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Effects of Subchronic Propofol Administration on the Proliferation and Differentiation of Neural Stem Cells in Rat Hippocampus

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ABSTRACT

Background: Although controversial, experimental data suggest the use of propofol may be associated with neurotoxicity. The mechanisms responsible for propofol neurotoxicity in animals are not yet clear.
Objective: This study aimed to determine the effects of propofol on the proliferation of neural stem cells in rat hippocampus and the mechanisms underlying these effects.
Methods: Forty-five adult male Sprague-Dawley rats were randomly divided into 5 groups: Control (N group), intralipid (V group), 30 mg/kg propofol (Prop30 group), 60 mg/kg propofol (Prop60 group), and 120 mg/kg propofol (Prop120 group). The rats in all groups received 5, once daily intraperitoneal injections. For each of the 5 days, the N group received 6 mL/kg normal saline, the V group received 6 mL/kg fat emulsion, the Prop30 group received 30 mg/kg propofol, the Prop60 group received 60 mg/kg propofol, and the Prop120 group received 120 mg/kg propofol. Memory function was scored daily using the Morris water maze test. Immunofluorescence staining was used to histologically monitor the proliferation and differentiation of the rats' hippocampal neural stem cells, and real time quantitative polymerase chain reaction and Western blotting were used to determine the expression of Notch3, Hes1, and Hes5.
Results: Compared with the N group, the Prop120 group exhibited reduced learning and memory, whereas there were no significant differences for the Prop60 group. The number of β-tubulin III^{+} cells increased in the Prop60 group, but decreased in the Prop120 group. Compared with the N group, the relative expression of Notch3 and Hes5 increased significantly in the Prop60 group, whereas this expression decreased in the Prop120 group.
Conclusions: These data demonstrate that repeated, subchronic (5 days) intraperitoneal injections of 60 mg/kg propofol can effectively promote rat hippocampal neural stem cells proliferation and differentiation, and that this is likely mediated by its effects on the Notch3-Hes5 pathway.

Introduction

Advances in medical technology and a growing focus on the provision of sedatives has resulted in the increased use of general anesthetics to diagnose and treat diseases, and to provide pain control and sedation in intensive care units as well as in emergency departments and other outpatient settings. Propofol, an intravenous anesthetic, is 1 such drug that acts via its effects on γ-aminobutyricacid A receptors.[1] However, as the clinical use of propofol has increased, its potential to cause neurotoxicity has raised concerns. Data from studies in a variety of experimental models suggest that propofol may cause developmental neurotoxicity, especially in a developing brain.[2–4] The primary manifestations of these adverse effects are histologic evidence of severe neuronal apoptosis and behavioral evidence of long-term learning and memory dysfunction. Several clinical studies have suggested that recurrent exposure to propofol anesthesia can considerably impair the learning, memory, and cognitive functioning of children younger than age 4 years.[5] Therefore, it is important to investigate the dose-dependent effects of propofol on long-term cogni-

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tive function, as well as the underlying mechanisms responsible for these effects.

The hippocampus plays a crucial role in memory processing. Multiple studies have linked the proliferation and differentiation of neural stem cells (NSCs) in the dentate gyrus of the hippocampus with the development of adult brain circuitry and the establishing of stable, lifelong behaviors. The synapses of these hippocampal NSCs regulate neural networks and influence cognitive performance via their connections.[6] Propofol has been shown to have the ability to destroy these neurons and prevent the maturation of dendrites, which may influence hippocampal neurogenesis and cause cognitive deficits.[7] Some evidence suggests that dysfunctional NSCs play a crucial role in propofol-induced changes in learning and memory dysfunction in newborn rats,[8,9] but the specific mechanisms responsible for these effects remain unknown.

Notch signaling is a highly conserved signaling pathway during evolution that regulates the differentiation and development of cells, tissues, and organs through the interactions between adjacent cells. Studies have confirmed that the Notch signaling pathway plays a critical role in the proliferation and differentiation of NSCs in both developing and adult brains.[10,11] This pathway relies on 5 Notch ligands (Delta 1–3 and Jagged 1, 2), 4 Notch receptors (Notch 1–4), and its downstream targets (Hes, Myc, p21, and Cyclin D3). Activation of the Notch signaling pathway is initiated by receptor-ligand binding, which induces Notch protein cleavage by presenilin and γ-secretase. This process releases the activated form of Notch, the Notch intracellular domain, which then enters the nucleus and induces the activation of its downstream target genes. These downstream target genes regulate cellular proliferation, differentiation, and apoptosis in the affected tissues.

Moreover, the Notch signaling pathway supports NSCs in maintaining their undifferentiated state and self-renewal ability across various developmental periods and disease states. Notch signaling is critical for the differentiation of NSCs into neurons and glial cells, making it a critical regulator of both embryonic and adult neurogenesis.[12] Previous studies have shown that the Notch1 and Notch3 proteins stimulate tumors and cell proliferation and neural differentiation in the central nervous system.[13–16] However, whether Notch3 and its downstream target genes are involved in the effects of propofol on the proliferation and differentiation of hippocampal NSCs remains unclear. This study was designed to explore the concentration-dependent effects of propofol on learning and memory in adult male rats, as well as its effects on the proliferation and differentiation of hippocampal NSCs. The role of the Notch signaling pathway in propofol-mediated destruction of learning and memory functions was examined. The data generated could provide a theoretical basis for the improved use of propofol in clinical practice.

