Long-term Fate Mapping to Assess the Impact of Postnatal Isoflurane Exposure on Hippocampal Progenitor Cell Productivity

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ABSTRACT

Background: Exposure to isoflurane increases apoptosis among postnatally generated hippocampal dentate granule cells. These neurons play important roles in cognition and behavior, so their permanent loss could explain deficits after surgical procedures.

Methods: To determine whether developmental anesthesia exposure leads to persistent deficits in granule cell numbers, a genetic fate-mapping approach to label a cohort of postnatally generated granule cells in Gli1-CreER^{T2}::GFP bitransgenic mice was utilized. Green fluorescent protein (GFP) expression was induced on postnatal day 7 (P7) to fate map progenitor cells, and mice were exposed to 6 h of 1.5% isoflurane or room air 2 weeks later (P21). Brain structure was assessed immediately after anesthesia exposure (n = 7 controls and 8 anesthesia-treated mice) or after a 60-day recovery (n = 8 controls and 8 anesthesia-treated mice). A final group of C57BL/6 mice was exposed to isoflurane at P21 and examined using neurogenesis and cell death markers after a 14-day recovery (n = 10 controls and 16 anesthesia-treated mice).

Results: Isoflurane significantly increased apoptosis immediately after exposure, leading to cell death among 11% of GFP-labeled cells. Sixty days after isoflurane exposure, the number of GFP-expressing granule cells in treated animals was indistinguishable from control animals. Rates of neurogenesis were equivalent among groups at both 2 weeks and 2 months after treatment.

Conclusions: These findings suggest that the dentate gyrus can restore normal neuron numbers after a single, developmental exposure to isoflurane. The authors' results do not preclude the possibility that the affected population may exhibit more subtle structural or functional deficits. Nonetheless, the dentate appears to exhibit greater resiliency relative to nonneurogenic brain regions, which exhibit permanent neuron loss after isoflurane exposure. **(Anesthesiology 2016; 125:1159-70)**

ALL commonly used anesthetics increase brain cell death in developing animals.[1] An analogous phenomenon has been described for anticonvulsant medications, many of which have mechanisms of action similar to that of anesthetics.[2,3] Prospective clinical studies are ongoing to establish whether anesthesia exposure in childhood is associated with long-term cognitive deficits. Early results from the general anesthesia and awake-regional anesthesia in infancy (GAS) study are encouraging, providing no evidence of neurocognitive deficits in children at 2 yr after less than 1 h exposure in infancy.[4] This is consistent with animal studies, which find little evidence for structural brain abnormalities after brief exposures. Retrospective clinical studies of longer or repeated exposures, on the other hand, have linked childhood anesthesia to subsequent language impairment, cognitive abnormalities, and learning disabilities[5–8] although not all groups have found deficits.[9,10] Prospective clinical studies will require several years to complete and are unlikely to cover all clinical scenarios, especially for prolonged exposure times. There is significant concern, therefore, that anesthesia exposure early in life may have long-term deleterious effects on the developing brain.

What We Already Know about This Topic

- It is well established that anesthetic exposure leads to death of granule cells in the dentate gyrus of the hippocampus.
- New cell generation (neurogenesis) occurs in the dentate gyrus throughout life.
- In 21-day-old rodents exposed to isoflurane, granule cell progenitors were labeled, and the balance between isoflurane-induced cell loss and new cell production was tracked to adulthood.

What This Article Tells Us That Is New

- As expected, isoflurane increased apoptosis of granule cells of the dentate gyrus. Changes in neurogenesis were not detected.
- There were no differences in the density of labeled granule cells in adult animals.
- The data are consistent with the premise that the neuron number in the dentate gyrus can be restored after anesthetic-induced cell loss. The mechanism remains to be found.

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Increased apoptotic cell death has been one of the most dramatic findings among anesthesia-exposed animals. Establishing whether there is a net loss of cells persisting into adulthood, however, has been challenging for three main reasons. First, the most vulnerable period for anesthetic exposure coincides with the period of naturally occurring apoptosis—a normal process that "prunes" excess neurons. Accelerated loss of neurons fated to die anyway could produce the well-characterized increase in apoptosis while still having no effect on final neuron numbers. By contrast, loss of neurons that should have survived to adulthood will reduce neuronal density in the mature brain. Traditional cell death markers cannot distinguish between these possibilities, and cell counts in adult animals have returned conflicting results.[11,12] A second complicating factor is the potential for compensatory neurogenesis among certain neuronal populations sensitive to anesthesia-induced death. Specifically, we and others have recently demonstrated that hippocampal dentate granule cells (DGCs) are especially vulnerable to anesthesia-induced neurotoxicity in 21-day-old (P21) mice,[13–15] a brain maturational stage comparable to human infants.[16] Granule cells, however, are produced throughout life in animals and humans,[17] so it is conceivable that the dentate could regenerate lost cells. Finally, within the dentate, there is the potential for loss of the progenitor cells responsible for adult neurogenesis. Progenitor cell loss would eliminate future generations of daughter cells, compounding neuronal loss well beyond the number of initially affected cells. The effect of such a loss is poorly captured by traditional approaches.

Given the importance of hippocampal granule cells for cognition,[18–20] we queried whether anesthesia produces a net deficit in their numbers. We genetically fate mapped a cohort of granule cell progenitors in developing mice by inducing persistent green fluorescent protein (GFP) expression among the population. Since all daughter cells of labeled progenitor cells express GFP, the net number of neurons produced can be counted. Changes in apoptosis or neurogenesis rates occurring over days, weeks, or even months, therefore, are revealed by the number of GFP-expressing cells.

Materials and Methods

Animals

All procedures conformed to National Institutes of Health (Bethesda, Maryland) and Cincinnati's Children Hospital Medical Center (Cincinnati, Ohio) guidelines for the care and use of animals. Sample sizes were estimated based on past experience with similar anatomical measures.[21] To generate animals for the current study, hemizygous Gli1-CreER^{T2} mice,[22,23] expressing a conditional, tamoxifen-inducible form of Cre recombinase, were crossed to homozygous CAG-CAT-EGFP reporter mice, which express enhanced green fluorescent protein (EGFP) under the control of the cytomegalovirus promoter.[24] A total of 36 Gli1-CreER^{T2}::GFP bitransgenic offspring from this cross were used for experiments. Mice were housed on a 14/10 (light/dark) cycle with access to food and water *ad libitum*. All animals were maintained on a C57BL/6 background.

