

# TNF- $\alpha$ receptor antagonist attenuates isoflurane-induced cognitive impairment in aged rats

NENGLI YANG<sup>1</sup>, YAFENG LIANG<sup>2</sup>, PEI YANG<sup>1</sup>, WEIJIAN WANG<sup>1</sup>, XUEZHENG ZHANG<sup>1</sup> and JUNLU WANG<sup>1</sup>

<sup>1</sup>Department of Anesthesiology, The First Affiliated Hospital of Wenzhou Medical University;

<sup>2</sup>Department of Pediatric Intensive Care Unit, The Second Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325000, P.R. China

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**Abstract.** Postoperative cognitive dysfunction (POCD), a common clinical in aged patients, is characterized by deficits in cognitive functions in patients following anesthesia and surgery. It has been demonstrated that isoflurane may lead to cognitive impairment in aged rats; however, effective clinical interventions for preventing this disorder are limited. Tumor necrosis factor (TNF)- $\alpha$  has been suggested to be involved in neuroinflammation as well as the development of POCD. Accordingly, the present study aimed to investigate whether TNF- $\alpha$  signaling is involved in the isoflurane-induced cognitive impairment in aged rats, and whether TNF- $\alpha$  receptor antagonist are able to attenuate isoflurane-induced cognitive impairment in aged rats. A population of 20-month-old rats were administered TNF- $\alpha$  receptor antagonist R-7050 or an equal volume of saline by intraperitoneal injection 12 h prior to exposure to isoflurane to model cognitive impairment following anesthesia in old patients. Then the rats were exposed to 1.3% isoflurane for 4 h. In the control group, rats showed impaired cognitive functions evaluated by Morris water maze assay after isoflurane exposure. Furthermore, isoflurane exposure induced marked upregulation of proinflammatory cytokines, including interleukin (IL)-1 $\beta$ , TNF- $\alpha$ , IL-6 and IL-8 in the hippocampus tissue. In the experimental group, intracisternal administration of TNF- $\alpha$  receptor antagonist R-7050 significantly attenuated isoflurane-induced cognitive impairment and upregulation of proinflammatory cytokines. Further investigation revealed that intracisternal administration of TNF- $\alpha$  receptor antagonist R-7050 notably suppressed isoflurane-induced activation of NF- $\kappa$ B and MAPK signaling. Collectively, the present results suggest that TNF- $\alpha$  receptor

antagonist may serve as a potential agent for the prevention of anesthesia-induced cognitive decline in aged patients.

## Introduction

Despite advances in perioperative care, a significant percentage of elderly patients experience postoperative cognitive dysfunction (POCD), which is a postoperative disorder of cognitive functions including memory, attention, speech and abstract thinking (1). As POCD has been associated with increased mortality in elderly patients, the development of potential preventive or therapeutic agents for POCD is of increasing significance (2).

Volatile anesthetics such as isoflurane may interact with neurodegenerative disease-associated proteins and induce processes that may be associated with Alzheimer disease neuropathology (3). Furthermore, clinical studies have highlighted the risk factors for POCD, including age of patients, type of surgery and the type of anesthesia (4). In addition, neuroinflammation has been demonstrated to be an important etiological factor for POCD. Lin *et al* have suggested that isoflurane induces neuroinflammation and causes cell injury in the hippocampus, which may contribute to cognitive impairment in aged rats (5). Besides, isoflurane can increase neuronal cell death vulnerability and decrease levels of acetylcholine in the brain tissues, which contributes to the development of cognitive dysfunction (6,7). Therefore, effective prevention of isoflurane-induced neuroinflammation shows promises for the treatment of POCD.

Tumor necrosis factor (TNF)- $\alpha$ , primarily secreted by macrophages, is a member of the TNF super-family (8-10). TNF- $\alpha$  plays an important role in the regulation of a number of biological processes including cell proliferation, apoptosis, differentiation, lipid metabolism and coagulation through binding to its receptors including TNFR1 and TNFR2 (8-10). Furthermore, TNF- $\alpha$  is also involved in a variety of diseases, such as autoimmune diseases, insulin resistance and cancer (8-10). In addition, through activation of inflammatory-related signaling pathways such as nuclear factor- $\kappa$ B (NF- $\kappa$ B) and c-jun N-terminal kinase (c-JNK), TNF- $\alpha$  is able to induce neuroinflammation, and isoflurane anesthesia significantly increases the expression levels of TNF- $\alpha$  in the brain tissues (11,12). Accordingly, TNF- $\alpha$  may be used as a potential target for the treatment of isoflurane-induced cognitive dysfunction.

