

Original Article

Sevoflurane inhibits the phosphorylation of ribosomal protein S6 in neonatal rat brain

Hong Tan, Chang-Lin Li, Lu Zhang, Zhou-Jing Yang, Xuan Zhao, Ying-Wei Wang

Department of Anesthesia and Critical Care, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai, China

Received August 16, 2015; Accepted August 25, 2015; Epub September 15, 2015; Published September 30, 2015

Abstract: Besides neurotoxic effects, inhaled anesthetics might have other adverse effects on the developing brain. Ribosomal protein S6 (rpS6), the first identified ribosomal protein undergoing phosphorylation, has important physiological functions in regulating protein synthesis, cell proliferation, and glucose homeostasis. To date, the function of sevoflurane on rpS6 phosphorylation is unclear. In our present study, we showed that sevoflurane anesthesia inhibited rpS6 phosphorylation in cerebral cortex and CA1 region of the hippocampus. The activity of Akt was detected to be reduced within both cortical and hippocampal regions in the brain with the treatment of sevoflurane. However, the treatment seemed to have no effect on the phosphorylation of mammalian target of rapamycin (mTOR), a downstream effector of Akt. Sevoflurane had a paradoxical effect on ERK activity in the hippocampus and cerebral cortex. Last but not the least, Sevoflurane increased PP1 activity in the cerebral cortex and hippocampus. Thus, the exposure to sevoflurane inhibited dramatically the phosphorylation of rpS6 in neonatal rat brains. The inhibitory effect of sevoflurane on rpS6 phosphorylation might be mediated by the suppression on AKT activity at an mTOR-independent manner and the enhancement of PP1 activity.

Keywords: Sevoflurane, ribosomal protein S6, cerebral cortex, hippocampus

Introduction

Neonatal exposure to anesthetics can cause neuronal apoptosis in the brain of animal models resulting in certain behavioral disorders [1-5]. It was reported that a combination of a few anesthetic drugs (midazolam, nitrous oxide, and isoflurane) could cause widespread neuronal apoptosis in the brain and subsequent cognitive deficits [2]. Previous studies revealed that three commonly used inhaled anesthetics (desflurane, isoflurane, and sevoflurane) all had similar significant neurotoxic profiles in neonatal animals besides their anesthetic effects [3]. It also has been suggested that inhaled anesthetics could inhibit neuronal activities, as well as exert their anesthetic effects mainly through ligand-gated ion channels including [gamma]-aminobutyric acid type A (GABA_A), glycine, serotonin and glutamate (kainate-sensitive) receptors [6]. Although a good amount of attention has been paid on the mechanisms underlying the neurotoxic effects of inhaled anesthetics, it

remains elusive how the inhaled anesthetics induce neuronal apoptosis in neonatal animals and humans. sevoflurane is currently widely applied in pediatric anesthesia due to its advantage. Previous studies indicated that sevoflurane could cause neurodegeneration in the developing brains of neonatal rodents [3, 7, 8]. However, the adverse effects of sevoflurane in neuronal cells *in vivo* have not been revealed entirely.

Ribosomal proteins are components of ribosomal subunits involved in the physiological process of protein translation. Ribosomal protein S6 (rpS6), the first identified ribosomal protein which can be phosphorylated, is a component of eukaryotic ribosomal 40S subunit [9, 10]. Evi Functional analyses of rpS6 phosphorylation suggested its role in the regulation of protein synthesis, cell proliferation, and glucose homeostasis [10-13]. The phosphorylation of rpS6 could be induced by multiple stimuli including growth factors, tumor promoting

agents, and mitogens. The inhibition of rpS6 phosphorylation was found mainly under stress conditions such as hypoxia, heat shock, and hyperosmolarity [10]. The effect of anesthetics on the regulation of ribosomal proteins is unknown. In our present study, we revealed that sevoflurane inhibits phosphorylation of rpS6 in the hippocampus and cerebral cortex of the neonatal rat brain, and explored the potential underlying mechanisms.

Materials and methods

Animals

All animal experiments were conducted according to the Principles of Laboratory Animal Care formulated by the National Committee of Medical Research, and the Guide for the Use of Laboratory Animals issued by the Institute of Laboratory Animal Resources of China. The study was approved by the Research Animal Care and Use Committee at Shanghai Jiao Tong University School of Medicine. The Sprague-Dawley rats used in this study were maintained under a 12-h light-dark cycle (lights on from 07:00 to 19:00) with room temperature at $22^{\circ} \pm 1^{\circ}\text{C}$. Food and water were available *ad libitum*.

Anesthesia treatment

On postnatal day 8 (P8), the rats were placed into a chamber and exposed to anesthetics. The total gas flow was 2 l/min, using 70% O_2 as a carrier. The oxygen and anesthetic agent fractions were measured by a gas analysis system (GE Healthcare). During the anesthetic exposure, the chamber was kept warmly at $37^{\circ} \pm 1^{\circ}\text{C}$ with an infrared heat lamp. Neonatal littermate rats were randomly assigned to receive the following treatments: 3% sevoflurane (Maruishi Pharmaceutical Co., Ltd, Japan) for 0.5 h, 1 h, 2 h and 4 h. After the anesthesia for 4 h, neonatal littermate rats were kept alive for 0.5 h and 2 h. In our pilot studies, anesthesia with 3% sevoflurane plus 70% O_2 as a carrier for 4 h does not alter blood gas and brain blood flow significantly.

Preparation of protein extracts

The hippocampi and cerebral cortex of the neonatal rats were quickly dissected out and homogenized in four volumes of homogenization buffer containing 50 mM Tris-HCl, pH 7.4,

150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate, protease inhibitor cocktail (Fermentas), and phosphatase inhibitors (10 mM Na_3VO_4 , 10 mM NaF). After homogenization, the homogenates were centrifuged at 12,000 rpm for 10 min at 4°C . The supernatant solutions were separated and stored at -70°C until use. Protein concentration in each sample was measured using a bicinchoninic acid protein assay kit (Pierce).