Bitransgenic Gli1-CreER^{T2}::GFP offspring were treated with 250 mg/kg tamoxifen (Sigma, USA) on P7 to activate Cre recombinase in Gli1-expressing neural progenitor cells,[23,25] leading to the persistent expression of GFP in these cells and all their progeny. On P21, animals were randomized to fasting in room air or 1.5% isoflurane (Isothesia; Henry Schein Animal Health, USA) in 30% oxygen for 6 h. Inspired anesthetic and oxygen concentrations were monitored using a gas analyzer (RGM 5250; Datex-Ohmeda, USA). Animals were housed in padded acrylic containers inside incubators warmed to 34°C during the 6 h. Rectal temperature measurements in a separate group of five mice revealed a gradual increase in temperature during the 6-h exposure, from 35.6 ± 0.3°C at 30 min to 38.0 ± 0.0°C at 6 h. These temperatures appear to be in the normal range.[26–28] Mice were killed either immediately after treatment or 60 days after treatment, generating four study groups: (1) P21 control: 3-week-old mice exposed to room air and perfused immediately (n = 7 [male = 3 and female = 4]); (2) P21 anesthesia: 3-week-old mice exposed to isoflurane and perfused immediately (n = 8 [male = 6 and female = 2]); (3) P81 control: mice exposed to room air on P21 and perfused 60 days later (n = 8 [male = 3 and female = 5]); and (4) P21 anesthesia plus 60 days: mice exposed to isoflurane on P21 and perfused 60 days later (n = 8 [male = 4 and female = 4]). Group numbers do not include three animals that died during anesthesia exposure, and two P81 control mice that died during the 2-month recovery period. For perfusions, mice were euthanized with an intraperitoneal injection of ketamine (20 mg/kg), acepromazine (0.5 mg/kg), and xylazine (1 mg/kg; triple cocktail prepared by the CCHMC Vivarium, USA) and then transcardially perfused with 0.1 M phosphate buffered saline (PBS) plus 1 U/ml heparin followed by 4% paraformaldehyde with 5% sucrose and 5% glycerol in PBS (pH 7.4). Brains were removed, postfixed overnight in the same fixative, cryoprotected in an ascending sucrose series (10%, 20%, and 30%) in PBS, and snap-frozen in 2-methyl-pentane at −25°C. Brains were sectioned sagittally at 60 μm on a cryostat (Microm HM 520 cryostat, Thermo Fisher Scientific, USA), and sections were mounted on gelatin-coated slides and stored at −80°C.

BrdU Pulse-chase Experiments

To determine whether anesthesia exposure altered cell proliferation, a group of C57BL/6 mice was exposed to isoflurane or room air on P21, in identical fashion to other animals in this study. After anesthesia exposure, animals received 5-Bromo-2'-deoxyuridine (BrdU; 100 mg/kg per dose; Sigma) on days 2, 4, 6, 8, and 10 after treatment, for a total of five doses.

Animals were euthanized and perfused 2 weeks after anesthesia exposure, on P35. A total of 11 animals were exposed to room air and 19 to isoflurane. There was no mortality in either group; however, one control and three anesthesia-treated mice were excluded for technical reasons (poor perfusion). Final groups included 10 control (P35, control; four male and six female) and 16 anesthesia-treated (P21 anesthesia plus 14 days; six male and 10 female) mice. Brains were prepared and sectioned as described for other animals in the study.

Immunohistochemistry

Sagittal sections between 0.60 and 0.84 mm lateral to the midline were used.[29] The slices were incubated in phosphate buffer (pH = 7.4) for 10 min. Slide-mounted sections were permeabilized for 4 h in 5% Tween-20 and 7.5% glycine in PBS on a shaker plate. Sections were blocked for 1 h at room temperature in 5% normal goat serum, 5% Tween-20, 0.75% glycine, and 0.5% nonfat dry milk in PBS before the primary antibodies were added.

Slides with up to four brain sections were immunostained with 1:1,000 chicken anti-GFP (ab13970; Abcam, USA) and 1:100 rabbit anti–caspase-3 (9661L; Cell signaling, USA) for the P21 groups or anti-GFP, 1:200 mouse anti-calretinin (MAB1568; Millipore, USA), and 1:200 rabbit anti-Ki67 (VP-K451; Vector Laboratories, USA) for the P81 groups. P35 groups were immunostained with anti–caspase-3, anti-calretinin, and anti-Ki67. Sections were incubated with the primary antibodies at room temperature overnight. The next day, sections were rinsed in 5% Tween-20 and 7.5% glycine in PBS and incubated in AlexaFluor 488 goat anti-chicken, AlexaFluor 594 goat anti-rabbit, AlexaFluor 594 goat anti-mouse, or AlexaFluor 647 goat anti-rabbit antibodies (Invitrogen, USA), as appropriate to match primary antibody species. All secondary antibodies were used at 1:750. Sections were washed in PBS, dehydrated in an ascending ethanol series, cleared in xylenes, and mounted with Krystalon (Harleco, Germany).

Brain sections from the P35 group were also immunostained for BrdU. Slides with up to four brain sections were permeabilized in 0.5% Igepal Tris–hydrochloric acid (HCl) buffer for 1 h at room temperature. Slides were then incubated at 37°C in 3 μg/ml protease (#53702; EMD Millipore, Merck KGaA, Germany), Tris–HCl with $CaCl_2$ buffer for 30 min, ice-cold 0.1 M HCl for 10 min, and 2 M HCl for 20 min. Slides were blocked in 5% normal donkey serum before the primary antibody was added. Slides were incubated overnight at 4°C with 1:200 rat anti-BrdU (#347580; Becton Dickinson, USA). The next day, slides were rinsed in 5% normal donkey serum and 0.5% Igepal in PBS and incubated in 1:750 AlexaFluor 488 donkey anti-rat antibodies. Sections were washed in PBS, dehydrated in an ascending ethanol series, cleared in xylenes, and mounted with Krystalon (Harleco).

GFP and calretinin immunostaining failed in one P81 control mouse. BrdU staining failed in one P21 anesthesia plus 14-day mouse. Calretinin staining failed in three P35 control mice and four P21 anesthesia plus 14-day mice. Immunostaining failures are attributed to technical problems with immunohistochemical procedures. In some of these cases, all available tissue from an animal was used such that it was not possible to repeat immunohistochemical stains. Decisions to exclude immunostained brain sections were made without knowledge of treatment group according to preestablished criteria (*e.g.*, tissue lost or damaged during processing, or positive controls are negative). Animal numbers (n) for each measure are reported in the text.

