Influence of sevoflurane exposure on mitogen-activated protein kinases and Akt/GSK-3β/CRMP-2 signaling pathways in the developing rat brain

YAFANG LIU[1*], CHUILIANG LIU[2*], MINTING ZENG[3], XUE HAN[1], KUN ZHANG[1], YANNI FU[1], JUE LI[1] and YUJUAN LI[1]

[1]Department of Anesthesiology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, Guangzhou, Guangdong 510120; [2]Department of Anesthesiology, Chancheng Center Hospital, Guangdong Medical College, Foshan, Guangdong 528030; [3]Department of Anesthesiology, Guangzhou Women and Children's Medical Centre of Guangzhou Medical University, Guangzhou, Guangdong 510523, P.R. China

Received October 19, 2016; Accepted October 20, 2017

DOI: 10.3892/etm.2017.5651

Abstract. Prolonged exposure to volatile anesthetics causes neurodegeneration in developing animal brains. However, their underlying mechanisms of action remain unclear. The current study investigated the expression of proteins associated with the mitogen-activated protein kinases (MAPK) and protein kinase B (Akt)/glycogen synthase kinase-3β (GSK-3β)/collapsin response mediator protein 2 (CRMP-2) signaling pathways in the cortices of neonatal mice following exposure to sevoflurane. Seven-day-old (P7) neonatal C57BL/6 mice were randomly divided into 2 groups and either exposed to 2.6% sevoflurane or air for 6 h. Terminal deoxyribonucleotide transferase mediated dUTP nick end labeling (TUNEL) staining, as well as the expression of activated caspase-3 and α-fodrin, was used to detect neuronal apoptosis in the cortices of mice. MAPK signaling pathways were investigated by detecting the expression of phosphorylated (p-) extracellular signal-regulated kinase 1/2 (ERK1/2), p-cyclic adenosine monophosphate response element-binding protein (CREB), p-p38, p-nuclear factor (NF-κB) and p-c-Jun N-terminal kinase (p-JNK). Akt/GSK-3β/CRMP-2 signaling pathways were assessed by detecting the expression of p-Akt, p-GSK-3β and p-CRMP-2 in the cortices of P7 mice 2 h following exposure to sevoflurane. The results demonstrated that sevoflurane significantly increased the apoptosis of cells in the retrosplenial cortex (RS), frontal cortex (FC) and parietal association cortex (PtA), increased the expression of cleaved caspase-3 expression and promoted the formation of 145 kDa and 120 kDa fragments from α-fodrin. Sevoflurane inhibited the phosphorylation of ERK1/2 and CREB, stimulated the phosphorylation of p38 and NF-κB, but did not significantly affect the phosphorylation of JNK. Furthermore, sevoflurane inhibited the phosphorylation of Akt, decreased the phosphorylation of GSK-3β at ser9 and increased the phosphorylation of CRMP2 at Thr514. These results suggest that multiple signaling pathways, including ERK1/2, P38 and Akt/GSK-3β/CRMP-2 may be involved in sevoflurane-induced neuroapoptosis in the developing brain.

Introduction

Exposure to general anesthetics during brain development may induce widespread apoptotic neurodegeneration in various mammalian species (1-4). Sevoflurane is an inhaled anesthetic commonly used in the clinic, particularly in pediatric medicine, due to its minimal airway reactivity and low blood/gas partition coefficient (5). Previous studies have indicated that sevoflurane causes biochemical changes, including apoptosis, amyloid-β accumulation and neuroinflammation in the hippocampus or cortex, and induces hippocampus-dependent and -independent cognitive dysfunction in developing mice (4,6,7). However, its underlying mechanisms of action remain unknown.

Mitogen-activated protein kinases (MAPKs) are a family of serine-threonine protein kinases that consist of three major members: Extracellular signal-regulated kinase 1/2 (ERK1/2), p38 MAPK and c-Jun N-terminal kinases (JNK) (8). MAPK signaling cascades serve crucial cellular roles under normal and pathological conditions, including nervous system development and neurodegeneration (9,10). Activation of the JNK and p38 pathways may contribute to apoptosis whereas the activation of ERK1/2 induces cell survival following central nervous system injury (11). ERK1/2-dependent phosphorylation of the cyclic adenosine monophosphate response element-binding protein (CREB) may lead to the transcriptional upregulation

Correspondence to: Professor Yujuan Li or Dr Jue Li, Department of Anesthesiology, Sun Yat-sen Memorial Hospital, Sun Yat-sen University, 107 Yanjiang West Road, Guangzhou, Guangdong 510120, P.R. China
E-mail: yujuan_04@hotmail.com
E-mail: leejue@foxmail.com

*Contributed equally

Key words: apoptosis, sevoflurane, mitogen-activated protein kinase, protein kinase B, developing brain

of the anti-apoptotic proteins Bcl-2 and brain-derived neurotrophic factor, which promote the survival and differentiation of neurons (12,13). It has been demonstrated that the transient suppression of ERK phosphorylation in neonatal mice causes marked apoptosis of brain cells and has profound long-term effects on brain function, including a reduction in long-term potentiation and memory impairments, and exhibiting no preference for interacting with animate vs. inanimate objects (14). Previous studies demonstrated that the inhaled anesthetic isoflurane suppresses ERK phosphorylation and increases the phosphorylation of p38, MAPK and JNK in the hippocampus of neonatal rats. Additionally, isoflurane increases neuronal apoptosis by activating the JNK and p38 MAPK pathways (15,16). However, it remains unclear exactly how sevoflurane affects the MAPK pathway.

