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Research Report

Quantitative assessment of new cell proliferation in the dentate gyrus and learning after isoflurane or propofol anesthesia in young and aged rats

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ABSTRACT

There is a growing body of evidence showing that a statistically significant number of people experience long-term changes in cognition after anesthesia. We hypothesize that this cognitive impairment may result from an anesthetic-induced alteration of postnatal hippocampal cell proliferation. To test this hypothesis, we investigated the effects of isoflurane and propofol on new cell proliferation and cognition of young (4 month-old) and aged (21 month-old). All rats were injected intraperitoneally (IP) with 50 mg/kg of 5-bromo-2-deoxyuridine (BrdU) immediately after anesthesia. A novel appetitive olfactory learning test was used to assess learning and memory two days after anesthesia. One week after anesthesia, rats were euthanized and the brains analyzed for new cell proliferation in the dentate gyrus, and proliferation and migration of newly formed cells in the subventricular zone to the olfactory bulb. We found that exposure to either isoflurane ($p=0.017$) or propofol ($p=0.006$) decreased hippocampal cell proliferation in young, but not in aged rats. This anesthetic-induced decrease was specific to new cell proliferation in the hippocampus, as new cell proliferation and migration to the olfactory bulb was unaffected. Isoflurane anesthesia produced learning impairment in aged rats ($p=0.044$), but not in young rats. Conversely, propofol anesthesia resulted in learning impairment in young ($p=0.01$), but not in aged rats. These results indicate that isoflurane and propofol anesthesia affect postnatal hippocampal cell proliferation and learning in an age dependent manner.

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1. Introduction

Since its introduction to medical practice, anesthetic agents have enabled surgeries to be performed with ease, ensuring analgesia, unconsciousness and amnesia. Nonetheless, growing clinical and laboratory data suggest that anesthetic agents have long lasting consequences that may lead to cognitive impairment (Moller et al., 1998a; Monk et al., 2008) particularly in elderly and pediatric patients.

Ten to fifteen percent of elderly patients suffer from difficulties with concentration and attention after anesthesia (Moller et al., 1998b). Furthermore, recent studies suggest an association between early exposure to general anesthesia and long-term impairment of cognitive function in pediatric patients (Wilder et al., 2009). This phenomenon has been observed in animal models. Culley et al. (2004a, 2004b) reported enduring deficits in spatial working memory of aged rats after exposure to isoflurane, nitrous oxide, or isoflurane-nitrous oxide anesthesia (Culley et al., 2004a, 2004b). Similarly, young rats exposed to isoflurane show persistent memory and learning deficits (Culley et al., 2004a; Jevtovic-Todorovic et al., 2003; Zhang et al., 2008).

Postnatal generation of neurons occurs throughout life and has been clearly demonstrated in two brain regions, the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus and the subventricular zone (SVZ) of the lateral ventricle where new neurons migrate into the olfactory bulb (OB) (Altman and Das, 1965; Eriksson et al., 1998; Gage, 2002; Ming and Song, 2005; Taupin, 2006a, 2006b). This process is more pronounced during early postnatal life and decreases with age (Galvan and Jin, 2007; Lazarov et al., 2010), which may make new cell proliferation at these time periods more susceptible to insults. Postnatal neurogenesis in the DG has been shown to be involved in learning and memory (Jessberger et al., 2009; Kempermann, 2002; Shors et al., 2002; Zhao et al., 2008). However, the effects of anesthetics on neurogenesis are not well understood, raising concerns regarding the effect of certain anesthetic agents on cognition (Fig. 1).

In this study, we investigated whether isoflurane and/or propofol affected learning and new cell proliferation in the brain of young and aged rats shortly after administration of anesthesia. We hypothesized that learning, as demonstrated by an olfactory association task, would be impaired following anesthesia and may result from an anesthetic-induced alteration of hippocampal new cell proliferation. Evaluation of new cell proliferation following anesthesia constitutes our first step of a more extensive study of the effects of anesthesia on neurogenesis.

