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BASIC SCIENCE ARTICLE

Multiple exposures to sevoflurane across postnatal development may cause cognitive deficits in older age

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BACKGROUND: The aim of the study was to determine the effects of repeated anesthesia exposure across postnatal development.
METHODS: Seventy-two newborn Sprague–Dawley rats were randomly divided into Sev group and Con-aged group. Sev groups were exposed to 2.6% sevoflurane for 2 h on postnatal day (P) 7, P14, and P21; the Con groups only received carrier gas for 2 h. Learning and memory were evaluated using the MWM test at P31 (juvenile), P91 (adult), and 18 months postnatally (aged). The relative expression of APP and Mapt mRNA was detected by RT-PCR, while Aβ, tau, and P-tau protein levels were analyzed by immunohistochemistry.
RESULTS: After repeated inhalation of sevoflurane, MWM test performance was significantly decreased in the Sev-aged group compared to the Con-aged group ($P > 0.05$). The relative expression of APP and Mapt mRNA was not significantly different between groups in each growth period ($P > 0.05$). The tau expression in the juvenile hippocampal CA1, CA3, and dentate gyrus regions increased markedly in the Sev group, while P-tau only increased in the hippocampal CA3 region in the Sev-adult group. The expression of tau, P-tau, and Aβ in the hippocampal regions was upregulated in the Sev-aged group.
CONCLUSIONS: Multiple exposures to sevoflurane across postnatal development can induce or aggravate cognitive impairment in old age.

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IMPACT:

- Whether multiple sevoflurane exposures across postnatal development cause cognitive impairment in childhood, adulthood, or old age, as well as the relationship between sevoflurane and the hippocampal Aβ, tau, and P-tau proteins, remains unknown.
- This study's results demonstrate that multiple exposures to sevoflurane across postnatal development do not appear to affect cognitive function in childhood and adulthood; however, multiple exposures may lead to a cognitive function deficit in old age.
- The underlying mechanism may involve overexpression of the tau, P-tau, and Aβ proteins in the hippocampus.

INTRODUCTION

Sevoflurane, a new type of inhalation anesthetic with quick inspiration, rapid induction, fine controllability, and an aromatic flavor, is widely used in pediatric anesthesia. However, the widespread use of sevoflurane has gained attention due to its reported association with neurotoxicity in the developing mammalian brain.[1,2] Mounting evidence indicates that sevoflurane and other inhaled anesthetics may be more likely to induce learning and memory deficits than intravenous anesthetics.[3]

One survey[4] indicates that one in seven children is exposed to at least one episode of general anesthesia by the age of 3 years and most of them receive sevoflurane anesthesia. Previous studies[5,6] have investigated the effects of sevoflurane on the cognitive function of neonatal and adult mice, respectively. The results showed that sevoflurane anesthesia can render neonatal mice more vulnerable to the development of Tauopathy and cognitive impairment. However, whether multiple sevoflurane exposures across postnatal development cause cognitive impairment in childhood, adulthood, or old age, as well as the relationship between sevoflurane and the hippocampal Aβ, tau, and P-tau proteins, remains unknown. In addition, whether sevoflurane exposure across postnatal development is a risk factor for the induction or aggravation of cognitive impairment in the elderly is unknown. Therefore, this study assesses learning and memory abilities at different growth stages after multiple sevoflurane exposures across postnatal development in rat models. This study also assesses the changes in hippocampal Aβ, tau, and P-tau expression at both the gene and protein levels to investigate repeated anesthesia exposure across postnatal development.

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MATERIALS AND METHODS

Animal resources

Pregnant rats were provided by the Experimental Animal Centre of Chongqing Third Military Medical University (SCXK 2012-0005). The current study (project no. 81360180) was approved by the Animal Experimental Ethics Committee of Zunyi Medical College, Zunyi, China on January 20, 2014.

A total of 72 newborn male and female Sprague–Dawley rats were randomly divided into two groups: the sevoflurane group (Sev group) and the control group (Con group). Each group was divided into three subgroups: juvenile, adult, and aged. The neonatal rats were housed with their mothers until postnatal day 21 (P21). Thereafter, the rats were housed in separate cages and fed with standard rat chow and tap water, at an ambient temperature of 22–24 °C, with a 12 h day/12 h night cycle.

