

## Original Article

# Effects of different concentration and duration time of isoflurane on acute and long-term neurocognitive function of young adult C57BL/6 mouse

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**Abstract:** Postoperative cognitive dysfunction (POCD) is a decline in cognitive performance after a surgery with anaesthesia. The exact reasons of surgery and/or anaesthesia resulting in POCD are unclear. The aim of this study is to investigate the effects of different concentration and duration time of isoflurane anaesthesia on cognitive performance and cellular mechanisms involved in learning and memory function. In present work, young adult male C57BL/6 mice (age: 8 weeks) were anaesthetized by different concentration isoflurane in 100% oxygen for different duration time (Mice in group I<sub>1</sub> received 0.7% isoflurane 0.5 h, mice in I<sub>2</sub> received 0.7% isoflurane 2 h, mice in I<sub>3</sub> received 1.4% isoflurane 2 h, and mice in I<sub>4</sub> received 1.4% isoflurane 4 h). Non-anaesthetized mice served as control group (I<sub>0</sub>). Spatial learning was assessed at 10 days post-anaesthesia in Morris water maze (MWM). Hippocampal protein expressions of activated caspase 3, NMDA receptor subunit NR2B, and extracellular-signal regulated kinase (ERK) 1/2 were evaluated 24 hours and 2 weeks post anaesthesia. Protein expression of activated caspase3 was detected acute elevated in I<sub>3</sub> (24 h post-anaesthesia) and acute and long-term elevated in I<sub>4</sub> (24 hours and 2 weeks post-anaesthesia). There was no significant difference between I<sub>1</sub>, I<sub>2</sub> and control group. Protein expressions of NR2B showed an acute and long-term increasement in I<sub>1</sub> and I<sub>2</sub>, decreasement in I<sub>4</sub>, and an acute decline, then returned to normal in I<sub>3</sub> compared to control group. The ratio of phospho-ERK1/2 to total-ERK showed an acute increasement in I<sub>1</sub> and I<sub>2</sub>, then came to normal 2 weeks post anaesthesia compared to control group, meanwhile, we detected an acute and long-term decline in I<sub>3</sub> and I<sub>4</sub>. In MWM test, mice in I<sub>1</sub> and I<sub>2</sub> showed cognitive improvement, mice in I<sub>3</sub> showed similar to control group, while mice in I<sub>4</sub> demonstrated cognitive impairment, which were approximately corresponding to the changes of protein expression of NR2B and activation of ERK1/2. The present data suggested the following: (1) Isoflurane may cause neurotoxicity by inducing caspase activation and apoptosis with the anesthetic concentration increased and duration prolonged. (2) Low concentration of isoflurane in 2 hours can induce a hippocampus-specific elevation of NR2B subunit composition and ratio of p-ERK1/2 to total ERK1/2, produce hippocampal-dependent cognitive improvement. While high concentration of isoflurane exceeding 4 hours may induce a decline of NR2B and ratio of pERK1/2 to ERK1/2, then result in cognitive impairment.

**Keywords:** Isoflurane, postoperative cognitive dysfunction (POCD), apoptosis, NR2B receptor, extracellular signal-regulated kinase (ERK)

## Introduction

Postoperative cognitive dysfunction (POCD) is a deterioration of cognitive performance after anesthesia (and/or surgery) presenting as impaired memory or concentration. Accumulating evidence showed that patients experiencing POCD had an increasing risk of death in the first year after surgery [1, 2]. Although elderly patients are at the greatest risk of developing POCD [3], POCD is also present in young

patients receiving non-cardiac surgery. Monk et al. [1] demonstrated that 36.6% of young patients (18-39 years old) and 30.4% of middle-aged patients (40-59 years old) showed POCD at hospital discharge, with symptoms continuing 5.7% and 5.6%, respectively, at 3 months after surgery. Steinmetz et al. [4] suggested that there were close relationship between POCD and inability to previous job. Anesthesia and surgery were reported as important risk factors of POCD [5-8]. It is well known that gen-

