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Sevoflurane induces temporary spatial working memory deficits and synaptic ultrastructure impairments in the hippocampus of neonatal rats

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Abstract. – OBJECTIVE: Exposure to volatile anesthetics in neonatal rats could induce neurotoxicity, learning deficits and abnormal social behaviors. The aim of this study is to investigate the potential neurotoxicity induced by sevoflurane.

MATERIALS AND METHODS: Postnatal day 7 (P7) Sprague-Dawley (SD) rats were continuously exposed to 2% sevoflurane plus 40% oxygen/air for 2 h. We used Morris water maze (MWM) to examine subsequent neurobehavioral performance. Transmission electron microscopy (TEM) was used to observe the histopathological changes in the hippocampus.

RESULTS: Neonatal exposure to 2% sevoflurane for 2 hours impaired short-term spatial working memory but not reference memory at P25. It induced synaptic ultrastructure impairments in the CA3 region of hippocampal, including fewer numbers of synapses, thinner thickness of postsynaptic dense, broader synaptic cleft width and smaller synaptic curvature. Our results also showed that all synaptic ultrastructure impairments and neurocognitive deficits had almost completely recovered at P53.

CONCLUSIONS: We showed that a single sevoflurane exposure to neonatal rats led to temporary spatial working memory deficits. It might be associated with synaptic ultrastructure impairments in the CA3 region of the hippocampus, including fewer numbers of synapses, thinner thickness of PSD and broader synaptic cleft width. Fortunately, all the neurotoxicity and neurocognitive deficits were reversible.

Key Words:
Sevoflurane anesthesia, Hippocampus, Synaptic ultrastructure, Working memory, Reference memory.

Introduction

Sevoflurane is one of the most commonly used volatile anesthetics during infants and children because of its low blood gas partition coefficient, without irritating the airway and rapid onset and offset[1]. A retrospective epidemiological study showed that early exposure to anesthesia and surgery could increase risks of neurocognitive impairments[2]. Similarly, several studies[3,4] revealed that exposure to sevoflurane in neonatal rats could induce neurotoxicity, learning deficits and abnormal social behaviors. These results exhibited us that sevoflurane might be detrimental to the developing brain. However, it is still unclear whether the neurotoxicity is permanent or not; the impaired nerve cell components are also ambiguous.

Synapses played a prominent role of almost all intercellular communication, matured 3 weeks later during the postnatal period in rats[5]. Synaptic plasticity in the CA3 region of the hippocampus, which requires normal synaptic transmission and release of neurotransmitters, is closely associated with learning and memory[6]. Normal and stable ultrastructure is essential for both synaptic plasticity and memory formation. The postsynaptic density (PSD), an electron-dense thickening of the postsynaptic membrane in glutamatergic excitatory synapses, participates in a signal process *via* glutamate receptor channels and some associated signaling proteins. In contrast, little PSD exists in inhibitory synapses[7].

We designed this study to investigate the neurotoxicity induced by sevoflurane. Neonatal rats were exposed to 2% sevoflurane for 2 hours. The morphological changes of synaptic ultrastructure in the CA3 region of the hippocampus were observed by electron microscope. In addition, we recorded neurobehavioral performance to evaluate the cognitive dysfunction induced by sevoflurane.

Materials and Methods

Animals

This study was approved by the Animal Ethics Committee of the Shandong University Animal

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Center. The Sprague-Dawley (SD) rats (all male) used in this study were provided by the Experimental Animal Center of the Shan Dong University. All the rats were housed under controlled illumination (12 h of light/dark, with light from 07:00 to 19:00), room temperature (22 ± 2°C) and room humidity (55 ± 5%) with free access to food and water.

Sevoflurane Exposure

Rats at postnatal day 7 (P7, 15-16 g) were randomly divided into the control group (n = 12) and the sevoflurane group (n = 12). The sevoflurane group was continuously exposed to 2% sevoflurane plus 40% oxygen/air for 2 hours in the anesthetizing chamber. The temperature in the sealed chamber was maintained at 38°C with a heating pad. Respiratory frequency and skin color of the rats were monitored during anesthesia. The pups were returned to their dams after righting reflex recovered. The control group was inhaled 40% oxygen/air in a similar chamber.

