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Effect of Hypercarbia and Isoflurane on Brain Cell Death and Neurocognitive Dysfunction in 7-day-old Rats

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Background: **Millions of neonates undergo anesthesia each year. Certain anesthetic agents cause brain cell death and long-term neurocognitive dysfunction in postnatal day (P)7 rats. Despite its intuitive appeal, a causal link between cell death and neurocognitive decline after anesthesia has not been established. If one existed, the degree of cell death would be expected to correlate with the degree of neurocognitive dysfunction caused by anesthesia. The authors therefore tested if cell death caused by various durations of isoflurane at 1 minimum alveolar concentration causes duration-dependent long-term neurocognitive dysfunction.**

Methods: **Isoflurane was administered to P7 rats at 1 minimum alveolar concentration for 0, 1, 2, or 4 h. To control for the respiratory depressant effects of anesthesia, a group of rats was treated with 4 h of carbon dioxide. Cell death was assessed by FluoroJade staining 12 h after the end of each intervention, and neurocognitive outcome was assessed 8 weeks later by using fear conditioning, spatial reference memory, and spatial working memory tasks.**

Results: **Widespread brain cell death was caused by 2 h and 4 h of isoflurane and by 4 h of carbon dioxide. The degree and distribution of thalamic cell death was similar in 4 h isoflurane-treated and 4-h carbon dioxide–treated rats. Only 4 h of isoflurane caused a long-term neurocognitive deficit affecting both spatial reference memory and spatial working memory. Working memory was improved in carbon dioxide–treated rats.**

Conclusion: **Isoflurane-induced brain cell death may be partly caused by hypercarbia. The inconsistencies between cell death and neurocognitive outcome suggest that additional or alternative mechanisms may mediate anesthesia-induced long-term neurocognitive dysfunction.**

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MILLIONS of neonates undergo anesthesia for surgical or diagnostic procedures each year.[1,2] Anesthesia kills neonatal brain cells of several animal species, including primates,[3–6] and causes long-term neurocognitive dysfunction.[3,5,6] Consequently, anesthesia-induced apoptotic brain cell death is a growing concern within the anesthesia community, the public, and regulatory authorities alike.[7] However, a causal link between anesthesia-induced brain cell death and anesthesia-induced long-term neurocognitive dysfunction is lacking. If such a causal relationship existed, the degree of anesthesia-induced brain cell death should predict the severity of anesthesia-induced neurocognitive dysfunction. We therefore studied if 0, 1, 2, or 4 h of isoflurane at 1 minimum alveolar concentration (MAC) kills brain cells of postnatal day (P)7 rats and causes long-term neurocognitive dysfunction. We previously observed that the arterial carbon dioxide tension of spontaneously ventilating isoflurane-treated P7 rats increases shortly after induction of general anesthesia; therefore, we also tested if hypercarbia alone affects cell death in the P7 rat brain and if it alters long-term neurocognitive function.

Pavlovian fear conditioning is a well-established method of studying learning and memory.[8,9] In a typical fear-conditioning experiment, a rodent is taught to associate a neutral cue (*e.g.*, a tone) with an aversive event (*e.g.*, a shock). After rodents have been trained with one or more pairings of tone and shock, they learn that tone predicts shock. Rodents will exhibit their innate fear response (i.e. freezing, immobility) even when the tone is presented without the shock. Memory of the association between the tone and shock is assessed by quantifying freezing behavior in response to the tone. In addition to learning that tone predicts shock, rodents also learn that the surrounding environment made up of visual, auditory, olfactory, and tactile cues (the context) also predicts the shock. When rodents are given multiple pairings of tone and shock, not only will they freeze in response to the tone, they will also freeze in response to the context in which the training took place.

The basolateral amygdala, located within the medial temporal lobe, is essential for the acquisition of conditional fear. The basolateral amygdala is the site at which convergence of information about the shock and its predictive cues occurs.[10,11] The hippocampus assembles the representation of the context and communicates it to the amygdala. The roles of these two structures have been well characterized. Lesions of the amygdala block

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fear to tone and context, whereas lesions of the hippocampus block only fear to context and leave fear to tone intact.[12,13] The differential contribution of the amygdala and hippocampus allows us to perform two different tests of memory after training: (1) measurement of freezing in response to tone in a second context distinct from the training context, providing an assessment of the effect of anesthesia on an amygdala-dependent, hippocampal-independent task; (2) measurement of freezing in response to the training context in the absence of tone or shock, providing an assessment of the effect of anesthetics on a hippocampal-dependent task.

In the spatial reference memory task of the Morris water maze, rats are trained to find a submerged platform to escape from the maze. Due to the absence of local visual or olfactory cues that would indicate platform position, rats have to use distal cues. This requires formation of an allocentric spatial representation of the maze, which depends on the hippocampus and the neocortex.[14,15] Both memory acquisition (learning) and memory retention can be tested in the Morris water maze. The spatial working memory task of the Morris water maze, which tests executive function, uses a submerged platform placed in a different location each day. This task requires integration of information from the hippocampus, the cortex and the thalamus, among others.[16–19]

Here we show that three interventions cause significant cell death in multiple brain regions, namely 2 h and 4 h of isoflurane as well as 4 h of carbon dioxide. Of these, only 4 h of isoflurane caused a neurocognitive deficit.

Materials and Methods

Animals

All experiments were conducted with approval from the Institutional Animal Care and Use Committee at University of California, San Francisco. Rats (n = 17) with litters containing male (n = 162) and female (n = 2) Sprague-Dawley rats (from Simonsen Laboratories, Gilroy, CA, and Charles River Laboratories, Davis, CA) were cross-fostered before starting the experiment. When pups were 7 days old, they were given anesthesia or sham anesthesia. After treatment, the three anesthesia groups and two control groups (4 h of sham anesthesia, 4 h of carbon dioxide) were distributed equally among litters.

Rat Anesthesia

Anesthesia was conducted as described elsewhere.[20] Briefly, isoflurane was administered to at least 10 rats anesthetized simultaneously. MAC was determined by tail clamping every 15 min. Physiologic variables were assessed, and temperature was controlled at 36.5°C.

Sham Anesthesia

Control rats were placed in the anesthesia glove box for 4 h without exposure to anesthetic agent but otherwise identical conditions as animals in the isoflurane group. Thirty rats were kept in a carbon dioxide–enriched atmosphere for 4 h to rule out hypercarbia as the cause of the effects observed in the anesthesia group. The fraction of inspired carbon dioxide was varied between 15 and 30% to mimic the carbon dioxide tension in the anesthesia groups. The assumption was made that as long as breathing did not appear depressed, arterial carbon dioxide tension would be less than 10 mmHg higher than inspired first carbon dioxide tension.[21] Cardiac puncture for blood draws in these rats was performed under local anesthesia (0.05 mm of 1% lidocaine) injected subcutaneously.

Tissue preparation and FluoroJade (FJ) staining was performed as described in the accompanying article in this issue of ANESTHESIOLOGY.[20]

Microscopy

FJ stains were scanned and photographed at 4× before a tile scan at 20× of areas with appreciable cell death. The brain was mapped according to Paxinos' atlas of the rat brain.[22] Data are presented as fold increase from control. Density of FJ-positive (FJ+) cells in control animals, representing physiologic cell death, is provided for reference. All FJ+ cells were counted in three slices of the structure of interest, and the results were averaged. The individually assessed thalamic nuclei combined in the analysis of P7 rats were the paracentral, centromedial, paraventricular, mediodorsal (central and lateral), intermediodorsal, rhomboid, reuniens (including ventral reuniens), and the lateral posterior (laterocaudal, laterorostral, mediocaudal, and mediorostral). Cortical layers 2-6 were combined and the hippocampal subregions analyzed were the cornu ammonis (CA)-1, CA-2, CA-3, pyramidal cell layer, and dentate gyrus.

