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Activation of the canonical nuclear factor-κB pathway is involved in isoflurane-induced hippocampal interleukin-1β elevation and the resultant cognitive deficits in aged rats

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ABSTRACT

Although much recent evidence has demonstrated that neuroinflammation contributes to volatile anesthetic-induced cognitive deficits, there are few existing mechanistic explanations for this inflammatory process. This study was conducted to investigate the effects of the volatile anesthetic isoflurane on canonical nuclear factor (NF)-κB signaling, and to explore its association with hippocampal interleukin (IL)-1β levels and anesthetic-related cognitive changes in aged rats. After a 4-h exposure to 1.5% isoflurane in 20-month-old rats, increases in IκB kinase and IκB phosphorylation, as well as a reduction in the NF-κB inhibitory protein (IκBα), were observed in the hippocampi of isoflurane-exposed rats compared with control rats. These events were accompanied by an increase in NF-κB p65 nuclear translocation at 6 h after isoflurane exposure and hippocampal IL-1β elevation from 1 to 6 h after isoflurane exposure. Nevertheless, no significant neuroglia activation was observed. Pharmacological inhibition of NF-κB activation by pyrrolidine dithiocarbamate markedly suppressed the IL-1β increase and NF-κB signaling, and also mitigated the severity of cognitive deficits in the Morris water maze task. Overall, our results demonstrate that isoflurane-induced cognitive deficits may stem from upregulation of hippocampal IL-1β, partially via activation of the canonical NF-κB pathway, in aged rats.

1. Introduction

Postoperative cognitive dysfunction (POCD) in the elderly has emerged as a major health concern [1]. It is associated with premature departure from the workforce, increased disability, and early mortality [2]. Unfortunately, the pathophysiology of POCD remains elusive.

The potential risk factors for POCD can be classified into three categories from the patient, the surgery or the anesthesia [1]. Many recent animal studies [3–6] and clinical observations [7,8] have supplied evidence that anesthetics, particularly inhalational anesthetics, may play a role in cognitive deficits. Isoflurane, an inhalation anesthetic that is widely used clinically, has been shown to induce POCD through cytokine-dependent neuroinflammatory mechanisms, in which isoflurane increases the production of interleukin 1 (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α in a rat or mouse brain [3,9]. Nevertheless, the exact signaling mechanisms by which isoflurane mediates increases in these proinflammatory cytokines remain to be elucidated.

Nuclear factor (NF)-κB is one of the most critical transcription factors involved in inflammation [10]. The NF-κB family comprises of RelA/p65, RelB, c-Rel, p50, and p52, RelA/p50 heterodimer being the most abundant and widely expressed [11]. Multiple proinflammatory signals activate NF-κB, mostly through inhibitor of NF-κB protein (IκB) kinase (IKK)-dependent phosphorylation of IκB, leading to IκB ubiquitination and degradation. This ultimately leads to nuclear translocation of NF-κB and induction of transcription of the target genes [11], including the proinflammatory cytokine IL-1β [10]. NF-κB signaling can be regulated by IκBs and IKKs through various routes. The most frequently observed is the canonical path-

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way, which is characterized by phosphorylation of IκBα on serine residues 32 and 36 and proteasome degradation, and nuclear translocation of RelA/p65 depending on the catalytic subunits IKKα/β [12].

Recent *in vivo* and *in vitro* studies have revealed that NF-κB activation may be involved in mediating the neuroinflammation in models of Alzheimer's disease [13,14], which has a similar molecular pathological mechanism to POCD. Therefore, the present study aimed to determine whether NF-κB signaling is involved in isoflurane-induced neuroinflammation and cognitive impairment *in vivo*. Specifically, we hypothesized that isoflurane would induce neuroinflammation through activation of the canonical NF-κB pathway in aged rats.

2. Materials and methods

2.1. Animals

Aged male Sprague–Dawley rats (20 months of age; weight: 550–600 g) were used for all experiments. They were bred and maintained under standardized housing conditions with food and water *ad libitum*. The experimental protocol was approved by the Peking University Biomedical Ethics Committee Experimental Animal Ethics Branch (Approval No. LA2012-38).

