



Research Article

Translocator Protein 18 kDa Involved in the Cognitive Impairment Induced by Isoflurane Inhalation Anesthesia

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Abstract: Background. Translocator protein 18 kDa (TSPO) plays a key role both in microglial activation and neuroinflammation. Postoperative cognitive decline (POCD), a notable hazard to both patients and society, maybe contribute to deficiently controlled neuroinflammatory processes initiated by anesthesia and surgery. So far it is unclear that whether TSPO is involved in the pathogenesis of POCD or not. Materials and Methods. Twelve adult rats and twelve aged rats were divided into control and isoflurane groups respectively. POCD was induced by a 4-hour exposure of 2% isoflurane. The memory retention capability was assessed by the Morris water maze trial, the mRNA and the protein expression of both TSPO and Iba1 were assessed by real-time quantitative PCR and Western Blot analysis separately. Results. Compared to the control group, the latency time to find platform was longer in the groups exposed to isoflurane ($p < 0.05$); the mRNA and the protein expression of both TSPO and Iba1 were correspondingly upregulated ($p < 0.05$); especially that the severity of cognitive decline and the degree of TSPO and Iba1 over-expression were significantly different between the adult and aged rats ($p < 0.05$); The twice times across the platform showed no significant difference among all the groups. Conclusions. Our study for the first time showed that TSPO may be involved in the pathogenesis of the cognitive decline induced by isoflurane anesthesia. Its role for being a biomarker and an interventional target of POCD deserves future investigation.

Keywords: Postoperative Cognitive Decline (POCD), Translocator Protein 18 kDa (TSPO), Neuroinflammation

1. Introduction

Translocator protein 18kDa (TSPO), a kind of five transmembrane domains expressed in particularly enriched (20- to 50-fold) in tissues in which steroid synthesize, such as adrenal, gonad and brain cells.¹ In the central nervous system, TSPO is usually expressed in microglia and activated astrocytes.^{2, 3} Once excitatory toxic compounds are injected, the expression of TSPO levels will be increased dose-dependently, which is closely related with the microglia activation.⁴ In some neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease, TSPO seems to be a sensitive biomarker of brain damage and

neuroinflammation.⁵ Further roles of TSPO in other processes, including inflammation, apoptosis, mitophagy, cancer, transport of porphyrin and have also been extensively investigated. But it remains a challenge, and an essential prerequisite for drug development, to establish the precise mechanism of TSPO's involvement in these neurodegenerative processes, especially in POCD.

POCD is defined as a later onset form of postoperative cognitive decline arising after anesthesia and surgery with significant consequences to both patients and the society.⁶ Advanced ages, prolonged anesthesia, extensive surgery, and perioperative complications are all among the recognized risk factors of POCD.⁷ However, the exact pathophysiology that

underlies POCD remains undefined. The current data primarily based on preclinical studies suggests that neuroinflammation is likely an important mechanism responsible for POCD.⁸⁻¹⁰ Increased expression of interleukins in mouse hippocampus undergoing minor surgery was associated with cognitive decline.¹¹ It has been shown recently that exposure to inhalational anesthetics, including isoflurane and sevoflurane,^{12, 13} induces neurodegenerative changes including neuronal apoptosis and elevated β -amyloid protein levels in mice. Impaired ability of spatial learning of aged mice was observed after isoflurane exposure, accompanied by overexpression of IL-1 β , TNF- α , and IFN- γ .¹⁴ Increasing number of studies has focused on the molecular mechanism of neuroinflammation in POCD, astrocytes activation have important roles in the central nervous system (CNS) during inflammation and experimental autoimmune encephalomyelitis (EAE) disease scores.¹⁵ Therefore, this study was designed to determine whether the exposure of 2% isoflurane (Iso) induces spatial learning deficit in rats of different ages, so as to explore the possible role of TSPO, the marker of astrocytes activation, in the development and progression of POCD induced by Isoflurane. In this study, we hypothesized that TSPO was associated with the neuroinflammation in the pathogenesis of POCD.

