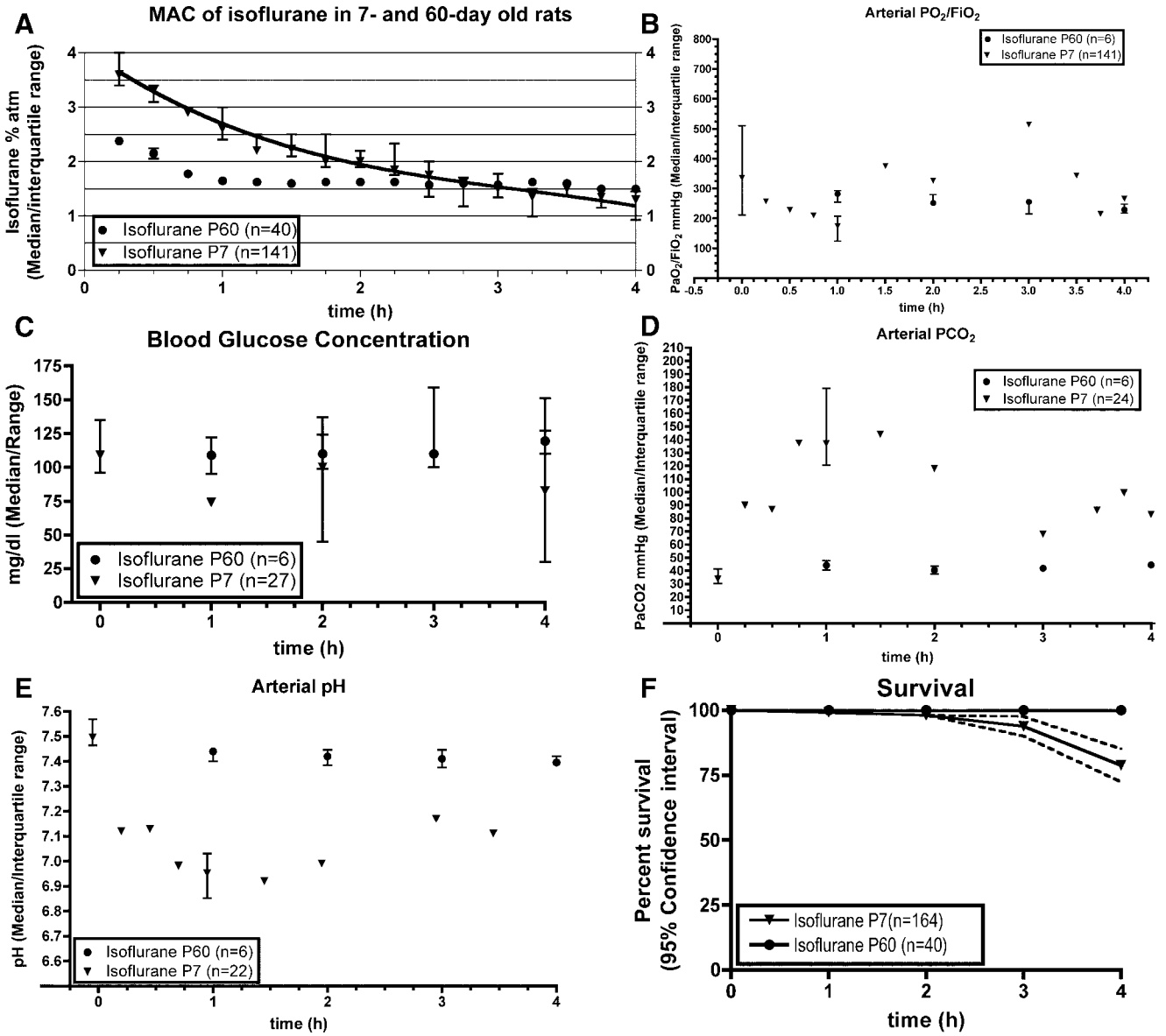

Fig. 1. Minimum alveolar concentration (MAC), physiologic variables, and survival during 4-h isoflurane in rats. MAC was determined using the response to tail clamping of ten or more simultaneously anesthetized rats. A second order polynomial fit was applied to the MAC data of postnatal day (P)7 rats (*A*). Blood withdrawn blindly from the left cardiac ventricle under general (*isoflurane groups*) or local anesthesia (*control*) was analyzed in a blood gas analyzer and oxygen tension (*B*), glucose concentration (*C*), carbon dioxide tension (*D*), and pH (*E*) were determined. Only one sample was obtained per P7 rat, whereas four blood samples were drawn from every P60 cardiorespiratory control rat (n = 6). Data are medians and interquartile ranges except glucose concentrations (median, range) and survival (*F*); mean, 95% confidence interval). Only those rats that awoke from anesthesia were considered survivors. Two rats in the P7 isoflurane group survived 4 h of isoflurane but died before awakening from anesthesia. None of the unanesthetized control animals died (not shown). FiO_2 = fraction of inspired oxygen; PCO_2 = partial pressure of carbon dioxide; PO_2 = partial pressure of oxygen.

38.6%, respectively, to a median of 4,473 (IQR 3,952–6,724) in P60 controls and 1,933 (IQR 895–4,020) in P60 isoflurane-treated rats ($P < 0.05$, Mann–Whitney U; fig. 4B) and 37,755 (IQR 33,683–42,997) in P7 controls *versus* 23,165 (IQR 16,387–32,913) in P7 isoflurane-anesthetized rats ($P < 0.05$, Mann–Whitney U; fig. 4C). As a control experiment, 300 mg/kg BrdU was injected 4 h before anesthesia in both groups. Since the availability of this dose of BrdU is less than 4 h,[14] the number of BrdU+ cells after anesthesia should not be affected. Indeed, we found that isoflurane had no effect on the number of cells labeled with BrdU in P60 (median [IQR]: control 2,446 [2,110–2,706]; isoflurane 2,142 [1,285–2,941]; $P > 0.05$, Mann–Whitney U; figure 4D) or P7 rats (median [IQR]: control 36,700 (34,000–44,504); isoflurane 36,596 (30,700–39,186); $P > 0.05$, Mann–Whitney U; fig. 4E).