2. Results

2.1. *Physiological Measurements*

Physiological parameters of rats exposed to anesthetics were recorded every thirty minutes during the procedure. The body temperature of the rats was maintained at 37 °C. Oxygen

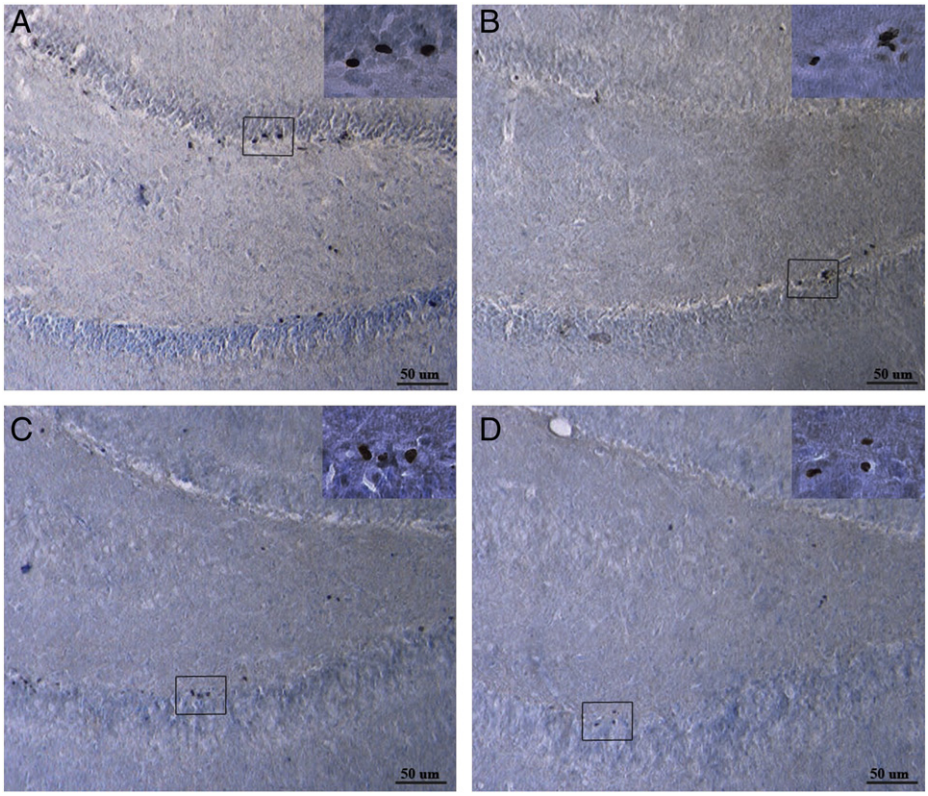

Fig. 1 – Immunohistochemical labeling of proliferating cells within the dentate gyrus (DG) with bromodeoxyuridine (A) Young intralipid (B) Young propofol (C) Young control (D) Young isoflurane.

saturation averaged 85% and 86%, for aged and young rats exposed to propofol respectively, and 94% and 95% for aged and young rats exposed to isoflurane respectively. Heart rate averaged 327 and 340 beats per minute for aged and young rats exposed to propofol respectively, and 297 and 328 beats per minute, respectively, for aged and young rats exposed to isoflurane. All physiological measurements of anesthetized rats remained within normal limits (Konze, 2007).

2.2. *New Cell Proliferation in the DG of Young Rats was Decreased by Isoflurane Anesthesia*

Fig. 2 shows the mean number of BrdU+cells in the DG of rats following room air (control) or isoflurane exposure. A two-way Analysis of Variance ANOVA ($F_{3,\ 27}=67.39$; $p=0.017$) revealed that, when compared to the control group, isoflurane anesthesia significantly decreased the number of BrdU positive (BrdU+) cells in the subgranular zone (SGZ) of the DG of young rats. There was not a statistical difference in the number of BrdU+cells between aged rats exposed to isoflurane and aged control rats ($p=0.084$). As previously reported (Garcia et al., 2004; Lazarov et al., 2010; Luo et al., 2006; Seki and Arai, 1995), significantly fewer number of BrdU+cells was found in aged as compared to young rats ($p<0.0001$).

2.3. *New Cell Proliferation in the DG of Young Rats was Decreased by Propofol Anesthesia*

New cell proliferation was assessed following propofol anesthesia (Fig. 3). A two-way ANOVA ($F_{3,\ 30}=51.13$; $p=0.006$) revealed that, when compared to the group infused with intralipid, propofol anesthesia significantly decreased the number of BrdU+cells in the DG of young rats. There was not a statistical difference in the number of BrdU+cells between aged rats infused with propofol and aged rats infused with intralipid ($p=0.181$).