eral anaesthesia interferes with memory function. But contradictory results have been reported. Some investigators reported anaesthesia-induced cognitive impairments in rats [9, 11], whereas others described cognitive improvements following anaesthetic exposure in rodents [9, 12, 13]. The exact mechanism is still unclear. Thus this study was designed to elucidate the role of inhalation anaesthetics in cognitive function. Inhalation anaesthetics, such as isoflurane, modulate NMDA-type glutamate receptors to produce analgesic and anaesthetic actions in the central nervous system (CNS) [14-19]. Due to the critical role of NMDA receptors in learning and memory processes [20], these receptors may play a role in anaesthesia-induced cognitive deficits in brain. We anaesthetized mice with isoflurane and investigated cognitive performance, and expression levels of activated caspase3, NMDA receptor subunit NR2B and downstream signaling pathways ERK1/2 in hippocampal brains 24 hours and 2 weeks post anaesthesia.

### Materials and methods

#### *Animals*

All mice were housed separately under standard laboratory conditions (12:12 light/dark cycle, 22°C, 60% humidity) and had free access to tap water and standard mouse chow. Prior to the investigations the mice were allowed to habituate to their new surroundings for at least three weeks after having been transferred from the breeder. The animal protocol was approved by the Standing Committee on Animals at Shanghai Tongji Hospital.

#### *Anesthesia*

Young male adult C57BL/6 mice received anaesthetic isoflurane plus 100% oxygen during anaesthesia. Sixty mice were randomly assigned to five groups (N = 12): (1) anaesthesia groups were composed of four groups including I<sub>1</sub>, I<sub>2</sub>, I<sub>3</sub>, and I<sub>4</sub>. Mice in I<sub>1</sub> group were exposed to 0.7% isoflurane (0.5 minimum alveolar concentration, MAC) 0.5 h, I<sub>2</sub> exposed to 0.7% isoflurane 2 h, I<sub>3</sub> exposed to 1.4% isoflurane (1MAC) 2 h, I<sub>4</sub> exposed to 1.4% isoflurane 4 h (2) control group (I<sub>0</sub>) received no isoflurane. The anaesthetic and oxygen concentrations were measured continuously (GE Datex-Ohmeda, Tewksbury, MA). The

temperature of the anaesthetizing chamber was controlled to maintain at 37 ± 0.5°C with a heating pad under the chamber. After recovery all mice were returned to their home cages. There was no mortality during or after anaesthesia.

