S6 inhibition contributes to isoﬂurane neurotoxicity in the developing brain

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HIGHLIGHTS

- Isoﬂurane induces S6, but not 4EBP1 inhibition.
- Isoﬂurane inhibits S6 activity via IGF-1/PI3K/Akt and IGF-1/MEK/ERK signaling pathways.
- S6 inhibition contributes to isoﬂurane-induced neuroapoptosis.
- S6 inhibition is involved in the isoﬂurane-induced synaptogenesis impairment.
- S6 inhibition is associated with spatial learning and memory decline caused by postnatal isoﬂurane exposure.

ABSTRACT

Postnatal isoﬂurane exposure leads to neurodegeneration and deﬁcits of spatial learning and memory in the adulthood. However, the underlying mechanisms remain unclear. Ribosomal protein S6 is demonstrated to play a pivotal role in control of cell survival, protein synthesis and synaptogenesis for brain development. In this study, the possible role of S6 and its upstream signaling pathways in the developmental neurotoxicity of isoﬂurane was evaluated using models of primary cultured hippocampal neurons and postnatal day 7 rats. We found that isoﬂurane decreased IGF-1 level and suppressed activation of IGF-1 receptor, sequentially inhibiting S6 activity via IGF-1/MEK/ERK and IGF-1/PI3K/Akt signaling pathways. S6 inhibition enhanced isoﬂurane-induced decreased Bcl-xL and increased cleaved caspase-3 and Bad, also reduced PSD95 expression and aggravated deﬁcits of spatial learning and memory. S6 activation could reverse the damages above. These results indicate that S6 inhibition, led by suppression of upstream IGF-1/MEK/ERK and IGF-1/PI3K/Akt signaling pathways, is involved in the neuroapoptosis, synaptogenesis impairment and spatial learning and memory decline caused by postnatal isoﬂurane exposure. S6 activation may exhibit protective potential against developmental neurotoxicity of isoﬂurane.

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1. Introduction

Since ketamine was fi rst reported to lead to neuroapoptosis and cognitive decline in the developing brain, the safety of general anesthetics has caused widespread concerns for anesthesiologists,
even the society (Ikonomidou et al., 1999). Hitherto, a majority of general anesthetics in pediatric anesthesia have been demonstrated to induce neurodegeneration in models of either neonatal rodents or nonhuman primates (Creeley et al., 2013; Loepke et al., 2009; Ramage et al., 2013). Isoflurane, a typical drug of inhalational anesthetics, is indicated to cause neuroapoptosis and this cell damages peak approximately 2 weeks after the cells are born in dentate granule of hippocampus (Hofacer et al., 2013; Loepke et al., 2009). Also, isoflurane interferes with acquisition of neuronal polarity, axon guidance and actin dynamics during the period of synaptogenesis (Lunardi et al., 2011; Mintz et al., 2013, 2012). However, the reason why isoflurane induces such changes and also causal relationship between cell damages and cognitive decline remain to be resolved.

Ribosomal protein S6, a downstream target of mTOR, promotes translation initiation via phosphorylation of eukaryotic translation initiation factor 4B (eIF4B) and translation elongation via phosphorylation of inhibitory eukaryotic elongation factor 2 (eEF2) kinase (Meyuhas and Dreazen, 2009). S6 activity is mediated by various cues such as neurotrophins and amino acids and downstream protein kinases such as mitogen-activated protein kinases (MAPK), Akt (protein kinase B; PKB) and adenosine monophosphate-activated protein kinase (AMPK) (Ruvinsky and Meyuhas, 2006). S6 has been proved to be pivotal in control of cell size, cell proliferation, protein synthesis, glucose homeostasis and cell survival (Meyuhas and Dreazen, 2009). Also, S6 plays an important role in the dendritic arborization, synaptogenesis and spine formation in the course of normal brain development and synaptic plasticity in the long-term potentiation (LTP), which is critical for memory (Antion et al., 2008; Cuesto et al., 2011; Jaworski et al., 2005).

Herein, the objective of present study was to investigate potential role of S6 and its upstream signaling pathways in the developmental neurotoxicity of isoflurane using models of primary hippocampal neurons and postnatal day 7 rats. The role of S6 in the neuroapoptosis, synaptogenesis impairment and decline of learning and memory caused by isoflurane exposure was particularly emphasized.