Confocal Microscopy and Histologic Analyses

All image collection and analyses were conducted by an investigator unaware of group assignment. GFP/caspase-3 double labeling (P21 groups) and GFP/calretinin/Ki67 triple labeling (P81 groups) were imaged using a Leica SP5 confocal system (Leica Microsystems, Germany) set up on a DMI 6000 inverted microscope (Leica Microsystems) equipped with a ×63 oil immersion objective (numerical aperture [NA], 1.4). Images were collected at 1-μm increments to generate three-dimensional confocal "z-stacks." Specifically, confocal image stacks through the z-depth were collected from the midpoint of both the upper and lower blades of the dentate cell body layer (two confocal z-stacks per animal, each with a field size of 240 × 240 μm). Image stacks were then imported into Neurolucida software (version 10.31; MBF Bioscience, USA) for quantification. For the P35 groups, calretinin, Ki67, and BrdU immunostained sections were imaged using a Nikon A1 confocal microscope (Nikon, Japan) equipped with a ×20, 0.75 NA objective. Images of an entire dentate gyrus from each mouse were collected at 1-μm increments through the z-depth of the tissue. Image sets were quantified using Imaris software (version 64 7.7.2; Bitplane, Switzerland) for calretinin and Ki67 and both Imaris and Neurolucida software (version 10.31; MBF Bioscience, USA) for BrdU-labeled sections (investigator-conducted BrdU counts using Neurolucida were used to validate the automated Imaris counting approach; Neurolucida counts are reported for BrdU). The number of caspase-3 immunopositive cells in the dentate gyri of P35 mice was counted using a DMI 6000 inverted microscope under a ×63 oil immersion objective (NA 1.4). Two to four dentate gyri per mouse were counted (damaged sections were excluded). The number of GFP-expressing hilar/molecular layer granule cells in the P81 groups was counted using an identical strategy. All cell-counting approaches used a variation of the optical dissector method to prevent bias due to changes in cell size.[14] Counts were normalized to the volume of dentate present in each region of interest. Cell numbers per hippocampal section were converted to density (mm^3) using the following equation: (number of cells per dentate/[DGC layer area × 20 μm volume]) × 10^9. No adjustments were made for tissue shrinkage.

Statistical Analysis

Data are presented as mean ± SEM. Data were tested for normality (Shapiro–Wilk) and equal variance (Brown–Forsythe). Parametric data were compared using two-tailed

Student's *t* tests. Data that failed tests for either normality or equal variance were either transformed to normalize the data or compared using the Mann–Whitney U test. For all analyses, statistical significance was determined using Sigma Stat software (version 12.3; Systat Software, USA). Specific statistical tests used are noted in the results. All parameters examined were statistically equivalent between male and female mice (data not shown), so data were pooled for analysis. Measurements from the upper and lower blades of the dentate gyrus were also found to be statistically equivalent for all parameters examined (data not shown), so upper and lower blade data sets were combined for simplicity. Statistical significance was accepted for $P < 0.05$.

Figure Preparation

Figures were prepared using Adobe Photoshop (CS5 Extended; Adobe, USA). Brightness and contrast were adjusted to optimize cellular detail. Identical adjustments were made to all images meant for comparison.

Results

Anesthetic Exposure Significantly Increases Cell Death among 2-week-old DGCs

GFP-expressing granule cells were evident in the dentate hilus and inner third of the DGC body layer in control and anesthetized animals immediately after exposure on P21, consistent with the known proliferation and migration patterns of these neurons.[30] The density and localization of GFP-expressing cells were similar in control and anesthesia-treated mice (fig. 1; P21 control, n = 7, 241,000 ± 26,000 GFP-positive cells per cubic millimeter; P21 anesthesia, n = 8, 243,000 ± 24,000 cells per cubic millimeter; $P = 0.961$, Student's *t* test), demonstrating that progenitor cell labeling was equivalent between the two groups. Exposure to isoflurane, however, led to a dramatic increase in the number of caspase-3–immunoreactive cells in the dentate gyrus (fig. 1), consistent with previous studies.[13,14] Moreover, quantification of caspase-3 immunolabeling in GFP-expressing cells revealed a five-fold increase in double-labeled cells relative to controls. Specifically, in 21-day-old control animals, 2.05 ± 0.75% of GFP-expressing cells were colabeled with caspase-3, while 11.03 ± 1.75% of cells in the P21 anesthesia group expressed caspase-3 (fig. 1; $P < 0.001$, Student's *t* test). Our results demonstrate that these young, approximately 2-week-old GFP-expressing cells are vulnerable to anesthesia-induced cell death.

GFP Expression Reveals the Morphology of Caspase-3–immunoreactive Cells

One advantage of the fate-mapping approach used here is that the morphology of dying cells can be readily assessed. In the P21 anesthesia group, GFP-expressing, caspase-3–immunoreactive granule cells exhibited morphologic changes consistent with cell degeneration. Specifically, cells had small, condensed cell bodies suggestive of pyknosis (fig. 2). Indeed, caspase-3–immunoreactive cells could be easily identified by this feature when examining GFP expression alone. When present, the processes of GFP-expressing, caspase-3–immunoreactive cells ran either parallel to the granule cell layer, indicative of type II progenitor cells, or projected perpendicularly into the granule cell layer. Processes of these latter cells lacked dendritic spines and terminated before reaching the outer molecular layer—morphologic features possessed by immature granule cells. Combined, these morphologic criteria suggest that anesthesia induces neuronal pyknosis and cell death in late progenitor and immature granule cells, a conclusion consistent with previous work showing that dying cells express the progenitor cell and early differentiation markers NeuroD1 and calretinin.[14]

Fate-mapped DGC Numbers Recover 60 Days after Anesthesia Exposure

Quantification of GFP-expressing cells in the dentate 60 days after isoflurane exposure revealed similar densities in

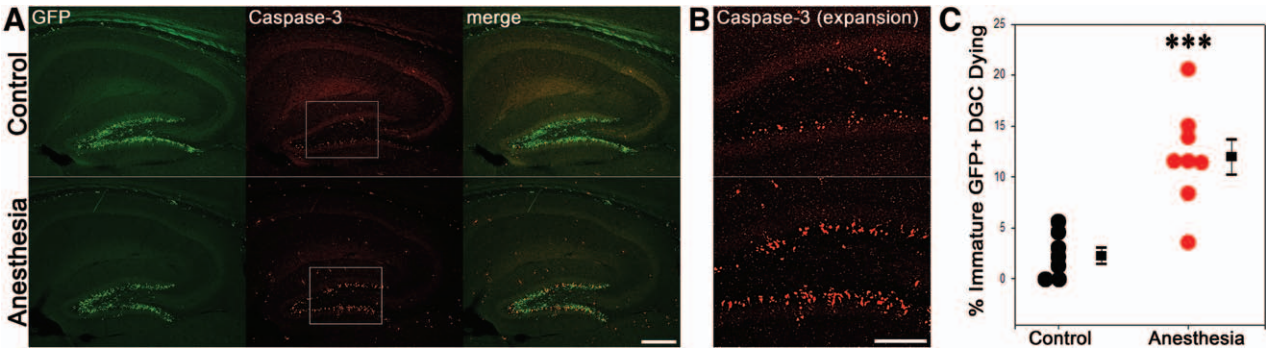

Fig. 1. (*A*) Anesthetic exposure increases caspase-3 expression in 2-week-old dentate granule cells (DGCs) immediately after exposure. Confocal maximum projections of hippocampal sections from representative 21-day-old mice exposed to 1.5% isoflurane (anesthesia) or room air (control) for 6 h. Sections are immunostained for green fluorescent protein (GFP) and caspase-3, a marker of apoptotic cell death. *Scale bar* = 250 μm. (*B*) *Boxed regions* in (*A*) are shown at higher resolution. *Scale bar* = 125 μm. (*C*) Scatterplot shows the percentage of GFP-expressing cells in the dentate gyrus that were also immunoreactive for caspase-3 immediately after exposure. Each dot represents one animal. *Boxes* are mean ± SEM. ***$P < 0.001$, Student's *t* test.

