



Comparison of Neurodegeneration and Cognitive Impairment in Neonatal Mice Exposed to Propofol or Isoflurane

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Abstract

Background: While previous studies have demonstrated neuronal apoptosis and associated cognitive impairment after isoflurane or propofol exposure in neonatal rodents, the effects of these two anesthetics have not been directly compared. Here, we compare and contrast the effectiveness of isoflurane and propofol to cause neurodegeneration in the developing brain and associated cognitive dysfunction.

Methods: Seven-day-old mice were used. Mice in the isoflurane treatment group received 6 h of 1.5% isoflurane, while mice in propofol treatment group received one peritoneal injection (150 mg/kg), which produced persistent anesthesia with loss of righting for at least 6 h. Mice in control groups received carrying gas or a peritoneal injection of vehicle (intralipid). At 6 h after anesthetic treatment, a subset of each group was sacrificed and examined for evidence of neurodegeneration, using plasma levels of S100 β , and apoptosis using caspase-3 immunohistochemistry in the cerebral cortex and hippocampus and Western blot assays of the cortex. In addition, biomarkers for inflammation (interleukin-1, interleukin-6, and tumor necrosis factor alpha) were examined with Western blot analyses of the cortex. In another subset of mice, learning and memory were assessed 32 days after the anesthetic exposures using the Morris water maze.

Results: Isoflurane significantly increased plasma S100 β levels compared to controls and propofol. Both isoflurane and propofol significantly increased caspase-3 levels in the cortex and hippocampus, though isoflurane was significantly more potent than propofol. However, there were no significant differences in the inflammatory biomarkers in the cortex or in subsequent learning and memory between the experimental groups.

Conclusion: Both isoflurane and propofol caused significant apoptosis in the mouse developing brain, with isoflurane being more potent. Isoflurane significantly increased levels of the plasma neurodegenerative biomarker, S100 β . However, these neurodegenerative effects of isoflurane and propofol in the developing brain were not associated with effects on inflammation or with cognitive dysfunction in later life.

Citation: Yang B, Liang G, Khojasteh S, Wu Z, Yang W, et al. (2014) Comparison of Neurodegeneration and Cognitive Impairment in Neonatal Mice Exposed to Propofol or Isoflurane. PLoS ONE 9(6): e99171. doi:10.1371/journal.pone.0099171

Editor: Daqing Ma, Imperial College London, Chelsea & Westminster Hospital, United Kingdom

Received: March 16, 2014; **Accepted:** May 12, 2014; **Published:** June 16, 2014

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data are included within the manuscript.

Funding: This work was supported by the National Institute of General Medical Science, National Institutes of Health, Baltimore, Maryland, K08 grant (1-K08-GM-073224, to HW) and R01 grant (1-R01GM084979-01, 3R01GM084979-02S1 to HW); March of Dimes Birth Defects Foundation Research Grant (12-FY05-62, to HW), White Plains, New York; and Department of Anesthesiology and Critical Care research funds, Perelman School of Medicine at the University of Pennsylvania. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Studies using a variety of animals ranging from rodents to rhesus monkeys have shown increased neuroapoptosis during postnatal brain development after exposure to intravenous or inhaled anesthetic agents [1–9]. General anesthetic (GA)-mediated apoptosis in the developing brain is correlated with an elevation of plasma S100 β , a neurodegenerative biomarker in blood [10,11].

Furthermore, some studies suggested that the anesthetic-mediated apoptosis in the developing brain may be associated with persistent learning deficits and social behavior dysfunction [1,2,12], although such an association could not be confirmed by other studies [10,13]. Most importantly, multiple exposures to general anesthetics in children under the age of 4 may be related to learning disabilities, including reading, language and math [14]. The mechanisms of general anesthetic-mediated apoptosis in the

developing brain are still not clear, although many hypotheses have been proposed, including the disruption of intracellular calcium homeostasis [13,15–26], activation of gamma-aminobutyric acid receptors and inhibition of N-methyl-D-aspartate receptors and associated impairment of synaptogenesis [1,25–29], activation of P75 neurotrophin receptors [5,30], regulation of cell cycle [31], and others [32,33]. There is increasing evidence suggesting that GAs may cause apoptosis and cognitive dysfunction by aggravating neuroinflammation [12,34–37], although surgery itself may cause cognitive dysfunction via increased inflammation [38–41].

General anesthetics, however, may not all affect neuroapoptosis in the developing brain or subsequent cognitive function with the same potency. This has important clinical implications for our pediatric patients [42,43]. Among the commonly used inhalational general anesthetics, isoflurane is the most widely reported to induce neurodegeneration in the developing brain and subsequent cognitive dysfunction in several animal models [1,2,13,28,30,44,45]. Similarly, the intravenous general anesthetic, propofol, at clinically relevant concentrations and durations, has also been shown to cause significant apoptosis in the developing brain [4,5,46,47], and associated cognitive dysfunction [7]. While comparisons of different inhaled anesthetics to induce neuroapoptosis in the developing brain and subsequent cognitive dysfunction have been undertaken [10,12,48,49], such a comparison has not been carried out between an inhalational anesthetic and propofol. Because propofol is the most commonly used induction agent for general anesthesia and isoflurane is the most widely studied inhalational general anesthetic, this study compares the potency of propofol and isoflurane to cause neurodegeneration in the developing rodent brain and associated changes in cognitive function.

