Etanercept, an inhibitor of TNF-α, prevents propofol-induced neurotoxicity in the developing brain

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A B S T R A C T

Propofol can induce acute neuronal apoptosis, neuronal loss or long-term cognitive impairment when exposed in neonatal rodents, but the mechanisms by which propofol induces developmental neurotoxicity are unclear. Recent studies have demonstrated that propofol can increase the TNF-α level in the developing brain, but there is a lack of direct evidence to show whether TNF-α is partially or fully involved in propofol-induced neurotoxicity. The present study shows that propofol exposure in neonatal rats induces an increase of TNF-α in the cerebral spinal fluid, hippocampus and prefrontal cortex (PFC). Etanercept, a TNF-α inhibitor, prevents propofol-induced short- or long-term neuronal apoptosis, neuronal loss, synaptic loss and long-term cognitive impairment. Furthermore, mTNF-α (precursor of TNF-α) expression in microglia is increased after propofol anaesthesia in either the hippocampus or PFC, but mTNF-α expression in neurons is only increased in the PFC. These findings suggest that TNF-α may mediate propofol-induced developmental neurotoxicity, and etanercept can provide neural protection. Microglia are the main cellular source of TNF-α after propofol exposure, while the synthesis of TNF-α in neurons is brain-region selective.

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1. Introduction

The developing brain is extremely sensitive and vulnerable to general anesthetics (Jevtić-Todorović et al., 2003; Yon et al., 2005). Accumulating studies have demonstrated that clinically used general anesthetics, which are N-methyl-D-aspartate (NMDA) receptor antagonists and/or γ-aminobutyric acid type A (GABA A) receptor agonists, can cause developmental neurotoxicity in a variety of animal species (Yon et al., 2005; Cattano et al., 2008; Rizzi et al., 2010; Zou et al., 2011). Retrospective clinical studies also demonstrate that children, at an early age, who receive multiple exposures of general anaesthesia have an increased risk to develop cognitive and behavioural impairment in adolescence (Wilder et al., 2009; Flick et al., 2011; Sprung et al., 2012).

Propofol, the most commonly used intravenous general anesthetic in the clinic, exerts its effect through potentiating the GABA A receptor. Single propofol exposure only induces neuronal apoptosis, while multiple propofol exposures can cause neuronal apoptosis and long-term cognitive dysfunction in rodents (Yang et al., 2014; Pesić et al., 2009; Gonzales et al., 2015; Yu et al., 2013; Karen et al., 2013). However, the mechanisms underlying propofol-induced developmental neurotoxicity need to be fully elucidated.

Neuroinflammation has been demonstrated to play an important role in neurodegenerative diseases, ischemic brain injury, and traumatic brain injury (TBI) (Clark et al., 2010; Watters and O’Connor, 2011; Chio et al., 2013). Tumour necrosis factor (TNF)-α is released at a very early stage in neuroinflammation, and it precedes other cytokines in concentration increases in TBI (Baratz et al., 2015). TNF-α is produced as a membrane-associated TNF-α (mTNF-α) protein (26 kDa), which can be cleaved into soluble 17-kDa TNF-α (sTNF-α) by the TNF-α converting enzyme; sTNF-α is released into the extracellular fluid (Jasmin and Ohara, 2010). It has been reported that general anesthetics like propofol can increase the TNF-α level in the developing brain (Popić et al., 2015), but there is a lack of direct evidence showing whether TNF-α is partly or fully involved in propofol-induced neurotoxicity in the developing brain.

TNF-α plays a vital role in neural development and cognitive function under physiological conditions; however, excessive TNF-α can induce neuronal apoptosis and cognitive impairment.

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(Pozniak et al., 2014; Donzis and Tronson, 2014). Etanercept, a TNF-α inhibitor, is a large fusion protein that consists of the extracellular domains of human TNF receptor 2 fragment coupled to an Fc region of human IgG. It primarily acts by binding to and neutralizing TNF-α (Mohler et al., 1993). It has been reported that etanercept, which is widely used in animal studies, can improve cognitive function in TBI- or streptozotocin-induced dementia (Pozniak et al., 2014; Kubra Ecioğlu et al., 2015), but it is unclear whether etanercept can prevent or attenuate propofol-induced neurotoxicity in the developing brain.

Microglia are the most important immune cells in the central nervous system (CNS). They can trigger neuroinflammation as the main cellular source of TNF-α (Kaur et al., 2013). Recently, it was reported in a vitro study that isoflurane could induce neurons to release TNF-α (Wu et al., 2012). However, the cells from which TNF-α originates in response to propofol anaesthesia in vivo need to be confirmed.