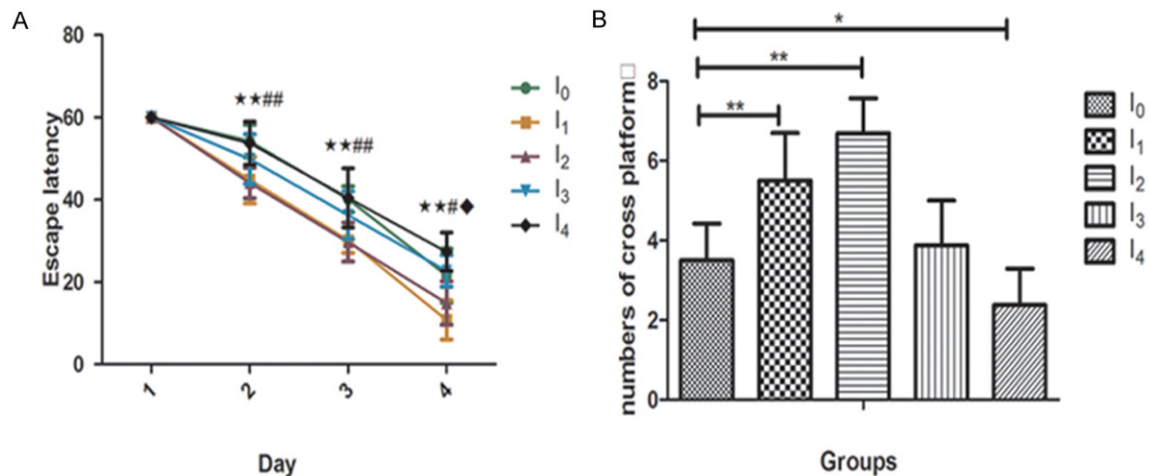
#### *Morris water maze*

10 days post anaesthesia, eight mice of each group were tested in Morris water maze. A round steel pool, 122 cm in diameter and 60 cm in height, was filled with water to a height of 1.0 cm above the top of a 10 cm diameter platform. The pool was covered with a blue curtain and was located in an isolated room with four visual cues on the wall of the pool. Water was kept at 20°C and opacified with titanium dioxide. The mice were tested in the Morris water maze (MWM) four times per day for 4 days [21]. Each day, animals were given four trials. The animal was placed randomly at each of four starting points (north, south, east, and west) and allowed 60 s to find the hidden platform and stay on it for 15 s. If the animal did not locate the platform within 60 s, it was gently guided to the platform and allowed to stay on it for 15 s. After each trial, the animal was placed in a cage and kept warm with an infrared heating lamp.

A video tracking system recorded the swimming motions of the animals, and the data were analyzed using motion-detection software for the MWM (Shanghai Mobile Datum Information Technology Co., Ltd.). At the end of the reference training, the platform was removed from the pool and the mouse was placed in the opposite quadrant. Each mouse was allowed to swim for 120 s, and the number of times the mouse swam across the platform area was recorded (numbers of cross platform).

#### *Antibodies and immunoblotting*

Caspase3, NR2B, pERK1/2, and ERK1/2 (all polyclonal antibodies at ratio 1:1000 from Abcam USA) and β-actin (1:3000, Sigma) were used. Animals were terminated by decapitation 24 h post-anaesthesia, and after behavioral testing (2 weeks post-anaesthesia). Control group was terminated at the same times. Animals bilateral hippocampi were quickly removed and dissected at 4°C in saline and fro-



**Figure 1.** Isoflurane anesthesia induced spatial learning changes 2 weeks post anesthesia. Animals were exposed to 0.5MAC or 1MAC isoflurane for different time. 10 days later the Morris Water Maze was performed. A. The escape latency in the I<sub>1</sub> and I<sub>2</sub> group decreased significantly, I<sub>4</sub> group increased compared with the control group I<sub>0</sub> (I<sub>1</sub> VS I<sub>0</sub> \**p* < 0.05, \*\**p* < 0.01; I<sub>2</sub> VS I<sub>0</sub> #*p* < 0.05, ##*p* < 0.01; I<sub>4</sub> VS I<sub>0</sub> ♦*p* < 0.05, ♦♦*p* < 0.01). B. I<sub>1</sub> and I<sub>2</sub> group increased numbers of cross platform significantly, while I<sub>4</sub> group decreased compared with the control group. There was no difference between group I<sub>3</sub> and control group (\**p* < 0.05, \*\**p* < 0.01).

zen in liquid nitrogen within 3 min of decapitation, and stored at -80°C. Samples (25 µg protein) were resolved on 8% sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% dry milk solution for 1 h and then incubated overnight at 4°C with anti-Caspase3, NR2B, pERK1/2 and ERK1/2 in the same 5% dry milk solution. Membranes were rinsed in Tween-TBS (TTBS) buffer and incubated in alkaline phosphatase-conjugated secondary antibodies (1:5000, Burlingame, CA) for 1 h at room temperature. The membranes were washed in TTBS by using the enhanced chemiluminescence method. Quantification of band density was performed using AlphaView Software3.4. Data were normalized to β-actin.

