

Inhibition of $\alpha 5$ GABAA receptors has preventive but not therapeutic effects on isoflurane-induced memory impairment in aged rats

Zi-Fang Zhao^{1, #}, Lei Du^{2, #}, Teng Gao³, Lin Bao¹, Yuan Luo⁴, Yi-Qing Yin^{1, *}, Yong-An Wang^{4, *}

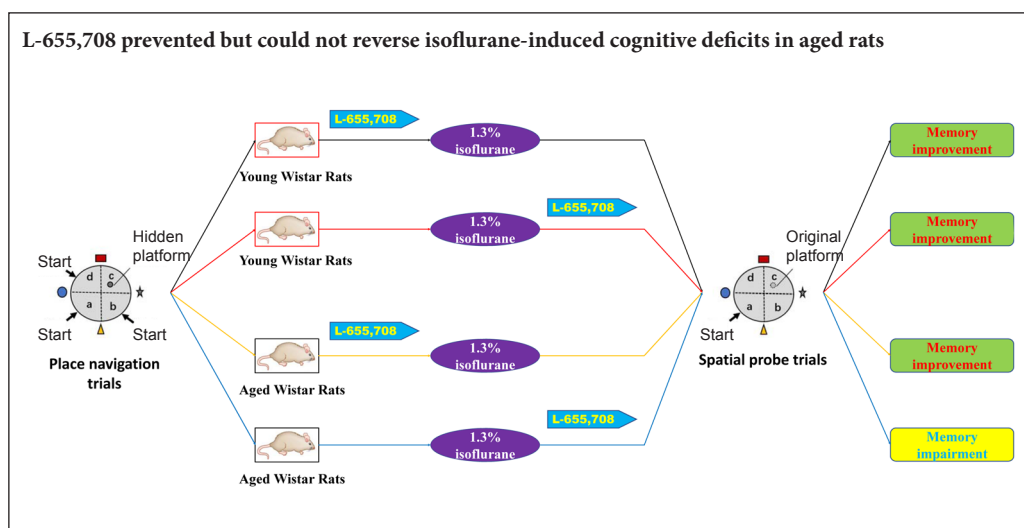
1 Department of Anesthesiology, China-Japan Friendship Hospital, Beijing, China

2 Department of Radiology, China-Japan Friendship Hospital, Beijing, China

3 Department of Anesthesiology, Beijing Shijitan Hospital Affiliated to Capital Medical University, Beijing, China

4 Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, Beijing, China

Graphical Abstract



*Correspondence to:

Yi-Qing Yin, M.D.,

yyq518@sina.com;

Yong-An Wang, M.D.,

yonganw@126.com.

#These authors contributed equally to this paper.

orcid:

0000-0002-1130-6676

(Yi-Qing Yin)

0000-0003-4939-7048

(Yong-An Wang)

doi: 10.4103/1673-5374.250621

Received: August 13, 2018

Accepted: November 13, 2018

Abstract

The $\alpha 5$ subunit-containing gamma-aminobutyric acid type A receptors ($\alpha 5$ GABAARs) are a distinct subpopulation that are specifically distributed in the mammalian hippocampus and also mediate tonic inhibitory currents in hippocampal neurons. These tonic currents can be enhanced by low-dose isoflurane, which is associated with learning and memory impairment. Inverse agonists of $\alpha 5$ GABAARs, such as L-655,708, are able to reverse the short-term memory deficit caused by low-dose isoflurane in young animals. However, whether these negative allosteric modulators have the same effects on aged rats remains unclear. In the present study, we mainly investigated the effects of L-655,708 on low-dose (1.3%) isoflurane-induced learning and memory impairment in elderly rats. Young (3-month-old) and aged (24-month-old) Wistar rats were randomly assigned to receive L-655,708 0.5 hour before or 23.5 hours after 1.3% isoflurane anesthesia. The Morris Water Maze tests demonstrated that L-655,708 injected before or after anesthesia could reverse the memory deficit in young rats. But in aged rats, application of L-655,708 only before anesthesia showed similar effects. Reverse transcription-polymerase chain reaction showed that low-dose isoflurane decreased the mRNA expression of $\alpha 5$ GABAARs in aging hippocampal neurons but increased that in young animals. These findings indicate that L-655,708 prevented but could not reverse 1.3% isoflurane-induced spatial learning and memory impairment in aged Wistar rats. All experimental procedures and protocols were approved by the Experimental Animal Ethics Committee of Academy of Military Medical Science of China (approval No. NBCDSER-IACUC-2015128) in December 2015.

Key Words: isoflurane; postoperative cognitive dysfunction; hippocampus; inverse agonist; $\alpha 5$ GABAA receptors; L-655,708; aged; Morris Water Maze; memory impairment; neural regeneration

Chinese Library Classification No. R453; R364

Introduction

Memory blockade and amnesic effects are important results of general anesthesia, and the majority of these effects disappear with the rapid elimination of general anesthetics. Unfortunately, learning and memory deficits, referred to as postoperative cognitive dysfunction (POCD) (Casella and Bimonte, 2017; Gao et al., 2017), may persist for months or can be permanent in patients following surgery and general anesthesia (Newman et al., 2001; Johnson et al., 2002; Gao et

al., 2017). Advanced age is a recognized risk factor of POCD (Liu et al., 2018; Monk et al., 2008). Despite improvements in perioperative care, a great number of elderly surgical patients experience POCD, which is associated with poor postoperative outcome and increased mortality (Moller et al., 1998; Gurlit and Möllmann, 2008; Coburn et al., 2010). Accordingly, the exploration of potential preventive or therapeutic methods for POCD in the aged population is of particular significance.

The gamma-aminobutyric acid type A receptors (GAB-

AARs) are critical inhibitory ion channel receptors in the mammalian central nervous system. Most intravenous and inhaled anesthetics cause memory blockade and amnesia by acting as positive allosteric modulators of GABAARs (Rudolph and Antkowiak, 2004).

$\alpha 5$ Subunit-containing GABAARs ($\alpha 5$ GABAARs) are a distinct subpopulation that are specifically distributed in the extrasynaptic area of hippocampal CA1 pyramidal neurons (Christie and de Blas, 2002; Serwanski et al., 2006), and their activation generates a specific tonic inhibitory current (Caraiscos et al., 2004b; Glykys and Mody, 2006; Chen et al., 2017). Zurek et al. (2014) found that etomidate increased the tonic current generated by $\alpha 5$ GABAARs and cell-surface expression of $\alpha 5$ GABAARs for at least 1 week. This increase in tonic current impaired learning memory. Consistently, Lecker et al. (2013) demonstrated that inverse agonists of $\alpha 5$ GABAARs, L-655,708 and MRK-016, alleviated the overactivity evoked by low-dose inhalational anesthetics. Pretreatment with L-655,708 fully prevented the isoflurane-induced memory deficits in mice aged 2–3 months. Therefore, hippocampal $\alpha 5$ GABAARs may be a potential target for post-operative memory impairment (Saab et al., 2010).