Thus, in the present study, we studied the role of etanercept in the neurotoxicity induced by multiple propofol exposures, and explored the cellular source of elevated TNF-α after propofol anaesthesia in neonatal rats.

2. Materials and methods

2.1. Animals

Postnatal day (P) 7 Sprague-Dawley male rat pups with their lactating dams were purchased from the Animal Center of Chongqing Medical University, China (permission number: SCXK 2012-0001). Eight rat pups per dam were housed in a 12/12 h light-dark cycle at 22 °C and allowed ad libitum access to food and water. Rat pups were weighed every day and weaned on P21. All animal experiments were approved by the Ethics Committee of Chongqing Cancer Institute and followed the Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by Ministry of Science and Technology of China. Every effort was made to minimize the number of animals used and their suffering.

2.2. Experimental protocol

In experiment 1, the effect of propofol on the sTNF-α level was studied. P7 rat pups received 3 intraperitoneal injections of 50 mg/kg propofol (10 mg/ml, Sigma-Aldrich Chemical Co, St. Louis, MO, USA) or an equal volume of vehicle (0.1%DMSO) from P7 to P9, at 24-h intervals. Rat pups in the control group underwent the same environmental conditions and maternal separation, but they did not undergo any procedures. The propofol dose was based on the published data (Pesić et al., 2009) and our preliminary experimental results. The duration of loss of the righting reflex after each intraperitoneal injection of 50 mg/kg propofol was 120 ± 13 min. During anaesthesia, all pups were placed on a heating pad, and their rectal temperature was maintained at 37 ± 1 °C, and oxygen saturation was monitored using pulse oximetry and maintained at 95% or so. After full recovery, the pups not intended for immediate sacrifice were allowed to return to their dams. Some pups from each group were sacrificed 6 h after propofol anaesthesia on P7, P8 and P9 for transcardial arterial blood gas analysis (n = 5). Some were sacrificed on P7, P8, and P9 (6 h after propofol anaesthesia), and on P10 or P11, the cerebral spinal fluid (CSF) in the lateral cerebral ventricle was collected for ELISA (n = 5). The hippocampus and prefrontal cortex (PFC) were dissociated for Western blot analysis (n = 5) to detect the sTNF-α level.

In experiment 2, etanercept (ETN, Amgen, USA), a TNF-α inhibitor, was used to explore the role of TNF-α in propofol-induced developmental neurotoxicity. ETN was administered intracerebroventricularly (i.c.v.) 30 min before propofol exposure on P7, which was followed by the other two i.p. injections of propofol on P8 and P9. In brief, pups were mounted in a stereotaxic apparatus under 3% sevoflurane anaesthesia for 5 min. Then ETN (5 μg/2 μl) was injected into the right lateral cerebral ventricle (2.0 mm posterior and 1.0 mm lateral to the bregma and 2.0 mm deep to the skull surface) (Li et al., 2014). Rat pups in the sham group received an equal volume of artificial cerebral spinal fluid (aCSF) i.c.v on P7, and they underwent the same environmental conditions without propofol exposure. Rats in the control or propofol group underwent treatment as mentioned in experiment 1. Some pups were sacrificed on P9 (6 h after propofol anaesthesia) and P35, and their brains were used for activated caspase-3 immunohistochemistry, Nissl staining and synaptophysin immunofluorescence (n = 5). The other pups were used for the Morris Water Maze (MWM) test from P36 to P41 (n = 12).

In experiment 3, P7 rat pups received 3 intraperitoneal injections of 50 mg/kg propofol from P7 to P9; they were sacrificed on P9 (6 h after propofol anaesthesia), and their brains were used for immunofluorescence (n = 5). We examined the cellular localization of mTNF-α and the densities of total microglia or activated microglia in the hippocampus and PFC after propofol anaesthesia.

2.3. ELISA

Pups were anesthetized with 4% sevoflurane for 3 min and CSF was collected as previously described (Al-Sarraf et al., 2000). The sTNF-α level in CSF was detected using a rat TNF-α ELISA kit (EMD Millipore, Darmstadt, Germany) according to the instruction of the manufacturer’s protocol.