Materials and Methods

Animals

The Institutional Animal Care and Use Committee at the University of Pennsylvania approved all experimental procedures and protocols used in this study. All efforts were made to minimize the number of animals used and their suffering. All C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were housed in a University of Pennsylvania animal facility in polypropylene cages and the room temperature was maintained at 22°C, with a 12-hour light-dark cycle. Mice had continuous access to water and food. Both male and female mice were used in the experimental and control aspects of this study.

Anesthesia exposure

For the isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoroethane) exposure studies, postnatal day 7 (P7) mice ($n = 22$) were placed in plexiglass chambers, resting in a water bath to maintain a constant environmental temperature of $37 \pm 0.5^\circ\text{C}$, and exposed to 1.5% isoflurane in humidified 30% oxygen/70% nitrogen for 6 h. Five liters of total gas flow were used to ensure a steady state of anesthetic gas and prevent accumulation of expired carbon dioxide within the chamber. Isoflurane, oxygen and carbon dioxide were monitored and maintained using IR absorbance (Ohmeda 5330, Detex-Ohmeda, Louisville, CO) as described in our previous study. Mice in the control group ($n = 18$) received humidified 30% oxygen balanced with nitrogen only for 6 h at room temperature. All animals were monitored and stimulated every 30 minutes to ensure reactivity. They were assessed constantly by observation to ensure the adequate spontaneous breathing same as in our previous study [10]. We did not monitor

blood gases in these treatments as previous study has demonstrated that 1.5% isoflurane for 2 hr did not affect arterial blood gas significantly [50]. 12 male and 10 female mice were used for isoflurane treatment group, while 10 male and 8 female were used for corresponding controls.

For propofol (2,6 diisopropylphenol) anesthesia, mice ($n = 20$) received one peritoneal injection of propofol in intralipid (150 mg/kg) and placed in the same anesthetic chambers as above with humidified 30% oxygen at 38°C . Mice in the vehicle control group ($n = 19$) received a peritoneal injection of intralipid (MP Biomedicals, Solon, OH) and placed in the same anesthetic chambers as above with humidified 30% oxygen at room temperature. The concentration of propofol (150 mg/kg) was determined in a pilot study to be sufficient for loss of righting for at least 6 h in P7 mice. Actually, all mice receiving one dose of propofol injection (150 mg/kg, IP) in the formal study maintained loss of righting for 6 hrs and most mice usually recovered the righting or woke up around 8 hr after propofol injection. This dose has also been determined to be the ED_{50} for propofol to maintain an adequate surgical plane of anesthesia, although up to 300 mg/kg, IP has been used for the study of propofol neurotoxicity in infant mouse [46]. Similarly, 1.5% isoflurane is about ED_{50} for P7 mice. The rectal temperature was periodically checked to ensure maintenance of body temperature at $37 \pm 0.5^\circ\text{C}$ using a thermometer (Fisher Scientific, Pittsburgh, PA). All mice in the propofol treatment group survived and 2 mice from isoflurane treatment group died. Compared with the control or vehicle mice, the anesthetized mice did not show significant changes in other behaviors after recovery (e.g., eating, drinking and body weight etc.). 11 male and 9 female mice were used for propofol treatment group, while 9 male and 10 female were used for vehicle controls.

A subset of animals from each experimental group was sacrificed 2 h after the anesthetic exposures for blood collection and biochemical assays. The remaining 54 mice were allowed to mature and underwent behavioral testing 32 days post-exposure, which are the required minimum age for mice to perform adequate Morris Water Maze (MWM) tests.

Measurement of plasma S100 β by ELISA

Two hours after the anesthetic exposures, P7 animals were deeply anesthetized with 3% isoflurane for less than 1 min and blood was collected from the left ventricle at the time of sacrifice. S100 β levels in the plasma were determined using Sangtec 100 ELISA kits (DiaSorin Inc, Stillwater, MN) following the manufacturer's protocol as we described previously [10,11]. Briefly, 0.1 ml of blood was centrifuged at 1500 RPM for 10 min to get the plasma. Plasma from each animal (50 μl) was mixed with 150 μl of tracer from the ELISA kit, incubated for 2 h, followed by 3,3',5,5' tetramethylbenzidine substrate and stop solution. The optical density was read at 450 nm and the concentrations of the samples were measured using a standard curve.

Brain tissue harvest

Mice were anesthetized briefly with inhalation of 2–3% isoflurane. The brains were harvested after the blood collection above from the P7 mice, and at the time of sacrifice after the behavioral testing, by intracardiac perfusion through the left ventricle and simultaneous exsanguination through the right atrium with ice-cold phosphate buffered saline (pH 7.4). The left cerebral cortex of each brain was dissected and immediately frozen in liquid nitrogen and stored at -80°C for Western blot analyses. The entire right hemisphere was post-fixed overnight in 4% paraformaldehyde in phosphate buffered saline, embedded in paraffin and serially sectioned (10 μm).