However, most existing studies of $\alpha 5$ GABAAR inverse agonists are limited to young animals, and relevant studies have scarcely focused on aging subjects. The aged population is more susceptible than the young to anesthetic-induced cognitive dysfunction and neurological damage (Magnusson et al., 2000; Wang et al., 2000; Culley et al., 2003; Jansson et al., 2004). For example, isoflurane and propofol affected post-natal hippocampal neurogenesis and cell proliferation in an age-dependent manner, and the alterations were significant in aged animals (Erasso et al., 2012, 2013). Therefore, whether the anesthesia-induced changes in hippocampal $\alpha 5$ GABAAR function are similar between young and aged animals and whether inverse agonists of $\alpha 5$ GABAARs have similar effects on postanesthetic memory impairments in aged and young subjects are issues that must be investigated. In the current study, we explored the effects of L-655,708, an inverse agonist of $\alpha 5$ GABAARs, on low-dose isoflurane-induced memory impairments in elderly rats. In addition, we investigated whether hippocampal $\alpha 5$ GABAARs are still a potential target for attenuating POCD in the aged population.

Materials and Methods

Animals

Male specific pathogen-free (SPF) Wistar rats, aged 3 months (average body weight 373.1 g) and 24 months (average body weight 790.5 g) provided by Vital River Lab Animal Technology Co., Ltd., China (animal license No.: SCXK (Jing) 2012-0001) were included in this study. The rats were housed separately in cages under standard laboratory conditions (lights on at 7:00 a.m.; lights off at 7:00 p.m., 22°C, 60% humidity) and were free to water and food.

All experimental procedures and protocols were approved by the Experimental Animal Ethics Committee of Academy of Military Medical Science of China (approval No. NBCD-SER-IACUC-2015128) in December 2015. All experimental

procedures described here were in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

Grouping

Both young (3-month-old) and aged (24-month-old) rats were randomly assigned to different treatment groups ($n = 8$) (Figure 1). Rats in the control group inhaled a mixture of oxygen and air (30% oxygen) for 1 hour. Rats in the ISO₂₄ group were anesthetized by inhaling 1.3% isoflurane (purchased from Hebei Yipin Pharmaceutical Co., Ltd., Hebei Province, China) for 1 hour. Rats in the pre-L group received L-655,708 (0.7 mg/kg in 10% dimethyl sulfoxide (DMSO) 2 μ L/g) subcutaneously 30 minutes before exposure to 1.3% isoflurane for 1 hour. Rats in the post-L group received L-655,708 subcutaneously 23.5 hours after 1.3% isoflurane exposure. Rats in these four groups were tested in a spatial probe trial at 24 hours after isoflurane exposure. Rats in the ISO₇₂ group were exposed to 1.3% isoflurane for 1 hour and then tested in the spatial probe trial at 72 hours after isoflurane exposure. We also tested the effects of DMSO on cognitive function in a separate experiment in which some rats (aged 3 months, $n = 7$) received subcutaneous injection of isovolumetric 10% DMSO.

L-655,708

L-655,708 (ethyl (13aS)-7-methoxy-9-oxo-11,12,13,13 atetrahydro-9H-imidazo [1, 5-a]pyrrolo[2,1c][1,4]benzodiazepine-1-carboxylate) used in the present trial was obtained from Sigma-Aldrich Co., St. Louis, MO, USA. For subcutaneous injection, we dissolved crystalline L-655,708 in 10% DMSO.

Anesthesia

Rats in the anesthesia and intervention groups were placed in an anesthetizing apparatus perfused with 30% oxygen [O₂] in air with or without 1.3% isoflurane delivered at a rate of 1 L/min. The real-time concentrations of isoflurane, oxygen, and carbon dioxide in the anesthetizing chamber were detected with a commercial Datex-Ohmeda Compact S/5 monitor (Datex-Ohmeda, Inc., Madison, WI, USA), and gas flow was regulated to maintain the desired concentrations. A warming blanket was used to avoid hypothermia during anesthesia. After treatment, the rats were removed from the chamber and allowed to recover in another heated clear chamber for 45 minutes before they were returned to the home cage. There was no mortality during or after the course of animal anesthesia.

Morris water maze test

The experimenters who performed the behavioral tests were blinded to group information. The water maze (Shanghai Xinruan Information Technology Co., Ltd., Shanghai, China) consisted of a circular pool (diameter, 150 cm; depth, 50 cm) filled with opaque water (kept at 22°C). Rats were repeatedly trained to swim from the water to a round escape platform (2.0 cm beneath the surface). The pool area was artificially separated into four imaginary quadrants: a, b, c

and d, with quadrant c as the target quadrant in the present study. The swimming motions of the animals were automatically recorded by a video computerized tracking system. The acquired data were processed using specific software for the Morris water maze (Shanghai Xinruan Information Technology Co., Ltd., Shanghai, China).

The Morris water maze test consists of place navigation trial and spatial probe trial. The place navigation trial helps animals to acquire specific spatial reference memory and the spatial probe trial tests the retention of acquired spatial memory. During the place navigation trial, each rat underwent four trials per day for 4 consecutive days. During the trial, each rat was gently put into the water from a stationary starting point and given 60 seconds to find the hidden platform. If the rat could not find the target within the designated time, the experimenter would gently guide it to get up the platform and stay there for 30 seconds. The rats that successfully found the target also stayed on the platform for 30 seconds before they were removed. Swim speed, the time spent to find the platform (escape latency), and the distance traveled were recorded. Escape latency is the primary indicator of spatial learning capability; shorter escape latency represents a better spatial learning ability.

Following the place navigation trial, rats received oxygen-air mixture, isoflurane anesthesia or L-655,708 according to the interventions designated for each group (Figure 1). Then spatial probe trials were conducted 24 hours (rats in the control, ISO₂₄, pre-L, and post-L groups) or 72 hours (rats in the ISO₇₂ group) after isoflurane anesthesia or sham to assess the influences of different treatments on the retention of acquired spatial memory of rats. In the spatial probe trial, the escape platform was removed, and rats were placed in the opposite quadrant (quadrant a) of the original target quadrant (quadrant c). Each rat was given 60 seconds to swim freely. The time spent for the first arrival at the original platform location (escape latency) and the time spent in quadrant c were acquired and used to evaluate memory retention. Shorter escape latency and/or longer time spent in quadrant c represent encouraging retention of spatial learning memory.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed to investigate the influence of isoflurane and L-655,708 on the mRNA expression of $\alpha 5$ GABA_ARs in the hippocampus. Following behavioral tests, rats were sacrificed, and hippocampal tissue was obtained and stored at -80°C immediately. Then the lysed tissue was homogenized by a homogenizer (Qiagen, Inc., Valencia, CA, USA) and then centrifuged. Total RNA was acquired from the supernatant and its concentration was measured using a Nano Drop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). $\alpha 5$ GABA_AR and β -actin primers were purchased from Tianyi Biotech, Beijing, China and sequence information was shown in Table 1. RT-PCR was conducted using the real-time PCR system with SYBR[®] Premix (TaKaRa, Inc., Dalian, China). $\alpha 5$ GABA_AR mRNA levels were standardized to β -actin levels as internal controls. PCR was conducted by 40 cycles at 96°C for 30 seconds, 55°C for 90 seconds, and 72°C for 60 seconds in a BioRad CFX96[™] Real-Time System C1000[™] Thermal Cycler (BioRad Laboratories, Shanghai, China). Threshold cycle (Ct) values were recorded and tested. The Ct values were defined as the amplification cycles when the fluorescent signal in the reaction system significantly exceeded the background fluorescence (threshold). The Ct values were negatively related to the tested mRNA level. Consequently, a lower Ct reflects a higher mRNA level (Dhar et al., 2002; Park et al., 2015).