Western blot analysis

The homogenates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Then, proteins were transferred onto polyvinylidenedifluoride membranes (Millipore). Antibodies against phospho-ERK (Cell Signal; 1:2000), ERK (Cell Signal; 1:1000), phospho-p38 (Cell Signal; 1:1000), p38 (Cell Signal; 1:1000), phospho-JNK (Cell Signal; 1:5000), JNK (Cell Signal; 1:1000), phospho-AKT (Cell Signal; 1:3000), AKT (Cell Signal; 1:3000), mTOR (Cell Signal; 1:1000), phospho-mTOR (Cell Signal; 1:1000), rpS6 (Cell Signal; 1:1000), phospho-rpS6 (Cell Signal; 1:1000), phospho-PP1 α (Epitomics; 1:1000) and actin (Santa Cruz; 1:10000) were used. The protein bands were visualized by enhanced chemiluminescence detection system (Millipore).

Histopathologic studies

The rats were anesthetized and perfused via the ascending aorta with saline followed by 4% paraformaldehyde and 0.1% picric acid in 0.1 M phosphate buffer (pH 7.4). Brains were then dissected out, and post-fixed in the same fixative for 1 day at 4°C , and then immersed in 20% sucrose in 0.1 M phosphate buffer for at least 2 day at 4°C . Coronal brain sections (20 μm thick) were cut using a cryostat and mounted on gelatin-coated glass slides. Then sections were stored at -20°C until use. Briefly, brain sections containing the hippocampus region were selected, endogenous peroxidase activity was quenched by the incubation with 3% H_2O_2 in methanol for 15 min. Permeabilization and blocking of non-specific binding were achieved using 0.2% Triton X-100 and 5% heat-inactivated donkey serum in phosphate-buffered saline (PBS). Primary antibodies were diluted in 5% donkey serum/PBS and incubated overnight at 4°C . The polyclonal antibodies

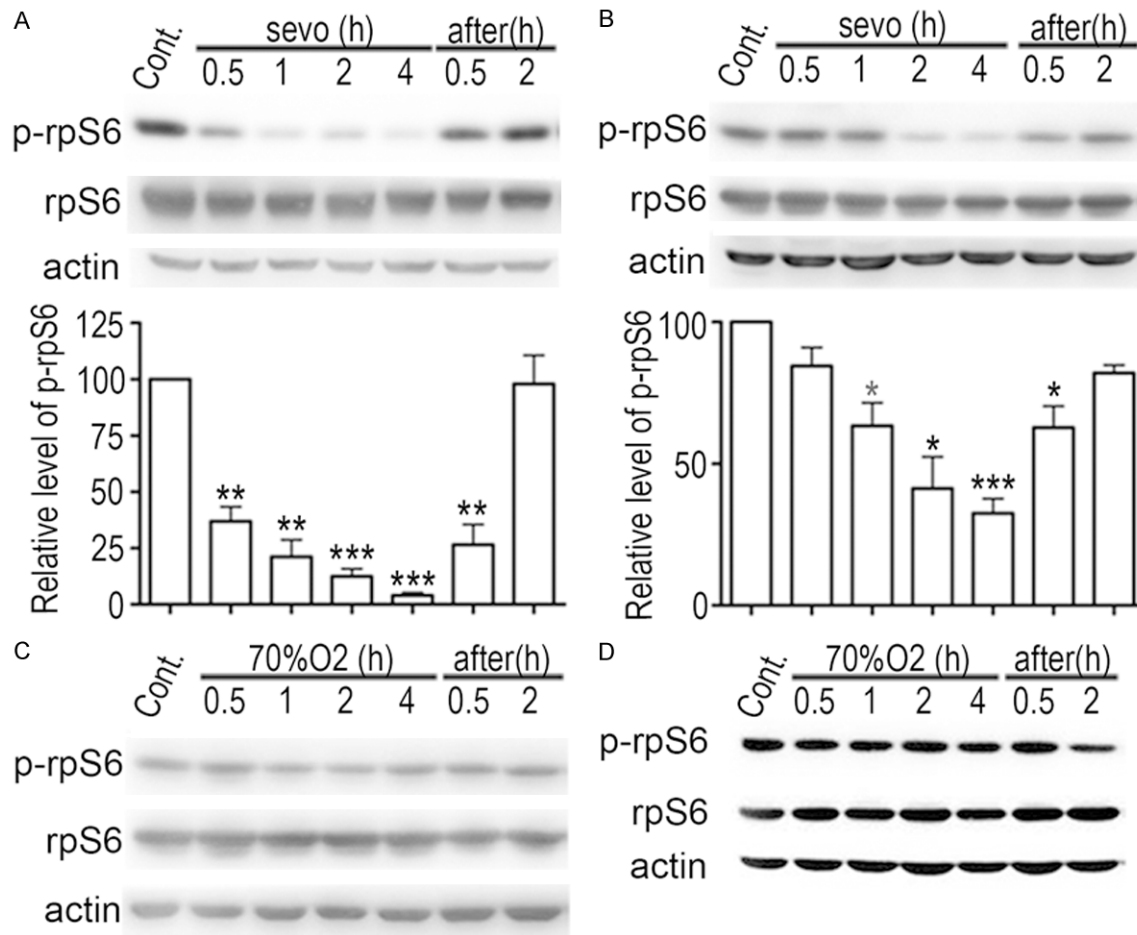


Figure 1. Anesthesia with 3% sevoflurane inhibits rpS6 phosphorylation with a time-dependent manner in the hippocampus and cerebral cortex of neonatal rat brains. Sevoflurane anesthesia inhibits rpS6 phosphorylation in cerebral cortex (A, above) and hippocampus (B, above) of the neonatal rats. Quantification of western blots shows that sevoflurane inhibits rpS6 phosphorylation in cerebral cortex (A, below) and hippocampus (B, below) with a time-dependent manner. The carrier (70% O₂) has on inhibitory effect on rpS6 phosphorylation in cerebral cortex (C) and hippocampus (D) of neonatal rats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus control.

against phospho-rpS6 (Cell Signal; 1:500) were used. Subsequent procedures were performed according to ABC method. Elite-ABC and DAB detection kits (Vector) were used according to the manufacturer's instructions. After the coloration, sections were finally mounted in 90% glycerol and cover-slipped. Tissue sections were examined under the light microscope.

