Lower Isoflurane Concentration Affects Spatial Learning and Neurodegeneration in Adult Mice Compared with Higher Concentrations

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ABSTRACT

Background: Volatile anesthetics such as isoflurane are widely used in clinical and research contexts. Concerns have been raised that the effects of these drugs on the central nervous system may result in long-term impairment after surgery or general anesthesia. Hence, this study aimed to detect how different isoflurane concentrations influence spatial learning and cell death in adult mice.

Methods: Fifty-two C57BL/6 mice were randomly divided in four groups. Mice in three groups were exposed to different concentrations of isoflurane (1, 1.5, and 2%) for 1 h; the control group was not exposed to anesthesia. Five mice per group were killed 3 h after anesthesia to perform histopathologic and immunohistochemical analyses (hematoxylin-eosin staining; caspase-3 activation). Eight mice per group were used for behavioral tests (open field, T-maze spontaneous alternation, and water maze) on subsequent days.

Results: There were no differences between groups in the T-maze spontaneous alternation test or in the open field (no confounding effects of stress or locomotion). The group anesthetized with 1% isoflurane performed worse in the water maze task on day 1 (550.4 ±162.78 cm) compared with the control group (400.1 ± 112.88 cm), 1.5% isoflurane (351.9 ± 150.67 cm), and 2% isoflurane (364.5 ± 113.70 cm; $P \le 0.05$) and on day 3 (305.0 ± 81.75 cm) compared with control group (175.13 ± 77.00 cm) and 2% isoflurane (204.11 ± 85.75 cm; $P \le 0.038$). In the pyramidal cell layer of the region cornu ammonis 1 of the hippocampus, 1% isoflurane showed a tendency to cause more neurodegeneration (apoptosis) (61.4 ± 26.40, profiles/mm^2) than the group with 2% of isoflurane (20.6 ± 17.77, profiles/mm^2; $P = 0.051$).

Conclusion: Low isoflurane concentration (1%) caused spatial learning impairment and more neurodegeneration compared with higher isoflurane concentrations. Results for mice receiving the latter concentrations were similar to those of control mice.

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What We Already Know about This Topic

❖ Anesthetic exposure may lead to an increased risk of cognitive impairment

What This Article Tells Us That Is New

❖ Low isoflurane concentration (1%) caused spatial learning impairment and a trend toward enhanced neurodegeneration compared with higher concentrations

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INHALATIONAL agents such as isoflurane have widespread application in clinical and research practice. In laboratory animal research, isoflurane is routinely used because it allows an easily maintained general anesthesia with fast recovery. However, there are still uncertainties about its
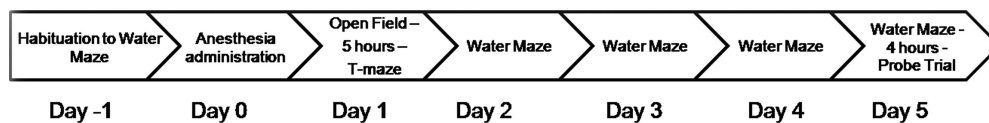
Fig. 1. Experimental protocol of the behavior tests.

mechanism of action and discussions about clinical implications of isoflurane anesthesia.

Inhalational anesthetics act mainly at two receptors, the *N*-methyl-D-aspartic acid receptor and the γ-aminobutyric acid A receptor, which are thought to be involved in cognitive processes.[1–2] Recent findings indicate that drugs acting by either inhibition *via* γ –aminobutyric acid receptors or by decreasing excitation through *N*-methyl-D-aspartic acid receptors induce widespread neuronal apoptosis in developing brain. Several studies show neurodegenerative effects of anesthetics in general[3–4] and isoflurane in particular[5–6] on the neural structure of immature animals. Results are contradictory, however, regarding cognitive impairment in adulthood in animals subjected to anesthesia during their infancy.[7–8]

A growing body of literature supports the view that humans and animals undergoing anesthetic procedure are exposed to an increased risk of cognitive impairment. In human patients, it has been shown that such cognitive impairment can last for periods longer than predicted (days or weeks after surgery).[9–10]

Culley *et al.*[11] showed that isoflurane was capable of producing sustained learning impairment in aged rats. However, they also showed that it improved the performance of previously learned tasks in young rats for periods of up to 2 months. Such a memory-enhancing effect in young animals had been demonstrated previously with the use of a nonspatial task 22 h after anesthesia administration.[12] A comparative study of rats aged 7 or 60 days showed that 4 h of isoflurane caused a permanent deficit of hippocampal-dependent but not hippocampal-independent learning and memory in 7-day-old rats, whereas 60-day-old animals showed a long-term improvement in spatial reference memory.[13]

Concerning the acquisition of new tasks, recent studies have demonstrated learning impairment 24 h and 2 weeks after anesthesia in aged rats,[14] whereas adult rats showed improved learning.[15] Although there seems to be an overall trend of cognitive impairment after anesthesia in aged animals, there are also contradictory results in which aged mice undergoing single or repeated episodes of isoflurane anesthesia had no immediate or long-lasting alterations at the cognitive level.[16] We have shown previously that low (1%) but not high (2%) isoflurane concentrations may impair the learning of spatial tasks in adult mice 28 h after anesthesia.[17]

This study aimed to study the influence of different depths of isoflurane anesthesia on cognition and potential brain-level neurodegeneration in adult mice.