Anesthetic treatment

All subjects were placed in a plastic, transparent anesthesia chamber (Guizhou Key Laboratory of Fundamental Research on Anesthesiology and Organ Protection) that was connected to an anesthesia device (Fabius Anesthetic Apparatus, Drägerwerk AG & Co, Lübeck, Germany), and both the inlet and the outlet were connected to a gas monitor (Vamos Variable Anesthetic Gas Monitor, Drägerwerk AG & Co). In the Sev groups, anesthesia was achieved with inhalation of 2.6% sevoflurane for 2 h on P7, P14, and P21; the carrier gas was oxygen 1 L/min and air 1 L/min. The Con groups only received carrier gas for 2 h. During anesthetic exposure, the rats were kept warm on an insulation blanket heated to 26–28 °C. Since it was necessary to prevent anoxia and carbon dioxide retention (leading to acid–base disturbances over the long duration of general anesthesia in the immature rats), soda lime (Intersurgical, UK, 1181619) was laid on the bottom of the chamber to absorb carbon dioxide, and a cotton pad was put on top to avoid burning the rats. The color of the rat's skin was observed during inhalation, and oxygen saturation was measured in six rats at random. Measurement intervals were every 30 min by pulse oximeter (Nonin Medical INC, 2500A).

After recovery from anesthesia, the rats were placed back in their original cages and maintained until the juvenile stage (P31–P37), adult stage (P91–P97), or aged stage (18 months old), at which points their behavior was assessed using the Morris water maze (MWM) test.

Morris water maze (MWM) test

The MWM test included three different assessments: acclimatization training, the place navigation test, and the spatial probe test. All trials were performed in a quiet room with indirect lighting. The apparatus was a circular black pool (diameter: 120 cm, height: 60 cm, and depth: 32 cm) that was filled with water (24–26 °C). To avoid the visual location of the platform and to obtain contrast for animal detection by a camera, 500 g of skim milk was dissolved in the water. The pool was divided into four identical quadrants, and a removable platform (diameter: 15 cm and height: 30 cm) was located underwater at the center of the fourth quadrant. A camera and lighting system was installed over the pool for data collection. Collected data were stored in a computer.

Acclimatization training

All rats were subjected to acclimatization training at P31, P91, and 18 months, for 1 day each time. Each rat was placed at a random position in the pool and was allowed to swim for 120 s. The aim of this was to adapt the rats to the environment and eliminate those who could not swim.

Place navigation test

Rats were subjected to the place navigation test for 5 consecutive days on the day after the acclimatization training. The rats were placed in the pool from different quadrants for each trial. In each trial, each rat was allowed to swim for 120 s until it climbed onto the platform. If a rat failed to locate the platform within this time, it was guided to the platform and allowed to remain there for 30 s. The time it took the rats to find the hidden platform from the point of entering the water was recorded as the escape latency.

Spatial probe test

In the spatial probe test, the platform was removed, and rats were placed in the quadrant opposite the original position of the platform and allowed to swim for 120 s. The percentage of time spent in the original platform quadrant was recorded as the retention time, and the number of times the rat entered into the original platform area was recorded. The rats were placed back into their heated cages following the completion of the swimming tests.

Immunohistochemical analyses

Six rats were randomly selected from each group and euthanized by intraperitoneal injection of 2% pentobarbital sodium (40 mg/kg) immediately after each MWM test. The brain was dissected and transferred into 4% paraformaldehyde solution and then embedded in paraffin after 24 h of fixation. Afterward, seven 2-μm-thick paraffin sections were prepared from each animal, using a microtome (Leica RM 2235, Wetzlar, Germany). A total of 252 slices were dried at 60 °C in an electrothermal incubator for 2 h. The expression of Aβ, tau, and P-tau proteins in hippocampal tissue was evaluated by immunohistochemistry using streptomycin–peroxidase. Beta-amyloid antibodies (Novus, America, NBP2-13075), anti-Tau antibodies (Abcam, England, ab32057), and anti-Tau (phospho S396) antibodies (Abcam, England, ab109390) were used in this study. The areas and integral optical densities of the hippocampal CA_1, CA_3, and dentate gyrus (DG) regions in serial sections taken from the same area were measured in microns using the image analysis software (Image-Pro plus 6.0; Media Cybernetics Inc., Rockville, MD). The average optical densities of the Aβ, tau, and P-tau proteins in the region as a reflection of expression levels were calculated and compared among the groups.

