

# Hippocampal Neuron Protecting Effect of Propofol Against Hypoxia/Reoxygenation via Inducing Nerve Growth Factor

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## Abstract

Propofol, an intravenous anesthetic agent, exhibits neuroprotective effects against cerebral ischemia–reperfusion injury. Here, we used a model of Hypoxia/Re-Oxygenation (H/R) injury to examine the hippocampal neuroprotective effect of propofol, and explored the role of Nerve Growth Factor (NGF) and NGF receptor TrkA in this action. Rat hippocampal neuron cells were subjected to H/R with different concentrations propofol, the viability and apoptosis of the cells were then determined by MTT assay and annexin V flow cytometry, respectively. Meanwhile, expression of NGF and TrkA were measured by RT-PCR and Western blot. The results showed that H/R significantly reduced viability and increased apoptosis of cultured hippocampal neuron cells, along with the significantly decreased expressions of NGF and TrkA. However, pretreatment of propofol recovered the expressions of NGF and the receptor TrkA, resulting in significantly decreased H/R-induced neurotoxicity. C-Jun N-terminal kinase (JNK) inhibitor suppressed the effect of propofol on the NGF expression, and the TrkA was decreased by PD98059, the Erk1/2 signal blocker. Administration of TrkA inhibitor altered the neuroprotective effect of propofol. These findings suggested the potential of propofol for protecting hippocampal neuron against H/R through at least partly, NGF/TrkA signaling pathway.

**Keywords:** Propofol; NGF; TrkA; Hypoxia/Reoxygenation; Hippocampal neuron

## Introduction

Propofol (2, 6-diisopropylphenol) is a short-acting, intravenously administered hypnotic agent commonly used in anesthesia and intensive care. Besides its classical anesthetic effect, propofol showed neuroprotective effects by attenuating caspase activation and thus apoptosis [1,2]. Moreover, Propofol was shown to attenuate the death and to promote neurogenesis of hippocampal neuron after oxygen deprivation or cerebral ischemia [3,4]. However, the detailed mechanisms remain obscure.

Nerve Growth Factor (NGF) is an evolutionarily conserved polypeptide neurotrophin, which plays a crucial role in sympathetic and sensory nervous systems [5]. Recent studies indicated that NGF prevents injury of cultured hippocampal and cortical neurons exposed to various insults [6,7]. NGF is known to bind TrkA receptor, which is a member of a large tyrosine kinase receptor family [8]. The binding of NGF to TrkA receptors induces their dimerization, followed by autophosphorylation of tyrosine residues within the intracellular kinase domain, which leads to the activation of signaling pathways such as the Phosphatidylinositol 3-Kinase (PI3K)/Akt and Mitogen-Activated Protein Kinase (MAPK)/ERK pathways and play a crucial role in regulating various processes in the brain, including neuronal proliferation, differentiation, development, migration, survival and long-term synaptic plasticity [9].

In this study, we hypothesized that propofol may protect hippocampal neurons against Hypoxia/Re-Oxygenation (H/R) by modulating the expression of NGF and TrkA receptor, and further explored the role of NGF and TrkA receptor in this action and the possible pathways involved.

## Materials and Methods

### Materials

Propofol were purchased from AstraZeneca Pharmaceutical, (Liaoning, China), MEK (MAPK/ERK) inhibitor PD98059, JNK (Jun

N-terminal kinase) inhibitor SP600125, and p38 MAPK inhibitor SB202190 were purchased from Calbiochem (San Diego, CA, USA). Highly selective TrkA inhibitor sc-311553 was purchased from Santa Cruz Biotech. Inc. (Dallas, TX, USA). Other reagents were of reagent grade and were used without further purification.

### Cell culture

Rat brain hippocampal neuronal cells (R-Hi-501) were purchased from Lonza Walkersville, Inc. (Walkersville, MD, USA). Cells were cultured in primary neuron basal medium containing 2 mM L-glutamine, 50 µg/ml gentamicin, 37 ng/ml amphotericin, and 2% neural survival factor (NSF)-1, at 37°C in an atmosphere of 5% CO<sub>2</sub>/95% air.