Materials and Methods

Animals and grouping

Forty-five male Sprague-Dawley rats weighing 200 to 250 g were obtained from the Laboratory Zoology Department at Kunming Medical University. The animals were kept in a temperature-controlled room (22°C ± 2°C) with a humidity of 45% to 50%, a 12-hour light/dark cycle, and access to food and water ad libitum. The animal care and study protocols were approved by the Animal Experimental Ethics Committee of Kunming Medical University (No. 2020005) and complied with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023, revised 1978). The rats were divided into 5 groups (n = 9 each) using a random number table and then assigned to either the normal group (N group), intralipid group (V group), 30 mg/kg propofol group (Prop30 group), 60 mg/kg propofol group (Prop60 group), and 120 mg/kg propofol group (Prop120 group).[17] The rats all in received 5, once daily intraperitoneal injections of 6 mL/kg normal saline for the N group, 6 mL/kg fat emulsion for the V group, 30 mg/kg propofol for the Prop30 group, 60 mg/kg propofol for the Prop60 group, and 120 mg/kg propofol for the Prop120 group. Only the person administering the intraperitoneal injections was privy to the randomized allocation. All other study staff members were blinded to the group assignment. All rats were monitored for any change in hemodynamic parameters (see the Supplemental Table).

Morris water maze test

All rats had their spatial learning and memory abilities assessed daily using the standard Morris water maze test (MWM) test[18] by an observer who was blinded the group assignment. The MWM experimental apparatus consisted of a black cylindrical water maze and a transparent plexiglass platform. The pool measured 120 cm in diameter and 60 cm in height, and was divided into 4 quadrants (I, II, III, and IV) with a 12-cm circular escape platform in quadrant III. The pool water was maintained at 1 cm below the platform, which was designed to allow rats to climb onto it after completing the maze. Each time a rat entered the water, its head was pointed toward the wall of the pool at the midpoint or edge of 1 of the quadrants. The reference outside the water maze remained unchanged throughout the experiments and all data collected were analyzed using the MWM software (Shenzhen RWD Life Science Inc, China). Training trials were performed 4 times per day for 4 consecutive days, and the water maze was cleaned daily to remove any olfactory cues. The elapsed time was recorded for each rat, how long it took the rat to complete the maze, starting when it entered the water and finishing when it climbed onto the platform. If a rat failed to find the platform within 2 minutes, it was led to the platform and left to stand there for 30 seconds, and its escape latency was recorded as 120 seconds. The arithmetic means of 4 latency periods were then used to evaluate the memory capacity of each animal. On the fifth day of MWM, the platform was removed, the rats were put into the water from any entry point of quadrant I, the swimming track (path) of the rats was recorded while they were searching for the escape platform in the pool within 120 seconds, and the percentage of their time in the target quadrant compared with the total swimming time (ie, the percentage of target quadrant time) was calculated, as was the number of crossings of the escape platform that had originally been placed in quadrant III.

Tissue preparation

One day after the final MWM test, the rats were put to death after inhalation of 2% 0.4L/min isoflurane,[19,20] and the hippocampal tissues of the brain were separated in an ice dish. Parts of the tissues were then washed with 0.9% normal saline and 4% paraformaldehyde and left in 4% paraformaldehyde for 24 hours at 4°C before being sectioned for immunofluorescence staining. The rest of the tissues were washed with 0.9% saline and used as the sample for the various biochemical analyses. Finally, the remaining tissue was stored at –20°C for any follow-up experiments.

Western blotting

A portion of the hippocampal tissues were lysed using a commercially available solution (Beyotime Institute of Biotechnology, Shanghai, China) and then centrifuged at 4°C, 12,000 rpm for 15 minutes to remove any unlysed tissue. The Bicinchoninic Acid Assay (BCA) protein assay kit (Beyotime Institute of Biotechnology) was used to assay protein concentrations according to manufacturer's instructions. Protein samples were separated by 15% SDS-

PAGE (SDS-PAGE protein loading buffer (5X), P0015L, Beyotime Institute of Biotechnology) before being transferred to polyvinylidene fluoride membranes (Merck Millipore, Burlington, Massachusetts) and blocked with 5% bovine serum albumin in Tris-Buffered Saline-Tween (TBS-T) for 40 minutes at room temperature. Antibodies against Notch3 (1:1000, rabbit, Abcam, Cambridge, UK), Hes1 (1:1000, mouse, Santa Cruz, California, USA), Hes5 (1:500, rabbit, Proteintech, Chicago,USA), and β-actin (1:2000, mouse, Bioss, Beijing, China) were added as appropriate and incubated overnight at 4°C. Gels were then washed thrice using TBST for 6 minutes each and incubated with a horseradish peroxidase-labeled secondary antibody at room temperature for 40 minutes, then washed 3 times in TBST for 15 minutes. They were then developed using an enhanced chemiluminescence Western blotting kit (Biosharp, Hefei, China) for one minute before imaging using a gel documentation system (Gel Doc XR+ system, Bio-Rad, California, USA). The mean gray value was measured using ImageJ software (National Institutes of Health, Washington, DC), and the relative protein expression was normalized against β-actin.[21]