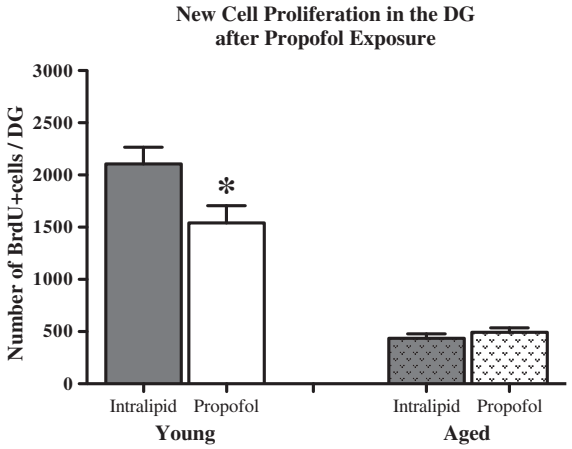

Fig. 3 – New cell proliferation in the DG of young rats was decreased by propofol anesthesia. Number of BrdU+cells in the dentate gyrus (DG) of young and aged rats, after 3 h of 35 mg/Kg/h of propofol anesthesia (propofol) or 35 mg/kg/h *p=0.0171.of 10% intralipid (intralipid). There was a statistically significant difference between young intralipid and young propofol-infused rats (*p=0.0062), but there was no difference in the aged groups.

We also found a decrease in new cell proliferation in aged, as compared to young, rats ($p<0.0001$) as expected.

2.4. *The Number of BrdU+cells in the Olfactory Bulb was not Altered by Either Isoflurane or Propofol Anesthesia*

To assess whether anesthesia produced an overall decline in cell proliferation, the number of BrdU+cells was counted in the granular and glomerular cell layer of the olfactory bulb (Figs. 4 and 5). No significant difference was found in the

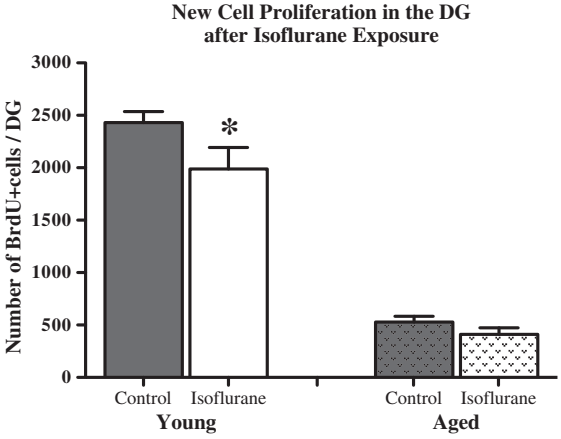

Fig. 2 – New cell proliferation in the DG of young rats was decreased by isoflurane anesthesia. Number of BrdU+cells in the dentate gyrus (DG) of young and aged rats, after 3 hours of 1.5% isoflurane anesthesia (isoflurane) or no isoflurane (control). There was a statistically significant difference between young control and young isoflurane-exposed rats (*p=0.0171), but not a statistically significant difference in the aged groups.

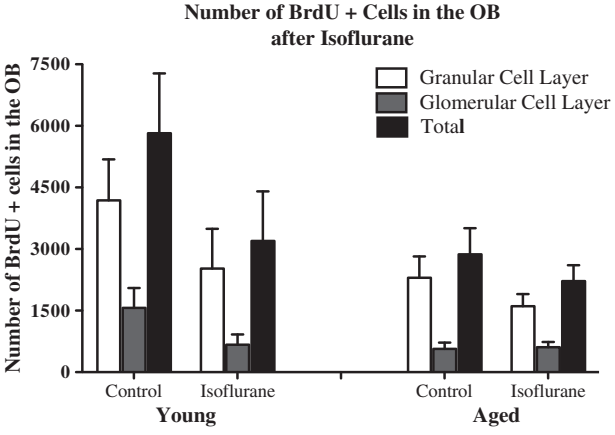

Fig. 4 – The number of BrdU+cells, that one-week had migrated to the olfactory bulb was not altered by isoflurane. Number of BrdU+cells in the granular and glomerular cell layer of the olfactory bulb (OB) of young and aged rats, one-week after 3 hours of 1.5% isoflurane anesthesia (isoflurane) or no isoflurane (control). There was not a statistically significant difference between the controls and the isoflurane-exposed rats.