Statistical analysis

Statistical comparisons were performed from four animals obtained in four independent experiments. Statistical analysis was performed using an unpaired two-tailed Student t test between two groups and one-way ANOVA plus Newman-Keuls multiple comparison test

among more than two groups. Summary data was represented as mean \pm SD. Differences between groups were considered to be significant with a P value of less than 0.05.

Results

Neonatal exposure to sevoflurane inhibited the phosphorylation of rpS6 in both cerebral cortex and hippocampus

To evaluate the impact of sevoflurane on the phosphorylation of rpS6 in neonatal rat brains, neonatal rats were examined by western blot after the treatment with 3% sevoflurane for 0.5 h, 1 h, 2 h, and 4 h. Data showed sevoflurane anesthesia inhibited rpS6 phosphorylation with

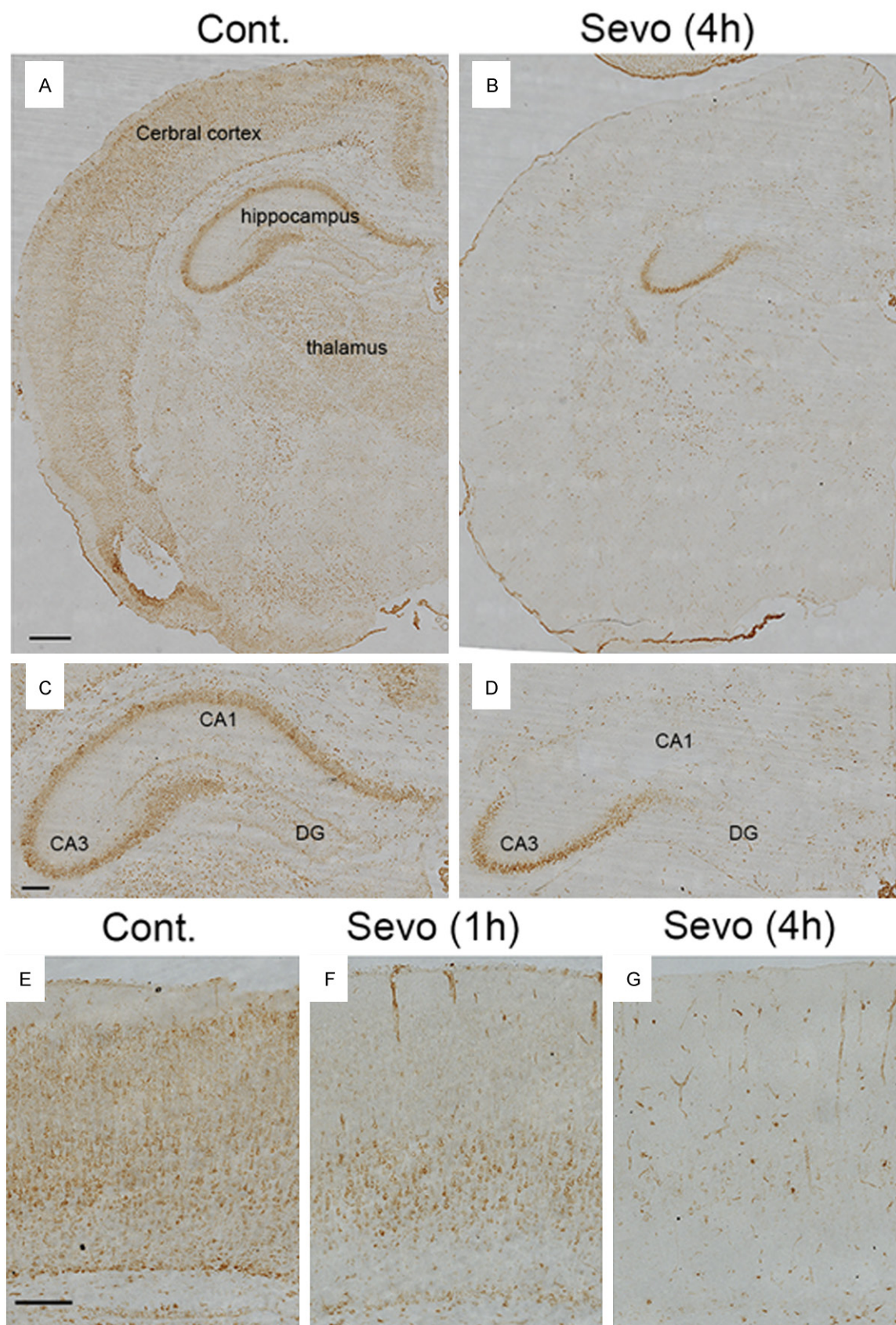


Figure 2. The distribution of p-rpS6⁺ cells in neonatal brains. (A) Representative light microscope images show the widespread distribution of p-rpS6⁺ cells in the neonatal brain. (B) Sevoflurane treatment for 4 h inhibits the phosphorylation of rpS6 in neonatal brains except CA3 region of hippocampus. Scale bar (A & B): 500 μ m. The magnified image of the hippocampus shows the intensity of p-rpS6⁺ staining in the control neonatal rat (C) and that exposed to sevoflurane for 4 h (D). Scale bar (C & D): 200 μ m. The magnified image of the cerebral cortex shows the expression of p-rpS6⁺ staining in control neonatal rat (E), that exposed to sevoflurane for 1 h (F), and that exposed to sevoflurane for 4 h (G). Scale bar (E-G): 100 μ m.