### Experimental design

Cells were assigned to followed groups: untreated control group, H/R group, in which cells were treated with 300 µM CoCl<sub>2</sub> (chemical anoxia analogue) for 1 hour, followed by normal medium for 24 hours; propofol groups, which the neurons were pretreated with different concentrations (10–100 µM) of propofol for 1 hour, then were treated the same as group CoCl<sub>2</sub>. Propofol was dissolved in intralipid (Yuheng

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Pharmic Company, Leshan, China), and intralipid was used as a vehicle agent for propofol.

### MTT assay

Hippocampal neurons cells were seeded in 96-well culture plates (3,000 cells/well). After overnight incubation, cells were pretreated with propofol (10-100  $\mu$ M) before H/R, followed by the MTT assay according to the manufacturer's instruction. The viability of the cells was expressed as the fraction of surviving cells relative to untreated controls. In some experiments, SP600125, PD98059, SB202190 or sc-311553 was incubated with hippocampal neuron cells for 24 h, and the cytotoxicity of each compound was examined by MTT assay.

### In vitro apoptosis assay

Hippocampal neurons plated in 12-well plates ( $1.0 \times 10^5$ /well) were subjected to each treatment and/or H/R, then apoptosis of hippocampal neurons after H/R was determined by a flow cytometric assay with annexin V, by using the Annexin V-FITC Apoptosis Detection kit (BD PharMingen, San Diego, CA).

### Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of NGF mRNA and TrkA mRNA expression

Total RNA from hippocampal neurons was extracted by using Trizol reagent (Invitrogen Co., Carlsbad, CA). Two microliters of total RNA was used for reverse transcriptase polymerase chain reaction (RT-PCR). The primers used for each mRNA were as follows: NGF: sense, 5'-GGCAGTGTCAAGGGAATGCGAAGTT-3'; antisense, 5'-CCAAGGGAGCAGCTTCTATCCTGG-3'; TrkA: sense, GGT ACC AGC TCT CCA ACA CTG AGG; antisense, CCA GA ACG TCC AGG TAA CTC GGT G; GAPDH was used as inner standard mRNA. The cycling parameters were as follows, 30 PCR cycles of 1 min for denaturing at 94°C, 1 min for annealing at 56–58°C, and 1 min for DNA synthesis at 72°C. PCR products then underwent electrophoresis on 1% agarose gels.

### Western blot analysis of NGF and TrkA protein levels

After each treatment and/or H/R, hippocampal neurons were harvested and centrifuged at 3,000 rpm for 5 min. The cell pellets were then homogenized with ice-cold homogenate buffer (20 mmol/l Tris-HCl, pH 7.4 plus 3 mg/ml PMSF and 3 mmol/l EDTA), and centrifuged at 10,000 rpm for 15 min at 4°C. The proteins in the supernatant were separated by electrophoresis with 12% SDS-polyacrylamide gels and transferred to immobilon polyvinylidene difluoride membranes (Millipore Co. Ltd., Bedford, MA). The following antibodies were used in this study: a rabbit polyclonal anti-NGF antibody and a rabbit polyclonal anti-TrkA antibody (Santa Cruz Biotech. Inc.), and a subsequent second antibody of goat anti-rat IgG (Beijing Zhongshan Biotech Co., Beijing, China). The protein band that reacted immunologically with the antibody was visualized by using the enhanced chemiluminescence system (ECL; Amersham Biosciences, Buck, UK).

In some experiments, 10 nM of TrkA inhibitor sc-311553 was used to elucidate the inducing pathway of TrkA.

### Statistical analysis

All data are presented as means  $\pm$  SD. Data were analyzed by one-way ANOVA followed by the Bonferroni t-test. The difference was considered statistically significant at  $p < 0.05$ .