Fear Conditioning

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

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After a 3-min baseline exploratory period in the chambers, rats received three tone (2000 Hz, 90 db)–shock (1 mA, 2 s) pairings separated by 1 min. Freezing, the absence of all movement except that necessary for respiration, is an innate defensive fear response in rodents and a reliable measure of learned fear.[12] Each animal's behavior was scored every 8 s during the observation period, and a percentage was calculated using the formula $100 \times f/n$, where f is the number of freezing events per rat and n is the total number of observations per rat.

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

Morris Water Maze Spatial Reference Memory Task

Training. A platform (diameter, 10.3 cm) was submerged in a circular pool (diameter, 180 cm; depth, 50 cm) filled with warm (24°C) opaque water. Two training sessions were administered each day (7 h apart). At the beginning of its first session, a rat was placed onto the platform for 60 s and then given three trials (intertrial interval, 15 s), in which it was released from one of six pseudorandomly assigned release points (facing the wall; see fig. 1). On subsequent sessions rats were not placed onto the platform for 60 s before the three trials. Animals were given 60 s to locate the hidden platform. If the rat did not locate the hidden platform in the allotted time, it was guided by the experimenter. In either case, the rat was removed from the platform after 15 s. To minimize any bias associated with platform location, equal numbers of rats in each group were assigned one of four platform locations for the duration of training (even numbers in fig. 1). Training sessions were administered until the rats were able to locate the hidden platform in less than 15 s averaged over a session after five or more

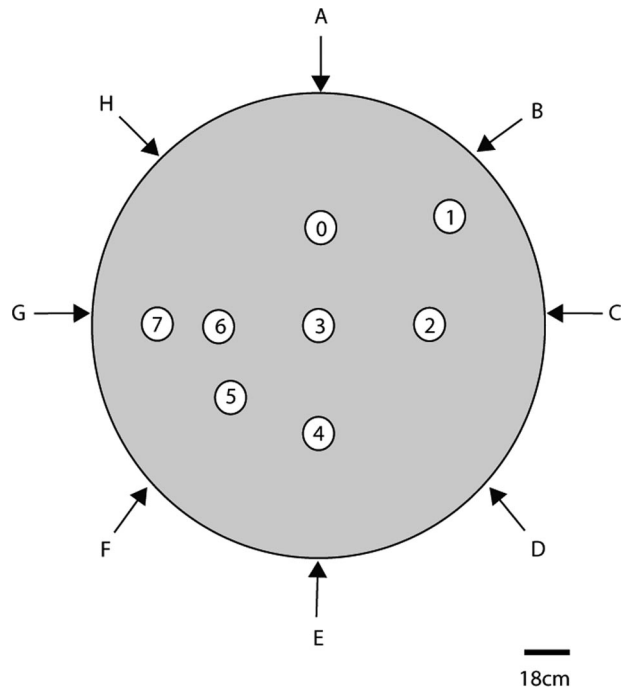

Fig. 1. Water Maze Setup. Water maze tank with escape locations and drop locations. *Numbers in the small circles* represent escape locations for the spatial reference memory task (0, 2, 4, and 6) and the spatial working memory task (1, 3, 5, and 7). *Arrows* on the periphery of the tank are the drop locations. Escape platform locations for the spatial reference memory task are the centers of the four quadrants. The escapes for the spatial working memory are halfway between spatial working memory escape locations and the wall of the tank on the border of two quadrants (*1*) or within a quadrant (*7*) or in the center of the maze (*3*) or on a line perpendicular to the border between two quadrants that transects one of the first two escape locations (*5*).

training sessions. Time to reach the platform (latency), path length, swimming speed, and time-integrated distance to the platform were analyzed using a EthoVision video tracking system (Noldus Instruments, Wageningen, Holland) set to analyze ten samples per second.

Probe Trial. To assess retention of the hidden platform location, a probe trial was administered with the platform removed from the tank 3 days after the last training session (when the learning criterion was achieved). During the 60-s probe trial, the proportion of time spent in the target quadrant *versus* each of the other quadrants was determined separately for each of two 30-s intervals, and the number of platform crossings was recorded and analyzed.

Cued Trials. After the probe trial, the appearance of the room was changed completely by relocating both the maze and a new set of remote spatial cues. To examine the presence of gross sensorimotor deficits, the escape platform was made visible by protruding it 1 inch above the water surface and marking its edge with bright red tape that could be seen clearly from the water surface but not by the tracking system. Rats were tested in three visible platform sessions (three trials per ses-

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sion) in which the platform was relocated each session.[23] The dependent variables recorded and analyzed during the hidden platform trials were also recorded and analyzed during visible platform trials.

Working Memory Water Maze Task

Training. For this task, the room was again completely rearranged by repositioning the tank and a new set of remote spatial cues. The platform was submerged 1 inch below the water surface in one of four platform positions (odd numbers in fig. 1) that were used pseudorandomly with each reoccurring on average every fourth session. This design sought to promote the use of hippocampal-dependent place strategies rather than taxon strategies.[23,24] One session was administered per day beginning with a 60-s free swim, during which the rat was allowed to explore the maze. If the platform was found, the rat was allowed to remain on it for 15 s. If the platform was not located within 60 s, the animal was guided to it and then allowed to remain on it for 15 s. Performance during the free swim was not scored. After the free swim, three trials were administered in which the rat was released from one of six pseudorandomly chosen locations facing the wall of the tank. The platform location was identical for all animals in a session, but the drop location was pseudorandomly varied to incorporate one short, one medium, and one long swim. This did not apply when the platform was located in the center of the pool, in which case all animals were placed in the pool at the same three drop locations. Training sessions were administered until the rats were able to locate the hidden platform in less than 15 s averaged over a session (after five or more training sessions). Time to reach the platform (latency), path length, and swimming speed were recorded with a EthoVision video tracking system (Noldus Instruments) set to analyze ten samples per second.

Testing. When the rats achieved criterion (day 12), indicating that they understood the rules of the task, a delay was inserted between the free swim and the subsequent trials to increase task difficulty. On day 13, a 1-h delay between the free swim and the three scored trials was introduced; on day 14, the delay was extended to 4 h. Time (latency) to reach the platform, time-integrated distance to platform, path length, and swimming speed were again recorded with the tracking system as described above (spatial reference memory training, working memory training). Performance on the first trial after the free swim on day 12 (1-min delay), day 13 (1-h delay), and day 14 (4-h delay) was used as a measure of working memory.

Statistical Methods

Data are reported as median and interquartile range for the control and as a median fold increase from control values for cell death experiments. To answer the question if isoflurane increases cell death dose-dependently, differences between the isoflurane groups and the control group were explored using a Kruskall-Wallis test with four degrees of freedom and revealed by a Dunn's correction for multiple comparisons to determine the threshold duration at which isoflurane causes a significant increase in cell death. To this end, the *post hoc* test compared each isoflurane group to the control group. To answer the question if hypercarbia causes cell death, differences between the hypercarbia and the control group were evaluated using the Wilcoxon signed rank test. To answer the question if the degree of cell death in the hypercarbia group differs from the degree of cell death in the 4-h isoflurane group, a Wilcoxon rank sum test was used. To assess the strength of the correlation between the distance from bregma in Paxinos' rat brain atlas and FJ+ cell density, the Spearmann rank correlation coefficient was used. Cell death data were analyzed using Prism for MacIntosh 4.0 (GraphPad Software Inc., San Diego, CA).

Fear conditioning freeze scores were expressed as medians (±interquartile range) and were analyzed both over time and collapsed for the tone or context portion of the test (to account for habituation to the stimulus). Group differences in the dependent variables (baseline freezing, postshock freezing, pretone freezing, tone-freezing, and context freezing) were explored using a Kruskall Wallis test with four degrees of freedom and revealed with a Dunn's correction for multiple comparisons. Fear conditioning data were analyzed using Prism for MacIntosh 4.0 (GraphPad Software Inc.).