Glycogen synthase kinase-3β (GSK-3β) functions in a wide range of cellular processes, including cell proliferation, differentiation, motility and apoptosis (17-19). It is one of the most important downstream targets of the protein kinase B (Akt) signaling pathway. Akt phosphorylating serine at position 9 in GSK-3β inhibits GSK-3β activity (20). In neurons, GSK-3β is involved in neuronal microtubule dynamics and determines axon/dendrite polarity by phosphorylating the downstream targets of GSK-3β, such as collapsin response mediator protein 2 (CRMP-2) (17,21,22). CRMP2 is involved in neuronal differentiation and axon growth via the binding of CRMP-2 and tubulin, which promotes microtubule assembly (23). In cultured neurons, CRMP2 has been demonstrated to be critical in axon specification, elongation and branching, thereby establishing and maintaining neuronal polarity (23). CRMP-2 has also been proved to co-localize with Numb and regulate Numb-mediated endocytosis, which is associated with axon growth (24). The binding of CRMP-2 to tubulin is inhibited following the phosphorylation of CRMP-2 by GSK-3β (21). Furthermore, it has been demonstrated that the Akt/GSK-3β/CRMP-2 signaling pathway serves important roles in the establishment of axonal-dendritic polarity *in vitro* (21) and in mediating axonal injury in the neonatal rat brain following hypoxia-ischemia *in vivo* (22). The inhibition of Akt signaling serves a critical role in isoflurane-induced neuroapoptosis in developing rats (25). Tao *et al* (26) also demonstrated that sevoflurane anesthesia stimulates Tau phosphorylation and activates GSK-3β in the hippocampus of young mice, causing cognitive impairment. However, it remains unknown how sevoflurane affects the Akt/GSK-3β/CRMP-2 pathway.

To determine the molecular mechanisms of neurotoxicity induced by anesthesia with sevoflurane, the current study investigated changes in the expression of proteins in the MAPK and Akt/GSK-3β/CRMP-2 signaling pathways in the cortices of 7-day-old neonatal mice.

Materials and methods

Animals. The current study was approved by the Animal Care Committee at Sun Yat-sen University (Guangzhou, China) and performed in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals (27). A total of 24 C57BL/6 male mouse pups, aged 7 days (P7) and weighing 3.5-4.5 g were obtained from Guangdong Medical Laboratory Animal Center (Guangdong, China; permission no. SCXK2011-0029). The pups were housed in the same cage as their mothers and were kept under temperature-controlled environmental conditions (26˚C) on a 14:10 constant light-dark cycle until P7. The mother mice had free access to food and water. The mouse pups at P7 were exposed to 2.6% sevoflurane (Jiangsu Hengrui Medicine Co., Ltd., Lianyungang, China) for 6 h [~1.0 minimal alveolar concentration (MAC) in P7 mice] in 50% oxygen in a temperature-controlled chamber, following a previously described protocol (n=12) (17). The control mice were exposed to normal air for 6 h under the same condition (n=12). The concentrations of anesthetic gas, oxygen and carbon dioxide in the chamber were measured using a gas analyzer (Datex-Ohmeda; GE Healthcare, Chicago, IL, USA). All animals were sacrificed 2 h following termination of sevoflurane/oxygen exposure and their cortices were used for western blotting (sevoflurane group, n=6; control group, n=6) or TdT-mediated dUTP nick end labeling (TUNEL) with fluorescent dye (sevoflurane group, n=6; control group, n=6).

Tissue preparation. Half of the mice in each group were used for western blotting and half of the mice for TUNEL studies. For western blotting, mouse pups were anaesthetized by inhaling 3% of sevoflurane until loss of the righting reflex (LORR), which indicated the mice had lost consciousness. Then the mice were sacrificed by decapitation. Cortices were isolated immediately on ice and then stored at -80˚C until use. For TUNEL studies, mouse pups were sacrificed by inhaling 3% of sevoflurane until LORR and perfused transcardially with ice-cold normal saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer for 10 min at 4˚C. Their brains were post-fixed in the same fixative for 48 h at 4˚C, and then paraffin embedded and sectioned into 6-μm-thick sections. As described in previous studies (15,16,25), at least three sections in the same plane of the hippocampus for each animal were selected to detect cells that exhibited positive TUNEL staining; all sections used in TUNEL were 100 μm apart and the sections were according to Figures 129-131 in the Atlas of the Developing Mouse Brain (28).

Western blotting. Western blotting was performed as previously described (15,16,25). Briefly, the protein concentration in each sample was determined using a BCA protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Sample proteins (40 μg/lane) were separated on 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% bovine serum albumin (Beyotime Institute of Biotechnology, Shanghai, China) in Tris-buffered saline with Tween-20 (TBST) at room temperature for 1 h. Membranes were subsequently incubated at 4˚C overnight with the following primary antibodies: Anti-cleaved caspase-3 (cat no. 9664) at 1:2,000 dilution, anti-α-fodrin (which contain SBDP145 and SBDP120 fragments; cat no. 2122) at 1:2,000 dilution, anti-phosphorylated-(p)-JNK (cat no. 4668) at 1:2,000 dilution, anti-JNK (cat no. 9252) at 1:2,000 dilution, anti-p-ERK1/2 (cat no. 4376) at 1:1,000 dilution, anti-ERK1/2 (cat no. 4695) at 1:1,000 dilution, anti-p-P38 (cat no. 4631) at 1:1,000 dilution, anti-P38 (cat no. 9212) at 1:1,000 dilution, anti-p-CREB (cat no. 9198) at 1:1,000 dilution, anti-p-nuclear factor-κB (NF-κB) (cat no. 3033) at 1:1,000