### Results

### Propofol increased the hippocampal neurons viability and decreased the apoptosis induced by H/R

As shown in Figure 1, H/R induced a decrease of hippocampal neurons viability, and an increase of apoptosis. However, the cell death induced by H/R was greatly inhibited by pretreatment with propofol; at the dose of 50  $\mu$ M propofol almost totally rescued the cells from H/R-induced cell death, though no significant protective effect was found at lower doses (Figure 1). Nevertheless, when dose was increased to 100  $\mu$ M, cell viability decreased and apoptosis increased significantly (Figure 1 and unshown data), suggesting propofol itself maybe toxic at high dose (e.g., > 100  $\mu$ M). The dose of 50  $\mu$ M was thus used in the following experiments. In addition, we did not find apparent protective effect by use of intralipid, suggesting the protective effect of is solely from propofol.

### Propofol pretreatment up-regulates the expression of NGF and TrkA mRNA and protein levels of NGF and TrkA by differential intracellular signaling pathways

Under the circumstance of H/R, expressions of NGF and TrkA were significantly decreased (Figure 2) which is positively related with the decrease of cell viability and increase of apoptosis (Figure 1). However, similar to the results showed in Figure 1A, pretreatment of propofol recovered the expression of NGF and TrkA (Figure 2).

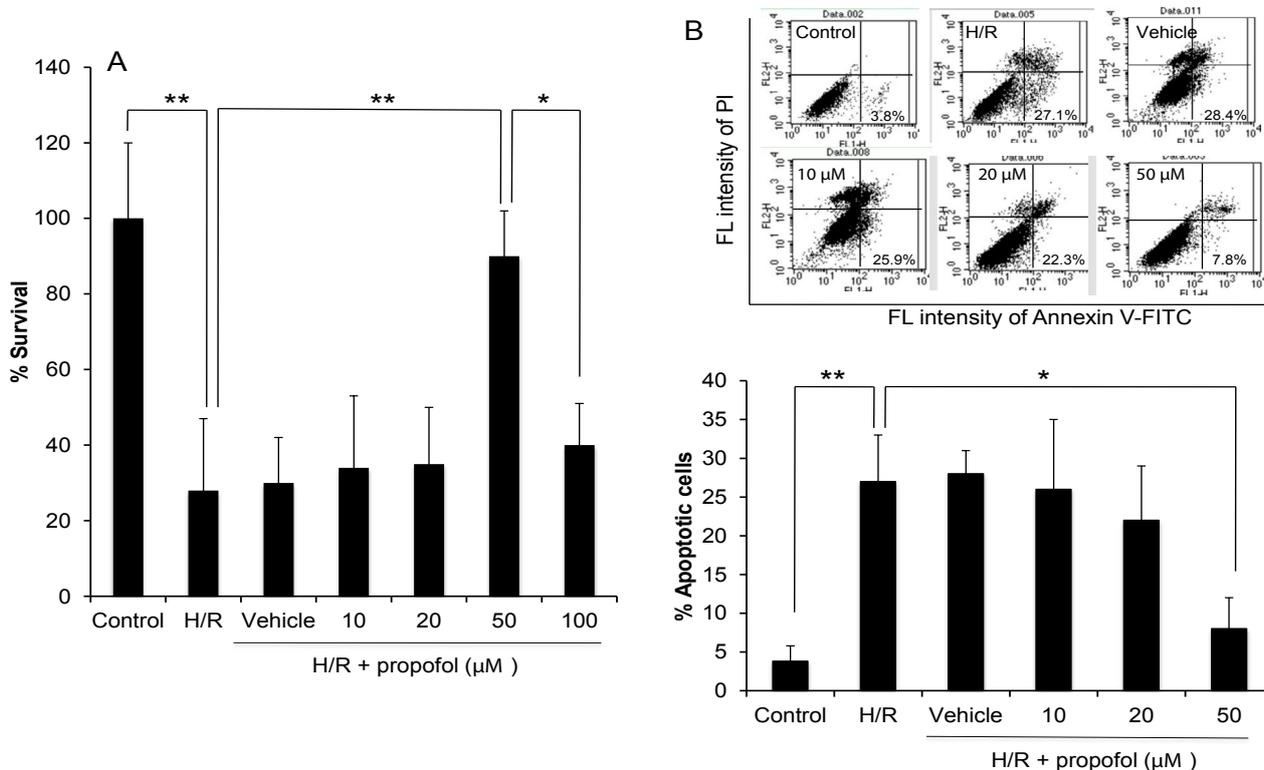
To further investigate the intracellular signaling pathways, cells were stimulated with 10  $\mu$ M of PD98059, 30  $\mu$ M of SP600125, or 30  $\mu$ M of SB202190 for 1h before treated with 50  $\mu$ M propofol. All these compounds alone did not show apparent effect on cell viability (Supplemental data Figure S1). However, expression of NGF protein in hippocampal neurons induced by propofol was markedly blocked by JNK inhibitor SP600125, whereas neither PD98059 nor SB202190 exhibited this activity (Figure 3A). Moreover, the expression of TrkA protein was blocked by PD98059 (Erk1/2 inhibitor) and SP600125, but not SB202190 (Figure 3B). In parallel, the viability of hippocampal neurons decreased significantly when SP600125 and PD98059 were added to the cells (Figure 3C).