Water maze data were expressed as means (± SE). In the water maze tasks, measures of latency, path length, and time-integrated distance to the platform were all highly positively correlated ($r > 0.74$; $P < 0.05$ within groups); therefore, time-integrated distances to platform are presented for clarity and economy. Group differences in the dependent variables (latency, path length, trials to criterion, swim speed, freezing scores) were explored using a two-way analysis of variance (ANOVA) and revealed using Newman-Keuls *post hoc* tests. When the dependent variable measures exhibited significant heterogeneity of variance, Mann–Whitney U tests were used. Data were analyzed using Statistica (V.6; StatSoft, Tulsa, OK) or Stata version 10.0 (StatCorp, College Station, TX). Alpha levels were set at 0.05.

Sample Size Calculation

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

For behavioral experiments, the sample size required to detect a 30% difference between groups at 80% power and a significance level of 0.05 was 12 animals per group. A group size of greater than 14 was chosen

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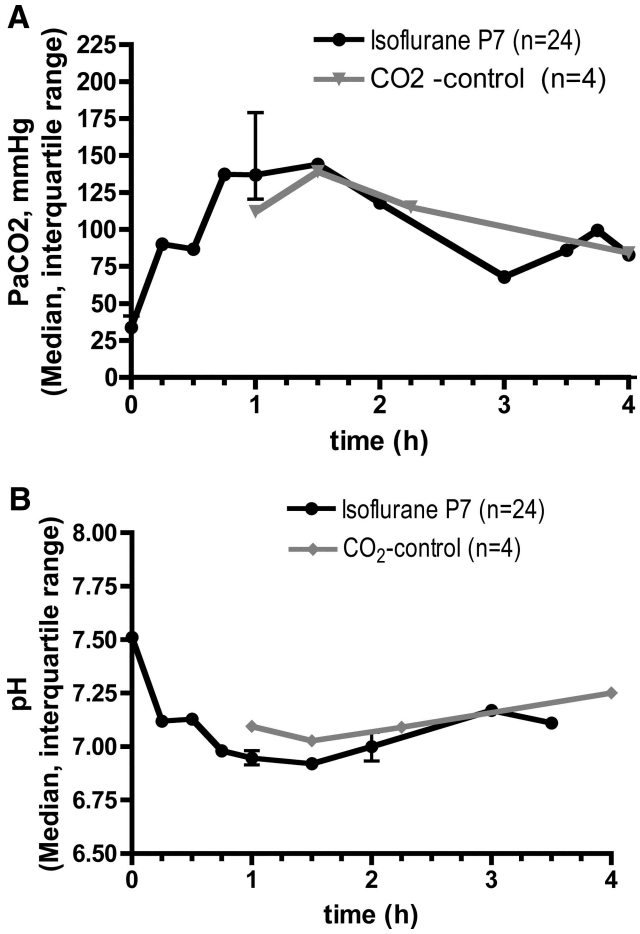

Fig. 2. Arterial carbon dioxide tension ($Paco_2$) and pH. Example of arterial carbon dioxide tension (*A*) and pH (*B*) over time in anesthetized postnatal day (P)7 rats (n = 141, 24 of which had one blood draw) and 21 carbon dioxide (CO_2) controls (n = 21, 4 of which had one blood draw). Data are medians and interquartile ranges.

to include a margin of error. SAS version 9 (SAS Institute, Cary, NC) Proc POWER was used for sample size calculations.

Results

Physiologic Variables: Early Hypercapnia — Delayed Mortality

The physiologic variables during this type of anesthetic are described in detail elsewhere.[20] Briefly, no hypoxia or hypoglycemia occurred during anesthesia in either group. Rapid and moderate hypercapnia (fig. 2A) and acidosis (fig. 2B) developed immediately after induction of general anesthesia. The mortality in rats anesthetized for 4 h and 2 h was 25%, with most animals dying during the last hour of anesthesia and two animals in the 2-h group dying after discontinuation of the anesthetic. The arterial carbon dioxide tensions and pH values of the carbon dioxide-control animals matched those of 4-h isoflurane–treated animals (fig. 2).

Substantial Thalamic Cell Death Caused by Isoflurane or Carbon Dioxide

Of all areas with detectable anesthesia-induced cell death, the thalamic nuclei were the most severely affected (fig. 3). After 4 h of isoflurane, the cell density of FJ+ cells was 45 to 360 times greater than control values in the lateral posterior and laterodorsal-ventrolateral thalamic nuclei, respectively. The effect of carbon dioxide on the thalamus varied by region. In the mediodorsal, laterodorsal, lateral posterior, reuniens, and central thalamic nuclei, the median amount of cell death caused by carbon dioxide was substantial (between 20-fold and 192-fold increase) and was not significantly different from that of the 4-h isoflurane group. In these regions, carbon dioxide caused more cell death than 1 h and 2 h of isoflurane, although the difference in these unplanned comparisons was not always statistically significant. In the submedius, rhomboid, and paraventral thalamic nuclei, carbon dioxide did not cause cell death, but 4 h of isoflurane did (table 1). Despite substantial cell death, the anteroventral and anteromedial thalamic nuclei were not included in the statistical analyses due to small size of usable samples for these areas.

Similar Rostrocaudal Thalamic Cell Death Gradient Caused by Isoflurane or Carbon Dioxide

The correlation between distance from bregma in Paxinos' atlas of the rat brain and FJ+ cell density in the lateral thalamic nuclei of both the 4-h isoflurane and carbon dioxide control groups was significant ($P < 0.01$ in 4-h isoflurane and $P < 0.001$ in carbon dioxide control, Spearmann rank correlation test; fig. 4). Neither the 1-h isoflurance group nor the 2-h isoflurane group had a similar distribution of cell death.

Hippocampal, Cortical, and Mamillothalamic Tract: Less Severe, Dose-dependent Cell Death Caused by at Least 2 h of Isoflurane

In the hippocampus (CA1-3 and dentate gyrus), the density of FJ+ cells was dependent on the duration of isoflurane exposure (fig. 5A). The median amount of cell death in the control group (1,802 cells/μl) did not differ significantly from the median cell death in the carbon dioxide control group (2,769 cells/μl). One hour of isoflurane had no significant effect on cell death, whereas 2 h of isoflurane caused a 2.5-fold increase in the density of FJ+ cells from controls, and 4 h caused a 5-fold increase. This suggests that isoflurane, but not carbon dioxide, causes mild hippocampal cell death, mostly in a dose-dependent manner, if administered for at least 2 h.

In layers 2–6 of the cortex, neither 1-h nor 2-h isoflurane treatment caused significant cell death, but 4 h of isoflurane did (fig. 5B). The maximum amount of cortical cell death was an approximately 7.5-fold increase from control levels caused by 4 h of isoflurane.