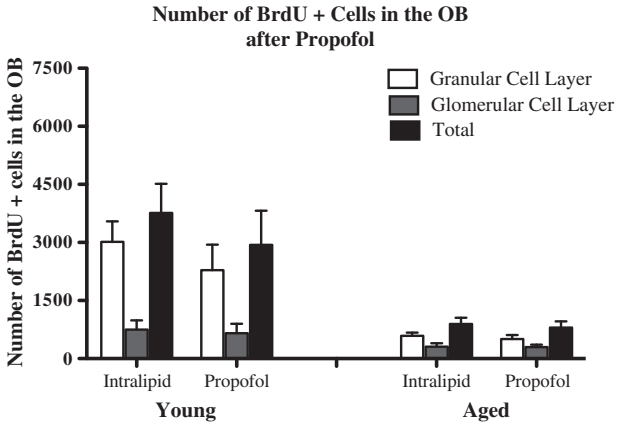

Fig. 5 – The number of BrdU+cells in the olfactory bulb was not altered by propofol anesthesia. Number of BrdU+cells in the granular and glomerular cell layer of the olfactory bulb (OB) of young and aged rats, after 3 hours of 35 mg/Kg/h of propofol anesthesia (propofol) or 35 mg/kg/h of 10% intralipid (intralipid). There was not a statistically significant difference between the controls and the isoflurane-exposed rats.

number of BrdU+cells in the glomerular or granule cell layers in rats exposed to either propofol or isoflurane anesthesia when compared with controls. However, a decrease in new cell proliferation and/or migration of the of newly formed cells in the subventricular zone to the olfactory bulb was observed in aged, as compared to young rats ($p<0.0001$) was found.

2.5. *Isoflurane Produced a Learning Impairment in Aged Rats*

Learning was assessed two days after rats were exposed to isoflurane or room air (control) (Fig. 6). Young rats learned the task quickly ($p<0.0001$), and their acquisition of the task was not affected by exposure to isoflurane ($p=0.495$). Aged rats also acquired the task ($p<0.0001$), though not as rapidly as young rats. Aged rats exposed to isoflurane showed a deficit in learning, as compared to the aged controls ($F_{3,\ 38}=21.11$; $p=0.044$) (Fig. 6A). Aged rats exposed to isoflurane made significantly more errors ($F_{1,60}=68.81$; $p<0.0001$) (Fig. 6B), and spent significantly more time in the non-rewarded quadrants ($F_{39,117}=1.88$; $p=0.0182$) (Fig. 6C) than aged controls.