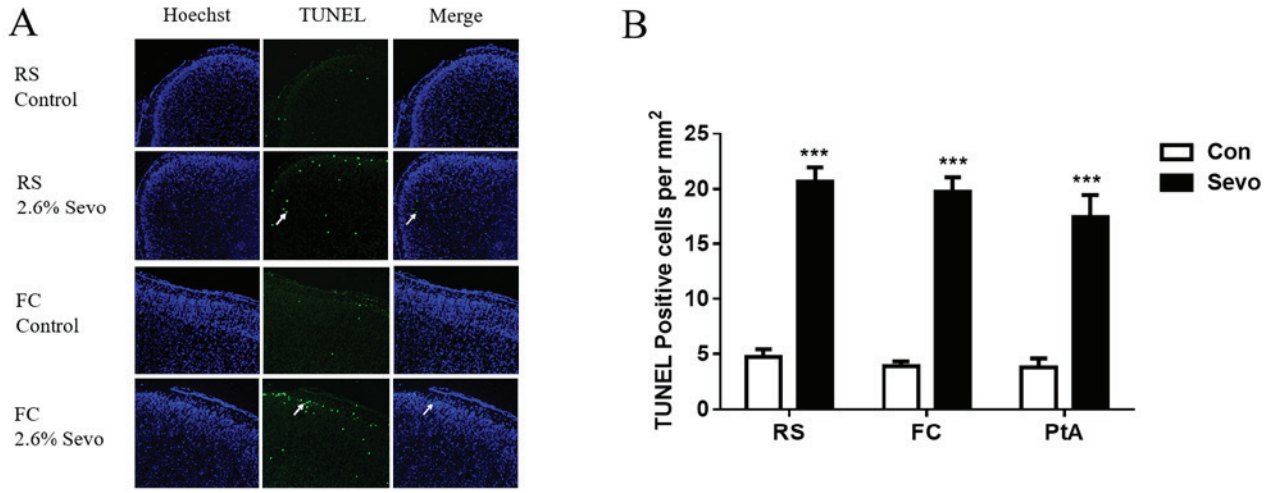

Figure 1. Sevoflurane increased the number of TUNEL positive cells in the cortices of P7 mice. Representative images of TUNEL staining in the (A) RS and FC regions of the cortices. Green staining indicated TUNEL-positive cells, blue staining indicated nuclear staining (magnification, x100). The white arrows indicate the TUNEL-positive cells. (B) Quantification of TUNEL positive cells in the RS, FC and PtA regions of the cortices. All results are presented as the mean ± standard deviation of the mean (n=6). ***P<0.001 vs. CON. TUNEL, Terminal deoxyribonucleotide transferase mediated dUTP nick end labeling; Con, control group; Sevo, sevoflurane group; RS, retrosplenial cortex; FC, frontal cortex; PtA, parietal association cortex; P7, 7-day-old neonatal mice.

dilution, anti-p-Akt (Ser 473) (cat no. 4060) at 1:2,000 dilution, anti-Akt (cat no. 4685) at 1:5,000 dilution, anti-p-GSK-3β (Ser 9) (cat no. 5558) at 1:2,000 dilution, anti-GSK-3β (cat no. 9315) at 1:2,000 dilution, anti-p-CRMP-2 (Thr 514) (cat no. 9397) at 1:2,000 dilution, anti-CRMP-2 (cat no. 9393) at 1:2,000 dilution and anti-β-actin (cat no. 3700) at 1:2,000 dilution (all Cell Signaling Technology, Inc., Danvers, MA, USA) and anti-p-GSK-3β (Ty 216) (cat no. ab75745; Abcam, Cambridge, USA) at 1:2,000 dilution. The membranes were washed with TBST three times and incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies (goat anti-mouse IgG, cat no. A0216; goat anti-rabbit IgG, cat no. A0208; 1:2,000; Beyotime Institute of Biotechnology) at room temperature for 1 h. The membranes were washed with TBST three times and visualized using an enhanced chemiluminescence detection system (cat no. 34580; Thermo Fisher Scientific, Inc.). Images were scanned using an Image Master II scanner (GE Healthcare) and were analyzed using Image Quant TL software (v2003.03, GE Healthcare). The band signals of p-ERK1/2, p-JNK, p-p38, p-Akt, p-GSK-3 and p-CRMP-2 were normalized to the bands of total ERK1/2, JNK, p38, Akt, GSK-3β and CRMP-2 from the same samples. The band signals of the other proteins were normalized to those of β-actin and the results in each group were normalized to that of the corresponding control group.

TUNEL assay. TUNEL was performed following a previously described protocol (15,16). A Dead End™ fluorometric TUNEL system (Promega Corporation, Madison, WI, USA) was used and staining following the manufacturer's protocol. Briefly, TUNEL labeling was conducted with a mix of 45 μl equilibration buffer, 5 μl nucleotide mix and 1 μl recombinant terminal deoxynucleotidyl transferase (rTdT) enzyme in a humidified, lucifugal chamber for 1 h at 37˚C, and then Hoechst 33258 (H-33258; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used to stain nuclei for 10 min at room temperature. The sections were protected by anti-Fade solution and mounted on glass coverslips with clear nail polish sealing the edges. Slides were protected from direct light during the experiment. The images of TUNEL positive cells in the retrosplenial cortex (RS), frontal cortex (FC) and parietal association cortex (PtA) areas were acquired by Ti-S inverted fluorescence microscope (Nikon Corporation, Tokyo, Japan) and analyzed using NIS-Elements Basic Research imaging processing and analysis software (version 3.0; Nikon Corporation). The density of TUNEL positive cells in the three cortical regions was calculated by dividing the number of TUNEL positive cells by the area of that brain region.

Statistical analysis. Sample size was calculated using PASS 11 software (NCSS, LLC, Kaysville, UT, USA) to achieve 80% power at a significance level of P<0.05. All data were determined to be normally distributed using the Shapiro-Wilk test and had no significant heterogeneity of variance as detected by Levene's test. GraphPad Prism 6.0 software (GraphPad Software Inc., La Jolla, CA, USA) was used to conduct all statistical analyses. Data were presented as mean ± standard deviation and were analyzed by Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

Results

Sevoflurane induces neuroapoptosis by activating caspase-3 and calpain in the cortices of developing mice. The results of preliminary experiments for arterial blood gas monitoring in the current study demonstrated that neonatal mice exhibited no hypoglycemia and acidosis during sevoflurane exposure. Neuronal apoptosis in the cortical RS, FC and PtA regions of P7 mice were detected by TUNEL (Fig. 1). Sevoflurane increased the number of apoptotic cells by 338.37% in RS, 409.78% in FC and 360.94% in PtA compared with controls (all P<0.001). In addition, changes in the expression of cleaved caspase-3 and α-fodrin (α-II-Spectrin) in the