2. Materials and Methods

2.1. Animals and Groups

All rats had unrestricted access to food/water in accordance with the Institutional Animal Care and Use Committee Procedures of Weifang Medical University. Twelve 3-month-old adult rats (weighing 200-220g) and twelve 20-month-old aged rats (weighing 470-550g) were purchased from Shandong Lukang Pharmaceutical Co., Ltd. All the rats were housed under a 12-hour light/dark cycle (lights on at 7:00 am). Room temperature was maintained at 20 \pm 2°C.

Twelve adult and twelve aged rats were divided into the control group (n=12, 6 adults and 6 aged) and the isoflurane group (n=12, 6 adults and 6 aged), respectively. The rats in the isoflurane group were exposed to 2% isoflurane (765865U, Abbott, American) for 4 hours in a chamber with 100% oxygen, the concentration of isoflurane was detected by gas detectors (Deatex-Ohmeda, America), while the rats in the control group only inhaled 100% oxygen under the same conditions. The mean rectal temperature of the animals was maintained at 37 \pm 0.5°C. The concentration of isoflurane and oxygen in the chamber were monitored through an anesthesia machine and adjusted accordingly.

2.2. Behavioral Analysis

The Morris water maze was used to evaluate the escape latency and memory retention capability of the rats.¹⁶ Behaviors were recorded by a video camera during the tests and then analyzed by an experimenter who was blind to the group. All behavioral tests were conducted during the dark (active) phase. All the rats were kept for 60 minutes for

familiarization with the environment after being transferred from their housing room to the testing laboratory.

The experiments were carried out in a dimly lit room. The stainless steel pool (150cm in diameter) was covered with a black curtain and the water (23 \pm 2°C) in the pool was maintained at room temperature. A platform, 1.5cm in diameter, was located according to the distant visual cues on the walls around the pool. Opaque water was filled in the pool with the movable platform submerged 2cm below the surface of the water. Every rat had undergone four times trials per day for five days. At the beginning of the trial, each rat was placed in the pool to search for the platform, with the searching duration limited to 90 seconds every time. The rat was allowed to stay on the platform for 15 seconds before the next trial if it completed the task precisely. The rats that failed to find the platform were gently guided to finish the task and allowed to stay on the platform for 15 seconds, too. A video tracking system was used to record the swimming motions of the rats. The escape latency (time used to reach the platform), an indicator of learning and spatial memory, was recorded for each trial. The trial was repeated 3 times for every rat in one day, and the average scores per day were used as the test scores of this day.

The probe test was used to evaluate the memory retention capability. The platform in the above procedure was removed from the pool in advance, and then the rats were placed in the quadrant opposite to the one where the platform had been previously located. The frequencies that the rats swam across the platform area in 90 seconds were recorded as the first time through platform test. According to every day's escape latency of these rats (the data didn't show), we found that after anesthesia; the difference of the behaviorists of the second day is most obvious. So, we projected the second time through platform test, and we evaluated the ability of recall based on that. At the second time, the rats were exposed to 2% isoflurane for 4 hours as before, either were the control groups respectively. Twenty-four hours following isoflurane anesthesia, the probe test was performed again and the frequency crossed platform quadrant was recorded as the second time through platform test. All the tests were repeated 3 times for each rat, and the average scores were used as the data of each rat.

2.3. Quantitative Real-Time PCR Analysis

Total RNA was isolated from the hippocampus tissue and cortex of the rats.^{17, 18} RNA extractions were performed with the guanidinium isothiocyanate/chloroform based technique (TRIZOL, Invitrogen, America) according to the manufacturer's protocol. The RNA samples from six individual animals each group were used to prepare cDNA for RT-PCR (real-time polymerase chain reaction) using the Thermo M-MLV Reverse Transcriptase (Biosci, Hangzhou, China), according to the protocol. The cDNA was quantified using the SYBR Green PCR master kit (Biosci, Hangzhou, China). The PCR primers being used are described in Table 1. Quantitative RT-PCR was performed using a Mx3005p QPCR system (Agilent, Stratagene, American) with the two-stage

program parameters as follows: 1 minute at 95°C, and then 40 cycles of 5 seconds at 95°C, and 30 seconds at 60°C. Normalized mRNA expression values were calculated following the mathematical model proposed by Livak et al. using the formula $2^{-\Delta\Delta Ct}$,¹⁵ the results were normalized by β -actin expression.