2. Materials and methods

2.1. Animals and reagents

The experimental protocol was approved by the Shanghai Jiao Tong University Ethics Committee for Care and Use of Laboratory Animals. Embryonic day 18 or postnatal day 7 Sprague-Dawley rats were purchased form SLAC Laboratory Animal (Shanghai, China). Animals were housed and bred in the facilities of Shanghai Jiao Tong University School of Medicine with food and water available ad libitum under a 12-h light/dark cycle at 22°C and terminated by adding 5 mL DMEM with 10% fetal bovine serum. The cell pellets were resuspended with DMEM supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 0.5 mM l-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and seeded onto multi-well culture plate precoated with 0.1 mg/mL poly-D-lysine. Following culture at 37°C in a humidified atmosphere of 5% CO2 for 4 h, the culture medium was replaced with Neurobasal medium containing 2% B27, 0.5 mM l-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin. On the second day, 5 μg/mL cytosine arabinoside was added into the medium to prevent glial cell proliferation. Later, half of medium was replaced every 2–3 days and neurons were used for experiments at 7–10 days in vitro (DIV).

2.2. Primary culture of hippocampal neurons

Hippocampal neurons were cultured following methods in the previous study (Wang et al., 2012). Briefly, E18 Sprague-Dawley pregnant rats were anesthetized with isoflurane and the embryos were removed. Hippocampi of embryos were digested with 0.05% trypsin for 10 min at 37°C and terminated by adding 5 mL DMEM with 10% fetal bovine serum. The cell pellets were resuspended with DMEM supplemented with 10% fetal bovine serum, 5% horse serum, 1 mM sodium pyruvate, 0.5 mM l-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin and seeded onto multi-well culture plate precoated with 0.1 mg/mL poly-D-lysine. Following culture at 37°C in a humidified atmosphere of 5% CO2 for 4 h, the culture medium was replaced with Neurobasal medium containing 2% B27, 0.5 mM l-glutamine, 1 mM sodium pyruvate, 100 U/mL penicillin and 100 μg/mL streptomycin. On the second day, 5 μg/mL cytosine arabinoside was added into the medium to prevent glial cell proliferation. Later, half of medium was replaced every 2–3 days and neurons were used for experiments at 7–10 days in vitro (DIV).

2.3. Isoflurane exposure and drug administration

The exposure protocol of isoflurane was established as described previously (Isaphanou et al., 2013; Loepke et al., 2009). For in vitro study, primary hippocampal cultures at day 7 received with or without 1.5% isoflurane in a gas mixture of 5% CO₂, 25% O₂ and 70% N₂ at 37°C in a tightly sealed plastic chamber. For in vivo study, postnatal day 7 rat pups were exposed to 1.5% isoflurane for 6 h in 50% oxygen. Control groups were performed in the same condition, except no isoflurane was flushed to the chamber. The gases used for in vitro and in vivo study were all delivered to the chamber at a flow rate of 21/min. The concentrations of isoflurane, CO₂, O₂ and N₂ were continuously monitored using a gas analyzer (Datex-Ohmeda, Inc., Louisville, CO, USA).

To investigate the role of S6 in the decline of learning and memory in the adulthood caused by isoflurane exposure, the S6 activator IGF-1 and S6 inhibitor PF-4708671 were administrated 30 min prior to exposure to 1.5% isoflurane for 6 h. The injections continued for another two days after isoflurane exposure. The concentration of human IGF-1 was adjusted to 10 μg/μL and the injections were performed subcutaneously at the dose of 1.8 μg/g body weight as described in the previous studies (Baldini et al., 2013; Landi et al., 2009). PF-4708671 was dissolved in DMSO and further diluted with soybean oil when drug administration began. A dose of 75 mg/kg body weight of PF-4708671 was delivered intraperitoneally as described in the study by Di et al. (2012). For comparison, control group or isoflurane group received intraperitoneal injection of equivalent volume of saline.

2.4. FITC Annexin V apoptosis detection

The procedures complied with the protocol of FITC Annexin V apoptosis detection kit (BD Biosciences, San Jose, CA, USA). FITC Annexin V positive and PI negative cells were considered to be apoptotic.