2.4. Tissue preparation

Pups were anesthetized with 4% sevoflurane for 3 min. For Western blot studies, the hippocampi and PFC were dissociated immediately after CSF collection and stored at −80 °C. For the immunohistochemistry experiments, Nissl staining and immunofluorescence, the pups were transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA) solution. The brains were removed and postfixed in 4% PFA solution for 24 h. The right halves of the brains were embedded in paraffin and cut into 4 μm thick sagittal sections for immunohistochemistry and Nissl staining. The left halves of the brains were cryoprotected with 25% sucrose solution until they sunk and were cut into 10 μm thick sagittal frozen sections for immunofluorescence. Three sections that contained the hippocampus and PFC (1.5–2.5 mm anterior to the bregma and 0.1–1 mm from the midline, Cichon et al., 2014) were selected from each pup.

2.5. Immunohistochemistry

Immunohistochemistry was performed as described in the previous study (Scallet et al., 2004). Paraffin sections were deparaffinized in xylene and rehydrated in graded ethanol, which was followed by antigen retrieval with 0.1 M sodium citrate buffer (pH 6.0) in a microwave oven for 20 min. After blocking with 0.3% hydrogen peroxide and 3% normal goat serum, sections were incubated with the primary rabbit polyclonal anti-activated caspase-3 antibody (1:100; Abcam Inc., Cambridge, MA, USA) overnight at 4 °C, they were then incubated with the second goat anti-rabbit IgG antibody and streptavidin-biotin complex (Histostain-plus kit, Boster, Wuhan, China) for 30 min at 37 °C respectively the next day. They were then colorized with DAB reagents (Beijing Zhongshan Biotechnology Co. Ltd., Beijing, China). Haematoxylin was used for nuclear staining. Negative sections were incubated with PBS instead of primary antibody. Activated caspase-3 positive cells
were counted from five microscopic areas in the hippocampal CA1 pyramidal cell layer or layers II–V of the PFC under 40x magnification by an investigator who was blinded to the experiments. The density of activated caspase-3 positive cells was obtained using the number of activated caspase-3 positive cells divided by the defined area (210 x 140 μm).

2.6. Nissl staining

Nissl staining was performed as previously described by Wang (Wang et al., 2012). The deparaffinized sections were incubated with Nissl staining solution containing cresyl violet (Beyotime Institute of Biotechnology, Shanghai, China) for 30 min at 37°C. The density of neurons was calculated with the method mentioned in the immunohistochemical experiment.

2.7. Immunofluorescence

Immunofluorescence was performed as previously described (Fogarty et al., 2013). After antigen retrieval, frozen sections were blocked with 3% normal goat serum and 0.1% Triton-X 100 in PBS for 1 h at 37°C. To detect synaptophysin positive puncta or Iba-1 positive cells, the sections were incubated with the primary monoclonal rabbit anti-synaptophysin antibody (1:250; EMD Millipore, Darmstadt, Germany) or polyclonal rabbit anti-Iba1 antibody (1:200, Wako, Japan) for 24 h at 4°C. They were then incubated with the second Alexa Fluor 555 goat anti-rabbit (1:200; Invitrogen, Eugene, OR, USA) or Alexa Fluor 488 goat anti-rabbit (1:200; Invitrogen) for 2 h in the dark at 37°C and counterstained with 0.5% DAPI (Sigma, USA) for 15 min at 37°C. To localize the cellular source of mTNF-α, the sections were incubated with the primary polyclonal rabbit anti-TNF-α antibody (1:50; Novus Biologicals, Littleton, CO, USA) for 24 h at 4°C, combined with one of the following antibodies: monoclonal mouse anti-NeuN antibody (1:500; Chemicon, Millipore, Billerica, MA) or polyclonal goat anti-Iba1 antibody (1:25; Novus Biologicals). The sections were then incubated with the second Alexa Fluor 555 goat anti-rabbit (1:200, Invitrogen) or Alexa Fluor 594 donkey anti-rabbit (1:200, Invitrogen) for 2 h at 37°C, combined with either Alexa Fluor 488 goat anti-mouse (1:200, Invitrogen) or Alexa Fluor 488 donkey anti-goat (1:200, Invitrogen). All images were taken under the confocal microscope (Leica TCS SP8, Wetzlar, Germany). Synaptophysin positive puncta in the radiation layer of hippocampal CA1 and layers II–V of the PFC were collected using a 63 x objective (NA 1.4), with a x 2 optical zoom, a 2-stack size of 1 μm, format with 1024 x 1024 pixels, and a field size of 92.06 x 92.06 μm. The other images were collected using a 40 x objective (NA 0.85), format with 1024 x 1024 pixels, and a field size of 291.57 x 291.57 μm. Analysis of synaptophysin images was performed using a similar approach that was described by Fogarty et al. (2013). In brief, the two-dimensional confocal images were imported into Imaris software (Bitplane, South Windsor, CT, USA) and visualized in the “surpass” view. Three-dimensional rendered images were reconstructed using the Filament Tracer function. The densities of total Iba-1 positive cells, activated Iba-1 positive cells, Iba-1-mTNF-α and NeuN-mTNF-α double positive cells in the hippocampus and PFC were calculated with the method that was mentioned in the immunohistochemical experiment.