2.4. Western Blot Analysis

Hippocampus and cortical tissues were collected after the rats were deeply anesthetized. The tissues were lysed with 1×RIPA buffer (Beyotime, Jiangsu, China) with 10% PMSF (Amresco, America) for 30 min on ice followed by vortexing for every 10 min. Total lysates were prepared by centrifugation at 4°C 12000g for 10 min. Total proteins were quantified by the BCA protein assay kit (Beyotime, Jiangsu, China). Thirty micrograms of the protein were separated by SDS-PAGE on 15% gels with BioStep™ Prestained Protein Marker (Tanon, Shanghai, China). Proteins were then transferred onto 0.2um PVDF membranes after blocking in TBST containing 5% nonfat dry milk for 2 hours at room temperature. The membranes were incubated at 4°C overnight with monoclonal goat anti-TSPO (1:100, Santa Cruze, America), goat anti-Iba1 (1ug/ml, Abcam) to visualize TSPO (18KDa), Iba1 (17KDa), β -actin (43KDa), respectively. Mouse anti- β -actin (1:1000, ZSGB-BIO, Beijing, China) was used as protein loading control for all tissues. Blots were then incubated with corresponding horseradish peroxidase-conjugated secondary antibody. Enhanced chemiluminescence signals were then visualized and imaged with the fully automatic gel imaging system (MINI K 3000). And the densities from each band were measured by Gel Analysis software.

2.5. Statistical Analysis

All numerical data were expressed in mean \pm standard deviation ($\bar{x} \pm SD$). The data were assessed by the completely random design of one-way analysis of variance (ANOVA) and Student's t-test. SPSS version 13.0 and GraphPad Prism software were used for all statistical analyses. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Escape Latency and Memory Retention Assessments

Compared with the control group, the escape latency of the aged rats in the isoflurane group was significantly longer ($p < 0.05$) and the time across the plat was significantly reduced ($p < 0.05$), suggesting that isoflurane anesthesia led to declined learning and memory ability. In contrast, there was no significant difference of the escaping latency in the adult rats between the control and isoflurane groups even though the escape latency of the young rats in the isoflurane group increased slightly ($p > 0.05$). The adult rats performed similarly ($p > 0.05$) in the trial of crossing the plat between the control and isoflurane groups (Figure 1).

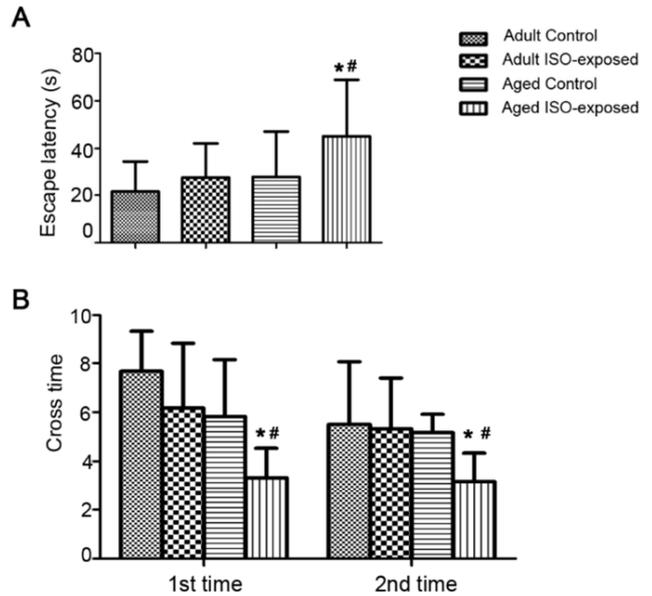


Figure 1

Figure 1. Escape latency and Memory retention capability comparison. (A) Spatial learning in the MWM (Morris water maze, MWM). Average escape latency (seconds) is shown in the maze. (B) Spatial memory in the MWM. Average time crossed platform quadrant two times are shown in the maze.

n=6, ISO, isoflurane, * $p < 0.05$ vs. Adult control; # $p < 0.05$ vs. Adult ISO-exposed.