Table 1 Details of the primers used for RT-PCR

Gene	Primer	Primer sequence (5'-3')	Product size (bp)
$\alpha 5$ GABA _A R	Forward	GAC TTT CCG ATG GAT GCC	188
	Reverse	GTG CTG GTG CTG ATG TTC TC	
β -Actin	Forward	GGC TGT ATT CCC CTC CAT CG	154
	Reverse	CCA GTT GGT AAC AAT GCC ATG T	

RT-PCR: Reverse transcription-polymerase chain reaction; $\alpha 5$ GABA_AR: $\alpha 5$ subunit-containing gamma-amino butyric acid type A receptors.

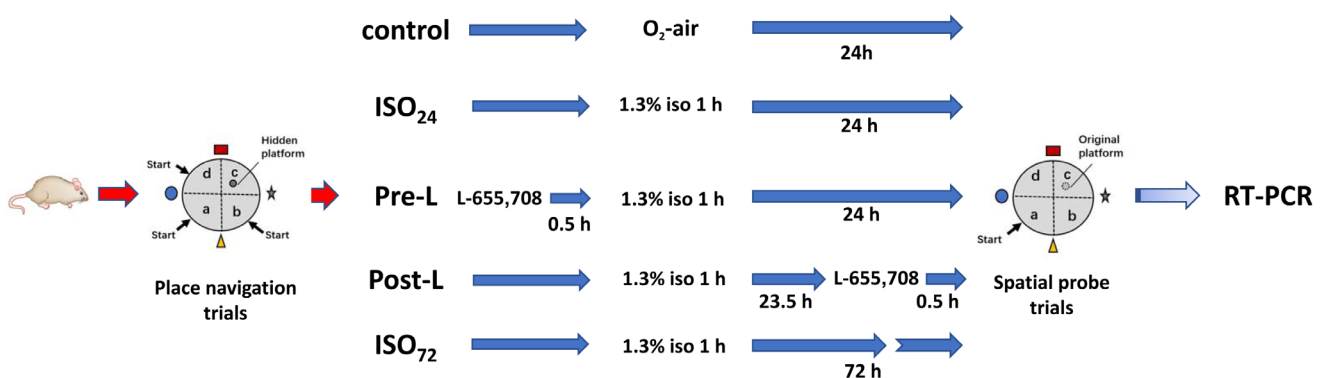


Figure 1 Experimental paradigm timeline.

Following the place navigation trials for 4 consecutive days, young and aged rats were randomly divided into five groups and received the corresponding treatment. Either 24 or 72 hours after anesthesia, the spatial probe trials were conducted to test the retention of spatial reference memory. After the probe trials, all rats were sacrificed, and RT-PCR was conducted to measure hippocampal $\alpha 5$ GABA_AR mRNA expression level. h: Hour(s); ISO: isoflurane; RT-PCR: reverse transcription-polymerase chain reaction; GABA_AR: gamma-amino butyric acid type A receptor; L-655,708.

Statistical analysis

All measurements were presented as the mean \pm SD and analyzed using SPSS 14.0 software (SPSS Inc., Chicago, IL, USA). The escape latency and swimming speed during the place navigation trials were analyzed by repeated measures one-way analysis of variance. Using two-way analysis of variance, age and treatment interactions in the spatial probe trials and RT-PCR were analyzed. Spatial probe trials and RT-PCR results were statistically compared between experimental and control groups using one-way analysis of variance followed by Tukey's *post hoc* test. A level of $P < 0.05$ was considered statistically significant.

Results

Age and treatment interactions

Two-way analysis of variance and RT-PCR assay (escape latency and time c were evaluated separately) revealed significant differences in spatial probe trial between two age groups ($P < 0.001$ for all) and among different treatment groups ($P < 0.001$ for all), but there was no significant difference in age and treatment interactions ($P > 0.05$ for all). One-way analysis of variance with Turkey's *post hoc* test was conducted for young cohort or aged cohort separately.

Rat performance in the place navigation trials

In the place navigation trial, both young and aged rats finished the learning task successfully, which was evidenced by gradually decreased escape latencies across the 4 days of training (Figure 2; $P < 0.05$). Consistent with the results of a previous study (Saab et al., 2010), DMSO injection had no impact on the acquisition or storage of spatial memory as demonstrated by the similar performance in the place navigation trial between DMSO-injected and control rats.

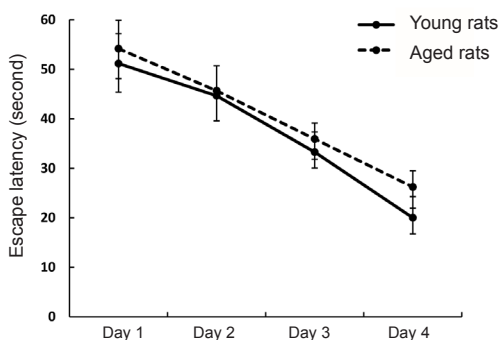


Figure 2 Performance of young (3 months old) and aged (24 months old) rats in the place navigation trials.

Data are expressed as the mean \pm SD ($n = 40$, repeated measures one-way analysis of variance). Escape latency: The time spent to discover the platform.

Effects of isoflurane and L-655,708 on spatial memory performance of rats in the spatial probe trial

Twenty-four hours after anesthesia, young rats showed significantly prolonged escape latency and decreased time c than control rats (Figure 3A and B; $P < 0.01$). However, at 72 hours after anesthesia, there was no significant difference in escape latency or time c between control and ISO₇₂ groups

(Figure 3A and B). These data indicate that rats exposed to isoflurane exhibited impaired spatial memory at 24 hours after anesthesia but not at 72 hours. The escape latency and time c in the pre-L and post-L groups were not significantly different from those in the control group (Figure 3C and D), but the escape latency was shorter and time c was longer in these groups when compared to the ISO₂₄ group (Figure 3C and D). These data indicate that subcutaneous administration of L-655,708 has preventive and therapeutic effects on isoflurane-induced spatial memory deficits. Escape latency was prolonged and time c was shortened in aged rats than in control rats, suggesting that spatial memory impairment occurred 24 hours after exposure to isoflurane (Figure 4A and B; $P < 0.001$). Rats in the ISO₇₂ group showed increased escape latency and decreased time c compared to control animals (Figure 4A and B; $P < 0.01$), indicating that the memory impairment of aged rats persisted at 72 hours after anesthesia. Escape latency was shorter and time c was longer in the pre-L group than in the ISO₂₄ group ($P < 0.05$) and there were no significant differences in escape latency and time c between pre-L and control groups (Figure 4C and D; $P > 0.05$). These suggest that subcutaneous pretreatment with L-655,708 prevented isoflurane-induced memory deficits.

