Propofol inhibits proliferation and induces neuroapoptosis of hippocampal neurons in vitro via downregulation of NF-κB p65 and Bcl-2 and upregulation of caspase-3

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Propofol is widely used in paediatric anaesthesia and intensive care unit because of its essentially short-acting anaesthetic effect. Recent data have shown that propofol induced neurotoxicity in developing brain. However, the mechanisms are not extremely clear. To gain a better insight into the toxic effects of propofol on hippocampal neurons, we treated cells at the days in vitro 7 (DIV 7), which were prepared from Sprague–Dawley embryos at the 18th day of gestation, with propofol (0.1–1000 μM) for 3 h. A significant decrease in neuronal proliferation and a remarkable increase in neuroapoptosis were observed in DIV 7 hippocampal neurons as measured by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay and apoptosis assay respectively. Moreover, propofol treatment decreased the nuclear factor kappaB (NF-κB) p65 expression, which was accompanied by a reduction in B-cell lymphoma 2 (Bcl-2) mRNA and protein levels, increased caspase-3 mRNA and activation of caspase-3 protein. These results indicated that downregulation of NF-κB p65 and Bcl-2 were involved in the potential mechanisms of propofol-induced neurotoxicity. This likely led to the caspase-3 activation, triggered apoptosis and inhibited the neuronal growth and proliferation that we have observed in our in vitro systems. Copyright © 2014 John Wiley & Sons, Ltd.

KEY WORDS—propofol; neurotoxicity; proliferation; neuroapoptosis; NF-κB; Bcl-2; caspase-3

INTRODUCTION

Advances in obstetric and paediatric surgery have resulted in an increasing amount of neonates, infants and children undergoing procedures requiring general anaesthesia. It means that millions of infants and young children are exposed annually to various anaesthetics. Although general anaesthetics have been safely used for surgery in adults for decades, growing pieces of evidence¹–³ suggested that general anaesthetic exposure during a period of increased synaptogenesis known as the ‘brain growth spurt’ in immature animals induced widespread neuronal cell death followed by long-term memory and learning abnormalities.

Propofol (2,6-diisopropylphenol), an alkyl phenol derivative that is dissolved in a lipid emulsion, is widely used in paediatric anaesthesia and intensive care practice because of its essentially short-acting anaesthetic effect. As numerous anaesthetics, the exact action mechanism of propofol is not completely known. It is considered that propofol can both interact with γ-aminobutyric acid type A (GABA_A) receptors and N-methyl-D-aspartic acid (NMDA) receptors.⁴,⁵ However, the combined application of GABA-enhancing and NMDA-blocking agents (such as midazolam, nitrous oxide and isoflurane), including alcohol, has been shown to induce widespread neuroapoptosis in the brain of newborn rodents and primates.¹,²,⁶,⁷ Propofol is one of the recently studied anaesthetics for addressing neurotoxicity issues in both animal models and animal-derived cell culture systems. Neuroapoptosis has been recognized as a vital mechanism of propofol-induced neurotoxicity in the developing brain. For instance, propofol (≥50 mg kg⁻¹) exposure significantly induced neuroapoptosis in 5 to 7-day-old mice.⁸ Creeley et al. recently also demonstrated that propofol general anaesthesia administered to pregnant rhesus monkeys induced apoptosis in the brain of foetal monkeys.⁹ In vitro experimental evidence from cultured neonatal animal neurons confirmed the in vivo findings.¹⁰ However, the potential mechanisms of the deleterious effects induced by propofol anaesthesia remain largely obscure.

The hippocampus, a critical centre for learning, memory forming, organizing and storing,¹¹,¹² has been implicated...
in the behavioural and learning impairments of anaesthetics. Therefore, we intend to observe the effect of propofol in the immature hippocampal neurons and to further explore its underlying mechanism. The immature hippocampal neurons were treated with different concentrations of propofol and analysed the cell viability, neuroapoptosis and the expression of nuclear factor kappaB (NF-κB) p65 subunit, B-cell lymphoma 2 (Bcl-2) and caspase-3. We hypothesized that propofol induced neurotoxicity via the NF-κB signalling pathway.