3.2. Real-time Quantitation PCR

Both Iba1 mRNA and TSPO mRNA were elevated in the tissues of the hippocampus and cerebral cortex in the isoflurane group compared with the control group ($p < 0.05$). Following isoflurane anesthesia, the Iba1 mRNA and the TSPO mRNA were upregulated significantly more in the aged rats than the adult rats ($p < 0.05$) (Figure 2).

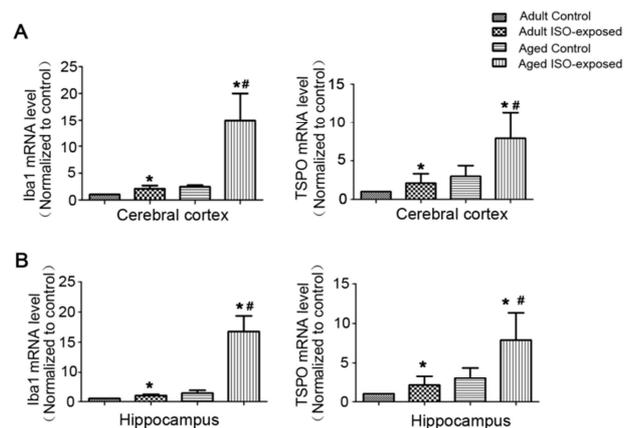


Figure 2

Figure 2. Expression of Iba1 mRNA and TSPO mRNA in cerebral cortex and hippocampus. (A) Iba1 mRNA and TSPO levels in cerebral cortex, (B) Iba1 mRNA and TSPO levels in the hippocampus.

ISO, isoflurane, n=6, * $p < 0.05$ vs. Adult control; # $p < 0.05$ vs. Adult ISO-exposed.

3.3. Western Blot

The expression of Iba1 and TSPO protein in isoflurane groups were both significantly upregulated compared with the control groups ($P < 0.05$), which is consistent with the mRNA analysis. The upregulation following isoflurane anesthesia is significantly more in the aged rats than the adult rats ($p < 0.05$) (Figure 3).

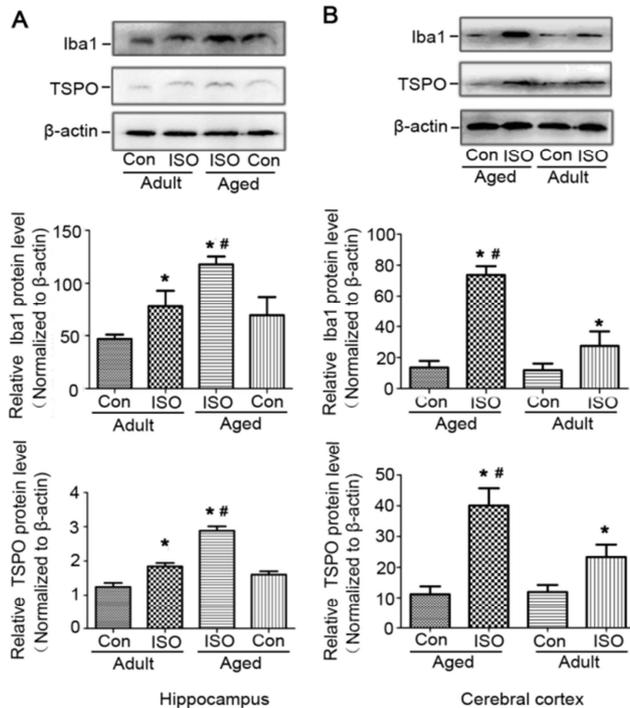


Figure 3

Figure 3. Expression of Iba1 and TSPO protein in cerebral cortex and hippocampus. (A) Iba1 and TSPO protein levels in hippocampus, (B) Iba1 and TSPO protein levels in the cerebral cortex.

ISO, isoflurane, Con, control, $n=6$, * $p < 0.05$ vs. Adult control; # $p < 0.05$ vs. Adult ISO-exposed.

Table 1. Primer sequence.