2.2. Experiment protocols

2.2.1. Experiment A

To study the effects of isoflurane exposure on the NF-κB signaling pathway activity, rats were randomly assigned to isoflurane ($n = 24$) or control ($n = 8$) groups and exposed to isoflurane or vehicle gas, respectively. The expression levels of hippocampal IL-1β and two glial cell activation markers, cluster of differentiation 11b (CD11b) and glial fibrillary acidic protein (GFAP), were dynamically examined at 1, 3, 6, 12, and 24 h after isoflurane exposure using enzyme-linked immunosorbent assay (ELISA) and Western blotting ($n = 4$ per time point), respectively. Meanwhile, the activation of NF-κB, including IκBα degradation and phosphorylation of IκBα and IKKα/β, was also assessed by Western blotting. NF-κB p65 nuclear translocation was observed at 6 h after anesthesia by immunofluorescence ($n = 4$ each).

2.2.2. Experiment B

To evaluate the role of the NF-κB signaling pathway in isoflurane exposure, the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) (Sigma–Aldrich, St. Louis, MO) was used for blocking studies. Rats were randomly assigned to control, ISO, PDTC + ISO, and PDTC groups ($n = 13$ per group). The rats in the PDTC + ISO and PDTC groups were both intraperitoneally administered with PDTC at 100 mg/kg in saline (total volume: 0.5 ml) 1 h before exposure to isoflurane or vehicle gas, respectively. The rats in the other two groups received an identical volume of saline. This dosing protocol of PDTC has been shown to effectively inhibit lipopolysaccharide-induced IκBα degradation and the resultant NF-κB activation in rats [15]. Subsequently, the rats in the ISO and PDTC + ISO groups received isoflurane exposure, while the rats in the other two groups were exposed to vehicle gas without anesthetic for an equivalent period of time.

At 6 h after anesthesia, four rats per group were euthanized for hippocampal harvesting. Half of each hippocampus was used for Western blotting analyses of the phosphorylated IKK (p-IKK), p-IκBα, and IκBα levels, and the other half was used for ELISA detection of the IL-1β levels ($n = 4$). Hippocampal-dependent spatial memory ability was evaluated using the Morris water maze (MWM) test ($n = 9$ per group).

2.3. Isoflurane exposure

The protocol for isoflurane exposure was based on our previous studies [16,17]. Briefly, rats were placed in a temperature-controlled, transparent anesthetic chamber. During exposure, the chamber was gassed with 1.5% isoflurane (Baxter Healthcare, Deerfield, IL) through a calibrated isoflurane vaporizer, carried by 100% oxygen for 4 h. Standard soda lime was placed at the bottom of the container to clear the carbon dioxide. The concentrations of isoflurane, oxygen, and carbon dioxide in the chamber were continuously analyzed with a gas monitor (Datex-Ohmeda, Louisville, CO). After anesthesia, the rats received 100% oxygen until they regained consciousness.

2.4. Blood gas analysis

To determine whether isoflurane anesthesia caused physiologic side effects such as hypoxia, hypercapnia, or hypoglycemia, five rats in the various treatment groups were selected as cardiorespiratory control animals (total: $n = 20$). Two milliliters of blood was immediately drawn by cardiac puncture at the end of the isoflurane exposure. Arterial blood gases (ABG) and blood glucose measurements were performed using a portable blood gas analyzer (OPTI Medical Systems, Roswell, GA) and an One Touch Ultra blood glucose monitoring system (Life Scan Inc., Milpitas, CA), respectively. The cardiorespiratory control rats were not used for any other part of the study.

2.5. ELISA

The homogenates from the hippocampus were centrifuged at 10,000×*g* for 10 min at 4 °C as described previously [14]. The concentration of IL-1β in the supernatant fluid was measured using an ELISA kit (Rapidbio Lab, West Hills, CA), according to the manufacturer's instructions. Each experimental condition was tested in three different wells and measured in duplicate.

2.6. Western blotting

Western blot analyses were performed to determine the expression levels of CD11, GFAP, p-IKKα/β, p-IκBα, and IκBα as previously described [18], with the following modifications. 60 μg protein per lane was separated by 10% SDS–PAGE. After transfer to membranes, the proteins were probed with the following primary antibodies: anti-CD11b (1:500; Millipore, Billerica, MA); anti-GFAP and anti-p-IκBα (1:1000; CST, Danvers, MA); anti-IκBα and anti-pIKK (1:1000; CST). Fluorescently labeled secondary antibodies (1:10,000; LI-COR Biosciences, Lincoln, NE) were used to detect the binding of the primary antibodies. The bound proteins were visualized by scanning the membranes in an Odyssey Infrared Imaging System (LI-COR Biosciences). The results for rats under the different experimental conditions were normalized by the mean values of the corresponding control animals.