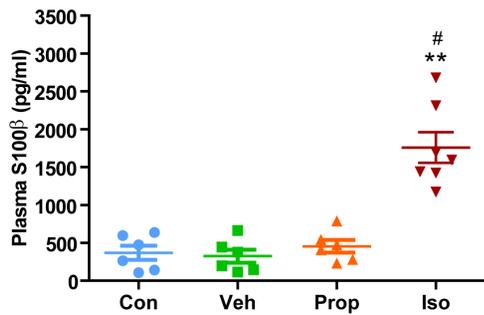


Figure 1. Isoflurane, but not propofol, significantly increases plasma S100 β level in P7 mice. Quantitation of ELISAs from the P7 cerebral cortex after 6 h exposures to isoflurane (n = 6), propofol (n = 6), 30% oxygen (control, n = 6), or intralipid (vehicle, n = 7) revealed that S100 β levels were only significantly increased after exposure to isoflurane. Data are expressed as mean \pm SD and were analyzed by one-way ANOVA followed by the student-Newman-Keuls post hoc test. **P < 0.01 compared to control, #P < 0.05 compared to propofol. Con (control), Veh (vehicle), Prop (propofol), Iso (isoflurane). doi:10.1371/journal.pone.0099171.g001

Western blot

As in our previous studies [10,13], frozen cortical brain tissue was homogenized on ice using immunoprecipitation buffer (10 mM Tris-HCl, Ph 7.4, 150 mM NaCl, 2 mM EDTA, AND 0.5% Nonidet P-40) plus protease inhibitors (1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 μ g/ml pepstatin A). The lysates were collected, centrifuged at 12,000 rpm for 10 min, and quantified for protein concentration with BCA protein assay kit (Pierce Biotechnology, Rockford, IL). To determine apoptosis in the brain after anesthetic exposures, caspase-3 levels were examined. Briefly, proteins from the P7 mouse cerebral cortex were separated by 12% gel electrophoresis and were transferred to a nitrocellulose membrane. The blots were incubated with a monoclonal antibody against cleaved caspase-3 (1:1000 dilution; Cell signaling technology, Boston, MA), then probed with horseradish peroxidase-conjugated secondary antibody. To study anesthesia-mediated neuroinflammation, we examined the protein levels of interleukin-1 (IL-1 β), interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF- α) by using antibodies to IL-1 β (1:1,000 dilution; Abcam, Cambridge, MA), IL-6 (1:1,000 dilution; Abcam, Cambridge, MA) and TNF- α (1:1,000 dilution; Abcam, Cambridge, MA). Detection was performed using the ECL-PLUS system and images were analyzed. Beta actin protein was used as a loading control. The band densities were measured with a GS-800 Densitometer (BIO-RAD, Hercules, CA) and Quantity One software (BIO-RAD version 4.5.0) and averaged for 4 replicates for each animal.

Immunohistochemistry in brain sections

As we described previously [10,13,51,52], brain sections were deparaffinized, rehydrated with graded alcohols, and washed in distilled water. The sections were incubated with anti-rabbit cleaved caspase-3 primary antibody (1/200, Cell Signaling Technology, Inc Danvers, MA, U.S.A) overnight and the next day incubated with secondary biotinylated goat anti-rabbit antibody (1/200, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 40 minutes, followed by incubation with the avidin-biotinylated peroxidase complex (Vectostain ABC-Kit, Vector Lab, Burlingame, CA) for 40 minutes. Negative control sections were incubated in blocking solution without primary antibody. Images were acquired and assessed at 20 \times using IP lab 7.0 software on an Olympus IX70 microscope (Olympus corporation,

Tokyo, Japan) equipped with a Cooke SensiCam camera (Cooke Corp., Romulus, MI). Three brain tissue sections corresponding to the Atlas of the Developing Mouse Brain at P6 Figure 131–133 [53] were chosen from each animal and analyzed for caspase-3 positive cells in the frontal cortex and hippocampal CA1 regions. Two investigators, blinded to the conditions, counted the number of caspase-3 positive cells using IPLab Suite v3.7 imaging processing and analysis software (Biovision Technologies, Exton, PA). The number of caspase-3 positive cells per mm² were quantified for each region. The numbers of animals examined in each group were as follows: isoflurane exposed (n = 6), controls (n = 6), propofol exposed (n = 6) and vehicle controls (n = 6).

Morris water maze

Learning and memory testing was conducted 32 days after the anesthetic exposures at P34 using the Morris Water Maze (MWM), as we described previously [10,13,51]. Briefly, the mice were first trained to escape from the pool (four 60 s trials per day for 5 days). A round plexiglass pool, 150 cm in diameter and 60 cm in height, was filled with water to a height of 1.5 cm above the top of the movable clear 15 cm diameter platform, which was flagged. The pool was covered with a white curtains, water was kept at a temperature between 26°C and 29°C with a pond heater and opacified with titanium dioxide. A video tracking system recorded the time for each animal to reach the platform and the data were analyzed using motion-detection software for the MWM (Actimetrics software, Evanston, IL). After every trial, each mouse was placed in a holding cage, under an infrared heat lamp, before returning to its home cage. The cued trials were used to determine any non-cognitive performance impairments (e.g. visual impairments, swimming difficulties).

For the reference memory trials (place trials), all mice received 4 trials per day for 5 days. The curtain was removed from around the pool to reveal numerous visual cues in the room and the submerged platform was hidden. For each trial, mice were placed in the pool at fixed starting points and allowed to search for the platform for up to 60 s. If a mouse did not find the platform within 60 s, it was gently guided to the platform and allowed to remain there for 30 s. Mice that found the platform also remained on it for 30 s before removal from the pool. The escape latency (time for each mouse to reach the hidden platform) was recorded. Spatial learning ability was reflected by the escape latency; the less time it took to reach the platform, the better spatial learning ability. The mice received two blocks of trials (two trials per block with 30 s apart, 60 s maximum for each trial, with 2 h rest between blocks) each day for 5 days.

