The American Journal of Chinese Medicine, Vol. 44, No. 4, 1–15
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Institute for Advanced Research in Asian Science and Medicine
DOI: 10.1142/S0192415X16500385

# Electroacupuncture Ameliorates Propofol-Induced Cognitive Impairment via an Opioid Receptor-Independent Mechanism

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Published

Abstract: While general anesthesia is known to induce cognitive deficits in elderly and pediatric patients, its influence on adults is less well-characterized. The present study was designed to evaluate the influence of propofol on the learning and memory of young adult rats, as well as the potential neuroprotective role of electroacupuncture (EA) in propofol-induced cognitive impairment. Intravenous anesthesia with propofol was administered to young adult male Sprague–Dawley (SD) rats for 6 h, and EA was administered three times before and after anesthesia. The Morris Water Maze (MWM) test was conducted to determine the rat's cognitive performance following the anesthesia treatment. Our results showed that propofol induced obvious cognitive impairment in young adult rats, which could be ameliorated by multiple EA treatments. Moreover, the decreased level of phosphorylated glycogen synthase kinase 3 β (pGSK-3β) in the CA1 region of the hippocampus accompanying the cognitive impairment was also reversed by EA treatment. Further experiments demonstrated that neither 2 nor 10 mg/kg (I.P.) naloxone blocked the effect of EA, indicating that the neuroprotective effect of EA on propofol-induced cognitive impairment was not mediated via the opioid receptors. The present study suggests that EA could ameliorate the cognitive impairment induced by prolonged anesthesia with propofol in young adult rats, which is likely associated with pGSK-3β levels in the CA1 independely of opioid receptors.

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These findings imply that EA may be used as a potential neuroprotective therapy for post-operative cognitive dysfunction (POCD).

*Keywords*: Electroacupuncture; Propofol; Post-Operative Cognitive Dysfunction; pGSK-3β; Opioid Receptor.

## Introduction

In recent years, the potential of anesthetics to act as neurotoxic factors in a number of pathological processes has attracted attention (Vutskits *et al.*, 2012; Jevtovic-Todorovic *et al.*, 2013). Propofol is widely used as a general anesthetic in operating rooms due to its rapid onset, and short recovery time. Although instances of cognitive dysfunction related to inhalational anesthetics have been known for a while (Ono and Maeshima, 1998; Simon *et al.*, 2001; Dijkstra and Jolles, 2002), the role of propofol in neurodegeneration, neuronal apoptosis and cognitive impairment has only been recently discovered (Fibuch and Wang, 2007; Yin *et al.*, 2014). These findings have raised vigorous debate and concern about the safety of the use of propofol in pediatric patients (Mellon *et al.*, 2007). Despite the large number of adult patients undergoing propofol anesthesia each year, the influence of propofol on cognitive functioning in adults still remains unclear.

GSK-3β is widespread in the adult brain and is particularly abundant in the hippocampus (Woodgett, 1990). GSK-3β not only plays crucial roles in many major cellular signaling pathways, but also controls the expression of numerous genes by regulating several transcription factors (Doble, 2003). Extensive experimental data have demonstrated that GSK-3β is associated with many neurological diseases, such as Alzheimer's disease (AD) and stroke (Kaytor and Orr, 2002). In this study, we tested the activity of GSK-3β by examining the levels of pGSK-3β in the hippocampus (Wei *et al.*, 2002), basolateral nucleus of amygdala (BLA) (Alkire and Nathan, 2005) and the prefrontal cortex (PFC) (Xu *et al.*, 2014), which were all previously shown to be vulnerable brain regions affected by general anesthesia.

To date, there is no effective treatment for post-operative cognitive dysfunction (POCD). Electroacupuncture (EA) has been used to treat a variety of diseases and symptoms in China, as well as throughout the world (Cabyoglu *et al.*, 2006), especially in analgesia, and anti-depression contexts, and in the rehabilitation of hemiplegia (Zhao, 2008; Gao *et al.*, 2011). Its role in treating POCD, however, is unclear. Moreover, previous studies have revealed that the endogenous opioid system plays an important role in pain relief with different frequencies of EA. Specifically, it was shown that EA stimulation with 2 Hz could induce the release of enkephalins and endorphins, while 100 Hz EA treatment could increase the content of dynorphins in the central nervous system. Further studies revealed that 2 Hz EA treatment exerted its effect via μ and $\delta$ opioid receptors, while the effect of 100 Hz EA treatment was exerted via κ opioid receptors (Han, 2003). Dynorphin is known to be distributed abundantly in the central nervous system, especially in the hippocampus and amygdala, which are important brain regions for learning and memory,

and presumably cognitive function (Borbely *et al.*, 2013). Considerable evidence has shown that opioid peptides have a complex role in mediating stress-coping actions and synaptic plasticity in the hippocampus (Ogren *et al.*, 2010). In agreement with these findings, it has been demonstrated that the metabolic disturbance of the endogenous opioid system is involved in the pathological process of several cognitive dysfunction diseases, such as AD (Cai and Ratka, 2012).