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*Correspondence to:* Professor Junlu Wang, Department of Anesthesiology, The First Affiliated Hospital of Wenzhou Medical University, 2 Fuxue Lane, Wenzhou, Zhejiang 325000, P.R. China  
E-mail: wenzhouwangjunlu@163.com

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The aim of the present study was to clarify whether TNF- $\alpha$  signaling is involved in the isoflurane-induced cognitive impairment in aged rats, and whether TNF- $\alpha$  receptor antagonist can attenuate isoflurane-induced cognitive impairment in aged rats. A population of 20-month-old rats were treated with isoflurane to model cognitive impairment following anesthesia in old patients.

## Materials and methods

**Animals and groups.** This study was approved by the Ethics Committee of Wenzhou Medical University (Wenzhou, China). Male 20-month-old Sprague-Dawley rats ( $n=50$ ) were purchased from Laboratory Animal Centre of Life Science Institute (Shanghai, China). All rats were housed separately under controlled temperature conditions ( $22\pm1^{\circ}\text{C}$ ) with a 12 h light/dark cycle, and allowed free access to standard rat chow and sterile water. A total of 25 rats were used for the experiments and separated into 5 groups ( $n=5$  per group). For the first experiment, the rats were separated into a control group and an isoflurane group. For the following experiments, 3 groups of rats were used: A control group ( $n=5$ ), an isoflurane + saline group ( $n=5$ ) and an isoflurane + R-7050 group.

**Exposure to anesthetic.** Rats were exposed to 1.3% isoflurane (Shanghai Yuyan Instruments Co., Ltd., Shanghai, China) in a humidified atmosphere with 30% oxygen carrier gas for 4 h. In the control group, rats were exposed to humidified 30% oxygen for 4 h. The 1.3% concentration, which is commonly performed in clinical practice, is the minimum alveolar concentration of isoflurane used in rats.

**Intracisternal administration of TNF- $\alpha$  receptor antagonist.** To determine whether TNF- $\alpha$  signaling is involved in the isoflurane-induced cognitive impairment in aged rats, rats received an administration of TNF- $\alpha$  receptor antagonist R-7050 (EMD Millipore, Billerica, CA, USA) prior to receiving anesthesia. Briefly, after the dorsal aspect of the skull was shaved and cleaned, a 27-gauge needle (Rysstech, Shanghai, China) attached via PE50 tubing (Rysstech) to a 25- $\mu\text{l}$  Hamilton syringe (Rysstech) was inserted into the cisterna magna. After confirming that the needle had entered the cisterna magna, 3  $\mu\text{l}$  R-7050 was administered. In the control group, rats received intracisternal administration of 3  $\mu\text{l}$  sterile physiological saline.

**Morris water maze (MWM) assay.** Each rat was placed on the platform in the center of the MWM (Shanghai Xinruan Information Technology Co., Ltd., Shanghai, China) for 30 sec, then released into water from an assigned release point. The rat was allowed to swim for 60 sec or until it landed on the platform. If the rat did failed to reach the platform within 60 sec, it was placed on the platform for a further 30 sec. Swimming distance and time to the platform were recorded using video tracking (Shanghai Xinruan Information Technology Co., Ltd.) and analyzed by MWM software (Shanghai Xinruan Information Technology Co., Ltd.).

**RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted using TRIzol<sup>®</sup> reagent (Thermo Fisher Scientific,

Inc., Waltham, CA, USA) from the hippocampus tissue of three rats randomly selected from each group. The rats were sacrificed by cervical dislocation under anesthesia with 10% chloral hydrate (0.3 ml/100 g; Dalian Meilun Biotech Co., Ltd., Dalian, China). The RNA was then reverse transcribed into a cDNA template using a PrimeScript<sup>™</sup> reverse transcription reagent kit (cat. no. 6110A; Takara Bio, Inc., Otsu, Japan). DNA polymerase was contained within the kit. The cDNA was amplified using a SYBR green qPCR assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for qPCR analysis using the ABI 7500 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction mixture contained 1  $\mu\text{l}$  cDNA, 10  $\mu\text{l}$  SYBR green PCR master mix, 2  $\mu\text{l}$  primer and 7  $\mu\text{l}$  H<sub>2</sub>O in a final reaction volume of 20  $\mu\text{l}$ . The PCR thermal cycling conditions were as follows: 95 $^{\circ}\text{C}$  for 5 min, 40 cycles of denaturation at 95 $^{\circ}\text{C}$  for 15 sec and annealing/elongation at 60 $^{\circ}\text{C}$  for 30 sec. The reaction was repeated 3 times and a negative control (no cDNA) and RT control (no RT) were used. The expression of target mRNA relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was calculated using crossing point (Cp) values and scaled relative to control samples set at a value of 1. The relative expression was calculated using the  $2^{-\Delta\Delta C_q}$  method (8). The primer sequences used were as follows: TNF- $\alpha$  forward, 5'-CATGATCCGAGATGTGGAAGTGGC-3' and reverse, 5'-CTGGCTCAGCCACTCAGC-3'; interleukin (IL)-1 $\beta$  forward, 5'-CATGATCCGAGATGTGGAAGTGGC-3' and reverse, 5'-CTGGCTCAGCCACTCAGC-3'; IL-6 forward, 5'-ACTCACCTCTTCAGAACGAATTG-3' and reverse, 5'-CCATCTTTGGAAGGTTCAAGTTG-3'; IL-8 forward, 5'-TTTTGCCAAGGAGTGCTAAGA-3' and reverse, 5'-AACCCTCTGCACCCAGTTTTC-3'.

**Western blot analysis.** Protein (100  $\mu\text{g}$ ) was extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Pierce; Thermo Fisher Scientific, Inc.). Protein concentration was determined using a Bradford DC protein assay (Bio-Rad Laboratories, Inc.). Subsequently, proteins were separated in 10% SDS-PAGE (Beyotime Institute of Biotechnology, Inc., Shanghai, China) and transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Inc.), which was then incubated with phosphate-buffered saline (PBS) containing 50 g/l skimmed milk at room temperature for 4 h. Next, the PVDF membrane was incubated with mouse anti-rat P65 NF- $\kappa\text{B}$  polyclonal antibody (1:100; cat. no. ab13594), mouse anti-rat p-c-JNK polyclonal antibody (1:100; cat. no. ab192200), rabbit anti-rat total-c-JNK monoclonal antibody (1:100; cat. no. ab179461), rabbit anti-rat p-p38 mitogen-activated protein kinase (MAPK) polyclonal antibody (1:200; cat. no. ab47363), mouse anti-rat total-p38 MAPK monoclonal antibody (1:200; cat. no. ab31828), rabbit anti-rat TNF- $\alpha$  polyclonal antibody (1:100; cat. no. ab6671), rabbit anti-rat IL-1 $\beta$  polyclonal antibody (1:200; cat. no. ab9722), mouse anti-rat IL-6 monoclonal antibody (1:200; cat. no. ab9324) and rabbit anti-rat IL-8 monoclonal antibody (1:100; cat. no. ab7747) (all Abcam, Cambridge, UK), at 37 $^{\circ}\text{C}$  for 1 h. After washing with PBS three times, the PVDF membrane was incubated with the goat anti-mouse (1:10,000; cat. no. ab97023) or goat anti-rabbit peroxidase-conjugated secondary antibody