METHODS

Primary culture of hippocampal neurons and the treatment

After obtaining approval from the Animal Care and Use Committee of the Guangxi Medical University, hippocampal tissues for primary cultures were prepared from Sprague–Dawley embryos at the 18th day of gestation as described previously,\(^1\)\(^2\) and we have made some improvement about it. In brief, the pregnant dams were killed, and the foetuses were removed quickly, placed on the ice and decapitated. The hippocampus of the foetal brain was rapidly dissected from the cortex in ice-cold phosphate buffer solution (PBS). Successively, after removing the meningeal tissues, the hippocampus was dissociated mechanically into small 1-mm\(^3\) pieces. Moreover, then, equal volume of 0.25% trypsin solution (Sigma, USA) was added to the dissected tissue and incubated at 37 °C for 15 min, mixing every 5 min. After removing the trypsin solution, 1 ml precooled foetal calf serum (Gibco, USA) was added into the tissue, mixed gently and incubated in a 37 °C water bath for 5 min to stop the digestion. After 5-min centrifugation (1000 rpm), the supernatant was discarded. Cells were washed for three times and resuspended in plating medium [Neurobasal medium (Gibco, USA) supplemented with 10% foetal calf serum, 0.2 mM glutamine, 100 U ml\(^{-1}\) penicillin and 100 U ml\(^{-1}\) streptomycin] and were transferred as 0.5-ml aliquot to a tube that contained 0.5 ml of 4% trypan blue. Cell suspensions were mixed thoroughly, and cell numbers were counted with the microscope. Then, cells were plated onto poly-L-lysine (Sigma, USA)-coated glass coverslips (30-mm diameter) in Petri dishes, and the seeding density was 5 × 10\(^5\) cells per dish. After 6-h incubation, the plating medium was exchanged for maintenance medium [Neurobasal medium with B-27 supplement (Gibco, USA), 0.2 mM glutamine, 100 U ml\(^{-1}\) penicillin and 100 U ml\(^{-1}\) streptomycin] at 37 °C, 5% CO\(_2\) in a humidified incubator for 6–8 days to reach confluence. Cells were fed every 3 days by replacing half of the medium.

Seven days later, cells were divided into the untreated control group, intralipid group and propofol groups. Subsequently, cells were incubated alone as control or with different concentrations of propofol (Disoprivan; Astra Zeneca, Italy) or 20% intralipid (Baxter, Guangzhou, China) for 3 h. Experimental pieces of evidence from in vitro and in vivo demonstrated that propofol could induce neurotoxicity when administered at low dose,\(^1\)\(^4\)\(^–\)\(^6\) but another study showed that only high doses and/or administration for prolonged periods caused neurotoxicity.\(^1\)\(^7\) Thus, in this study, we treated cells with propofol at the concentrations of 0 (control), 0.1, 1, 10, 100 or 1000 μM, or with an intralipid vehicle. The cell viability was checked by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The apoptotic cells were also detected by apoptosis assay. The NF-κB p65, Bcl-2 and caspase-3 levels were analysed by reverse transcription PCR (RT-PCR) and Western blot after propofol exposure.