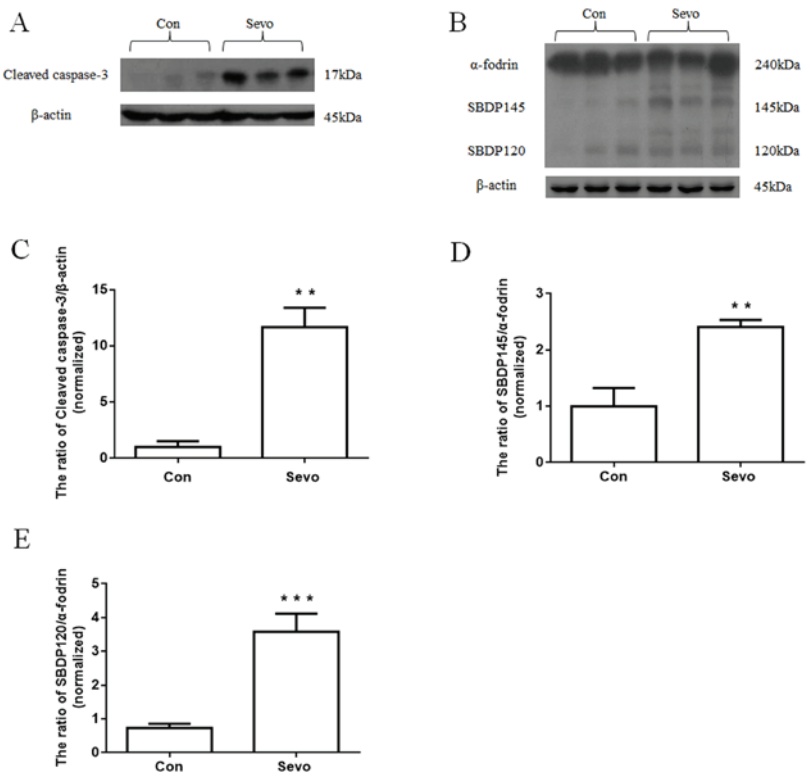

Figure 2. Sevoflurane increased the expression of cleaved caspase-3, SBDP145 and SBDP120 fragments of α-fodrin in the cortices of P7 mice. Representative western blots of (A) cleaved caspase-3 and (B) α-fodrin. Quantitative analysis of (C) cleaved caspase-3, (D) SBDP145 and (E) SBDP120 expression. All results are presented as the mean ± standard deviation of the mean (n=6). **P<0.01 and ***P<0.001, vs. Con. CON, control group; Sevo, sevoflurane group; P7, 7-day-old neonatal mice.

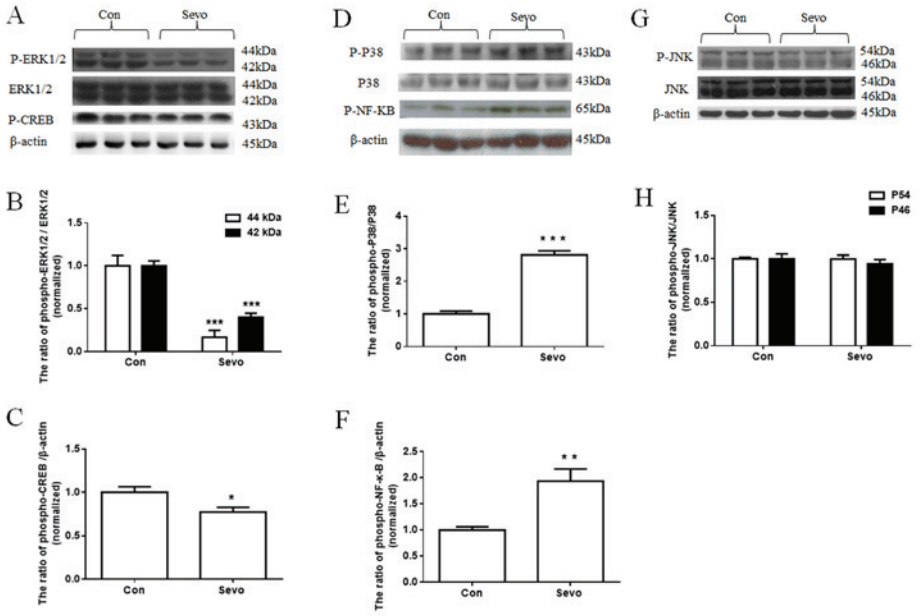

Figure 3. Sevoflurane inhibited the phosphorylation of ERK1/2 and CREB, increased the phosphorylation of p38 and NF-κB, and did not alter JNK activation in the cortex of P7 mice. Representative Western blots of (A) ERK1/2, p-ERK1/2 and p-CREB, (D) p-p38, P-38 and p-NF-κB and (G) JNK and p-JNK. Quantitative analysis of (B) p-ERK1/2 (44 and 42 kDa), (C) p-CREB, (E) p-p38, (F) p-NF-κB (F) and (H) p-JNK. The results are presented as the mean ± standard deviation of the mean (n=6). *P<0.05, **P<0.01, ***P<0.001 vs. CON. CON, control; SEVO, sevoflurane; NF-κB, nuclear factor κB; p-, phosphorylated; CREB, cyclic adenosine monophosphate response element-binding protein; JNK, c-Jun N-terminal kinase; ERK1/2, extracellular signal-regulated kinase 1/2.

cortices of mice were assessed by western blotting (Fig. 2). Sevoflurane anesthesia significantly increased the expression of cleaved caspase-3 protein expression by 11.66-fold. (P<0.01; Fig. 2A and C). To examine whether sevoflurane anesthesia influences calpain activity, the expression of α-fodrin in the cortex was measured. Cleavage of the 320 kDa full-length