Materials and Methods

All procedures were carried out under personal and project licenses approved by the National Institutional

Animal Care Committee, Direcção Geral de Veterinária (Lisbon, Portugal).

Animals

Fifty-two 2–3-month old male C57BL/6 mice bred in the animal facility of the institute (F1-F2 offspring of animals bought from Charles River Laboratories, Barcelona, Spain) were distributed equally among 13 cages (Makrolon type II cage, Tecniplast; Dias de Sousa, Alcochete, Portugal). Each cage was provided with standard corncob litter (Probiológica, Lisbon, Portugal), a piece of tissue paper, and a cardboard tube. Water and rodent pellets (4RF25-GLP Mucedola S.R.L., Settimo Milanese, Italy) were provided *ad libitum*. The animals were kept in a room with controlled temperature (21 ± 1°C) and humidity (55%). Lights were kept on a 12-h light/dark cycle, with lights off at 4:00 PM.

Anesthesia

Mice were randomly assigned into four treatment groups: unanesthetized control animals (control), animals treated with low (1%) isoflurane concentration (IF1%), animals treated with an intermediate (1.5%) isoflurane concentration (IF1.5%), and animals treated with a high (2%) isoflurane concentration (IF2%). Five mice per group were killed 3 h after anesthesia to perform histopathologic and immunohistochemical analyses. Eight mice per group were used for behavioral tests. A time line is presented in figure 1.

The animals were placed individually in the acrylic induction chamber, and anesthesia was induced with 4% isoflurane (Isoflo; Abbot Laboratories Ltd, Queenborough, Kent, United Kingdom; Esteve Farma Lda., Carnaxide, Portugal) in 100% oxygen with a delivery rate of 5 l/min until loss of righting reflex. This isoflurane concentration reduces the time required for the induction and hence reduces the stress caused to the animal. As soon as loss of consciousness was observed the animals were placed in dorsal recumbence inside a plastic resealable-zipper storage bag (zip bag) and the concentration was decreased to the respective treatment concentration. The zip bag contained three holes: the first to pass the wires for measuring heart rate and rectal temperature, the second for the vaporizer connected to isoflurane, and the last to exit gas (connected to the capnograph, which allows control of gas). After passing the wire, the holes were well sealed with adhesive tape. To introduce the animal, the zip was simply opened and then closed. This is a refinement that reduces the dead space and facilitates the monitoring of gas compared with the acrylic box chamber conventionally used for induction.

Anesthesia was then maintained with isoflurane in 100% oxygen with a flow of 1 l/min. A cuff and a transducer con-

nected to a pressure meter (LE 5001; Panlab, Barcelona, Spain) were placed on the tail base to measure the pulse rate. Pulse and respiratory rate were recorded at intervals of 10 min. Body temperature was maintained at 37°C by a homeo-thermic blanket connected to a rectal thermal probe (50-7061-F; Harvard Apparatus, Ltd., Kent, United Kingdom) and placed under the zip bag. Animals were anesthetized for 1 h, during which no stimuli were applied and background noise was reduced to a minimum. Isoflurane concentration was monitored and controlled in the exhausted air with an agent gas monitor (Datex Capnomac Ultima, Helsinki, Finland). After 1 h of anesthesia, all animals received 100% oxygen until recovery of righting reflex. No mice were re-strained during anesthesia. The animals from the control group were not anesthetized; however, they were manipu-lated and placed inside the induction chamber for 1 min (the average time until loss of righting reflex in anesthetized ani-mals) where 100% of oxygen was delivered, to also mimic the airflow. To avoid isolation stress in the nonanesthetized con-trol animals, they were then returned to their home cages.

Open Field

The open field test allows the evaluation of stress levels and exploration activity. The apparatus consisted of a circular arena with a diameter of 1 m made of gray polypropylene and surrounded by a 30-cm wall. The arena was placed in the center of a brightly lit room. The test began 19 h after anes-thesia. Each animal was released in the center of the arena and allowed to explore it for 20 min. At the end of testing, the number of fecal boli was counted, and the arena was cleaned with alcohol at 70% to avoid the presence of olfactory cues. The test was recorded with a camera placed above the appa-ratus and collected into a computer with a multicamera vig-ilance system (GV-800/8; GeoVision, Taipei, Taiwan). The video analysis was carried out with the program VideoMot 2 (TSE-Systems, Bad Homburg, Germany), which measured several parameters: total speed, total distance walked, num-ber of visits to each region, and the parameters distance walked, and percentage of time spent in each region. The regions were defined in the program analysis as center and periphery, with the limit between the two regions at 45 cm from the center of the apparatus.

T-maze Spontaneous Alternation

T-maze spontaneous alternation test is a tool to access work-ing memory,[18] a transient nonarchival memory that operates from seconds to a few minutes.[19] Working memory is pri-marily dependent on the prefrontal cortex; it lasts as long as the electrical activity of the prefrontal cells of the cortex persists.[20] The procedure used tested nonspatial working memory, because the trials were consecutive without enough time for the animal to be guided by spatial cues,[21] and the maze was opaque to prevent animals from using extramaze cues.