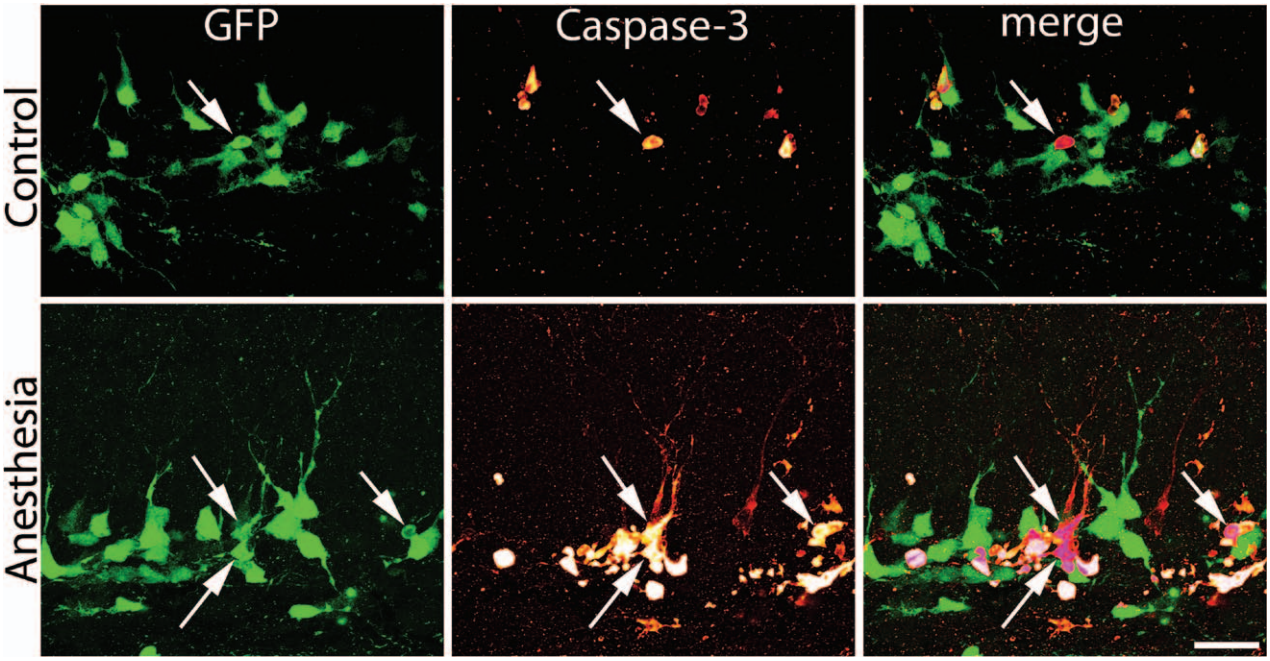

Fig. 2. Green fluorescent protein (GFP) reveals the cellular morphology and integrity of dentate granule cells in control and anesthesia-exposed mice. Images show representative cells after a 6-h exposure to room air or isoflurane. Isoflurane dramatically increased caspase-3 immunoreactivity. *Arrows* denote double-labeled cells. In the anesthesia-treated animal, the two cells in the center of the image have short, aspiny dendrites projecting into the dentate molecular layer; morphologic features of immature granule cells. Morphology suggests cellular disintegration. *Scale bar* = 20 μm.

81-day-old control mice and 81-day-old mice exposed to isoflurane at P21 (fig. 3; P81 control, n = 7, 245,000 ± 33,000 GFP-expressing cells per cubic millimeter; P21 anesthesia plus 60 days, n = 8, 250,000 ± 22,000 cells per cubic millimeter; $P = 0.915$, Student's *t* test). These findings may reflect regeneration of this population, presumably by increased proliferation of surviving, GFP-expressing progenitor cells. Alternatively, anesthesia exposure might have simply accelerated the death of newborn cells fated to undergo natural apoptosis at a later stage.

The Cohort of Fate-mapped GFP-expressing Granule Cells Exhibits Similar Rates of Proliferation 60 Days after Anesthesia Exposure

It is conceivable that the observed anesthesia-induced loss of immature granule cells in 21-day-old mice might produce a lasting alteration in adult granule cell neurogenesis since neurogenesis is very sensitive to a variety of physiologic and pathologic stimuli.[31] To explore this possibility, we colabeled GFP-expressing neurons with the proliferative cell marker Ki67 and the immature granule cell marker calretinin. No significant differences were found in the percentage of GFP-positive granule cells that coexpressed either Ki67 or calretinin 60 days after exposure (fig. 4). In P81 controls, 4.49 ± 1.62% (n = 8) and 17.94 ± 1.41% (n = 7) of GFP-labeled cells expressed Ki67 or calretinin, respectively. In the P21 anesthesia plus 60 days, 5.99 ± 1.55% of GFP-labeled cells expressed Ki67 (n = 8; $P = 0.517$; Student's *t* test compared with control) and 21.65 ± 3.06% expressed calretinin (n = 8; $P = 0.291$, Student's *t* test compared with control). These findings indicate that the cohort of granule cell progenitors labeled with GFP exhibits normal proliferation rates at this time point. These findings also demonstrate active proliferation within the GFP-expressing cell population in these animals, consistent with the possibility that neurons lost to anesthesia exposure may be replaced by subsequent neurogenesis.

Mice Exposed to Anesthesia on P21 Show No Evidence of Granule Cell Migration Defects in Adulthood

Postnatally generated granule cells are produced in the subgranular zone, located immediately below the granule cell body layer, and migrate short distances (tens of microns) to occupy final positions in the inner third of the granule cell layer. Smaller numbers of granule cells migrate to the middle and outer thirds of the cell body layer in healthy animals. A variety of brain insults, such as seizures, can disrupt granule cell migration. Under pathologic conditions, large numbers of granule cells can migrate in the wrong direction to reside ectopically in the dentate hilus or they can migrate too far, traveling through the granule cell layer to take final positions in the dentate molecular layer.[32–34] To determine whether anesthesia exposure disrupted granule cell migration, the number of GFP-expressing granule cells in the dentate hilus and molecular layer was determined. The number of GFP-expressing ectopic cells in the hilus (P81 control, n = 8, 3.31 ± 0.57 cells per hippocampal section; P21 anesthesia plus 60 days, n = 8, 4.25 ± 0.85; $P = 0.372$, Student's *t* test