Immediately after the place trials and 24 h later, probe trials were conducted to evaluate memory retention. The platform was removed from the pool and the mouse was placed in the opposite quadrant. Each mouse was allowed to swim 60 s and the time spent in each quadrant and the swim speed were recorded and analyzed. The data are expressed as the percent time spent in each of the four quadrants.

Statistical analysis

ELISAs, western blots and immunohistochemical assays were analyzed using one-way ANOVA followed by post hoc student-Newman-Keuls test. Behavioral studies were analyzed with two-way ANOVA with repeated measures followed by the Bonferroni Multiple Comparison Test using pair-wise comparisons. There were no missing data for the variables during the data analyses. Values of P < 0.05 were considered statistically significant. GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA) was used for all statistical analysis and graph generation.

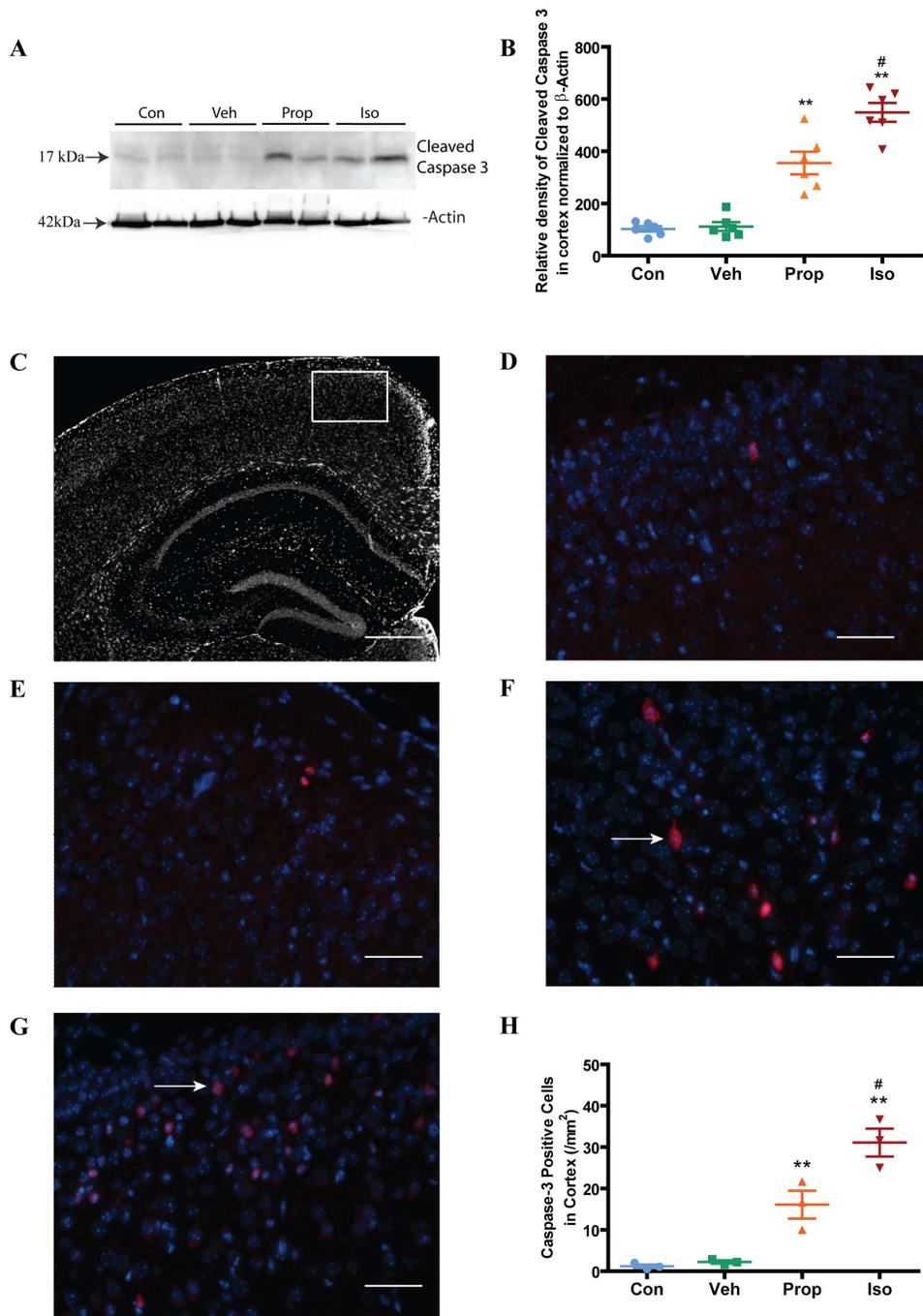


Figure 2. Isoflurane induced significantly greater apoptosis than propofol in cortex of P7 mice. (A) Representative western blot from cortex after exposures to isoflurane or propofol in P7 mice. (B) Quantification of the Western blots showed that though both isoflurane and propofol exposures significantly increased apoptosis in the cortex, isoflurane was more potent. (C) Image of the area sampled in the cortex (white rectangle) for cleaved-caspase-3 positive cells. Scale bar = 600 μ m. (D and E) Examples of cortical images from control and vehicle groups, respectively. Scale bar = 50 μ m. (F and G) Examples of caspase-3 positive cells (red stained, white arrows) in the cortex of P7 mice exposed to isoflurane and propofol, respectively. Scale bar = 50 μ m. (H) Quantitation of the number of caspase-3 positive cells per area from the various experimental groups showed that both isoflurane and propofol significantly increased apoptosis in the cortex and that isoflurane had a significantly greater effect than propofol. All Western blot assay data are expressed as mean \pm SD (n = 6 in each group, 4 replicates per animal) and were analyzed by one-way ANOVA followed by Newman-Keuls post hoc test. The immunohistochemical data are expressed as number per area and analyzed by one-way ANOVA followed by Newman-Keuls post hoc test, mean \pm SE (n = 6 in each group, 3 sections per animal). ** P < 0.01 compared to control or vehicle respectively, # P < 0.05 compared to the propofol group. Con (control), Veh (vehicle), Prop (propofol), Iso (isoflurane). doi:10.1371/journal.pone.0099171.g002