Morris Water Maze (MWM) Test

The MWM was used to test spatial working memory and reference memory in P21-P25 rats and P49-P53 rats. The MWM apparatus (smart 2.5; Shenzhen Rwd Life Science Co., Ltd., Shenzhen, China) used in the study consisted of a circular, light-blue swimming pool with dimensions as follows: diameter 120 cm; wall height 50 cm. It was filled with tap water to a depth of 30 cm. The water temperature was carefully maintained at 23 ± 2°C. The pool was divided into four quadrants (North-West (NW), North-East (NE), South-West (SW), and South-East (SE)) according to the Super Maze system to quadrisect the pool. A removable squared escape platform (10 cm×10 cm) was positioned in the SE zone, with the center 30 cm away from the wall and 1 cm below the level of the water, to be invisible to the swimming rat. The pool was placed in an experimental room lit by bright white light (about 200 lux). An automatic video tracking system (SuperMaze Video Tracking System, Shanghai, China) recorded animal movements in the pool, which provided the escape latency, path length and the time spent in each quadrant. Animals were submitted to the following training protocol according to a protocol described by Plescia et al[8]. Place learning with multiple trials (days 1-4): place learning consisted of training the rats to escape from the water by reaching a hidden platform placed in the SE zone where it was maintained throughout the experimental session. Rats were placed into the pool facing the walls of each quadrant, in the following starting point order: SW, NW, NE and SE. Each animal underwent four trials per day over four days consecutively, and was allowed to swim until the escape platform was found (escape latency), for a maximum of 120 s. When the platform was reached, they stayed for 15 s. The rats did not find the platform within the required time would be guided to the platform and remain for 15 s to strengthen memories. Animals were returned to their home cages and briefly warmed under a heating lamp during the 5 min inter-trial intervals. The parameters were recorded as follows: escape latency (s) as a measure of acquisition and retrieval of the spatial information necessary to reach the platform location, and path length (m) as an additional element in search strategies. Trial duration, reinforcement time on the platform and other experimental conditions were as before. Probe (day 5): animals completed the 4-day place-learning task were placed in the pool for 120 s on the fifth day without the goal platform and the number of platform crossings was recorded. When rats finished the MWM test, they were killed to prepare histological specimens.

Transmission Electron Microscopy (TEM)

Rats (n = 4) in each group were killed respectively for transmission electron microscopy observation at P8, P25 and P53. Animals were anesthetized with 10% chloral hydrate 4 mL/kg by intraperitoneal injection and transcardially perfused with saline through the left cardiac ventricle until the liver turned white and clear liquid outflowed right auricle. Then, the animals were perfused with 4% paraformaldehyde for 25-30 min until the liver hardened. The hippocampus was removed and cut into 1 mm^3 tissue pieces. The samples were rinsed in cold Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA) and placed in 2.5% glutaraldehyde at 4°C for 2 h. The tissue was rinsed in buffer and post-fixed with1% osmium tetroxide for 2 h. Then, the tissue was rinsed with distilled water before undergoing a graded ethanol dehydration series and was infiltrated using a mixture of half acetone and half resin overnight at 4°C. The tissue was embedded 24 h later in resin and cured fully, in turn, as follows: 37°C overnight, 45°C for 12 h and 60°C for 24 h. After that, 70-nm sections were cut and stained with 3% uranyl acetate for 20 min and

0.5% lead citrate for 5 min. Ultrastructure changes of synapses in the hippocampus were observed under TEM (Philips, EM208S, Eindhoven, Netherlands). We counted the number of synapses per 100 μm^2 and observed the ultrastructure, which included synaptic cleft width, pre- and postsynaptic density. To do this, we quantitatively analyzed the experimental results by the Imaging J Software (NIH, Bethesda, MD, USA).

Statistical Analysis

All of the data are expressed as the mean ± standard deviation, and we performed the statistical tests using Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA). The comparison between groups was made using One-way ANOVA test followed by Post-Hoc Test (Least Significant Difference). Escape latency from MWM training test between groups was analyzed by a repeated measure two-way ANOVA. Zone time, platform crossing, escape latency and path length from MWM probe test between groups were analyzed by an unpaired *t*-test. $p < 0.05$ was considered to be statistically significant.

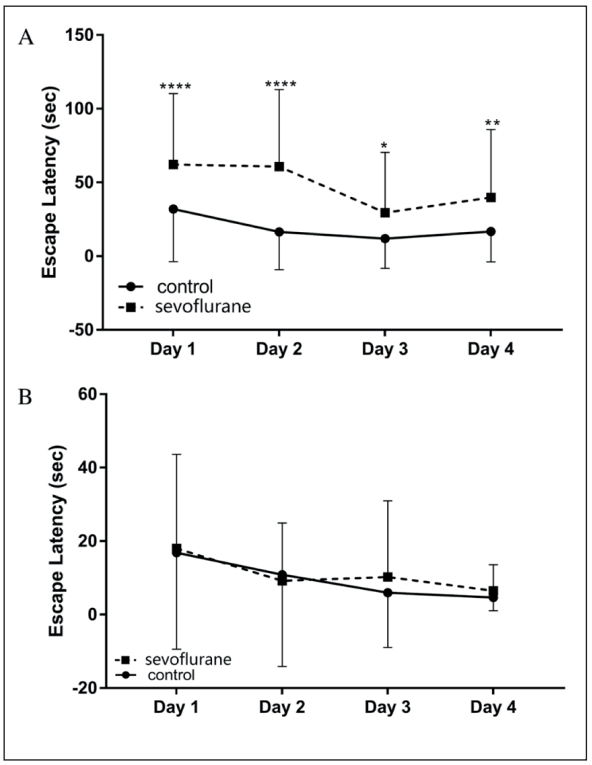

Figure 1. Effects of neonatal sevoflurane exposure at P7 on spatial working memory ability of P21-24 rats ***A***, and P49-52 rats ***B***, in Morris water maze test ($n = 4$ per group). Place navigation trials were performed to examine rats' learning ability and measure rats' spatial information acquisition by the escape latency. The data showed the time (sec) which rats required to find the platform from different quadrants.