Apparatus. The T-shaped maze was made of gray acrylic sheets and consisted of a starting arm and two choice arms,

each measuring 10 × 60 × 20 cm (width, length, height). A removable cardboard central partition extending from the center of the back of the T into the start arm was added; thereby, the animal was prevented, after choosing the arm, from making a distant inspection of the other arm.[22] The T-maze was kept at the same location throughout the study.

Test. T-maze spontaneous alternation sessions began 24 h after the anesthetic procedure. Each animal was placed in the starting arm with a sliding door made of hard cardboard blocking the access to the choice arms. The trials started when the experimenter lifted the door and ended when the animal choose and was blocked in one of the arms; entrance was recorded when the animal entered with all four paws in the selected arm. The first trial was a forced-choice trial in which one of the arms was blocked. The subsequent 14 trials were free-choice trials with both arms open and the animal was allowed to choose. After a choice, the animal was con-fined in the selected arm until it made contact with the closed door, which marked the end of the trial. The door of the selected arm was opened and the door of the other arm was closed, so that the mouse, when leaving the selected arm, would return to the start arm. Animals had a maximum time of 2 min to perform each trial; after that the animal was gently pushed to the start arm. The T-maze was cleaned with alcohol at 25% between trials to avoid the presence of odor cues. The latency of first entry on the arm, the side choice, and the percentage of alternation was recorded.

Morris Water Maze

This Water Maze protocol, with a 4-day training period fol-lowed by a probe trial, evaluates long-term/reference mem-ory, long-term storage of information that involves a se-quence of specific molecular processes in the CA1 area of hippocampus and its connections.[20]

Apparatus. The maze consisted of a circular gray polypro-pylene tank with a diameter of 1 m and a depth of 0.5 m. The tank was filled with water to a 35-cm depth, and its temper-ature was kept constant at 24°C. A transparent acrylic plat-form (12 × 9 cm) was placed in the maze, which was then divided into four imaginary quadrants, and four starting po-sitions (north, south, east, west) were determined at the in-tersections of the quadrants. To ensure a constant surround-ing environment, curtains were placed in one side of the room to cover the benches. Geometrical figures (extramaze visual cues) were placed on the curtains at approximately 1 m above the edge of the pool. The features from the other sides were maintained throughout all the experiment. Video re-cordings were analyzed using the same methods described for the open field test.

Habituation. During the habituation session, the day before anesthesia, animals were allowed to see the platform; it was raised above the water level and a colored flag was placed on top of it. They were habituated to the water using a four-trial procedure in which the position of the platform and the starting point were changed for each trial. A trial began when the animal was released in the water. The mouse was allowed

to swim a maximum of 90 s to locate the platform; if after this time it failed, the experimenter would lead it to the platform. The animal was then allowed to rest on the platform for 10–15 s before being picked up by the experimenter. This shaping method allowed the mice to habituate to the water and to learn how to escape from it by climbing onto the platform.

Acquisition. The animals were allowed to recover for 41 h after anesthesia to avoid any confounding influence of residual anesthetic. Then they were tested in the water maze to assess spatial reference memory for 4 consecutive days. The platform was submerged and was not visible to the animals for the entire acquisition phase. The procedure consisted of six trials during which the platform location was kept constant but the starting position was semirandomly changed for each trial. In the semirandomization schedule, the four positions/imaginary quadrants of the tank are used; in the first four trials, the animal started from each of the four different quadrants, and the other two trials were randomly chosen.[23] Each mouse was given 90 s to locate the platform and allowed 10–15 s to rest on it before being removed by the experimenter and placed under a heating infrared lamp. Intertrial interval was set at 15 min. The parameters analyzed to assess performance were latency and distance traveled to find the platform and swim speed.

Probe Test. The probe test, run in the last day, 2 h after the last test trial, consisted of one trial in which animals were released from the same point. The platform was removed and the mouse was allowed to swim in the maze for 90 s. The parameter measured was time spent in each quadrant expressed in percentage as a measure of spatial accuracy. All behavioral tests were performed and analyzed by the same researcher (open field and T-maze spontaneous alternation by Ms. Valentim and water maze task by Mr. Di Giminiani), blind to the anesthetic procedures that the animals had undergone.

Histopathologic (Hematoxylin-Eosin) and Immunohistochemical (Caspase-3 Activation) Studies

Three hours after anesthesia, the mice were killed by cervical dislocation, and the brains were removed. Brains were fixed for 48 h in 4% buffered paraformaldehyde (phosphate-buffered saline [PBS], pH 7.4, 0.1 M), cut in 2 mm thick coronal sections, processed, and embedded in Paraplast (Hypercenter XP and Histocenter 2; Shandon, Burlingame, CA). For each block, two serial sections 4 μm thick were made making coronal cuts at the level of bregma −2.30 mm/interaural 1.50 mm.[24] One was stained with hematoxylin and eosin for a general observation of cellular death, and the other was used for caspase-3 detection.