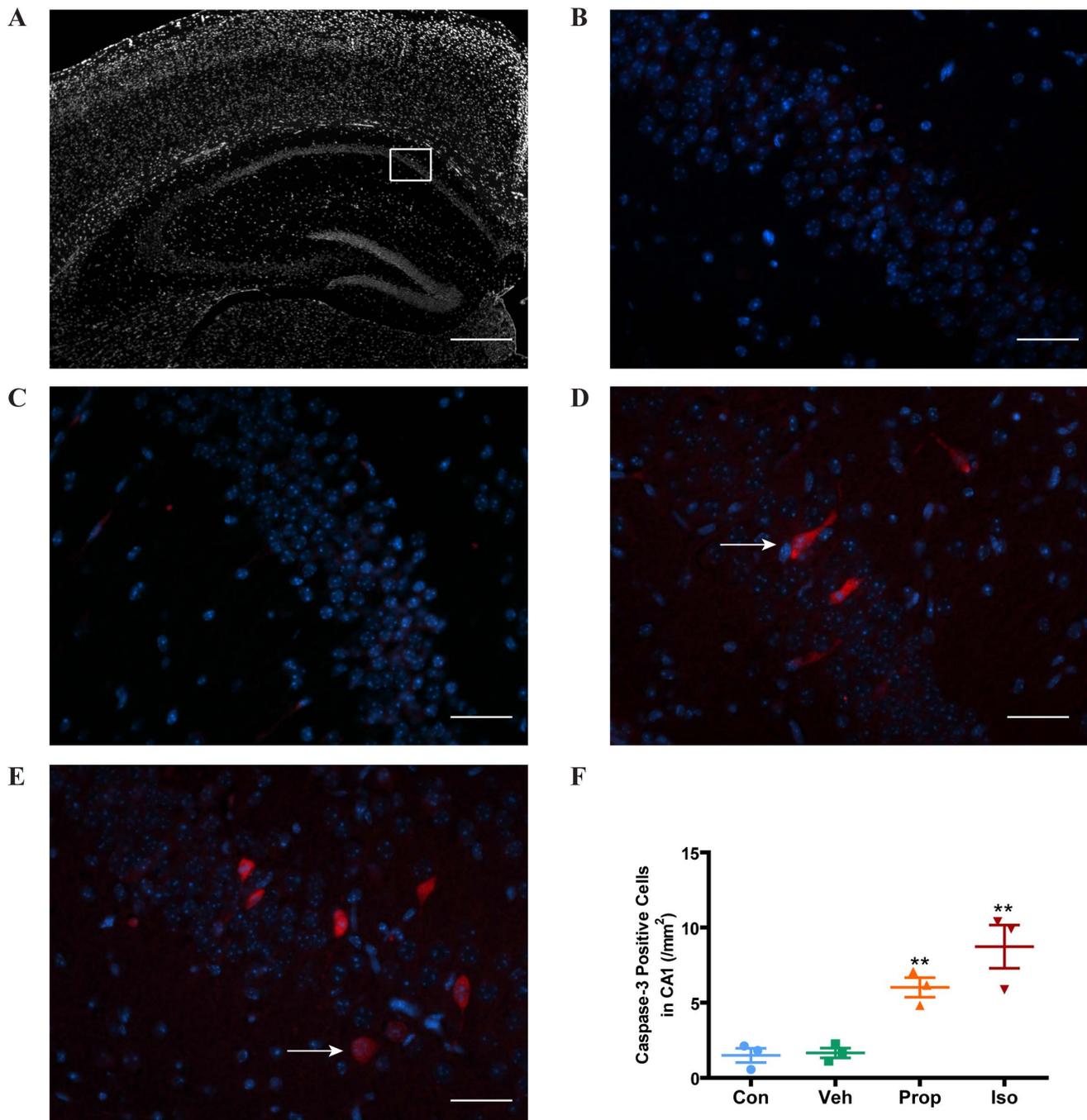


Figure 3. Isoflurane and propofol induced apoptosis in the hippocampal CA1 region of P7 mice. (A) Area of the hippocampal CA1 region sampled is demonstrated with the white rectangle. Scale bar = 600 μ m. (B and C). Representative images of the hippocampal CA1 region from control and vehicle control groups, respectively. Scale bar = 50 μ m. (D and E). Examples of caspase-3 positive cells (red stained, white arrows) are demonstrated in the hippocampal CA1 region after isoflurane or propofol treatments, respectively. Scale bar = 50 μ m. (F) Quantitation of the caspase-3 positive cells per mm^2 from the experimental groups showed that both isoflurane and propofol significantly increased apoptosis in the hippocampus compared to the control groups. Data are expressed as mean \pm SE (n = 6 in each group, 3 sections per animal) and were analyzed by one-way ANOVA followed by Newman-Keuls post hoc test. ** P < 0.01 compared to control or vehicle respectively. doi:10.1371/journal.pone.0099171.g003

Animal numbers in each experimental group are listed in the figure legends. Biochemical assay data were expressed as mean \pm SD and behavioral data were expressed as mean \pm SEM.