**Statistical analysis**

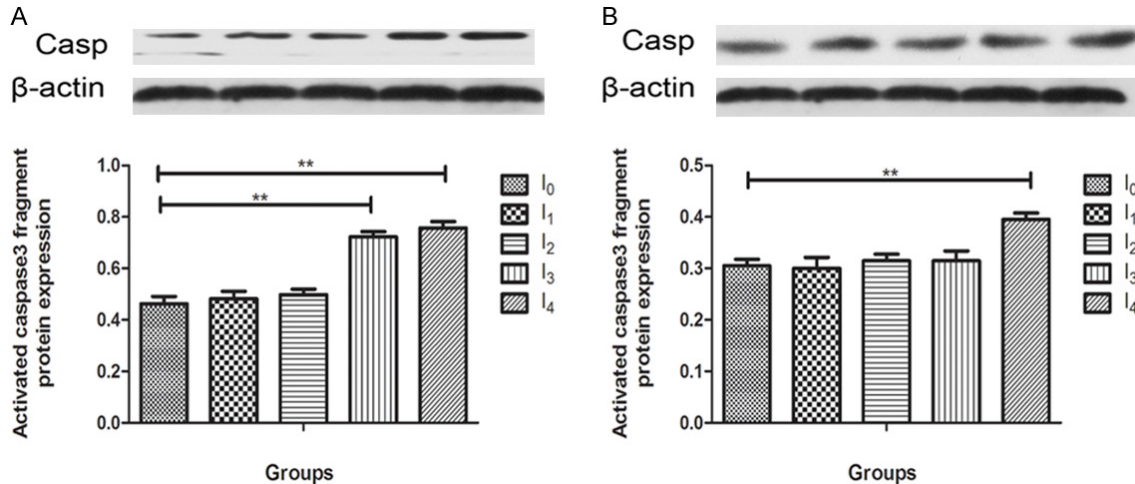
Data were expressed as mean ± SD. Statistical comparisons between experimental and control groups were made using one-way analysis of variance (ANOVA) with least Significant Difference (LSD) post hoc testing. The escape latency and swimming speed were analyzed by one-way analysis of variance with repeated measures. A statistical software package for the Social Sciences (SPSS) 14.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. *P*-values for significance used were *P* < 0.05.

**Results**

*Isoflurane anesthesia induced spatial learning changes 10 days post anesthesia*

The mice were tested 10 days after anesthesia (N = 8). A comparison of the time that each mouse took to reach the platform during reference training (escape latency) were recorded. We found that 0.5MAC isoflurane anesthetic exposure decreased the escape latency in I<sub>1</sub> and I<sub>2</sub> groups as compared to those in the control group (*P* < 0.05), while 1MAC isoflurane prolonged the escape latency in I<sub>4</sub> compared to those in the control group (*P* < 0.05)(**Figure 1A**). A comparison of the times that each mouse crossed the location of the absent platform at the end of reference training (numbers of cross platform) indicated that anesthesia groups I<sub>1</sub> and I<sub>2</sub> increased the platform crossing times while I<sub>4</sub> group decreased those as compared with the control group (**Figure 1B**). There was no difference between the I<sub>3</sub> and control group. There was no significant difference in mouse swimming speed between the mice in the isoflurane anesthetic groups and the mice in the control group (data not shown). These data suggest that the different concentration and duration of isoflurane exposure in mice may induce cognitive change in the mice approximately 2 weeks after anesthesia.

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**Figure 2.** Effect of Isoflurane anesthesia on early and late caspase 3 activation in the hippocampus of mouse brain. A. 24 hours post anesthesia, I<sub>3</sub> and I<sub>4</sub> group induced caspase-3 cleavage (activation) compared with control group ( $P < 0.01$ ). I<sub>1</sub> and I<sub>2</sub> group caused a lesser degree of caspase-3 activation compared with control group ( $P > 0.05$ ). B. 2 weeks post anesthesia, I<sub>4</sub> group induced caspase-3 activation clearly ( $P < 0.05$ ). There was no difference between the rest groups. There was no significant difference in amounts of  $\beta$ -actin [Data are presented as means, error bars represent SD, One-way ANOVA and least Significant Difference (LSD) were performed,  $*p < 0.05$ ,  $**p < 0.01$ ].

Given the findings that exposures to isoflurane in young adult mice might induce cognitive change, we next investigated the underlying mechanisms.