For immunohistochemical analysis, a streptavidin-biotin immunoperoxidase method was used. In brief, the sections were deparaffinized, hydrated, washed in PBS, quenched in

3% hydrogen peroxide for 20 min, and washed in H_2O. For antigen retrieval, microwave treatment was used (two times for 5 min each in citrate buffer, pH 5). After washing in PBS, the sections were incubated in blocking solution for 5 min (Ultra V Block, UltraVision Detection System; Thermo Fisher Scientific, Waltham, MA), followed by overnight incubation in rabbit anti–caspase-3-antibody (CPP32 Ab-4, Rabbit Polyclonal Antibody; Thermo Fisher Scientific) diluted 1:100 in PBS at 4°C. For negative control, one extra brain section was incubated in PBS; as a positive control, a section of newborn and regression stage mouse thymus was processed and analyzed in the same way as the brain. The next day, the sections were washed in PBS and incubated in biotinylated goat anti-polyvalent antibody (UltraVision Detection System) for 10 min at room temperature. After washing in PBS, sections were incubated in streptavidin peroxidase complex (UltraVision Detection System) for 10 min at room temperature. To detect the immunoreactions, a diaminobenzidine (Sigma, Madrid, Spain) solution (0.01% 2,3-diaminobenzidine with 0.01% H_2O_2) was used. Finally, the slides were washed in H_2O, counterstained with Gill hematoxylin, and dehydrated.

Tissue sections were examined with a microscope (Eclipse E600; Nikon, Tokyo, Japan) equipped with a digital camera (DXM 1200; Nikon). The hippocampus, retrosplenial cortex, and visual cortex were photographed using a 20× objective (numerical aperture, 0.50); to avoid overlapping, the position of each field was recorded using the x-y coordinates of the microscope stage. Digitized images with a resolution of 3840 × 3072 pixels (3382.34 cm²) with the final magnification of 960× were exported to Adobe Photoshop CS3 (Adobe Systems Inc., Mountain View, CA). Cells showing a clear positive immunoreaction in the retrosplenial cortex, visual cortex, pyramidal cell layer of the cornu ammonis (CA) 1 and CA3 areas of hippocampus, and in the granular layer of the dentate gyrus, were directly and manually counted.

Statistical Analysis

The animals were randomly distributed into the groups using a web site service.** We used analysis of variance (ANOVA) and Student t test to analyze parametric data, whereas nonparametric data were evaluated with the Kruskal–Wallis and Mann–Whitney tests.

Physiologic Parameters. Respiratory and pulse rate were analyzed by repeated-measures ANOVA with Bonferroni corrections for multiple comparisons between anesthetic treatment and within groups (time).

Behavioral Tests. The number of visits to the periphery of the open field and the alternation rate in the T-maze spontaneous alternation were analyzed by Kruskal–Wallis test, whereas one-way ANOVA was used to evaluate total speed and distance, distance walked, and percentage of time spent in each region of the open field and to evaluate normalized data on latency to enter one of the arms of the T-maze (reciprocal transformation). To access spontaneous alternation

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** Random.org-True random number service. Available at: http://www.random.org. Accessed November 3, 2008.

within groups, Students t test for difference from chance (50%) was carried out. Performance in the water maze test (distance and time to find the platform) was analyzed by repeated-measures two-way ANOVA with Bonferroni corrections for multiple comparisons between anesthetic treatment and within groups (days as repeated measures factor)—two factors. If differences were found between treatment groups, Student unpaired t test would be used to analyze on which days these differences were detected. Pearson correlation test was used to study correlation between latency and time to find the platform in the water maze. Because the data on the percentage of time spent in each quadrant of the water maze during probe trial were normally distributed, one-way ANOVA was used to detect differences between groups. To assess learning of the platform location within each group, we used one-sample t test between the percentage of time spent in each quadrant and the chance level percentage of time (25%). Slopes of the regression lines of the performance in the water maze were analyzed by one-way ANOVA for main effect of group.

Neurodegeneration Analyses. The number of dead cells per square millimeter of brain in the retrosplenial cortex, visual cortex, and pyramidal cell layer of CA3 field by hematoxylin-eosin staining and caspase-3 activation were analyzed by Kruskal–Wallis test. This test was also used to evaluate the number of cells in the granular layer of the dentate gyrus activated by caspase staining and the number of pyramidal cells of CA1 field stained by hematoxylin-eosin. One-way ANOVA was used to analyze the number of granular cells of the dentate gyrus marked with hematoxylin-eosin and the number of caspase-3 activated cells in the pyramidal cell layer of CA1 field of the hippocampus; Tukey test was used as a *post hoc* test.

All hypotheses were two-tailed tested and statistical significance was considered to be reached at $P \leq 0.05$. All results were analyzed by using Microsoft Office Excel 2003 (Microsoft Corporation, Redmond, WA) for data acquisition and SPSS 16.0 for Windows (SPSS Inc., Chicago, IL) for statistical analysis. Data are presented as mean $\pm$ SD because all data shown were parametric.

Results

Anesthesia. Group IF2% had significantly lower respiratory rate compared with groups IF1% ($P < 0.0001$) and IF1.5% ($P < 0.0001$) at all time points except point zero, where the only significant difference was between groups IF2% and IF1.5%. There was no significant interaction between time and treatment. Heart rate data from two animals were eliminated from the analysis because of technical problems with the equipment. Group IF2% had significantly lower heart rate compared with groups IF1% ($P = 0.012$) and IF1.5% ($P = 0.003$); there were no differences detected between groups IF1% and IF1.5%. No significant interaction between time and treatment was observed.