Cell viability and proliferation assay

The MTT assay was used to determine the viability of hippocampal neurons. This assay is based on the reduction of MTT (Sigma, USA) to a blue formazan product. Briefly, the cells were plated onto 96-well plates coated with poly-D-lysine at a density of 1 × 10\(^5\) cells per well and treated as described previously. Control cells were kept in Neurobasal medium. There were six parallel samples for each group. After incubation for 12, 24, 36, 48, 60 and 72 h, 20 μl MTT was added to each well, followed by incubation at 5% CO\(_2\) and 37 °C. After 4 h, the supernatant was discarded, and each well was exposed to 200 μl of dimethyl sulfoxide to solubilize the formazan product. After 10-min oscillation, the optical density of each sample was determined at a wavelength of 490 nm (A\(_{490}\)) by a 96-well OpsyS MR™ microplate reader (Thermo Labsystems, Chantilly, VA, USA) and Revelation™ QuickLink software. The blank control wells with medium only were set as zero absorbance controls. The percentage of cell survival was calculated using the background-corrected absorbance: % cell proliferation=A\(_{490}\) of experimental well/A\(_{490}\) of untreated control well ×100%. The growth curves of hippocampal neurons in each group [A\(_{490}\) versus time (h)] were then plotted. All experiments were performed for at least three times.

Apoptosis assay by flow cytometry

Cells were treated as described previously. Cell death was further analysed by double staining with FITC-labelled annexin V and propidium iodide (PI) using the annexin V-FITC apoptosis detection kit (Jingmei Biotech Co., Shenzhen, China). Both floating and aberrant cells were collected and plated onto a six-well plate at 1 × 10\(^5\) cells per well. Cells were washed with PBS and collected by trypsinization. After centrifugation for 5 min at 4 °C, cells were resuspended and washed with PBS to test tubes, in which 5 μl of PI were added, and gently vortexed before incubation for 30 min at 4 °C in the dark. After adding PI to each tube for 10 min, the cells were analysed by flow cytometry using the FACSCalibur system (BD Biosciences, CA, USA). The annexin V-FITC-negative/PI-negative population was considered to include all normal healthy cells. Annexin V-FITC-positive/PI-negative cells were regarded as a measure of early apoptosis. Annexin V-FITC-positive/PI-positive population was considered to represent late
apoptotic or necrotic cells, and annexin V-FITC-negative/PI-positive cells were considered to include necrotic cells.

Quantitative real-time RT-PCR analysis
Total cellular RNA was isolated from the days in vitro 7 (DIV 7) primary hippocampal neurons in different groups using TRIzol Reagent (Invitrogen, USA) as recommended by the manufacturer’s instructions. RNA was reverse transcribed to single-strand complementary DNA (cDNA) using a RevertAid First Strand cDNA Synthesis Kit (Fermentas International, Inc., Canada) according to the manufacturer’s instructions. Reverse transcription was carried out at a final volume of 20 μl comprised of 2 μg RNA, 1-μl random primer and diethylpyrocarbonate-treated water added to the sample to a volume of 12 μl, incubated at 70 °C for 5 min and chilled on ice. Then, 4 μl of 5x reaction buffer, 1 μl of RiboLock™ Ribonuclease, 2 μl of 10 mM deoxynucleotide triphosphate mix and 1 μl of RevertAid™ M-MuLV Reverse Transcriptase were added. The reaction was allowed to proceed at 25 °C for 5 min and 42 °C for 60 min and then heated at 70 °C for 10 min. Quantitative real-time PCR was conducted with a PTC 200 real-time PCR reactor (MJ Research, Fremont, CA, USA) for SYBR green PCR master mix. Individual primers were as follows: Bcl-2, forward 5′-GCTACGAGTGGGATACGTGAGA-3′ and reverse 5′-GAACCTCAAGAAGGCGACAATC-3′; caspase-3, forward 5′-ATGTGAGATCGACTAAACCTCA-3′ and reverse 5′-TGCTCTATACCGAGTGCCAG-3′ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-ACAGCACAAGGTGTGGGAC-3′ and reverse 5′-TTTGAGGTTGCGAGCAGCTT-3′. The PCR conditions were 94 °C for 3 min, 94 °C for 45 s, 57.8 °C (Bcl-2) or 62.4 °C (caspase-3) for 1 min and 72 °C for 30 s, for a total of 35 cycles, with a final extension for 10 min at 72 °C. After PCR reaction, 4.5 μl of the amplified product was electrophoresed in 1.5% agarose gelatin gels buffered with 1× Tris/borate/EDTA. Relative gene expression quantifications were calculated according to the comparative Ct method using GAPDH as an internal standard. In all cases, each PCR trail was performed with triplicate samples and repeated for least three times.