### *Effect of isoflurane anesthesia on caspase 3 activation in the hippocampus of mouse brain*

Accumulating studies suggest that anesthetic neurotoxicity may result from caspase3 activation in the CNS [22, 23]. In our study, to determine caspase 3 activation levels of isoflurane anesthetic exposure, animals were terminated by decapitation 24 hours post-anesthesia ( $N = 4$ ) (**Figure 2A**) and after behavioral testing (2 weeks post-anesthesia) ( $N = 4$ ) (**Figure 2B**). Hippocampal tissues from experimental groups were subjected to western blot analysis with antibodies against caspase3. Representative blots for hippocampus are shown. Caspase 3 activation was measured by caspase 3 fragment (17 kD) quantification. 24 h post-anesthesia, caspase 3 was detected elevated in I<sub>3</sub> and I<sub>4</sub> ( $p < 0.01$ ) compared with the control group. 2 weeks post anesthesia caspase3 was still increased in I<sub>4</sub> group compared to the control mice ( $p < 0.01$ ). There were no significant difference between other experimental groups and control group at 24 h and 2 weeks post anesthesia.

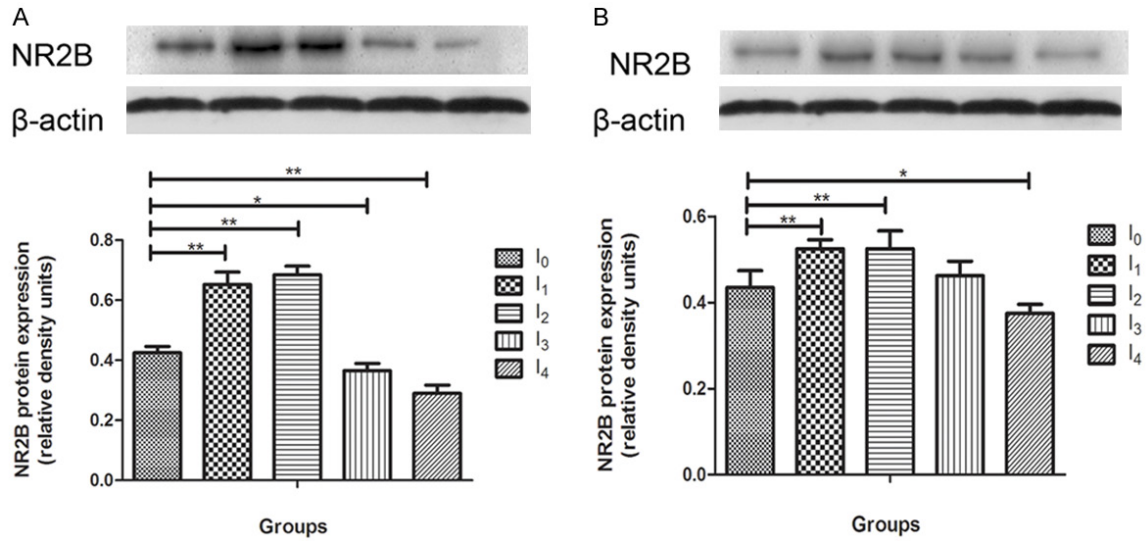
### *Effect of isoflurane anesthesia on protein expression levels of NMDA receptor subunits NR2B in the mouse hippocampal brain*

In **Figure 3**, hippocampal brain samples from different groups mice were immunoblotted for NMDA receptor subunits NR2B to determine changes in protein expression as a result of anesthesia exposure 24 hours ( $N = 4$ ) and 2 weeks post-anesthesia ( $N = 4$ ). We found that the mice treated with 0.5MAC anesthesia in I<sub>1</sub> and I<sub>2</sub> demonstrated an acute increase 24 hours post anesthesia ( $p < 0.01$ , **Figure 3A**) and a long-term increase at 2 weeks post-anesthesia compared to naïve mice ( $p < 0.01$ , **Figure 3B**). We also found that the mice treated with 1MAC anesthesia in I<sub>3</sub> ( $p < 0.05$ ) and I<sub>4</sub> ( $p < 0.01$ ) groups indicated an acute decrease in NR2B protein expression levels (**Figure 3A**), while a long-term decrease in I<sub>4</sub> 2 weeks post anesthesia compared to naïve mouse in the hippocampus ( $p < 0.05$ , **Figure 3B**).