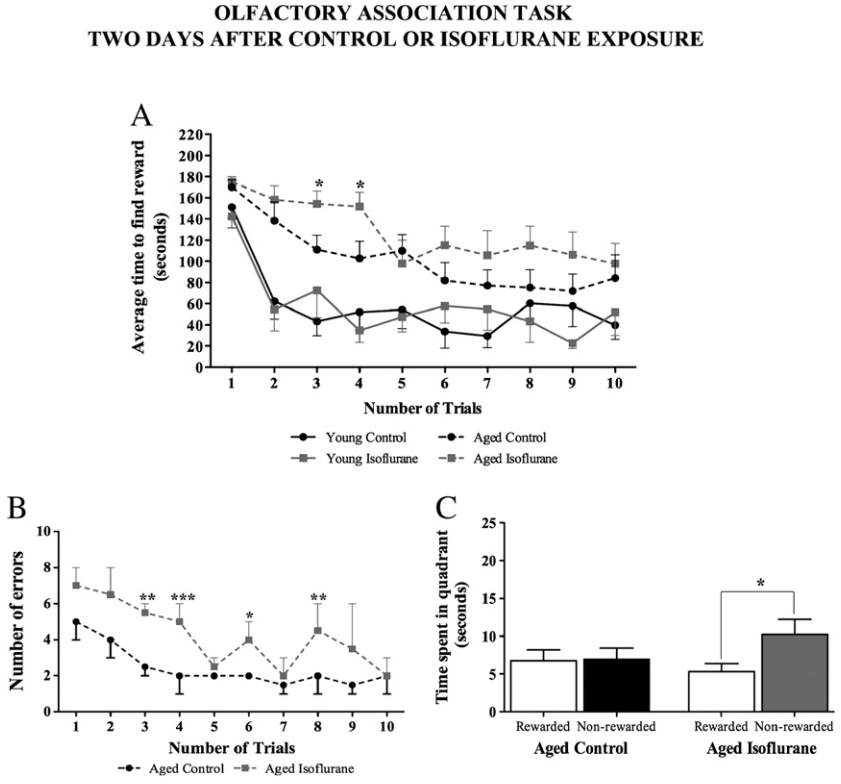

Fig. 6 – Isoflurane caused learning impairment in aged rats. This graph shows the performances, during the olfactory association task, of rats exposed to either isoflurane or room air (control). (A) Average time of young and aged rats to find reward after being placed in the cage. All rats acquired the behavioral task ($p<0.0001$), though aged rats not as rapid as young rats. Aged rats exposed to isoflurane took more time to find the reward than their respective controls ($p=0.0443$). Post-hoc analysis showed that aged rats exposed to isoflurane performed significantly worse in trials 3 and 4 than aged control rats (*$p<0.05$). (B) Error rates of aged rats before finding the reward during each trial. Aged rats exposed to isoflurane made significantly more errors than their respective controls ($p<0.0001$). Post-hoc analysis showed that aged rats exposed to isoflurane performed significantly worse in trials 3, 4, 6, and 8 (*$p<0.05$ **$p<0.01$ ***$p<0.001$). (C) Average time spent by aged rats in the rewarded vs. the non-rewarded quadrants during the behavioral task. Aged rats exposed to isoflurane spent significantly more time in the non-rewarded vs. the rewarded quadrant (*$p=0.0182$).

2.6. *Propofol Produced a Learning Impairment in Young Rats*

Learning was assessed two days after rats were infused with propofol or intralipid (control) (Fig. 7). Young control rats learned the task quickly ($p<0.0001$). However, young rats infused with propofol took significantly longer to find the reward, as compared to young controls ($F_{3,\ 40}=17.58$; $p=0.01$) (Fig. 7A). Young rats infused with propofol made significantly more errors than young controls ($F_{1,\ 12}=6.49$; $p=0.026$) (Fig. 7B), and spent more time in the non-rewarded vs. the rewarded quadrants ($F_{3,\ 296}=4.71$; $p=0.0038$) (Fig. 7C). Aged rats also acquired the task ($p<0.0001$), though not as rapid as young rats. Learning in the aged group exposed to propofol was not impaired when compared to the aged control group ($p=0.067$) (Fig. 7A).

3. Discussion

3.1. *Effect of Isoflurane and Propofol on New Cell Proliferation in the Brain*

Our study reveals three interesting results. First, as previously reported (Garcia et al., 2004; Lazarov et al., 2010; Luo et al., 2006; Seki and Arai, 1995), we confirmed a decrease in new cells proliferation in the DG of aged rats as compared to young rats. Similarly, we found a decrease in the number of BrdU+ cells in the OB indicating a decrease in new cell proliferation and/or migration to the OB of aged rats as compared to young rats.

Second, three hours of isoflurane or propofol anesthesia affected DG cell proliferation after exposure in young rats, while new cell proliferation in the DG of aged rats was unaffected, suggesting an age dependent susceptibility. Cell proliferation in the young DG, although occurring at the higher rate, seems more susceptible to propofol and isoflurane anesthesia than DG cell proliferation in the aged brain (Shruster et al., 2010). Similar studies by other groups have also shown an alteration of DG cell proliferation after isoflurane anesthesia in young animals (Sall et al., 2009; Stratmann et al., 2009a, 2009b; Zhu et al., 2010). There are several possible explanations for the observed decrease in the DG new cell proliferation induced by isoflurane or propofol. For example, it may be due to a decrease in the proliferative activity of progenitor cells through temporary cell cycle arrest, permanent cell cycle exit, or direct toxicity of isoflurane or propofol to the progenitor cells in the DG. Studies by other groups have found no alterations on new cell proliferation after isoflurane or propofol anesthesia