2.8. Western blot

Western blots were performed as previously described (Li et al., 2013). The tissues were homogenized in RIPA buffer. The homogenates were centrifuged at 12,000g for 10 min at 4°C and the supernatants were collected and stored at -80°C. Protein concentrations were determined using the BCA protein assay (Bio-Rad, Hemel Hempstead, Herts, UK). Each protein sample of 30 micrograms was separated on a 10% sodium dodecyl sulfate polyacrylamide gel by electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes. Nonspecific protein binding was blocked with 5% bovine serum albumin in Tris-buffered saline Tween-20 (TBS-T) for 30 min at 37°C. The membranes were incubated with the primary goat polyclonal anti-TNF-α antibody (1:1000; Santa Cruz Biotechnology, USA) overnight at 4°C, and then incubated with rabbit anti-goat IgG-HRP (1:1000; Neobioscience Technology Co., Ltd., Beijing, China) or anti-β-actin (1:1000; Neobioscience Technology Co., Ltd.) for 2 h at 37°C. The protein bands were detected by enhanced chemiluminescence and photographed. The results were analysed using an image acquisition system (Fusion FX7, Vilbert Lourmat, France).

2.9. Morris water maze test

Spatial learning and memory tests were performed using the Morris Water Maze test (MWM) according to the protocol (Yorhues and Williams, 2006). In brief, a round pool (150 cm in diameter, 60 cm in height) was filled with water (22 ± 1°C) containing ink to hide the platform. The platform (9 cm in diameter) was submerged 1.0 cm below the water surface and four visual cues were placed on the pool wall. A continuous video tracking system recorded the swimming motions of each rat and data were analysed using MWM motion-detection software (Zhenghua biotech Co., Ltd., Huabei, China). In the acquisition trials, each rat received 4 trials per day for 5 days. Rats were placed in the water facing the wall in a random starting position and allowed to search for the platform within 90s. The time for each rat to reach the platform (escape latency) was recorded. If the rat did not find the platform within 90s, it was guided to the platform and allowed to stay on it for 15s, and the escape latency was recorded as 90s. On day 6, the platform was removed and the probe trial was performed. Rats were placed in the farthest starting point from the previous platform location and allowed to swim for 90s. The number of times that rats crossed the previous platform position (platform crossing times) was recorded.

2.10. Statistical analysis

Statistical analysis was performed with SPSS (version 22, IBM, New York, NY, USA). The data for the acquisition trial in the MWM were analysed using a repeated-measures general linear model of ANOVA followed by post hoc Bonferroni test. The data for the probe trial in the MWM were expressed as median (interquartile range) and analysed using Kruskal–Wallis test. Other data were expressed as mean ± SEM and were analysed using Student’s t-test, one-way or two-way ANOVA. Post hoc pairwise comparisons with Bonferroni correction (simple main effects tests) were performed when appropriate. P value less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of propofol on the body weight, and respiratory or metabolic function

We did not find any differences in the daily weight gain among the groups (data not shown). The results of the blood-gas analysis on P7, P8 and P9 showed that propofol exposures at doses of 50 mg/kg i.p. did not cause significant respiratory or metabolic distress (Table 1). No rat pups died during propofol anaesthesia.
Table 1
Arterial blood physiological parameters in the P7, P8 and P9 pups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Vehicle</th>
<th>Propofol</th>
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<tr>
<td></td>
<td>P7</td>
<td>P8</td>
<td>P9</td>
</tr>
<tr>
<td>pH</td>
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<td>7.32±0.03</td>
<td>7.31±0.04</td>
</tr>
<tr>
<td>pCO₂</td>
<td>49.4±4.6</td>
<td>48.8±3.7</td>
<td>48.5±4.1</td>
</tr>
<tr>
<td>pO₂</td>
<td>91.0±4.8</td>
<td>90.4±5.2</td>
<td>91.5±3.8</td>
</tr>
<tr>
<td>Glucose</td>
<td>7.9±0.4</td>
<td>8.1±0.7</td>
<td>8.2±0.3</td>
</tr>
<tr>
<td>Lactate</td>
<td>3.0±0.3</td>
<td>2.8±0.2</td>
<td>3.1±0.4</td>
</tr>
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</table>

Data were expressed as mean±SEM (n=5). Units of PO2 or PCO2: mmHg; Units of glucose or lactate: mM.