Open Field Test. No differences were detected between groups on the parameters measured in the open field.

T-maze Spontaneous Alternation. None of the groups alternated in a manner significantly different from chance (50%), and there were no significant differences between the groups.

Water Maze Test. Mice anesthetized with IF1% showed a significantly worse performance in the water maze. The distance traveled to reach the platform was significantly longer in the IF1% group (550.4 $\pm$ 162.78 cm) (fig. 2A) than in control mice (400.1 $\pm$ 112.88 cm; $P = 0.05$), group IF1.5% (351.9 $\pm$ 150.67 cm; $P = 0.024$) and group IF2% (364.5 $\pm$ 113.70 cm; $P = 0.019$) on day 1 and significantly longer (305.0 $\pm$ 81.75 cm) than control mice (175.13 $\pm$ 77.00 cm; $P = 0.006$) and group IF2% (204.11 $\pm$ 85.75 cm; $P = 0.038$) on day 3, whereas larger variation on day 2 cancelled out statistical significance. Likewise, on days 1, 3, and 4 (23.8 $\pm$ 7.80, 13.5 $\pm$ 4.25, and 9.0 $\pm$ 3.11 s, respectively), IF1% mice showed significantly longer latency to find the platform (fig. 2B) than control mice (16.1 $\pm$ 5.11, 7.68 $\pm$ 3.10, and 5.7 $\pm$ 2.36 s, respectively; $P \leq 0.035$). Distance and time to find the platform did not show exactly the same statistical differences; however, these two measures are highly correlated ($P < 0.0001$). There were no differences between the slopes of the curves representing the performance of the different groups, neither for distance nor latency to find the platform. There was no treatment effect on swim speed; thus, no motor impairment was present. On the probe trial, all groups spent significantly more time than expected by chance (25%) in the target quadrant ($P \leq 0.01$) and significantly less time on the opposite quadrant ($P \leq 0.004$) (fig. 3), indicating that they had learned the task. There was no difference detected between groups. The simple concentration-response curve for water maze performance (mean of distance of all days) did not reveal a linear relation but a peak corresponding to impaired performance after 1% isoflurane anesthesia (fig. 4).

Brain Analyses. Regarding histopathology (hematoxylin-eosin stain), there were no significant differences detected in the number of dead cells between groups in any of the brain regions studied. Immunohistochemical analyses showed a similar result concerning the number of apoptotic cells (activated for caspase-3). However, IF1% mice tended to present more apoptotic neurodegeneration (61.4 $\pm$ 26.40) than the group IF2% (20.6 $\pm$ 17.77) in the pyramidal cell layer of the field CA1 of hippocampus ($P = 0.051$) (fig. 4). This tendency was not detected between the other groups or in the other brain regions studied. The simple concentration-response curve (apoptotic neurodegeneration) shows a shape similar to that for the water maze task; a peak is observed for 1% isoflurane concentration (more apoptosis), whereas 0, 1.5, and 2% isoflurane revealed a similar caspase-3–activated positive profile in the CA1 region of the hippocampus (fig. 4).

Discussion

The outcome of this study shows that 1 h of isoflurane anesthesia at a low concentration (1%) impaired performance in
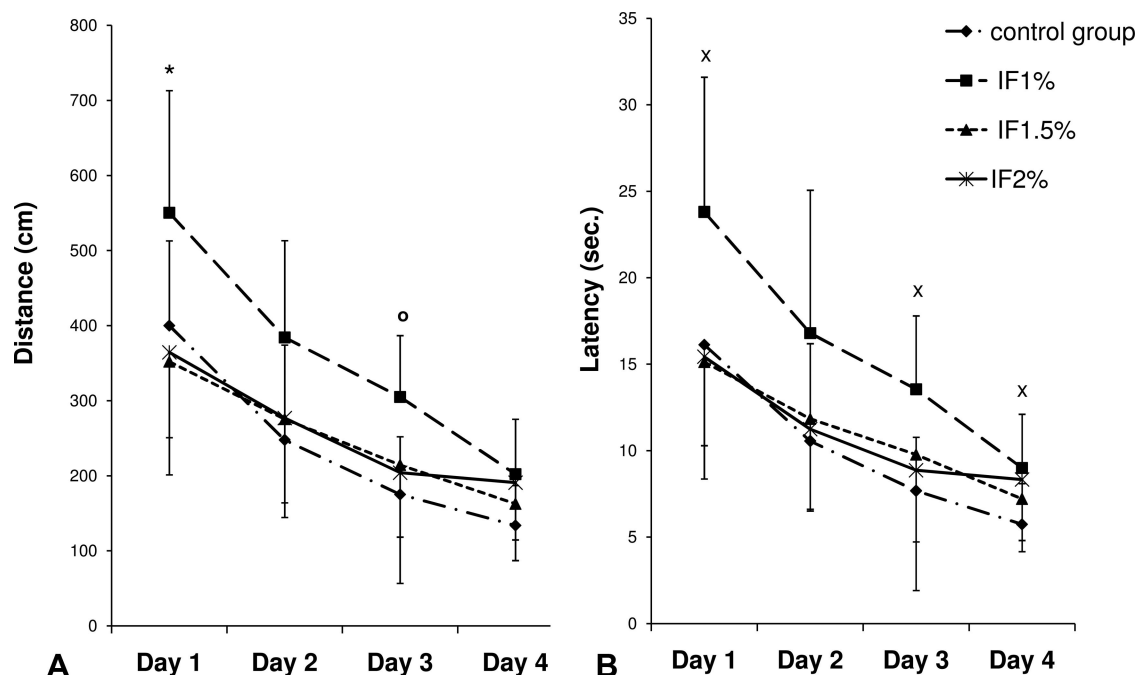
Fig. 2. Distance traveled (*A*) and latency (*B*) to find the platform in the water maze test during 4 consecutive days after a 41-h postanesthesia recovery period. n = 8 mice. Data are expressed as mean ± SD; * $P \leq 0.05$, IF1% versus the other groups; ° $P \leq 0.03$, IF1% *versus* control and IF2%; × $P \leq 0.038$, IF1% versus control group. IF1% = 1% isoflurane; IF1.5% = 1.5% isoflurane; IF2% = 2% isoflurane.