2.7. Morphology

Tissue preparation and immunofluorescence staining of brain sections were performed as previously described [17] using an anti-NF-κB p65 primary antibody (1:50; CST) and a fluorescein isothiocyanate-labeled secondary antibody (1:200; Abcam, Cambridge, UK). The nuclei were counterstained with 4,6-diamidino-2-phenyl-indole (1:5000; Roche, Mannheim, Germany).

2.8. MWM test

As previously described [14], four groups with 9 rats per group were tested for memory using the MWM test by investigators blinded to the group conditions. The rats received four training trials daily for 5 consecutive days. During each trial, the rats were gently placed in the water facing the wall of the maze at one of the four equally spaced start positions (north, south, east, or west). The time it took to locate the submerged platform (defined by the latency cut-off time of 120 s) and the swimming velocity were recorded. On day six, a series of probe trials were conducted, whereby the platform was removed. The platform site crossovers and the percentage of time spent in the previous platform quadrant in a 90-s period were determined.

2.9. Statistical analysis

For statistical analyses, SPSS 14.0 for Windows (SPSS, Chicago, IL) was used. The values for physiological parameters and data obtained by Western blotting analysis and ELISA were expressed as the mean ± SD, and analyzed by one-way analysis of variance (ANOVA), followed by a least square difference multiple comparison test. Data collected from the behavioral studies were expressed as the mean ± SEM, and analyzed by two-way repeated-measures ANOVA, with Bonferroni post hoc analysis. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Physiologic parameters after isoflurane exposure

There were no significant differences in the ABG values and blood glucose concentrations among the four treatment groups immediately after the 4-h exposure to 1.5% isoflurane (Supplementary Table 1). These data reduce the possibility that the isoflurane-induced neurodegeneration in the hippocampus was caused by the above-mentioned physiologic side effects.

3.2. Isoflurane exposure induces IL-1β upregulation, but not neuroglia activation

IL-1β expression was significantly increased after isoflurane exposure. Specifically, it was increased at 1 h after anesthesia, peaked at 3 h, persisted until 6 h, and then decreased to the baseline levels at 12 h after anesthesia (Fig. 1).

Neuroglial activation could be both a cause and an effect of increased IL-1β hippocampal expression [19]. Increased expressions of specific markers, such as CD11b and GFAP, have been respectively associated with the activation of microglia and astrocytes [20]. Consequently, we examined the impacts of isoflurane exposure on microglial and astrocyte activation by comparing the levels of CD11b and GFAP in the hippocampus (Fig. 1B). Compared with control rats exposed to vehicle gas, there were no significant differences in the CD11b and GFAP contents in the hippocampus of rats exposed to isoflurane at a series of time points after the anesthesia.

3.3. Isoflurane exposure activates the canonical NF-κB signaling pathway

Compared with the control group, the expression of p-IKKα/β protein was 1.51 times greater at 3 h after isoflurane exposure and 1.78 times greater at 6 h after isoflurane exposure (Fig. 2B). The IκBα phosphorylation levels from 3 to 12 h after isoflurane exposure were higher than those in the control group (Fig. 2C). In contrast to the pattern of p-IKKα/β changes, decreases in the total protein levels of IκBα, an indicator of NF-κB activation, were detected from 3 to 6 h after isoflurane exposure (Fig. 2D). To investigate the downstream nuclear translocation of NF-κB p65, immunofluorescence analyses at 6 h after isoflurane exposure were conducted. The results showed that the increased NF-κB p65 (green) was mainly localized in the neuronal nuclei in the hippocampal dentate gyrus region (arrowheads), compared with the control group (Fig. 2E). These changes indicated a transient NF-κB activation with a threshold time of 1–3 h after a 4-h isoflurane challenge.