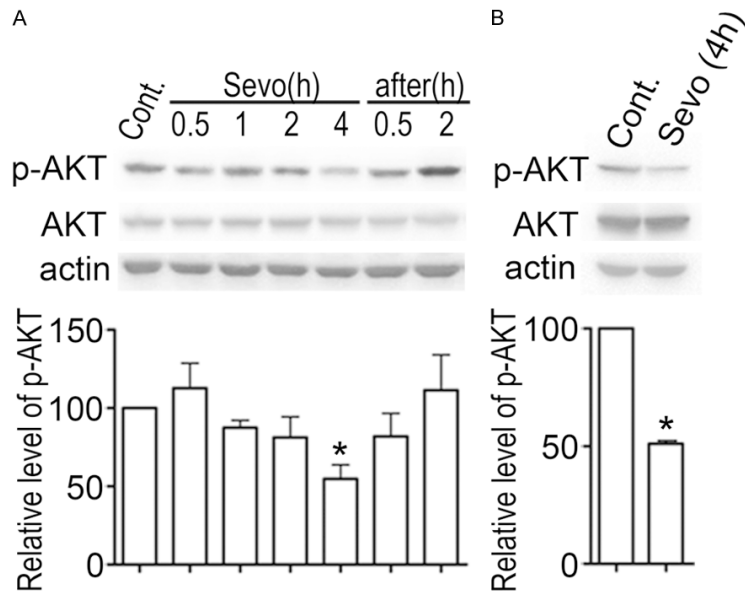


Figure 3. Anesthesia with 3% sevoflurane inhibits AKT phosphorylation in the cerebral cortex and hippocampus of the neonatal rat brain. Western blot data demonstrated sevoflurane anesthesia inhibits AKT phosphorylation in cerebral cortex (A, above) and hippocampus (B, above) of the neonatal rat. Quantification data showed the inhibitory effect in the cerebral cortex (A, below) and hippocampus (B, below). *P<0.05 versus control.

a time-dependent manner in both hippocampus and cerebral cortex of neonatal brains. In the neonatal rat cerebral cortex, the decrease of the level of phosphorylated rpS6 protein (p-rpS6) became notable after sevoflurane anesthesia for 0.5 h (Figure 1A). The level of rpS6 phosphorylation came down to the bottom (about 5% of the control level) after sevoflurane anesthesia for 4 h (Figure 1A). In neonatal rat hippocampus, the inhibitory effect became significant after sevoflurane anesthesia for 1 h (Figure 1B). The level of rpS6 phosphorylation came down to the bottom (about 30% of the control level) after sevoflurane anesthesia for 4 h (Figure 1B). Thus the inhibitory effect of sevoflurane on the phosphorylation of rpS6 in neonatal cerebral cortex was stronger than in the hippocampus. The inhibitory effect became weaker in the hippocampus and cerebral cortex after the remove of sevoflu-

rane anesthesia (Figure 1A & 1B). And the level of phospho-rpS6 recovered within 2 h after the removal of sevoflurane anesthesia (Figure 1A & 1B). As a control, the treatment with the carrier (70% O₂) had no effect on the level of phospho-rpS6 in the hippocampus and cerebral cortex (Figure 1C & 1D).

To explore the localization of phospho-rpS6 in the brain, the neonatal rats were treated for the immunohistochemical studies. Immunostaining data showed that phospho-rpS6 positive signals were distributed widely in neonatal brains including cerebral cortex, hippocampus, thalamus and other regions (Figure 2A). After sevoflurane anesthesia for 4 h, the expression of phospho-rpS6 positive signals decreased dramatically (Figure 2B).

The inhibitory effects of sevoflurane anesthesia on rpS6 phosphorylation in the hippocampus and cerebral cortex were emphasized in the present study. In neonatal hippocampus, the signals of phospho-rpS6 positive neurons were stronger in CA1 and CA3 region than those in DG region (Figure 2A & 2C). The intensity of phospho-rpS6 positive signals in CA1 region of the hippocampus was decreased remarkably after sevoflurane anesthesia for 4 h (Figure 2B & 2D). Note that the intensity of phospho-rpS6 positive signals in CA3 region was not affected (Figure 2B & 2D). In cerebral cortex, the phospho-rpS6 positive signals were detected in lamina II~VI neurons under normal conditions (Figure 2A & 2E). After sevoflurane anesthesia for 1 h, the p-rpS6 positive signals were localized mainly in lamina V and the bottom of lamina VI, and the signals in other laminas were not significantly reduced (Figure 2F).

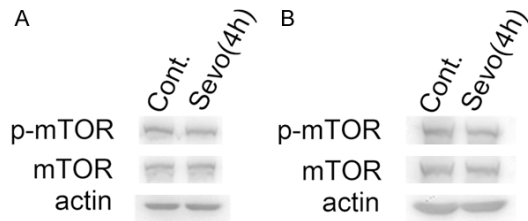


Figure 4. Sevoflurane anesthesia had no effect on mTOR phosphorylation in cerebral cortex and hippocampus of the neonatal rat's brain. Western blot data demonstrate that sevoflurane anesthesia did not affect mTOR phosphorylation in cerebral cortex (A, above) and hippocampus (B, above) of neonatal rats. Quantification data were shown here as well - cerebral cortex (A, below) and hippocampus (B, below).

After sevoflurane anesthesia for 4 h, the p-rpS6 positive signals were inhibited in lamina V of the cerebral cortex (**Figure 2B & 2G**). Only very little p-rpS6 positive signals were found in the cerebral cortex of neonatal brains (**Figure 2B & 2G**). Thus these data demonstrated that neonatal exposure to sevoflurane inhibited the phosphorylation of rpS6 with a time-dependent manner in cerebral cortex, CA1 region of the hippocampus and other brain regions. However, sevoflurane treatment had no significant effect on rpS6 phosphorylation in CA3 region of the neonatal hippocampus.