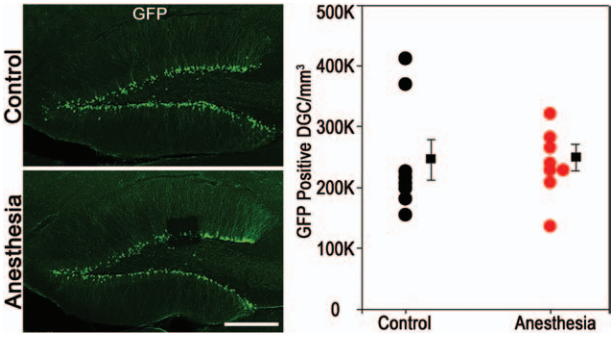

Fig. 3. Anesthesia-induced apoptotic cell death in 2-week-old dentate granule cells (DGCs) does not lead to long-term diminution in neuronal density in this population. Confocal maximum projections show immunostaining for green fluorescent protein (GFP) in representative 2-month-old animals exposed to 6 h of 1.5% isoflurane (anesthesia) or room air (control) on postnatal day 21. No differences between control and anesthesia-treated mice were found. *Scale bar* = 250 μm. Scatterplot shows the density of GFP-expressing cells in the dentate granule cell body layer. *Circles* represent individual animal means, while *squares* reflect group means ± SEM. The two groups were statistically equivalent (P = 0.915, Student's t test).

compared with control) and molecular layer (P81 control, n = 8, 1.38 ± 0.26 cells per hippocampal section; P21 anesthesia plus 60 days, n = 8, 1.88 ± 0.61; P = 0.464, Student's t test compared with control) was similar between groups, suggesting that cells born after anesthesia treatment followed normal migration patterns (fig. 5).

Neurogenesis Is Not Increased in the Weeks after Anesthesia Exposure

To determine whether the recovery in GFP-expressing granule cell numbers reflected increased proliferation in the dentate, a second group of mice was treated with the S-phase proliferative marker BrdU 2, 4, 6, 8, and 10 days after isoflurane treatment on P21. This BrdU injection protocol covers a period during which increased cell proliferation was previously demonstrated after adult exposure to isoflurane.[35] Mice were euthanized 14 days after anesthesia treatment on P35. No difference in the density of BrdU-stained cells was evident between the two groups (fig. 6; P35 control, n = 10; P21 anesthesia plus 14 days, n = 15; P = 0.771, Student's t test), suggesting that proliferation was not increased during the time period of BrdU injections.

The P35 control and P21 anesthesia plus 14 day groups were also immunostained for the proliferative cell marker Ki67. Ki67 provides a snapshot of cycling cells at the time the animals were perfused (P35). No difference in the density of Ki67-positive cells in the dentate gyrus was evident between the two groups (fig. 6; P35 control, n = 10; P21 anesthesia plus 14 days, n = 16; Student's t test, P = 0.904), indicating similar numbers of proliferating cells at the time of euthanasia.

Finally, the P35 control and P21 anesthesia plus 14 day groups were immunostained with the immature granule cell marker calretinin, which is expressed in granule cells roughly 14 to 28 days old. Calretinin staining provides a measure of neurogenesis rates 2 to 4 weeks earlier. No differences in the number of calretinin-labeled cells were evident between groups (fig. 6; P35 control, n = 7; P21 anesthesia plus 14 days, n = 12; Student's t test, P = 0.964).

Anesthesia-treated Mice Show a Trend Toward Reduced Apoptosis 2 Weeks after Anesthesia Exposure

The recovery in granule cell numbers 2 months after isoflurane exposure could reflect increased neurogenesis or decreased apoptosis (or both). To explore the possibility that survival might be enhanced after isoflurane treatment, P35 control and P21 anesthesia plus 14 day groups were immunostained for activated caspase-3. Anesthesia-treated mice showed a nonsignificant trend toward reduced density of caspase-3 immunoreactive neurons in the dentate (fig. 6; P35 control, n = 10; P21 anesthesia plus 14 days, n = 16; Student's t test on square root transformed data, P = 0.075). We interpret this trend cautiously, however, as it is partly driven by the presence of an outlier in the control group (P = 0.201 with outlier removed).

Discussion

In the current study, we examined anesthesia-induced cell death among immature hippocampal granule cells produced by a cohort of Gli1-expressing granule cell progenitors. GFP expression among progenitor cells was induced on PD7, and animals were exposed to isoflurane on PD21. GFP-labeled immature granule cells, therefore, would be up to 2 weeks old at the time of exposure. Isoflurane treatment produced a five-fold increase in apoptosis among GFP-labeled daughter cells, confirming that this age range is vulnerable to anesthesia-induced cell death. However, despite the loss of GFP-expressing cells immediately after isoflurane exposure, no differences in the density of GFP-labeled cells were evident 60 days later, when the animals were young adults. This finding demonstrates that isoflurane treatment does not produce persistent cell loss among the cohort of GFP-labeled neurons. Integration of the GFP-labeled granule cells was also grossly normal, with no evidence of migration defects among the population. Finally, we did not observe any change in the rate of hippocampal neurogenesis between isoflurane-treated animals and controls either 2 weeks or 2 months after exposure. Taken together, these findings indicate that the murine hippocampus can restore neuron numbers after anesthesia exposure, possibly *via* modest reductions in apoptosis that occur below levels of detection.

Cell Fate Mapping to Assess the Impact of Anesthesia Exposure

A key strength of the fate-mapping approach utilized here is that it provides an accurate measure of net neurogenesis/apoptosis rates over the entire experimental time window. Other approaches commonly used to assess the impact of anesthetic neurotoxicity—such as BrdU labeling, cell death markers, and immunohistochemical markers of neurogenesis