Results

Single Exposure to Sevoflurane Impaired Short Spatial Working Memory but Not Reference Memory in Juvenile Rat

During place navigation in P21-P24 rats, the MWM test data showed that the escape latency in the sevoflurane group was significantly longer than the control group ($p < 0.0001$, $p < 0.0001$, $p< 0.0379$, $p < 0.0028$; Figure 1A). In the spatial probe test on P25 rats, an unpaired *t*-test showed that there was no significant difference between the two groups in target quadrant residence time, the number of platform crossings, the escape latency and swimming distance ($p = 0.5617$, $p = 0.3602$, $p = 0.6091$, $p = 0.2896$; Figure 2).

Single Exposure to Sevoflurane Did Not Impair Spatial Learning or Memory in Adult Rat

During place navigation in P49-P52 rats, the MWM test data showed that the escape latency did not differ significantly between the sevoflurane and control groups ($p = 0.9949$, $p = 0.9813$, $p = 0.6071$, $p = 0.9721$; Figure 1B). In the spatial probe test on P53 rats, the unpaired *t*-test showed that there was no significant difference between the two groups in target quadrant residence time, the number of platform crossings, the escape latency and swimming distance ($p = 0.6502$, $p = 0.2133$, $p = 0.7082$, $p = 0.9166$; Figure 3).

Single Exposure to Sevoflurane Induces Synaptic Ultrastructure Impairments in the CA3 Region of the Hippocampus

The synaptic ultrastructure in the CA3 region of the hippocampus was examined using the TEM. Compared to the control group, the sevoflurane group rats showed a fewer number of synapses, thinner thickness of PSD, broader synaptic cleft width and smaller synaptic curvature at P8 (Table I, Figure 4). Compared to the control group, the sevoflurane group rats showed fewer numbers of synapses, thinner thickness of PSD and broader synaptic cleft width at P25. All the differences were statistically significant ($p < 0.05$; Table I, Figure 5). Compared to the control group, the sevoflurane group rats showed that there was no significant difference about