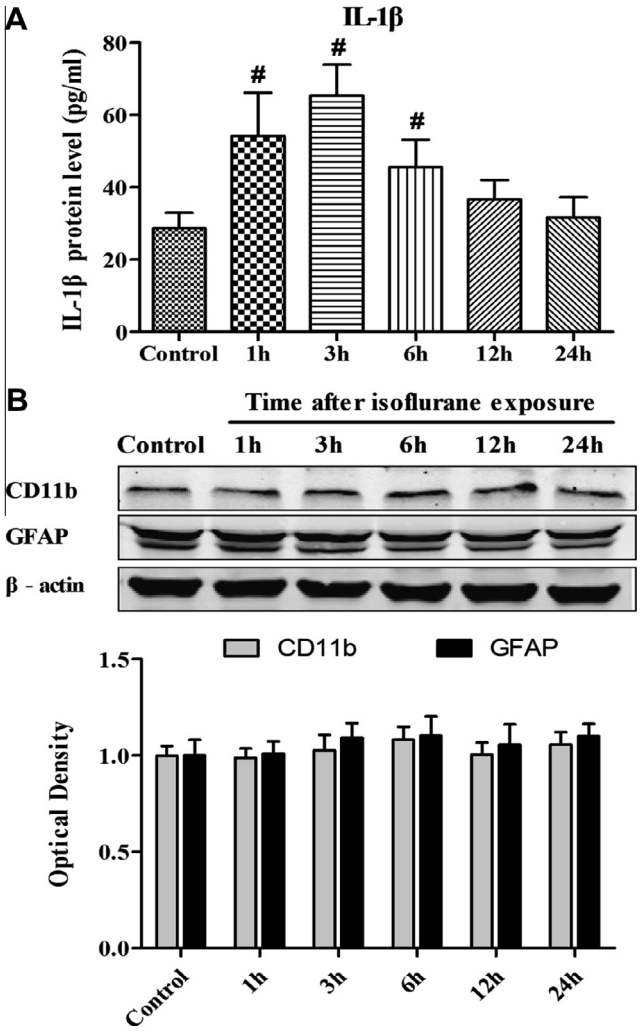

Fig. 1. Effects of isoflurane exposure on the levels of IL-1β, CD11b, and GFAP in the hippocampus. Twenty-month-old rats were exposed to 1.5% isoflurane or vehicle gas for 4 h. (A) The hippocampal IL-1β expression changes significantly over time after isoflurane exposure. (B) Compared with control rats, no significant changes in the levels of CD11b and GFAP are observed at any time points. The values are given as means ± SD ($n = 4$) for each condition. $^{\#}$ $p < 0.05$, vs. control group.

3.4. PDTC suppresses isoflurane-induced NF-κB activation and IL-1β upregulation

To determine whether the canonical NF-κB pathway was involved in the hippocampal interleukin-1β elevation after isoflurane exposure, the inhibitory effects of PDTC were evaluated at 6 h after isoflurane exposure. Western blotting demonstrated that PDTC pretreatment significantly prevented the isoflurane-induced increase in p-IKKα/β and p-IκBα and the decrease in IκBα protein levels (Fig. 3A). Similarly, PDTC also markedly suppressed the in-