Based on evidence from previous studies, it is reasonable to speculate that EA might be capable of alleviating propofol-induced cognitive impairment. To verify this hypothesis, we first investigated the cognitive impairment induced by propofol anesthesia in young adult rats, and then examined the protective effect of EA on propofol anesthesia-induced cognitive impairment. Additionally, we investigated whether the effect of EA was related to the activation of GSK-3β and whether it was mediated by opioid receptor.

## Materials and Methods

### *Animals*

Ten-week-old male SD rats were chosen for the present study. All rats were provided and maintained by the Laboratory Animal Center of the Peking University Health Science Center. Rats were housed 3 per cage in a room and the circadian rhythm of the room (12:12 h light: dark cycle) was controlled by a light that automatically turned on at 8.00 a.m. The relative humidity of the room was maintained at 45–55% while the temperature was kept at $20 \pm 2$°C. All rats had *ad libitum* access to food and water. Permission for the animal experiments was obtained from the local committee of animal use and protection, and the experimental operations were conducted according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

### *Anesthesia*

Rats were intravenously injected with propofol (2, 6-diisopropylphenol, 5 mg/kg/h) for 6 h. This dose was chosen based on the results from pilot experiments, which showed that 5 mg/kg propofol per hour could induce stable anesthesia (i.e. rats did not show corneal reflex or muscular reflex) without leading to any inhibition of circulation and respiration. The procedure of anesthesia was conducted in a quiet room and a heat lamp was used to keep the body temperature of rats at approximately 37°C. During the process of anesthesia, the rectal temperature, respiration and heart rate of rats were continuously monitored.

### *EA Treatment*

We followed the protocol of Wan *et al.* (2001) for EA stimulations. Rats were restrained in 16-cm long, cylindrical plastic restrainers which only allowed the rat's hind legs to extend out. The needles used for EA stimulations were made of stainless steel, and had diameters of 0.3 mm and 3 mm in length. Needles were bilaterally inserted in acupoint

ST36 and acupoint SP6, which were located in the hind legs of rat, and were fixed there with adhesive plaster. These needles were connected to an HANS electronic pulse generator (LH 800; Beijing University of Aeronautics and Astronautics), which could provide electrical stimulations at a constant current to rats in bilateral acupoint ST36 and acupoint SP6 through the needles. The whole EA stimulation procedure was conducted for 30 min. The intensity of stimulation started at 0.5 mA, and was increased by 0.5 mA every 10 min to 1.5 mA. The stimulating frequency of EA alternated between 2 Hz and 100 Hz (0.6 ms at 2 Hz and 0.2 ms at 100 Hz). Electrical stimulation was applied in the EA and EA + Pro groups only; rats in the other groups were restrained in the restrainers for 30 min with needles in acupoint ST36 and acupoint SP6 but no electrical current was applied.

To verify the role of opioid receptors, 84 rats were divided into seven groups. Twenty minutes before the EA treatment, 2 mg/kg naloxone was intraperitoneally injected to block μ and $\delta$ opioid receptors, while 10 mg/kg naloxone was used to block κ opioid receptor.

*Morris Water Maze Test*

The dimensions of the tank used in the experiment were as follows: a diameter of 140 cm and height of 60 cm. During the training and test, water at $21 \pm 2°C$ was filled in the tank to a height of 35 cm. To obscure background stimuli and provide external visual cues, a white curtain with four groups of marks was drawn around the circumference of the tank. The platform was made of plexiglas, with a diameter of 10 cm. During the hidden platform training, the top of the platform was 1.5 cm under the water surface, so that the platform was invisible to the rats, which always keep their heads above the water surface while swimming. A video-tracking system connected to a computer was used to observe the movement of the rats in the tank and to record relevant data. For data analysis, the maze was divided into 4 quadrants (the target quadrant was the one containing the platform) to calculate the cognitive performances of the rats during the MWM training.

The water maze test protocol consisted of two training phases, a probe trial and reversal training. Firstly, the visible platform water maze training was performed. The aim of this stage was to assess the nonspatial learning and memory abilities of rats and to exclude any rats that did not show motivation of swimming. The platform was placed in the center of the tank with a flag on top and rats were placed in each quadrant one time per day for two consecutive days. For each trial, rats that were able to find the platform within 60 s would be left on the platform for another 10 s, rats that did not find the platform within 60 s would be guided onto the platform and left there for another 10 s.

For the hidden platform training, the main purpose was to examine the spatial learning and memory ability of the rats. In this phase, the platform was moved into the middle of one quadrant of the tank and rats were sequentially placed in the other three quadrants to search for the invisible platform one time per day for 6 days. A probe test was conducted on the next day to assess the memory of the rats. In this test, rats were allowed to search in the tank for 60 s after being placed in a random quadrant.

Subsequently, the reversal platform water maze training was performed to assess the cognitive flexibility of rats. The platform was moved into the opposite quadrant, and rats

were trained to find the new location of the platform in the following 4 days as described above.