Delayed Increase in Proliferation in P60 but Not in P7 Rats

To assess if the isoflurane-induced decrease in progenitor proliferation observed in both age groups re-

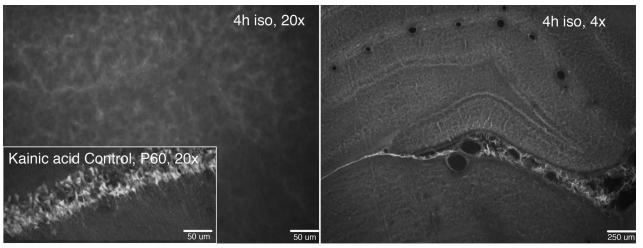

Fig. 2. Isoflurane does not induce cell death in postnatal day (P)60 rats. FluoroJade-stained coronal rat brain sections harvested 12 h after 4 h of isoflurane administered at P60. Every twelfth slice was examined throughout the entire rostrocaudal extent of the brain, and all slides contained one kainic acid control slice to ensure that the stain was working. Unlike in a kainic acid-treated P60 control rat (*inset in the left panel*) showing extensive neurodegeneration in the CA-1 of the hippocampus, there was no Fluoro Jade staining in the brain of isoflurane-treated or unanesthetized P60 rats.

covers over time, we labeled progenitors with BrdU 4 days after anesthesia. Twelve hours after the last of two BrdU injections on day 4 after anesthesia, the number of BrdU+ cells was increased in the P60 isoflurane-treated animals compared to unanesthetized P60 controls by 235% ($P < 0.05$, Mann–Whitney U; fig. 5A). In P7 isoflurane-treated rats, the number of BrdU+ cells was decreased by 21.4% at the same time point compared to controls ($P < 0.05$, Mann–Whitney U; fig. 5B).

No Difference in New Granule Cell Number 4 Weeks Later

The effect of isoflurane on the number of new granule cell neurons was assessed 4 weeks after BrdU injection before anesthesia or on the fourth day after anesthesia in both age groups. Neither the acute increase in the proportion of BrdU+ cells that assumed a neuronal lineage in P60 rats nor the prolonged decrease in progenitor proliferation in P7 rats resulted in a change in the number of BrdU+ cells coexpressing the mature neuronal marker NeuN 28 days later ($P > 0.05$, data not shown). At this time point, 58% (range, 47–63%) of BrdU+ cells in controls coexpressed the mature neuronal marker NeuN *versus* 52% (range, 40–66%) in the isoflurane ($P > 0.05$, data not shown).

To assess if the delayed peak in progenitor proliferation in P60 rats gives rise to new granule cell neurons, BrdU was injected twice daily on days 4–7 after anesthesia. Twenty-eight days after the last injection, the proportion of BrdU+ cells coexpressing NeuN (fig. 5C) was significantly less in P60 isoflurane-treated animals (median 6.3%, IQR 3.1–23.8%) than in controls (median 54.2%, IQR 7.8–79.6%; $P < 0.05$, Mann–Whitney U; fig. 5D). However, the total number of new granule cells at this time point was again not different between P60 isoflurane-treated and control animals ($P > 0.05$; fig. 5E).