In contrast, administration of L-655,708 at 23.5 hours after isoflurane anesthesia did not affect the escape latency or time c during the search for the escape platform. In the post-L group, rats showed a significantly longer escape latency and shorter time c than control rats (Figure 4C and D; $P < 0.01$ and $P < 0.001$), indicating that administration of L-655,708 at 23.5 hours after anesthesia had no effects on impaired memory.

Effects of isoflurane and L-655,708 on rat hippocampal $\alpha 5$ GABAAR expression as assessed by RT-PCR

In young rats, $\alpha 5$ GABAAR expression in the hippocampus was significantly increased 24 hours after anesthesia compared to that in the control rats but returned to control level at 72 hours after anesthesia (Figure 5A; $P < 0.001$ and $P > 0.05$). There was no significant difference in $\alpha 5$ GABAAR expression between control and pre-L groups (Figure 5B; $P > 0.05$), indicating that treatment with L-655,708 at 0.5 hours prior to isoflurane exposure prevented the change in hippocampal $\alpha 5$ GABAAR expression. Furthermore, in young rats, $\alpha 5$ GABAAR expression in the post-L group was not significantly different from that in the ISO₂₄ group (Figure 5B; $P > 0.05$). In the aged groups, $\alpha 5$ GABAAR mRNA expression in the hippocampus was significantly lower at 24 hours after 1.3% isoflurane anesthesia than that in the control group, and this trend persisted at least 72 hours after anesthesia (Figure 5A; $P < 0.05$).

Similarly, there was no significant difference in hippocampal $\alpha 5$ GABAAR mRNA expression between aged control and aged pre-L groups (Figure 5B; $P > 0.05$). These observations indicate that prophylactic administration of L-655,708 was also able to prevent the alterations in $\alpha 5$ GABAAR expression in the aged hippocampus. No significant difference in hippocampal $\alpha 5$ GABAAR mRNA expression was observed between aged ISO₂₄ and post-L groups (Figure 5B; $P > 0.05$).

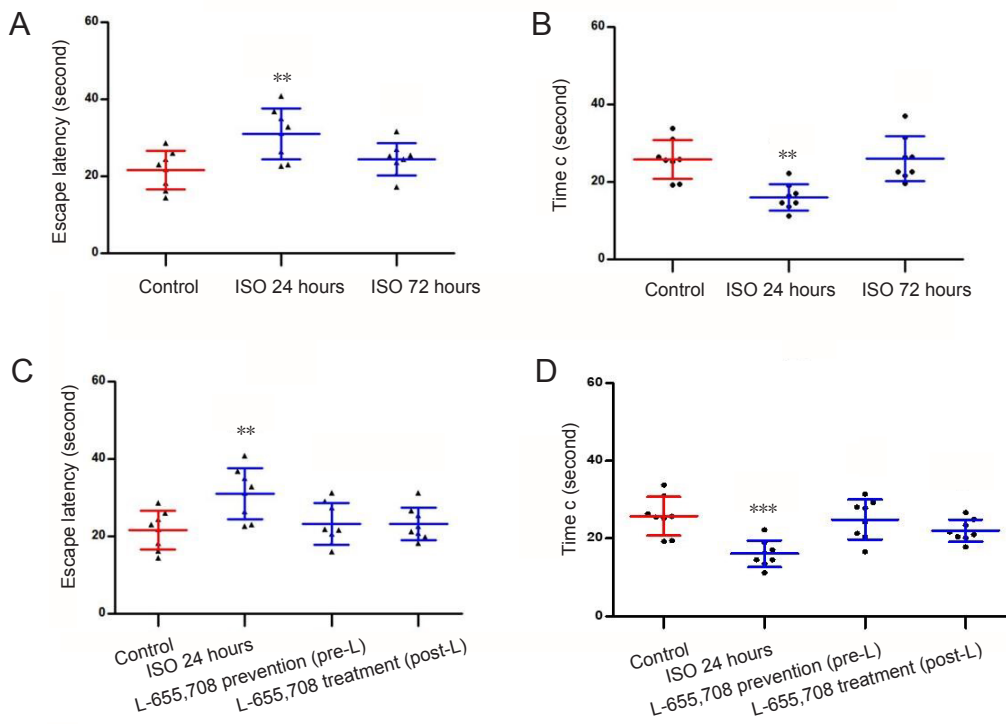


Figure 3 Performance of young (3 months old) rats in the Morris maze test.

(A, B) Effects of isoflurane (ISO) on the escape latency (A) and time c (B) of young rats in the spatial probe trials. (C, D) Effects of L-655,708 on the escape latency (C) and time c (D) of young rats in the spatial probe trials. (D) Effects of L-655,708 on the time c of young rats in the spatial probe trials. (** $P < 0.01$, *** $P < 0.001$, vs. control group. Data are expressed as the mean \pm SD (8 rats in each group, one-way analysis of variance with Turkey's *post hoc* test). Escape latency: The time spent before the first arrival at the original platform location; time c: the time spent in quadrant c.

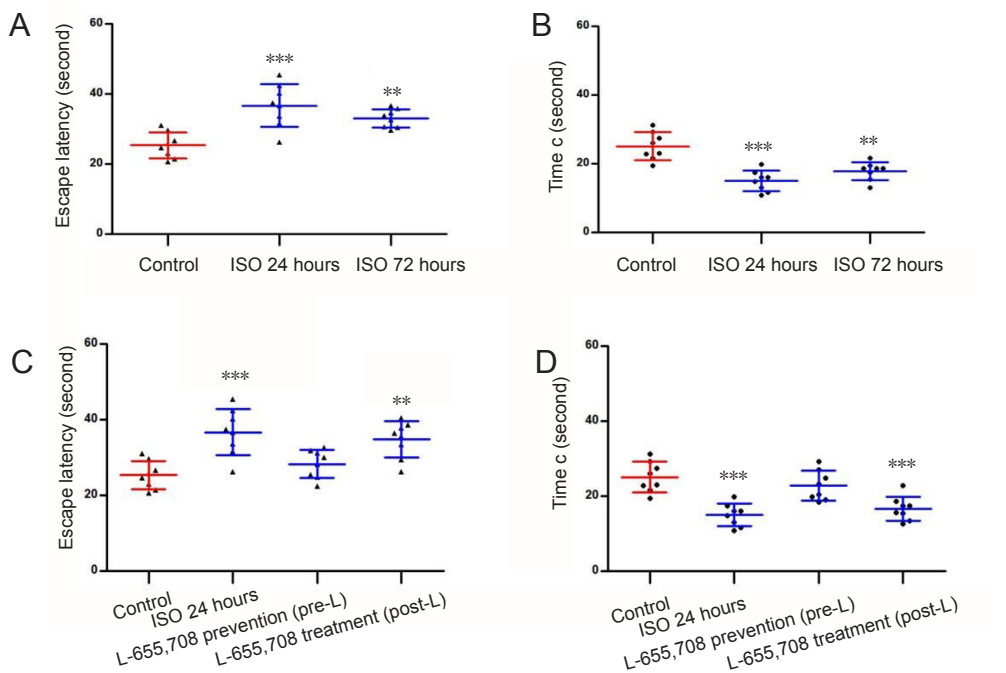


Figure 4 Performance of aged (24 months old) rats in the Morris maze test.