Results

Isoflurane but not propofol significantly increased plasma S100 β

S100 β has been shown to be a useful biomarker for the detection of anesthetic-mediated neurodegeneration [10,11]. As

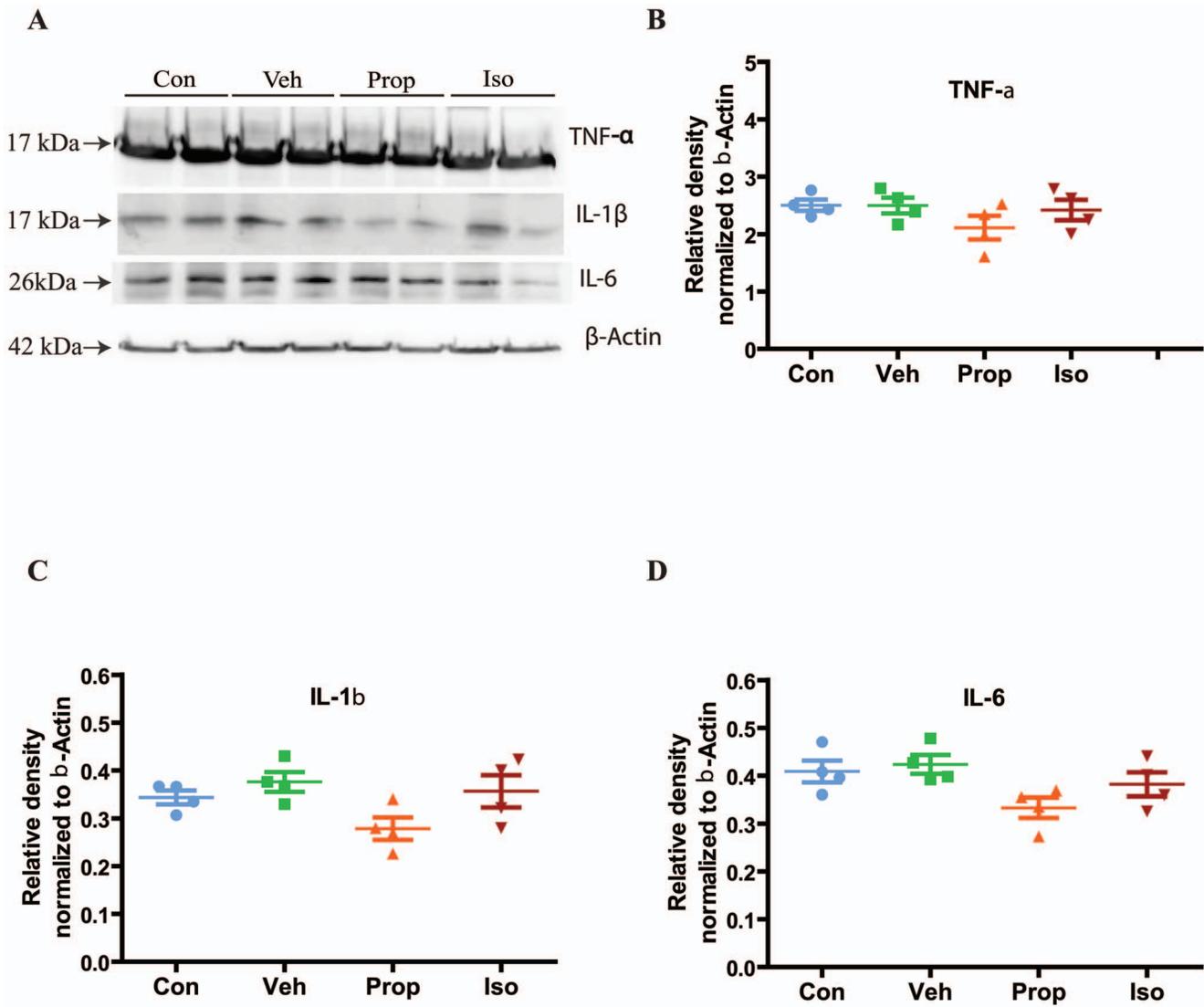


Figure 4. Effects of isoflurane and propofol on inflammation. (A) A representative Western blot of TNF- α , IL-6 and IL-1 β levels in the P7 cortex immediately after anesthetic exposures. IL = interleukin; TNF = tumor necrosis factor. (B–D) Quantification of the western blot data normalized to β -actin for TNF- α , IL-6 and IL-1 β levels respectively in the mouse cortex at P7. Data are expressed as mean+SD ($n = 6$ in each group, 4 replicate blots per animal) and were analyzed by one-way ANOVA followed by Newman-Keuls post hoc test. doi:10.1371/journal.pone.0099171.g004

demonstrated in Fig. 1, exposure to 1.5% isoflurane for 6 h in P7 mice significantly increased plasma S100 β levels compared to controls, while exposure to propofol for 6 h did not increase plasma S100 β levels significantly.