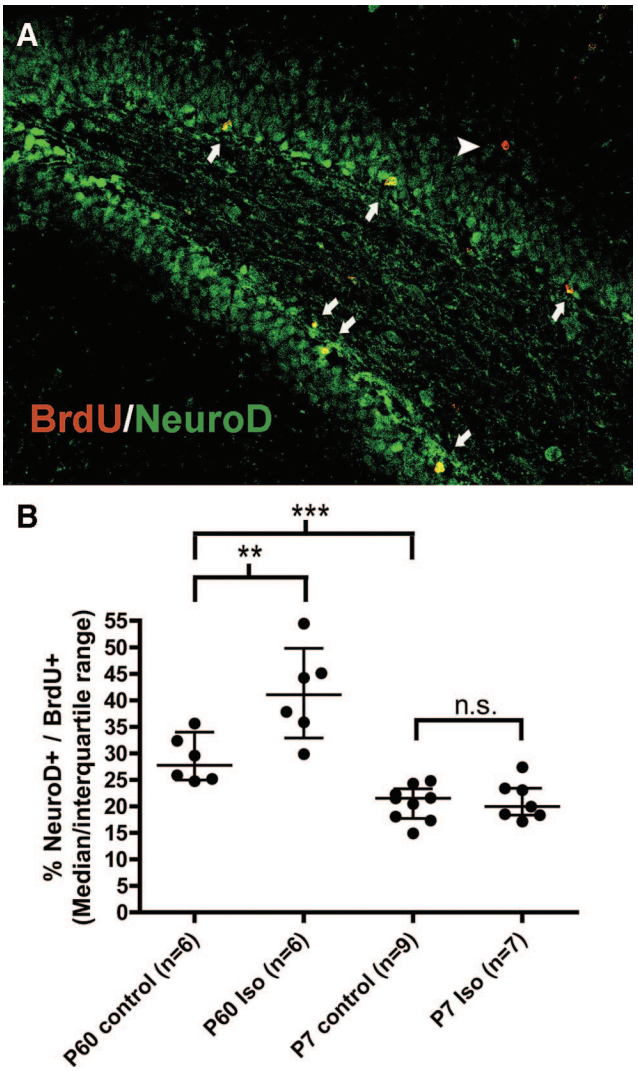

Fig. 3. Isoflurane (Iso) increases neuronal differentiation in the dentate gyrus of postnatal day (P)60 but not of P7 rats. After injection of 5-bromodeoxyuridine (BrdU) 4 h before the start of a 4 h of isoflurane anesthetic, the proportion of BrdU+ cells (*A*, *red*) that expressed the early neuronal marker NeuroD (*A*, *green*) was increased by isoflurane in P60 but not in P7 rats (*B*). The microscopic image in fig. 4A (20× objective) is a 3D-reconstruction of an image stack of a coronal section from an isoflurane-treated animal acquired on a grid confocal microscope. The *filled arrows* point to BrdU+/NeuroD+ double-labeled cells in the subgranular zone of the dentate gyrus of the hippocampus. The *arrowhead* points to a BrdU+/ NeuroD– cell. This image shows a greater than average proportion of double-labeled cells. Data are medians ± interquartile ranges. ** $P < 0.01$, *** $P < 0.001$, Mann–Whitney U-test.

Fear Conditioning

No difference was observed in fear conditioning performance 4 months after 4 h of isoflurane administered to P60 rats (n = 14) compared to their respective controls (n = 13) (data not shown). After 4 h of isoflurane at P7, fear conditioning was tested 15 and 26 days after anesthesia and in a separate cohort of rats at 5 months after anesthesia. No deficit was evident at the two early time points (fig. 6, A and B), but the freeze scores of isoflurane-treated rats were signif-

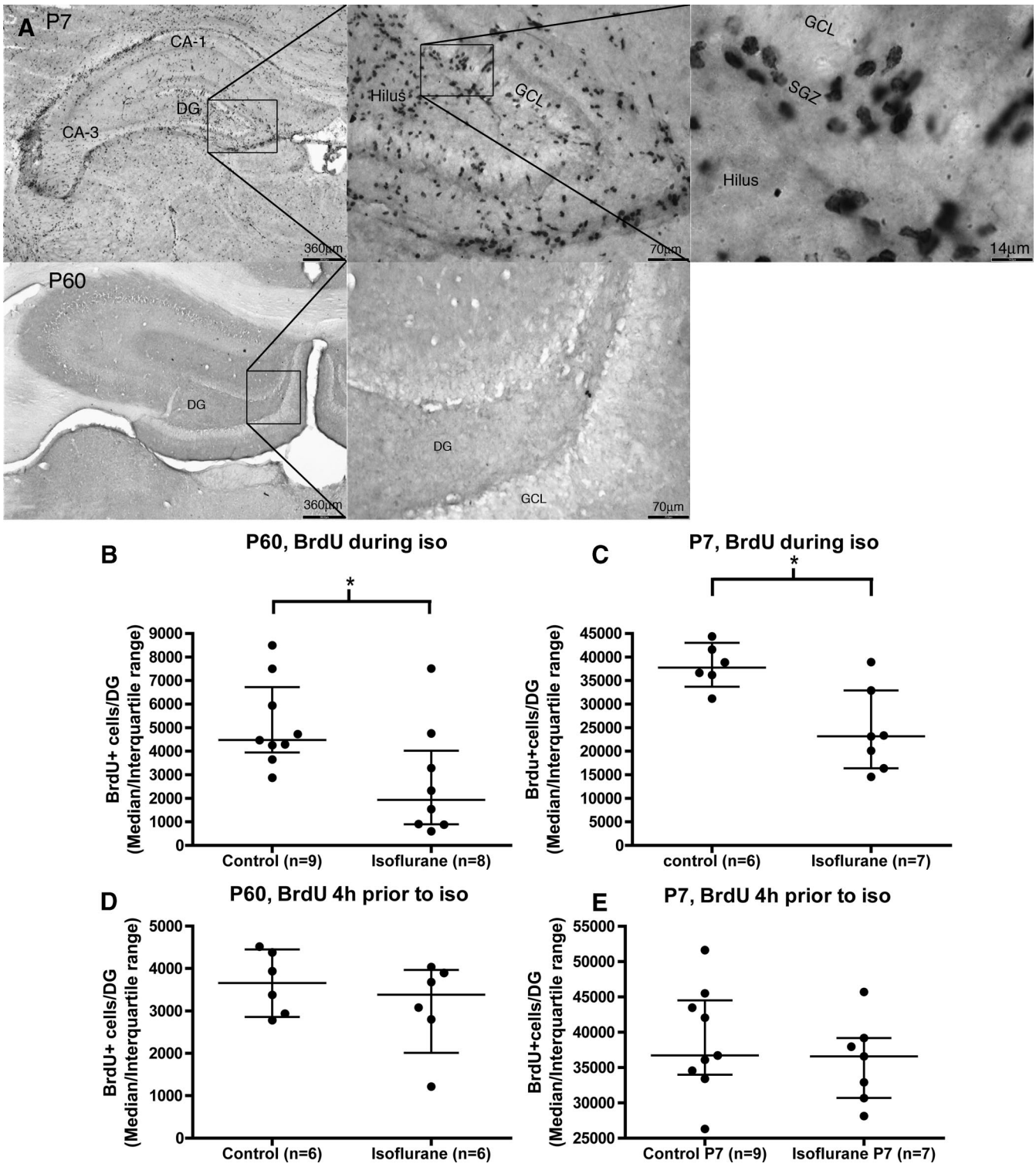

Fig. 4. Isoflurane (iso) decreases progenitor proliferation in postnatal day (P)7 and P60 rats. P60 and P7 rats were injected intraperitoneally with 5-bromodeoxyuridine (BrdU) during and 4 h before anesthesia with 4 h of isoflurane. Example of BrdU+ cells in the dentate gyrus (DG) of the hippocampus imaged with 4×, 20×, and 100× objective lenses (*A*). The number of BrdU+ cells was decreased after BrdU injection during anesthesia in both age groups (*B, C*) and was not affected by isoflurane at either age when BrdU was injected 4 h before anesthesia (*D, E*). * $P < 0.05$, Mann–Whitney U test. CA = cornu ammonis; GCL = granule cell layer; n.s. = not significant; SGZ = subgranular zone.

icantly lower than those of unanesthetized controls at 5 months in the hippocampal-dependent context portion but not the hipppocampal-independent tone portion of the test (fig. 6C).