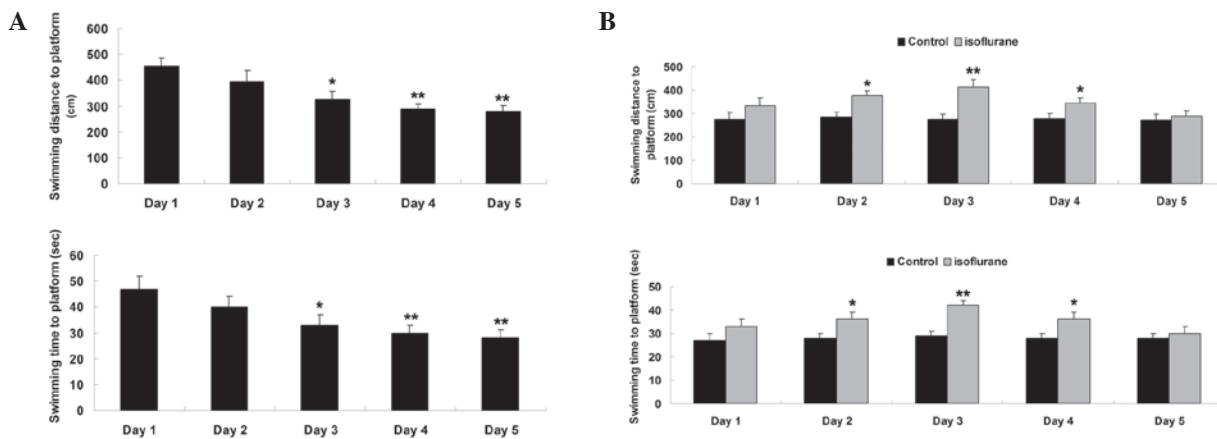


Figure 1. Swimming distance and time to the platform in a Morris water maze test were used to evaluate rat spatial memory function. (A) Prior to exposure to isoflurane, all aged rats were trained in Morris water maze for 5 days. \* $P < 0.05$  vs. Day 1; \*\* $P < 0.01$  vs. Day 1. (B) Morris water maze assay was performed on days 1-5 after exposure to isoflurane. Control rats did not receive isoflurane. \* $P < 0.05$  vs. Control; \*\* $P < 0.01$  vs. Control.

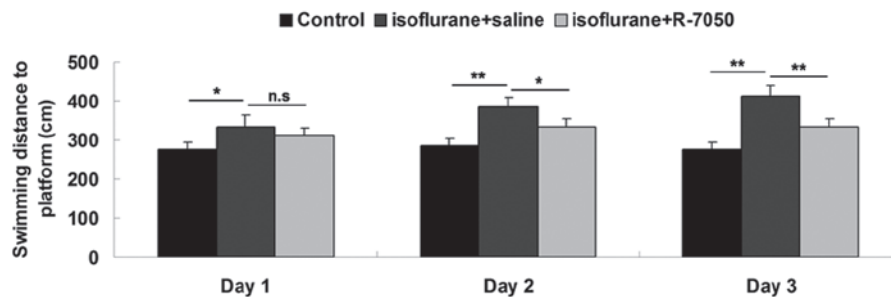


Figure 2. Morris water maze assay was performed on days 1-3 after exposure to isoflurane in rats receiving intracisternal administration of R-7050 or saline before exposure to isoflurane. Control rats did not receive isoflurane. \* $P < 0.05$ ; \*\* $P < 0.01$ ; n.s., not significant.

(1:10,000; cat. no. ab6721; both Abcam) at room temperature for 1 h. Chemiluminescence detection was performed using an ECL Western Blotting kit (cat. no. 32109; Pierce; Thermo Fisher Scientific, Inc.).

**Statistical analysis.** Data was expressed as the mean  $\pm$  standard deviation. Differences between two groups were determined using Student's t-test. Statistical analysis was performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Aged rats show cognitive dysfunction following exposure to isoflurane.** All aged rats were trained in MWM for 5 days prior to exposure to isoflurane. Swimming distance and time spent in the MWM were used to evaluate their spatial memory function. The data showed that swimming distance and time to the platform in MWM were notably reduced during the 5 days of training (Fig. 1A). These results suggest that the rat spatial memory is gradually developed.

Then, the MWM assay was performed on days 1-5 after exposure to isoflurane. As shown in Fig. 1B, the swimming distance and time to the platform were increased on days 1-3 after exposure to isoflurane when compared with the control group, suggesting that the spatial memory was impaired after

exposure to isoflurane. From day 4 after exposure to isoflurane, the swimming distance and time to the platform were gradually reduced. On day 5 after exposure to isoflurane, the swimming distance and time to the platform showed no significant difference when compared with the control group (Fig. 1B). These findings suggest that their impaired spatial memory was gradually recovered.

**TNF- $\alpha$  receptor antagonist attenuates isoflurane-induced cognitive dysfunction in aged rats.** To reveal the role of TNF- $\alpha$  in isoflurane-induced cognitive dysfunction in aged rats, intracisternal administration of TNF- $\alpha$  receptor antagonist R-7050 was performed prior to rat exposure to isoflurane. The swimming distance and time to the platform on days 1-3 after exposure to isoflurane. Following the intracisternal administration of R-7050, the swimming distance and time to the platform were notably reduced, when compared with the rats in the isoflurane + saline group, although they remained higher than the control group in which the rats did not receive isoflurane anesthesia (Fig. 2). These data show that the intracisternal administration of TNF- $\alpha$  receptor antagonist R-7050 notably attenuated isoflurane-induced cognitive dysfunction in aged rats.