Western blot analysis
To analyse variation in protein levels, cellular proteins were extracted from the DIV 7 primary hippocampal neurons in different groups. At the end of incubation, cells were rinsed with ice-cold PBS and lysed in 150 μl radioimmunoprecipitation assay buffer (Beyotime Biotech Co., Shanghai, China) with a 1% protease inhibitor cocktail. The cell lysates were cleared by centrifugation at 12,000 rpm for 30 min at 4 °C. The supernatant fraction was stored at −80 °C and used for the protein determination of NF-kB p65, Bcl-2, activated caspase-3 and GAPDH.

After protein concentration in the supernatant fluid of the lysate was measured by the bicinchoninic acid protein assay (Beyotime Biotech Co., Shanghai, China), equal amounts of proteins were loaded onto 12% sodium dodecyl sulphate PAGE. The gels were transferred to polyvinylidene fluoride microporous membranes (0.22-μm pore size, Millipore, Bedford, USA) followed by electrophoretic separation. Membranes containing the transferred proteins were blocked with 5% skim milk in TBS containing 0.1% Tween-20 (TBST) for 2 h at room temperature (RT) and then incubated with a dilution of primary antibody, i.e. anti-p65 antibody (1:200; Santa Cruz Biotechnology, Inc., USA), anti-Bcl-2 antibody (1:1000; Cell Signaling Technology, USA) or anti-activated-caspase-3 antibody (1:800; Cell Signaling Technology, USA) overnight at 4 °C. The membranes were washed thoroughly with TBST for three times and incubated with horseradish-peroxidase-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, Inc., USA) for 30 min at RT. Finally, the blots were washed, and immunolabelling was detected with a chemiluminescence detection system (Pierce, USA) and visualized on an X-ray film. GAPDH (1:10000; Santa Cruz Biotechnology, Inc., USA) was used as the loading control.

Statistical analysis
Data were expressed and graphed as mean±standard error of the mean analysed by SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA) and Origin version 7.5 software programmes (OriginLab Co., Northampton, MA, USA). Multiple comparisons were performed with a one-way analysis of variance (ANOVA), followed by Dunnett’s post hoc test as appropriate. Differences in the MTT cell viability were analysed with two-way ANOVA tests followed by a post hoc test. P values <0.05 were considered to be significant.

RESULTS
Propofol inhibited hippocampal neuron growth and proliferation
To determine whether propofol regulates the proliferation of hippocampal neurons, we examined the effects of propofol on cell viability by an MTT analysis as described previously. As shown in the cell viability curves (Figure 1A), control cells and 20%-intralipid-treated cells proliferated over the observed time periods. In contrast, cell viability was reduced in cells treated with propofol at all tested concentrations (0.1–1000 μM). A two-way ANOVA with group and time as factors yielded a significant effect of group (F= 79.806, P < 0.001), time (F= 50.037; P < 0.001) and the interaction (F=12.252, P < 0.001). Moreover, we noticed that cells treated with higher concentration of propofol (1000 μM) proliferated less. The reduction in cell viability was observed starting from 12 h after treatment and became more pronounced as treatment time was extended. Post hoc Tukey contrasts (P < 0.001) indicated that the 1000-μM-propofol group was significantly different from the others. This result indicated that propofol at high concentrations has been shown to have a strongly negative effect on neuron viability. Control cells exhibited about 1.37-fold, 1.51-fold, 1.65-fold, 1.68-fold and 4.33-fold (P = 0.014, P = 0.001,
Figure 1. The effects of propofol on cell survival as assessed by the MTT assay. (A) The growth curves of developing rat hippocampal neurons (DIV 7) in the control group, intralipid group and propofol groups. Cells were treated with various concentrations (0.1–1000 μM) of propofol for 3 h. At 12, 24, 36, 48, 60 and 72 h after treatment, cell viability was detected by the MTT assay. The relative cell viability was calculated using control (culture with complete medium) as a normalizer. The growth curves showed that propofol groups have lower cell viability compared with the control group (two-way ANOVA, effect of group, F = 79.806, P < 0.001; time, F = 50.037, P < 0.001 and the interaction, F = 12.353, P < 0.001). (B) The mean cell proliferation rates of hippocampal neurons in propofol groups were significantly lower compared with those in the control group and intralipid group. *P < 0.05, **P < 0.01, ***P < 0.001 versus the control group. Mean ± SD (n = 5)