*Immunofluorescent Staining*

After MWM training, rats were sacrificed by fast transcardial perfusion, with 250 mL of 0.9% saline to rinse off the blood under chloral hydrate anesthesia (17.5%, 300 mg/kg). Then, 250 ml of 4% paraformaldehyde was perfused transcardially to fix the tissues. The brains were removed and immersed in 4% paraformaldehyde overnight at 4°C for further fixation. The brains were immersed in 20% sucrose until complete infiltration of sucrose, and then immersed with 30% sucrose for another 7 days for further cryoprotection.

For immunostaining of pGSK-3β, brains were prepared as frozen cross-sections of 30 μm in thickness. Sections were immersed with 5% normal goat serum for 60 min at room temperature to block non-specific antigens, and then were incubated with anti-pGSK-3β (Ser-9) monoclonal antibody (1:1,000; CST, Danvers, USA) and anti-NeuN mouse polyclonal (1: 600; CST, Danvers, USA) overnight at 4°C. After the sections were rinsed in PBS 3 times, they were incubated with fluorochrome-conjugated secondary antibodies for 1–2 h with slight agitation in a cassette at room temperature. Finally, a fluorescence microscope was used to examine the stained sections (Leica CTR 5000; Germany).

*Western Blotting*

The brains of rats were obtained after the rats were decapitated following the last MWM test. The brains were then frozen in a mixture of isopentane and drikold (−70°C) and then stored at −80°C. To obtain tissues of CA1, a sliding freezing microtome was used to cut the brains into sections of 50 μm in thickness, and then 16 gauge needles were used to dig out brain tissues of CA1 from those sections. The obtained tissues were homogenized in RIPA lysis buffer (Beijing Applygen Technologies Inc., Beijing, China) with a 1× complete protease inhibitor cocktail (Beijing Applygen Technologies Inc., Beijing, China). Homogenates were centrifuged to obtain the sediments that contained total protein. Fifty μg of protein sample were used for immunodetection with rabbit antisera to pGSK-3β (1:1000) or total GSK-3β (1:1000). After film exposure, membranes were incubated in stripping buffer (Beijing Applygen Technologies Inc., Beijing, China) with slight agitation for 40 min at 45°C. Anti-β-actin (1:5000, Zhongshan Biotechnology, Beijing, China) was used as an internal reference. Finally, an analysis system (Total Lab 2.01, Phoenix, UK) was used to quantify the band intensities by densitometry.

*Statistical Analysis*

Statistical analyses were performed using the Prism 6.0. All data were displayed as mean ± SEM. Cognitive performances in the MWM training phases were analyzed using a two-way ANOVA (repeated measure). The experimental group and training day were

taken as sources of variance of spatial memory, and the Bonferroni test was used for post-hoc comparisons. A one-way ANOVA was performed for the probe test and western immunoblots, and Newman-Keul's test was used for post-hoc comparisons. A Pearson correlation was performed to determine whether there was a relationship between spatial learning performance and pGSK-3β levels. The accepted level of significance was $P < 0.05$.

## Results

### *The Effect of Propofol Anesthesia on Learning and Memory in Young Adult Rats*

Twenty-four rats were randomly divided into 2 groups: the control group and the propofol-treated group. After receiving a tail vein injection of saline or propofol, respectively, the two groups underwent the MWM test to examine the influence of propofol anesthesia on