Water Maze Spatial Reference Learning and Memory Retention Tasks

When rats that had received 4 h of isoflurane on P60 were tested in the water maze, the isoflurane group showed a trend

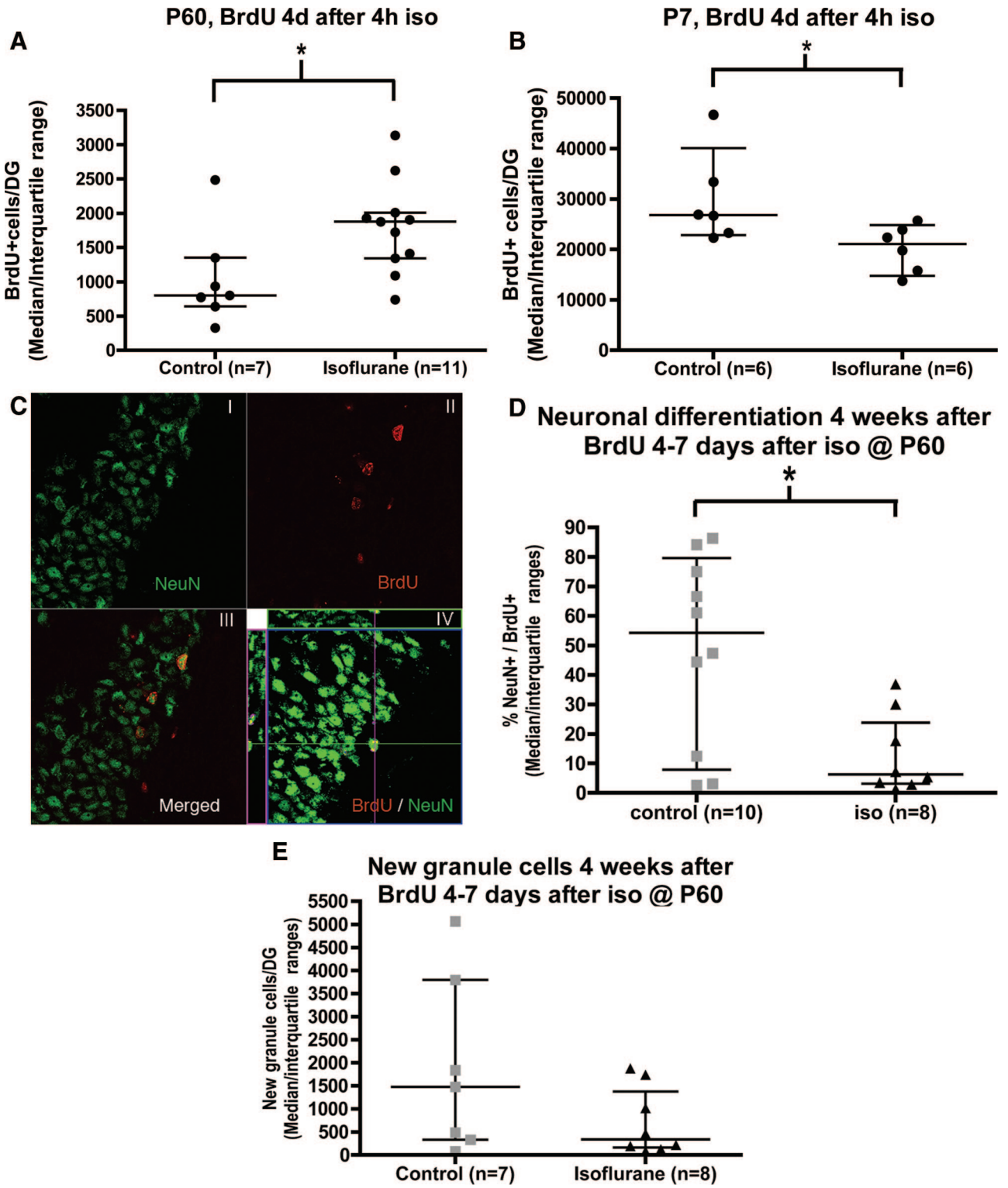

Fig. 5. Compensatory increase in progenitor proliferation in postnatal day (P)60 but not in P7 rats. Four days after isoflurane (iso) anesthesia, 5-bromodeoxyuridine (BrdU) was injected twice with a 12-h interval followed by a 12-h survival after the last injection. Progenitor proliferation was increased in P60 (*A*) but not in P7 rats (*B*). To determine if this peak in proliferation in P60 animals is neurogenic, BrdU was injected 4–7 days after anesthesia, and colocalization of BrdU with the mature neuronal marker neuron-specific nuclear protein (NeuN) was assessed 28 days later (*C*). *Panel IV* in (*C*) (63×) shows orthogonal images in the *xz* and *yz* planes. Significantly fewer cells labeled with BrdU on days 4–7 after isoflurane coexpress NeuN 4 weeks later compared to controls (*D*). This did not affect the total number of new granule cells 28 days after BrdU injections on postanesthesia days 4–7 (*E*). *$P < 0.05$, Mann–Whitney U test. DG = dentate gyrus.