Primer	Sequence(5'→3')	Amplification size (BP)
TSPO-F	GCCCGCTTGCTGTATCCTTA	88
TSPO-R	CTCGCCGACCAGAGTTATCA	
Iba1-F	TCCTGAAGCGAATGCTGGA	87
Iba1-R	GAGCCACTGGACACCTCTCTAAT	
β -actin-F	CAGCCTTCCTTCCTGGGTAT	107
β -actin-R	CTGTGTTGGCATAGAGGCTT	

F: sense primer; R: downstream primers.

4. Discussion

The results of our study showed that prolonged isoflurane anesthesia leads to cognitive impairment in both adult and aged rats with the severity of functional abnormality age dependent. Isoflurane anesthesia also led to the upregulated expression of mRNA and protein of both Iba1 and TSPO, with

the degree of upregulation also depending on the age. Overall, our study suggests that the translocator protein (18 kDa) may involve in the pathogenesis of cognitive decline induced by prolonged isoflurane anesthesia.

It was previously reported that 1.5% isoflurane exposure for 4 hours led to impaired spatial learning and memory in aged rats, accompanied by impaired hippocampal autophagy following a transient activation.¹⁹ Our results are consistent with the previous report except that we used 2% instead of 1.5% isoflurane for 4 hours and both adult and aged rats were used in our study.^{19(p507)} Other inhalational anesthetics such as sevoflurane can also lead to cognitive decline in rodents.¹⁷ Research focusing on the mechanism of cognitive impairment after anesthesia and surgery bears clear significance because the cognitive function is one of the most basic and important neurological functions of the brain, and it is a basic indicator of the development of human intelligence.^{6(p148)}

The precise mechanism underlying the cognitive impairment induced by inhalational anesthesia in rodent remains unclear even though it appears both hippocampus and age dependent.^{19(p509)} The Iba1 gene is specifically expressed in microglia in brain cells. Upon activation of microglia due to inflammation, expression of Iba1 is upregulated allowing discrimination between physiological and activated microglia.^{21(p8)} Our research also showed that Iba1 expression was elevated both in hippocampus and cerebral cortex after Isoflurane exposure, which also indicated that the neuroinflammation played role in POCD, and our results for the first time suggest that TSPO may be involved in the pathogenesis of cognitive decline induced by isoflurane anesthesia, which is associated with the neuroinflammation. In the central nervous system, microglia provides the first defense against damage and disease and contributes to the support of neuronal viability and regeneration.^{20 (p768), 22} However, chronic microglial activation may endanger the neuronal cells and play a role in neurodegenerative processes.²³ The marked and prolonged increase in TSPO expression in neural cells after injury or in neurological disorders suggests that it may be involved in the pathogenesis of neuronal damage or disease.

Accumulating evidence suggests neuroinflammation may play an important role in the pathogenesis of POCD. The inflammatory signal cascades can lead to the release of cytokines that impair the integrity of the blood–brain barrier, with resultant macrophage migration into the hippocampus and memory impairment as a consequence.²⁴ The level of inflammatory factors including TNF- α , IL-6, and IL-1 β is significantly increased in the rat brains after isoflurane anesthesia.²⁵ Notably, TSPO also regulates the viability and functions of immune cells, including lymphocytes and macrophages, and virtually all cell types of the immune system expressing TSPO.²⁶ Our results showed that the expression of both TSPO and Iba1 were upregulated in hippocampus and cerebral cortex after isoflurane anesthesia. However, how TSPO overexpression in the key brain regions, its effect on the immune system, and its interaction with the neuroinflammatory process remain to be better defined.

In summary, TSPO overexpression in hippocampus and cerebral cortex is associated with cognitive decline induced by prolonged isoflurane anesthesia in aged rats, suggesting that it may play a role in the pathogenesis of POCD. TSPO is a potential biomarker of POCD and a new pharmacological target for POCD prevention and treatment. Further study is needed to better understand its interaction with other inflammatory processes and the immunological system in the central nervous system.

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Conflict of Interest

None of the authors have any conflicts of interest associated with the work presented in this manuscript

Rui Zhang and Shanshan Zou contributed equally to this article.

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