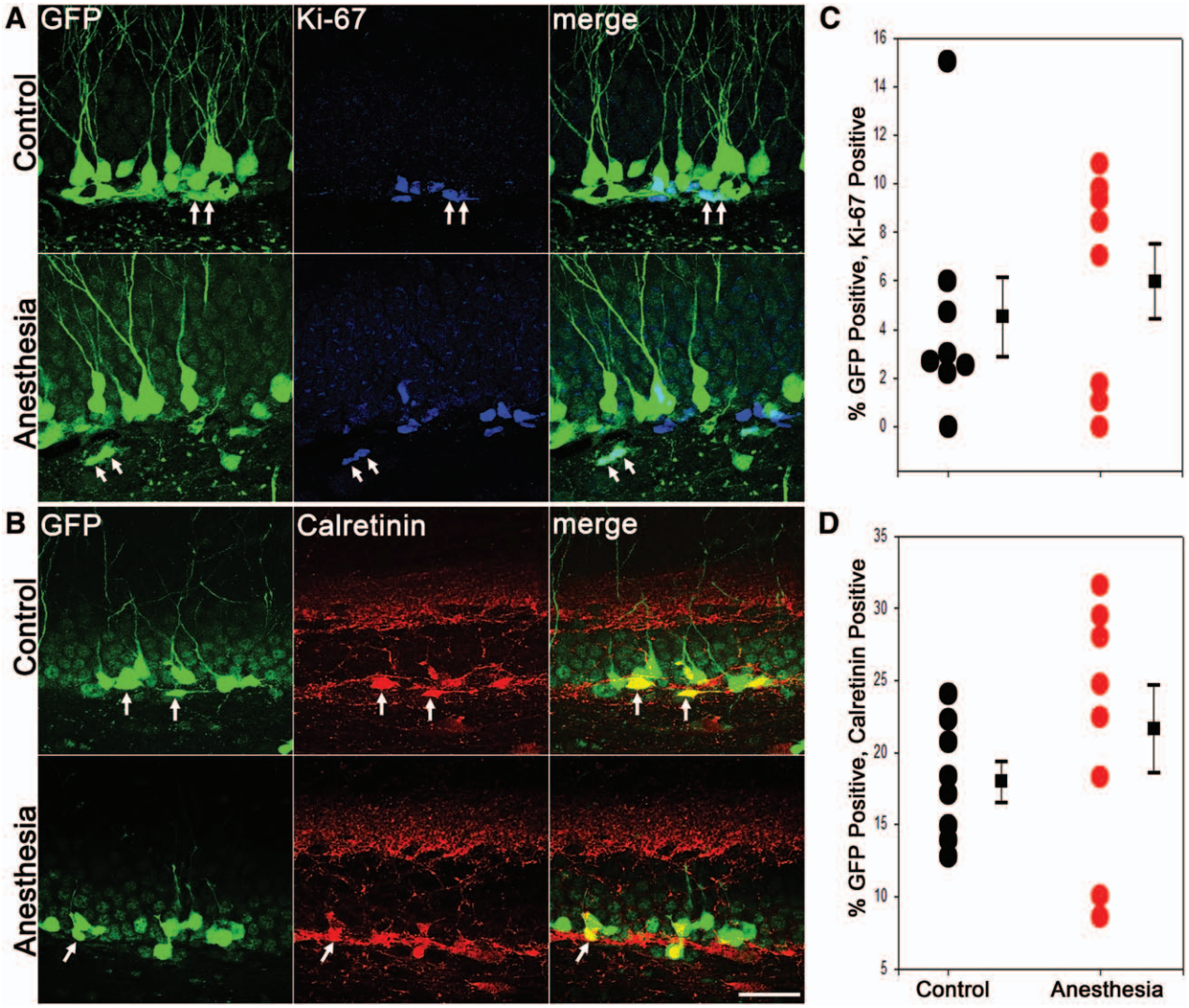

Fig. 4. Anesthesia-induced apoptotic cell death in 2-week-old dentate granule cells does not lead to alterations in neuronal proliferation in this population. Confocal maximum projections show green fluorescent protein (GFP) plus Ki67 (*A*) and GFP plus calretinin (*B*) immunostaining in the hippocampal granule cell layer. Double-labeled cells are denoted by *arrows*. *Scale bar* = 30 μm. Scatterplots show the percentage of GFP-expressing cells double-labeled with Ki67 (*C*) or calretinin (*D*). *Circles* represent individual animal means, while *squares* reflect group means ± SEM. The two groups were statistically equivalent for both Ki67 and calretinin.

—only provide snapshots of neurogenesis/cell death rates at the time the animal is killed, significantly limiting conclusions about the net effects of anesthesia exposure. Repeated histologic assessments at different time points after anesthetic exposure can mitigate these limitations, but still risk missing critical windows of vulnerability during time periods not examined. Moreover, traditional approaches poorly capture a second source of neuronal elimination—loss of progenitor cells. Specifically, although caspase-3 immunostaining can detect the death of a single progenitor cell, it provides no information about the number of daughter cells the progenitor might have produced had it survived. Finally, there is evidence from *in vitro* studies of human neural progenitor cell lines that isoflurane can inhibit cell proliferation.[36] In order to capture changes in cell proliferation and the aforementioned effects on cell loss, fate mapping provides an attractive measure of net progenitor cell productivity (*e.g.*, the number of daughter cells that were produced and survived to the experimental endpoint).

Reduced Natural Apoptosis Post Anesthesia May Restore Neuron Numbers in the Dentate Gyrus

We have previously demonstrated that postnatally generated granule cells are selectively vulnerable to anesthesia-induced cell death about 2 weeks after they are generated using BrdU birth dating and immunohistochemical phenotyping techniques.[14] The fate-mapping approach utilized here confirms our previous findings, demonstrating significant loss of immature granule cells after anesthesia exposure.

Our previous work did not reveal whether the cell death observed immediately after isoflurane exposure captured the

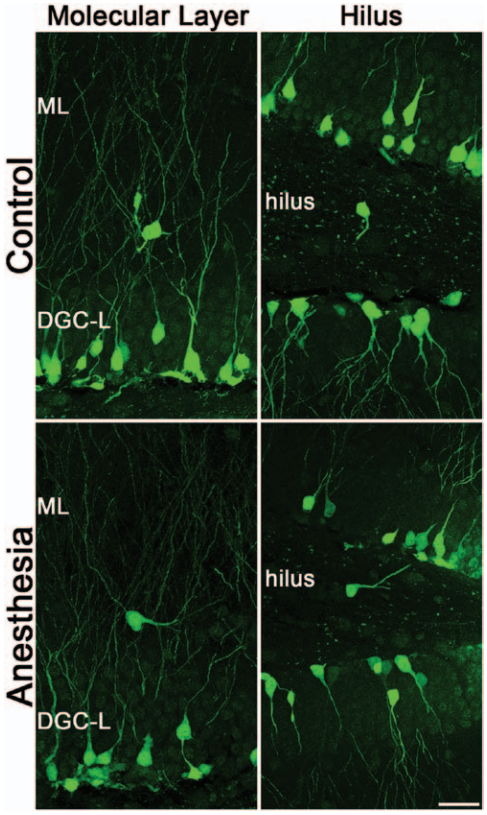

Fig. 5. Confocal maximum projections from postnatal day 81 (P81) control and P21 anesthesia plus 60-day mice show green fluorescent protein (GFP)–expressing cells misplaced to either the dentate molecular layer (ML) or hilus. No differences in the frequency of ectopic cells were evident between groups, and the overwhelming majority of GFP-labeled cells were correctly localized to the granule cell body layer (DGC-L). *Scale bar* = 25 μm.

bulk of the cell loss; whether it was merely the "tip of the iceberg," with secondary waves of cell loss occurring hours, days, or even weeks later; or whether cell numbers recovered. We now demonstrate an absence of detectable change in the density of GFP-expressing cells in adult animals treated with isoflurane on P21. This result reveals that the dentate is able to reestablish appropriate neuron numbers during the 2-month interval after the insult. This result partly contrasts with the work by Zhu *et al.*[37] (2010) in rats, who found a modest decrease in granule cell numbers 10 weeks after four, 35-min isoflurane exposures between days 14 and 17. Whether the different treatment paradigm, species, or previous exposure accounts for the opposing results remains to be determined, but all could be critical variables. Notably, even earlier, P7 treatment with isoflurane has been shown to decrease neurogenesis and lead to learning deficits in adulthood,[38] while treatment of 12-week-old rats does not appear to alter proliferation.[39] Together, these findings suggest that the window of vulnerability for long-term reductions in cell numbers closes before P21. Restated, the most pronounced loss occurs around P7, more modest loss is evident after exposure a week later (P14), and P21 exposure produces damage that is qualitatively adult like, impacting neurogenic brain regions, which then recover. Quantitatively, P21 exposure produces greater acute cell loss than observed in adults, reflecting the larger population of vulnerable, immature neurons present during this developmental period. Whether the greater degree of acute cell loss at P21 produces any subtle changes that are distinct from adults remains to be determined.