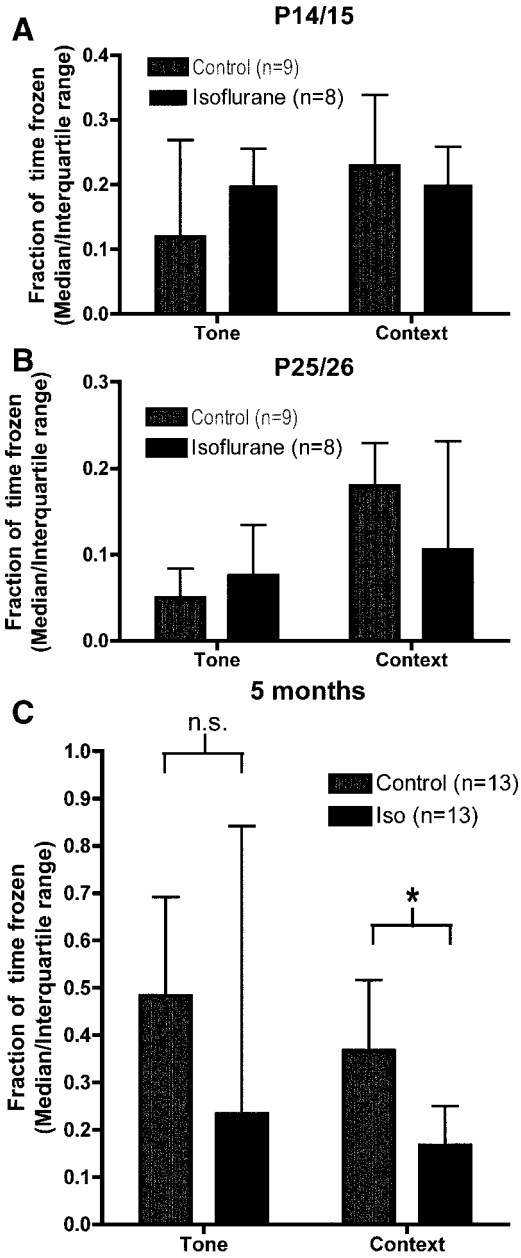

Fig. 6. Longitudinal assessment of fear conditioning performance after 4 h of isoflurane (Iso) on postnatal day 7. Rats underwent fear conditioning 4 h of isoflurane (Iso) on postnatal day 7 to test how well the association between a footshock and either a tone or an environment (*context*) is formed. The shock-context association is hippocampal-dependent, whereas the shock-tone association is not.13 Freeze scores, a measure of fear reflecting the degree of associative learning at 15 days (*A*) and 26 days (*B*) were not different between groups for either the tone or the context portion of the test. At 5 months of age, (*C*) isoflurane-treated rats have a hippocampal-dependent learning deficit as indicated by freeze scores in the context portion of the test that were significantly lower in the isoflurane group than in the control group ($P < 0.05$, Mann–Whitney U test). Data are medians ± interquartile ranges.

towards better performance in the spatial reference task ($P > 0.08$, fig. 7A) and spent a significantly greater proportion of time searching for the removed platform in the target quadrant than unanesthetized controls (fig. 7B).

Both the isoflurane group and the control group learned at a steady but slow rate when spatial reference memory was tested in the Morris water maze on postnatal days 16–23 after 4 h of isoflurane on P7 (fig. 7C). It took a total of 7 sessions of cued trials for learning curves to flatten out so the training flag could be removed from the platform. There was no difference between groups in performance of cued trials, place trials, for which the platform is hidden, or the probe trial, for which the platform is removed from the tank (fig. 7, C and D). The same animals were retested 6 weeks after the anesthetic, at which point their performance was much improved. Again there was no difference in time-integrated distance to the escape platform in cued or place trials between groups (fig. 7E). However, rats that had received isoflurane at 7 days of age spent significantly less time searching for the platform in the target quadrant during the probe trial (fig. 7F). When a different set of animals was tested 8 months after administration of 4 h of isoflurane on P7, place trial performance was significantly worse in the isoflurane-treated animals (fig. 7G), whereas probe trial performance was not affected (fig. 7H).

Discussion

The main finding of this study is an anesthesia-induced, delayed-onset, permanent deficit of hippocampal-dependent but not hippocampal-independent learning and memory in P7 rats. In contrast, isoflurane in P60 rats causes a long-term improvement in spatial reference memory. In P7 rats, isoflurane caused a decrease in progenitor proliferation lasting for at least 4 days, and it increased neuronal differentiation in P60 rats.

Neurogenesis in the DG of the hippocampus continues for life in animals and humans (for review, see Abrous *et al.*[6]). The advantage of sustaining a dividing precursor pool in the subgranular zone is retention of a unique form of adaptability. The cellular plasticity conferred by neurogenesis allows the DG to select those new neurons that allow it to best meet a functional demand.[10,15–18] The DG encodes episodic memory, and DG neurogenesis is crucial in this process.[8–10,18,19] Increases in neurogenesis improve hippocampal learning (for review see Kempermann[20]), whereas disruption of neurogenesis impairs hippocampal learning.[21–23] For example, progenitors are extremely sensitive to the effects of radiation (reviewed by Monje *et al.*[24]), and brain irradiation causes hippocampal dysfunction in animals.[22,23,25–27] In humans, brain irradiation causes a delayed onset, progressive hippocampal deficit; depending on the age at treatment and dose of radiation, it can also affect general cognition.[28–30] However, memory suffers to a degree greater than predicted by intelligence quotient scores.[29] A cause-effect relationship between radia-