(A, B) Effects of isoflurane on the escape latency (A) and time c (B) of aged rats in the spatial probe trials. (B) Effects of isoflurane on the time c of aged rats in the spatial probe trials. (C, D) Effects of L-655,708 on the escape latency (C) and time c (D) of aged rats in the spatial probe trials. (** $P < 0.01$, *** $P < 0.001$, vs. control group. Data are expressed as the mean \pm SD (8 rats in each group, one-way analysis of variance with Turkey's *post hoc* test). Escape latency: The time spent before the first arrival at the original platform location. Time c: The time spent in quadrant c.

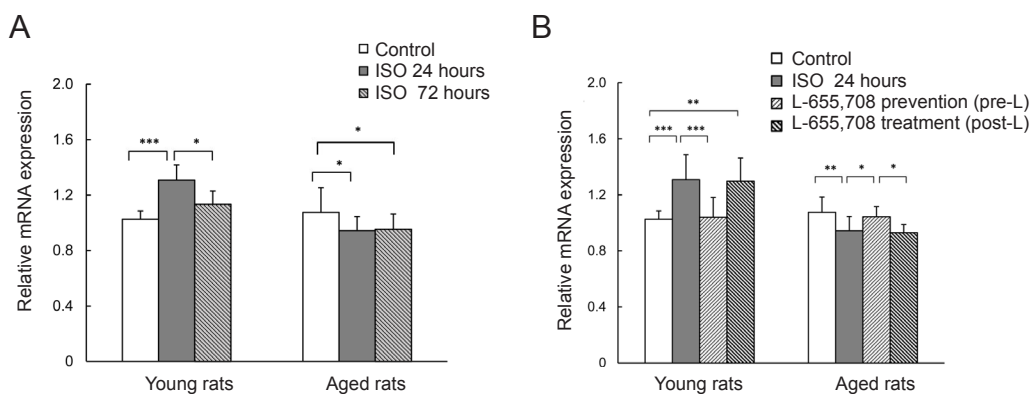


Figure 5 Hippocampal $\alpha 5$ GABAAR mRNA expression determined by RT-PCR.

(A) Effects of isoflurane anesthesia on hippocampal $\alpha 5$ GABAAR expression in young and aged rats. (B) Effects of L-655,708 on hippocampal $\alpha 5$ GABAAR expression in young and aged rats. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Data are expressed as the mean \pm SD (8 rats in each group, one-way analysis of variance with Turkey's *post hoc* test). $\alpha 5$ GABAARs: $\alpha 5$ subunit-containing gamma-aminobutyric acid type A receptors; RT-PCR: reverse transcription-polymerase chain reaction.

Discussion

Results from this study showed that 1.3% isoflurane exposure was associated with post-anesthetic spatial reference memory loss and that memory impairment persisted longer in aged rats. Administration of an inverse agonist of $\alpha 5$ GABAARs, L-655,708, before or after anesthesia was able to reverse the memory loss in young rats, while in aged groups, only application before exposure had an effect.

GABAARs are often increased by general anesthetics at high concentrations, but $\alpha 5$ GABAAR is specifically enhanced by low-dose inhaled anesthetic (isoflurane) (Caraiscos, 2004a). Saab et al. (2010) found that 1.3% isoflurane-induced memory impairment in young mice was reversed by L-655,708. Therefore, we kept the concentration of isoflurane to be 1.3%, though the minimal alveolar concentrations of isoflurane were different in mice ($1.34 \pm 0.10\%$) and rats ($1.46 \pm 0.06\%$) (Mazze et al., 1985). In this study, impaired memory retention was both observed 24 hours and 72 hours after anesthesia in aged rats. Aged subjects have been demonstrated to be more vulnerable to anesthetics-induced cognitive deficits than young subjects, and clinical studies have also verified that advanced age is a risk factor for POCD (Culley et al., 2003, 2004; Callaway et al., 2015). Furthermore, we found that subcutaneous injection of L-655,708 0.5 hours before or 23.5 hours after anesthesia enhanced the cognitive performance of young rats exposed to isoflurane anesthesia, indicating that a preventive or therapeutic dose of L-655,708 was able to reverse the low-dose isoflurane-induced memory deficits, which is in accordance with the results from another study (Saab et al., 2010). In aged rats, prophylactic administration of L-655,708 before anesthesia improved cognitive performance. However, administration after isoflurane exposure was unable to reverse the post-anesthetic memory impairment, indicating that L-655,708 has preventive but not therapeutic effects on cognitive memory dysfunction caused by low-dose isoflurane in aged rats.

$\alpha 5$ GABAARs are of particular interest due to its specific distribution in the hippocampus (Martin et al., 2009; Olsen and Sieghart, 2009). $\alpha 5$ GABAARs are highly sensitive to GABA and show a slow desensitization rate (Soh and Lynch, 2015), allowing them to mediate the tonic inhibition regulated by the low-concentration ambient GABA that exists outside of hippocampal neuronal synapses (Caraiscos et al., 2004b; Glykys and Mody, 2006). Alterations in the activity and density of hippocampal $\alpha 5$ GABAARs partially contribute to the amnesic effects of general anesthetics (Cheng et al., 2006; Martin et al., 2009). Zurek et al. (2014) found that isoflurane increased the activity and cell-surface expression of hippocampal $\alpha 5$ GABAARs, thereby impairing cognitive performance. Moreover, transgenic $\alpha 5$ GABAAR knockout mice show resistance to the memory-blocking effects of inhaled isoflurane and sevoflurane (Zurek et al., 2012). $\alpha 5$ GABAAR activity and tonic inhibitory current in hippocampal pyramidal neurons have been shown to be preferentially potentiated by low-concentration isoflurane and etomidate and thus produce memory-blocking effects (Caraiscos, 2004a). Consistently, pharmacological inhibi-

tion of $\alpha 5$ GABAARs has been found to improve cognition. Negatively modulating the activity of $\alpha 5$ GABAARs by pretreatment with $\alpha 5$ IA in human subjects specifically reversed the decline in the capability to recall a word list after alcohol drinking (Nutt et al., 2007; Atack, 2010). Furthermore, the anesthetic-induced potentiation of $\alpha 5$ GABAARs was significantly attenuated by inverse agonists in a concentration-dependent manner (Lecker et al., 2013). In a series of newly published studies (Pálvölgyi et al., 2016; Etherington et al., 2017; Gacsályi et al., 2017), a potent competitive antagonist of $\alpha 5$ GABAARs, S44819, enhanced hippocampal synaptic plasticity *in vitro* and improved cognitive function.