Neonatal exposure to sevoflurane inhibited AKT phosphorylation in cerebral cortex and hippocampus

Two families of ribosomal S6 kinases were identified due to the great effort deployed by several laboratories. One is the RSK (90 kDa ribosomal S6 kinase) family, the other is S6K (70 kDa ribosomal S6 kinase) family [10]. S6K was the predominant rpS6 kinases in somatic cells [14]. The activation of S6K was mainly mediated by class I phosphoinositide 3-kinase (PI3K)/AKT pathway [10]. And the activation of RSKs was primarily mediated by Ras/mitogen-activated protein kinase (MAPK) pathway. AKT belongs to the AGC family of serine/threonine kinases [15]. Its activation is through PI3K. After activation, active AKT exerts an influence on its substrates through phosphorylation. PI3K/AKT signal pathway played an important role in regulating many biological processes including cellular metabolism, cell survival, apoptosis and others [16]. In our study, we

intended to determinate whether the inhibitory effect of sevoflurane anesthesia on rpS6 phosphorylation was mediated by the inhibition of PI3K/AKT pathway. Thus we measured the phosphorylation of AKT in neonatal rat brains by western blots to evaluate the hypothesis. Our data showed that neonatal exposure to sevoflurane anesthesia induced a significant inhibition of AKT activity in neonatal brains. In the cerebral cortex, AKT phosphorylation was not significantly reduced after the exposure to sevoflurane for 2 h, but was decreased remarkably after four hours (**Figure 3A**). AKT phosphorylation recovered to basal level after the remove of sevoflurane anesthesia (**Figure 3A**). Similarly, sevoflurane treatment for 4 h also suppressed the phosphorylation of AKT in the hippocampus of the neonatal brain (**Figure 3B**). As a control, the phosphorylation of AKT in the cerebral cortex and hippocampus of the neonatal brain was not affected by the treatment with 70% O₂ (data not shown).

Neonatal exposure to sevoflurane had no effect on mTOR activity in cerebral cortex and hippocampus

Mammalian target of rapamycin (mTOR) is one of the protein kinases related to PI3K. mTOR is a component of two distinct mTOR complexes, rapamycin-sensitive mTOR complex 1 (mTORC1) and rapamycin-insensitive mTOR complex 2 (mTORC2). And S6K is phosphorylated by mTORC1 [17, 18]. PI3K/AKT acts as the upstream of mTOR and phosphorylates mTOR at Ser2448. After the activation of mTOR, it activates S6K and inactivates 4E-BP1, the inhibitor of eIF4E [17, 18]. We hypothesized that sevoflurane treatment could suppress mTOR activity by inhibiting AKT activity. However, our data showed that sevoflurane anesthesia had no significant effect on the phosphorylation of mTOR in cerebral cortex (**Figure 4A**) and hippocampus (**Figure 4B**) of the neonatal rat brain.

Neonatal exposure to sevoflurane had a paradoxical effect on ERK phosphorylation in cerebral cortex and hippocampus

RSKs are minor rpS6 kinases [19]. Following the stimulation with growth factors, RSK is phosphorylated by the activated Ras/MAPK cascade. In mammals, three major MAPK path-

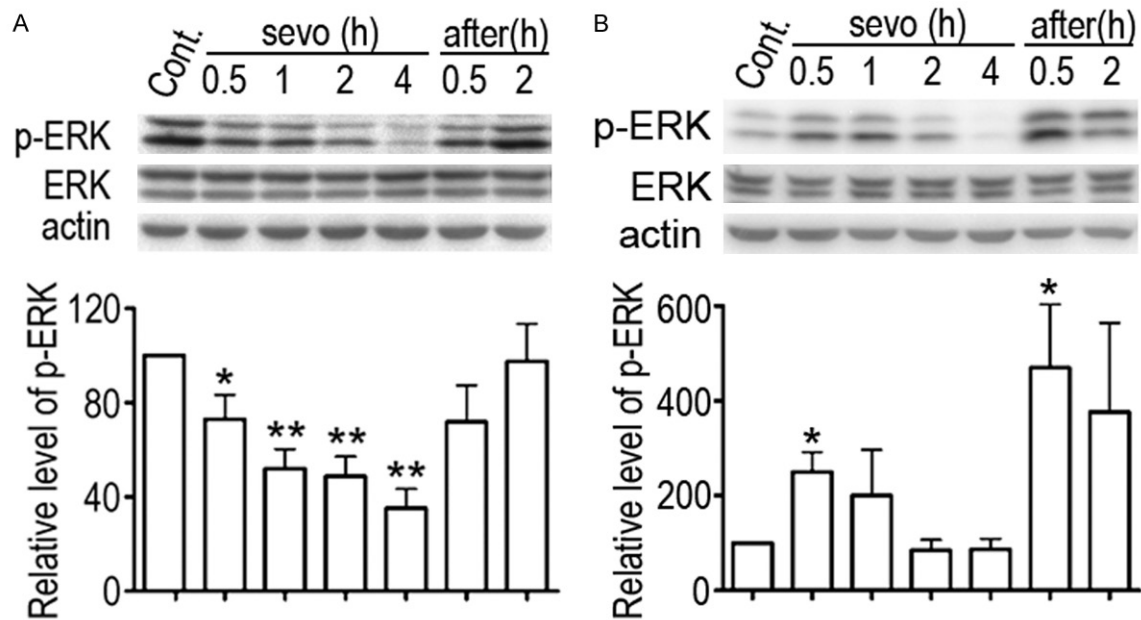


Figure 5. Anesthesia with 3% sevoflurane had a paradoxical effect on ERK activity in the cerebral cortex and hippocampus of neonatal rat brains. Western blot data shows sevoflurane anesthesia inhibits ERK phosphorylation at a time-dependent manner in cerebral cortex (A), but increases ERK activity in hippocampus (B). * $P < 0.05$, ** $P < 0.01$ versus control.

ways have been identified: extracellular signal-regulated kinase (ERK), c-JUN N-terminal kinase (JNK) and p38 kinase [20]. And ERK is the major MAPK that involves in the phosphorylation of RSK. We also presumed that the inhibitory effect of sevoflurane on the phosphorylation of rpS6 in neonatal rat brains was at least partially mediated through the inhibitory effect of sevoflurane on ERK pathway. In our study, we evaluated the effects of sevoflurane on three MAPK pathways in neonatal rat brains by western blots. Our data showed that sevoflurane anesthesia had a paradoxical effect on ERK activity. In cerebral cortex, sevoflurane anesthesia induced an inhibition of ERK phosphorylation with a time-dependent manner (**Figure 5A**). The inhibitory effect recovered 2 h after the removal of sevoflurane. In comparison, the treatment with sevoflurane induced an upregulation of ERK activity in the hippocampus after sevoflurane exposure for 0.5 h, and recovered to basal level after anesthesia for 2 h (**Figure 5B**). ERK activity was upregulated again at 0.5 h after the removal of sevoflurane anesthesia for 4 h. Then ERK phosphorylation recovered again 2 h after the termination of anesthesia (**Figure 5B**). As a control, the treatment with the carrier (70% O_2) had no effect on ERK signal

pathway in hippocampus and cerebral cortex of neonatal brains (data not shown). Thus these data demonstrated that sevoflurane anesthesia had a paradoxical effect on ERK activity in hippocampus and cerebral cortex of neonatal brains.