**TNF- $\alpha$  receptor antagonist suppresses the isoflurane-induced expression of proinflammatory cytokines in the hippocampus tissue of aged rats.** It has been demonstrated that

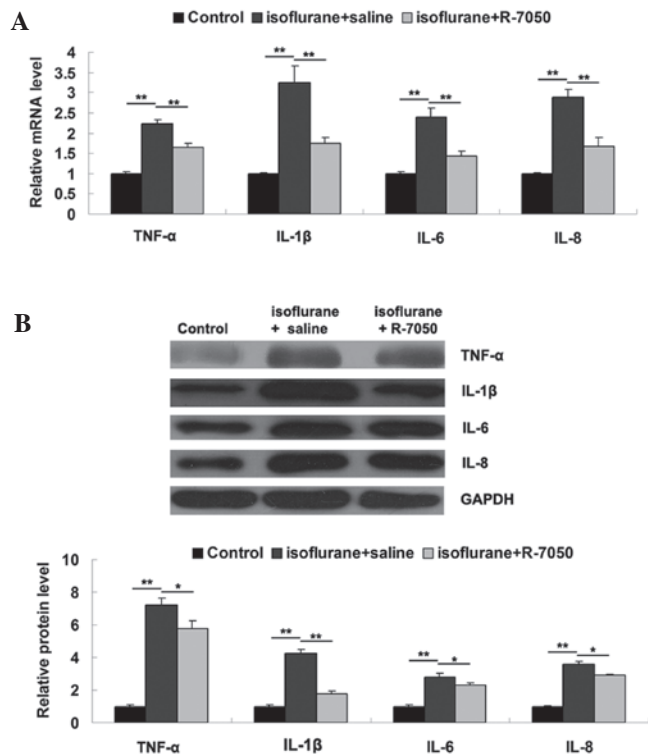


Figure 3. (A) On day 3 after exposure to isoflurane, quantitative polymerase chain reaction was performed to determine the mRNA expression of proinflammatory cytokines in the hippocampus tissue of aged rats receiving intracisternal administration of R-7050 or saline before exposure to isoflurane. \*\* $P < 0.01$ . (B) On day 3 after exposure to isoflurane, western blot analysis was performed to determine the protein expression of proinflammatory cytokines in the hippocampus tissue of aged rats receiving intracisternal administration of R-7050 or saline before exposure to isoflurane. \* $P < 0.05$ ; \*\* $P < 0.01$ . Control rats did not receive isoflurane. TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; IL, interleukin.

proinflammatory cytokines, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8, play key roles in POCD, and their expression levels may be notably upregulated after surgery and anesthesia (5). To further reveal the underlying mechanisms, we evaluated the expression levels of these proinflammatory cytokines in the hippocampus tissue of aged rats in each group by performing RT-qPCR and western blot analyses. As shown in Fig. 3A and B, the mRNA and protein levels of these proinflammatory cytokines were significantly increased on day 3 after exposure to isoflurane, when compared to the control group. However, intracisternal administration of R-7050 significantly attenuated the isoflurane-induced upregulation of these proinflammatory cytokines, suggesting that inhibition of TNF- $\alpha$  effectively inhibits the isoflurane-induced expression of proinflammatory cytokines in the hippocampus tissue of aged rats.

*TNF- $\alpha$  receptor antagonist suppresses isoflurane-induced activation of p38 MAPK signaling in the hippocampus tissue of aged rats.* MAPKs signaling pathways have been demonstrated to be involved in TNF- $\alpha$ -mediated inflammatory responses (9). Therefore, we further determined the involvement of c-JNK and p38 MAPK signaling pathways in hippocampus tissue of aged rats on day 3 after exposure to isoflurane. As demonstrated in Fig. 4, the phosphorylated protein levels of c-JNK and p38 MAPK were significantly