### Propofol protect hippocampal neuronal subjected to H/R through NGF/TrkA signaling

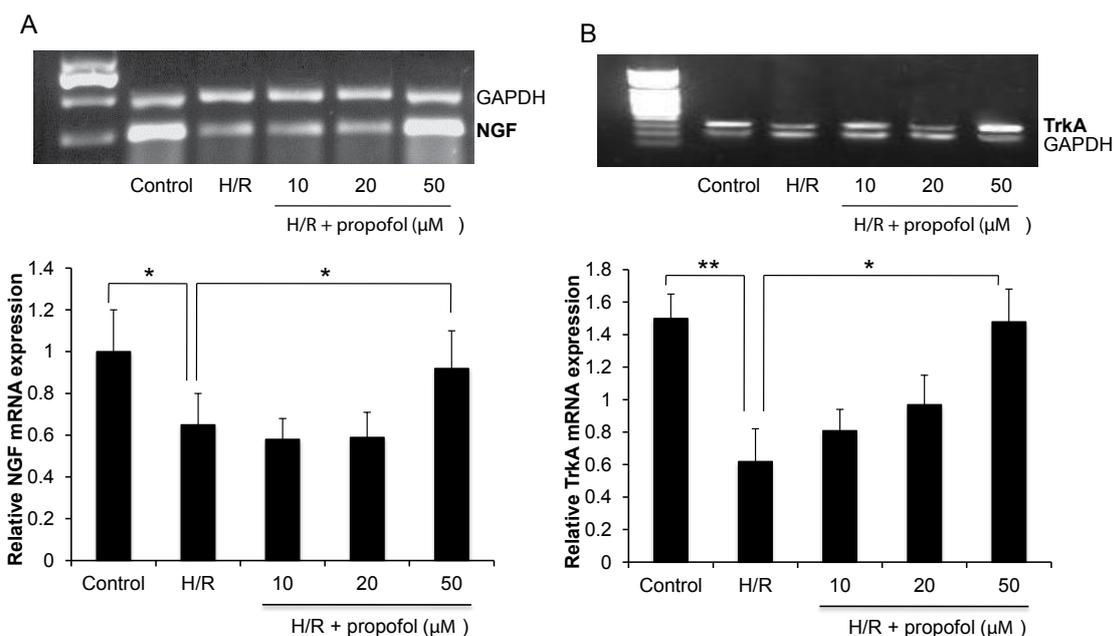
As showed in Figure 4, in the presence of TrkA inhibitor sc-311553, expression of TrkA greatly decreased (Figure 4A), and consequently the neuroprotective effect of propofol against H/R induced injury in hippocampal neuronal was also significantly nullified (Figure 4B). In addition, sc-311553 itself did not show cytotoxicity to the cells (Supplemental data Figure S1).