Real-time polymerase chain reaction

For real-time polymerase chain reaction (RT-PCR) analysis of the gene expression of the amyloid protein precursor (APP) and the microtubule-associated protein tau (Mapt), hippocampal samples were obtained as they were for the immunohistochemical analyses. After weighing, each sample was preserved with 500 mL of RNAriso (TaKaRa, Japan, AK9801) plus solution at −80 °C. The total RNA was extracted from frozen hippocampal tissue samples and the purity of the isolated RNA in each case was verified by the OD 260/280 ratio, spectrophotometrically. Then a RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA) was used to produce cDNA at 37 °C for 15 min, 85 °C for 5 s, and hold at 4 °C. Afterward, the material was PCR-amplified using an SYBR Green I Master kit and CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA), using the primers listed. The amplification conditions were as follows: initial denaturation at 95 °C for 30 s, followed by 40 cycles each consisting of 95 °C for 35 s and 60 °C for 30 s. Relative quantification of gene expression against β-actin was achieved using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

SPSS Statistics 20 (SPSS, Chicago, IL) was used to analyze the data. All data were expressed as mean ± standard deviation (SD). Repeated-measures analysis of variance was used for comparison of repeated measurement data in the MWM test among the groups. Comparisons of measured data among the groups were completed using one-way analysis of variance and least-significant difference analysis after a homogeneity test of variance. P values < 0.05 were considered statistically significant.

RESULTS

Behavior test results in different growth stages after multiple sevoflurane exposures across postnatal development

The weight of the rats increased with age, and there was no statistically significant difference between the groups in any of the growth periods (Fig. 1). All rats breathed steadily, no cyanosis was found, and the oxygen saturation held firm between 96 and 100% during sevoflurane inhalation. No rat was unable to swim in the acclimatization training, and no rat died during the MWM tests.

As shown in Fig. 2, after subjecting rats to 5 days of place navigation testing, the escape latency tended to shorten gradually in both groups over time. Compared with the first day, the escape latency over the next 4 days was significantly shorter in both the Sev group ($^{*}P < 0.05$) and the Con group ($^{\#}P < 0.05$). In the Sev-adult group, the escape latency on the second day was longer than that on the fifth day ($^{\dagger}P < 0.05$) while the escape latency on the second day was longer than that on the fourth and fifth days in the Con-adult group ($^{+}P < 0.05$). There were no further significant differences in latency over the next few days in both

the Sev and Con groups ($P > 0.05$). These results indicate that all rats had formed a stable spatial reference memory. There were no obvious inter-group differences among the different growth stages ($P > 0.05$).

In the spatial probe test (Fig. 3), there were no significant differences in the number of crossings over the quadrant originally containing the platform and the percentage of original platform quadrant retention time between the Con- and Sev-juvenile groups ($P > 0.05$) or adult groups ($P > 0.05$). However, both the number of crossings and the retention time were significantly shortened in the Sev-aged group when compared with the Con-aged group ($P < 0.05$).

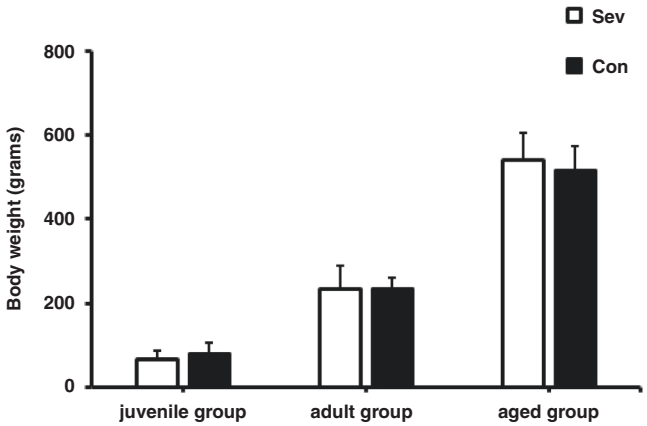

Fig. 1 The weight of rats increased with age; there was no statistically significant difference between groups in any of the growth stages. Error bars are standard deviation.

Effects of multiple exposures to sevoflurane across postnatal development on APP and Mapt levels

The amplification curve and corresponding melting curve of each index of the RT-PCR suggested that the Ct value was reliable. There was no significant difference in the relative expression of APP and Mapt mRNA between the groups in each growth period ($P > 0.05$, Fig. 4).