Immunofluorescence staining

Coronal hippocampal slices were fixed in 4% paraformaldehyde for 10 minutes, before being washed in phosphate buffered saline, blocked in 5% goat serum and 0.3% Triton X-100, and incubated with the appropriate primary antibody (Nestin, 1:500, mouse, Abcam; Ki67, 1:200, rabbit, CST, Massachusetts, USA) overnight at 4°C. These sections were then washed 3 times in phosphate buffered saline and incubated for 1 hour at room temperature in the appropriate secondary antibody (DyLight 488, 1:100, green goat anti-rabbit, Abbkine, California; DyLight 594, 1:100, red goat anti-mouse, Abbkine, California, USA) for visualization. Finally, the sections were counterstained with 4',6-diamidino-2-phenylindole (Beyotime Institute of Biotechnology) and observed using a fluorescence microscope (DMi8; Leica, Solms, Germany). ImageJ software quantified the number of single- and double-positive cells in the hippocampal dentate gyrus.

Quantitative reverse transcriptase polymerase chain reaction

Total RNA was extracted from the sample tissues using the Total RNA Extraction Kit (R1200-50T; Solarbio, Beijing, China), which was then used as a template for cDNA synthesis using a RevertAid First Strand cDNA Synthesis kit (catalog No.: K1622; Thermo Fisher Scientific, Waltham, Massachusetts). Quantitative reverse transcriptase polymerase chain reaction was performed using SYBR Green Master Mix in a DNA thermal cycler (CFX96; Bio-Rad) and β-actin served as the internal control. Relative mRNA expression levels were normalized to the internal control and calculated using the $2-\Delta\Delta CT$ method.

Sample size

The sample size calculation was based on the primary outcome of the variance of the numbers of $Nestin^+/Ki67^+$ between N and Prop60 groups. Based on this preliminary finding, using the online sample size statistics software on the biostatistics network (https://www.cnstat.org/), 8 male rats in each group were required, assuming a 2-sided type I error of 0.05 and a power of 80%. Allowing for 10% of the study rats to not be useable, 45 male rats were required (9 per group).

Statistical analysis

The data were analyzed using SPSS 26.0 software (IBM SPSS Inc, Armonk, New York) and values were presented as the mean (SD). Statistical significance between multiple groups was determined using ANOVA followed by a Bonferroni correction for multiple comparisons and defined at $P < 0.05$.

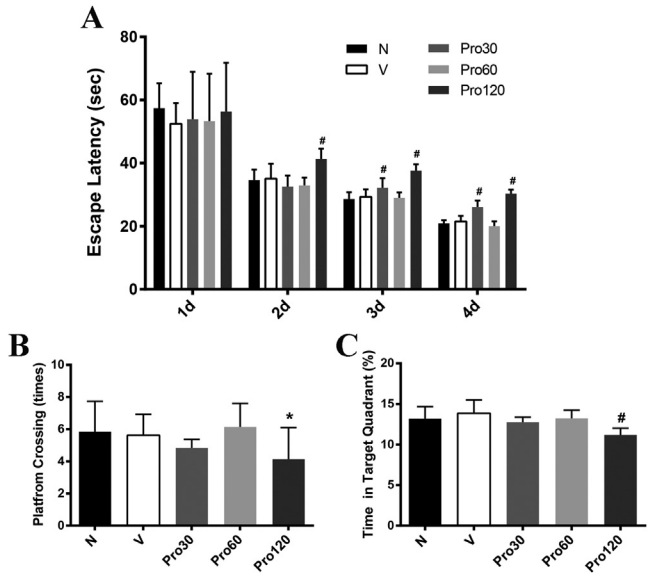

Figure 1. Effects of different doses of propofol on learning and memory function. (A) The escape latency for each group. (B) The platform crossing times for each group. (C) The percentage of time spent in target quadrant of each group. Data are presented as mean (SD). **Compared with Group N, $P < 0.01$.

Results

Hemodynamic changes

Over the 5 days, there were no significant difference in hemodynamic parameters in any of the different groups (see the Supplemental Table).

Effects of different doses of propofol on learning and memory function

Over the 4 days of training, no animals took more than 120 seconds to find the platform. And the escape latency consistently decreased in each, with no statistically significant differences between the N, V, and Prop60 groups ($P > 0.05$). However, between the second and fourth day of training, the escape latency of rats in the Prop120 group increased significantly ($P < 0.01$). In addition, on the third and fourth days of training, the escape latency of rats in the Prop30 group was significantly prolonged when compared with that of the rats in the N group ($P < 0.01$) (Figure 1A). In the Prop30 and Prop60 groups, there was no statistically significant difference in the number of platform crossings or the time spent in the target quadrant within 120 sec ($P > 0.05$) when compared with the N group. However, in the Prop120 group, the number of platform crossings and the time spent in the target quadrant was significantly reduced ($P < 0.01$) when compared with the N group (Figure 1B and 1C). These results imply that the subchronic (ie, 5 days) intraperitoneal propofol dose of 60 mg/kg had a negligible effect on the cognitive capacities of these adult male rats.