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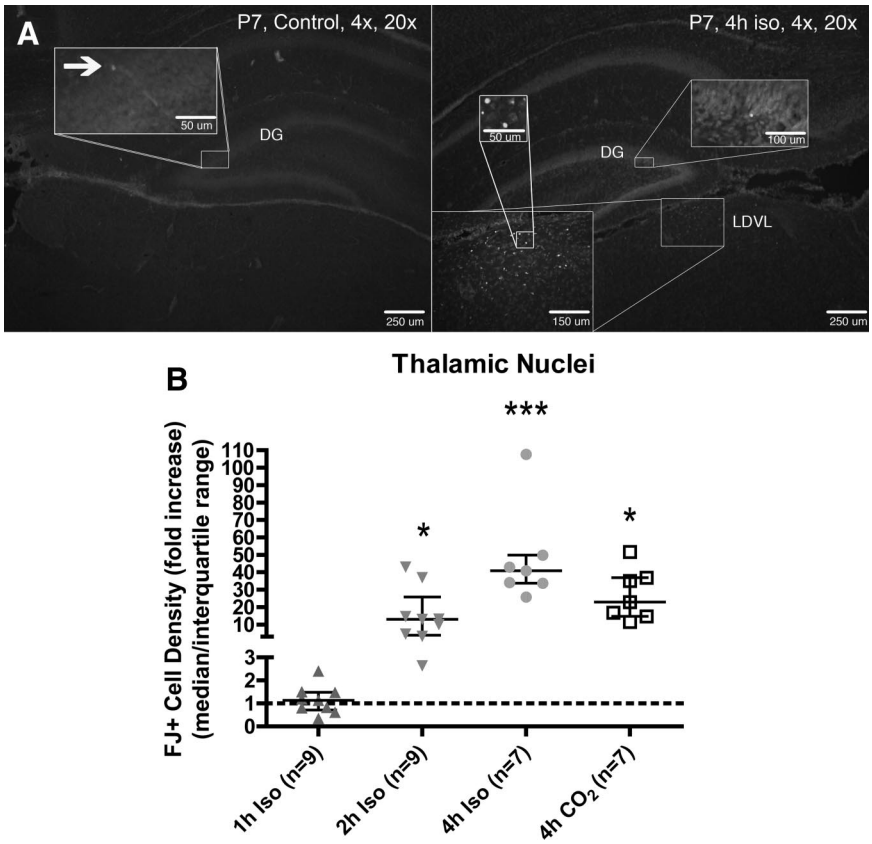

Fig. 3. Cell death in the thalamic nuclei. Example of FluoroJade–stained coronal brain sections from rats anesthetized with isoflurane (Iso) for 0 h (*Control*) and 4 h at postnatal day (P)7 (*A*). Unanesthetized rats had some naturally occurring cell death (*arrow*, for amount by area see table 1). Four-hour isoflurane significantly increased cell death in the thalamic nuclei (*A, B*) and mildly increased hippocampal cell death (*A*, also see Fig. 5A) as well as in other brain regions not shown here. For a detailed analysis of separate subregions, see table 1. Data are median fold increase from control group median (*dotted line*) with interquartile ranges. The raw cell death numbers in the control group can be found in table 1. *$P < 0.05$, ***$P < 0.001$, Kruskall-Wallis test with Dunn's correction for multiple comparisons. CO_2 = carbon dioxide; DG = dentate gyrus; LDVL = laterodorsal ventrolateral thalamic nucleus.

In the mammillothalamic tract, there was no significant increase in cell death in the carbon dioxide–treated or the 1-h isoflurane–treated group. However, both the 2-h and 4-h isoflurane treatments significantly increased cell death (fig. 5C).

Additional regions implicated in the type of deficit observed here, such as the fornix, entorhinal, perirhinal, or parahippocampal cortices, or mamillary bodies,[25] had little to no visible cell death in any of the groups.

Neurocognitive Outcome Inconsistent with Cell Death Data

Fear Conditioning.

Training. Baseline freezing before the onset of the first tone-shock presentation during training was generally low and significantly less in the 2-h and 4-h isoflurane groups than in either regular controls or carbon dioxide controls ($P < 0.01$, fig. 6A). Administration of the shock elicited robust and increasing postshock freezing, indicating the formation of short-term memory (fig. 6B).

Tone Test. On the next day, the pretone baseline freezing scores were again low; however, unlike baseline freezing before training the previous day, no differences were found between groups (fig. 6C). Tone freezing scores showed high interindividual variability and were not different between groups (fig. 6D) and there was no effect of time (not shown).

Context Test. Context freezing scores were again highly variable, and there was no effect of treatment (fig. 6E). There was no effect of order of test, *i.e.*, tone test first *versus* context test first (not shown).

Morris Water Maze Spatial Reference Memory

ANOVA revealed a significant treatment × session effect ($F_{(4,52)} = 1.79$; $P = 0.001$; fig. 7A) on latency to the hidden platform. A *post hoc* test showed that, although all groups acquired the hidden platform position at about the same rate, the 4-h isoflurane group did perform slightly worse (session 2; $P < 0.05$) and the carbon dioxide group significantly better than the control group (sessions 2 and 3; $P < 0.01$) in the first few sessions. Interestingly, there was a treatment effect on number of sessions required to achieve learning criterion (15 s latency averaged over three trials) ($F_{(4,81)} = 2.89$; $P = 0.027$; fig. 7B). Rats exposed to isoflurane for 4 h required significantly more training sessions to achieve learning criterion than all other groups did ($P < 0.05$, Newman–Keuls *post hoc* test). There was also a treatment effect on swim speed ($F_{(4,52)} = 2.27$; $P < 0.0001$; fig. 7C). Surprisingly, rats exposed to carbon dioxide neonatally swam significantly slower than the other groups over the first 5 sessions ($P < 0.001$), despite exhibiting better performance in earlier sessions. Given that these animals performed as well as or better than other groups, their slower swim speeds likely reflect reduced anxiety rather than impaired sensorimotor ability. The data show that prolonged neonatal exposure (4 h but not 2 h or 1 h) to isoflurane produces mild cogni-

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Table 1. Brain Cell Death as a Function of Isoflurane Dose (Minimum Alveolar Concentration × h)

		Median Fold Increase from Control				*P*	
Control, FJ+ cells/μl		1 h Iso	2 h Iso	4 h Iso	4 h CO_2	a	b
Hippocampus	1,447, 1,802, 3,691	1.2	2.49*	4.78‡	1.54*	0.0002	0.0006
GCL CA1	670.3, 1215, 1848	1.27	1.37	3.01	1.72	NS	NS
GCL CA2	332.2, 771.9, 2,509	1.73	5.26	11.65	3.94	NS	NS
GCL CA3	188, 931.1, 2,397	1.01	3.5	6.71*	1.33	0.0059	0.0022
DG	1,985, 3,194, 6,038	1.8	1.94*	2.94†	1.11	0.0015	0.0023
PCL CA1-3	817.4, 1,750, 3,684	0.96	3.23*	6.54†	1.59*	0.0002	NS
Thalamic nuclei	258.7, 773.7, 1,286	1.14	12.91*	40.84‡	22.88*	< 0.0001	NS
Laterodorsal							
LDDM	215, 450.1, 1,837	0.9	12.73	152.6†	65.61*	< 0.0001	NS
LDVL	0, 0, 235.1	0.43	131.4*	361.0†	192.5*	< 0.0001	NS
Mediodorsal (IMD, MDL, MDM, MDC)	261.8, 674.9, 1,634	0.49	7.26	57.52†	21.48	< 0.0001	NS
Paraventral (PVA, PV, PVP)	0, 0, 305.4	0.93	35.52*	167.7‡	18.86	0.0004	0.0061
Rhomboid (Rh)	0, 0, 2,063	0.41	5.52	30.56†	15.61	0.0024	NS
Submedius (Sub, SubD, SubV)	0, 347.8, 3,222	1	29	121.5‡	33.01	0.0001	0.0095
Reuniens (Re, RRe, VRe)	0, 139.9, 765.4	1.3	43.56	101.4†	110.9	0.0003	NS
Central (CM, CL, PC)	455.3, 754.2, 1,054	0.92	12.5	49.93†	22.35*	< 0.0001	NS
Lateral posterior (LPLR, LPMC, LPMR)	526.3	1.1	5.23	44.56†	58.3*	< 0.0001	NS
Cortex	977, 2,169, 3,208	1.158	1.753	6.95‡	4.567	< 0.0001	0.042
Cortex layers 2-3	646.9, 1,568, 2,344	0.91	1.86	8.47‡	1.73	0.0003	0.0061
Cortex layers 4-6	1,307, 2,770, 4,072	1.18	1.5	6.81‡	2.35	0.0002	0.0061
Mammillothalamic tract (mt)	0, 2,463, 5,053	0	7.16*	28.36*	6.14	0.001	0.016

Data for controls are 25th percentile, median, 75th percentile; data for all other groups are fold increases from control medians. *P*-values reflect (*a*) Krukall-Wallis test used to answer whether isoflurane increases cell death dose-dependently and (*b*) Wilcoxon rank sum test used to answer whether the degree of cell death caused by 4 h of isoflurane differs from the degree of cell death caused by 4 h of CO_2. Symbols in the isoflurane columns denote the significance level of the differences between the isoflurane groups and the control group (Dunn's correction for multiple comparisons). Symbols in the carbon dioxide column indicate the significance level of the difference between the carbon dioxide group and the control group (Wilcoxon signed rank test).