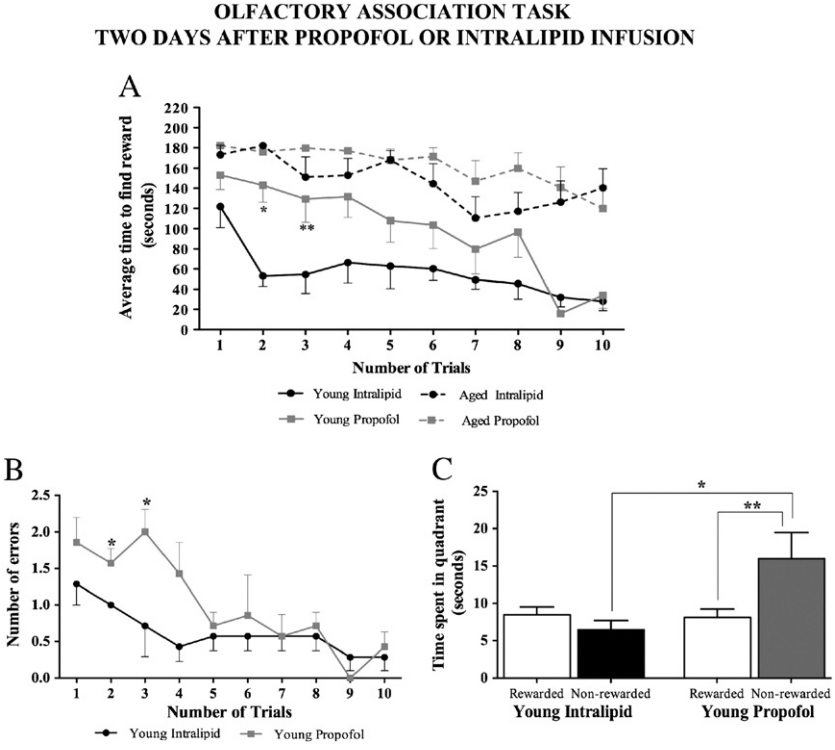

Fig. 7 – Propofol caused learning impairment in young rats. This graph shows the performances, during the olfactory association task, of rats infused with either propofol or intralipid. (A) Average time of young and aged rats to find reward after being placed in the cage. All rats acquired the behavioral task ($p<0.0001$), though aged rats not as rapid as young rats. Young rats infused with propofol took more time to find the reward than their respective controls ($p=0.0099$). Post-hoc analysis showed that young rats infused with propofol performed significantly worse in trials 2 and 3 (*$p<0.05$ **$p<0.01$) (B) Error rates of young rats before finding the reward during each trial. Young rats infused with propofol made significantly more errors than their respective controls ($p<0.0001$). Again, Post-hoc analysis showed that young rats infused with propofol performed significantly worse in trials 2 and 3 (*$p<0.05$). (C) Average time spent by young rats in the rewarded vs. the non-rewarded quadrants during the behavioral task. Young rats infused with propofol spent significantly more time in the non-rewarded quadrants as compared to the rewarded quadrant ($p=0.0167$). They also spent significantly more time in the non-rewarded quadrants as compared to young control rats (exposed to intralipid) (*$p=0.0038$).**

(Lasarzik et al., 2006; Tung et al., 2008). These studies used adult rats rather than young and aged rats, a different rat strain (Sprague Dawley), different BrdU doses (200 mg/Kg), and a different timing of the BrdU injection (before or during anesthesia). In our study, we chose to use the F344 strain because it is widely used in cognitive aging studies (Jennifer L. Bizon, 2009). We also used 3 h of anesthesia because it is the average time for common surgical procedures. We also chose to use 50 mg/kg of BrdU because it has been shown to reliably label the entire population of proliferative cells (Burns and Kuan, 2005) without unwanted toxic side effects (Sekerkova et al., 2004).

Third, we found that while isoflurane or propofol anesthesia resulted in a decrease in the number of Brdu+cells in the DG of young rats, the number of Brdu+cells in the in the granular and glomerular cell layer of the olfactory bulb (OB) was unaffected. In this study, we wanted to examine whether the anesthetic agent effect was confined to the DG or if there was a generalized effect on progenitor cell proliferation in the brain with anesthesia. Our results suggest that isoflurane and propofol have a specific effect on new cell proliferation in the DG, but not on the proliferation and/or migration of cells to the OB from the SVZ. A reduction in new cell proliferation in the DG, but not in the SVZ has been reported after low-dose irradiation of the heads of adult rodents (Mizumatsu et al., 2003; Snyder et al., 2005; Tada et al., 2000). These results and our results suggest that new cell proliferation in the DG may be more susceptible to disruption than new cell proliferation in the SVZ.

3.2. *Effect of Isoflurane and Propofol Anesthesia on Learning*

Learning impairment has been observed in aged rats (18–20 month old) following exposure to anesthesia using the 12-arm radial arm maze. When studying the effect of anesthesia on old memory retrieval, aged rats were impaired while adult rats improved their performance (Culley et al., 2003). On the other hand, when rats were tested for new memory formation 2 weeks after exposure to anesthesia, aged rats were impaired (Culley et al., 2004a) while adult rats (6 month old) showed no impairment (Crosby et al., 2005). In our study, aged rats in both the control and the isoflurane-exposed groups learned the task. Nevertheless, aged rats in the control group took less time to reach the reward and made fewer errors during the task than aged rats exposed to isoflurane.

Therefore, learning was impaired by the anesthetic. When looking at the effect of isoflurane on cognition of young rats under the same testing conditions, we found that learning was unaffected by isoflurane exposure. Our study, supports previous findings of cognitive impairment after isoflurane anesthesia (Culley et al., 2003, 2004a, 2004b).