Effects of multiple exposures to sevoflurane across postnatal development on Aβ, tau, and P-tau protein levels

Immunohistochemical staining showed that the cells in the CA_1, CA_3, and DG regions of the hippocampus were tightly arranged, with morphological and structural integrity, and the blue-stained nuclei were round or oval under a high-power microscope. Tan-stained Aβ protein was expressed in the nucleus and cytoplasm, brown-stained tau protein was widely distributed in the cytoplasm and dendrites, while pale, brown-stained P-tau protein was distributed only in the nuclei (Fig. 5).

In the juvenile and adult stages, the average optical density of Aβ in various regions of the hippocampus was not significantly different between the Sev and Con groups ($P > 0.05$; Fig. 6Ia, b); however, it was significantly increased in the CA_1 and DG regions in the Sev-aged group when compared with the Con-aged group ($P < 0.05$, Fig. 6Ic). Tau protein expression in the CA_1, CA_3, and DG regions was markedly increased in the Sev groups compared with

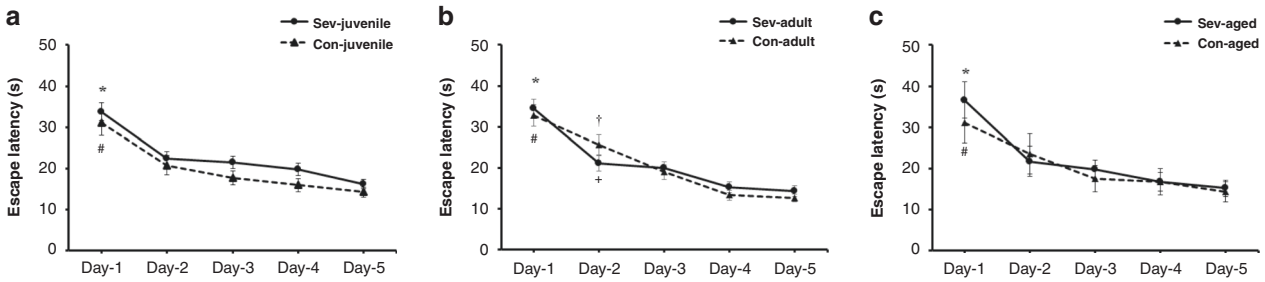

Fig. 2 The escape latency in the Morris water maze test tended to shorten gradually in both groups over time. a Place navigation test in juvenile. **b** Place navigation test in adult. **c** Place navigation test in aged. Compared with the first day, the escape latency over the next 4 days was significantly shortened within both groups ($^{*,\#}P < 0.05$); in Sev-adult group, the escape latency on the second day was longer than that on the fifth day ($^{\dagger}P < 0.05$) while the escape latency on the second day was longer than that on the fourth and fifth days in the Con-adult group ($^{+}P < 0.05$); no significant difference was observed for the remaining days in both the groups ($P > 0.05$); There were no obvious inter-group differences among the different growth stages either ($P > 0.05$). Error bars are standard deviation.

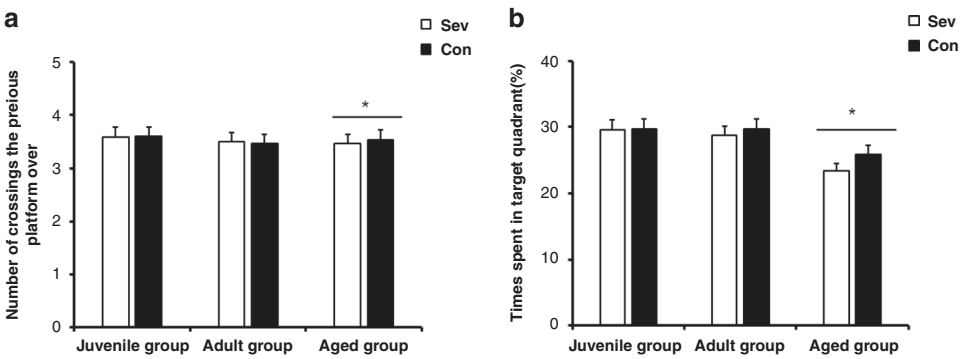

Fig. 3 The result of spatial probe test. There were no significant differences in **a** the numbers of crossings over the original of platform-containing quadrant or **b** the retention time (percentage of time spent in the quadrant originally containing the platform) between the Con-juvenile group and the Sev-juvenile group ($P > 0.05$). In addition, there was no significant difference between adult groups ($P > 0.05$). However, both the number of crossings and the retention time were significantly less in the Sev-aged group than in the Con-aged group ($^{*}P < 0.05$). Error bars are standard deviation.