L-655,708 possesses 107-, 61-, and 54-fold greater selectivity for the $\alpha 5$ subunit than $\alpha 1$, $\alpha 2$ or $\alpha 3$ subunit-containing GABAARs, respectively (Quirk et al., 1996). A previous study suggested that L-655,708 preferentially suppressed the hippocampal $\alpha 5$ GABAA receptor-mediated tonic currents (Casula et al., 2001). Interestingly, upon exploration of the effects of L-655,708 in aged rats, we found that these effects differed from the effects of L-655,708 on isoflurane-induced memory deficiency in young rats. To investigate this issue, we performed RT-PCR and determined that, compared to a sham exposure, exposure to 1.3% isoflurane increased $\alpha 5$ GABAAR mRNA expression in the young hippocampus but decreased the expression in the aged hippocampus 24 hours after anesthesia. The increased expression of $\alpha 5$ GABAAR in the young rats returned to baseline 72 hours after anesthesia. However, in aged rats, reduced expression of hippocampal $\alpha 5$ GABAARs was detected at both 24 hours and 72 hours after anesthesia. Importantly, $\alpha 5$ GABAAR mRNA expression was at baseline level in both the young and aged rats that received subcutaneous L-655,708 0.5 hours before anesthesia. The activity and expression of hippocampal $\alpha 5$ GABAARs are integrally linked to normal spatial reference memory. Therefore, the opposite alterations in hippocampal $\alpha 5$ GABAAR expression observed in young and aged rats could both result in spatial memory impairment. Isoflurane anesthesia induced a decrease of $\alpha 5$ GABAAR expression in aged hippocampus. Consequently, treatment with the inverse agonists was not able to reverse the imbalance between the excitatory receptor system and inhibitory receptor system that was evoked by anesthesia. For this reason, L-655,708 was unable to reverse the memory impairment in the aged group that received L-655,708 after anesthesia exposure. Koh et al. (2013) found that positive allosteric modulators of $\alpha 5$ GABAARs, compound 6 and compound 44, improved cognition in aged rats with memory impairment. Therefore, whether the administration of $\alpha 5$ GABAAR agonists is able to reverse the isoflurane-induced memory impairment in aged subjects should be explored in a future study.

A question raised here is why hippocampal $\alpha 5$ GABAAR expression was differentially affected in young and aged rats. We speculate that neuroinflammation may explain the alterations in hippocampal $\alpha 5$ GABAARs in young rats. Volatile anesthetics can result in noninfectious neuroinflammation and the consequent excessive production of proinflammatory cytokines, including interleukin (IL)-1 β , IL-6, and tumor

necrosis factor- α (Cibelli et al., 2010; Wu et al., 2012; Kong et al., 2013; Zhang et al., 2013). The overexpression of these proinflammatory cytokines is involved in POCD. For example, IL-1 β activates p38 MAPK, which is associated with the phosphorylation of radixin (an actin-binding protein that regulates the surface expression of $\alpha 5$ GABAARs) (Srinivasan et al., 2004; Koss et al., 2006; Loebrich et al., 2006). Wang et al. (2012) found that IL-1 β increased the surface expression of hippocampal $\alpha 5$ GABAARs and tonic currents in young mice, which were associated with impairments in contextual fear memory. Therefore, the alterations in the expression of $\alpha 5$ GABAARs that occurred in young rats might be results of an acute inflammatory reaction induced by isoflurane anesthesia. However, aged subjects are more susceptible to POCD and neurological damage than young subjects (Magnusson et al., 2000; Wang et al., 2000; Culley et al., 2003; Jansson et al., 2004). The hippocampus is especially vulnerable to aging, consequently leading to cognitive disorders including memory impairments (McLay et al., 1997). Isoflurane might cause hippocampal neuron injury or apoptosis and a subsequent decline in $\alpha 5$ GABAAR expression, thereby disturbing the balance of the excitatory and inhibitory receptor systems involved in spatial memory (Zhang et al., 2012; Kong et al., 2013; Wang et al., 2016). The up-regulation of proinflammatory cytokines influences the synaptic plasticity of the hippocampus and triggers neurodegeneration. Therefore, we speculate that the reduction in hippocampal $\alpha 5$ GABAAR expression in aged rats might be the result of hippocampal damage caused by isoflurane exposure. This abnormality in $\alpha 5$ GABAAR expression persisted at least 72 hours after anesthesia. Interestingly, our behavioral investigation showed that a preventive dose of L-655,708 before anesthesia avoided the deterioration in cognitive memory both in young and aged rats. We propose that prophylactic injection of L-655,708 at the dose applied in our study may protect the hippocampus from neuroinflammation and proinflammatory cytokine-induced injury. Therefore, investigations into the mechanism behind this effect are of interest.

The present study has several limitations. First, the group size of rats is small for the cognitive behavior tests, although similar animal number has been used in some other studies (Koh et al., 2013; Lin et al., 2012). Second, simple RT-PCR data is insufficient to well elucidate the actual change of hippocampal $\alpha 5$ GABAAR. Therefore, it is of necessity to profoundly explore relevant mechanism using larger group size and more experimental data (western blotting, immunohistochemical or electrophysiological data). Moreover, the mechanism by which isoflurane influences the function of hippocampal neurons and the regulation of $\alpha 5$ GABAAR expression remains to be determined. We speculate that the isoflurane-induced abnormal release of proinflammatory cytokines is involved in the regulation of hippocampal $\alpha 5$ GABAAR expression. However, the associations between the inflammatory reaction, pathological lesion, hippocampal $\alpha 5$ GABAAR expression and memory impairment that occur after isoflurane exposure remain to be verified.

In conclusion, we found in the present study that administration of the inverse $\alpha 5$ GABAAR agonist before but not after anesthesia was able to reverse the low-dose (1.3%) isoflurane-induced spatial reference memory impairments in aged Wistar rats, which was different from that observed in young rats. In addition, the differential effects between young and aged rats might result from the converse alterations in the expression of hippocampal $\alpha 5$ GABAARs caused by isoflurane anesthesia.

Author contributions: Study design: YQY and ZFZ; experiment implementation: ZFZ, LD, TG, LB, and YL; data analysis: YQY, ZFZ, and LD; paper writing: ZFZ and LD; paper review and editing: YQY and YAW. All authors approved the final version of this paper.

Conflicts of interest: The authors declare that there are no conflicts of interest associated with this manuscript.

Financial support: None declared.

Institutional review board statement: All experimental procedures and protocols were approved by the Experimental Animal Ethics Committee of Academy of Military Medical Science of China (approval number: NBCD-SER-IACUC-2015128) in December 2015. All experimental procedures described here were in accordance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals.

Copyright license agreement: The Copyright License Agreement has been signed by all authors before publication.

Data sharing statement: Datasets analyzed during the current study are available from the corresponding author on reasonable request.

Plagiarism check: Checked twice by iThenticate.

Peer review: Externally peer reviewed.

Open access statement: This is an open access journal, and articles are distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as appropriate credit is given and the new creations are licensed under the identical terms.