Propofol is widely used among pediatric and geriatric patients. However, there are few studies on its effect on cognitive function in an animal model. In one study, propofol anesthesia in aged rats (18 month old) did not affect spatial memory assessed by the 12-arm-radial maze (Lee et al., 2008). Similarly, in our study, propofol anesthesia had no effect on learning in aged rats using the olfactory association task. However, we found that, similar to isoflurane, propofol had an age dependent effect, though opposite to that found with isoflurane. Propofol produced learning impairment in young rats.

In summary, we found that isoflurane and propofol anesthesia produced learning impairments as assessed by our olfactory behavioral paradigm that are age, and agent dependent. The effect on progenitor cell proliferation was age dependent and seems to be specific to the DG. Furthermore, propofol in young animals impaired learning and decreased progenitor cell proliferation.

In summary, we found a learning impairment on young rats exposed to propofol and a decreased in new cell proliferation in DG of these animals, we also found a learning impairment in aged rats exposed to isoflurane, but no decrease in new cell proliferation in these animals. New cell proliferation is only the first step of postnatal neurogenesis, which is a process that includes proliferation, fate specification of adult neural stem cells, cell migration, axonal and dendritic development, and synaptic integration of newborn neurons (Duan et al., 2008; Ming and Song, 2005). Therefore, new cell proliferation may not be the most relevant measurement of the animal's learning capacity since these newly formed cells are unlikely to be directly involved in learning and memory at the 2 day time point at which we tested these rats. Our results suggest that the ability to learn is not correlated with concurrent new cell proliferation. Therefore, the effect of anesthesia on cognitive function is likely related to the maturation process of newly formed cells in the dentate gyrus, and more detailed studies on the effect of these two anesthetic agents on the entire process of neurogenesis are necessary.

4. Experimental Procedure

4.1. *Animals*

All experiments were conducted in accordance with the National Institute of Health Guide and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use committee of the University of South Florida, College of Medicine, Tampa, Fl. To evaluate the effects isoflurane and propofol on the developing and aging brain, rats of two different ages were used for this study: 4-month old (young) and 21-month old (aged) male Fischer-344 rats (Jennifer L. Bizon, 2009) (Harlan Sprague Dawley, Indianapolis, IN). All rats were acclimated and handled for two weeks prior to the beginning of the experimental procedures.

4.2. *Isoflurane Anesthesia*

One cohort of rats was randomly assigned to four different groups (n=12 per group): young rats exposed to 1.5% isoflurane delivered in 2 L/min O_2 via nose cone for 3 h, young rats exposed to a mixture of air and 2 L/min O_2 in the cage for 3 hours, aged rats exposed to 1.5% isoflurane delivered in 2 L/min O_2 via nose cone for 3 h, and aged rats exposed to a mixture of air and 2 L/min O_2 in the cage for 3 hours. Isoflurane (Forane, Ohmeda Caribe, NJ, USA) was delivered via a standard anesthesia vaporizer.

4.3. *Propofol Anesthesia*

A separate cohort of rats was randomly assigned to four different groups breathing air (n=10 per group): young rats that

received 35 mg/kg/hr of propofol or 10% intralipid (control) for 3 hrs, and aged rats that received 35 mg/kg/hr of propofol or 10% intralipid for 3 h. Unanesthetized rats were placed in a restrainer and a tail vein catheter was implanted and attached to a syringe pump (model 11 Plus; Harvard Apparatus). Propofol (Diprivan; AstraZeneca) or 10% intralipid (Fresenius Kabi; Sweden), which served as control, was continuously administered for 3 h. Intralipid is the commercial formulation for the solvent of propofol (Astrazeneca, 2011).

4.4. *Physiological Measurements*

Body temperature, oxygen saturation and heart rate were measured in anesthetized rats throughout the period of isoflurane or propofol anesthesia. The body temperature of these rats was maintained at 37 °C with a thermostatically controlled heating pad. Similar physiological measurements were not made on control animals.

4.5. *BrdU Injections*

To evaluate the effects of isoflurane and propofol on new cell proliferation in the brain, the thymidine analog 5-Bromo-2-deoxyuridine (BrdU; Sigma-Aldrich Munich, Germany, 5 mg/mL dissolved in 0.9% saline) was prepared fresh before use and injected intraperitoneally (50 mg/kg) (Burns and Kuan, 2005) to all groups immediately following the anesthesia or control procedure and 18 hours after the first injection.