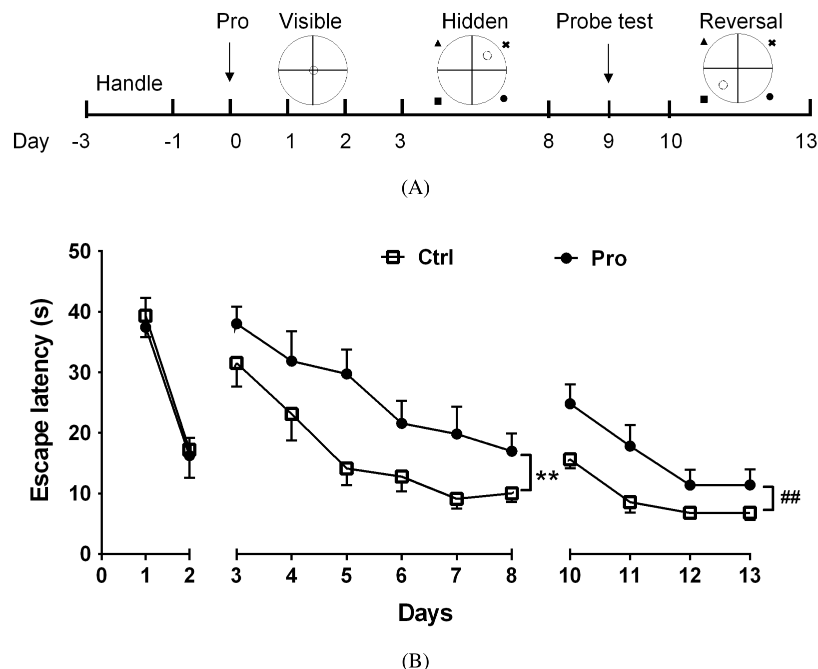


Figure 1. Effect of propofol anesthesia on learning and memory abilities in young adult rats. (A) Experimental procedure of anesthesia and MWM test. (B) Escape latencies during visible, hidden and reversal platform water maze training. Propofol had no influence on the escape latency during visible platform training. The propofol-treated group showed longer escape latency than the control group during hidden and reversal platform water maze training. ($n = 12$, $^{**}p < 0.01$ during hidden platform water maze training, $^{\#\#}p < 0.01$ during reversal platform water maze training by two-way RM ANOVA test). (C) Searching time in the target quadrant. Propofol-treated group spent less time in the target quadrant when compare to the control group. ($n = 12$, $^{*}p < 0.05$ by $t$-test). (D) Platform crossings during the probe test. Propofol-treated rats crossed the platform area less than control group ($n = 12$, $^{*}p < 0.05$ by $t$-test). Ctrl = Control, Pro = propofol. Data are shown as a mean ± SEM.

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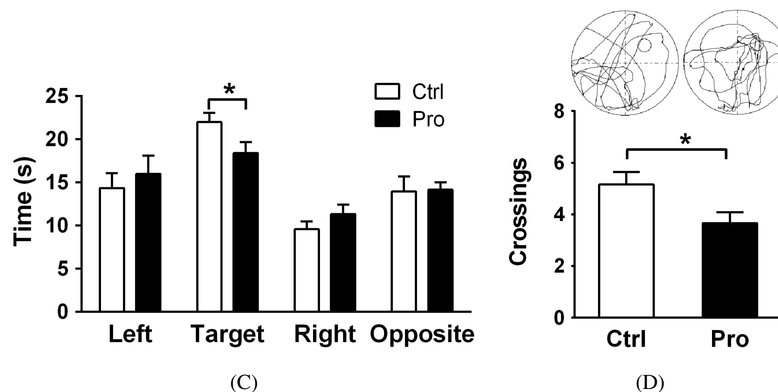


(C) (D)

Figure 1. (*Continued*)

spatial learning and memory. As shown in Fig. 1A, the difference of escape latencies between the propofol-treated group and the control group was not significant during the visible platform training (Fig. 1B, $F_{1,22} = 0.1078$, $p = 0.7458$), indicating that the non-spatial learning and memory ability of the rats was intact after propofol-treatment. In contrast, rats that were treated with propofol exhibited significantly impaired learning abilities compared with the control group during the hidden platform water maze training (Fig. 1B, $F_{1,22} = 8.231$, $p = 0.0089$). Furthermore, the dwell time in the target quadrant (Fig. 1C, $p = 0.0445$) and the platform crossing times (Fig. 1D, $p = 0.0263$) of propofol-treated rats were also significantly decreased following the probe test, while the dwell time in the other three quadrants did not differ significantly. These data suggested that propofol treatment impaired the spatial learning and memory abilities of rats. In the following reversal trials, the escape latency of the propofol-treated group was longer than the control group ($F_{1,22} = 8.119$, $p = 0.0093$), indicating that propofol treatment also impaired the cognitive flexibility of rats.

*The Effect of Propofol Anesthesia on the Expression of pGSK-3β in the CA1*

Immunofluorescent staining showed that the fluorescence intensity of pGSK-3β in the CA1 region was decreased after propofol treatment, without significant change in total GSK-3β (Fig. 2A). There were no significant changes in pGSK-3β in the BLA and PFC (data not shown). In accordance with immunofluorescent staining, western blotting showed that the level of pGSK-3β in the CA1 region was significantly decreased by propofol treatment (Fig. 2B, $p = 0.0467$), while the expression of total GSK-3β remained unchanged (data not shown). These results indicated that propofol treatment induced a significant decrease in the levels of pGSK-3β in the CA1. Furthermore, Pearson correlation analysis demonstrated that the pGSK-3β expression level of rats was positively correlated with their platform crossings in the MWM probe test in the propofol-treated group (Fig. 2C, $p = 0.0319$), suggesting that the activity of GSK-3β was negatively correlated with the cognitive performance of rats.

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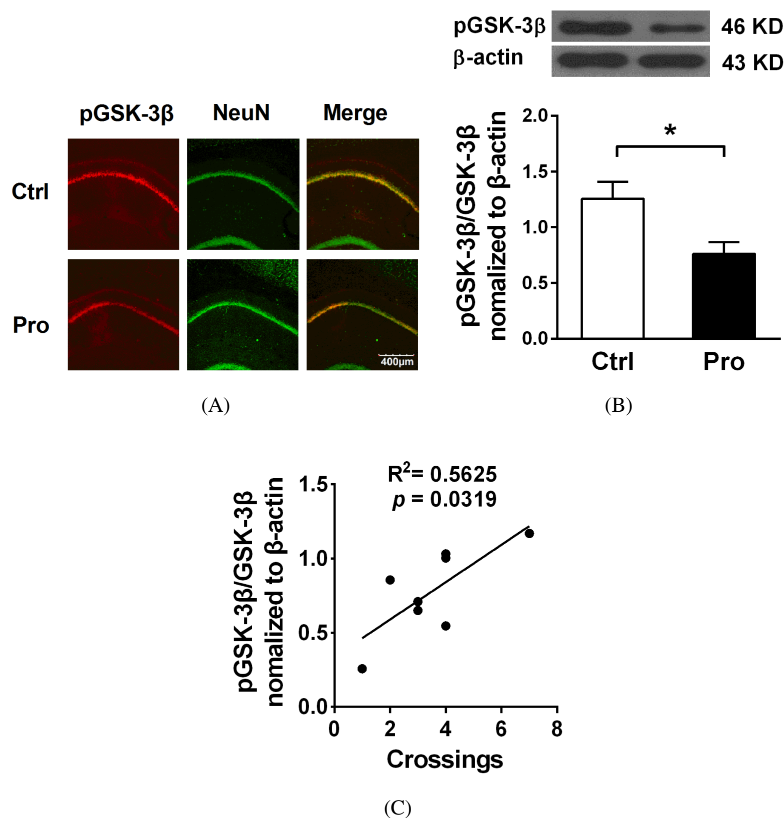