### Discussion

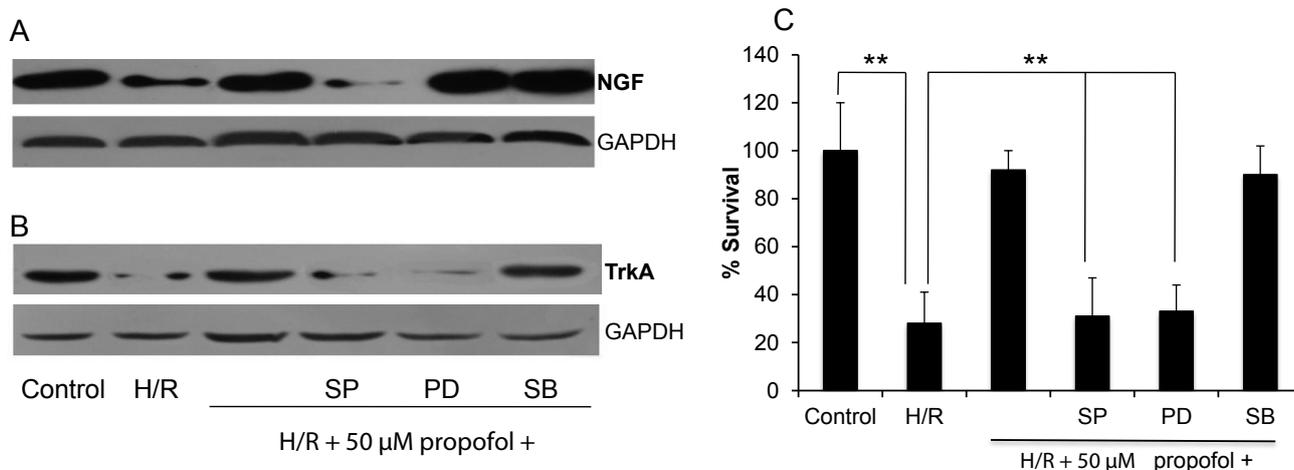
The major finding of this study is that propofol pretreatment at concentration of 50  $\mu$ M exerted remarkable protective activity on hippocampal neurons against H/R-induced cell injury (Figure 1), probably through NGF/TrkA signaling pathway as propofol significantly prevented H/R-induced decrease in NGF and TrkA (Figure 2). Namely, propofol induced NGF expression as a consequence of the JNK signal transduction pathway because the JNK-specific inhibitor suppressed the expression of NGF induced by propofol, whereas it triggered the activation of TrkA probably via PI3K/Akt pathway because the finding that this function was blocked by the Erk1/2 inhibitor (Figure 3). We further confirmed that NGF/TrkA signaling is involved, at least partly, in the regulation of neuroprotection by propofol because in the presence of TrkA inhibitor sc-311553, expression of TrkA greatly decreased,



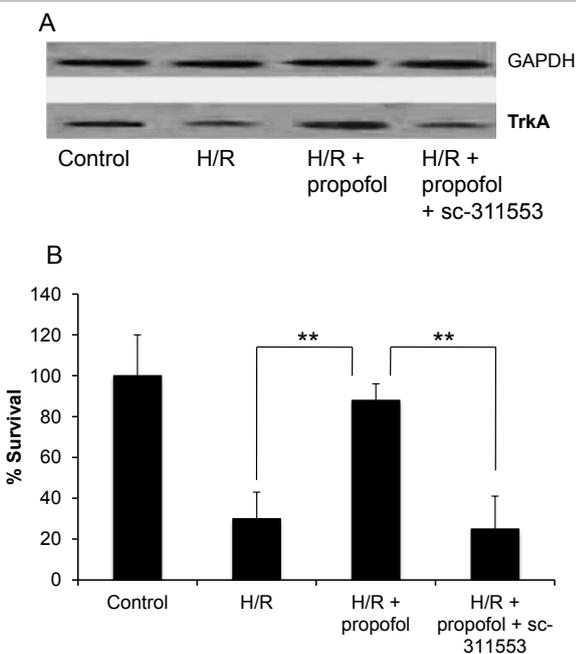
**Figure 1:** H/R-induced cytotoxicity on hippocampal neurons and the protective effect of propofol. Cell viability (A) was measured by MTT assay, and apoptosis was determined by a flow cytometric assay with FITC-annexin V in which the cells with high FITC fluorescence as shown in the right quad gate in the histogram were considered apoptotic cells (B). Intralipid of 10% in which propofol was dissolved, was used as the vehicle. Values are means ± SD. \* p < 0.05, \*\* p < 0.01.