References

- Atack JR (2010) Preclinical and clinical pharmacology of the GABA A, receptor $\alpha 5$ subtype-selective inverse agonist $\alpha 5$ 1A. *Pharmacol Ther* 125:11-26.
- Callaway JK, Jones NC, Roysse AG, Roysse CF (2015) Memory impairment in rats after desflurane anesthesia is age and dose dependent. *J Alzheimers Dis* 44:995-1005.
- Caraiscos VB, Newell JG, You-Ten KE, Elliott EM, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (2004a) Selective enhancement of tonic GABAergic inhibition in murine hippocampal neurons by low concentrations of the volatile anesthetic isoflurane. *J Neurosci* 24:8454-8458.
- Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (2004b) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by $\alpha 5$ subunit-containing γ -aminobutyric acid type A receptors. *Proc Natl Acad Sci U S A* 101:3662-3667.
- Cascella M, Bimonte S (2017) The role of general anesthetics and the mechanisms of hippocampal and extra-hippocampal dysfunctions in the genesis of postoperative cognitive dysfunction. *Neural Regen Res* 12:1780-1785.
- Casula MA, Bromidge FA, Pillai GV, Wingrove PB, Martin K, Maubach K, Seabrook GR, Whiting PJ, Hadingham KL (2001) Identification of amino acid residues responsible for the $\alpha 5$ subunit binding selectivity of L-655,708, a benzodiazepine binding site ligand at the GABA_A receptor. *J Neurochem* 77:445-451.
- Chen X, Keramidis A, Lynch JW (2017) Physiological and pharmacological properties of inhibitory postsynaptic currents mediated by $\alpha 5\beta 1\gamma 2$, $\alpha 5\beta 2\gamma 2$ and $\alpha 5\beta 3\gamma 2$ gabaa receptors. *Neuropharmacology* 125:243-253.
- Cheng VY, Martin LJ, Elliott EM, Kim JH, Mount HT, Taverna FA, Roder JC, Macdonald JF, Bhambri A, Collinson N, Wafford KA, Orser BA (2006) Alpha5GABA receptors mediate the amnestic but not sedative-hypnotic effects of the general anesthetic etomidate. *J Neurosci* 26:3713-3720.
- Christie SB, Blas AL (2002) Alpha5 subunit-containing GABA(A) receptors form clusters at GABAergic synapses in hippocampal cultures. *Neuroreport* 13:2355-2358.
- Cibelli M, Fidalgo AR, Terrando N, Ma D, Monaco C, Feldmann M, Takata M, Lever IJ, Nanchahal J, Fanselow MS, Maze M (2010) Role of interleukin-1 β in postoperative cognitive dysfunction. *Ann Neurol* 68:360-368.
- Coburn M, Fahlenkamp A, Zoremba N, Schaelte G (2010) Postoperative cognitive dysfunction: incidence and prophylaxis. *Anaesthesist* 59:177-184.