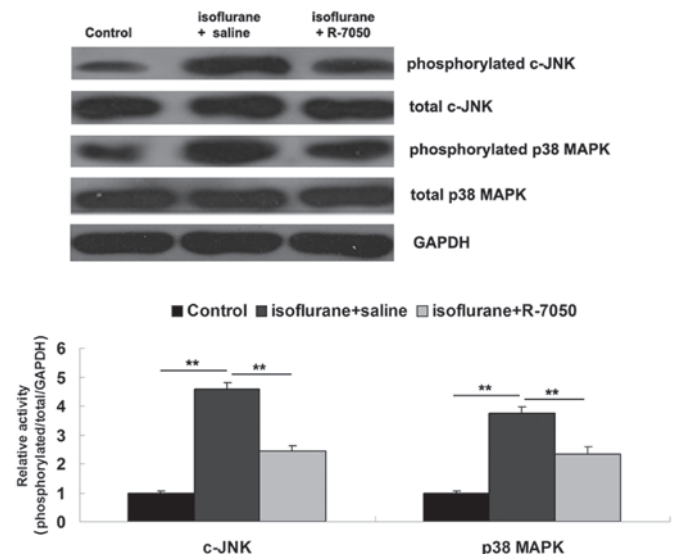


Figure 4. On day 3 after exposure to isoflurane, western blot analysis was performed to determine the total protein and phosphorylated protein levels of c-JNK and p38 MAPKs in the hippocampus tissue of aged rats with intracisternal administration of R-7050 or saline. Control rats did not receive isoflurane. \*\* $P < 0.01$ . c-JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

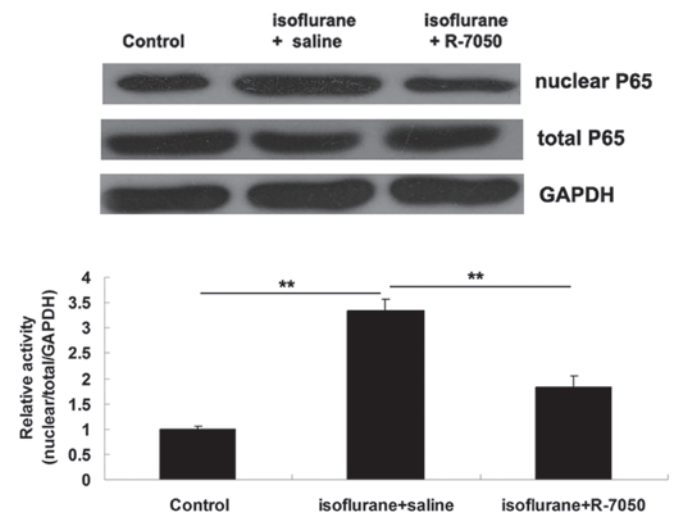


Figure 5. On day 3 after exposure to isoflurane, western blot analysis was performed to determine the protein level of P65 in nuclear as well as total P65 in the hippocampus tissue of aged rats with intracisternal administration of R-7050 or saline. Control rats did not receive isoflurane. \*\* $P < 0.01$ .

increased on day 3 after exposure to isoflurane when compared to the control group, suggesting that the c-JNK and p38 MAPK signaling pathways in hippocampus tissue were activated by isoflurane. However, intracisternal administration of R-7050 significantly attenuated the isoflurane-induced upregulation of phosphorylated protein levels of c-JNK and p38 MAPK in hippocampus tissue, suggesting that inhibition of TNF- $\alpha$  suppressed isoflurane-induced activation of MAPKs signaling in the hippocampus tissue of aged rats.

*TNF- $\alpha$  receptor antagonist suppresses the isoflurane-induced activation of NF- $\kappa$ B signaling in the hippocampus tissue of aged rats.* NF- $\kappa$ B is known to function as a downstream signaling

molecule of TNF- $\alpha$  (9). Accordingly, we further examined the activity of NF- $\kappa$ B signaling in the hippocampus tissue of aged rats in each group. The protein expression levels of NF- $\kappa$ B P65 in nucleus was significantly upregulated on day 3 after exposure to isoflurane in the hippocampal tissue, when compared with the control group, indicating that NF- $\kappa$ B signaling was activated (Fig. 5). However, intracisternal administration of the TNF- $\alpha$  receptor antagonist R-7050 significantly attenuated the isoflurane-induced activation of NF- $\kappa$ B signaling in the hippocampus tissue of aged rats (Fig. 5).