a spatial test, whereas mice that received intermediate (1.5%) and high (2%) concentrations behaved similarly to control mice, 41 h after anesthesia administration. Moreover, there was a tendency of increased apoptosis observed at the hippocampal level in animals subject to low concentration of isoflurane anesthesia, sacrificed 3 h after anesthesia.

The observation of impaired performance in a spatial learning and memory task after low concentration anesthesia is in agreement with our previous preliminary results where only light isoflurane anesthesia impaired acquisition in the

T-maze test, a simple spatial task.[17] However, when the cognitive measure used was performance in the radial arm maze, no difference between different depth of isoflurane anesthesia was detected.[25] Nevertheless, taken together, the results of the three different studies clearly reinforce the idea that high concentrations of isoflurane anesthesia are not associated with cognitive deficits.

The effects of depth of anesthesia are still relatively unexplored; however, a study in humans focusing on postoperative cognitive dysfunction partly supports our results.[26] Patients undergoing deeper anesthesia showed improved recovery of cognitive functions (processing speed) compared with more lightly anesthetized patients. Although the clinical
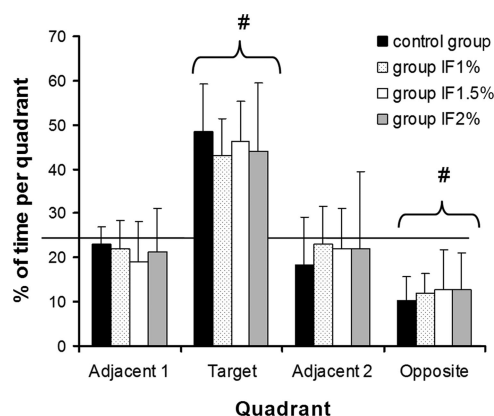
Fig. 3. Percentage of time spent on each imaginary quadrants during the probe trial of the water maze test 5 days postanesthesia. n = 8 mice. Bars represent means, vertical lines represent SDs, and the horizontal line represents chance performance (25%); # $P \leq 0.01$ for 25% of chance. IF1% = 1% isoflurane; IF1.5% = 1.5% isoflurane; IF2% = 2% isoflurane.
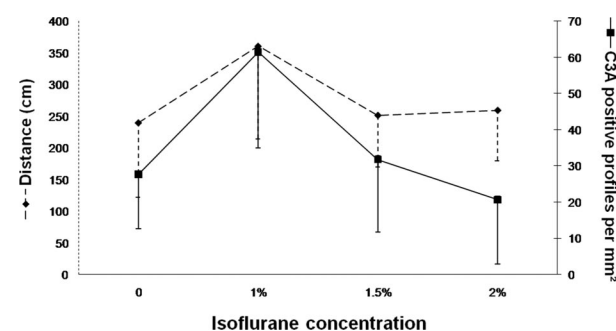
Fig. 4. Concentration-response curves for water maze performance (mean of 4 days of the distance traveled to find the platform in centimeters) (♦), and the caspase-3 activation positive profile per mm² in the pyramidal cell layer of the cornu ammonis 1 area of the hippocampus (■). n = 8 mice for water maze task and n = 5 mice for caspase-3 activation. Data expressed as mean ± SD. C3A = caspase-3 activation.

study and our experimental studies address different aspects of cognition, they indicate a common potential effect on the cognitive capacity. Human clinical studies may have problems in evaluating the real effect of one drug/anesthesia because of pharmacokinetic interactions and in differentiating the effects of anesthesia from those of surgery and hospitalization.[27] In our study, these confounding variables were not present.

In the present study, the only behavioral difference observed between groups was in water maze task performance. This impairment was detected in both distance traveled and latency to reach the escape platform comparing the 1% isoflurane group with the other groups. These two measures were highly correlated, and there was no treatment effect on swimming speed, excluding the possibility that a motor impairment in any of the groups could have influenced the results. The absence of a treatment effect on open field behavior is also relevant to exclude the possibility that treatment groups differed in stress or anxiety in a way that could have influenced performance in the water maze task.[28] Thus, the impairment in performance most likely reflects a true impairment in spatial cognition. However, rather than a general and persisting effect on learning and memory, the results indicate that the main consequence of low-concentration anesthesia was impaired performance of acquisition 41 h after anesthesia. The effect was transient; animals in this treatment group performed at control levels on the fourth day of water maze testing. This is further supported by the observation that all animals showed evidence of having learned the task when tested in the probe trial on day 4. Hence, there was no evidence of long-term effects on spatial cognition. The absence of differences in the T-maze spontaneous alternation may be due to the less active role of the hippocampus in this nonspatial protocol.