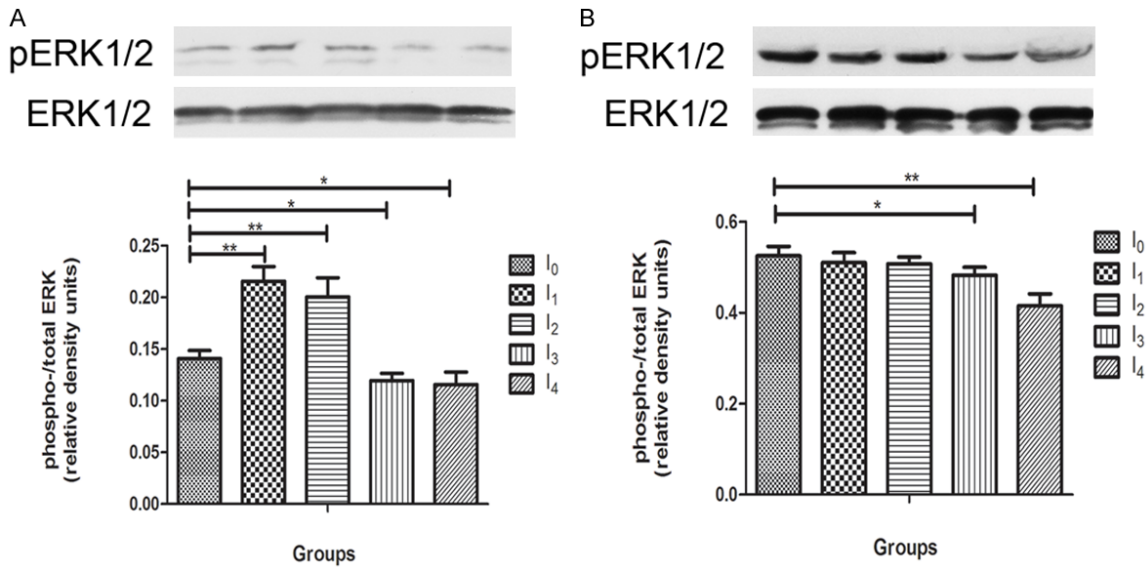
### *Effect of isoflurane anesthesia on ERK1/2 activation in the mouse hippocampal brain*

Activation of ERK1/2, a downstream kinase of NMDA receptors, is required for hippocampal dependent spatial learning. Hippocampal brain samples from all groups were immunoblotted

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**Figure 3.** Effect of isoflurane anesthesia on early and late protein expression of NMDA receptor subunits NR2B in the hippocampus of mouse brain. A. 24 hours post anesthesia,  $I_1$  and  $I_2$  group increased NR2B protein expression significantly ( $P < 0.05$ ),  $I_3$  and  $I_4$  group decreased compared with control group ( $P < 0.05$ ). B. 2 weeks post anesthesia,  $I_1$  and  $I_2$  group increased NR2B protein expression continuously,  $I_4$  decreased continuously compared with control group. There was no difference between group  $I_3$  and control group [Data are presented as means, error bars represent SD, One-way ANOVA and LSD analysis were performed,  $*p < 0.05$ ,  $**p < 0.01$ ].