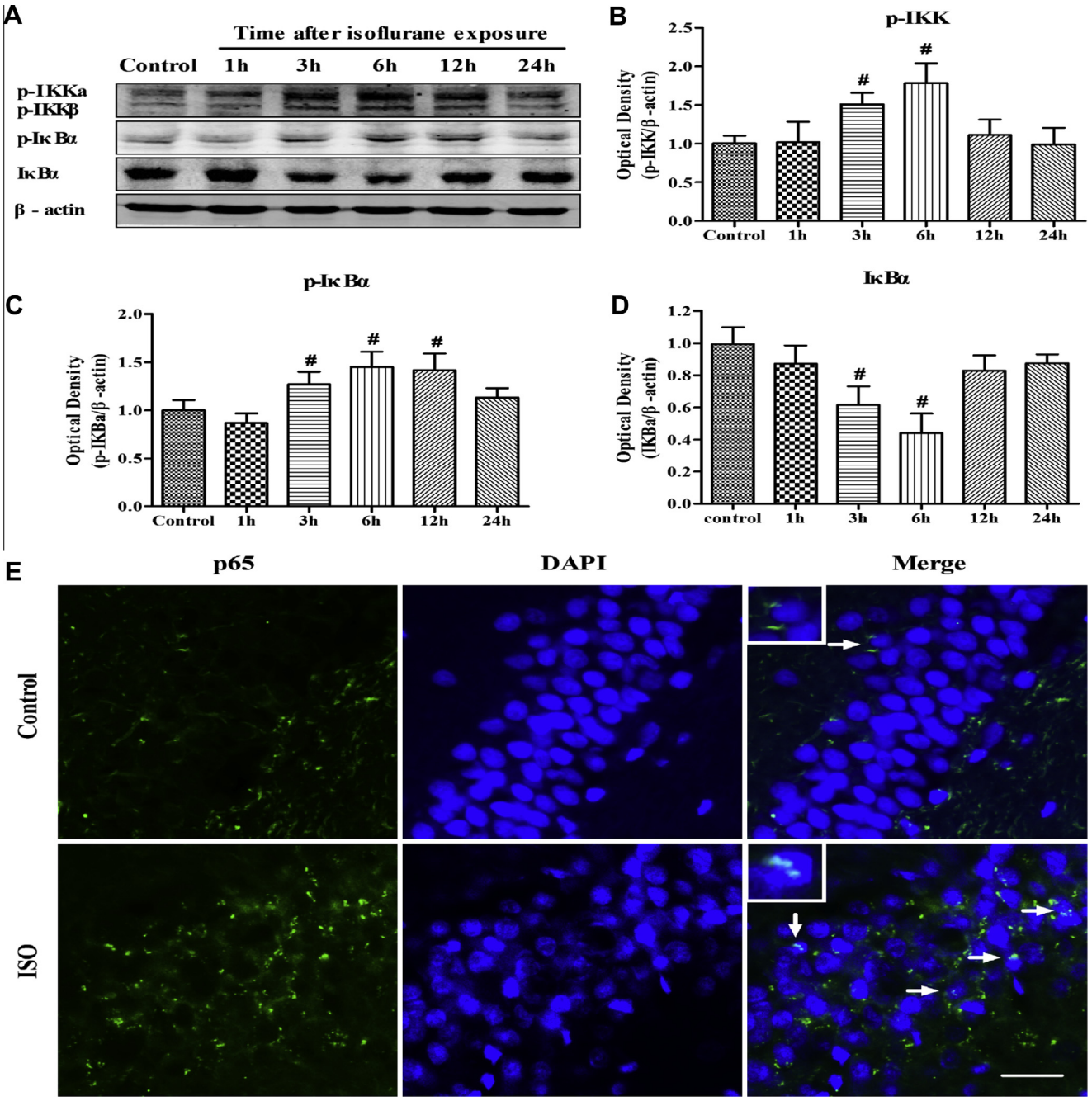

Fig. 2. Effects of isoflurane exposure on the canonical NF-κB signaling pathway activity. Twenty-month-old rats were exposed to 1.5% isoflurane or vehicle gas for 4 h. (A) Representative Western blotting images. Kinetics of isoflurane-induced changes in the levels of p-IKK (B), p-IκBα (C), and IκBα (D) in the hippocampus of aged rats. (E) Immunofluorescence analysis of the p65 subunit of NF-κB reveals its increased nuclear localization in the hippocampal dentate gyrus region at 6 h after isoflurane exposure. NF-κB p65, green; cell nuclei, blue. Magnification, ×400 (inset, ×1000). Scale bar, 20 μm. Values are given as means ± SD ($n = 4$) for each condition. $^{\#}p < 0.05$, vs. control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

crease in hippocampal IL-1β levels (Fig. 3B). When given alone, PDTC had no effect on the expression of p-IKKα/β, p-IκBα, IκBα and IL-1β at 6 h after isoflurane.

3.5. Isoflurane exposure-induced cognitive impairment is attenuated by PDTC

As shown in Fig. 4A, both the repeated factor (days) and the over-group factor (treatment) significantly affected the latency of the rats to locate the hidden platform ($p < 0.001$). However, no interactive effect between days and treatment was found. Statistical analyses showed that on days 4 and 5, the isoflurane-exposed rats took longer to find the platform. There was no significant difference in the latencies between the control and PDTC groups. All of the rats appeared to swim normally and no differences were observed in the swimming speeds among the four groups (Fig. 4B).

In the probe test, the time spent in the platform target area by the rats in the ISO group was much shorter than that of the rats in the control group (Fig. 4C), and the number of platform crossings was lower (Fig. 4D), thus validating the memory impairments after