Figure 2. Effect of propofol anesthesia on the level of pGSK-3β. (A) Confocal micrographs of the CA1 region of the hippocampus stained with NeuN (green) and pGSK-3β (red) (100X). (B) The level of pGSK-3β was significantly decreased in the CA1 in propofol-treated group. ($n = 8$, *$p < 0.05$ by $t$-test). (C) Correlation analyses of pGSK-3β concentrations in the CA1 and platform crossings during the probe test of propofol-treated group. ($n = 8$, $p = 0.0319$ by Pearson's correlation analysis). Data are shown as a mean $\pm$ SEM.

*The Effect of EA Treatment on Propofol-Induced Cognitive Impairment*

EA (2/100 Hz) was conducted for 6 days, as illustrated in Fig. 3A. During the visible platform training, there was no difference in escape latencies among the propofol-treated, EA (EA + pro)-treated and control group (Fig. 3B, $p > 0.05$), indicating EA had no influence on the non-spatial learning and memory of rats. During the hidden and reversal platform water maze training, EA treatment rescued the prolonged escape latencies induced by propofol back to the same level as the control (Fig. 3B, $p < 0.05$). In addition, the dwell time in the first (target) quadrant (Fig. 3C, $p < 0.05$) and platform crossings (Fig. 3D, $p < 0.05$) of rats in the EA-treated group during the probe test were increased significantly. Taken together, these results suggest that the 2/100 Hz EA treatment could ameliorate propofol-induced cognitive impairment.

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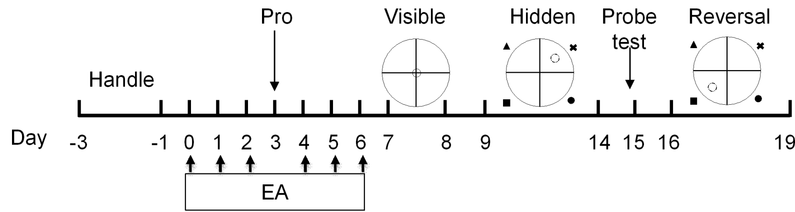


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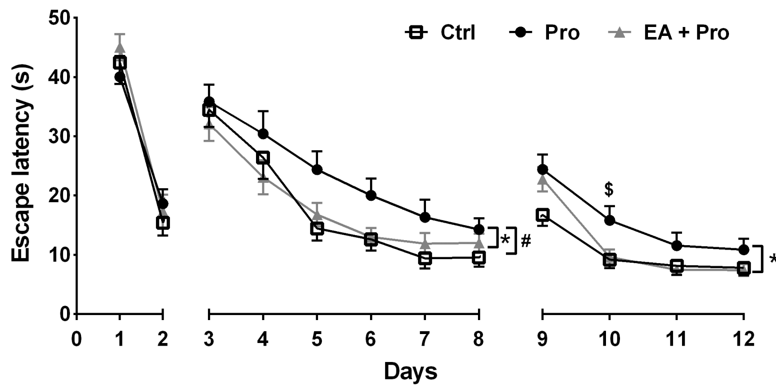


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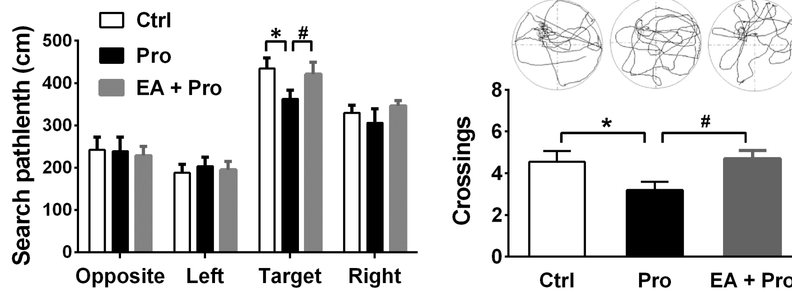



(C) (D)