![Fig. 1](image)

**Fig. 1.** Effect of propofol on the sTNF-α levels in the CSF, hippocampus and PFC. (A) Changes of sTNF-α in the CSF. (B, D) Representative Western blot results of sTNF-α in the hippocampus (B) and PFC (D). (C, E) Quantification of the sTNF-α protein levels (ratio to β-actin) in hippocampus (C) and the PFC (E). Data are expressed as mean±SEM, n=5. **P<0.01, ***P<0.001 vs control.

3.2. Propofol increased the sTNF-α levels in the CSF, hippocampus and the PFC

To investigate the effect of propofol on the extracellular TNF-α level in the central nervous system of neonatal rats, the soluble TNF-α (17-kD) level in CSF was measured using ELISA (two-way ANOVA: F_{group(2,60)}=42.93, P<0.0001; F_{age(4,60)}=5.17, P=0.001; F_{group×age(8,60)}=4.09, P=0.001). Post hoc tests revealed that the sTNF-α level in propofol group was increased by 1.6-fold on P7 compared with control, and it remained elevated on P8, P9 and P10 (P<0.0001, P=0.0001, and P=0.004, respectively), the levels returned to the control level on P11 (P>0.05, Fig. 1A). The sTNF-α protein levels in the hippocampus and PFC were measured using Western blot. In the hippocampus (two-way ANOVA: F_{group(2,60)}=54.21, P<0.0001; F_{age(4,60)}=4.38, P=0.004; F_{group×age(8,60)}=2.83, P=0.01), the sTNF-α protein level was increased in propofol group on P7 (P<0.0001), P8 (P<0.0001), P9 (P<0.0001), and P10 (P=0.008), it returned to control level on P11 (P>0.05, Fig. 1B and C). Similarly, in the PFC (two-way ANOVA: F_{group(2,60)}=71.66, P<0.0001; F_{age(4,60)}=4.21, P=0.005; F_{group×age(8,60)}=3.46, P=0.002), the sTNF-α protein level was elevated in propofol group on P7, P8, P9, or P10 (P<0.0001, P<0.0001, P=0.0001, and P=0.002, respectively), and the levels recovered on P11 (P=0.371, Fig. 1D and E). There were no differences in the changes in the sTNF-α level between the sham and control groups. These results suggest that propofol increases the extracellular TNF-α level in the developing brain.

3.3. Etanercept prevented propofol-induced neuronal apoptosis and neuronal loss

Next, we investigated the effect of etanercept on propofol-induced neuronal apoptosis and neuronal loss. In the hippocampal CA1 (two-way ANOVA: F_{group(3,32)}=164.30, P<0.0001; F_{age(1,32)}=265.72, P<0.0001; F_{group×age(3,32)}=89.61, P<0.0001), activated caspase-3 positive cells were increased in propofol
Fig. 2. Etanercept prevented propofol-induced neuronal apoptosis (A,B) Images of the defined area (black rectangle) in hippocampal CA1 (A) and the PFC (B). Scale bar = 200 μm. (C) Representative images of activated caspase-3 positive cells (brown stained, black arrows) on P9 in the defined area. Scale bar = 20 μm. (D,E) Quantification of the densities of activated caspase-3 positive cells on P9 and P35 in hippocampal CA1 (D) and the PFC (E). ETN: Etanercept. Data are expressed as mean ± SEM, n = 5. *P < 0.05, **P < 0.01, ***P < 0.001 vs control. #P < 0.05. ##P < 0.01, ###P < 0.001 vs ETN.

The hippocampal CA1 (two-way ANOVA: Fgroup(3,32) = 9.98, P < 0.0001; 
Fage(1,32) = 20.71, P < 0.0001; 
Fgroup × age(3,32) = 0.52, P = 0.674), compared with control, propofol decreased the densities of neurons on P9 and P35 (79.4 ± 7.7 vs 126 ± 10.2 cells/mm², P = 0.012; 
59.8 ± 7.1 vs 91.4 ± 4.5 cells/mm², P = 0.024, respectively). There were no differences between etanercept-pretreated rats and control rats on P9 or P35 (P = 0.037, P = 0.035, respectively, Fig. 3A and B). The protective effect of etanercept was observed in the PFC as well (two-way ANOVA: Fgroup(3,32) = 11.37, P < 0.0001; 
Fage(1,32) = 24.95, P < 0.0001; 
Fgroup × age(3,32) = 0.52, P = 0.673), etanercept reduced propofol-induced neuronal loss to control levels on P9 and P35 (Fig. 3A and C). There were no differences between the sham and control groups in the densities of activated caspase-3 positive cells or neurons under Nissl staining. These results suggest that etanercept can prevent propofol-induced short- or long-term neuronal apoptosis and neuronal loss.