P = 0.001, P = 0.003 and P < 0.001 respectively) higher mean proliferation rates than propofol-treated cells (Figure 1B). We also observed that cells in propofol groups grew slower than control cells and 20%-intralipid-treated cells, consistent with the concentration of propofol and indicative of a suppressive effect of propofol on hippocampal neuron growth and survival.

Propofol induced neuroapoptosis in hippocampal neurons

Next, we studied whether propofol induced rat DIV 7 hippocampal neuron apoptosis. Cells were treated with different concentrations of propofol as indicated. After 3 h, cells were stained with annexin V/FITC and PI before flow cytometric analysis. The dual-parameter fluorescent dot plots (Figure 2A) showed that the viable cells were in the lower left quadrant, and the apoptotic cells were in the right quadrant. As shown in Figure 2B, no significant difference was observed in cell apoptosis between intralipid-treated cells (39.52 ± 2.66%) and untreated control cells (35.59 ± 2.86%; P = 0.055). By contrast, propofol treatment starting at 0.1 μM induced cell apoptosis, doubling that of untreated cells. The total percent of apoptotic neurons was directly related to propofol concentration. When treated with 0.1–1000 μM propofol, cell apoptotic rate reached 62.29 ± 2.95, 66.37 ± 3.86, 70.61 ± 4.91, 71.14 ± 4.97 and 82.93 ± 4.94% respectively (P < 0.001, all). Consistent with the proliferation assay, the results revealed that propofol induced neuroapoptosis.

Propofol downregulated NF-κB p65 and Bcl-2 and upregulated caspase-3

To investigate whether propofol acts on the NF-κB signaling pathway, NF-κB p65 from hippocampal neurons was analysed by Western blotting using antibodies against NF-κB p65 (Figure 4A). Western blot analysis revealed that NF-κB p65 levels, but not the other subunits of NF-κB, were lower in propofol-treated cells compared with those in control cells and 20%-intralipid-treated cells (P = 0.002, P = 0.047, P < 0.001, P < 0.001, P < 0.001 respectively; Figure 4B). These results suggested that propofol was specifically associated with lower levels of p65, which is a subunit of multifunctional transcription factor NF-κB that regulates the expression of genes involved in numerous normal cellular activities including cell survival and apoptosis.18,19