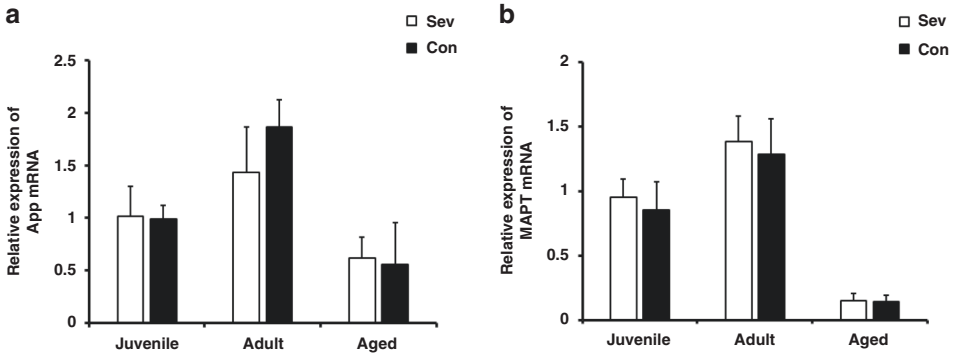

Fig. 4 The relative expression of APP and Mapt mRNA in hippocampal. The relative expression of **a** APP and **b** Mapt mRNA was not significantly different between groups in each growth period ($P > 0.05$). Error bars are standard deviation.