3.4. Etanercept prevented propofol-induced synaptic loss

We then explored whether etanercept attenuated propofol-induced synaptic loss. In hippocampal CA1 (two-way ANOVA: Fgroup(3,32) = 10.28, P < 0.0001; 
Fage(1,32) = 15.42, P < 0.0001; 
Fgroup × age(3,32) = 0.004, P = 1.0), the densities of synaptophysin positive puncta were decreased by 38.6% and 31.2% in propofol group on P9 and P35 compared with the control group (P = 0.029, P = 0.035, respectively), but there were no differences between ETN group and control on P9 or P35 (P > 0.05, Fig. 4A, B and C). Similar results were observed in the PFC (two-way ANOVA: Fgroup(3,32) = 13.89, P < 0.0001; 
Fage(1,32) = 22.75, P < 0.0001; 
Fgroup × age(3,32) = 1.81, P = 0.166), and post hoc tests revealed that the densities of synaptophysin positive puncta in propofol group were decreased on P9 and P35 compared with the control densities (P = 0.015, P = 0.001, respectively). This observation could be
reversed to control levels by etanercept pretreatment (Fig. 4A, B and D). These results suggest that etanercept can prevent propofol-induced short- or long-term synaptic loss.

3.5. Etanercept improved the long-term cognitive function impaired by propofol

The Morris Water Maze test was used to evaluate spatial learning and memory. In the acquisition trial, the escape latencies were reduced during daily training in all groups ($F_{3,40,149.48} = 87.77$, $P < 0.0001$), and the between-subjects effect of the treatment group ($F_{3,44} = 10.66$, $P = 0.0001$) was significant. Post hoc Bonferroni test revealed that propofol increased the escape latencies on Day 2 ($P = 0.017$), Day 3 ($P < 0.0001$), Day 4 ($P < 0.0001$) and Day 5 ($P < 0.0001$) compared with control. Etanercept significantly reduced propofol-induced increase in the escape latencies ($P = 0.038$, $P = 0.003$, $P = 0.005$, $P = 0.006$, respectively, Fig. 5A) from Day 2 to Day 5. In the probe trial, propofol significantly decreased the platform crossing times compared with control ($P < 0.0001$, Fig. 5B), while etanercept attenuated propofol-induced decreases in the platform crossing times ($P = 0.037$, Fig. 5B). Moreover, escape latencies and platform crossing times in ETN group were not different from those in the control group ($P > 0.05$).

3.6. The cellular source of propofol-increased TNF-α

We next examined the cellular localizations of mTNF-α. Double immunofluorescence revealed that mTNF-α was expressed in microglia either in hippocampus or in the PFC, but it was only expressed in neurons in the PFC after propofol anaesthesia (Fig. 6A). Quantified results showed that the cells with coexpression of Iba-1 (a marker of microglia) and mTNF-α level in the hippocampus ($t_{(8)} = −7.889$, $P < 0.0001$, Fig. 6B) and PFC ($t_{(8)} = −6.624$, $P < 0.0001$, Fig. 6D) were significantly increased after propofol anaesthesia compared with control. Similar results were observed for the cells with coexpression of NeuN (a marker of neuron) and mTNF-α after propofol anaesthesia in the PFC ($t_{(8)} = −6.65$, $P < 0.0001$, Fig. 6E), but not in the hippocampus ($t_{(8)} = −0.408$, $P = 0.69$, Fig. 6C). These results suggest that propofol-induced mTNF-α expression in neurons has brain region selectivity. Microglia were detected by Iba-1 positive staining, in which both resting and activated microglia could be recognized. From the previous report, activated microglia were defined according to the morphological criteria (swelling of the cell body with a diameter cutoff more than 7.5 μm and decrease in the distal ramifications, Batchelor et al., 1999). Compared with control, the densities of total microglia in the hippocampus and PFC were increased after propofol anaesthesia ($t_{(8)} = −3.038$, $P = 0.016$; $t_{(8)} = −5.193$, $P = 0.001$, respectively). Similar results were observed for activated microglia in the two regions after propofol anaesthesia ($t_{(8)} = −5.456$, $P = 0.001$; $t_{(8)} = −4.802$, $P = 0.001$, respectively, Fig. 7A and B).