**Figure 2:** Induction of NGF and TrkA expression in hippocampal neurons subjected to H/R by propofol. Propofol (50 μM) significantly increased the NGF (A) and TrkA expression (B) after H/R. Data are means ± SD. \* p < 0.05, \*\* p < 0.01.



**Figure 3:** Effect of JNK inhibitor SP600125, Erk1/2 inhibitor PD98059, and the p38MAPK inhibitor SB202190 on NGF and TrkA expression (A, B), and their influence on the protective effect of propofol against neuron H/R injury (C). Cells were treated with the above inhibitors in the presence of propofol (50 μM) in H/R model, after which the cells were subjected to Western blot. The viability of cells upon above mentioned treatment was measured by MTT assay. \*\*  $p < 0.01$ .



**Figure 4:** The effect of TrkA inhibitor sc-311553 on TrkA protein expression (A) and propofol induced neuroprotection (B). Treatment protocol is same as that in Figure 3. Data are presented as means  $\pm$  SD. \*\*  $p < 0.01$ .

and consequently the neuroprotective effect of propofol against H/R induced injury in hippocampal neurons was also significantly nullified (Figure 4).

Animal models are commonly used to study brain ischemia/reperfusion injury. However, in this study, we utilized an *in vitro* model, by inducing the cell hypoxia with  $\text{CoCl}_2$ , to investigate the cerebral protection at the cell level.  $\text{CoCl}_2$  is a generally accepted chemical hypoxia modeling material, which can induce the up regulation of the hypoxia inducible factor 1- $\alpha$  (HIF-1 $\alpha$ ), sequentially regulating

the expression of many effector genes [10]. It is thus considered a convenient *in vitro* ischemia/reperfusion model.

Hippocampus plays a critical role in learning and memory in humans and animals. Recently, a growing number of evidences have demonstrated that propofol improved learning and spatial memory functions [11,12], indicating that hippocampus is one of the target structures of propofol in brain. This has been further confirmed by *in vivo* studies showing that propofol was able to attenuate hippocampal neuron death and promote neurogenesis after oxygen deprivation or cerebral ischemia [4,13]. Consistent with these findings, the present study, revealed a significant increase in hippocampal neuron cell survival during H/R after pretreatment with 50 μM propofol.

Several mechanisms are implicated in hippocampal neuroprotective effects of propofol, including the upregulation of cleaved-caspase-3, c-fos as well as Bcl-2 [14,15], which leads to the decrease of hippocampal neuron apoptosis and death. In addition to these mechanistic explanations, our findings demonstrated that propofol protected hippocampal neurons subjected to H/R by inducing NGF expression (Figure 2A).

NGF is the prototype of the neurotrophin family of growth factor molecules, it regulates the growth, development and plasticity of selective neuronal populations in the nervous system [5-9,16]. It acts through binding and activating specific cell surface TrkA receptor and p75 neurotrophin (p75NTR) receptor [17,18]. Through TrkA receptor, neurotrophins activate many intracellular signaling pathways including those controlled by Ras, leading to the activation of MAPK, phospholipase C (PLC)- $\gamma$ 1, and PI3K/Akt, thereby affecting both development and function of the nervous system [19]. The protective effect of propofol may, at least partly, be mediated by induction of NGF and thus activation of TrkA receptor, which was supported by the fact that inhibition of TrkA receptor signaling with TrkA inhibitor prevented the survival function of propofol (Figure 4).