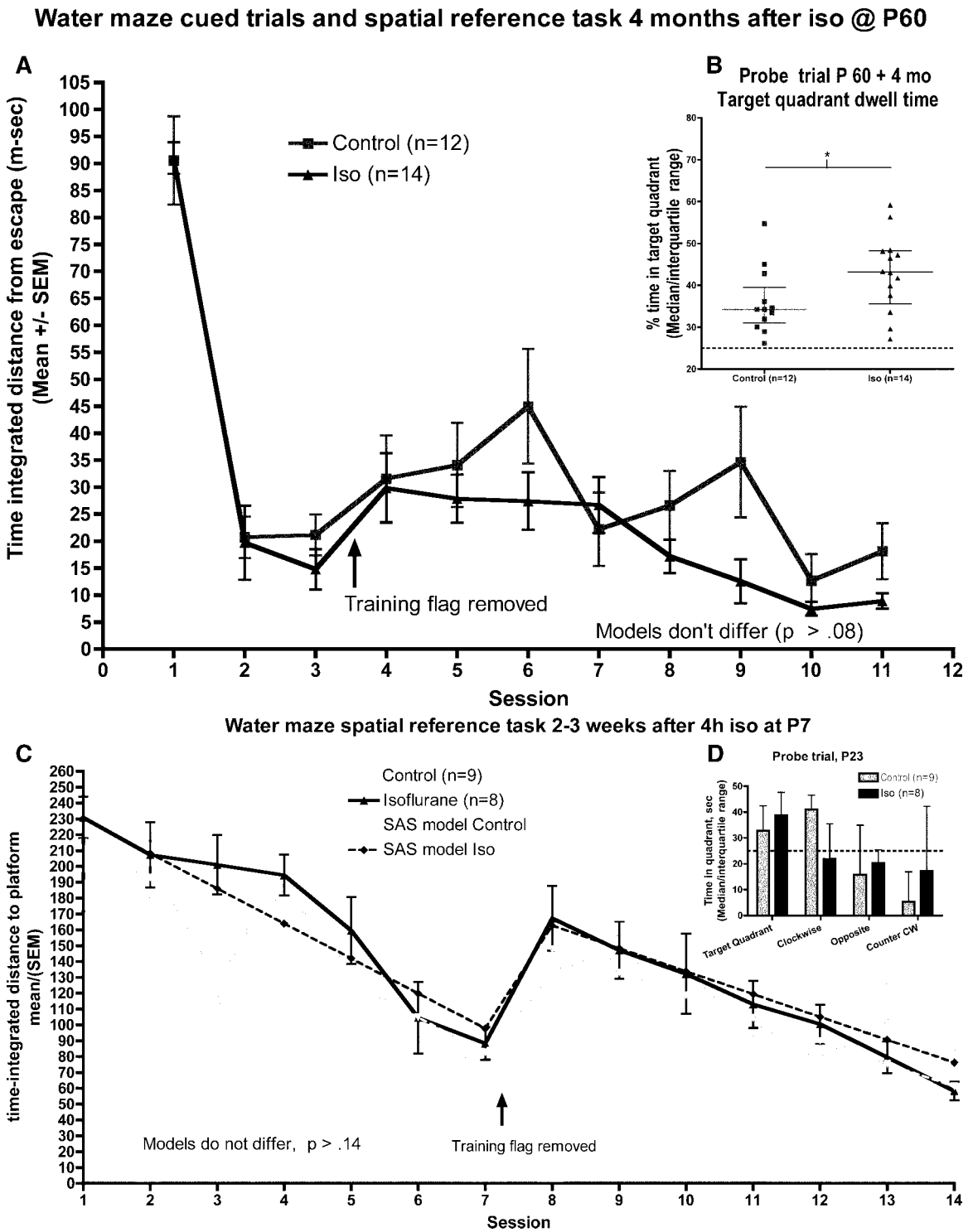

Fig. 7. Spatial learning and memory after 4 h of isoflurane (Iso) on postnatal day (P)7 or P60. Rats received 4 h of isoflurane at P7 or P60 and underwent assessment of spatial reference memory acquisition and retention in the Morris water maze. Memory acquisition was assessed in cued trials and place trials by measuring time-integrated distance to the escape platform. During cued trials, the submerged platform was marked with a flag, which was removed when learning curves flattened out. During these place trials, rats navigated to the hidden platform using remote spatial cues. Memory retention was assessed 24 h later in a probe trial during which the platform is removed from the pool. In P60 rats, isoflurane tended to improve spatial reference memory acquisition (*A*) and significantly improved memory retention (*B*). When isoflurane is administered at P7, memory acquisition from P16 to P22 (*C*) and memory retention on P23 is unaffected (*D*). Six weeks after anesthesia, memory acquisition is unchanged by isoflurane (*E*); however, during the probe trial (*F*), target quadrant dwell time was reduced in isoflurane-treated rats ($P < 0.05$, Mann–Whitney U). Eight months after anesthesia, time-integrated distance to platform during the first session of place trials reverted to a significantly greater degree in isofluane-treated rats than in controls (*G*). Probe trial performance was unaffected (*H*). * $P < 0.05$, Mann–Whitney U.