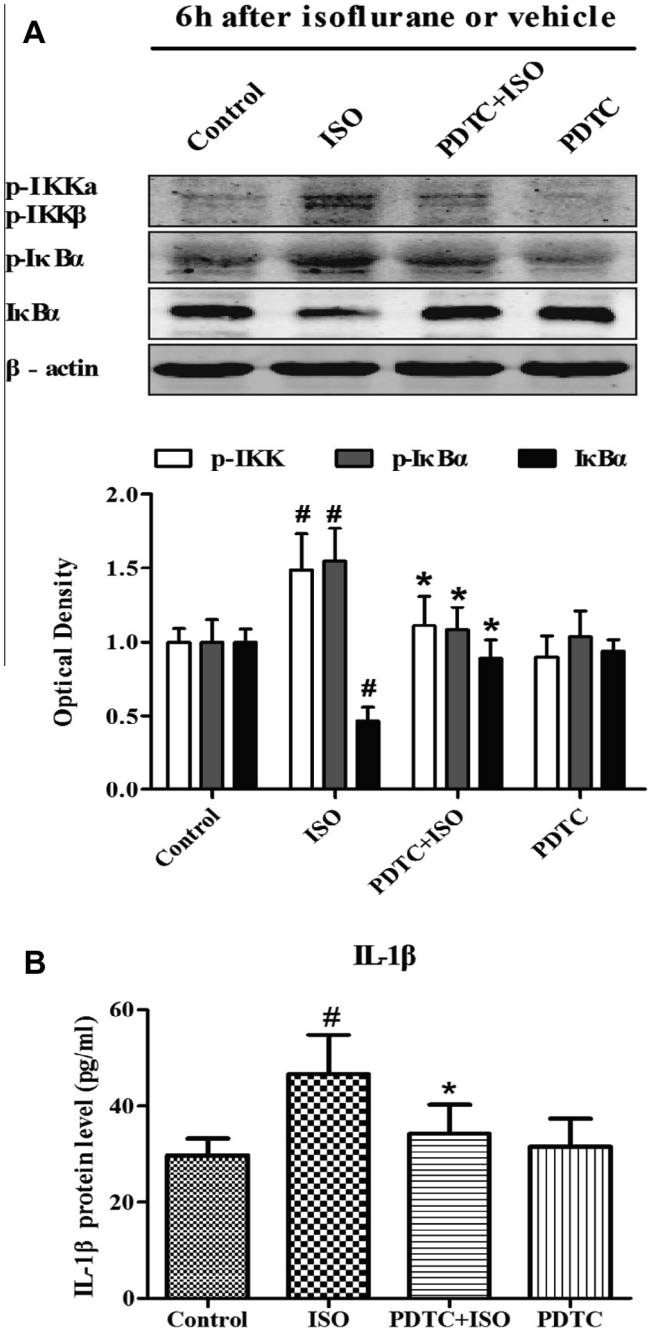

Fig. 3. PDTC inhibits isoflurane-induced NF-κB activation and IL-1β elevation in the hippocampus. Twenty-month-old rats were exposed to 1.5% isoflurane or vehicle gas in the presence or absence of PDTC for 4 h. PDTC (100 mg/kg) was injected intraperitoneally at 1 h before exposure. (A) PDTC blocks the isoflurane-induced increases in the p-IKK and p-IκBα levels and decrease in the IκBα level at 6 h after isoflurane exposure. (B) PDTC suppresses the increase in hippocampal IL-1β levels 6 h after isoflurane. Values are given as means ± SD ($n = 4$) for each condition. # $p < 0.05$, vs. control group. * $p < 0.05$, vs. ISO group.

a 4-h isoflurane exposure. After PDTC pretreatment, the percentage time that the rats in the PDTC + ISO group spent in the target quadrant was much greater than that of the rats in the ISO group (Fig. 4C), which was consistent with the searching swimming paths in the probe trial test (Fig. 4E). However, there were no significant differences between the rats in the ISO and PDTC + ISO groups with respect to the numbers of platform crossings (Fig. 4D).

4. Discussion

The major focus of the present study was to determine the role of NF-κB signaling in mediating isoflurane-induced neuroinflammation and spatial memory deficits *in vivo*. We demonstrated that a 4-h isoflurane exposure induced elevation of the proinflammatory cytokine IL-1β, but not neuroglial activation, in the hippocampus of aged rats. The canonical NF-κB signaling pathway was transiently activated, as evidenced by marked upregulation of p-IKKα/β and p-IκBα, degradation of IκBα, and nuclear translocation of NF-κB p65 at 6 h after isoflurane exposure. Inhibition of NF-κB by PDTC suppressed the downstream IL-1β expression and mitigated the isoflurane-induced cognitive dysfunction.

As one of the NF-κB target genes, IL-1β is under the regulation of NF-κB signaling and is thought to be a classical marker of neuroinflammation [10]. It is uniformly reported to be increased after isoflurane exposure in aged rats [5,21]. Here, we found that there was an increase in the IL-1β levels in the hippocampus from 1 to 6 h after isoflurane exposure, accompanied by a transient NF-κB activation. Specifically, we observed phosphorylation of IκB on serine residues 32 and 36 (from 3 to 12 h) and proteasome degradation (from 3 to 6 h) after anesthesia in the hippocampus. The immunofluorescence staining also revealed an increase in nuclear translocation of p65, a hallmark of the activated canonical pathway. In contrast, inhibition of NF-κB signaling by PDTC suppressed the IL-1β gene expression, demonstrating that isoflurane increased the IL-1β levels via activation of the canonical NF-κB pathway.