Effects of different doses of propofol on the proliferation and differentiation of hippocampal NSCs

Because of the essential role of the hippocampus in memory processing, the effects of propofol on the proliferation and differentiation of hippocampal NSCs were studied. A significant decrease

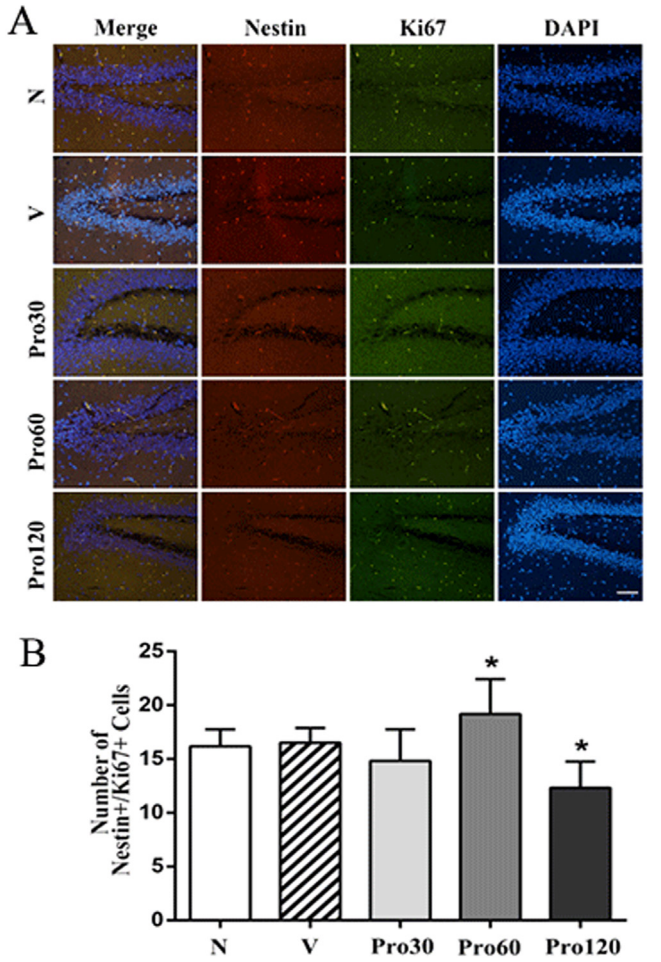

Figure 2. Effects of propofol on the proliferation of hippocampal neural stem cells (NSCs). (A) Nestin (*red*) and Ki67 (*green*), combined with 4',6-diamidino-2-phenylindole staining (*blue*) on hippocampal NSCs of dentate gyrus in adult rat. Scale bar is 100 μm. (B) Nestin/Ki67 double-positive cells distributed in the dentate gyrus of adult rats. Data are presented as mean (SD). *Compared with Group N, $P < 0.05$.

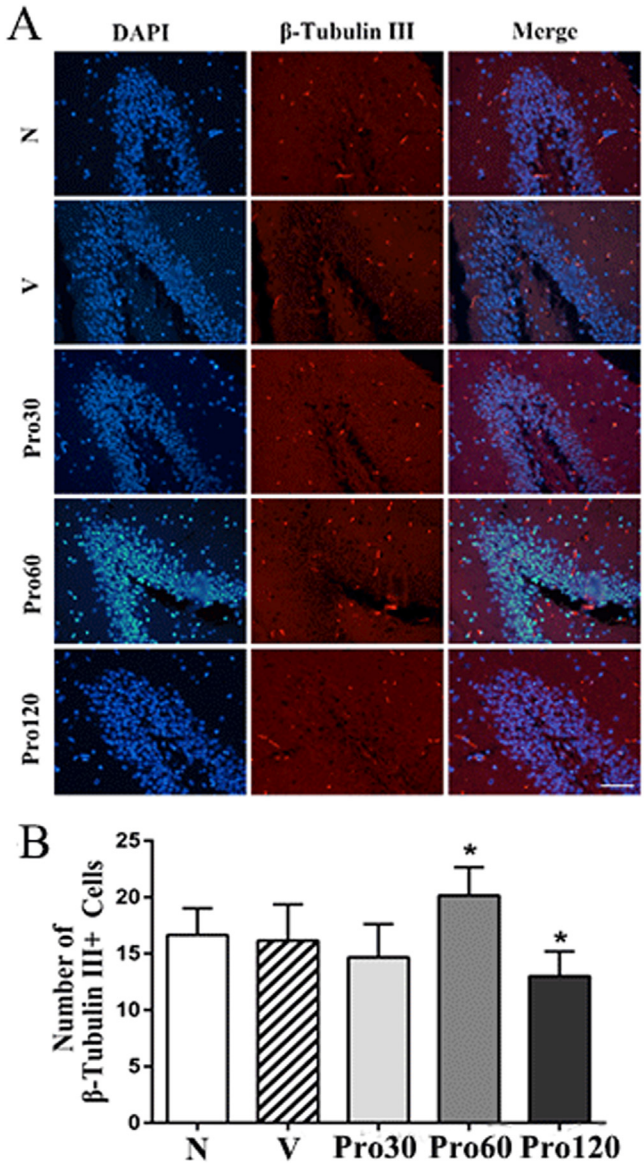

Figure 3. Effects of propofol on neonatal hippocampal neurons. (A) β-Tubulin III (*red*) combined with 4',6-diamidino-2-phenylindole staining (*blue*) on hippocampal neural stem cells of dentate gyrus in adult rats. Scale bar is 20 μm. (B) The β-tubulin-positive cells distributed in the dentate gyrus of adult rats. Data are presented as mean (SD). *Compared with Group N, $P < 0.05$.