In the subsequent experiments, the expression of two apoptosis-related factors, Bcl-2 and caspase-3, was examined. Bcl-2, a downstream gene in the NF-κB signalling pathway, is an anti-apoptotic gene that plays a key role in promoting cell survival,20 while caspase-3 is proapoptotic and is considered to be an ‘executioner’ caspase that cleaves a large number of substrates during apoptosis as an indicator of apoptotic cells.21 Consistent with the loss in cell viability and NF-κB p65, levels of Bcl-2 were significantly reduced by 69.4, 66.2, 67.4, 60.6 and 63.7% in mRNA (P < 0.001, all; Figure 3A and 3B) and 50.7, 57.3, 64, 33.3 and 29.3% by 69.4, 66.2, 67.4, 60.6 and 63.7% in mRNA (P = 0.04, P < 0.001, P = 0.01, P < 0.001, P < 0.001 respectively; Figure 3C and 3D). Subsequently, we detected the expression of activated caspase-3 proteins (Figure 4E); the outcome indicated that propofol induced caspase-3 activation as evidenced by production of an increase of active fragments (17 kDa). More specifically, compared with untreated control, primary hippocampal neurons treated for 3 h with 0.1–1000 μM of propofol displayed a significant 1.54-fold, 1.71-fold, 1.86-fold, 2-fold and 1.88-fold (P = 0.041, P = 0.028, P < 0.001, P < 0.001, P < 0.001 respectively; Figure 4F) increase of activated caspase-3.
Figure 2. Percentages of apoptotic cells analysed by flow cytometry. (A) Developing rat hippocampal neurons (DIV 7) were exposed to propofol (0.1–1000 μM) and assayed for percentage of cells induced into apoptosis via annexin V/PI flow cytometry. Data represent the results of at least three independent experiments. The dual-parameter fluorescent dot plots reflect the viable neurons and the apoptotic neurons. An apoptotic fraction is seen in the lower right quadrant by fluorescence-activated cell sorting analysis. (B) Following treatment with propofol, a greater percentage of hippocampal neurons were induced into apoptosis compared with untreated cells. The total apoptotic rates in propofol groups were significantly higher than those in the control group and intralipid group. *P < 0.05, **P < 0.01, ***P < 0.001 versus the control group. Mean ± SD (n = 5).
levels respectively. No such changes were observed between the untreated control group and intralipid group ($P = 0.875$).

**DISCUSSION**

In this study, we investigated the toxic effects of propofol on the hippocampal neurons from embryos at the 18th day of gestation. Our major finding is that a 3-h-long exposure of immature hippocampal neurons to propofol, at a wide range of concentrations (0.1–1000 μM), triggered a robust neuroapoptosis, which may contribute to inhibit cell growth and proliferation at an *in vitro* developmental age (DIV 7). On the basis of RT-PCR and Western blot data, we showed that propofol decreased NF-κB p65 subunit expression, which was accompanied by a reduction in Bcl-2 mRNA and protein levels, increased caspase-3 mRNA and activation of caspase-3 protein. Therefore, we assumed that all of them contributed to neuroapoptosis that was observed by the apoptosis assay following propofol treatment in our study. It means that propofol-induced neurotoxicity is probably involved in the NF-κB signalling pathway.

To study propofol-induced neurotoxicity, we mimicked developmental principles to obtain hippocampal neurons of rats that were similar to human neurons at morphological and structural levels. We then used multiple methods to analyse multiparametric indicators of neurotoxicity inferring mechanisms of cell death and covering a wide spectrum of cytopathological changes including cell viability, apoptosis and related factor production. The data from MTT and apoptosis assays revealed that significant inhibition in neuronal growth and proliferation, and a remarkable increase in apoptotic neurons was observed with propofol at the concentrations of 0.1–1000 μM. These findings are similar to another report recently in which propofol has been shown to suppress proliferation of Eca-109 cells in part by promoting apoptosis.22 Additionally, 5–100 μM propofol was found to inhibit neural stem cell growth and induced cell apoptosis of E14–16 embryos in a dose-dependent manner, which is in agreement with the results from *in vivo* studies10. For instance, administration of propofol (25–300 mg·kg$^{-1}$) triggers a robust neuroapoptosis response in the infant mouse brain, and the minimal effective dose for causing a significant response was 50 mg·kg$^{-1}$.8 There is also evidence25 that 2 h of prenatal (gestational day 18) propofol exposure led to apoptosis and neuron deletion in the offspring rat in CA1 and CA3 regions of the hippocampus, as well as induced neurotoxicity and persistent learning deficit in the offspring.