4. Discussion

The mechanisms of developmental neurotoxicity induced by general anesthetics are being extensively investigated nowadays. Although propofol exerts its efficacy of general anesthesia through GABA<sub>A</sub> receptor potentiation, it has been recently reported that GABA<sub>A</sub> receptor is not involved in propofol-induced neuronal apoptosis in the developing brain (Pearn et al., 2012).

The present study demonstrates that multiple propofol exposures induce elevations of TNF-α level in the CNS of neonatal rats, as well as short- or long-term neurotoxicity. In the present study, we only focused on two brain regions, the hippocampus and PFC, because these two regions are particularly vulnerable to anaesthesia-induced neurodegeneration (Jevtović-Todorović et al., 2003; Satomoto et al., 2009; Briner et al., 2011) and appear to be associated with the processing of allocentric and egocentric spatial information in rats (Yoon et al., 2008).

General anesthetics, such as isoflurane, sevoflurane, propofol, and ketamine, can induce neuroinflammation in the developing brain (Wu et al., 2012; Shen et al., 2013; Popić et al., 2015; Zheng et al., 2015). TNF-α plays a very important role in this process and precedes other cytokines in concentration elevations (Wajant
Fig. 4. Etanercept prevented propofol-induced synaptic loss (A, B) Representative images of two-dimensional (red, A) and three-dimensional (3D renderings, B) synaptophysin positive puncta on P9 in hippocampal CA1 and the PFC. Nuclear staining (blue) with DAPI. Scale bar = 10 μm. (C,D) Quantification of synaptophysin positive puncta densities on P9 and P35 in hippocampal CA1 (C) and the PFC (D). ETN: Etanercept. Data are expressed as mean ± SEM, n = 5. *P<0.05, **P<0.01 vs control. #P<0.05 vs ETN. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Etanercept prevented propofol-induced long-term cognitive impairment. (A) Etanercept reduced the increase in the escape latencies induced by propofol. ETN: Etanercept. Data are expressed as mean ± SEM, n = 12. *P<0.05, **P<0.001 vs control. #P<0.05 vs ETN. (B) Etanercept attenuated the decrease in the platform crossing times induced by propofol. Data are expressed as median (interquartile range), n = 12. ***P<0.0001 vs control, #P<0.05 vs ETN.
In the present study, propofol induces TNF-α elevations in the CSF, hippocampus, and PFC. However, an increase in TNF-α may also occur in other brain regions, as a result, the effect of propofol on TNF-α level in other brain regions will need to be studied in the near future. Accumulating studies have demonstrated that TNF-α contributes to the development and progression of neurodegenerative diseases (Lyman et al., 2014). Our study is the first to demonstrate that etanercept, a TNF-α inhibitor, can prevent propofol-induced neurotoxicity in the developing brain, which suggests that TNF-α may mediate propofol-induced developmental neurotoxicity. Additionally, inhibition of TNF-α could become a promising therapeutic strategy.

The role of TNF-α upregulation in neuronal death has been reported in cerebral ischemia and TBI. Propofol-induced developmental neuronal apoptosis occurs via both intrinsic and extrinsic pathways (Yon et al., 2005). TNF-α activates TNF-α receptor type 1 (TNFR1) and causes neuronal apoptosis through the TNFR1-associated death domain (TRADD) and TNFR1-associated factor 2
(TRAF2) recruiting enzymes, caspases-8 is activated as a downstream target and the extrinsic apoptotic pathway can be initiated (Wajant et al., 2003). In addition, TNF-α can induce mitochondrial dysfunction and cytochrome c release in cultured HT-22 cells (Doll et al., 2015), so that the intrinsic pathway may also be involved in TNF-α-induced apoptosis. High TNF-α levels can also inhibit neurogenesis by inducing death of neural precursor cells (NPCs) or inhibiting cell differentiation (Koehane et al., 2010; Bernardino et al., 2008). Together with the pro-apoptotic effect of TNF-α on mature neurons, this may explain the short- and long-term neuronal density reduction in our study. Emerging evidence has shown that neuronal apoptosis is an insufficient contributing factor to long-term cognitive impairment. It was reported that TNF-α could inhibit synaptic plasticity and long-term potentiation (LTP) by activating the p38MAPK signalling pathway in a dose-dependent manner (Trancredii et al., 1992; Cunningham et al., 1996; Butler et al., 2004). Synaptic dysfunction induced by neuroinflammation may in turn cause synaptic loss or neuronal apoptosis, and cognitive impairment may ultimately occur. Furthermore, there are some direct evidences to show that exaggerated TNF-α expression by either genetic or pharmacological interventions is detrimental to the cognitive function (Bjugstad et al., 1998; Matsumoto et al., 2002; Fiore et al., 1996), but the intracellular downstream cascade of TNF-α contributing to propofol-induced neurotoxicity needs to be elucidated in the near future.