in the numbers of Nestin$^+$/Ki67$^+$ (Figure 2) and β-tubulin III$^+$ (Figure 3) cells in the Prop120 group ($P < 0.05$) when compared with N group was noted; however, the numbers of these 2 kinds of cells were significantly increased in the Prop60 group compared with the N group ($P < 0.05$). These results suggest that the subchronic (ie, 5 days) 60 mg/kg IP propofol dose may have had little effect on the proliferation and differentiation of hippocampal NSCs, and therefore a minimal influence on memory processing in these adult male rats.

Effects of different doses of propofol on the expression of Notch3, Hes1, and Hes5

The effects of propofol on hippocampal NSCs and the mechanisms underlying these changes were examined. The relative mRNA and protein expression of Notch3 significantly increased in the Prop60 group when compared with the N group ($P < 0.05$). In the Prop120 group, this relative expression decreased, but the difference was not significant ($P > 0.05$) (Figure 4). The relative expression of Hes5 significantly increased in the Prop60 group compared with the N group (Figure 5), but decreased in the Prop120 group ($P < 0.05$). The relative expression of Hes1 was not significantly different between any of these groups ($P > 0.05$). These results indicate that in rats given a subchronic (ie, 5 days) intraperitoneal dose of 60 mg/kg propofol, the Notch3-Hes5 signaling pathway had a minimal role in controlling the proliferation and differentiation of hippocampal NSCs (Figure 6).

Discussion

Propofol is a commonly used clinical anesthetic given by multiple routes of administration, the most common being intravenous continuous pump injection. However, considering the large differences between animal experiments and clinical applications and the methods used in many published animal model studies, intraperitoneal injections were selected for use in this study.[17,22,9] About 7 minutes after receiving 60 mg/kg IP propofol, the male rats lost their reflexes and reached an anesthetized state. After about 13 minutes, the reflexes were gradually recovered, consistent with results of another, somewhat similar study.[23]

Assisted spatial learning and memory tests can be used to evaluate the spatial learning and exploration capacity of different an-