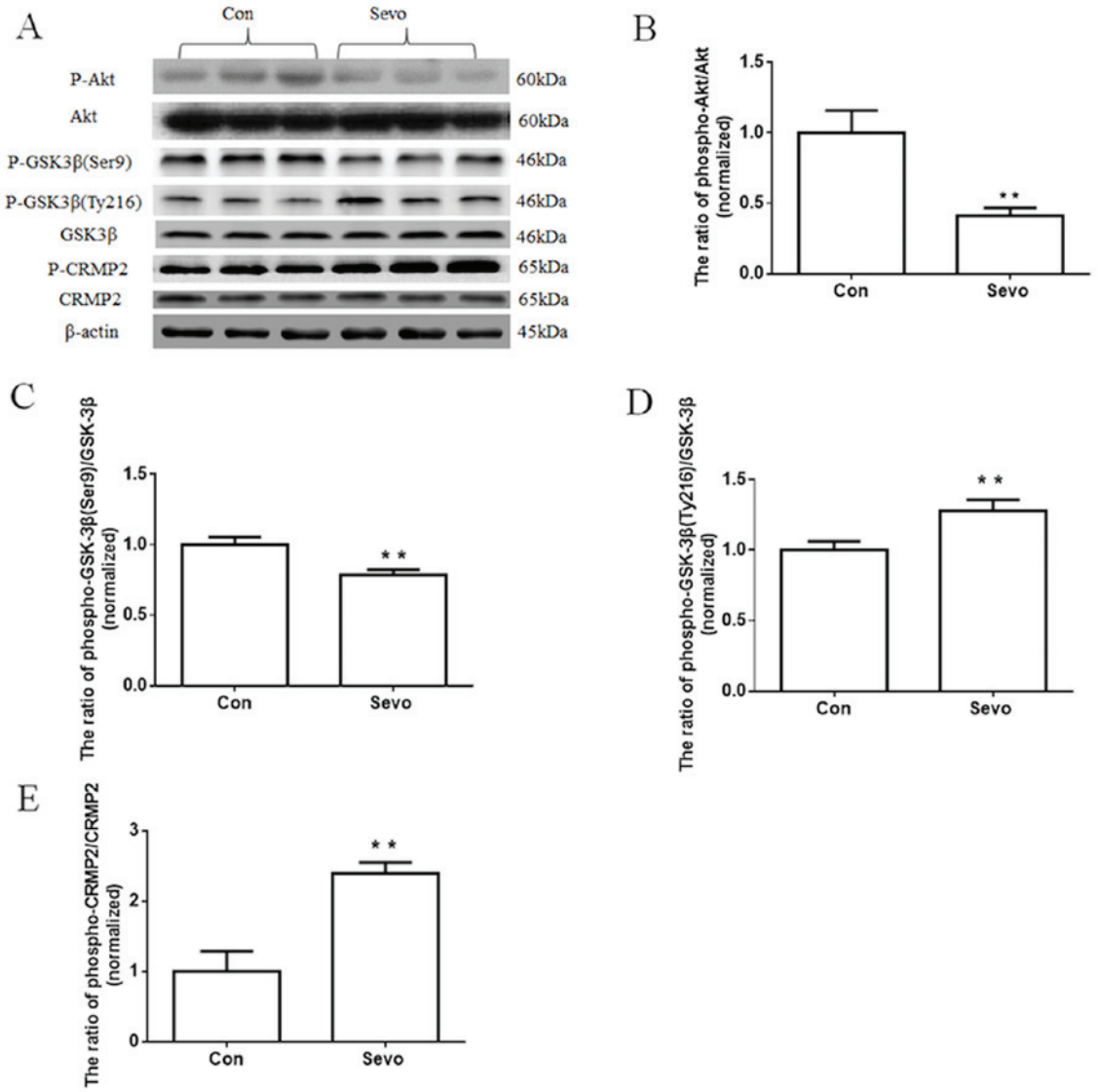

Figure 4. Sevoflurane inhibited the activity of the Akt/GSK-3β/CRMP2 pathway. Sevoflurane reduced the phosphorylation of Akt and GSK-3β (Ser 9) and increased phosphorylation of GSK-3β (Ty216) and CRMP2 (Thr 514) in the cortex of P7 mice. (A) Representative western blots of p-Akt, p-GSK-3β (Ser 9), p-GSK-3β (Ty216) and p-CRMP2 (Thr 514); Quantitative analysis of (B) p-Akt, (C) p-GSK-3β (Ser 9), (D) p-GSK-3β (Ty216) and (E) p-CRMP2 (Thr 514). Results are presented as the mean ± standard deviation of the mean (n=6). **P<0.01 vs. Con. P7, 7-day-old neonatal mice; Con, control group; Sevo, sevoflurane group; p-, phosphorylated; GSK-3β, glycogen synthase kinase-3β; Akt, protein kinase B; CRMP2, collapsin response mediator protein 2.

α-fodrin by calpain leads to the formation of the 145 kDa fragment (known as Spectrin breakdown product 145; SBPD145), which served as a relative measure of calpain activity, whereas the appearance of an additional 120 kDa fragment (also known as Spectrin breakdown product 120; SBPD120) served as an indicator of caspase-3 activity (29). The amount of the 145 kDa and 120 kDa protein fragment significantly increased by 140.1% (P<0.01; Fig. 2B and D) and 324.3% (P<0.001; Fig. 2B and E), respectively, immediately following termination of sevoflurane anesthesia.

The effect of sevoflurane on MAPK signaling pathways. Sevoflurane significantly decreased the expression of p-ERK1/2 at 44 kD by 73.9% and p-ERK1/2 at 42 kD by 47.0% (P<0.001; Fig. 3A and B). To further analyze ERK1/2 activity, the phosphorylation of CREB, one of the substrates of ERK1/2, was examined. A 22.6% decrease (P<0.05; Fig. 3A and C) in p-CREB expression was observed in the cortex following anesthesia with sevoflurane. By contrast, sevoflurane significantly increased the expression of p-p38 by 204.5% (P<0.001; Fig. 3D and E) and its downstream substrate p-NF-κB by 58.9% (P<0.01; Fig. 3D and F). The expression of p-JNK remained unchanged in the cortices following exposure to sevoflurane (P=0.0665; Fig. 3G and H). Furthermore, the expression of ERK1/2, JNK and p38 exhibited no significant differences between sevoflurane and control rats (data not shown).

The effects of sevoflurane on the Akt/GSK-3β/CRMP-2 pathway. To determine whether the Akt/GSK-3β/CRMP-2 signaling pathway is involved in sevoflurane-induced neuroapoptosis, the expression of Akt, GSK-3β and CRMP-2, and their phosphorylation were assessed in the cortex following anesthesia with sevoflurane. Western blot analysis demonstrated that sevoflurane inhibits Akt activity as, following sevoflurane treatment; the expression of p-Akt was significantly reduced

by 58.9% compared with the control (P<0.01; Fig. 4A and B). Sevoflurane activated GSK-3β by significantly reducing the expression of p-GSK-3β at Ser 9 by 21.45% (P<0.01; Fig. 4A and C) and significantly increasing the expression of p-GSK-3β at Ty216 by 28.02% (P<0.01; Fig. 4A and D). Furthermore, the expression of p-CRMP2 at Thr 514, which reflects GSK-3β activity, was significantly increased by 198.42% following sevoflurane anesthesia compared with the control (P<0.01; Fig. 4A and E). The expression of Akt, GSK-3β and CRMP-2 did not differ significantly between rats in the sevoflurane and control groups (data not shown).

Discussion

In the current study, it was demonstrated that 6 h exposure to 2.6% sevoflurane significantly increases neuronal apoptosis in the cortices of P7 mice. The activation of calpain and caspase-3 contributed to this neuronal apoptosis. Sevoflurane suppressed the phosphorylation of ERK1/2 and CREB, and promoted the phosphorylation of p38 and NF-κB, but did not influence JNK phosphorylation following 6-h exposure. Furthermore, sevoflurane inhibited the activity of the Akt/GSK-3β/CRMP-2 pathway by reducing the phosphorylation of Akt and GSK-3β, and increasing the phosphorylation of CRMP-2.