In the present study, acute elevation of hippocampal IL-1β occurred at 1 h after isoflurane exposure. This preceded the initial activation of NF-κB at 3 h after isoflurane exposure. Interestingly, the pattern of these changes was not consistent with previous observations by Li et al. [5], in which aged rats demonstrated immediate elevation of hippocampal IL-1β and degradation of IκBα at 0 h after isoflurane exposure. The aspect of whether these inconsistencies arise through differences in the anesthetic exposure methods (e.g., vehicle gas) or anesthetic durations (e.g., 4 vs. 6 h) remains unclear at this time. The delayed kinetics of NF-κB induction and earlier IL-1β translation in response to isoflurane exposure further suggest that other transcription factors, such as cyclic AMP [22] and activating protein-1 [23], may also participate in this inflammatory process. Further research on this issue may be warranted.

Nevertheless, the expressions of CD11b, an indicator of reactive microglia, and GFAP, an astrocyte marker, were both unchanged by isoflurane exposure. These findings indicate that mild neuroinflammation characterized by an increase in IL-1β expression after isoflurane exposure may not be sufficient to induce activation of microglia or astroglia in the aged rat brain. These results are similar to previous data obtained with mouse models [3,19].

Both neuroprotective and neurotoxic effects of isoflurane have been extensively reported in the literature. However, the factors that determine the direction of its effects remain obscure [24]. The level and duration of isoflurane exposure appear to be determining factors [24,25]. We chose 1.5% isoflurane because it represents the minimum alveolar concentration (MAC) of isoflurane over 4 h in aged rats (1 MAC is the concentration at which 50% of animals do not move in response to a standardized stimulus) [26]. The determination of anesthetic duration was based on a previous rat study, in which a 4-h exposure to 1 MAC isoflurane resulted in cognitive deficits in the MWM test [4]. Similar to our previous report [16] and other related animal studies [3–6], exposure of aged rats to 1.5% isoflurane for 4 h in the present study caused deficits in their spatial learning and memory, as manifested by the longer escape latency, less time spent in the target quadrant, and fewer original platform crossings in the MWM test.