**Figure 4.** Effect of isoflurane anesthesia on ERK1/2 activation in the hippocampus of mouse brain. Animals were terminated at 24 hours post-anesthesia (A) and 2 weeks post-anesthesia (B) for antibodies against phospho-ERK1/2 and ERK1/2, followed by quantification of the ratio of phospho-ERK over total ERK to determine ERK activation. A.  $I_1$  and  $I_2$  groups increased ERK activation significantly compared with control group ( $P < 0.01$ ),  $I_3$  and  $I_4$  groups decreased ERK activation compared with control group ( $P < 0.05$ ). B. At 2 weeks post anesthesia,  $I_3$  and  $I_4$  decreased ERK activation continuously ( $P < 0.05$ ). There were no difference between group  $I_1$ ,  $I_2$  and control group. [Data are presented as means, error bars represent SD. One-way ANOVA and LSD analysis were performed,  $*p < 0.05$ ,  $**p < 0.01$ ].

for phospho-ERK1/2 and total ERK1/2 to determine changes in activation of ERK1/2, the ratio of phospho- to total-ERK, following iso-

flurane anesthesia at early (N = 4) and long-term time point (N = 4). An increase in ratio of phospho- to total-ERK was detected in 0.5MAC

anesthesia treated groups  $I_1$  and  $I_2$  ( $p < 0.01$ ), meanwhile, a decrease was detected in 1MAC anesthesia treated groups  $I_3$  and  $I_4$  ( $p < 0.01$ ) compared to the control group 24 hours post anesthesia (Figure 4A). The decrease trend in 1MAC anesthesia treated groups  $I_3$  ( $p < 0.05$ ) and  $I_4$  ( $p < 0.01$ ) continued till 2 weeks post-anesthesia. While the mice in 0.5MAC groups ( $I_1$  and  $I_2$ ) became normal as control group (Figure 4B).

### Discussion

There are conflicting opinions about the effect of anesthetic inhalation on cognitive function. Previous studies suggested cognitive impairment following exposure to anesthetic inhalation, while recently other studies report cognitive improvements following anesthetic exposure. The spatial reference memory version of MWM is a standard task used to assess hippocampal-dependent spatial learning in rodents [21]. Spatial acquisition and retention was assessed using a water maze navigational task. In this study, MWM was adopted to assess spatial learning and memory of mice. We found that, compared with the control group, the mice receiving 0.5MAC isoflurane 0.5 or 2 hours performed cognitively better, the mice receiving 1MAC isoflurane 2 hours performed cognitive similar, the mice receiving 1MAC isoflurane 4 hours performed worse. This indicated that isoflurane may affect spatial learning and memory with a concentration- and duration-dependent manner.

It is well known that the hippocampus plays a vital role in learning and memory processes and is a known target for the modulatory actions of drugs. There may be an interaction between neural apoptosis and anesthetic insult that result in cognitive dysfunction. Xie et al. [22, 23] found that isoflurane may cause neurotoxicity by inducing caspase activation and apoptosis in H4 human neuroglioma cells and in the brain tissues of 5 month-old WT mice. Zhang et al. [24] suggested isoflurane, but not desflurane, induced reactive oxygen species (ROS) accumulation, which then facilitated opening of mitochondrial permeability transition pore (mPTP). Opening of mPTP could cause decreases in levels of mitochondrial membrane potential (MMP), and consequently reduction in adenosine-5'-triphosphate (ATP) levels, leading

to neurotoxicity (e.g., caspase 3 activation) and finally impairment of learning and memory. In this study, no change was detected 24 hours post-anesthesia in activated caspase 3 in the mice exposed to 0.5MAC isoflurane for 0.5 or 2 hours. However, an increase in activated caspase 3 was detected 24 hours post-anesthesia in the mice exposed to 1MAC isoflurane 2 or 4 hours. The latter increased continuously until 2 weeks post-anesthesia. Thus, we concluded that with the anesthetic concentration increased and duration prolonged, isoflurane may cause neurotoxicity by inducing caspase activation and apoptosis.