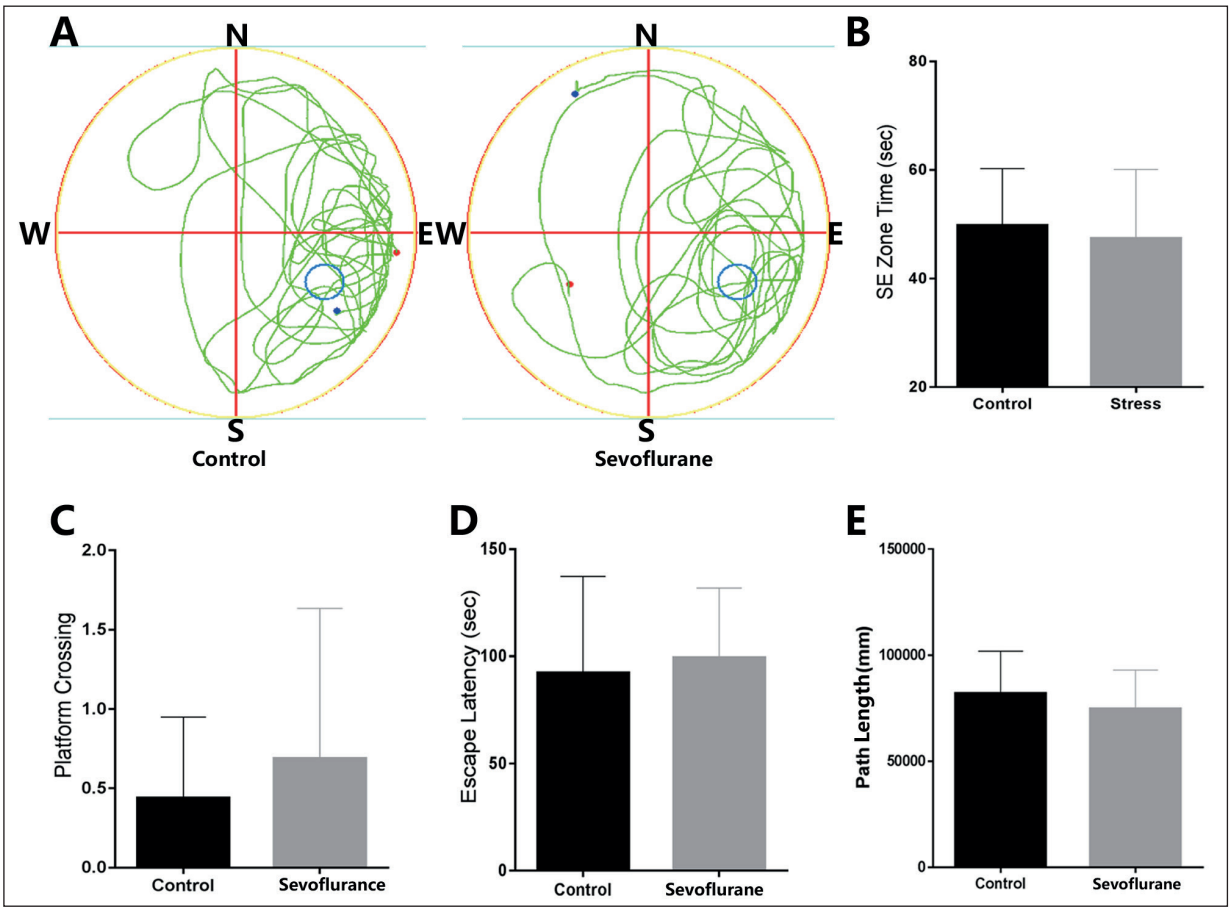

Figure 2. Effects of neonatal sevoflurane exposure at P7 on spatial reference memory ability of P25 rats in Morris water maze test (n = 4 per group). Spatial probe trials were performed to examine rats' spatial reference memory ability. The data showed the representative swimming path ***A***, the residence time (sec) on target quadrant ***B***, the number of platform crossing ***C***, the escape latency to find the hidden platform ***D,*** and the swimming paths length (mm) ***E,*** during the probe trial within 120 s for each group.

the number of synapses, the thickness of PSD, synaptic cleft width and synaptic curvature at P53 ($p > 0.05$, Table I, Figure 6). Besides, compared to the sevoflurane group rats at P53, the sevoflurane group rats showed fewer numbers of synapses, thinner thickness of PSD and broader synaptic cleft width at P8 ($p < 0.05$, Table I, Figure 4, Figure 6). There was no significant difference in the thickness of the presynaptic membrane between all groups either ($p > 0.05$, Table I).

Discussion

In current work, we exposed 2% sevoflurane to postnatal day 7 SD rat pups for a 2 h duration and evaluated the effect on developing brain. Compared with 3.28%, which is the minimum alveolar concentration of sevoflurane in neonatal rats[9], 2% sevoflurane is too low to inhibit respiration and circulation.

Synaptic plasticity, which requires normal synaptic transmission and normal release of neurotransmitters, is closely associated with brain functions such as learning and memory[6]. Amrock et al[10] have demonstrated that single or multiple neonatal sevoflurane exposures reduced both synaptic density and presynaptic mitochondrial localization. Xiao et al[11] showed that sevoflurane exposure of neonatal rats decreased numbers of synapses, widened synaptic clefts and other pathological features. Here TEM photomicrographs exhibited temporary ultrastructure changes of synapses in the sevoflurane groups, including a fewer number of synapses, broader synaptic clefts and the thinner thickness of the PSD. This might because volatile anesthetics