* $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

CL = central lateral thalamic nuclei; CM = central medial thalamic nuclei; DG = dentate gyrus; GCL = CA1-3 granule cell layer cornu ammonis 1-3; FJ+ = FluoroJade-positive; IMD = intermediodorsal thalamic nucleus; Iso = isoflurane; LDDM = laterodorsal dorsomedial thalamic nucleus; LDVL = laterodorsal ventrolateral thalamic nucleus; LPLR = lateral posterior lateral rostral thalamic nucleus; LPMC = lateral posterior mediocaudal thalamic nucleus; LPMR = lateral posterior mediorostral thalamic nucleus; MAC = minimum alveolar concentration; MDC = mediodorsal central thalamic nucleus; MDL = mediodorsal lateral thalamic nuclei; MDM = mediodorsal medial thalamic nucleus; mt = mamillothalamic tract; NS = not significant; PC = paracentral thalamic nucleus; PCL CA1-3 = pyramidal cell layer cornu ammonis 1-3; PV = paraventral thalamic nucleus; PVA = paraventral anterior thalamic nucleus; PVP = paraventral posterior thalamic nucleus; Re = reuniens thalamic nucleus; Rh = rhomboid thalamic nucleus; RRe = retroreuniens thalamic nucleus; Sub = submedius thalamic nucleus; SubD = submedius dorsal thalamic nucleus; SubV = submedius ventral thalamic nucleus; VRe = ventral reuniens thalamic nucleus.

tive impairment in the standard Morris water maze task. Surprisingly, neonatal carbon dioxide exposure does not impair but appears to improve performance in this task. The groups all performed equally well on visible platform trials ($F_{(4,8)} = 1.40$; $P > 0.05$; data not shown), suggesting that sensorimotor ability and motivation to solve the task was not adversely affected by neonatal exposure to anesthesia. Three days after the last spatial reference memory trial, the platform was removed from the pool and target quadrant dwell time was assessed for 60 s. All groups spent about the same amount of time searching the target quadrant ($F_{(4,12)} = 0.03$; $P > 0.05$; fig. 7D) and made similar numbers of platform crossings ($F_{(4,75)} = 0.54$; $P > 0.05$). These data indicate that neonatal exposure to isoflurane did not adversely affect memory for the platform location once it was learned.

Working Memory

Latency data show that all groups performed, on average quite well on working memory task training trials (sessions 1–12; fig. 8A). Although the rats exposed to carbon dioxide neonatally appeared to outperform the control group on training trials, an ANOVA demonstrated that there was no significant treatment × sessions effect on latencies in this task ($F_{(4,44)} = 1.14$; $P > 0.05$; fig. 8A). An ANOVA on sessions to criterion (15 s latency) revealed that, on average, all groups required about the same number of sessions to reach criterion ($F_{(2,80)} = 2.16$; $P > 0.05$; control, 5.70 ± 0.24; carbon dioxide, 5.11 ± 0.08; 1-h isoflurane, 6.0 ± 0.25; 2-h isoflurane, 5.73 ± 0.28; 4-h isoflurane, 5.87 ± 0.26 sessions), which supports this finding. Interestingly, the swim speed differences between groups that were observed in the spatial reference memory task disappeared on training sessions in this task ($F_{(4,44)} = 1.26$; $P > 0.05$) such that all groups swam as slowly as the carbon dioxide group did in the spatial reference memory task. This further suggests that the carbon dioxide–treated group had lower anxiety levels than the other groups in the first task but that the other groups had reduced anxiety levels with extensive training. When we increased task difficulty by asking the rats to remember the novel platform location for 1 h (session 13) and then 4 h (session 14), group differences emerged in latency to relocate the

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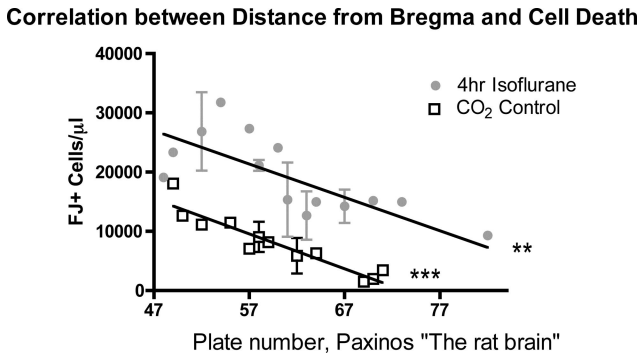

Fig. 4. Distribution of thalamic cell death is similar after 4 h of isoflurane and 4 h of carbon dioxide (CO_2). The density of cell death in the rostral portion is greater than in the caudal portion of the lateral thalamic nuclei in both the 4-h isoflurane–treated group and the 4-h carbon dioxide group. Lateral thalamic nuclei included in analysis are laterodorsal dorsomedial thalamic nuclei (LDDM), laterodorsal ventrolateral thalamic nuclei (LDVL), lateral posterior lateral rostral thalamic nuclei (LPLR), lateral posterior lateral caudal thalamic nuclei (LPLC), lateral posterior mediocaudal thalamic nuclei (LPMC), and lateral posterior mediorostral thalamic nuclei (LPMR). ** $P < 0.01$, *** $P < 0.001$, Spearman rank correlation test. FJ+ = FluoroJade-positive.

novel platform location learned on the previous trial ($F_{(4,8)} = 2.00$; $P < 0.05$; fig. 8B). *Post hoc* analyses revealed that at the 1-h delay, rats exposed neonatally to carbon dioxide performed significantly better ($P = 0.001$), whereas rats exposed neonatally to isoflurane for 4 h tended to perform worse ($P = 0.10$); however, no group differences emerged when a 4-h delay was inserted between the free swim trial and next trial. Swim speed differences (fig. 8C) emerged between groups ($F_{(4,8)} = 5.07$; $P = 0.00002$), with rats previously exposed to carbon dioxide swimming more slowly than the other groups and outperforming them on the session in which a 1-h delay was inserted between the free swim and the next trial ($P = 0.001$).

Discussion

There are several main findings of this study. First, isoflurane at 1 MAC causes brain cell death in P7 rats when administered for at least 2 h. Beyond this threshold, brain cell death in most areas is dose-dependent, with dose being defined as MAC × h. Substantial cell death is caused by 4 h of isoflurane, especially in the thalamic nuclei. This extends previous findings of anesthesia-induced brain cell death[3–6,26–29] to a single volatile agent used at a clinically relevant concentration of 1 MAC.