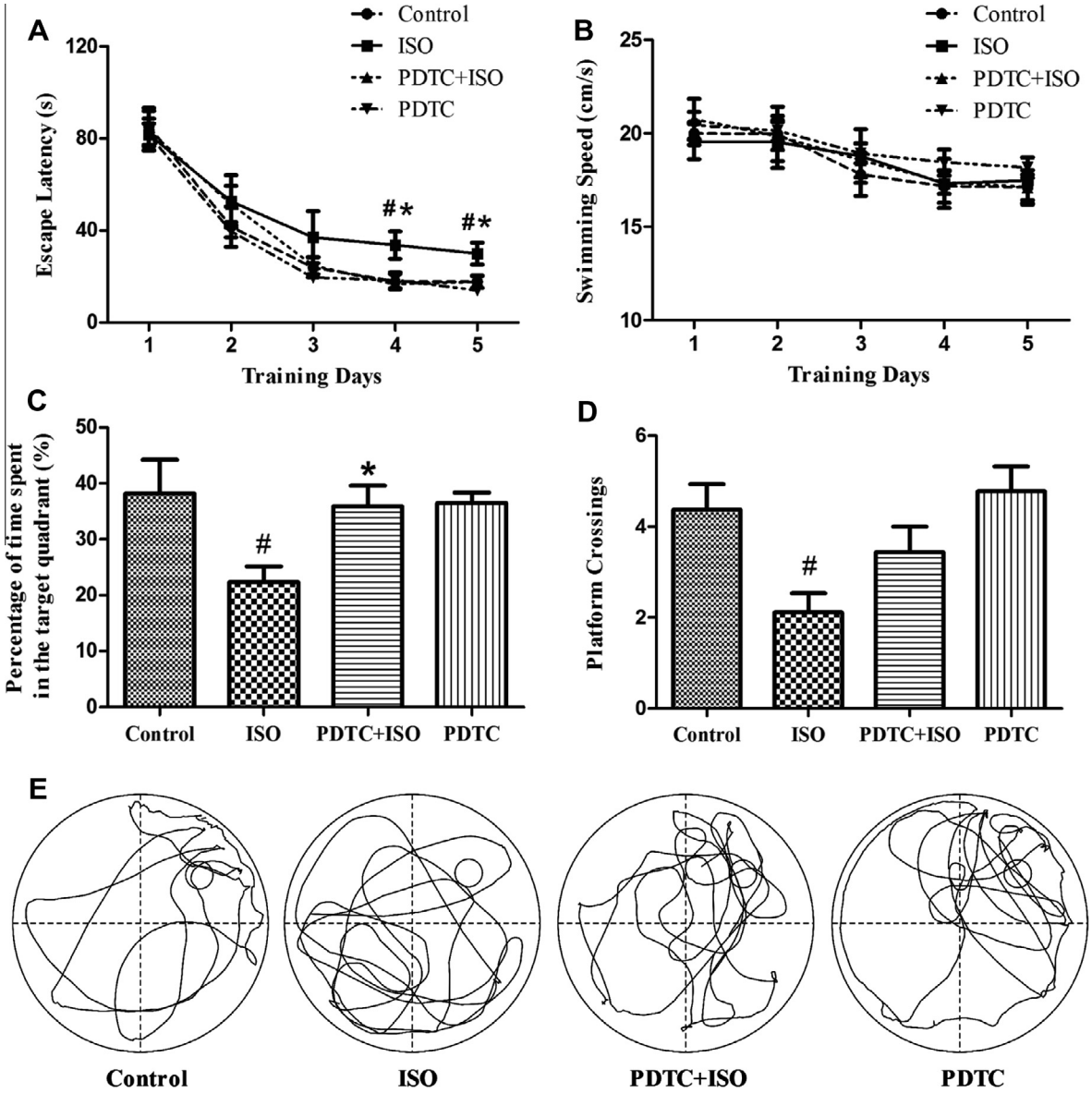

Fig. 4. PDTC pretreatment mitigates the isoflurane-induced spatial memory impairment. Twenty-month-old rats were exposed to 1.5% isoflurane or vehicle gas in the presence or absence of PDTC for 4 h. PDTC (100 mg/kg) was injected intraperitoneally at 1 h before exposure. (A, B) Acquisition trials demonstrating the latency for the rats to reach the platform (A) and the swimming speed (B), measuring spatial information acquisition. (C, D) Probe trials demonstrating the time spent in the target quadrant (C) and the number of original platform crossings (D), measuring memory retention capabilities. (E) Representative searching swimming paths of four aged rats with different treatments in the probe trial tests. Results were presented as means ± SEM ($n = 9$). $^{\#}p < 0.05$, vs. control group. * $p < 0.05$, vs. ISO group.

Recently, Zhang et al. [27] showed increases in the nuclear p65 level and transcriptional binding activity of NF-κB in cultured H4 human neuroglioma cells and mouse microglia after isoflurane treatment. However, the *in vivo* function of NF-κB in mediating neuroinflammation after isoflurane anesthesia and its role in the development of isoflurane-induced spatial memory impairment have not been established. Reportedly, isoflurane does not induce impairment of learning and memory in IL-1β-deficient mice [3]. In agreement with this, we found that inhibition of hippocampal IL-1β elevation by PDTC, an NF-κB pathway inhibitor capable of crossing the blood–brain barrier [28], occurred in parallel with improvements in the behavioral outcomes. These findings suggest that hippocampal IL-1β elevation may play, at least in part, a role in isoflurane-mediated cognitive impairment in aged rats. Collectively, our study first establishes an *in vivo* linkage between canonical NF-κB activation, inflammatory gene expression, and the development of cognitive deficits induced by isoflurane.

Furthermore, it should be emphasized that the duration of memory impairment in aged animals after isoflurane did not parallel the duration of hippocampal IL-1β elevation. This does not necessarily imply that neuroinflammation has no contribution to isoflurane-induced cognitive dysfunction, since hippocampal neuronal apoptosis [5,6], amyloid pathology [29], cholinergic dysfunction [17], and synaptic ultrastructure impairment [21], etc. were postulated to be associated with isoflurane-induced cognitive dysfunction. This phenomenon may be caused by detrimental effects of isoflurane in the early phase, so that the above-mentioned events subsequently occur and lead to the delayed cognitive dysfunction.

In conclusion, our *in vivo* data suggest a key role for the canonical NF-κB signaling pathway in mediating isoflurane-induced neuroinflammation. Defining the detailed mechanisms by which upregulation of p-IKK and p-IκBα and downregulation of IκBα mediate NF-κB activation and lead to the initiation/amplification

of inflammatory processes may provide clues toward the mechanism of isoflurane-induced cognitive dysfunction, and indicate precise targets for prophylaxis and treatment.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.003.

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