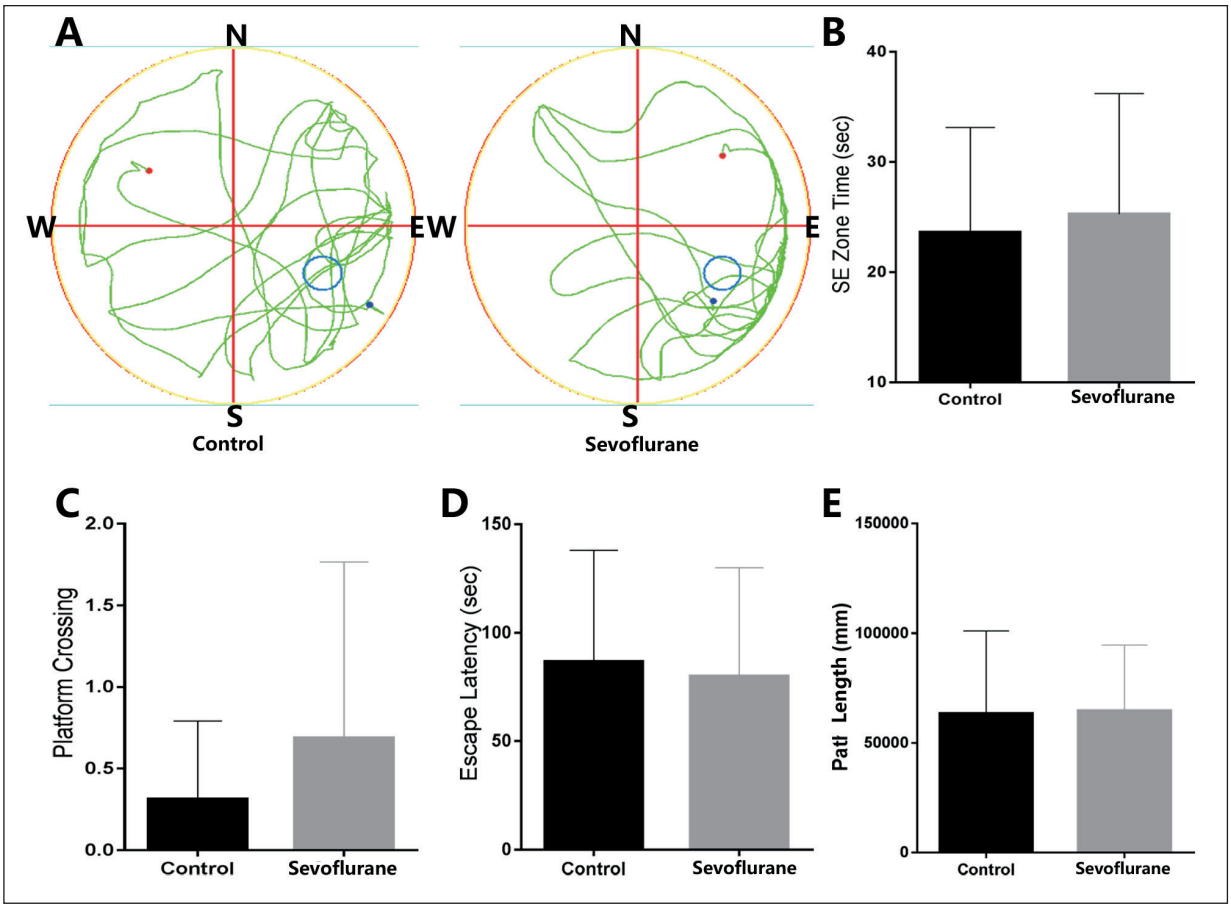

Figure 3. Effects of neonatal sevoflurane exposure at P7 on spatial reference memory ability of P53 rats in Morris water maze test ($n = 4$ per group). Spatial probe trials were performed to examine rat' spatial reference memory ability. The data showed the representative swimming path ***A***, the residence time (sec) on target quadrant ***B***, the number of platform crossing ***C***, the escape latency to find the hidden platform ***D,*** and the swimming paths length (mm) ***E,*** during the probe trial within 120 s for each group.

blocked actin-based motility in dendritic spines during the synaptic growth-spurt period[12]. Then, the ultrastructural changes have returned to normal at P53. This might attribute to a high degree of plasticity of young mice brain[13].

The main function of glutamatergic excitatory synapses is to store information and transmit signals in learning and memory[14]. Signals transduction takes place at the PSD[15]. The PSD which prominently exists in glutamatergic excitatory

Table I. Structural parameters of the synaptic interface in the CA3 regions of hippocampus (N = 20 synapses).

	Control (P8)	Sevoflurane (P8)	Control (P25)	Sevoflurane (P25)	Control (P53)	Sevoflurane (P53)
Numbers of synapses (100 μm^2)	36.17 ± 2.08	20.18 ± 3.32*	35.18 ± 1.98	28.91 ± 2.96*$^{\#}$	35.67 ± 2.16	34.93 ± 3.07$^{\#}$
PSD thickness (nm)	67.79 ± 4.06	39.18 ± 5.06*	70.21 ± 4.70	60.10 ± 5.20$^{\#}$	72.37 ± 6.21	70.89 ± 6.77$^{\#}$
Synaptic cleft width (nm)	18.11 ± 1.80	23.76 ± 2.08*	18.21 ± 1.70	22.10 ± 1.20*	18.17 ± 1.60	17.27 ± 1.00$^{\#}$
Presynaptic membrane (nm)	21.06 ± 2.01	19.97 ± 2.11	21.59 ± 1.89	20.21 ± 2.96	23.41 ± 2.08	23.13 ± 2.07
Synaptic curvature	1.19 ± 0.10	0.77 ± 0.11*	1.21 ± 0.11	1.10 ± 0.12$^{\#}$	1.28 ± 0.13	1.27 ± 0.10$^{\#}$

Data were presented as the means ± SEM. *: $p < 0.05$, Sevoflurane (P8) *vs*. Control (P8), Sevoflurane (P25) *vs*. Control (P25) (One-way ANOVA); $^{\#}$: $p < 0.05$, *vs*. Sevoflurane (P8) (Student-Newman-Keuls test). N: the number of synapses, PSD: postsynaptic density.