Second, 4 h of carbon dioxide alone causes substantial cell death in most thalamic nuclei. Furthermore, the degree and rostrocaudal distribution of thalamic cell death was similar to the degree and rostrocaudal distribution of cell death caused by 4 h of isoflurane, suggesting that part of the isoflurane-induced cell death may be caused by isoflurane-induced hypercarbia. The brain as a whole and structures within it grow directionally from caudal to rostral and from inside to outside.[30] It follows that cells in certain regions are of similar postmitotic age. One example of this is cortical development, where older neurons are located in deeper layers and younger

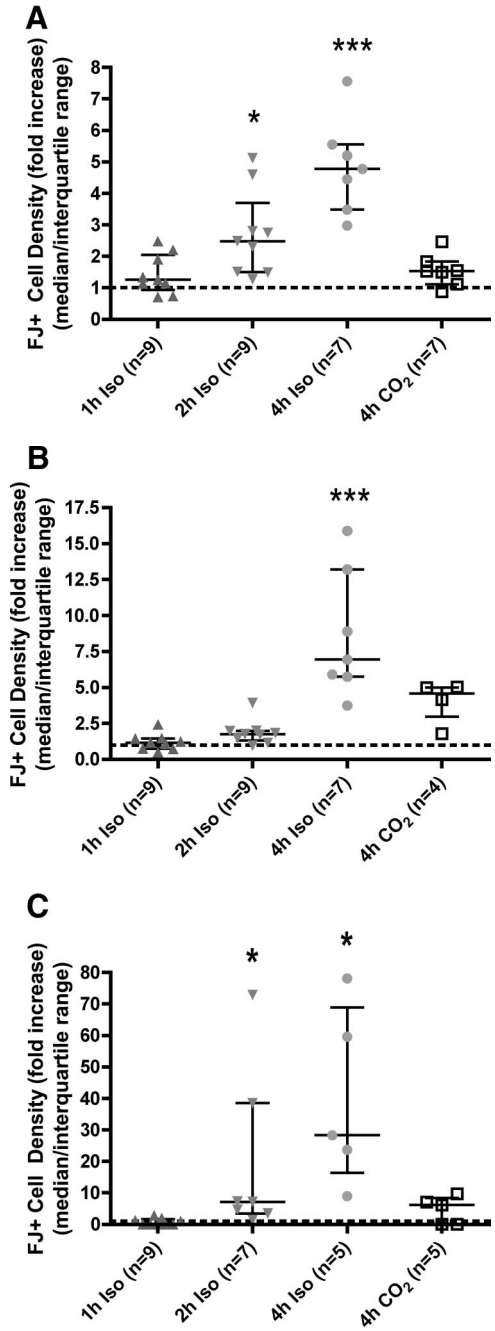

Fig. 5. Cell death in the hippocampus, cortex, and mammillothalamic tract. Density of FluoroJade-positive (FJ+) cells in the hippocampus (*A*), cortical layers 2–6 (*B*), and the mammillothalamic tract (*C*). For a detailed analysis of cell death in each of the subregions, see table 1. Data are median fold increases from control group median (*dotted line*) with interquartile ranges. The raw cell death numbers in the control group can be found in table 1. * $P < 0.05$, *** $P < 0.001$, Kruskall-Wallis test with Dunn's correction for multiple comparisons. CO_2 = carbon dioxide; Iso = isoflurane.

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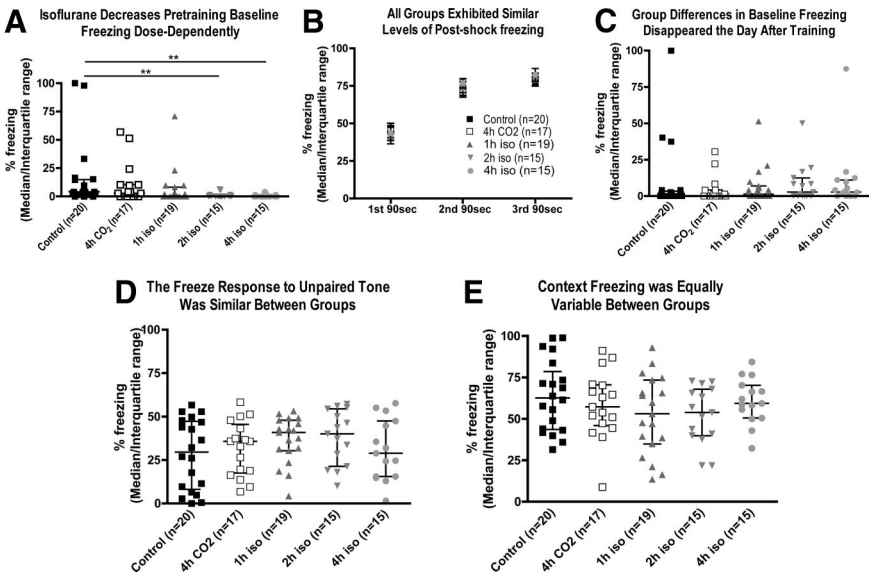

Fig. 6. Fear conditioning performance 8 weeks after 0-h, 1-h, 2-h, or 4-h isoflurane (iso) or 4-h carbon dioxide (CO_2) at postnatal day (P)7. Isoflurane given to P7 rats for 2 h or 4 h decreased baseline freezing (*A*) but not pretone test freezing (*C*). Tone-shock pairings resulted in short-term memory formation (*B*). There was a difference in neither the hippocampal-independent tone portion of the test (*D*) nor the hippocampal-dependent context portion (*E*). Data are medians with interquartile ranges. ** $P < 0.01$, Kruskall-Wallis test with Dunn's correction.

ones more superficially located. If the vulnerability of immature neurons to anesthetic toxicity were a function of postmitotic age of cells, for example, the age at which immature neurons are naturally eliminated from the brain for failure to make appropriate connections with other cells,[31,32] one would expect clustering of anesthesia-induced cell death in areas that contain more cells at this vulnerable stage.

Therefore, the rostrocaudal gradient of FJ+ cell density in the lateral thalamic nuclei of both the 4-h isofluane and 4-h carbon dioxide treatment groups suggests that the two insults may affect the same cell type. However, this study does not provide direct evidence of the identity of the cells killed by carbon dioxide or isoflurane. It is possible that a different kind of cell is affected despite similar degree of cell death and similar rostrocaudal pattern of cell death caused by 4 h of isoflurane or 4 h of hypercarbia.

Third, of the three interventions causing cell death (4 h of isoflurane, 2 h of isoflurane, 4 h of carbon dioxide), only 4 h of isoflurane caused a long-term neurocognitive deficit, which was evident in spatial reference memory and spatial working memory tasks but not in fear conditioning tasks. We chose the neurocognitive test battery on the basis of the relatively well-established knowledge about the anatomic regions involved in these tasks[12,16–19,25,33,34] and on their different requirements for motivation at the hands of different levels of aversiveness promoting behavior in these tasks.

Surprisingly, carbon dioxide, which caused cell death in certain areas of the brain, improved spatial reference memory and spatial working memory, and we can offer no explanation for this counterintuitive finding. As in the thalamus, 2 h of isoflurane caused significant cell death in the mamillothalamic tract and mild hippocampal cell death but no neurocognitive deficit. While confirming previous findings of long-term neurocognitive dysfunction after neonatal anesthesia in rodents,[3,5,6] the inconsistencies between brain cell death and long-term neurocognitive outcome evident in this study suggest that isoflurane-induced cell death alone might not be sufficient to cause isoflurane-induced neurocognitive dysfunction. Furthermore, a deficit due to cell death should be evident immediately after cell loss with some recovery over time, especially in the highly plastic neonatal brain. As reported in an accompanying article in this issue, we found the nature of the deficit to be just the opposite with a delayed onset and a progressive course to at least one third of a rat's life span.[20]