Etanercept, a TNF-α inhibitor, specifically binds to and neutralizes TNF-α, and it can attenuate cognitive and behavioural deficits in a variety of models such as TBI and Alzheimer’s disease (AD) (Baratz et al., 2015; Clark et al., 2010). Additionally, a prospective open-label pilot study has demonstrated that AD patients who receive peri-spinal etanercept therapy exhibit cognitive improvements, whereas untreated patients have progressive cognitive decline (Tobinick and Gross, 2008). In the present study, etanercept could prevent propofol-induced neurotoxicity in the developing brain. As etanercept cannot cross the blood-brain barrier, it was administered by intracerebroventricular injection in this study. The dose of etanercept was chosen based on the published data in which 5 μg of etanercept (administered i.c.v.) could relieve LPS-induced anxiety-like behaviour (Camara et al., 2015).

Microglia are the main source of TNF-α in neuroinflammation, but neurons have been reported to produce TNF-α in a murine model of malarial encephalopathy (Medana et al., 1997). Recent studies have demonstrated that inhalation anesthetics can cause cultured cortical neurons to release TNF-α (Wu et al., 2012). In the present study, propofol can cause microglial proliferation and activation, and mTNF-α expression is increased in microglia in both the hippocampus and PFC, while its expression is only increased in neurons in the PFC (and not in the hippocampus) after propofol exposure, this finding suggests that mTNF-α synthesis in neurons is probably brain region-dependent in the developing brain. However, it is unclear whether astrocytes are involved in TNF-α synthesis and release after propofol anaesthesia. The mechanisms by which propofol induces TNF-α synthesis and release from microglia or neurons have not been elucidated till now. P2 × 7 receptor, a purinergic receptor, is predominantly expressed in microglia, and it can be expressed in neurons of the developing brain as well (Anderson and Nedergaard, 2006). The release of TNF-α can be modulated by P2 × 7 receptor, and general anesthetic agents can potentiate or induce P2 × 7-mediated currents in microglia (Liu and Dai, 2009; Nakashii et al., 2007), but whether P2 × 7 receptor in developing brain is involved in propofol-induced TNF-α synthesis and release needs to be corroborated.

We selected two time points (P9 and P35) in the present study, and found that etanercept could provide a protective effect against propofol-induced short-term (P9) or long-term (P35) neurotoxicity. Neuronal apoptosis and synaptic loss were both induced by propofol on P35, the time just before the MWM test, so it is meaningful to study the role of long-term neuronal apoptosis and synaptic loss in long-term cognitive impairment induced by propofol in the near future.

It has been confirmed that poor nutrition in early life can decrease the synapse density and promote cognitive dysfunction (Granados-Rojas et al., 2004; Lister et al., 2005). In the present study, there were no significant differences in the daily weight gain between the pups in each group, and the blood-gas analysis showed that rat pups receiving propofol anaesthesia developed mild respiratory acidosis, but there were no significant differences in pCO2 among the groups. Hence, it is reasonable to rule out the nutritional status and mild respiratory acidosis as contributing factors to the neurotoxicity induced by propofol. Additionally, only male rats were included in the present study and we could not address whether there were any sex-related differences. This is important, as it has been reported that female rats with single postnatal isoflurane exposure have greater deficits in spatial working memory (Murphy and Baxter, 2013); however, a recent study has demonstrated that isoflurane exposure in newborn rats induces long-term cognitive dysfunction in males but not in females (Lee et al., 2014), and it has been reported that there are no sex differences in spatial memory retention performance in the Morris Water Maze test after anesthetic-exposure in neonatal rats (Boscolo et al., 2013).

In conclusion, propofol exposures in neonatal rats induce an increase of TNF-α in the CNS, short- or long-term neuronal apoptosis, neuronal loss, synaptic loss, and long-term cognitive impairment. While etanercept provides neuro protection against propofol-induced developmental neurotoxicity, which suggests that TNF-α may mediate propofol-induced neurotoxicity in the developing brain. Microglia are the main source of TNF-α after propofol anaesthesia, while TNF-α synthesis in neurons is brain-region selective.

Conflict of interest

All authors declared that they have no conflicts of interest.

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