It has been demonstrated that sevoflurane induces neuroapoptosis, which is dependent on the depth of the anesthesia and is time- and brain region-specific (7,30,31). Previous studies have indicated that isoflurane induces more neurotoxicity than equivalent doses of sevoflurane (25,32). Exposure of P7 rats to 1% sevoflurane for 2 h did not result in severe neuroapoptosis, whereas increased concentrations of sevoflurane caused neuroapoptosis or cognitive dysfunction in the developing brain (31,33). In the current study, it was demonstrated that exposure of P7 rats to 2.6% sevoflurane (~1 MAC) activates capsase-3 dependent apoptosis, as indicated by the increase in cleaved 19/17-kDa caspase-3 subunits and generation of the α-fodrin 120 kDa fragments (31,33). Furthermore, the relative activity of calpain, manifested as changes in the level of proteolytic fragment in the 145 kDa of α-fodrin, was also increased following sevoflurane exposure in the cortices of P7 rats. Calpains are a family of cysteine proteases activated by calcium and autolytic processing. It has been demonstrated that the pathological activation of calpain leads to cytoskeletal protein breakdown, the loss of structural integrity, dysfunctions of axonal transport and eventually, neuronal cell death (34). Activated calpain may also bind to the apoptosis induced factor (AIF) and thus mediate the caspase-independent apoptotic pathway (35). Indeed, our previous study determined that sevoflurane increases the expression of AIF in the cortex of P7 rats (36). Therefore, it is possible that sevoflurane induces neuroapoptosis by activating the caspase-dependent and -independent pathways.

Sevoflurane induces abnormal social behaviors and cognitive dysfunctions in developing animals. CREB activation by phosphorylation is essential in the process of memory formation and maintenance by inducing the expression of genes that are essential for learning (13). Hardingham *et al* (37) demonstrated that depolarization-stimulated CREB phosphorylation is dependent on activation of the ERK and calcium-dependent protein kinase (CaMK) signaling pathways. The ERK pathway is responsible for the late phase of depolarization-stimulated CREB phosphorylation and the CaMK cascade regulates the early transient phase (38). The results of the current study demonstrate that sevoflurane decreases ERK phosphorylation and the phosphorylation of downstream CREB, indicating that sevoflurane may inhibit CREB activation by decreasing the phosphorylation of ERK. It has been demonstrated that the suppression of ERK phosphorylation is critically involved in the mechanism underlying sevoflurane-induced toxicity in the developing brain and that lithium or N-stearoyl-L-tyrosine attenuates anesthetic-induced neuroapoptosis by upregulating the ERK pathway. The results of the present study indicate that the ERK-CREB pathway may be involved in sevoflurane-induced neurotoxicity and cognitive dysfunctions.

Neuroinflammation contributes to volatile anesthetic-induced cognitive deficits and it has been demonstrated that isoflurane induces learning impairment by activating the NF-κB pathway and upregulating the expression of hippocampal interleukin-1β in rodents (39,40). Sevoflurane also increases levels of the pro-inflammatory cytokine interleukin-6 and tumor necrosis factor-α in the developing mouse brain (7). Anti-inflammatory therapy significantly attenuated the cognitive impairments induced by sevoflurane in young and aged rats (7,41). Our previous study demonstrated that isoflurane induces neuroapoptosis by activating the p38-NF-κB signaling pathway in the brain of developing rats (16). In the present study, it was demonstrated that sevoflurane activates the p38 pathway, as demonstrated by the increase in the phosphorylation of p38 and its downstream substrate NF-κB. However, further studies are required to identify whether the p38-NF-κB pathway is involved sevoflurane-induced neuroapoptosis and neuroinflammation. Unlike isoflurane, sevoflurane does not promote the phosphorylation of JNK, which is consistent with the results of a study by Wang *et al* (42) demonstrating that inhibition of JNK does not attenuate sevoflurane-induced neuroapoptosis. Furthermore, sevoflurane induces astrocytic dysfunction by inactivating the JAK/STAT pathway in the hippocampus of neonatal rats (43). These results suggest that the inhibition of the JNK pathway may contribute to neurotoxicity of sevoflurane.

The use of general anesthesia may increase the risk of Alzheimer's disease (AD). Isoflurane or sevoflurane promotes AD neuropathogenesis by inducing caspase activation, accumulation of β-amyloid (Aβ) and overt tau hyperphosphorylation (5,44,45). GSK-3 activation is a critical step in the cascade of detrimental events that occur during the development in AD, preceding the neurofibrillary tangles and neuronal death pathways (19). It has been demonstrated that sevoflurane induces Tau phosphorylation and GSK-3β activation in the hippocampus of developing mice (26). GSK-3β catalytic kinase activity is regulated by the differential phosphorylation of serine/threonine residues, including Ser 21 and Ser 9, which have an inhibitory effect, and tyrosine residues such as Tyr 279 and Tyr 216, which have an activating effect. CRMP-2, a phospho-protein involved in axonal outgrowth and microtubule dynamics, is aberrantly phosphorylated at Thr514 by GSK-3β in the brain of patients with AD (46). In the current study, sevoflurane decreased the phosphorylation of Akt and its downstream protein GSK-3β at Ser9 and also enhanced the phosphorylation of GSK-3β at Ty216, suggesting that sevoflurane activates GSK-3β. This

effect was confirmed by the effect of sevoflurane on CRMP2 phosphorylation at Thr514, which is one of the downstream sites of GSK-3β. Wang *et al* (47) suggested that the suppression of CRMP-2 hyperphosphorylation ameliorates β-amyloid-induced cognitive dysfunction and hippocampal axon degeneration. However, it remains unknown whether the suppression of CRMP-2 hyperphosphorylation ameliorates neurotoxicity of sevoflurane in the developing brain.