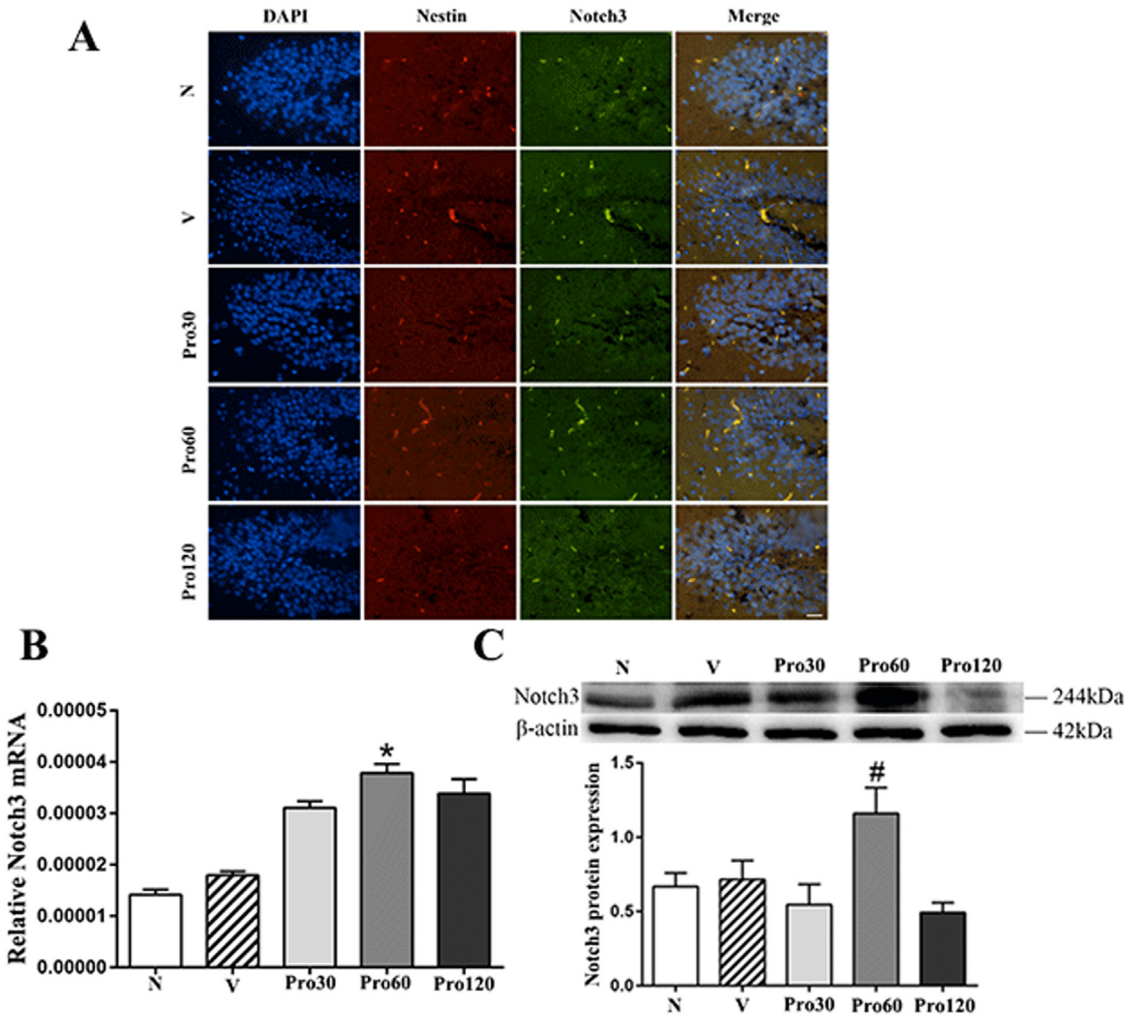

Figure 4. Effects of propofol on the expression of Notch3. (A) Nestin (*red*) and Notch3 (*green*), combined with 4',6-diamidino-2-phenylindole staining (*blue*) on hippocampal neural stem cells of dentate gyrus in adult rats. Scale bar is 20 μm. (B) The relative expression level of Notch3 mRNA in the dentate gyrus of adult rat was evaluated by quantitative polymerase chain reaction. (C) The protein expression profile of Notch3 was analyzed by Western bolt technique. Data are presented as mean (SD). **Compared with Group N, $P < 0.01$.

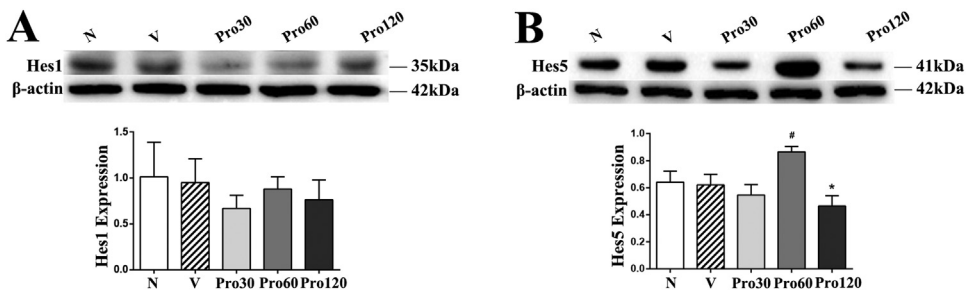

Figure 5. Effects of propofol on the expression of Hes1 and Hes5. (A) The expression level of Hes1 in the dentate gyrus of adult rats. (B) The expression level of Hes5 in the dentate gyrus of adult rats. Data are presented as mean (SD). *Compared with Group N, $P < 0.05$. **Compared with Group N, $P < 0.01$.

imals, including various rodents. The MWM test can be used to study the neurocognitive function, learning and memory capacity, and escape latency of rats, which was defined in these experiments as a reduction in the number of platform crossings and the percentage of time spent in the target quadrant. Results of the MWM test has been used to evaluate learning and memory impairment in animals.[24] Data from this study showed that the learning and memory ability of rats in the Prop120 group decreased when compared with the N group, but there was no significant difference in these values for the Prop30 or Prop60 groups, suggesting that <60 mg/kg IP propofol may not result in damage to learning and memory performance of adult male rats.

NSCs exhibit multidirectional differentiation potential and self-renewal. Neurogenesis refers to the proliferation and differentiation of NSCs into a variety of other cell types under specific conditions in which they perform different neural functions. The neurogenesis of these NSCs within the hippocampal dentate gyrus plays an important role in learning and memory capacity.[25] A Ki67 anti-