To explore possible mechanisms by which appropriate cell numbers might be restored, calretinin and Ki67 immunostaining was conducted at the 2-month time point, and calretinin, Ki67, BrdU, and caspase-3 labeling was examined in an additional group of animals collected 2 weeks after exposure. Ki67 labels actively proliferating cells, providing a snapshot of cell division at the animals' time of death, and calretinin labels 2- to 4-week-old granule cells,[40] providing measures of neurogenesis around the time of anesthesia treatment. The cell birth dating marker BrdU was injected between 2 and 10 days after isoflurane exposure to assess neurogenesis during this period. A wide variety of stimuli can induce hippocampal neurogenesis,[41–43] including neuronal loss,[43–45] so restoration of cell numbers by increased neurogenesis is quite plausible.

Despite a multipronged approach to gather information about neurogenesis rates at numerous time points after isoflurane exposure, no evidence of increased neurogenesis was found. Although we cannot exclude the possibility that neurogenesis is increased at remaining time points not examined here, it seems increasingly likely that the injury caused by P21 isoflurane exposure does not induce a dramatic burst of neurogenesis in the dentate. It remains possible that a modest, but prolonged, increase in neurogenesis replaces lost neurons. Such an increase might be sufficient to replace the lost cells while still remaining below detection thresholds of the techniques used here.

A second possibility that cannot be fully excluded by the present findings is that there is a modest increase in the survival of newborn granule cells. Granule cells are produced in significant excess, and many undergo apoptosis under normal conditions. Inactive granule cells are preferentially eliminated.[46] Anesthesia-induced loss of granule cells could be naturally offset by increased neuronal activity funneled to the remaining cells, thereby enhancing their survival. Such a mechanism might operate over weeks to months, making it difficult to detect.

Significance and Limitations of the Current Findings

It remains possible that subtle changes in granule cell structure or function might lead to long-lasting deficits in hippocampal-dependent tasks. Indeed, Briner *et al.*[47] demonstrated a persistent increase in spine density among cortical neurons after exposure to propofol in rats at 15, 20, and 30 days, and Mintz *et al.*[48] have shown that isoflurane can disrupt axonal targeting in cultured neocortical slices. Additional studies to characterize the morphology and physiology of exposed granule cells will be needed to determine whether they function normally.[49]

A question worth considering is whether a temporary loss of 2-week-old hippocampal granule cells might produce lasting changes in brain function. Even though our findings support the conclusion that these lost neurons are ultimately replaced, it

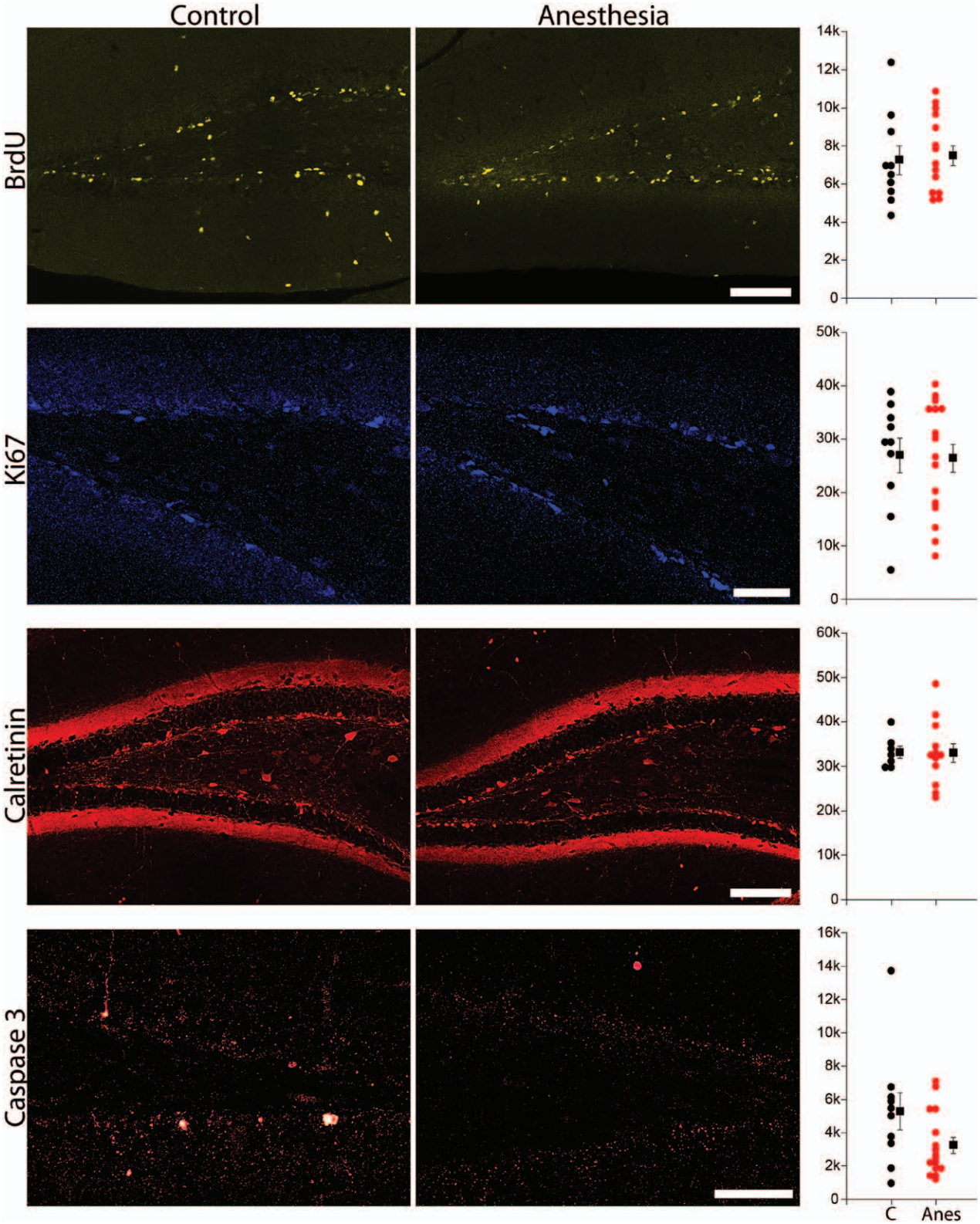

Fig. 6. 5-Bromo-2'-deoxyuridine (BrdU), Ki67, calretinin, and caspase-3 labeling in postnatal day 35 (P35) control (C) and P21 anesthesia plus 14-day (Anes) mice. *Scale bars* = 100 μm (BrdU and calretinin) and 50 μm (Ki67 and caspase-3). Scatterplots shows the density of labeled cells in the dentate granule cell body layer for each corresponding image set. *Circles* represent individual animal means, while *squares* reflect group means ± SEM. The two groups were statistically equivalent for all measures ($P > 0.05$, Student's *t* test).