In our study, it suggested that with the increase of isoflurane concentration and duration time, caspase3 was elevated significantly, meaning increased apoptosis, but in behavioral test, the group exposed to 1MAC anesthesia 2 h was not impaired in spatial memory ability compared to control group. This means neural apoptosis may cause side effect on learning and memory. While there are many factors in the process of learning and memory, and neural apoptosis was not a decisive factor. Due to the critical role of NMDA receptors in learning and memory processes [20], these receptors may play a role in anesthesia-induced cognitive deficits. Previously, in cortical neurons, NMDA-gated currents mediated by NR2B-containing receptors were more sensitive to isoflurane than currents mediated by NR2A-containing receptors [25]. NR2B subunit plays a key role in synaptic plasticity, synaptogenesis, excitotoxicity, memory acquisition and learning. Genetic overexpression of the gene encoding NR2B in adult fore-brains led to mice with facilitated LTP induction and improved learning and memory in a variety of behavioural tasks, whereas a hippocampal NR2B deficit impaired spatial learning [26]. Rammes and Zhao reported that high levels of NMDA receptor subunit NR2B protein expression corresponded to improved spatial learning performance in 4-5 month-old mice [13, 27]. Our findings found the two groups receiving 0.5MAC isoflurane 0.5 h or 2 h detected obvious increase of NMDA receptor subunit NR2B protein expression at early and late time points corresponding to better behavioral performance. While the group receiving 1MAC isoflurane 2 h or 4 h were detected marked reduction of NR2B protein expression at early time point, and the 4 h group continued

lower NR2B protein expression till 2 weeks post anesthesia.

Extracellular signal-regulated protein kinase 1/2 (ERK1/2) belong to subfamily of MAPKs, a prototype of MAPKs, they are activated via phosphorylation on Thr202 and Tyr204. The NMDAR have been demonstrated to positively regulate ERK1/2 phosphorylation. It has also been reported that NMDA receptor-dependent ERK activation is mainly mediated by NR2B-containing receptors [28]. Once activated, ERK1/2 translocate from the cytosol to the nucleus to activate specific transcription factors, leading to inducible gene expression. A noticeable role of active ERK1/2 is its strong regulation of gene expression. As an information superhighway between the surface receptor and the nucleus, ERK1/2 effectively link environmental inputs to genomic responses. Through regulating specific sets of transcriptional events, ERK1/2 modulate expression levels and thus functions of various key synaptic proteins. This process influences many forms of synaptic plasticity, including LTP and LTD, cellular models of learning and memory. It was reported that the ERK1/2 protein expression in hippocampus was closely related with hippocampus dependent spatial learning and memory task such as MWM [29, 30]. Our findings identify an increase induced by 0.5MAC isoflurane treated groups ( $I_1$  and  $I_2$ ) and a reduction caused by 1MAC isoflurane treated groups ( $I_3$  and  $I_4$ ) in hippocampal ratio of p-ERK/ERK at early time (24 hours post anesthesia). Two weeks later, there was no difference between 0.5MAC isoflurane treated groups and control group. Meanwhile, 1MAC isoflurane treated groups were still detected lower ratio of p-ERK/ERK. From our results, we considered that the change of activation of ERK induced by 0.5MAC anesthesia continued not as long as the NR2B. The effect of anesthesia on activation of ERK gradually came to normal. As for 1MAC groups we speculated that they may also be in the process of recovery especially the  $I_3$  given that the behavioral performance.

Taken together, our findings suggested low concentration of isoflurane (0.5MAC) exposure in 2 hours did not induce caspase3 activation and neural apoptosis significantly, while induced increase of hippocampal NMDA receptor subunit NR2B protein expression and ERK1/2

activation contributing to the improvement of spatial learning performance. However, high concentration of isoflurane(1MAC) exposure in 2 hours can cause caspase3 activation and neural apoptosis obviously, meanwhile, inhibited hippocampal NMDA receptor subunit NR2B protein expression and ERK activation at early time point (24 h post anesthesia), then NR2B became normal 2 weeks post anesthesia resulting in no cognitive impairment. Furthermore, high concentration of isoflurane exposure exceeding 4 hours may induce caspase3 activation, inhibit NR2B protein expression and ERK1/2 activation for 2 weeks since anesthesia, corresponding to the impairment of spatial learning performance. Thus our study indicate that isoflurane may affect spatial learning and memory with a concentration- and duration-dependent manner resulting from multiple reasons including neural apoptosis, changes of NR2B receptor and its downstream signaling pathways ERK1/2 in hippocampus.

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### Disclosure of conflict of interest

None.

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