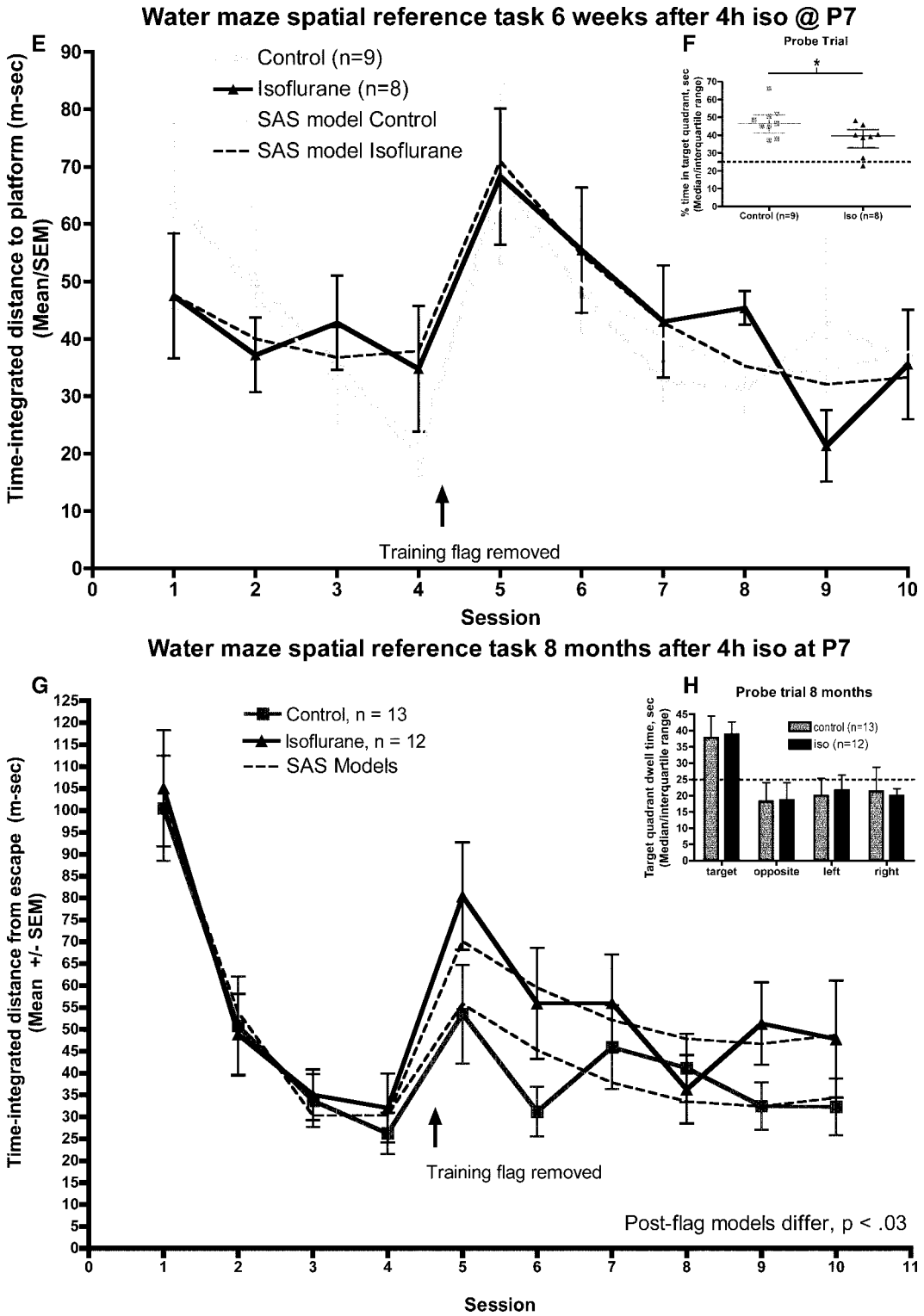

Fig. 7. (*Continued*).

tion-induced decrease in neurogenesis and the cognitive decline in humans, however, has not been proven.

We interpret the deficit caused by 4 h of isoflurane in P7 rats as progressive in nature, in that we were unable to demonstrate a deficit until 6 weeks after anesthesia, but we found it consistently from that point on in both the current and a previous investigation.[2] The deficit is initially rather subtle and can be brought out by severe increases of task difficulty.[2] At 5 months, however, even the less demanding fear conditioning task, as used in this investigation, clearly shows the hippocampal deficit.

This is inconsistent with recent reports that fear conditioning is not sensitive to changes in progenitor proliferation.[8,9] The interval between the interventions and functional assessment in these studies were 4 weeks and 6 weeks, respectively, and it is possible that the deficit in a less demanding task is not obvious at this time.[8,9] Consistent with this, we previously did not find any effect on fear conditioning 8 weeks after 4 h of isoflurane, when spatial reference memory and spatial working memory tasks were clearly impaired in rats that had received neonatal isoflurane anesthesia.[2]

The hippocampal-type deficit observed here refers to impaired context freezing but not tone freezing 5 months after isoflurane; context freezing requires the hippocampus, whereas tone freezing does not.[13] Likewise, spatial reference memory, which was impaired 6 weeks and 8 months after isoflurane, requires an intact hipppocampus, among other brain structures, whereas cued trial performance, which was unaffected by isoflurane, does not require the hippocampus.[31] Hippocampal neurogenesis is restricted to the DG, at least in P60 rats. It follows that an anesthetic effect on neurogenesis would affect primarily DG function as opposed to general hippocampal function. For DG neurogenesis to be a possible candidate mechanism for anesthesia-induced long-term neurocognitive dysfunction, it is required that the DG dysfunction alone produces a hippocampal-type deficit. This requirement seems to be fulfilled. Keith *et al.*[32] injected colchicine into the DG, causing a DG lesion and sparing other hippocampal regions. This produced impairment in spatial reference memory and spatial working memory but not cued trial performance. In other words, a DG lesion caused a hippocampal-type deficit. Fear conditioning was not assessed in that investigation. Notably and expectedly, the hippocampal deficit caused by cell death in the DG improved over 6 weeks,[32] whereas the hippocampal deficit caused by isoflurane described here seems to get progressively worse.

Hence, both the type of deficit caused by isoflurane in P7 rats and its time course seem to be compatible with neurogenesis as a cause, although proof of a cause-effect relationship will have to be established in future investigations.