Besides the ERK pathway, we also evaluated the effect of neonatal exposure to sevoflurane on JNK and p38 signal pathways. Our data demonstrated that sevoflurane anesthesia had no effect on either JNK (**Figure 6A & 6B**) or p38 (**Figure 6C & 6D**) pathways in cerebral cortex and hippocampus of neonatal brains.

Neonatal exposure to sevoflurane enhanced the phosphorylation of PP1 in cerebral cortex and hippocampus

The phosphorylation of rpS6 is regulated by the kinases (S6Ks and RSKs) and the opposing phosphatases. Direct evidence showed that protein Phosphatase 1 (PP1) antagonized the phosphorylation of rpS6 [21]. We presumed that sevoflurane anesthesia might be able to alter the activity of PP1. Our data showed that sevoflurane anesthesia enhanced the phosphorylation of PP1 α in cerebral cortex (**Figure**

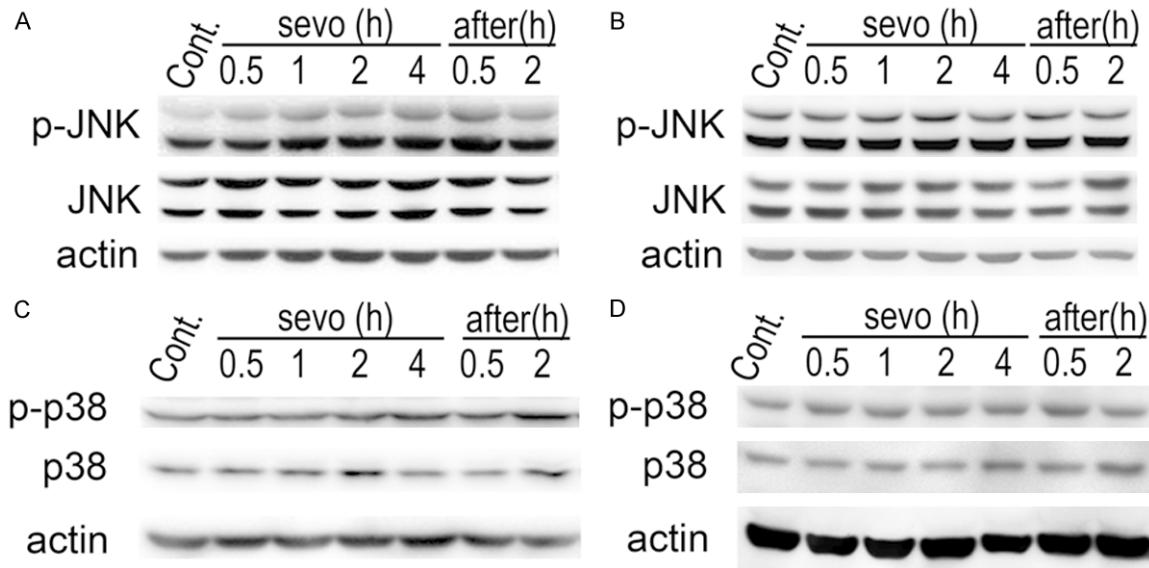


Figure 6. Anesthesia with 3% sevoflurane had no effect on the phosphorylation of JNK or p38 in the cerebral cortex and hippocampus of neonatal rat brains. Western blot data demonstrated sevoflurane anesthesia had no effect on JNK phosphorylation in cerebral cortex (A) and hippocampus (B) of neonatal rats. Western blot data demonstrate sevoflurane anesthesia had no effect on p38 phosphorylation in cerebral cortex (C) and hippocampus (D) of neonatal rats.

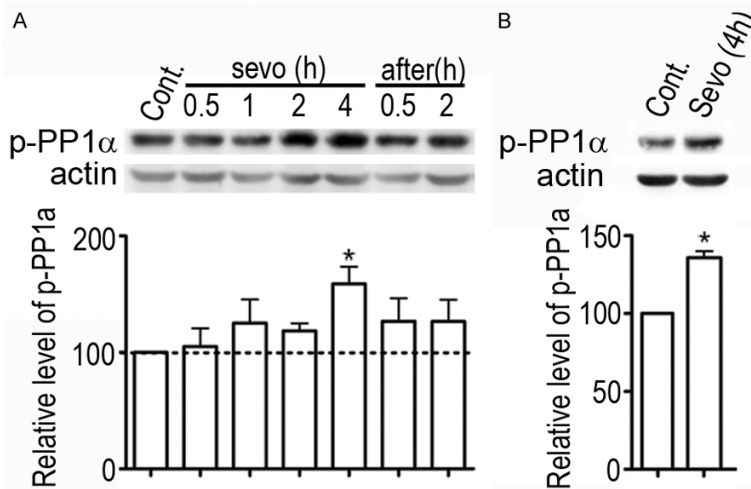


Figure 7. Anesthesia with 3% sevoflurane enhanced PP1 activity in cerebral cortex and hippocampus of neonatal rat brains. Western blot data demonstrated sevoflurane anesthesia increased the phosphorylation of PP1 in cerebral cortex (A) and hippocampus (B) of neonatal rats. * $P < 0.05$ versus control.

7A) and hippocampus (Figure 7B) of neonatal rat brains after the anesthesia for 4 h.