Isoflurane induced greater apoptosis than propofol in the developing brain

Caspase-3 is cleaved during the process of apoptosis and the cleaved caspase-3 is a well accepted biomarker for cell death by apoptosis. We investigated the effects of exposure to isoflurane or propofol on cleaved caspase-3 in the cortex and hippocampus in 7 day-old mice. We found that while both isoflurane and propofol significantly increased cleaved caspase-3 levels in the cortex determined by Western blot (Fig. 2 A and B) and immunohistochemistry (Fig. 2 C–G), isoflurane was more potent than propofol. Similarly, immunohistochemical analysis of the hippocampal CA1

region (Fig. 3) showed that both isoflurane and propofol significantly increased cleaved caspase-3.

Effects of isoflurane and propofol on inflammation

Given that recent studies have suggested that isoflurane may induce neuroinflammation which may be associated with cognitive impairment [12,35,48], we examined with Western blot assays (Fig. 4A) the effects of isoflurane and propofol on the pro-inflammatory cytokines, TNF- α (Fig. 4B), IL-1 β (Fig. 4C) and IL-6 (Fig. 4D), in the cortex at P7. Neither isoflurane nor propofol significantly changed these markers of inflammation.

Effects of isoflurane and propofol on cognitive function

The Morris Water Maze test was used to evaluate potential learning and memory deficits after exposure to isoflurane and propofol in mice 32 days after anesthesia exposure at P7. The cued and reference memory trials demonstrated no significant physical

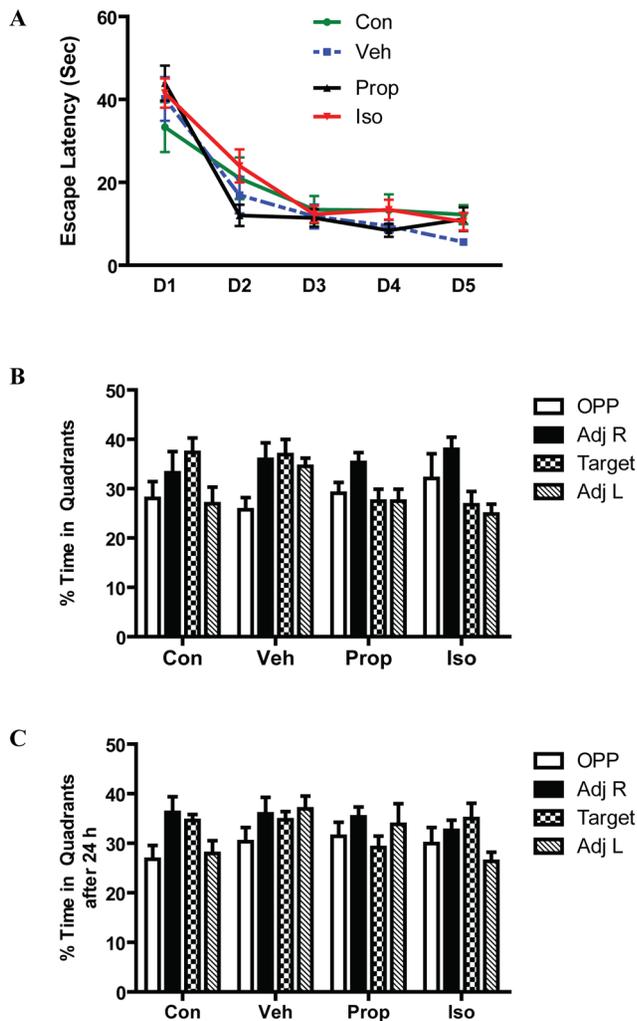


Figure 5. Effects of isoflurane and propofol on cognitive function. (A) The mean escape latency over 5 consecutive days (D) of place trials showed no significant difference in all groups. Probe trials were conducted to measure memory retention. The percent time spent in each quadrant was determined immediately (B) or 24 hr (C) after the last place trial. Data were analyzed by two-way ANOVA with repeated measures. All data represent mean \pm SEM. Con, Control (n=12), Veh, vehicle (n=12), Prop, propofol (n=14), Iso, isoflurane (n=16). doi:10.1371/journal.pone.0099171.g005

impairments in four groups (data not shown). The escape latency in place trials, shown in Fig. 5A, indicated that the average time to reach the submerged platform did not differ significantly after exposure to isoflurane or propofol. Probe trials demonstrated no statistical difference in retention memory either immediately (Fig. 5B) after the place trials or 24 h later (Fig. 5C). In addition, there were no significant differences in swim speed during the probe trials (data not shown).

Discussion

This study showed that even though isoflurane and propofol significantly increased neuroapoptosis in the rodent developing brain, this was not associated with immediate changes in neuroinflammation or subsequent cognitive dysfunction.

Commonly used general anesthetics have been reported to cause apoptosis in the developing brain and subsequent cognitive