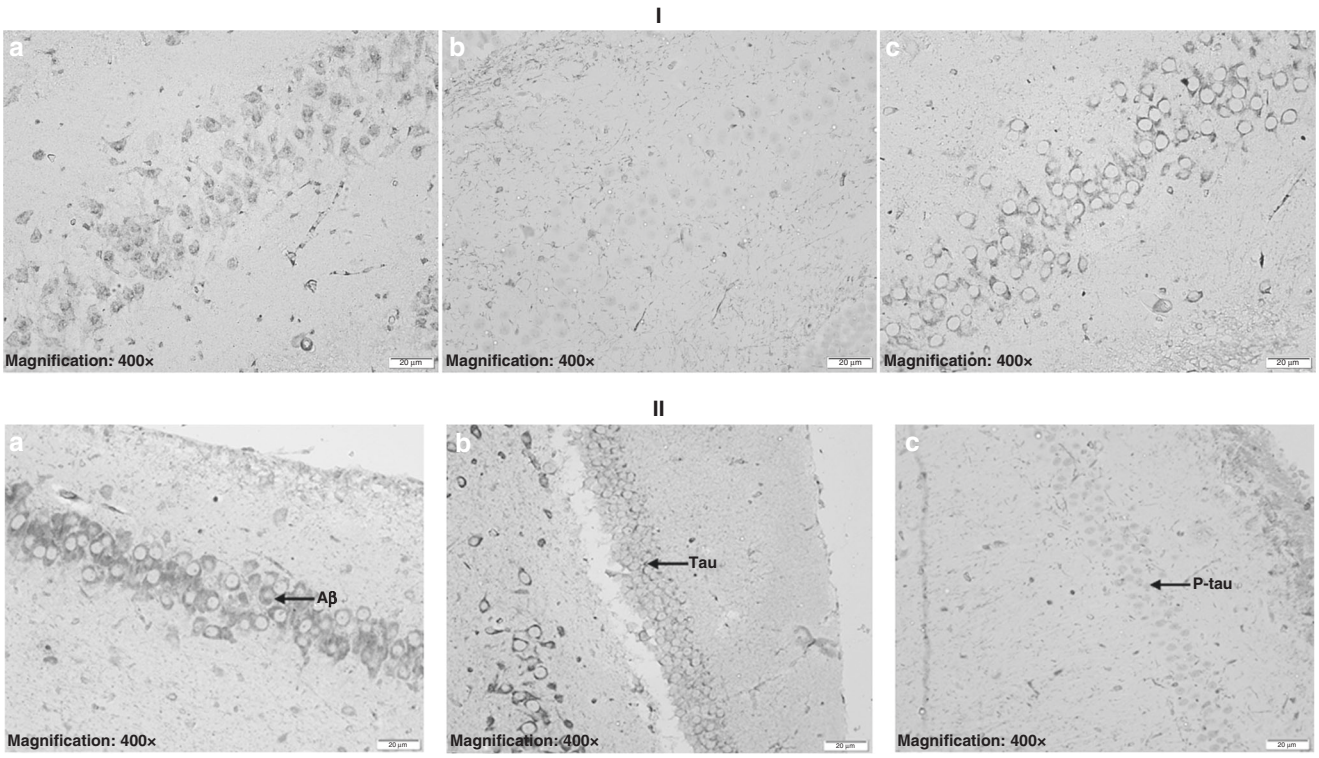

Fig. 5 Immunohistochemical techniques of aβ, tau, and P-tau in hippocampus. I: **a** is Aβ in CA3 area, **b** shows p-tau in CA3 area, and **c** shows tau in CA3 area. II: immunohistochemical staining of **a** Aβ, **b** tau, and **c** P-tau in the hippocampus. Aβ protein was expressed in the nucleus and cytoplasm, tau protein was expressed in the cytoplasm and dendrites, while P-tau protein was only distributed in the nucleus. Scale bars 20 μm.

the Con groups in the juvenile and aged stages ($P < 0.05$, Fig. 6IIa–c), but there were no significant differences between the Sev-adult and Con-adult groups in any of the hippocampal regions ($P > 0.05$, Fig. 6IIb). In addition, the average optical density of P-tau was significantly increased in all regions in the Sev-aged group compared with the Con-aged group ($P < 0.05$). In addition, the average optical density of P-tau in the Sev-adult group increased when compared with the Con-adult group only in the CA_3 region ($P < 0.05$); there was no significant difference between groups in the juvenile stage ($P > 0.05$, Fig. 6III).

DISCUSSION

With the widespread application of sevoflurane in pediatric anesthesia,[7] the potential accompanying neurotoxicity and cognitive impairment has been the subject of much speculation, particularly in the context of infants whose brains are in a stage of rapid growth and development.[8,9] A previous study[10] found that multiple exposures to general anesthesia before the age of 3 years were associated with an increased risk of cognitive impairment, including learning disabilities and attention deficit; However, it is unclear whether the cause is the mode of anesthesia or anesthetic drugs. The peak neurotoxicity associated with general anesthesia occurs during rapid neurodevelopment and synaptogenesis.[11] In humans, this period ranges from the third trimester to several years postnatally, and up until P14 in rats;[12] in this study, rats were exposed to sevoflurane inhalation on P7, P14, and P21, so the current study involves only two exposure during peak synaptogenesis and one beyond that timing. The concentration of sevoflurane was 2.6%, based on minimum alveolar concentration values for neonatal rats and clinical application.[13–15] In addition, to avoid the effect of high-concentration oxygen[16] or hypothermia[17]