Discussion

Sevoflurane is one of the most commonly used anesthetics in pediatric surgeries. And the development of the brain in the infant stage is

essential. Thus it is important and urgent to understand deeply the toxic effects of those anesthetics in the neonatal brain and the underlying mechanisms. Previous studies demonstrated that neonatal exposure to inhaled anesthetics induced neuronal apoptosis in the brain [2, 3, 7, 8]. However, the underlying mechanisms are still unknown. In the present study, we showed, for the first time, that sevoflurane anesthesia suppressed rpS6 activity by inhibiting its phosphorylation in the developing neonatal rat brain. In the cerebral cortex and hippocampus of neonatal rats, sevoflurane suppressed AKT activity and

enhanced PP1 activity. However, sevoflurane had no significant effect on mTOR activity. Sevoflurane had a paradoxical effect on ERK activity that sevoflurane suppressed ERK activity in cerebral cortex, but it enhanced ERK activity in the hippocampus. Based on above-mentioned data, we proposed that the inhibitory effect of sevoflurane anesthesia on rpS6

activity was mediated by the enhancement of PP1 activity and the suppression of AKT activity through mTOR pathway. The role of ERK activity remained unclear.

Our data demonstrated that rpS6 phosphorylation in the neonatal brain was negatively regulated with the application of sevoflurane anesthesia. Sevoflurane suppressed rpS6 phosphorylation significantly with a time-dependent manner in cerebral cortex, hippocampus CA1 region, and other brain regions of the neonatal rat brain (**Figures 1 & 2**). Notably, sevoflurane had no significant inhibitory effect on the phosphorylation of rpS6 in CA3 region (**Figure 2B & 2D**). In the cerebral cortex, the neurons in lamina V were less sensitive to sevoflurane anesthesia than those in lamina II, III, IV and VI (**Figure 2F**). The difference of the sensitivity to sevoflurane needs to be investigated in the future. The inhibition of rpS6 phosphorylation was mainly found under stress conditions such as hypoxia, heat shock, and hyperosmolarity [10]. The inhibitory effect of sevoflurane on rpS6 phosphorylation indicated that sevoflurane anesthesia was a stress condition to the neonatal brain. The rpS6 protein is the first identified ribosomal protein that can be phosphorylated. The phosphorylation of rpS6 is important for its function in modulating the protein translation. The inhibitory effect of sevoflurane on rpS6 phosphorylation indicated that protein translation be potentially inhibited by sevoflurane anesthesia.

There are two families of kinases that phosphorylate rpS6 (S6K family and RSK family). S6Ks are the predominant kinases to phosphorylate rpS6. S6K is the downstream target of PI3K/AKT cascade [22], while RSK is the downstream target of MAPK cascade [19]. Our present data demonstrated that sevoflurane anesthesia induced the significant inhibition of AKT phosphorylation in cerebral cortex and hippocampus of neonatal brains (**Figure 3**). And the inhibitory effect of sevoflurane anesthesia on AKT phosphorylation was weaker than the effect on rpS6 phosphorylation. Since mTOR is downstream target of PI3K/AKT cascade, and can activate S6K, we also measured the effect of sevoflurane anesthesia on mTOR activity. Our data shown that sevoflurane anesthesia had no significant effect on the phosphorylation at Ser2448 of mTOR in cerebral cortex and hippocampus of neonatal brains (**Figure 4**).

Although hypoxia suppressed the phosphorylation of rpS6 by reducing mTOR activity [23], our data indicated that sevoflurane anesthesia inhibited rpS6 phosphorylation in the neonatal rat brain through the suppression of AKT activity independent of mTOR activity.

The RSK is directly activated by ERK1/2 in response to extracellular stimuli including neurotrophins, chemokines [19]. Our data demonstrated sevoflurane anesthesia had a paradoxical effect on ERK activity in the hippocampus and cerebral cortex of neonatal brains. Sevoflurane anesthesia enhanced the phosphorylation of ERK1/2 in hippocampus (**Figure 5B**), but suppressed the phosphorylation in cerebral cortex (**Figure 5A**). Our data also showed that sevoflurane had no effect on the activity of p38 or JNK (**Figure 6**). In cultured cerebral cortex neuron, sevoflurane induced ERK phosphorylation by activating protein kinase C (PKC) α and β II [24]. Sevoflurane and isoflurane also enhanced PKC activity in rat synaptosomes extracted from cerebral cortex [25]. And in cultured cortical and hippocampal neurons 5 days *in vitro*, isoflurane induced JNK phosphorylation [26], a downstream effector of p75 activation. The difference in ERK activation might be attributed to distinct experimental conditions. Our present data were derived from animal (*in vivo*) but not from *in vitro* culture experiments. The inconsistency in ERK activation between hippocampus and cerebral cortex remained unclear. The difference in JNK activation might be attributed to the different anesthetics. Sevoflurane had no significant effect on JNK activation, while isoflurane activated JNK.

Protein phosphatase-1 (PP1), an abundant phosphatase in the brain localizes in synaptic terminals, and negatively regulated synaptic strength [27]. Here, our data demonstrated that sevoflurane anesthesia enhanced the PP1 α activity in cerebral cortex and hippocampus of neonatal rat brain (**Figure 7A & 7B**). S6K was reported to be a substrate of PP1 [21]. Thus sevoflurane might inhibit the S6K activity by the enhancement of PP1 activity.

In conclusion, Sevoflurane inhibited the rpS6 activity in the neonatal rat brain (cerebral cortex, CA1 region of hippocampus, and other regions except CA3 region of the hippocampus).

pus). Sevoflurane inhibited AKT activity and enhanced PP1 activity in both cerebral cortex and hippocampus, which might underlie the inhibitory effect of sevoflurane anesthesia on rpS6 phosphorylation in neonatal rat brains.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Beijing) Grant No. 81171169, and Shanghai New 100-Talent Program Grant No. XBR2011023.

Disclosure of conflict of interest

The authors declare that there is no conflict of interest.