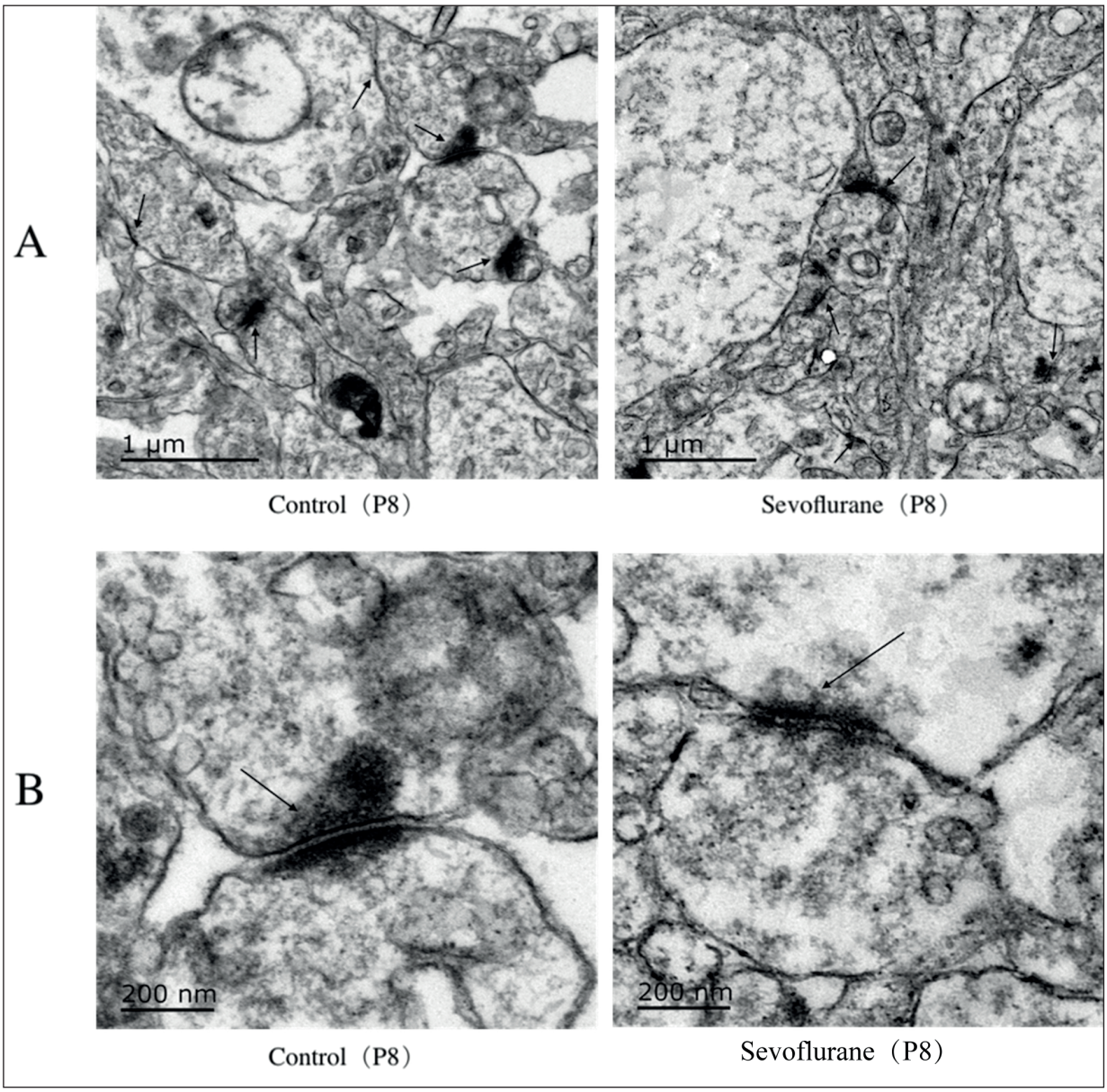

Figure 4. Effects of sevoflurane on synaptic ultrastructure in the CA3 region of the hippocampus in TEM analysis on P8 rats. ***A,*** Representative pictures (X 3000) showed the difference on the number of synapses (4-6 synapses) in per area (100 μm^2) in the two groups. (Black arrows count the number of synapse) Scale bars = 1 μm. ***B,*** Control group: synaptic structural integrity, vesicles clear and dense; sevoflurane group: vesicles dispersed, structure blurred. Representative pictures (X 10000) showed the difference on the thickness of postsynaptic dense, the synaptic cleft width, presynaptic membrane and synaptic curvature in two groups. (Black arrows show the synaptic linkages) Scale bars = 200 nm.

synapses participates in a signal process via glutamate receptor channels and some associated signaling proteins[7]. We found that thickness of PSD thinned severely, then recovered gradually and returned almost to normal level at P53. The missing ingredients were not yet clear. Recently Lu et al[16] showed that sevoflurane reduced postsynaptic density-95 protein (PSD-95) by activating the ubiquitination-proteasome pathway and led to cognitive impairment in young mice. The PSD-95 is one of the membrane-associated guanylate kinase family (MAGUK) which plays a crucial role in synaptic plasticity[17]. The PSD-95 is also abounding in PSD and acts as an excitatory postsynaptic marker[18]. However, it was unclear whether there are other components to the loss in the PSD. Further studies were needed to explore it.