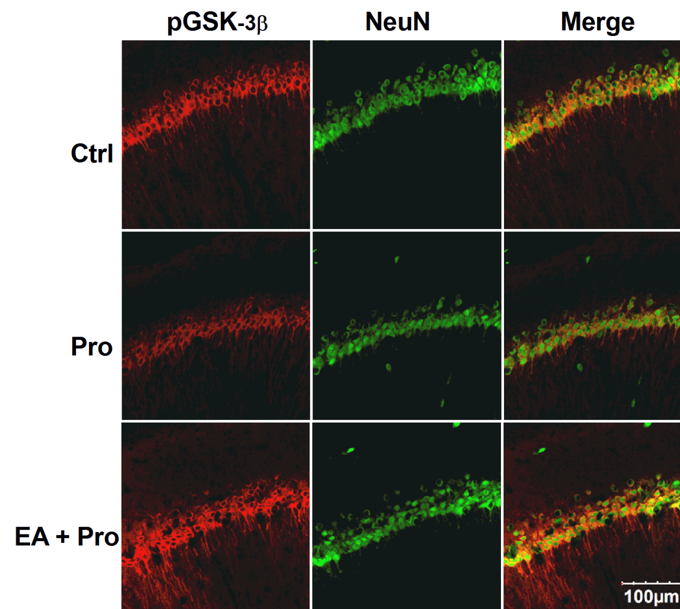
Figure 3. Effect of EA treatment on propofol-induced cognitive impairment in young adult rats. (A) Experimental procedure of propofol anesthesia, EA treatment and MWM test. (B) Escape latencies during visible, hidden and reversal platform water maze training. Neither propofol nor EA had any effect on the escape latency during visible platform training. Propofol group showed longer escape latency than control and EA groups during hidden and reversal platform water maze training. ($n = 20$, $^*p < 0.05$ compared with control group; $^\#p < 0.05$ compared with EA group, two-way ANOVA, $^\$p < 0.05$ compared with EA group by Bonferroni *post-hoc* test). (C) Searching time in the target quadrant during the probe test. Propofol-treated rats spent less time in the target quadrant than control and EA groups. ($n = 20$, $^*p < 0.05$ compared with control group; $^\#p < 0.05$ compared with EA group by one-way ANOVA, Newman-Keuls *post-hoc* test). (D) Platform crossings during the probe test. Rats in propofol-treated group crossed the platform area less than the control and EA groups. ($n = 20$, $^*p < 0.05$ compared with control group; $^\#p < 0.05$ compared with EA group by one-way ANOVA, Newman-Keuls *post-hoc* test). Data are shown as mean $\pm$ SEM.

*The Effect of Propofol and EA on the pGSK-3β Level in the CA1*

The propofol-induced decrease in the fluorescence intensity of pGSK-3β in the CA1 was reversed by EA treatment (Fig. 4A). Furthermore, the level of pGSK-3β in the CA1 was significantly decreased by propofol as mentioned in Fig. 2B, and this change was reversed by EA treatment ($p < 0.05$, Fig. 4B). Meanwhile the total GSK-3β protein expression was unchanged (data not shown). These results indicate that EA treatment could reverse the decrease in pGSK-3β induced by propofol in the CA1. However, the Pearson correlation analysis showed that the relation between pGSK-3β levels and the platform crossings in the EA-treated group was not significant (Fig. 4C, $R^2 = 0.6054$, $p = 0.0684$).

*The Effect of Propofol, EA and Naloxone on Learning and on Memory Abilities in Young Adult Rats*

Twenty minutes before the EA treatment, 2 mg/kg naloxone was intraperitoneally injected to block the μ and $\delta$ opioid receptors, while 10 mg/kg naloxone was used to block the




(A)

Figure 4. Effect of propofol and EA on the level of pGSK-3β in the CA1. (A) Confocal micrographs of the CA1 stained for NeuN (green) and pGSK-3β (red) (500X). (B) Propofol induced a significantly decrease of pGSK-3β in the CA1, which could be reversed by EA treatment. ($n = 6$, $*p < 0.05$ compared with control group; $^{###}p < 0.001$ compared with the EA + Pro group, one-way ANOVA, Newman-Keuls *post-hoc* test). (C) Correlation analysis of pGSK-3β in the CA1 and platform crossings during probe test of EA treatment group ($n = 6$). Data are shown as mean ± SEM.