Address correspondence to: Yingwei Wang, Department of Anesthesia and Critical Care, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, No. 1665 Kongjiang Road, Shanghai, 200092, China. Tel: +862125078700; E-mail: wangyingwei@yahoo.com

References

- [1] Olney JW, Young C, Wozniak DF, Jevtovic-Todorovic V and Ikonomidou C. Do pediatric drugs cause developing neurons to commit suicide? *Trends Pharmacol Sci* 2004; 25: 135-139.
- [2] Jevtovic-Todorovic V, Hartman RE, Izumi Y, Ben-shoff ND, Dikranian K, Zorumski CF, Olney JW and Wozniak DF. Early exposure to common anesthetic agents causes widespread neurodegeneration in the developing rat brain and persistent learning deficits. *J Neurosci* 2003; 23: 876-882.
- [3] Istaphanous GK, Howard J, Nan X, Hughes EA, McCann JC, McAuliffe JJ, Danzer SC and Loepeke AW. Comparison of the neuroapoptotic properties of equipotent anesthetic concentrations of desflurane, isoflurane, or sevoflurane in neonatal mice. *Anesthesiology* 2011; 114: 578-587.
- [4] Kodama M, Satoh Y, Otsubo Y, Araki Y, Yonamine R, Masui K and Kazama T. Neonatal desflurane exposure induces more robust neuroapoptosis than do isoflurane and sevoflurane and impairs working memory. *Anesthesiology* 2011; 115: 979-991.
- [5] Zhang Y, Xu Z, Wang H, Dong Y, Shi HN, Culley DJ, Crosby G, Marcantonio ER, Tanzi RE and Xie Z. Anesthetics isoflurane and desflurane differently affect mitochondrial function, learning, and memory. *Ann Neurol* 2012; 71: 687-698.
- [6] Krasowski MD and Harrison NL. General anesthetic actions on ligand-gated ion channels. *Cell Mol Life Sci* 1999; 55: 1278-1303.
- [7] Satomoto M, Satoh Y, Terui K, Miyao H, Takishima K, Ito M and Imaki J. Neonatal exposure to sevoflurane induces abnormal social behaviors and deficits in fear conditioning in mice. *Anesthesiology* 2009; 110: 628-637.
- [8] Lu Y, Wu X, Dong Y, Xu Z, Zhang Y and Xie Z. Anesthetic sevoflurane causes neurotoxicity differently in neonatal naive and Alzheimer disease transgenic mice. *Anesthesiology* 2010; 112: 1404-1416.
- [9] Ventimiglia FA and Wool IG. A kinase that transfers the gamma-phosphoryl group of GTP to proteins of eukaryotic 40S ribosomal subunits. *Proc Natl Acad Sci U S A* 1974; 71: 350-354.
- [10] Meyuhas O. Physiological roles of ribosomal protein S6: one of its kind. *Int Rev Cell Mol Biol* 2008; 268: 1-37.
- [11] Ruvinsky I and Meyuhas O. Ribosomal protein S6 phosphorylation: from protein synthesis to cell size. *Trends Biochem Sci* 2006; 31: 342-348.
- [12] Ruvinsky I, Sharon N, Lerer T, Cohen H, Stolovich-Rain M, Nir T, Dor Y, Zisman P and Meyuhas O. Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev* 2005; 19: 2199-2211.
- [13] Volarevic S and Thomas G. Role of S6 phosphorylation and S6 kinase in cell growth. *Prog Nucleic Acid Res Mol Biol* 2001; 65: 101-127.
- [14] Chung J, Kuo CJ, Crabtree GR and Blenis J. Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. *Cell* 1992; 69: 1227-1236.
- [15] Pearce LR, Komander D and Alessi DR. The nuts and bolts of AGC protein kinases. *Nat Rev Mol Cell Biol* 2010; 11: 9-22.
- [16] Manning BD and Cantley LC. AKT/PKB signaling: navigating downstream. *Cell* 2007; 129: 1261-1274.
- [17] Wang X and Proud CG. The mTOR pathway in the control of protein synthesis. *Physiology (Bethesda)* 2006; 21: 362-369.
- [18] Zoncu R, Efeyan A and Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. *Nat Rev Mol Cell Biol* 2011; 12: 21-35.
- [19] Anjum R and Blenis J. The RSK family of kinases: emerging roles in cellular signalling. *Nat Rev Mol Cell Biol* 2008; 9: 747-758.
- [20] Johnson GL and Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002; 298: 1911-1912.

Sevoflurane for neonatal rat's brain

- [21] Hutchinson JA, Shanware NP, Chang H and Tibbetts RS. Regulation of ribosomal protein S6 phosphorylation by casein kinase 1 and protein phosphatase 1. *J Biol Chem* 2011; 286: 8688-8696.
- [22] Yap TA, Garrett MD, Walton MI, Raynaud F, de Bono JS and Workman P. Targeting the PI3K-AKT-mTOR pathway: progress, pitfalls, and promises. *Curr Opin Pharmacol* 2008; 8: 393-412.
- [23] Liu L, Cash TP, Jones RG, Keith B, Thompson CB and Simon MC. Hypoxia-induced energy stress regulates mRNA translation and cell growth. *Mol Cell* 2006; 21: 521-531.
- [24] Hasegawa J, Takekoshi S, Nagata H, Osamura RY and Suzuki T. Sevoflurane stimulates MAP kinase signal transduction through the activation of PKC alpha and beta1 in fetal rat cerebral cortex cultured neuron. *Acta Histochem Cytochem* 2006; 39: 163-172.
- [25] Moe MC, Berg-Johnsen J, Larsen GA, Kampen-haug EB and Vinje ML. The effect of isoflurane and sevoflurane on cerebrocortical presynaptic Ca²⁺ and protein kinase C activity. *J Neurosurg Anesthesiol* 2003; 15: 209-214.
- [26] Head BP, Patel HH, Niesman IR, Drummond JC, Roth DM and Patel PM. Inhibition of p75 neurotrophin receptor attenuates isoflurane-mediated neuronal apoptosis in the neonatal central nervous system. *Anesthesiology* 2009; 110: 813-825.
- [27] Morishita W, Connor JH, Xia H, Quinlan EM, Shenolikar S and Malenka RC. Regulation of synaptic strength by protein phosphatase 1. *Neuron* 2001; 32: 1133-1148.