Regarding the mechanisms of propofol inducing NGF expression, it has been known that NGF expression in astrocytes is mediated by signaling through the MAPKs system [19]. Several studies have proved that JNK, a member of the MAPK family, is activated by a variety

of cellular stresses and extracellular signals [20,21], leading to the regulation of many genes, resulting in cell proliferation, transformation and tissue regeneration [22]. However, may other studies also proved that inhibiting the activation of JNK may be neuroprotective [23,24]. In the present study, a JNK inhibitor abolished the effect of propofol (Figures 3A and 3C), indicating that the JNK pathway may be involved in neuroprotective function of propofol. Such a regulation discrepancy in terms of JNK pathways may be owing to the difference in agent properties, target genes, cell types, and/or culture conditions of normoxia or H/R, which warrants further investigations. Moreover, it should be noted that multiple mechanisms are known to be involved in the neuronal protection of propofol against brain ischemia, such as reducing the cerebral metabolic rate of oxygen, removing oxygen free radicals and lipid peroxide, as well as modulating the uptake of glutamate and its binding properties to glutamate receptors [25-27]. GABAA receptor that is the major target of propofol to exert its anesthetic effect, is also known to regulate the expression of neurotrophic factor receptor [28], thus it may also be involved in the protective mechanisms of propofol, which warrants further investigations.

In conclusion, we described here that propofol may have a therapeutic potential for the treatment of brain ischemia/reperfusion injury, by increasing endogenous neurotrophin generation and through an NGF/TrkA signaling pathway.