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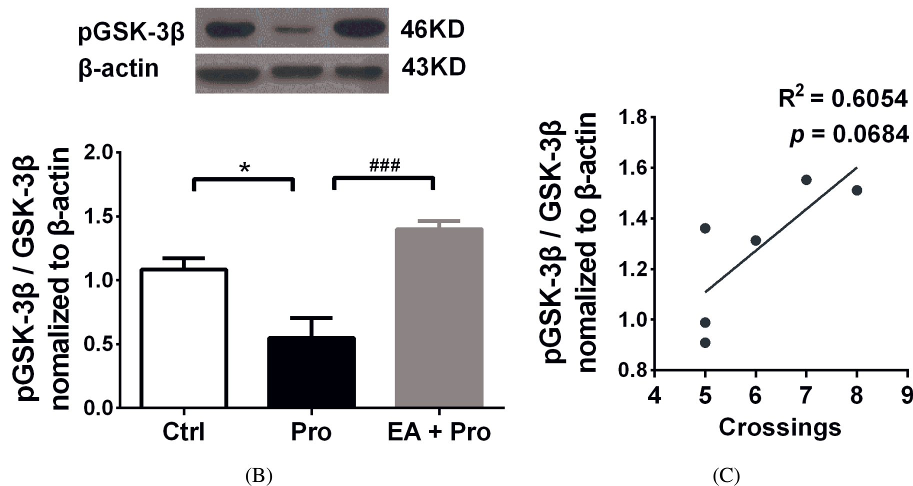
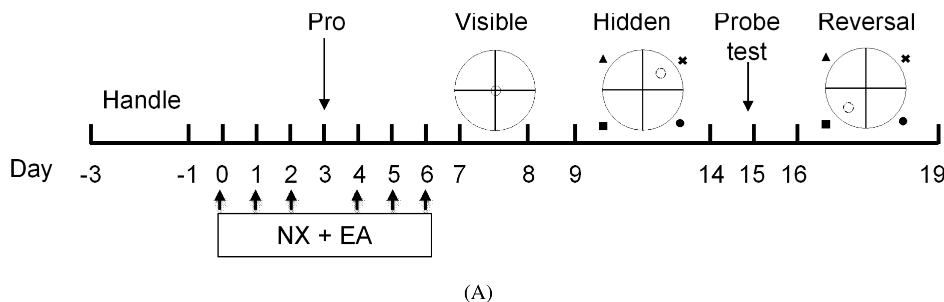



(B) (C)

Figure 4. (*Continued*)

κ opioid receptor (Fig. 5A). However, neither 2 mg/kg nor 10 mg/kg naloxone blocked the ameliorative effect that EA treatment had on propofol-induced cognitive impairment (Figs. 5B and 5C), indicating the effect of EA treatment was not opioid receptor-dependent. However, rats in the Pro + NX group showed a significant shorter escape latency than




(A)

Figure 5. Effect of propofol, EA and naloxone on learning and memory abilities in young adult rats. (A) Experimental procedure of propofol anesthesia, EA treatment, naloxone treatment and MWM test. (B) The effect of 2 mg/kg naloxone on escape latency during hidden platform water maze training. In accordance with our date above, Propofol group showed longer escape latency than control and EA groups during hidden and reversal platform water maze training ($n = 12$, $^*p < 0.05$ compared with control group; $^{\#}p < 0.05$ compared with the EA group, two-way ANOVA, Bonferroni *post-hoc* test). There was no significance difference between the EA + Pro group and the EA + Pro + NX group ($n = 12$, $p > 0.05$ by two-way ANOVA, Bonferroni *post-hoc* test). (C) The effect of 10 mg/kg naloxone on escape latencies during hidden platform water maze training. There was no significance difference between the EA + Pro group and the EA + Pro + NX group ($n = 12$, $p > 0.05$ by two-way ANOVA, Bonferroni *post-hoc* test); However, the Pro + NX group showed shorter escape latency than the Pro group during hidden platform water maze training ($n = 12$, $^{\$}p < 0.05$ by two-way ANOVA, Bonferroni *post-hoc* test). NX = Naloxone. Data are shown as a mean ± SEM.

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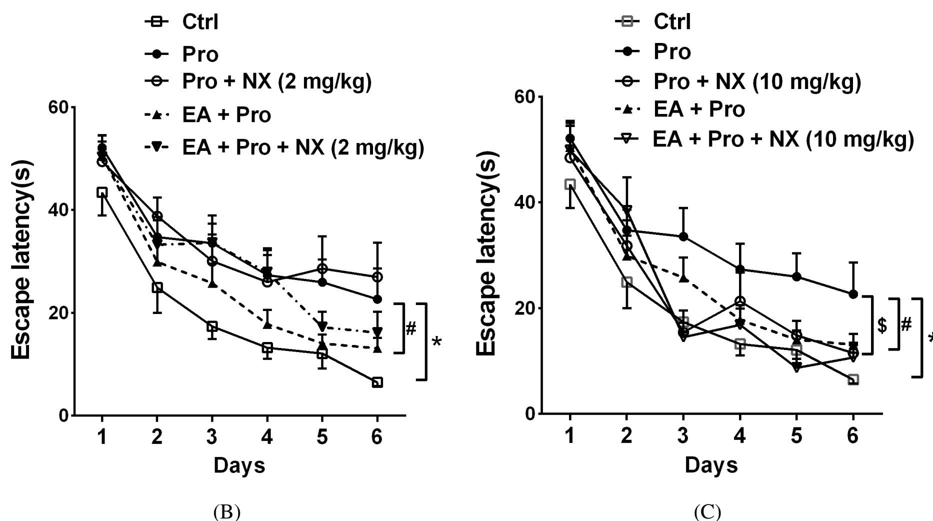


Figure 5. (*Continued*)

the Pro group (Fig. 5C, $p < 0.05$), suggesting that multi-treatment with 10 mg/kg naloxone may have a potential cognitive benefit effect on propofol-treated rats.