4.6. *Cognitive assessment*

Cognitive function, as defined by learning a new task, was assessed using an odor association paradigm that required the rats to make odor-reward associations using foraging for palatable food, resulting in rapid acquisition with minimal stress and fear (Eichenbaum, 1998). Rats performed the odor discrimination task two days after exposure to either isoflurane or propofol anesthesia. Rats were initially habituated to a 97 cm^2 black Plexiglas box, shaped to receive treats in the box, and food deprived the night prior to behavioral testing. Two odors were presented in adjacent corners of the box. A treat was placed next to one odor while the second odor was not paired to a reward (Fortin et al., 2002). The rat was placed in the middle of the box facing away from the odors and reward; then allowed to explore the novel odors and find the reward. The position of the odor/reward pair was randomized between trials. Each rat received ten learning trials (180 seconds cut off) to identify the odor paired to the reward and find the reward. An Etho Vision computerized tracking and movement analysis system (Noldus Information Technology) was used to collect animal trajectories as time spent to find reward, number of error made and time spent in the rewarded vs. the non-rewarded quadrant. A blinded individual performed the behavioral experiment, and all equipment used for behavioral testing was wiped with 70% ethanol between animal uses to eliminate olfactory trails.

4.7. *Tissue Preparation and BrdU Immunohistochemistry*

One week after anesthesia, rats were deeply anesthetized with pentobarbital (50 mg/kg, i.p.) and transcardially perfused with 100 ml saline. The brain and olfactory bulbs (OB) were removed, and immersion fixed in 4% paraformaldehyde for 5 days, and then placed in 20% sucrose. Frozen sagittal sections (30 μm) through the entire DG and OB were collected serially. Sections were pretreated with 50% formamide/2X Saline-Sodium Citrate (SSC) at 65 °C for 2 h, rinsed in 2X (SSC), incubated in 2 N HCL for 30 min at 37 °C, and then washed with borate buffer (pH 8.5), followed by phosphate buffered saline (PBS). Subsequently, endogenous peroxidase activity was quenched with 3% H_2O_2 solution in PBS. Sections were blocked for 1 hour in 3% normal horse serum and 0.25% Triton X-100 in PBS (PBS-TS). Sections were incubated overnight with mouse-anti-rat-BrdU (1:100; Roche) in PBS-TS. The following day, sections were washed in PBS, incubated for one hour in biotinylated secondary antibody (horse anti-mouse IgG rat adsorbed 1:200; Vector Laboratories, Burlingame, CA) in PBS-TS, and washed in PBS before incubation for 1 hour in avidin-biotin substrate (ABC kit, Vector Laboratories, Burlingame, CA). Sections were washed in PBS for 10 minutes and reacted with 3,3´- diaminobenzidine tetrahydrochloride (DAB) solution (Thermo Scientific, Rockford, IL). Sections were then mounted onto glass slides, dehydrated, and coverslipped with mounting medium (Cytoseal 60,Stephens Scientific, Riverdale) (Bachstetter et al., 2010).

4.8. *Microscopy*

Quantification of BrdU+cells was performed using an Olympus IX71 microscope, with a 40X objective (1330X final magnification) by an individual blinded to the treatment of the animals. A modified unbiased stereological method was used to estimate the number of BrdU+cells in the subgranular zone of the DG and the granular and glomerular layers of the OB. Eleven to thirteen equally spaced sections throughout the medial-lateral extent of the DG were collected per animal. Similarly, five to six equally spaced sections were collected throughout the medial-lateral extend of the OB. Because BrdU+cells are a rare event, all BrdU+cells in the DG and OB were counted in each of the collected sections per animal. The number of BrdU+cells in each DG and OB examined was summed for individual animals and the sum from each animal was then multiplied by the section spacing to estimate the total number of BrdU+cells These data were then used to calculate group means for estimates of total BrdU+cells in the DG and OB (Mouton, 2002). Labeled cells that were greater than 1 cell diameter away from the subgranular zone were not included in the count. Only cells that had a clearly defined nuclear outline, with speckled or solid labeling over the nucleus, were considered BrdU+(Fig. 1).

4.9. *Statistical Analysis*

The number of BrdU+cells in the DG and OB was analyzed using a two-way analysis of variance (ANOVA). For behavioral data, the average time to find the reward and the number of errors were each analyzed using a two-way ANOVA for repeated measures. The time spent in the rewarded vs. non-rewarded quadrant was analyzed using a one-way ANOVA. Bonferroni post-hoc analysis was used following all statistically significant ANOVA results. All results are presented as the mean±SEM.

Statistical comparison of the data was performed using GraphPad Prism version 5.00 for Mac (GraphPad Software, San Diego California USA, www.graphpad.com).

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