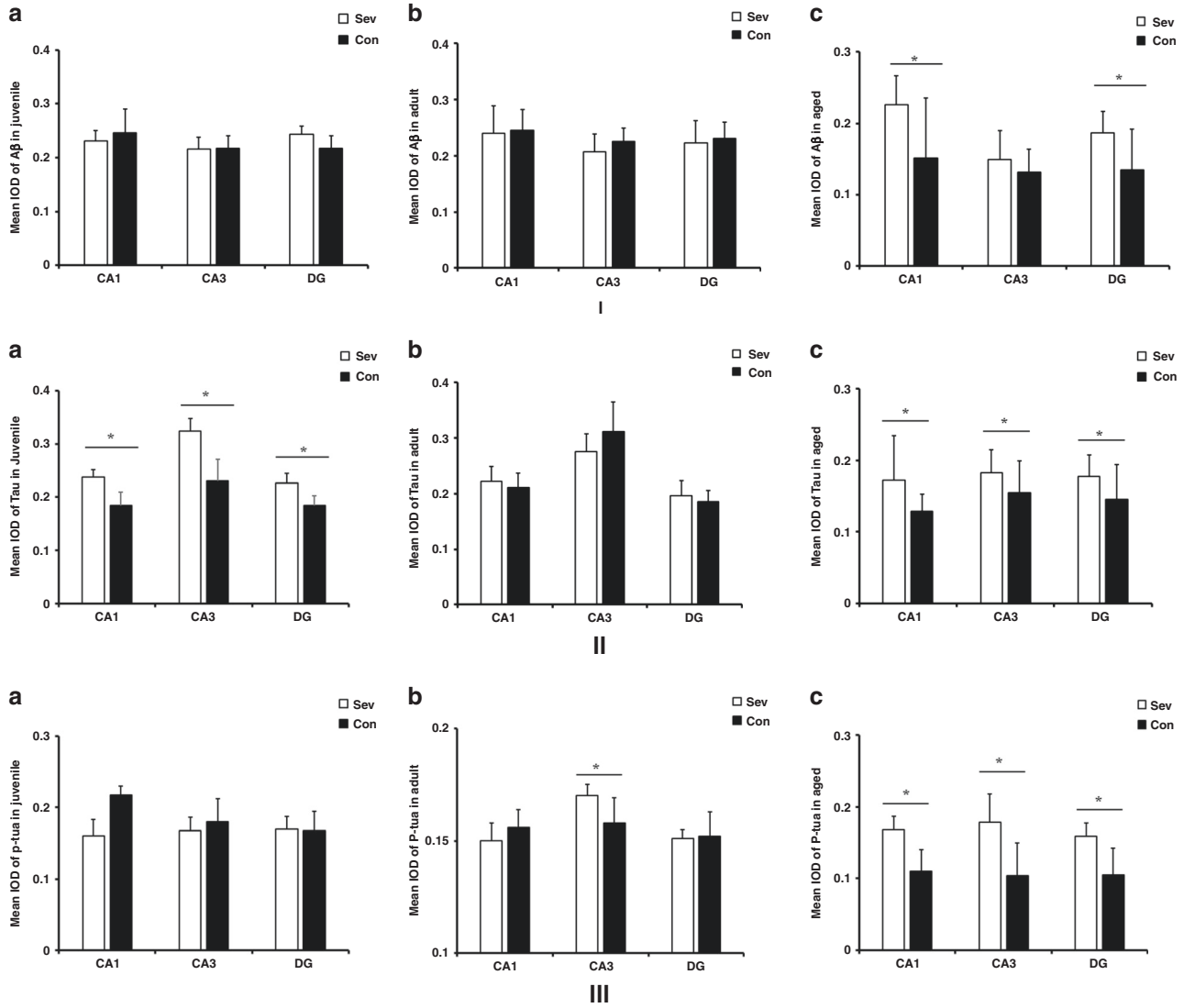

Fig. 6 The average optical density of Aβ, tau and P-tau in various regions of hippocampus. I: the average optical density of Aβ in various regions of hippocampus. There was no significant difference between groups in the **a** juvenile and **b** adult stages. **c** Aβ was significantly increased in the CA1 and dentate gyrus (DG) regions in the Sev-aged group (*$P < 0.05$). Error bars are standard deviation. II: the average optical density of tau protein in various regions of the hippocampus: it increased markedly after multiple exposures to sevoflurane in infancy in the **a** juvenile and **c** aged stages (*$P < 0.05$), but there was no significant difference between the **b** Sev-adult group and Con-adult group ($P > 0.05$). Error bars are standard deviation. III: the average optical density of P-tau protein in various regions of the hippocampus: there was no significant difference in the average optical density of P-tau between groups **a** in the juvenile stage ($P > 0.05$). **b** The Sev-adult Group showed an increase in P-tau only in the CA3 region, as compared to the Con-adult group (*$P < 0.05$). **c** The average optical density of P-tau was significantly increased in all regions of the hippocampus in the Sev-aged group as compared to the Con-aged group (*$P < 0.05$). Error bars are standard deviation.

on learning and memory, oxygen 1 L/min and air 1 L/min were used as the carrier gases and the temperature of the anesthesia chamber was kept constant.

The MWM test is commonly used to evaluate cognitive function in rodents. Various test protocols are available for assessing cognitive function.[18] The place navigation test and spatial probe test are the most classic experimental methods used with the MWM and, respectively, represent learning and memorizing abilities in rodents. In this study, the weight of the rats within each growing period was not statistically significantly different between groups. This ensured that there were no motor ability differences between the groups at each stage. In addition, all rats were able to swim during the acclimatization training, removing any confounding influences. In the place navigation test, over time, this study found that the search strategy for each group of rats changed from blind, inefficient, and random searching to a more straight-line type of search, indicating that the rats gradually developed a stable spatial learning ability.

The escape latency in the place navigation test reflects the ability for spatial information gathering; increased escape latency has a high correlation with impaired ability. However, in this study, the escape latency between the Sev group and the Con group showed no significant difference, suggesting that multiple exposures to sevoflurane across postnatal development do not damage long-term declarative memory.