Cell death, as assessed by hematoxylin-eosin and caspase-3 staining, was not different between groups in the different brain regions. However, animals subject to the light isoflurane concentration tended to have a higher number of apoptotic cells in the pyramidal cell layer of the CA1 of hippocampus than those treated with the high isoflurane concentration. Indeed, recent studies have shown that drugs modifying receptor activity in the central nervous system induce neuronal apoptosis, including a neurotoxic effect of isoflurane at a cellular level, especially in the neonatal brain.[5,6,29] Infant mice exposed to isoflurane under the minimal alveolar concentration (sub-MAC) for 1 h suffered neuroapoptosis.[6] Although the concentration used in the study of Johnson *et al.*[6] is different from that used in our study, both concentrations, 2% for pups in the Johnson *et al.*[6] study and 1% for adult mice in our study, are sub-MAC values. MACs for pups and for adults are, respectively, 2.24%[6] and 1.3%.[30] Therefore, our results corroborate and expand Johnson's results by demonstrating apoptosis also in adult mice after sub-MAC isoflurane anesthesia. Furthermore, the results are in agreement with those from a recent study[31] that observed no long-term cognitive deficits or neurotoxicity af-

ter 4 h of isoflurane anesthesia at MAC concentrations in aged rats.

When a cell enters into apoptosis, the caspase-3 is activated and reaches a detectable peak. We choose the timing of tissue collection for the study of caspase-3 activation in accordance with the rapid clearance of apoptotic cells in the mouse brain. Xie *et al.*[32] found a considerable amount of activated caspase-3 6 h after anesthesia, a moderate activation 12 h after anesthesia, and no activation at 24 h.[32] The study of caspase-3 activation 2–3 h after the use of a potentially neurotoxic substance is common in results from other works.[33]

The tendency of increased apoptosis in the CA1 hippocampal region in mice subject to 1% isoflurane anesthesia is in line with the impaired performance of these mice in the spatial water maze task (fig. 4). The hippocampus plays a major role in spatial learning,[34] and its synaptic transmission is altered by the majority of agents used in general anesthesia.[5] The relation between spatial learning performance and distribution of receptor binding sites over hippocampal regions may provide an explanation why the CA1 region was specifically affected. Zilles *et al.*[35] found a negative correlation between the escape latency of the water maze and the density of [3H]muscimol binding sites, the ligand of the γ-aminobutyric acid A receptor on which isoflurane acts. Although [3H]muscimol binding sites were found in all hippocampal subregions, generally, the density was highest in CA1 and dentate gyrus.[35]

Processes other than apoptosis may have contributed to the water maze result, such as inflammatory processes. These have been described to influence memory acquisition during anesthesia and surgery; however, our animals did not suffer any surgical stimulus and the effects of anesthesia on inflammatory responses is not clear. There are data suggesting that anesthesia with propofol and fentanyl promotes proinflammatory immune responses,[36] that with thiopental and midazolam impair neutrophil functions,[37] whereas that with isoflurane enhances timely resolution of acute inflammation processes.[38] A study by Loop *et al.*[39] showed that sevoflurane and isoflurane induce apoptosis in T lymphocytes, increasing mitochondrial membrane permeability and caspase-3 activation in a dose-dependent manner. In this sense, and if inflammatory processes would have contributed to our results, these should promote a deficit in the 2% group and not in the 1% group as it was observed.

Some studies showed that isoflurane may be neurotoxic; however, its neurotoxicity mechanisms are not clear or fully understood. The literature indicates mechanisms related to calcium homeostasis, apoptosis activation, or age-sensitive suppression of neuronal stem cell proliferation or differentiation.[40] Studies have shown that cell death processes induced by *N*-methyl-D-aspartic acid antagonists and γ-aminobutyric acid mimetic drugs involves translocation of Bax protein to mitochondrial membranes, where it disrupts membrane permeability, allowing extramitochondrial leakage of cytochrome *c*, followed by a sequence of changes culminat-