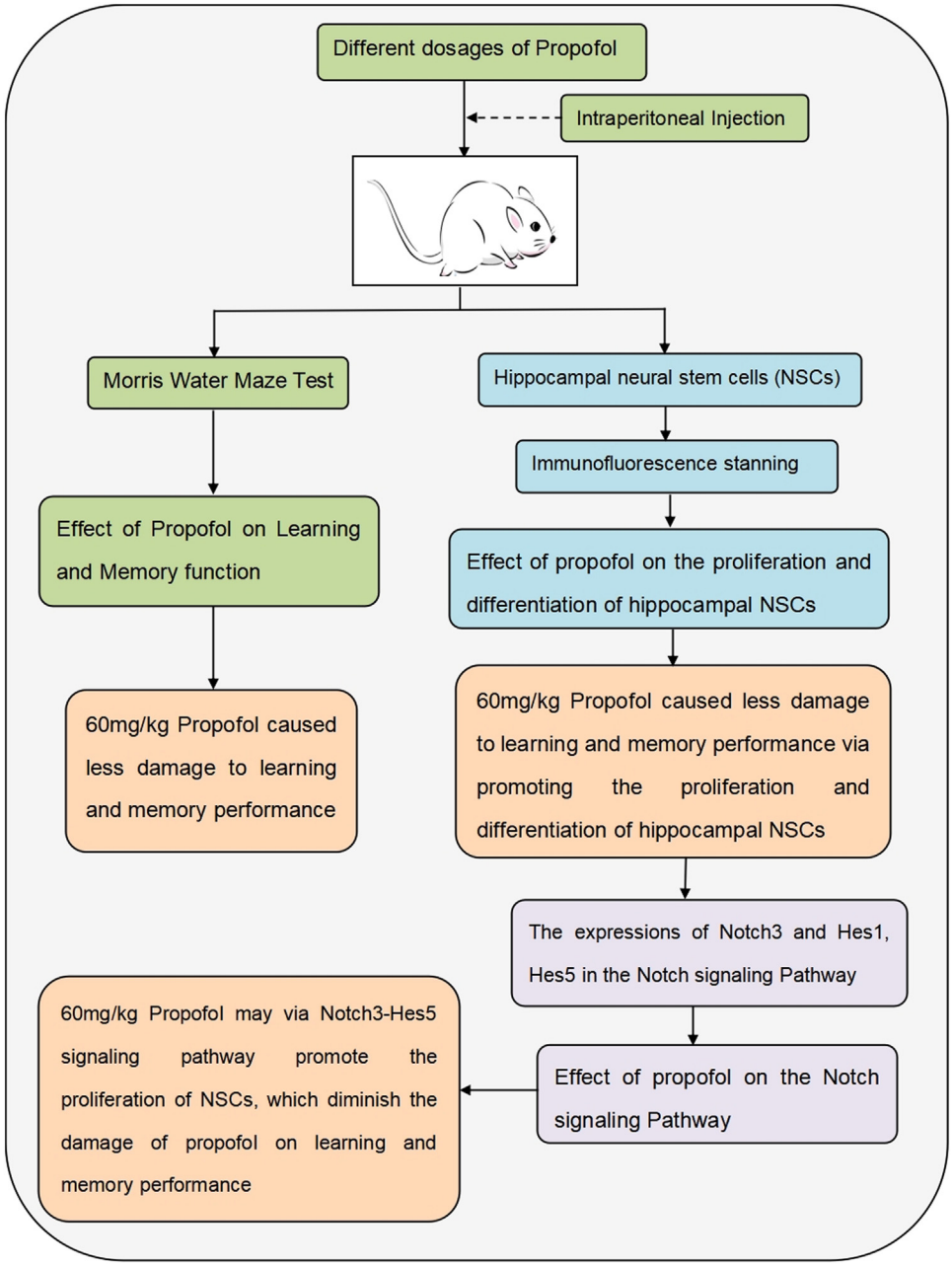

Figure 6. The possible effects and underlying mechanism of propofol on the memory processing of the adult rats.

body was used to evaluate the proliferative activity of NSCs in the adult male rat hippocampal dentate gyrus because Ki67 is known to be expressed in proliferating cells and can be identified throughout the majority of the mitotic cycle.[26] These results suggest that subchronic treatment with 60 mg/kg IP propofol actually may promote NSC proliferation, whereas a higher, 120 mg/kg IP propofol dose inhibited their proliferation. Previous studies have reported that propofol may inhibit the proliferation of NSCs in the hippocampus in a dose-dependent manner,[27,28] and some research teams have used NSCs from the adult hippocampus of Fisher 344 rats to demonstrate that low concentrations of propofol (10 mM) had no effect on NSC activity, but slightly increased concentrations (50 mM) increased their proliferation.[29]

In addition, in this study the number of newborn neurons (β-tubulin III$^+$ cells) in adult male rats was found to be increased after receiving the subchronic 60 mg/kg IP propofol, but decreased after receiving the subchronic 120 mg/kg IP propofol. Sall et al,[30] also reported that propofol at clinically relevant concentrations may promote NSC differentiation into neurons, but that when propofol exceeds a critical concentration (7.1 mM), it can kill NSCs. Another study also reported that propofol promoted NSC differentiation into neurons only at certain concentrations.[31] These findings suggest that even long-term exposure to propofol at certain doses may have little effect on the neurocognitive function, at least of rats. This phenomenon may be explained by the fact that propofol depending on its concentration or exposure over time can either promote or inhibit NSC proliferation in the hippocampal dentate gyrus and their differentiation into hippocampal neurons.