Of the areas affected by cell death, the hippocampus, cortex, and thalamus make contributions to the type of function tested 8 weeks after anesthesia.[12,16–19,25,33] Although, as argued above, thalamic cell death may not fully explain the long-term cognitive deficit, it is possible that the relatively minor hippocampal and/or cortical cell death contribute to the neurocognitive deficit observed here. It is also theoretically possible that a certain threshold of cell death, which was reached only by 4 h of isoflurane, is required to produce the deficit. Another possible interpretation of the results is that the mild degree of cortical cell death that was caused only by 4 h of isoflurane is responsible for the neurocognitive findings. Besides these remote possibilities, there may be additional or even alternative mechanisms that could mediate anesthesia-induced long-term cognitive dysfunction; two attractive possibilities in this regard include a potential anesthetic effect on neurogenesis or on synaptic morphology and function. These potential mechanisms represent two forms of brain plasticity, namely cellular and synaptic plasticity. In support of these hypotheses are the findings that the pattern of a deficit in spatial reference memory but no deficit in contextual fear conditioning has been found 4 weeks after inhibition of progenitor proliferation in mice[35] and 6 weeks after specifically ablating adult-born nestin-expressing

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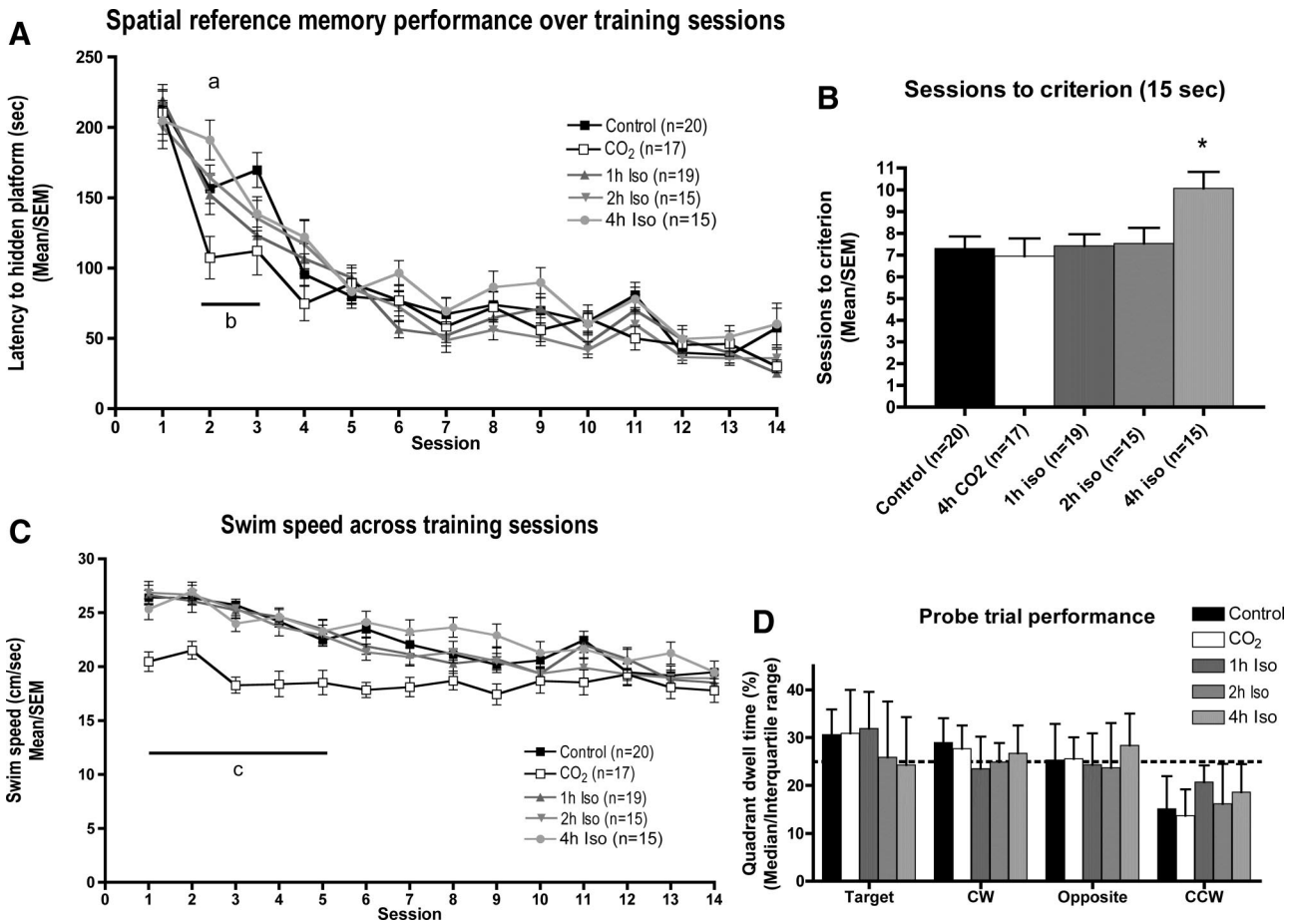

Fig. 7. Spatial reference memory. Latency to find a hidden platform as a function of group and session (*A*). Data are averages of three trials per session. The comparison marked by *A* indicates worse performance ($P < 0.05$, Newman-Keuls *post hoc* test) of the 4-h isoflurane (iso) group compared to controls, and the comparisons marked by *B* reveal better performance by carbon dioxide (CO_2)-treated animals ($P < 0.01$, Newman-Keuls). Sessions to criterion (reaching the platform within 15 s) (*B*) and swim speed (*C*). The comparisons marked by *C* reveal a slower swim speed for carbon dioxide–treated rats compared to all other groups ($P < 0.05$, ANOVA, Newman-Keuls *post hoc* test) during the first five sessions. Data are means ± SEM. Three days after the final spatial reference memory trial, the escape platform was removed and animals were reintroduced into the pool. The quadrant from which the platform was removed is the target quadrant. Data are quadrant dwell times expressed as percent of time spent in the target quadrant during the entire 60-s trial *versus* all other quadrants (*D*). Data are medians/interquartile ranges. The dotted line marks chance performance. CW = clockwise; CCW = counterclockwise. * $P < 0.05$ *versus* control.

precursors,[36] suggesting that only certain tasks (like the more demanding water maze task) may be affected at this time. Furthermore, a single exposure of P7 mice to alcohol, which mechanistically resembles anesthetic agents,[37] decreases dentate gyrus neurogenesis in adulthood,[38,39] which is consistent with our findings of a decrease in progenitor proliferation for at least 4 days after isoflurane anesthesia in P7 rats.[20] Anesthetic agents have been shown to affect dendritic morphology,[40–42] and emerging data extend these findings to anesthetic effects on short-term[43,44] and long-term synaptic morphology.[44] These are just two plausible target mechanisms by which anesthesia could affect the developing brain for a duration that far exceeds the pharmacologic action of the anesthetic.

Limitations of this study are plentiful. For example, FJ staining, although validated against other methods of cell death detection as extremely sensitive,[45,46] is not specific for apoptotic or necrotic cell death.[45,46]

The model of anesthesia used in this study is more likely a model of anesthesia plus surgery. Tail clamping causes tissue damage, inflammation, and scar formation, which could be a potential confounder. This is both a weakness from a mechanistic standpoint and a strength from a translational standpoint because anesthesia without surgery is administered to children only relatively rarely. In addition, a clinically relevant anesthetic endpoint, MAC, was used in this study, which enhances translatability, within the narrow confines of the limitations imposed by species differences between rats and humans. Hence, despite efforts to duplicate a clinically relevant scenario, by far the most important limitation of this type of study is the undefined applicability of the presented data to human anesthesia.