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#### References

1. Wu GJ, Chen WF, Hung HC, Jean YH, Sung CS, et al. (2011) Effects of propofol on proliferation and anti-apoptosis of neuroblastoma SH-SY5Y cell line: new insights into neuroprotection. *Brain Res* 1384: 42-50.
2. Bayona NA, Gelb AW, Jiang Z, Wilson JX, Urquhart BL, et al. (2004) Propofol neuroprotection in cerebral ischemia and its effects on low-molecular-weight antioxidants and skilled motor tasks. *Anesthesiology* 100: 1151-1159.
3. Iijima T, Mishima T, Akagawa K, Iwao Y (2006) Neuroprotective effect of propofol on necrosis and apoptosis following oxygen-glucose deprivation--relationship between mitochondrial membrane potential and mode of death. *Brain Res* 1099: 25-32.
4. Lasarzik I, Winkelheide U, Stallmann S, Orth C, Schneider A, et al. (2009) Assessment of postischemic neurogenesis in rats with cerebral ischemia and propofol anesthesia. *Anesthesiology* 110: 529-537.
5. Lykissas MG, Batistatou AK, Charalabopoulos KA, Beris AE (2007) The role of neurotrophins in axonal growth, guidance, and regeneration. *Curr Neurovasc Res* 4: 143-151.
6. Shimohama S, Ogawa N, Tamura Y, Akaike A, Tsukahara T, et al. (1993) Protective effect of nerve growth factor against glutamate-induced neurotoxicity in cultured cortical neurons. *Brain Res* 632: 296-302.
7. Shigeno T, Mima T, Takakura K, Graham DI, Kato G, et al. (1991) Amelioration of delayed neuronal death in the hippocampus by nerve growth factor. *J Neurosci* 11: 2914-2919.
8. Lewin GR, Barde YA (1996) Physiology of the neurotrophins. *Annu Rev Neurosci* 19: 289-317.
9. Kaplan DR, Miller FD (2000) Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol* 10: 381-391.
10. Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM, et al. (2004) Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. *J Biol Chem* 279: 40337-40344.
11. Dong J, Min S, Wei K, Li P, Cao J, et al. (2010) Effects of electroconvulsive therapy and propofol on spatial memory and glutamatergic system in hippocampus of depressed rats. *J ECT* 26: 126-130.
12. Luo J, Min S, Wei K, Li P, Dong J, et al. (2011) Propofol protects against impairment of learning-memory and imbalance of hippocampal Glu/GABA induced by electroconvulsive shock in depressed rats. *J Anesth* 25: 657-665.
13. He J, Huang C, Jiang J, Lv L (2013) Propofol exerts hippocampal neuron protective effects via up-regulation of metallothionein-3. *Neurol Sci* 34: 165-171.
14. Xi HJ, Zhang TH, Tao T, Song CY, Lu SJ, et al. (2011) Propofol improved neurobehavioral outcome of cerebral ischemia-reperfusion rats by regulating Bcl-2 and Bax expression. *Brain Res* 1410: 24-32.
15. Cui Y, Ling-Shan G, Yi L, Xing-Qi W, Xue-Mei Z, et al. (2011) Repeated administration of propofol upregulated the expression of c-Fos and cleaved-caspase-3 proteins in the developing mouse brain. *Indian J Pharmacol* 43: 648-651.
16. Barbacid M (1994) The Trk family of neurotrophin receptors. *J Neurobiol* 25: 1386-1403.
17. Kaplan DR, Miller FD (1997) Signal transduction by the neurotrophin receptors. *Curr Opin Cell Biol* 9: 213-221.
18. Patapoutian A, Reichardt LF (2001) Trk receptors: mediators of neurotrophin action. *Curr Opin Neurobiol* 11: 272-280.
19. Galve-Roperh I, Haro A, Díaz-Laviada I (1997) Induction of nerve growth factor synthesis by sphingomyelinase and ceramide in primary astrocyte cultures. *Brain Res Mol Brain Res* 52: 90-97.
20. Kyriakis JM, Avruch J (2001) Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 81: 807-869.
21. Nishina H, Wada T, Katada T (2004) Physiological roles of SAPK/JNK signaling pathway. *J Biochem* 136: 123-126.
22. Wada T, Penninger JM (2004) Mitogen-activated protein kinases in apoptosis regulation. *Oncogene* 23: 2838-2849.
23. Borsello T, Clarke PG, Hirt L, Vercelli A, Repici M, et al. (2003) A peptide inhibitor of c-Jun N-terminal kinase protects against excitotoxicity and cerebral ischemia. *Nat Med* 9: 1180-1186.
24. Carboni S, Hiver A, Szyndralewicz C (2004) AS601245 (1,3-benzothiazol-2-yl (2-[[2-(3-pyridinyl) ethyl] amino]-4 pyrimidinyl) acetonitrile): a c-Jun NH2-terminal protein kinase inhibitor with neuroprotective properties. *J Pharmacol Exp Ther* 310: 25-32.
25. Wilson JX, Gelb AW (2002) Free radicals, antioxidants, and neurologic injury: possible relationship to cerebral protection by anesthetics. *J Neurosurg Anesthesiol* 14: 66-79.
26. Cervantes M, Ruelas R, Chávez-Carrillo I, Contreras-Gomez A, Antonio-Ocampo A (1995) Effects of propofol on alterations of multineuronal activity of limbic and mesencephalic structures and neurological deficit elicited by acute global cerebral ischemia. *Arch Med Res* 26: 385-395.
27. Illievich UM, Zornow MH, Choi KT, Strnat MA, Scheller MS (1994) Effects of hypothermia or anesthetics on hippocampal glutamate and glycine concentrations after repeated transient global cerebral ischemia. *Anesthesiology* 80: 177-186.
28. Fukui M, Nakamichi N, Yoneyama M, Yoshida K, Ozawa S, et al. (2008) Up-regulation of ciliary neurotrophic factor receptor expression by GABAA receptors in undifferentiated neural progenitors of fetal mouse brain. *J Neurosci Res* 86: 2615-2623.