In P60 rats, we found that isoflurane-induced increase in neuronal differentiation was associated with an initial decrease in progenitor proliferation, which we interpret as a secondary decrease in proliferation due to cell cycle exit associated with neuronal lineage selection. This interpretation is speculative because the study was not designed to examine if isoflurane causes cell cycle arrest or cell cycle exit. The subsequent increase in progenitor proliferation may be a compensatory response to maintain the progenitor pool size. We base this hypothesis on the finding that only a very small proportion of progenitors labeled from 4–7 days after anesthesia assumed a mature neuronal phenotype 28 days later. The identity of the nonneuronal BrdU+ cells, however, remains to be determined.

Interestingly, in P7 rats we were unable to demonstrate an effect of isoflurane on neuronal differentiation. This is surprising given that GABA depolarizes both neonatal[33,34] and adult progenitors,[34,35] causing cell cycle exit and neuronal lineage selection.[35–37] In the P7 rat brain, the DG has only just formed[38] and the switch from the excitatory to the inhibitory GABA-acid-A ($GABA_A$) receptor phenotype has not yet occurred (reviewed by Ben-Ari *et al.*[39]). Therefore, we expected isoflurane, a predominantly GABAergic anesthetic, to cause cell cycle exit and neuronal differentiation of progenitors in P7 rats. It is possible that our labeling protocol missed the neurogenic event, but we repeated the experiments with a BrdU-injection at the end of the 4-h anesthetic followed by 8-h survival and were again unable to show an effect of isoflurane on neuronal differentiation (not shown). This is despite the fact that isoflurane has been shown to induce neuronal differentiation and cell cycle exit of neural progenitors harvested from 2-day-old rats *in vitro*.[40] We therefore guardedly interpret the decrease in progenitor proliferation in P7 rats as a primary decrease, *i.e.*, one that is not secondary to an increase in neuronal differentiation. One possible mechanism for a primary decrease in progenitor proliferation would be isoflurane toxicity in neural precursors, as has been suggested to occur after alcohol exposure.[41] However, isoflurane is not toxic to neural progenitors in culture.[40] It is unclear if the isoflurane-induced decrease in progenitor proliferation at P7 represents cell cycle exit or cell cycle arrest and if progenitor proliferation eventually recovers. It is also unclear if the isoflurane-induced effects on progenitor proliferation occurs in other areas of the developing brain, such as the subventricular zone of adult animals or the cortex or thalamic nuclei of immature rats, where a decrease in progenitor proliferation could augment isoflurane-induced cell death.[2,42]

Study Limitations

The anesthetic model used here combines a clinically relevant isoflurane concentration with a supramaximal pain stimulus causing tissue injury. This, in fact, models anesthesia plus surgery, which is a strength from a translational standpoint and a weakness from a mechanistic standpoint. The effects of anesthesia will have to be distinguished from those of inflammation caused by tissue injury.

One of the key findings of this study is a decrease in progenitor proliferation caused by isoflurane in 7-day-old rats. We do not know the time course of the isoflurane-effect on proliferation in neonatal animals for more than 4 days after anesthesia. This would be important for understanding if and how an isoflurane effect on neurogenesis affects long-term cognitive outcome of anesthe-

sia. For example, if the degree of progenitor proliferation at the time of functional testing correlated with performance in neurocognitive tests, a stronger case could be made for a causal link between this aspect of neurogenesis and functional outcome.

The fact that no sustained change in the number of new neurons was observed after a decrease in progenitor proliferation in both groups and an increase in neuronal differentiation in P60 rats could be interpreted as the absence of an effect of isoflurane on neurogenesis altogether. While this is certainly a possibility, another interpretation is that the absolute number of new neurons 4 weeks after anesthesia is not a primary determinant of function. This is suggested by findings of a close link between cell proliferation and cell elimination,[18,43–48] a means to tightly control the absolute granule cell number. Specifically, the rate of cell death changes with the rate of cell birth.[43] The reverse may also be true,[18] although this is controversial.[49,50] Either way, neuronal turnover in the DG confers a recently recognized form of cellular plasticity through incorporation of neurons that best meet a functional demand. The total number of granule cells may not be as important for hippocampal function as efficient neuronal turnover, as illustrated by impairment in hippocampal function by inhibition of progenitor proliferation,[9,22,23] but interestingly also by inhibition of apoptotic cell death in the DG.[18] In an elegant study, Dupret *et al.*[18] demonstrated that cell death of immature neuronal stages is necessary for spatial learning and memory and that performance in spatial reference memory tasks correlates with the amount of cell death of BrdU-labeled cells and inversely correlates with the number of new granule cells. Other examples of a dissociation between granule cell number and function include the findings that a decrease in progenitor proliferation causing functional impairment did not affect the total number of dentate granule cells[9] or that prevention of the decrease in depression-induced hippocampal volume by antidepressant drugs[51] occurs with a concomitant increase in the rate of apoptosis,[44] with the end result being normalization of hippocampal volume.[51] Collectively, these findings suggest that not all neurogenesis-related interventions affect function by merely increasing or decreasing the number of new dentate granule cells.

Our data currently represent nothing more than an association between an isoflurane effect on certain aspects of neurogenesis and anesthesia-related long-term neurocognitive outcome. As is true for other proposed mechanisms of anesthesia-induced long-term neurocognitive decline, the exact nature and strength of the relationship between this proposed target mechanism and anesthesia-induced neurocognitive dysfunction requires further study.

Applicability of the data to human anesthesia is limited. It is entirely unclear if anesthesia-induced cognitive impairment occurs in humans. No evidence currently exists to support this notion, although the consistency with which deficits of learning and memory after anesthesia in immature rats have been demonstrated is concerning and warrants concerted investigative efforts.

We conclude that 4-h isoflurane induced a long-term neurocognitive improvement in P60 rats and a delayed-onset, progressive, persistent deficit in hippocampal function in P7 rats. The associated findings of isoflurane-induced increase in neuronal differentiation in P60 rats and decrease in progenitor proliferation in P7 rats may play a role in the long-term cognitive outcome of anesthesia.

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