presumably still takes several weeks to regenerate the lost cells. Newborn granule cells transiently exhibit a variety of unique physiologic properties during a period occurring approximately 3 to 6 weeks after they are generated. These properties include increased excitability, lower inhibition, and enhanced long-term potentiation.[31] The functional significance of this unique population of granule cells remains controversial; however, numerous lines of evidence suggest that they are important for memory and cognition. Loss of this population of cells—even temporarily—might have a profound impact on the hippocampal circuit. This could be particularly relevant for the developing brains of young children. Consistent with this idea, experimental manipulations that transiently deplete adult-generated granule cells have been shown to produce a variety of behavioral deficits, including disruption of hippocampal-dependent memory,[50–52] impaired responses to antidepressant medications,[53–55] and altered responses to convulsant drugs.[56,57] Deficiencies in recollection memory have recently been observed 5 to 10 yr after anesthesia and surgery in infancy,[58] possibly reflecting long-term consequences of transient hippocampal disruption. Animal and human studies designed to detect such transient deficits should be carefully timed with regard to the exposure period to optimize the chances of observing an effect.

In summary, exposing 3-week-old mice—comparable in brain development to human infants—to a clinically relevant dose of isoflurane for 6 h increased apoptotic cell death among 2-week-old DGCs. Fate mapping the exposed population, however, did not reveal a reduction in neuronal numbers after the animals reached adulthood. Taken together, these findings indicate that overt neuronal loss may not be a significant consequence of P21 anesthesia exposure in this regenerative population. The results leave open the possibility, however, that more subtle changes in neuronal structure or function may still occur in dentate.

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Competing Interests

The authors declare no competing interests.

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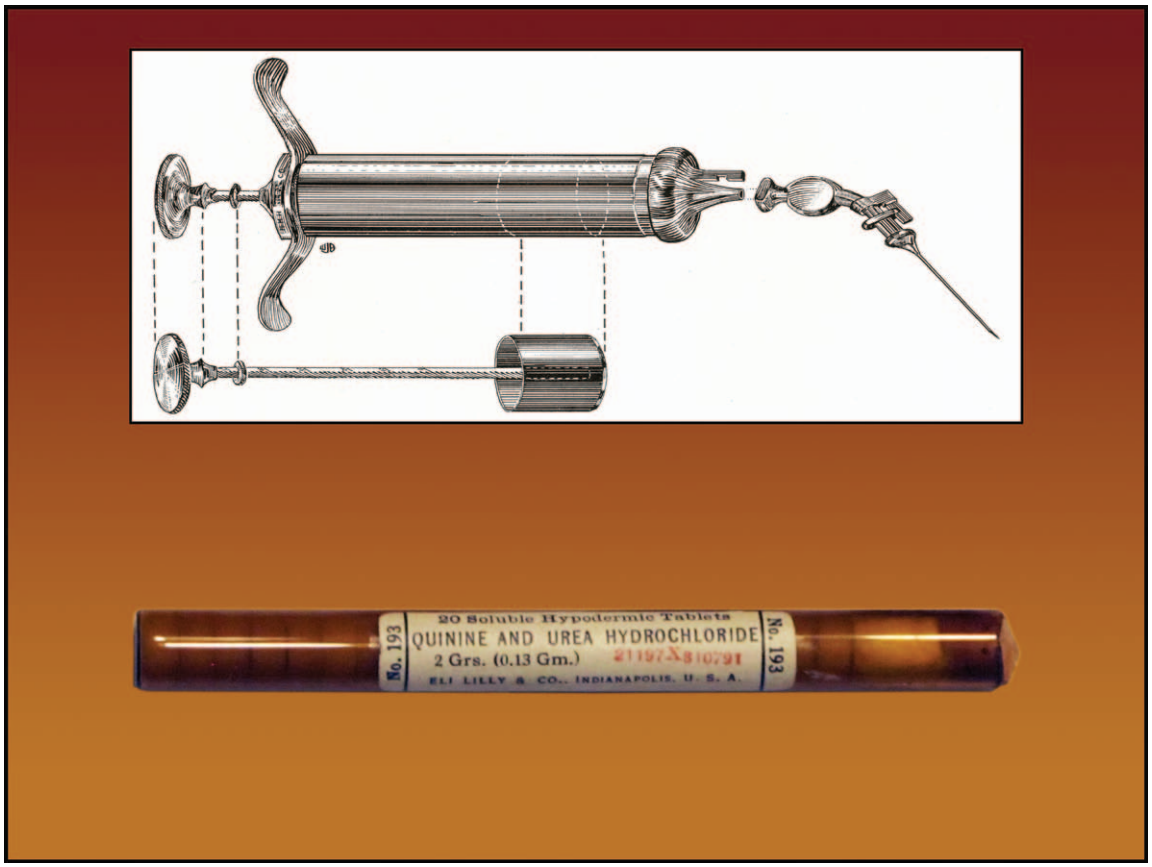
Moynihan Syringe for Quinine and Urea Local Anesthesia



In his 1914 masterwork *Anoci-Association*, Cleveland surgeon George W. Crile, M.D. (1864 to 1943), depicted (*top*) the "Moynihan Syringe for the Infiltration of Quinin[e] and Urea Hydrochlorid[e]." His book was published seven years after the first publication about long-lasting local anesthesia and analgesia following injection of a mixture of quinine with urea. Manufactured by Eli Lilly & Company of Indianapolis, the glass tube (*bottom*) contained "20 soluble hypodermic tablets" with 2 grains or 0.13 grams of Quinine-Urea mixture. Although it was a long-acting local anesthetic and analgesic, the quinine-urea mixture could delay wound healing if directly injected into wound edges. And that is why Dr. Crile recommended use of a syringe with a long offset needle such as Moynihan's for infiltrating "*at a Distance* from the Incision." (Copyright © the American Society of Anesthesiologists' Wood Library-Museum of Anesthesiology.)

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