The current study had several limitations. Firstly, the changes in the expression of proteins were only evaluated 2 h following sevoflurane exposure and were not observed over a longer duration, which may improve understanding regarding the changes in the expression of these proteins. Additionally, specific inhibitors of p38 or GSK-3β were not used. These may be useful in determining the exact mechanism of some signaling pathways following exposure to sevoflurane. Additionally, synaptic morphology and the behavior of animals following exposure to sevoflurane was not assessed in the current study. These evaluations may contribute to clarifying the effects of these pathways on sevoflurane-induced long-time cognitive impairment.

In conclusion, the results of the current study demonstrated that sevoflurane induces changes in the expression of proteins that serve an important role in the brain development of neonatal animals. The proteins identified suggest that sevoflurane may disturb neuronal migration, differentiation and energy metabolism in the brains of neonatal rats, which may contribute to its neurodegenerative effects.

Acknowledgements

The present study was supported by National Natural Science Foundation of China (grant no. 81371259), the Natural Science Foundation of Guangdong Province (grant no. 2016A030313251), the Science and Technology Program of Gagungdong Province (grant nos. 2014A020212147 and A2013206), the Science and Technology Planning Project of Guangzhou, China (grant no. 201605122118121) and the Medical Science and Technology Program of Foshan (grant no. 20130841).

References

1. Brambrink AM, Evers AS, Avidan MS, Farber NB, Smith DJ, Zhang X, Dissen GA, Creeley CE and Olney JW: Isoflurane-induced neuroapoptosis in the neonatal rhesus macaque brain. Anesthesiology 112: 834-841, 2010.
2. Zheng H, Dong Y, Xu Z, Crosby G, Culley DJ, Zhang Y and Xie Z: Sevoflurane anesthesia in pregnant mice induces neurotoxicity in fetal and offspring mice Anesthesiology 118: 516-526, 2013.
3. Paule MG, Li M, Allen RR, Liu F, Zou X, Hotchkiss C, Hanig JP, Patterson TA, Slikker W Jr and Wang C: Ketamine anesthesia during the first week of life can cause long-lasting cognitive deficits in rhesus monkeys. Neurotoxicol Teratol 33: 220-230, 2011.
4. Zhang X, Xue Z and Sun A: Subclinical concentration of sevoflurane potentiates neuronal apoptosis in the developing C57BL/6 mouse brain. Neurosci Lett 447: 109-114, 2008.
5. Goa KL, Noble S and Spencer CM: Sevoflurane in paediatric anaesthesia: A review. Paediatr Drugs 1: 127-153, 1999.
6. Dong Y, Zhang G, Zhang B, Moir RD, Xia W, Marcantonio ER, Culley DJ, Crosby G, Tanzi RE and Xie Z: The common inhalational anesthetic sevoflurane induces apoptosis and increases beta-amyloid protein levels. Arch Neurol 66: 620-631, 2009.
7. Shen X, Dong Y, Xu Z, Wang H, Miao C, Soriano SG, Sun D, Baxter MG, Zhang Y and Xie Z: Selective anesthesia-induced neuroinflammation in developing mouse brain and cognitive impairment. Anesthesiology 118: 502-515, 2013.
8. Ravingerová T, Barancík M and Strnisková M: Mitogen-activated protein kinases: A new therapeutic target in cardiac pathology. Mol Cell Biochem 247: 127-138, 2003.
9. Roux PP and Blenis J: ERK and p38 MAPK-activated protein kinases: A family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev 68: 320-344, 2004.
10. Pearson G, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K and Cobb MH: Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. Endocr Rev 22: 153-183, 2001.
11. Liu AL, Wang XW, Liu AH, Su XW, Jiang WJ, Qiu PX and Yan GM: JNK and p38 were involved in hypoxia and reoxygenation-induced apoptosis of cultured rat cerebellar granule neurons. Exp Toxicol Pathol 61: 137-143, 2009.
12. Riccio A, Ahn S, Davenport CM, Blendy JA and Ginty DD: Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons. Science 286: 2358-2361, 1999.
13. Lonze BE and Ginty DD: Function and regulation of CREB family transcription factors in the nervous system. Neuron 35: 605-623, 2002.
14. Yufune S, Satoh Y, Takamatsu I, Ohta H, Kobayashi Y, Takaenoki Y, Pagès G, Pouysségur J, Endo S and Kazama T: Transient Blockade of ERK Phosphorylation in the critical period causes autistic phenotypes as an adult in mice. Sci Rep 5: 10252, 2015.
15. Li Y, Wang F, Liu C, Zeng M, Han X, Luo T, Jiang W, Xu J and Wang H: JNK pathway may be involved in isoflurane-induced apoptosis in the hippocampi of neonatal rats. Neurosci Lett 545: 17-22, 2013.
16. Liao Z, Cao D, Han X, Liu C, Peng J, Zuo Z, Wang F and Li Y: Both JNK and P38 MAPK pathways participate in the protection by dexmedetomidine against isoflurane-induced neuroapoptosis in the hippocampus of neonatal rats. Brain Res Bull 107: 69-78, 2014.
17. Yoshimura T, Kawano Y, Arimura N, Kawabata S, Kikuchi A and Kaibuchi K: GSK-3beta regulates phosphorylation of CRMP-2 and neuronal polarity. Cell 120: 137-149, 2005.
18. Martin M, Rehani K, Jope RS and Michalek SM: Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. Nat Immunol 6: 777-784, 2005.
19. Takashima A: GSK-3 is essential in the pathogenesis of Alzheimer's disease. J Alzheimers Dis 9 (3 Suppl): S309-S317, 2006.
20. Cross DA, Alessi DR, Cohen P, Andjelkovich M and Hemmings BA: Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature 378: 785-789, 1995.
21. Yoshimura T, Arimura N, Kawano Y, Kawabata S, Wang S and Kaibuchi K: Ras regulates neuronal polarity via the PI3-kinase/Akt/GSK-3beta/CRMP-2 pathway. Biochem Biophys Res Commun 340: 62-68, 2006.
22. Xiong T, Tang J, Zhao J, Chen H, Zhao F, Li J, Qu Y, Ferriero D and Mu D: Involvement of the Akt/GSK-3β/CRMP-2 pathway in axonal injury after hypoxic-ischemic brain damage in neonatal rat. Neuroscience 216: 123-132, 2012.
23. Inagaki N, Chihara K, Arimura N, Menager C, Kawano Y, Matsuo N, Nishimura T, Amano M and Kaibuchi K: CRMP-2 induces axons in cultured hippocampal neurons. Nat Neurosci 4: 781-782, 2001.
24. Nishimura T, Fukata Y, Kato K, Yamaguchi T, Matsuura Y, Kamiguchi H and Kaibuchi K: CRMP-2 regulates polarized Numb-mediated endocytosis for axon growth. Nat Cell Biol 5: 819-826, 2003.
25. Li Y, Zeng M, Chen W, Liu C, Wang F, Han X, Zuo Z and Peng S: Dexmedetomidine reduces isoflurane-induced neuroapoptosis partly by preserving PI3K/Akt pathway in the hippocampus of neonatal rats. PLoS One 9: e93639, 2014.
26. Tao G, Zhang J, Zhang L, Dong Y, Yu B, Crosby G, Culley DJ, Zhang Y and Xie Z: Sevoflurane induces tau phosphorylation and glycogen synthase kinase 3β activation in young mice. Anesthesiology 121: 510-527, 2014.
27. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals: Guide for the Care and Use of Laboratory Animals. 8th edition. National Academies Press, Washington, DC, 2011.
28. Paxinos G, Halliday G, Watson C, Koutcherov Y and Wang H: Atlas of the developing mouse brain at E17.5, P0 and P6. Academic Press, New York, NY, 2006.