The molecular mechanisms by which propofol affects the proliferation and differentiation of NSCs in the hippocampal dentate gyrus was investigated by evaluating the expression of key proteins in the Notch signaling pathway. An increasing number

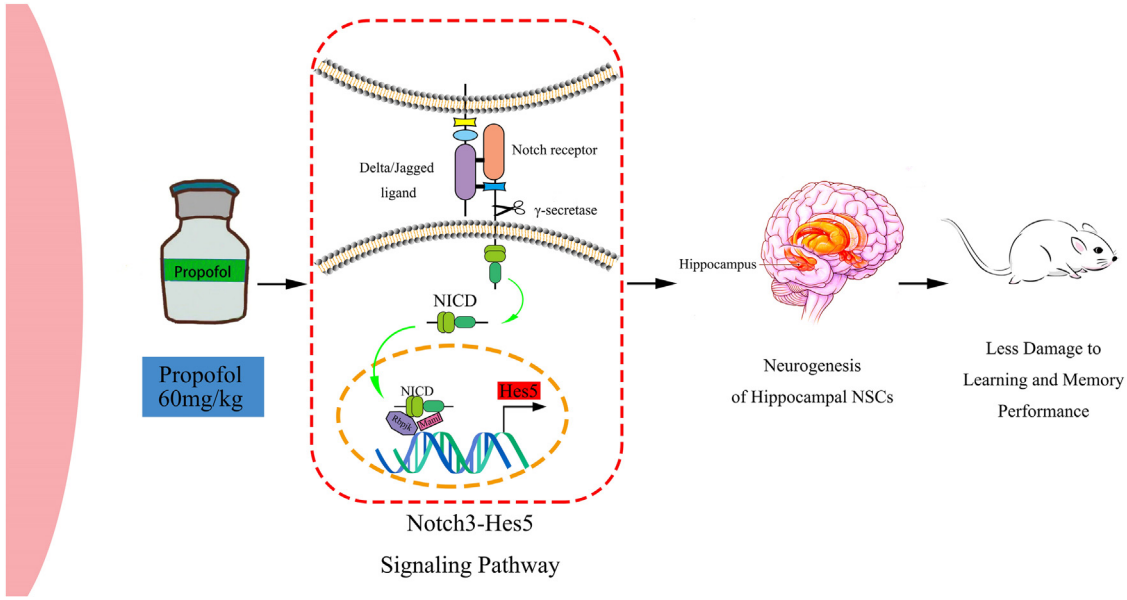

Figure 7. The flowchart diagram of the current study.

of studies have confirmed that the Notch signaling is a highly conserved key pathway regulating signal transmission between cells. The expression of Notch proteins is closely associated with the occurrence and development of cerebral ischemia–reperfusion injuries[32] as well as with the treatment of epileptic seizures.[33] The expression of Notch3 was significantly upregulated in response to higher propofol exposure. Previous studies have shown that different Notch proteins control the state of NSC proliferation in different regions of the brain. Similarly, a recent study found that the Notch proteins regulate the neurogenesis of NSCs in a region-specific manner.[34] Other studies have demonstrated that the expression of Notch3 proteins is related to learning and memory dysfunction in adult rats with cerebral autosomal dominant arteriopathy, subcortical infarcts, and leukoencephalopathy disease.[35] In this report, the upregulation of Notch3 expression was also shown to inhibit hippocampal neuronal cell apoptosis and promote NSC neurogenesis.[36]

In addition, this study showed that a 60 mg/kg IP propofol dose significantly increased the expression of Hes5, but when the dose was increased to 120 mg/kg, the expression of Hes5 was decreased. However, there was no obvious difference found in the expression of Hes1 between the groups. Many studies have shown that Hes5, a primary effector molecule of the Notch signaling pathway, plays an important role in regulating NSC proliferation and differentiation,[37] and that inducing the expression of Hes5 can increase hippocampal neurogenesis.[38] A recent study also suggested that Fluoxetine has neuroprotective effects in relieving lipopolysaccharide (LPS)-induced depression-like and motor behaviors by inhibiting the expression of Hes5.[39] These findings are consistent with the results of this study, which demonstrated that the Notch3-Hes5 signaling pathway may be involved in the effect of propofol on memory processing.

Limitations

This study has several limitations. First, only adult male rats were included in the study. Therefore, the results may not be generalizable to newborn rats, which are known to be more sensitive to propofol neurotoxicity. Second, male and female rats may react differently to the same doses of propofol. Third, a positive control was not included in this study. Fourth, considerable amounts of lipid emulsion were not included in this study, which could diminish or eliminate any possible lipid effects. Nevertheless, the present study is the first to demonstrate that biphasic effects on the Notch3-Hes5 signaling pathway may explain the reduced memory processing impairment in adult male rats at a dose of 60 mg/kg IP propofol.

Conclusions

The data from this study provide insights into the mechanisms responsible for the influence of different doses of propofol on memory processing. These data demonstrate that repeated, subchronic (5 days) intraperitoneal injections of 120 mg/kg propofol but not 60 mg/kg cause damage to learning and memory performance in male rats that were associated with increases in the proliferation and differentiation of NSCs in the hippocampus via effects on the Notch3-Hes5 signaling pathway (Figure 7).

Conflicts of Interest

The authors have indicated that they have no conflicts of interest regarding the content of this article.

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C. Chang was responsible for conceptualization, study design, data collection, and writing the original draft. W. Bai was responsible for study design, writing the original draft, data collection, and formal analysis. J. Li was responsible for supervision, writing, review, and editing. S. Huo was responsible for project administration, visualization, investigation, review, and editing. T. Wang was responsible for conceptualization, methodology, project development, review, and editing. J. Shao was responsible for conceptu-

alization, methodology, project development, and ensuring scientific accuracy of the manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.curtheres.2023.100691.

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