ing in activation of caspase-3.[6,41] Because of this neurotoxicity and because isoflurane acts on the central nervous system by a reversible intoxication process, it would be expected that high concentrations provoke a greater effect on cognition. Why isoflurane at a lower concentration should have a potential noxious effect on learning ability remains unclear. At relevant clinical concentrations, isoflurane also has been reported to act as a neuroprotective agent[42] by inhibiting necrotic processes, such as in ischemia and hypoxia.[43–44] The exact mechanism leading to a reduction of neurotoxicity is not very clear, but there are several proposed mechanisms: activation of adenosine triphosphate-dependent potassium channels, up-regulation of nitric-oxide synthase, reduction of excitotoxic stressors and cerebral metabolic rate, augmentation of periischemic cerebral blood flow, and up-regulation of antiapoptotic factors, including mitogen-activated protein kinases.[45] Overall, high concentrations of isoflurane might lead to decreased cellular degeneration, causing less damage to the central nervous system. Moreover, general deep anesthesia may mimic natural slow-wave sleep that has a less disruptive effect on cognition than does light anesthesia.[46] Other potential explanations for the impairments at low rather than high anesthesia concentrations may be related to a recent proposed theory. In the developing brain, the lack of synaptic feedback may result in apoptosis as a result of failure to form synaptic connections. Likewise, nonanesthetized neurons that generate action potentials but do not receive a synaptic feedback, because the neurons they are connected with are anesthetized, may be at higher risk for cell death. So, the ratio of nonanesthetized to anesthetized neurons should be higher in less anesthetized individuals.[47] Therefore, at lower concentrations of anesthetics, more nonanesthetized neurons are surrounded by anesthetized neurons. If this situation is indeed harmful, larger apoptosis is to be expected than at a deeper level of anesthesia. This idea is supported by observations from Lenz *et al.*,[48] who showed that anesthetics may differentially affect the metabolism of different brain subregions.

The background of this study is the increasing concern over potential cognitive impairments after anesthesia[49–51] and contradictory results regarding effects of age.[14–15,52] The vast majority of studies of potential neurodegenerative effects of anesthesia were conducted in very young and/or old animals,[5–7,11,14,50] and there is an overall lack of studies in adults. Nevertheless, the basic mechanisms of neurodegeneration are similar for the different ages; the young brain and the old brain are more sensitive to chemical insults (such as anesthesia). The young brain is still in the period of brain growth in which physiologic cell death is a regular phenomenon; redundant or unsuccessful synapses and neurons are deleted by apoptosis, which enhances the capacity for plasticity and contributes to the stabilization of circuits in the developing brain system. Hence, if exposed to insult, immature neurons are more prone to trigger cell death than adult neurons.[53] The old brain, on the other hand, is more sensitive as a result of different aging processes, including reduced oxidative phosphorylation, increased frequency of mutations, and manifestation of silent genes as well as an increase of free radicals and reduced levels of free radical scavengers (vitamin C and E, melatonin).[48] Although aged and young animals are, in principle, more prone to suffer injury, this does not mean that the adult brain is not damaged by anesthetics.[52,54]

The central aim was to evaluate the effects of depth of isoflurane anesthesia on the central nervous system in young adult animals. In line with some previous experimental studies, we found that light depth of anesthesia caused a short-term impairment in spatial cognitive performance until 4 days after anesthesia compared with 2% isoflurane concentration and control, and tended to result in more apoptosis in the CA1 field of the hippocampus, 3 h after anesthesia, than high isoflurane concentration in adult mice. Although performed at different times, the association between low *versus* high concentrations of anesthesia and impairment is supported by behavioral and immunohistochemical data.

The results cannot be explained by clinical or physiologic parameters, in that the study was carried out in healthy age-matched animals not undergoing surgery, or by drug interactions, because only one anesthetic agent was administered. The physiologic parameters collected were the respiratory rate and heart rate; the measurement of other parameters such as blood pressure or arterial oxygen and carbon dioxide would imply the use of invasive techniques. However, this was practically and ethically impossible in the present experiment, because animals in the 1% treatment group were anesthetized at sub-MAC,[30] making it impossible to apply invasive techniques on them. These results suggesting that light isoflurane anesthesia may have effects in the central nervous system have not been described previously in animal models and may have important clinical implications.

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Valentim *et al.*

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ANESTHESIOLOGY REFLECTIONS

Hall's Engraving of Morton Etherizing Frost
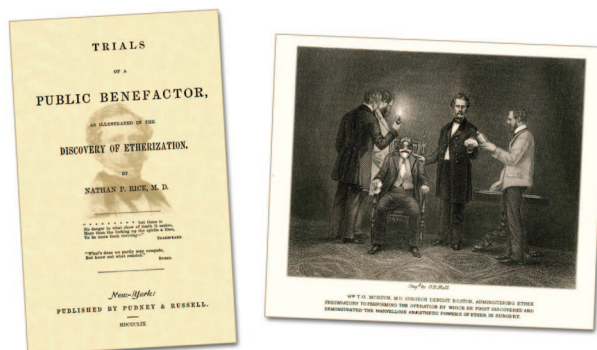
The title page (*above left*) of the 1890 edition of *Trials of a Public Benefactor*, introduces readers to Dr. Nathan P. Rice's efforts to credit dentist William T. G. Morton as the world's first surgical etherizer. The book features G. R. Hall's classic engraving (*above right*) with a caption reading "Wm T. G. Morton, M.D. Surgeon Dentist, Boston, administering ether preparatory to performing the operation by which he first discovered and demonstrated the marvellous [*sic*] anaesthetic powers of ether in surgery." That caption is not exactly accurate. Indeed, Morton's etherization of the seated merchant, Eben Frost, for dental extraction occurred on September 30, 1846, 6 yr before Morton, a medical school dropout, received his honorary M.D. from the Washington University of Baltimore, Maryland. (Copyright © the American Society of Anesthesiologists, Inc. This image appears in color in the *Anesthesiology Reflections* online collection available at www.anesthesiology.org.)

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Valentim *et al.*