Several possible molecular pathways have been shown to be involved in the apoptosis that was induced by propofol in the developing brain,15,24–26 but the mechanistic details have yet to be established. NF-κB, a ubiquitous nuclear transcription factor, is widely known for its pivotal roles in cell death, cell division and survival pathways.18,19 Similar to other organ systems, functional NF-κB complexes existed in essentially all kinds of cells in the central nervous system (CNS), covering neurons, astrocytes, microglia, and oligodendrocytes.27–30 In vertebrates, the NF-κB family is comprised of five members, namely, RelA (also known as p65), RelB, c-Rel, p50 and p52. They exist in various combinations of homodimers and heterodimers, and their expression and regulation in the hippocampal neurons may contribute to learning-associated synaptic reorganization and memory formation.31–33 The predominant transcriptionally active form of NF-κB in the nervous system is the p65/p50 heterodimer, of which the RelA or p65 subunit is indispensable for cell survival.

Recently, pieces of evidence are emerging that blocking of NMDA receptors may suppress the NF-κB signalling and lead to neurotoxicity in the hippocampus. For example, previous research implied that NMDA receptor antagonist MK-801 could reverse the increased NF-κB p65 expression on the contralateral hippocampus in constriction sciatic nerve injury rats.34 Moreover, MK-801 (1 μM) pretreatment completely blocked NF-κB DNA binding activity in cerebellar neurons *in vitro*.35 The mechanism by which propofol affects CNS has been largely unclear now, but several studies have indicated that propofol have both NMDA receptor blocking and GABA$_A$ receptor enhancing properties.4–6 Similarly, it means that propofol can also interfere with the NF-κB p65 expression and NF-κB activity. In the present study, we confirmed that propofol downregulated NF-κB p65 expression, and we deduced that, at least to a certain degree, it inhibited transcriptional activation of NF-κB. Moreover, the decrease of NF-κB p65 that modulates NF-κB activity may be the factor, at least in part, contributing for propofol triggering the apoptotic cascade in the immature hippocampal neurons.36 Our results are in accordance with the findings from Hsing, who found a significant reduction of NF-κB p65 in RAW264.7 murine macrophages in an *in vitro* model after propofol exposure.37

As a transcription factor, NF-κB is known to influence the expression of a broad array of genes. Our data showed that the decrease in NF-κB p65 was accompanied by remarkable attenuation in Bcl-2 mRNA and protein, while caspase-3 mRNA was significantly increased in the hippocampal neurons. To further assess the role of caspase-3 in the cascade triggered by the propofol exposure, we measured the appearance of active caspase-3 fragments. Propofol application also increased the release of 17-kDa caspase-3 fragments in the immature hippocampal neurons, and the minimum concentration was 0.1 μM.

Bcl-2, which acts downstream of the NF-κB signalling pathway, is widely used to evaluate cell survival following an apoptotic stimulus. Early indications suggest that in embryonic neurons, NF-κB translocation stimulated by tumour NF alpha promotes neuron survival,38,39 possibly by inducing the expression of anti-apoptotic proteins such as Bcl-2 and Bcl-x.40 The important contribution of Bcl-2 to apoptotic processes in hippocampal neurons is now especially well documented.41 Moreover, several studies have linked the reduction of Bcl-2 expression to the neuronal damage in the CNS after exposure to anaesthetics.42–44 Apoptosis is strictly controlled by multiple gene families, including not only the Bcl-2 but also the caspase families. Bcl-2 is an upstream inhibitor of caspase-3; it can migrate from the cytoplasm to mitochondria, which are distributed in a manner that is consistent
with mitochondrial release of cytochrome c and caspase.\textsuperscript{45} In vitro data showed that knockdown of Bcl-2 induced caspase-3 cleavage upregulated in Jurkat T cells.\textsuperscript{46} Conversely, the activation of caspase-3 is considered the terminal event preceding cell death that plays an extremely important role in neuronal apoptosis. While the involvement of caspase-3 in propofol-induced cell death in the immature neurons (DIV 4–7) was demonstrated in two studies,\textsuperscript{14,15} a series of animal experiments also have confirmed that propofol may trigger caspase-3 activation in the hippocampus of developing brain and led to a widely hippocampal neuron apoptosis,\textsuperscript{16,22} these damages may finally cause persistent learning/memory impairment.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.
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