## Discussion

In this study, we demonstrated that propofol anesthesia could induce spatial learning and memory impairments in young adult rats, which was accompanied by a decrease in pGSK-3β in the CA1 region of the hippocampus. Multi-EA treatments could ameliorate propofol-induced cognitive impairment in the rats, which co-occurred with the reversal of the downregulation of pGSK-3β by EA. The opioid receptor antagonist naloxone could not block the effect of EA, implying EA treatment is opioid receptor-independent.

It is well established that propofol impairs the induction and maintenance of long-term potentiation (LTP) in the CA1 of hippocampus (Wei *et al.*, 2002), which plays a pivotal role in the information processing underlying spatial cognition and learning. However, the potential functional consequences of propofol anesthesia in the animal models have not been fully examined. In the current study, MWM tests revealed that propofol anesthesia impaired spatial learning and memory in young adult rats, as well as their cognitive flexibility. These findings were of practical significance as propofol has been often recommended as a preferred anesthetic for an overwhelming majority of surgeries in adults due to its rapid pharmacological effects and presumed cerebral physiology beneficial effects. In this context, our results have raised concern about the safety of using propofol in young adults.

The participation of propofol in neuron apoptosis and neurodegeneration has been proposed as a critical mechanism of its neurotoxicity (Tu *et al.*, 2011; Pearn *et al.*, 2012;

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Yu *et al.*, 2013). GSK-3β is not only a key modulator of apoptosis but is also involved in many neurodegenerative diseases, such as AD (Ma, 2014). Therefore, we speculated that the change in GSK-3β activity might play a role in propofol-induced neurotoxicity. In the present study, we found that propofol significantly reduced the expression of pGSK-3β in the CA1 region of hippocampus rather than the BLA or PFC. The levels of pGSK-3β were positively correlated with the cognitive performance of rats. This finding is in line with a previous study, which demonstrated that propofol upregulated pGSK-3β in macrophages (Hsing *et al.*, 2012). Further studies are required to determine whether GSK-3β exerts specific effect on propofol-induced cognitive impairment.

EA is considered to be a complementary therapy for various diseases, and it may improve the cognitive and behavioral outcome of cerebral injuries (Gao *et al.*, 2011). Guo *et al.* has previously found that EA pretreatment protected rats from cerebral ischemia/reperfusion injury by increasing the level of pGSK-3β (Ser-9) (Guo *et al.*, 2015). We then hypothesized that EA treatment could ameliorate propofol-induced cognitive impairment and GSK-3β might be involved in this process. The present study verified the conjecture that EA treatment not only ameliorated the impaired learning and memory performance induced by propofol, but also reversed the decrease of pGSK-3β induced by propofol in the CA1. The data demonstrate that EA possesses neuroprotective potential for anesthesia-related neurotoxicity, however, confirmation of the underlying molecular mechanisms still awaits the results of further studies.

There is considerable biochemical evidence tying the analgesic effect of EA to opioid receptors (Han, 2003). Recently studies have found that opioid receptors also participated in neuroprotection by mediating the phosphorylation of GSK-3β. Olianas *et al.* found that activation of opioid receptors induced a rapid increase of pGSK-3β *in vitro*. Furthermore, the inhibition of GSK-3β activity had powerful neuroprotective effects on gentamicin-induced neurotoxicity (Olianas *et al.*, 2011). μ opioid receptors have been shown to be involved in the cytoprotective effect, which is mediated by phosphoinositide 3-kinase (PI3K)/GSK-3β signaling (Goldsmith *et al.*, 2013). Hence, we hypothesized that opioid receptors-mediated changes in GSK-3β activity were involved in the ameliorative effect of EA treatment on propofol-induced cognitive impairment. Importantly, our results demonstrated that neither 2 mg/kg nor 10 mg/kg naloxone blocked the effect of EA treatment, which is different from previous studies. The result suggests that the neuroprotective effect of EA treatment on propofol-induced cognitive impairment is opioid receptor-independent and the widespread influence of EA might be the best explanation to this discrepancy. Previous studies have shown that the effect of EA treatment was not only modulated through opioid receptors, but also closely tied to the endocannabinoid system, sympathetic-catecholamine system and 5-HT system (Cabyoglu *et al.*, 2006). Therefore, the effect of EA treatment in our animal model is more likely to be mediated by the above systems and further studies are required to clarify it. It is, nevertheless, important to note that 10 mg/kg naloxone reduced the escape latency of propofol-treated rats. The complex function of opioid receptors in mediating stress-coping actions and spatial learning be the most likely explanation for this (Borbely *et al.*, 2013). The exact role of κ opioid receptors in our model needs to be further studied as well.

In conclusion, this study demonstrates that EA treatment ameliorates the cognitive impairment induced by prolonged anesthesia with propofol in young adult rats, which is likely to be associated with the pGSK-3β in the CA1 in an opioid receptor-independent way. These results are in line with a previous series of observations demonstrating the neuroprotective effect of EA treatment. Given the increasing number of surgeries and scarcely any therapeutic measures to POCD, our findings might also provide the first potential safe treatment for POCD.

## Acknowledgments

This work was supported by the National Basic Research Program of China (2013CB531905) and a grant from the National Natural Science Foundation (31271163).

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