## Discussion

Neuroinflammation is known to play a key role in the development of POCD as well as other diseases in the central nervous system. Among the risk factors of POCD, anesthesia has been well studied recently (13). For example, Callaway *et al* showed that desflurane anesthesia induced memory impairment in rats, which was age- and dose-dependent (14). Liu *et al* investigated the effects of different concentrations and duration times of isoflurane administration on acute and long-term neurocognitive function in young mice, and suggested that isoflurane may cause neurotoxicity by inducing caspase activation and apoptosis with increased anesthetic concentration and prolonged duration (15). A single injection of emulsified isoflurane causes reversible learning and memory dysfunction in adult rats, and the downregulation of brain-derived neurotrophic factor expression may be involved in the isoflurane-induced cognitive impairment (16). Furthermore, in streptozotocin-induced diabetic rats, isoflurane anesthesia also induces cognitive dysfunction (11). The present results suggest that isoflurane caused impaired cognitive function, as demonstrated by increased swimming distance and time to the platform in an MWM assay. Yang *et al* compared the roles of propofol and isoflurane in the neurodegeneration and cognitive impairment in neonatal mice, and found that both caused significant apoptosis in the developing brain, with isoflurane being more potent (17). In addition, isoflurane significantly increased the levels of the plasma neurodegenerative biomarker, S100 $\beta$  (18).

Neuroinflammation has been demonstrated to be a key factor for the progression of cognitive dysfunction after surgery and/or anesthesia (19). During the development of POCD, the expression levels of various proinflammatory cytokines has been reported to be significantly increased, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 (18). For example, Yu *et al* (18) showed that splenectomy induced a transient cognitive deficiency in elderly rats, accompanied by notable upregulation of TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 expression in the hippocampal tissues. Moreover, it has been reported that these proinflammatory cytokines further led to the activation of microglial cells and caused neuroinflammation responses and brain injury (20,21). The present data show that exposure to isoflurane led to increased productions of these proinflammatory cytokines in the hippocampus tissue of aged rats, which is consistent with previous studies. For instance, Li *et al* found that isoflurane anesthesia increased the hippocampal levels of IL-1 $\beta$  and TNF- $\alpha$  (22). Furthermore, Zhang *et al* found that isoflurane-induced neuroinflammation by promoting the expression of IL-6 (23).

TNF- $\alpha$  mediates various biological processes by binding to its receptors (24,25). Two TNF- $\alpha$ -mediated signaling pathways have been found to be involved in inflammation, including MAPKs and NF- $\kappa$ B (24,25). As a member of MAPKs family, c-JNK has been found to be function as a downstream effector of TNF- $\alpha$  (24,25). Kassardjian *et al* found that c-JNK mediated the effect of caspases and NF- $\kappa$ B in the TNF- $\alpha$ -induced inhibition of Na<sup>+</sup>/K<sup>+</sup> ATPase in HepG2 cells (26). Furthermore, TNF- $\alpha$  reduces Na<sup>+</sup>/K<sup>+</sup> ATPase activity by activating JNK in LLC-PK1 cells (27). In addition, the activation of the p38 MAPK is involved in the TNF- $\alpha$ -induced upregulation of inflammation responses (28). In the present study, the inhibition of TNF- $\alpha$  suppressed the isoflurane-induced activation of the c-JNK and p38 MAPK signaling pathways in the hippocampus tissue of aged rats. In addition, TNF- $\alpha$  has been found to lead to the activation of NF- $\kappa$ B signaling, which may result in the abundant expression of cytokines involved in inflammation (29,30). Zhu *et al* have found that NF- $\kappa$ B signaling is involved in bisphenol A-induced TNF- $\alpha$  expression in microglial cells (31). The present findings showed that the inhibition of TNF- $\alpha$  suppressed the activation of the NF- $\kappa$ B signaling pathway, accompanied by the downregulation of proinflammatory cytokines in the hippocampus tissue of aged rats after exposure to isoflurane, suggesting that the inhibitory effect of TNF- $\alpha$  antagonist on isoflurane-induced cognitive decline may occur via the suppression of NF- $\kappa$ B signaling in aged rats.

In conclusion, the present study showed that exposure to isoflurane led to cognitive decline and neuroinflammation in aged rats. Further investigation suggested that inhibition of TNF- $\alpha$  significantly attenuated isoflurane-induced cognitive dysfunctions, which may occur via the mediation of the MAPK and NF- $\kappa$ B signaling pathways. Therefore, TNF- $\alpha$  may become a potential molecular target for the prevention of POCD.

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