Concern that anesthesia-induced cell death in the rat brain could translate to human anesthesia-induced long-term cognitive dysfunction is based on the following line of reasoning. Neurons receive survival signals from other

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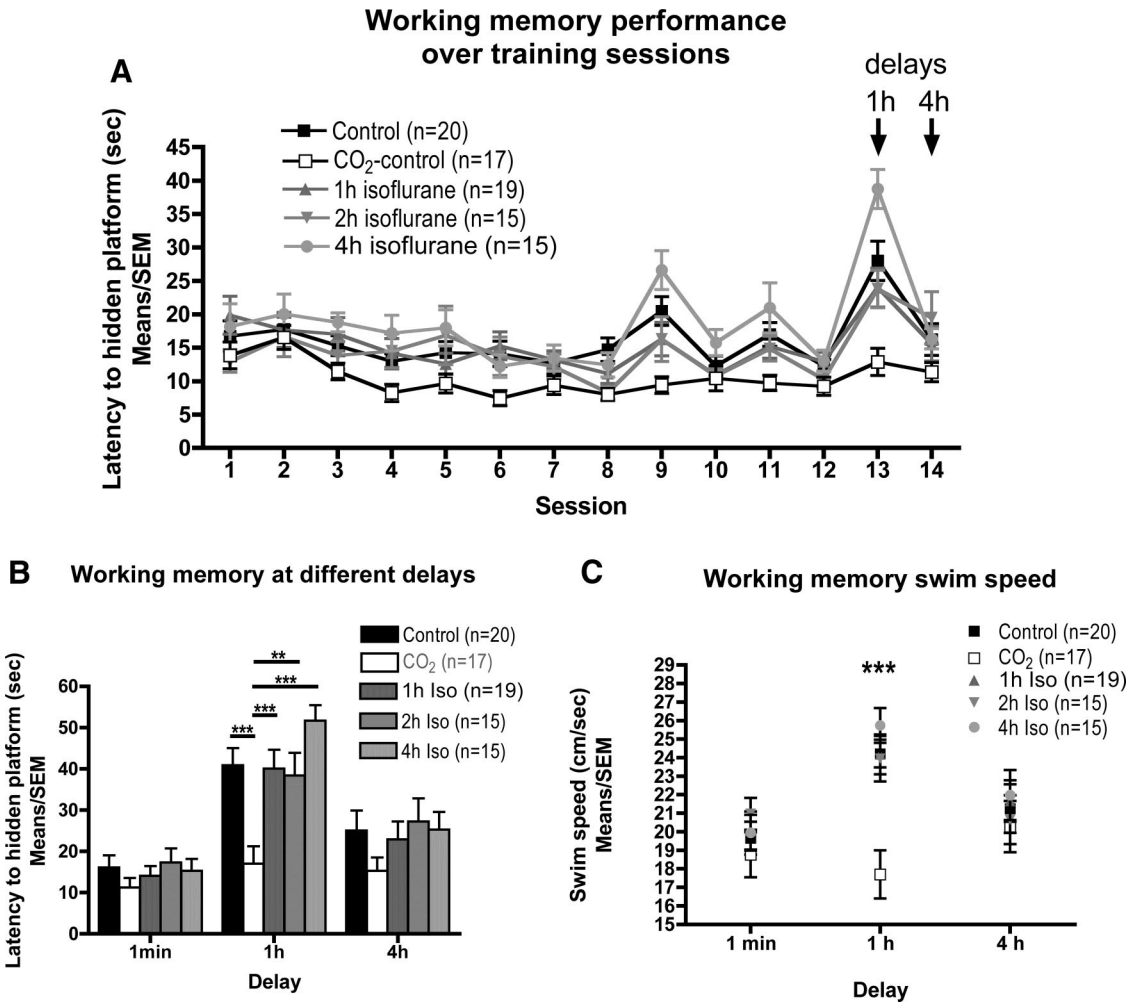

Fig. 8. Spatial working memory. Latency to find a hidden platform by sessions and groups (*A*). The platform was moved to a new location every session. Data are averages of three trials per session presented as means ± SEM. Introduction of delays of 1 h (*session 13*) and 4 h (*session 14*) between the unscored 60-s free swim and the first of three scored trials increased task difficulty (*B*) and affected swim speed (*C*). Data are latencies during the first trial of each session and average swim speeds expressed as means ± SEM. ** $P < 0.01$, *** $P < 0.001$, ANOVA/Newman-Keuls *post hoc* test. CO_2 = carbon dioxide; Iso = isoflurane.

neurons to which they are synaptically connected.[31,32] During a vulnerable period, assumed to correspond to the period of synaptogenesis,[3,47–50] anesthesia interrupts crosstalk between neurons, causing withdrawal of survival signals and neuronal death.[3,5,6,26,27,29,45,47–55] Synaptogenesis occurs in the human brain until several years after birth.[56] Anesthesia-related cell death caused during the period of synaptogenesis causes neurocognitive dysfunction in rodents[3,5,6]; if the same mechanism were operational in humans anesthesia could cause brain cell death and neurocognitive dysfunction until several years after birth.

The current work challenges the assumption that cell death necessarily causes neurocognitive dysfunction. Other assumptions in the above line of reasoning may also be questioned, namely the conjectures that the period of vulnerability is defined by the peak of synaptogenesis and that the dead cells are mature neurons.

It has been held that vulnerability to developmental anesthetic toxicity, which occurs at around P7 in rodents,[48,51] corresponds to the period of synaptogenesis.[27,29,48,51–54] The contribution most frequently cited in support of this is a review of brain weight gain during development across different species.[56] Although that work speaks to the timing of the brain growth spurt, it does not relate to synaptogenesis *per se*. The peak of synaptogenesis in rodents may not occur until P 11–16, and synaptogenesis seems to last until at least P32.[57,58] The period of vulnerability to anesthesia-induced cell death probably does not extend past roughly P10[53] to P14[47]; therefore, the connection between synaptogenesis and vulnerability to anesthesia-induced cell death is questionable.

A variety of anesthetic agents have caused brain cell death in various species including monkeys[1,3,5–7,26,27,29,45,47–54] but are these cells mature neurons? Due to the short gestational period (21.5 days) of rats, the most mature neurons in the structures affected by cell death of P7 rats are 16–17 days of age, with the majority being even younger.[59] Even relatively mature neurons in P7 rats differ from fully

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mature neurons in that they are likely to have smaller Na^+-currents,[61] a smaller total dendritic length,[60] an immature γ-aminobutyric acid reuptake system,[61] immature chloride extrusion, and they are consequently depolarized by γ-aminobutyric acid, not hyperpolarized like mature neurons (for review see Ben-Ari *et al.*[62]). Therefore, the possibility should be considered that the pathoanatomic correlate of developmental anesthetic toxicity may be an immature neuronal stage rather than a mature neuron. The postmitotic age of cells killed by anesthesia must be defined by means other than morphologic criteria or markers of mature neurons, none of which provide a meaningful temporal resolution or functional correlate.[60] Knowledge of the postmitotic age of cells subject to anesthetic toxicity would facilitate a more focused search of the mechanisms involved in anesthesia-induced cell death.

As a result of the discrepancies discussed above reflecting the lack of a convincing mechanism for anesthesia-induced neurocognitive decline in rodents, it may be premature to speculate if and when the human brain is vulnerable to developmental anesthetic toxicity.

Even if anesthesia does kill cells in the immature human brain, it is unclear if neurocognitive dysfunction would necessarily follow. The current results suggest that the two outcomes are less tightly linked than previously thought and that other mechanisms may at least contribute to the long-term cognitive outcome of neonatal anesthesia. This is not intended to downplay the potential problem. The finding of anesthesia-induced long-term neurocognitive dysfunction in animals warrants both concern and investigative efforts to assess if the problem occurs in humans. In the meantime, mechanistic studies in *in vitro* and *in vivo* models are also required to better inform us of the type of cognitive deficit, if any, that anesthesia might cause in humans as well as the cellular or molecular mechanisms that mediate it.

In conclusion, the current findings suggest that hypercarbia may play a role in anesthesia-induced brain cell death and that cell death alone is unlikely to account for the long-term neurocognitive decline after neonatal anesthesia in rats. The mechanism of anesthesia-induced neurocognitive dysfunction after neonatal anesthesia and its relevance to clinical neonatal anesthesia remain undefined and require further study.

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