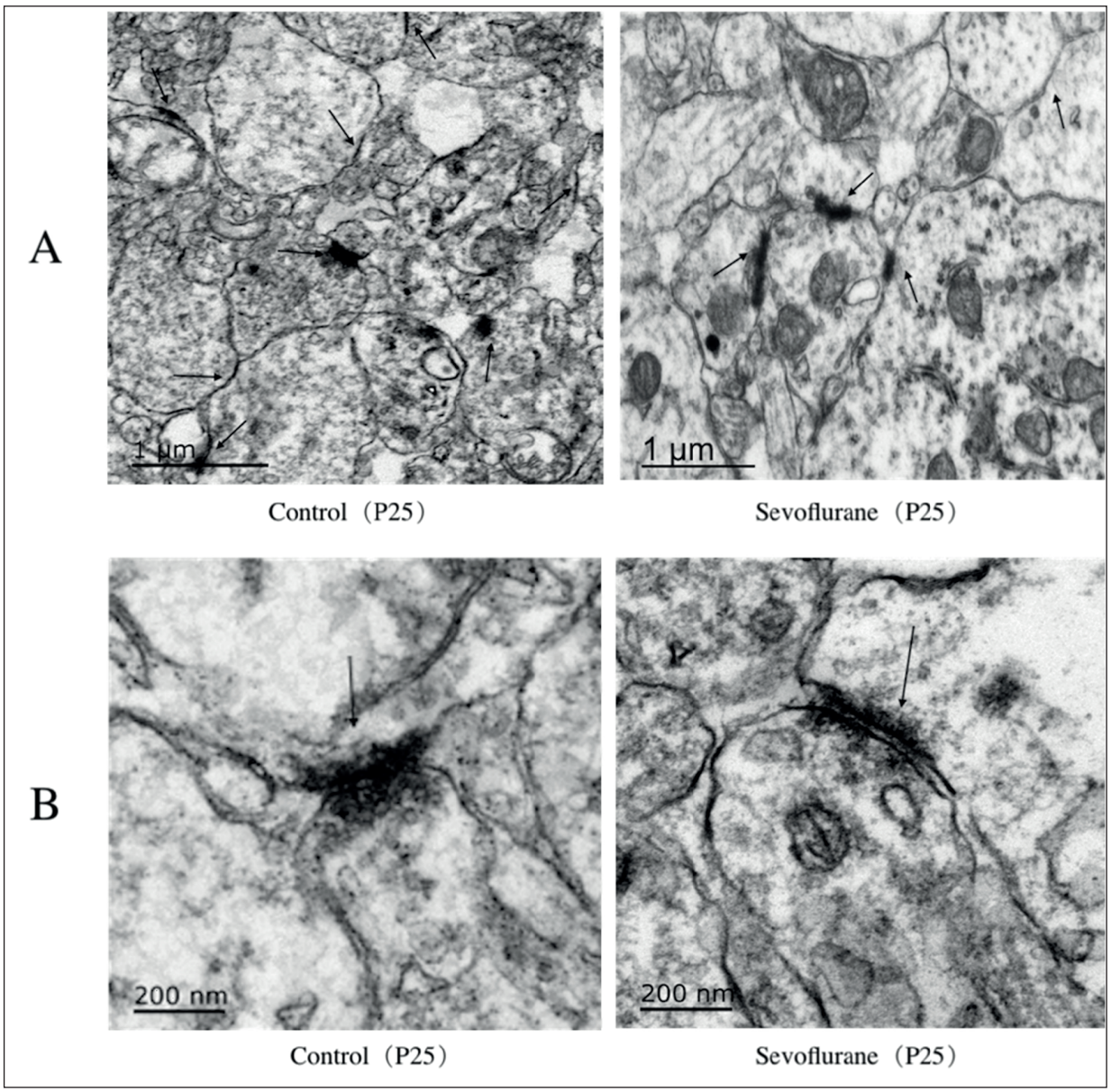

Figure 5. Effects of sevoflurane on synaptic ultrastructure in the CA3 regions of the hippocampus in TEM analysis on P25 rats. ***A,*** Representative pictures (X 3000) showed the difference on the number of synapses (4-6 synapses) in per area (100 μm^2) in the two groups. (Black arrows count the number of synapse) Scale bars = 1 μm. ***B,*** Control group: synaptic structural integrity, vesicles clear and dense; sevoflurane group: vesicles dispersed, structure blurred. Representative pictures (X 10000) showed the difference on the thickness of postsynaptic dense, the synaptic cleft width, presynaptic membrane and synaptic curvature in the two groups. (Black arrows show the synaptic linkages), Scale bars = 200 nm.

In addition, we observed normal presynaptic membrane and widened synaptic clefts after sevoflurane exposure by TEM. The presynaptic membrane contains lots of synaptic vesicles which carry the neural signals[19]. The synaptic cleft, between pre- and postsynaptic membrane, consists of trans-synaptic complexes that form extensive lateral connections[20]. The widened synaptic clefts may affect signal transduction between pre- and postsynaptic membrane.

Scholars[21,22] demonstrated that inhalational-anesthetic induced a neurocognitive deficit in both spatial working memory and spatial reference memory. In the current work, the MWM test showed that the sevoflurane group had a longer escape latency during spatial training days but no