29. Vanags DM, Pörn-Ares MI, Coppola S, Burgess DH and Orrenius S: Protease involvement in fodrin cleavage and phosphatidylserine exposure in apoptosis. J Biol Chem 271: 31075-31085, 1996.
30. Zheng SQ, An LX, Cheng X and Wang YJ: Sevoflurane causes neuronal apoptosis and adaptability changes of neonatal rats. Acta Anaesthesiol Scand 57: 1167-1174, 2013.
31. Xiao H, Liu B, Chen Y and Zhang J: Learning, memory and synaptic plasticity in hippocampus in rats exposed to sevoflurane. Int J Dev Neurosci 48: 38-49, 2016.
32. Liang G, Ward C, Peng J, Zhao Y, Huang B and Wei H: Isoflurane causes greater neurodegeneration than an equivalent exposure of sevoflurane in the developing brain of neonatal mice. Anesthesiology 112: 1325-1334, 2010.
33. Wang SQ, Fang F, Xue ZG, Cang J and Zhang XG: Neonatal sevoflurane anesthesia induces long-term memory impairment and decreases hippocampal PSD-95 expression without neuronal loss. Eur Rev Med Pharmacol Sci 17: 941-950, 2013.
34. Vosler PS, Brennan CS and Chen J: Calpain-mediated signaling mechanisms in neuronal injury and neurodegeneration. Mol Neurobiol 38: 78-101, 2008.
35. Lu JR, Lu WW, Lai JZ, Tsai FL, Wu SH, Lin CW and Kung SH: Calcium flux and calpain-mediated activation of the apoptosis-inducing factor contribute to enterovirus 71-induced apoptosis. J Gen Virol 94: 1477-1485, 2013.
36. Li Y, Liu C, Zhao Y, Hu K, Zhang J, Zeng M, Luo T, Jiang W and Wang H: Sevoflurane induces short-term changes in proteins in the cerebral cortices of developing rats. Acta Anaesthesiol Scand 57: 380-390, 2013.
37. Hardingham GE, Arnold FJ and Bading H: Nuclear calcium signaling controls CREB-mediated gene expression triggered by synaptic activity. Nat Neurosci 4: 261-267, 2001.
38. Wu GY, Deisseroth K and Tsien RW: Activity-dependent CREB phosphorylation: Convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway. Proc Natl Acad Sci USA 98: 2808-2813, 2001.
39. Cao L, Li L, Lin D and Zuo Z: Isoflurane induces learning impairment that is mediated by interleukin 1β in rodents. PLoS One 7: e51431, 2012.
40. Li ZQ, Rong XY, Liu YJ, Ni C, Tian XS, Mo N, Chui DH and Guo XY: Activation of the canonical nuclear factor-κB pathway is involved in isoflurane-induced hippocampal interleukin-1β elevation and the resultant cognitive deficits in aged rats. Biochem Biophys Res Commun 438: 628-634, 2013.
41. Gong M, Chen G, Zhang XM, Xu LH, Wang HM and Yan M: Parecoxib mitigates spatial memory impairment induced by sevoflurane anesthesia in aged rats. Acta Anaesthesiol Scand 56: 601-607, 2012.
42. Wang WY, Wang H, Luo Y, Jia LJ, Zhao JN, Zhang HH, Ma ZW, Xue QS and Yu BW: The effects of metabotropic glutamate receptor 7 allosteric agonist N, N'-dibenzhydrylethane-1, 2-diamine dihydrochloride on developmental sevoflurane neurotoxicity: Role of extracellular signal-regulated kinase 1 and 2 mitogen-activated protein kinase signaling pathway. Neuroscience 205: 167-177, 2012.
43. Wang W, Lu R, Feng DY and Zhang H: Sevoflurane Inhibits Glutamate-Aspartate Transporter and Glial Fibrillary acidic protein expression in hippocampal astrocytes of neonatal rats through the Janus Kinase/Signal Transducer and activator of transcription (JAK/STAT) pathway. Anesth Analg 123: 93-102, 2016.
44. Liu XS, Xue QS, Zeng QW, Li Q, Liu J, Feng XM and Yu BW: Sevoflurane impairs memory consolidation in rats, possibly through inhibiting phosphorylation of glycogen synthase kinase-3β in the hippocampus. Neurobiol Learn Mem 94: 461-467, 2010.
45. Dong Y, Wu X, Xu Z, Zhang Y and Xie Z: Anesthetic isoflurane increases phosphorylated tau levels mediated by caspase activation and Aβ generation. PLoS One 7: e39386, 2012.
46. Ni MH, Wu CC, Chan WH, Chien KY and Yu JS: GSK-3 mediates the okadaic acid-induced modification of collapsin response mediator protein-2 in human SK-N-SH neuroblastoma cells. J Cell Biochem 103: 1833-1848, 2008.
47. Wang Y, Yin H, Li J, Zhang Y, Han B, Zeng Z, Qiao N, Cui X, Lou J and Li J: Amelioration of β-amyloid-induced cognitive dysfunction and hippocampal axon degeneration by curcumin is associated with suppression of CRMP-2 hyperphosphorylation. Neurosci Lett 557: 112-117, 2013.