- Culley DJ, Baxter M, Yukhananov R, Crosby G (2003) The memory effects of general anesthesia persist for weeks in young and aged rats. *Anesth Analg* 96:1004-1009.
- Culley DJ, Baxter MG, Crosby CA, Yukhananov R, Crosby G (2004) Impaired acquisition of spatial memory 2 weeks after isoflurane and isoflurane-nitrous oxide anesthesia in aged rats. *Anesth Analg* 99:1393-1397.
- Dhar AK, Roux MM, Klimpel KR (2002) Quantitative assay for measuring the Taura syndrome virus and yellow head virus load in shrimp by real-time RT-PCR using SYBR Green chemistry. *J Virol Methods* 104:69-82.
- Erasso DM, Camporesi EM, Mangar D, Saporta S (2013) Effects of isoflurane or propofol on postnatal hippocampal neurogenesis in young and aged rats. *Brain Res* 1530:1-12.
- Erasso DM, Chaparro RE, Rio CEQ, Karlowski R, Camporesi EM, Saporta S (2012) Quantitative assessment of new cell proliferation in the dentate gyrus and learning after isoflurane or propofol anesthesia in young and aged rats. *Brain Res* 1441:38-46.
- Etherington LA, Mihalik B, Pálvölgyi A, Ling I, Pallagi K, Kertész S, Varga P, Gunn BG, Brown AR, Livesey MR, Monteiro O, Belelli D, Barkóczy J, Spedding M, Gacsályi I, Antoni FA, Lambert JJ (2017) Selective inhibition of extra-synaptic $\alpha 5$ -GABA A receptors by S44819, a new therapeutic agent. *Neuropharmacology* 125:35-38.
- Gacsályi I, Móricz K, Gigler G, Wellmann J, Nagy K, Ling I, Barkóczy J, Haller J, Lambert JJ, Szénási G, Spedding M, Antoni FA (2017) Behavioural pharmacology of the $\alpha 5$ -GABA A receptor antagonist s44819: enhancement and remediation of cognitive performance in preclinical models. *Neuropharmacology* 125:30-38.
- Gao ZX, Rao J, Li YH (2017) Hyperbaric oxygen preconditioning improves postoperative cognitive dysfunction by reducing oxidant stress and inflammation. *Neural Regen Res* 12:329-336.
- Glykys J, Mody I (2006) Hippocampal network hyperactivity after selective reduction of tonic inhibition in GABA A receptor $\alpha 5$ subunit-deficient mice. *J Neurophysiol* 95:2796-2807.
- Gurlit S, Möllmann M (2008) How to prevent perioperative delirium in the elderly? *Z Gerontol Geriatr* 41:447-452.
- Jansson A, Olin K, Yoshitake T, Hagman B, Herrington MK, Kehr J, Permert J (2004) Effects of isoflurane on prefrontal acetylcholine release and hypothalamic Fos response in young adult and aged rats. *Exp Neurol* 190:535-543.
- Johnson T, Monk T, Rasmussen LS, Abildstrom H, Houx P, Korttila K, Kuipers HM, Hanning CD, Siersma VD, Kristensen D, Canet J, Ibañez MT, Moller JT (2002) Postoperative cognitive dysfunction in middle-aged patients. *Anesthesiology* 96:1351-1357.
- Koh MT, Rosenzweig-Lipson S, Gallagher M (2013) Selective GABA(A) $\alpha 5$ positive allosteric modulators improve cognitive function in aged rats with memory impairment. *Neuropharmacology* 64:145-152.
- Kong F, Chen S, Cheng Y, Ma L, Lu H, Zhang H, Hu W (2013) Minocycline attenuates cognitive impairment induced by isoflurane anesthesia in aged rats. *PLoS One* 8:e61385.
- Koss M, Pfeiffer GR, Wang Y, Thomas ST, Yerukhimovich M, Gaarde WA, Doerschuk CM, Wang Q (2006) Ezrin/radixin/moesin proteins are phosphorylated by TNF- α and modulate permeability increases in human pulmonary microvascular endothelial cells. *J Immunol* 176:1218-1227.
- Lecker I, Yin Y, Wang DS, Orser BA (2013) Potentiation of GABAA receptor activity by volatile anaesthetics is reduced by $\alpha 5$ GABAA receptor-prefering inverse agonists. *Br J Anaesth* 110:i73-i81.
- Lin D, Cao L, Wang Z, Li J, Washington JM, Zuo Z (2012) Lidocaine attenuates cognitive impairment after isoflurane anesthesia in old rats. *Behav Brain Res* 228:319.
- Liu X, Wang WJ, Wu XM, Xie H (2018) Effect of target controlled infusion of propofol on perioperative hemodynamics and awakening period in elderly patients. *Zhongguo Zuzhi Gongcheng Yanjiu* 22:4835-4840.
- Loeblich S, Bähring R, Katsuno T, Tsukita S, Kneussel M (2006) Activated radixin is essential for GABA A receptor $\alpha 5$ subunit anchoring at the actin cytoskeleton. *EMBO J* 25:987-999.
- Magnusson KR, Scanga C, Wagner AE, Dunlop C (2000) Changes in anesthetic sensitivity and glutamate receptors in the aging canine brain. *J Gerontol A Biol Sci Med Sci* 55:B448-B454.
- Martin LJ, Bonin RP, Orser BA (2009) The physiological properties and therapeutic potential of $\alpha 5$ -GABA A receptors. *Biochem Soc Trans* 37:1334-1337.
- Mazze RI, Rice SA, Baden JM (1985) Halothane, isoflurane, and enflurane MAC in pregnant and nonpregnant female and male mice and rats. *Anesthesiology* 62:339-341.
- McLay RN, Freeman SM, Harlan RE, Ide CF, Kastin AJ, Zadina JE (1997) Aging in the hippocampus: interrelated actions of neurotrophins and glucocorticoids. *Neurosci Biobehav Rev* 21:615-629.
- Moller JT, Cluitmans P, Rasmussen LS, Houx P, Rasmussen H, Canet J, Rabbitt P, Jolles J, Larsen K, Hanning CD, Langeron O, Johnson T, Lauen PM, Kristensen PA, Biedler A, van Beem H, Fraidakis O, Silverstein JH, Beneken JEW, Gravenstein JS (1998) Long-term postoperative cognitive dysfunction in the elderly: international study of post-operative cognitive dysfunction study. *Lancet* 351:857-861.
- Monk TG, Weldon BC, Garvan CW, Dede DE, van der Aa MT, Heilman KM, Gravenstein JS (2008) Predictors of cognitive dysfunction after major non-cardiac surgery. *Anesthesiology* 108:18-30.
- Newman MF, Kirchner JL, Phillips-Bute B, Gaver V, Grocott H, Jones RH, Mark DB, Reves JG, Blumenthal JA, Neurological Outcome Research Group and the Cardiothoracic Anesthesiology Research Endeavors Investigators (2001) Longitudinal assessment of neurocognitive function after coronary-artery bypass surgery. *N Engl J Med* 344:395-402.
- Nutt DJ, Besson M, Wilson SJ, Dawson GR, Lingford-Hughes AR (2007) Blockade of alcohol's amnesic activity in humans by an $\alpha 5$ subtype benzodiazepine receptor inverse agonist. *Neuropharmacology* 53:810-820.
- Olsen RW, Sieghart W (2009) GABA A receptors: subtypes provide diversity of function and pharmacology. *Neuropharmacology* 56:141-148.
- Pálvölgyi A, Etherington LA, Mihalik B, Ling I, Pallagi K, Kertész S, Gunn BG, Brown AR, Livesey MR, Belelli D, Barkóczy J, Varga P, Spedding M, Gacsályi I, Lambert JJ, Antoni FA (2016) Selective targeting of extra-synaptic $\alpha 5$ -GABA A receptors by S44819 (Egis-13529), a novel competitive GABA A receptor inhibitor compound. *Soc Neurosci Abstr* 124:16.
- Park Y, Kim E, Lee HS, Moon HJ, Yoon JH, Kwak JY (2015) Real-time PCR cycle threshold values for the brafv600e mutation in papillary thyroid microcarcinoma may be associated with central lymph node metastasis: a retrospective study. *Medicine*. 94:e1149.
- Quirk K, Blurton P, Fletcher S, Leeson P, Tang F, Mellilo D, Ragan CI, McKernan RM (1996) [3H]L-655,708, a novel ligand selective for the benzodiazepine site of GABA A receptors which contain the $\alpha 5$ subunit. *Neuropharmacology* 35:1331-1335.
- Rudolph U, Antkowiak B (2004) Molecular and neuronal substrates for general anaesthetics. *Nat Rev Neurosci* 5:709-720.
- Saab BJ, MacLean AJB, Kanisek M, Zurek AA, Martin LJ, Roder JC, Orser BA (2010) Short-term memory impairment after isoflurane in mice is prevented by the $\alpha 5$ γ -aminobutyric acid type A receptor inverse agonist L-655,708. *Anesthesiology* 113:1061-1071.
- Serwanski DR, Miralles CP, Christie SB, Mehta AK, Li X, De Blas AL (2006) Synaptic and non-synaptic localization of GABA A receptors containing the $\alpha 5$ subunit in the rat brain. *J Comp Neurol* 499:458-470.
- Soh MS, Lynch JW (2015) Selective modulators of $\alpha 5$ -containing GABA A receptors and their therapeutic significance. *Curr Drug Targets* 16:735-746.
- Srinivasan D, Yen JH, Joseph DJ, Friedman W (2004) Cell type-specific interleukin-1 β signaling in the CNS. *J Neurosci* 24:6482-6488.
- Wang DS, Zurek AA, Lecker I, Yu J, Abramian AM, Avramescu S, Davies PA, Moss SJ, Lu WY, Orser BA (2012) Memory deficits induced by inflammation are regulated by $\alpha 5$ -subunit-containing GABA A receptors. *Cell Rep* 2:488-496.
- Wang HL, Liu H, Xue ZG, Liao QW, Fang H (2016) Minocycline attenuates post-operative cognitive impairment in aged mice by inhibiting microglia activation. *J Cell Mol Med* 20:1632-1639.
- Wang Y, Kikuchi T, Sakai M, Wu JL, Sato K, Okumura F (2000) Age-related modifications of effects of ketamine and propofol on rat hippocampal acetylcholine release studied by in vivo brain microdialysis. *Acta Anaesthesiol Scand* 44:112-117.
- Wu X, Lu Y, Dong Y, Zhang G, Zhang Y, Xu Z, Culley DJ, Crosby G, Marcantonio ER, Tanzi RE, Xie Z (2012) The inhalation anesthetic isoflurane increases levels of proinflammatory TNF- α , IL-6, and IL-1 β . *Neurobiol Aging* 33:1364-1378.
- Zhang L, Zhang J, Yang L, Dong Y, Zhang Y, Xie Z (2013) Isoflurane and sevoflurane increase interleukin-6 levels through the nuclear factor-kappa B pathway in neuroglioma cells. *Br J Anaesth* 110:82-91.
- Zhang Y, Xu Z, Wang H, Dong Y, Shi HN, Culley DJ, Crosby G, Marcantonio ER, Tanzi RE, Xie Z (2012) Anesthetics isoflurane and desflurane differentially affect mitochondrial function, learning, and memory. *Ann Neurol* 71:687-698.
- Zurek AA, Bridgwater EM, Orser BA (2012) Inhibition of $\alpha 5$ γ -aminobutyric acid type A receptors restores recognition memory after general anesthesia. *Anesth Analg* 114:845-855.
- Zurek AA, Yu J, Wang D-S, Haffey SC, Bridgwater EM, Penna A, Lecker I, Lei G, Chang T, Salter EWR, Orser BA (2014) Sustained increase in $\alpha 5$ GABA A receptor function impairs memory after anesthesia. *J Clin Invest* 124:5437-5441.

C-Editor: Zhao M; S-Editors: Wang J, Li CH; L-Editor: Song LP; T-Editor: Liu XL