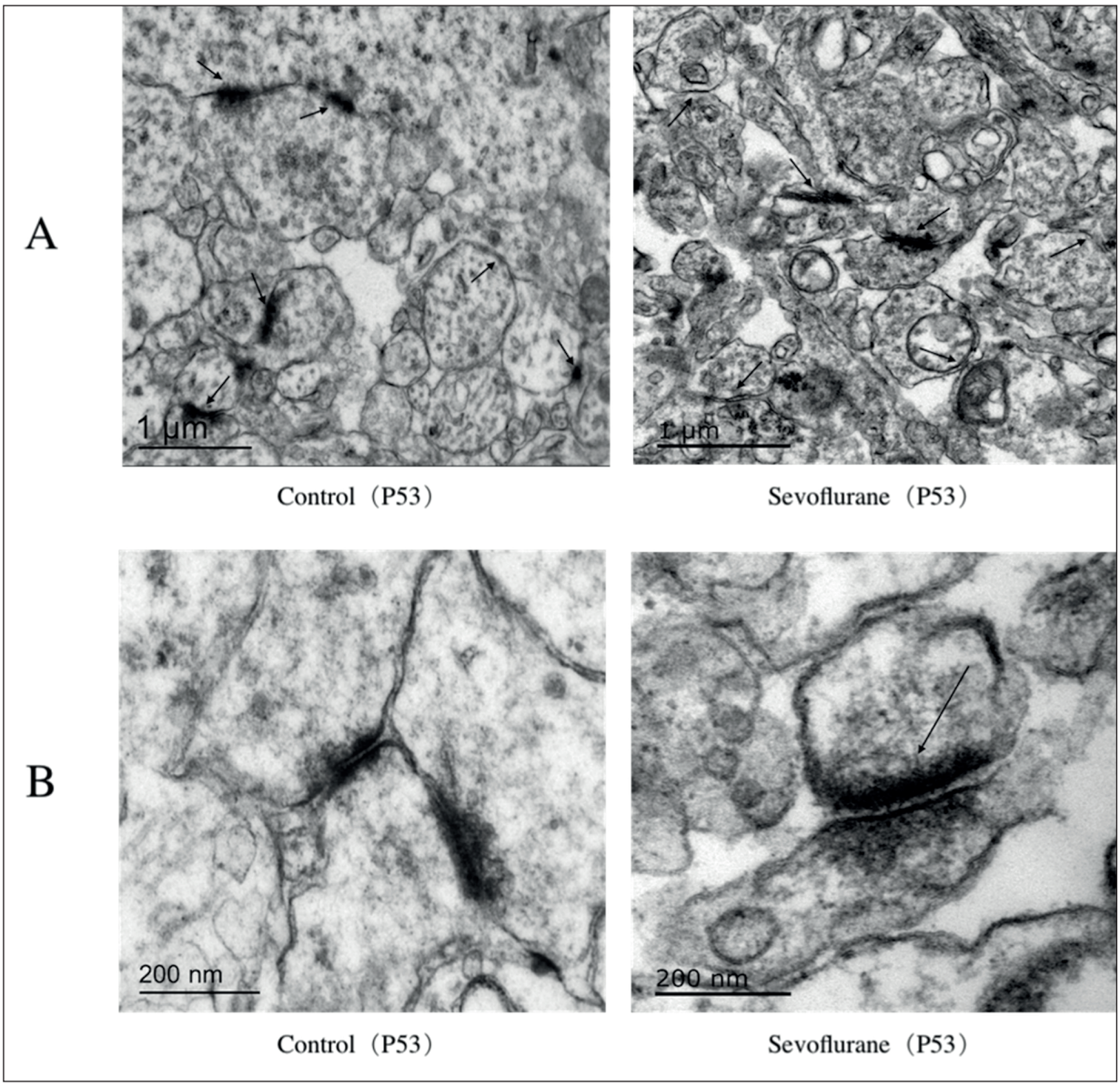

Figure 6. Effects of sevoflurane on synaptic ultrastructure in the CA3 regions of the hippocampus in TEM analysis on P53 rats. ***A,*** Representative pictures (X 3000) showed the difference on the number of synapses (4-6 synapses) per area (100 μm^2) in the two groups. (Black arrows count the number of synapse), Scale bars = 1 μm. ***B,*** Control group: synaptic structural integrity, vesicles clear and dense; sevoflurane group: vesicles dispersed, structure blurred. Representative pictures (X 10000) showed the difference on the thickness of postsynaptic dense, the synaptic cleft width, presynaptic membrane and synaptic curvature in the two groups. (Black arrows show the synaptic linkages), Scale bars = 200 nm.

significant difference in target quadrant residence time during spatial probe tests at P25. These results indicated that sevoflurane exposure to neonatal rats led to spatial working memory deficits. The deficits were consistent with synaptic ultrastructural impairments in the CA3 region of the hippocampus. Interestingly, inhaled sevoflurane only induced spatial working memory deficits but did not affect reference memory. It was possible because developmental sevoflurane treatment decreased N-methyl-D-aspartic acid receptor subunit NR2A through extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling[23]. NR2A was indispensable to rapidly acquire spatial working memory but not incremental spatial reference memory[24]. In accordance with the results observed by TEM, the spatial working memory deficits returned to normal at P53.

Conclusions

We showed that a single 2% sevoflurane exposure for 2 h to rats at P7 led to temporary spatial working memory deficits. It might be associated with synaptic ultrastructure impairments in the CA3 region of the hippocampus, including fewer numbers of synapses, the thinner thickness of PSD and broader synaptic cleft width. Fortunately, all the neurotoxicity and neurocognitive deficits were reversible.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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