The spatial probe test is mainly used to assess the memory retrieval capacity; it reflects the extraction of the memory information built up in the navigation experiment. Data collected during the test demonstrated that repeated sevoflurane inhalation across postnatal development led to a significant decrease in the

number of entries into the original platform area and the percentage of time spent in the quadrant originally containing the platform quadrant when the rats had reached old age. In childhood and adulthood, however, all the rats exhibited a normal spatial memory.

Given the differences in the results of the place navigation test and the spatial probe test, the results indicate that repeated sevoflurane anesthesia across postnatal development may affect spatial association and recall exploring ability (rather than causing a declarative memory deficit) in the elderly. In turn, this may be a triggering factor for cognitive impairment in the elderly.

The hippocampus is an important structure associated with memory and learning.[19,20] It is divided into the CA_I, CA_3, and DG regions according to cell morphology and function. Studies have shown that partial or complete loss of memory is closely related to hippocampal damage and that a possible mechanism is excessive accumulation of Aβ and hyperphosphorylation of the tau protein.[21] Therefore, changes in the Aβ, tau, and P-tau protein levels and the corresponding genes in the CA_I, CA_3, and DG regions were detected in this study by immunohistochemistry and RT-PCR, respectively. It was found that multiple exposures to sevoflurane across postnatal development only increased the expression of tau protein in the hippocampus of juvenile rats; thereafter, P-tau increased in the hippocampus CA_3 region in the Sev-adult group. However, there were no differences in Mapt or APP mRNA (or in MWM performance) between age-matched groups. Based on the results, this study concludes that repeated sevoflurane inhalation across postnatal development led to a transient increase in tau protein expression but not sufficiently enough to influence cognition. This observation agreed with the results of Ozer et al.[22] Another study also studied the effect of sevoflurane on Tau protein expression in neonatal mice and found that repeated exposures to sevoflurane induce brain Tau phosphorylation in neonatal but not in adult mice, and induced cognitive impairment in the MWM test in newborn mice, which is contrary to our results.[6] In old age, the expression of tau, P-tau, and Aβ proteins in the rat hippocampal regions was upregulated in the Sev group while the rats' performance in the spatial probe test demonstrated a significant decrease. Therefore, it is proposed that multiple exposures to sevoflurane across postnatal development may lead to cognitive deficits during old age (i.e., sevoflurane anesthesia across postnatal development may induce or aggravate cognitive impairment in old age). There was no significant difference found in the expression of APP and Mapt mRNA between groups, and the effect of sevoflurane on tau and P-tau expression was more marked than the effect on Aβ expression. This result is in contrast to the view that tau may lie downstream of APP/Aβ;[23] nevertheless, these proteins may form part of a crucial, converging pathway.

The current study did have limitations. First, arterial oxygen saturation was not evaluated during the period of inhalation because the rats were too small to draw enough blood samples. Although no significant decrease in oxygen saturation and no bluish skin tinge was observed during inhalation, some rats may have had hypoxia. Second, the concentration of sevoflurane was chosen as it was in a frequently used safe range. It remains unknown whether lower or higher concentrations will lead to other changes.

CONCLUSIONS

This study's results demonstrate that multiple exposures to sevoflurane across postnatal development do not appear to have an effect on cognitive function in childhood and adulthood; however, multiple exposures may lead to a cognitive function deficit in old age. Therefore, sevoflurane exposure across postnatal development may be a risk factor that induces or aggravates cognitive impairment in the elderly. The underlying mechanism may involve overexpression of the tau, P-tau, and Aβ proteins in the hippocampus.

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AUTHOR CONTRIBUTIONS

Participated in experimental manipulation and manuscript writing: Y.Z.; data analysis and interpretation: C.Z.; Participated in experimental manipulation, collection and assembly of data: Y.W. C.T. J.R. and M.W.; Drawing Images and final approval of undefined manuscript: D.L. Conception, design and administrative support: Z.Z.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This study was conducted in accordance with the Declaration of Helsinki and with approval from the Animal Ethics Committee of Zunyi Medical College, Zunyi, China on January 20, 2014 (Project No. 81360180), Animal Ethics Committee of Zunyi Medical University, Zunyi, China on March 11, 2019 (No. (2019)2-044), Animal Ethics Committee of Zunyi Medical University, Zunyi, China on February 18, 2021 (No. (2021) 2-172).

ADDITIONAL INFORMATION

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