dysfunction [1,2,48,49]. One strategy to minimize these possible detrimental effects of GAs is to use a less neurotoxic GA in the more vulnerable developing brain. Many studies have compared the potency of isoflurane, sevoflurane and desflurane to cause neurodegeneration in the brain, with controversial results [10,12,48,49,54,55]. Some studies demonstrated a higher potency of isoflurane to cause neuroapoptosis compared to sevoflurane [10] or desflurane [48], while others could not confirm these findings [49,54]. The variation may be caused by different ways to determine the minimal alveolar concentration (MAC), either with [49,54] or without [10,12,48] a stimulus to pinch the tail during anesthesia exposure, as well as different animal species and experimental protocols [10,12,48,49,54,55]. Compared to inhalational anesthetics, fewer studies have examined the neurotoxicity of the commonly used intravenous anesthetic, propofol. Recent studies suggest that propofol, like inhalational anesthetics, also induced apoptosis of neurons and oligodendrocytes during brain development in rhesus macaques [4], rats [7,47] and mice [46], and are associated with cognitive dysfunction, especially after repeated exposures [7]. The mechanism of propofol-mediated apoptosis in the developing brain is still unclear, but it is likely to share common mechanisms with inhalational general anesthetics, such as GABA receptor activation [25], P75 neurotrophin receptor activation [5,30], and intracellular calcium dysregulation by over activation of inositol trisphosphate (InsP_3) receptors [13,15,56]. Few studies have directly compared the potency of neurotoxicity of propofol and an inhalational anesthetic in the developing brain, although exposure to sevoflurane has poorer cognitive outcomes than propofol in elderly patients with mild cognitive dysfunction [57]. We have found propofol to be less effective than isoflurane in activating InsP_3 receptors and therefore propofol may induce less over-activation of the InsP_3 receptor and less apoptosis accordingly [13,15,19]. Results from this study suggest that propofol is less potent than isoflurane in causing neurodegeneration in the developing brain, evidenced by no changes in plasma S100 β levels and less significant caspase-3 activation in the cortex. The possible clinical implications of these results need to be further studied.

Recent studies suggest that inflammation may play an important role in GA-mediated neurodegeneration, especially its association with cognitive dysfunction [12,35,48]. Interestingly, GAs also have different potencies in affecting neuroinflammation and, thus, cognitive dysfunction [12,48]. However, the present study demonstrates significant apoptosis induced by isoflurane and propofol without significant changes in inflammation, suggesting other mechanisms may play important roles in GA-induced apoptosis in this animal model. Although initial studies suggested an association between isoflurane-mediated apoptosis in the developing brain and subsequent cognitive dysfunction [1,2], further studies could not confirm this association, at least at low concentrations of isoflurane [10,13,58]. On the other hand, GA-mediated inflammation has been demonstrated to correlate well with cognitive dysfunction [12,48]. This hypothesis is supported by the close association between surgery induced inflammation and postoperative cognitive dysfunction [38–41]. Results from this study also support these findings, in that while both propofol and isoflurane caused significant apoptosis in the developing brain, this did not correlate with neuroinflammation or later cognitive dysfunction. Further studies including surgery are needed to confirm that inflammation during brain development may play a more important role in GA-mediated cognitive dysfunction than apoptosis. It has been shown that propofol may exert its neuroprotective effects in animal models of ischemia [59], trauma [60] and pressure stress [61] by limiting microglia activation. In

contrast, propofol treatment alone without other stresses factors did not induce inflammation [62], and demonstrated significant induction of apoptosis in the infant mouse brains in the current study. The role of microglia activation in propofol induced apoptosis in the developing brains need to be studied furthermore in the future.

It has been widely accepted that general anesthetics cause significant apoptosis in the developing brain in various species. Our previous studies [10,11] have demonstrated a close relationship between isoflurane induced apoptosis in the rodent developing brain and the elevation of plasma S100 β , a sensitive but not specific neurodegenerative biomarker, to detect brain damage induced by various stresses [63,64]. Similar to our previous studies [10,11], the plasma S100 β measured in this study most likely is reflective of the exposure to isoflurane because no surgeries or other significant stresses had been applied to these animals. However, while propofol significantly induced apoptosis in the developing brain, it failed to increase plasma S100 β levels. This could be due to S100 β not being as sensitive of a marker of neurodegeneration in the developing brain as caspase-3. Further studies in both animals and human beings are needed to investigate the value of plasma S100 β as a biomarker to detect anesthetic-mediated neurodegeneration in the developing brain.

Our study has several limitations. We did not measure the actual plasma concentrations of isoflurane and propofol as it is technically difficult to place an intravenous line and obtain blood in P7 mice. Likewise, we did not carry out continuous intravenous infusions of propofol to maintain a constant concentration of propofol in the blood. Instead, we used the ED₅₀ dose to maintain a surgical plane of anesthesia and shown to cause significant

apoptosis in the mouse developing brain [46]. We only exposed the mice to anesthesia, without surgical stimulation, which does not truly mimic the clinical scenario. Recent studies have suggested that surgery, rather than anesthesia, plays an important role in postoperative cognitive dysfunction, possibly by promoting neuroinflammation [40,52]. To minimize the use of animals, we did not do a dose-response study for the isoflurane and propofol treatments, which may provide more insight into their effects on apoptosis, inflammation and cognitive function.

In summary, we have demonstrated that a onetime 6 h clinically relevant exposure to isoflurane or propofol induced significant apoptosis in the mouse P7 developing brain, with isoflurane being more potent, which was not associated with significant changes in neuroinflammation or cognitive function.

Acknowledgments

The research work was performed in the lab of Dr. Huafeng Wei and should be attributed to the Department of Anesthesiology, University of Pennsylvania, Philadelphia, Pennsylvania, USA. The authors appreciate the technical support from Qingcheng Meng, Ph.D., Senior Research Scientist, and the support from Maryellen Eckenhoff, M.D., Research Associate, Roderic Eckenhoff, M.D. and Lee Fleisher, M.D., Professors, Department of Anesthesiology and Critical Care, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA.

Author Contributions

Conceived and designed the experiments: HW. Performed the experiments: BY GL SK ZW WY. Analyzed the data: BY GL DJ WY. Contributed to the writing of the manuscript: BY HW.

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