2.5. ELISA

Postnatal day 7 rats were inhaled with or without 1.5% isoflurane for 6 h and after treatment the hippocampi were harvested. The expression levels of IGF-1 in the hippocampal supernatants were measured with an ELISA kit (R&D Systems, USA).
2.6. Immunoﬂuorescence staining

After treatment with isoflurane, postnatal day 7 rats were anesthetized with an overdose pentobarbital (100 mg/kg i.p.) and transcardially perfused with saline, followed by cold 4% paraformaldehyde in phosphate buffered saline (Wang et al., 2012, 2014). The neonatal brain were removed, postﬁxed, dehydrated and cut into 25 µm thick coronal sections. The sections were simultaneously permeabilized and blocked with 10% donkey serum, 1% BSA and 0.3% Triton X-100 in phosphate buffered saline for 1 hour and incubated with polyclonal rabbit anti-p-S6 (Ser235/236) antibody (1:100 dilution) and monoclonal mouse anti-β-Tubulin-III antibody (1:200 dilution) overnight at 4 °C. The sections were then incubated with Alexa Fluor 488 donkey anti-rabbit and 594 donkey anti-mouse (Invitrogen™, Life Technologies Co., Grand Island, NY, USA) for 1 h at 37 °C. After immunostaining, the sections were mounted with a drop of anti-fade mounting medium (Beyotime, Shanghai, China) and stored in dark for observation with confocal microscopy.

Fig. 1. Effect of isoflurane on mTOR pathway in vitro and vivo. (A) Primary hippocampal neurons (7 DIV) were treated with 1.5% isoflurane for 6 h, 12 h and 24 h and after treatment the cultures were harvested for determining activation level of the mTOR pathway. (B) Bar graph summarizes experiments as shown in (A). The band intensities of p-S6 and p-4EBP1 are normalized to total proteins S6 and 4EBP1 respectively. The percentages with control group are calculated and compared. (C) Postnatal day 7 rat pups were inhaled with or without 1.5% isoflurane for 6 h. The hippocampi were harvested 0 h, 3 h, 6 h, 12 h and 24 h after treatment for Western blot analysis. (D) Bar graph shows experiments in (C). (E and F) After isoflurane exposure, the brains of rat pups were ﬁxed and immunostained with β-tubulin III, p-S6 and Hoechst 33342 for p-S6 location. Scale bar for (E) is 20 µm and scale bar for (F) is 200 µm. The red square is present to indicate expression change of p-S6 in the CA3 area of hippocampus in the control group and isoflurane group. All data are presented as mean ± S.E.M., n = 6 for each group, ***P < 0.001 versus Ctrl group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
2.7. Western blot

The neonatal cultures and hippocampi of postnatal day 7 rats were lysed in ice-cold RIPA buffer supplemented with PMSF, protease and phosphatase inhibitor cocktail solution as described previously (Shen et al., 2013). The protein concentrations of samples were determined by the BCA assay. Thirty to sixty micrograms of protein of each sample were loaded on an 8–15% SDS-PAGE gel and electrotransferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% nonfat milk and incubated with primary antibodies against p-IGF-1 receptor β (Tyr1135/1136), ERK1/2, p-ERK 1/2 (Thr202/Tyr204), Akt p-Akt Ser473, S6, p-S6 (Ser235/236), p-4EBP1 (Thr37/46), 4EBP1, cleaved caspase 3, Bad Bcl-xL, PSD95 (1:1000 dilution) and Gephyrin (1:200 dilution) with gentle agitation overnight at 4°C. The immunoreactive protein bands were detected and visualized with the ImageQuant LAS 4000 Mini (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). Densitometric measurement of band intensity was analyzed using Image J software.

2.8. Open-field test

The open-field test was performed to determine spontaneous locomotor activity of rats as described previously (Walsh and Cummins, 1976). The apparatus consisted of a square plywood arena (100 × 100 × 40 cm). The rats were placed into the center of open field and allowed to explore it for 5 min. During the interval between each trial, the open field was cleaned with 70% ethyl alcohol to strip any olfactory cues. The number of line crossings over a 5 min period was recorded to measure the locomotor activity.

2.9. Morris water maze test

The spatial learning and memory of young rats exposed to isoflurane at postnatal day 7 (P7) was measured using Morris water maze test as described previously (Li et al., 2007). Briefly, P49 rats from control and experimental groups were trained for place trials and probe trials in a tank 180 cm in diameter and 50 cm in depth, with a hidden 10-cm-diameter platform 1.5 cm below the surface of water in one quadrant. The place trials were conducted four times daily for five successive days and the probe trials were performed on the sixth day. For each place trial, rats were randomly placed into four quadrants to swim freely for a maximum of 60 s. If the platform was not found, the rats were guided to the platform and stayed for 20 s. The latency to find platform, swim speed and swim distance were recorded. For probes trials, the platform was removed and the rats were introduced to the quadrant opposite to where the platform located and allowed to swim for 60 s. Spatial retention was measured by time spent in the target quadrant compared to the rest quadrants.

2.10. Statistics

The data were expressed as mean ± S.E.M. The protein expression and apoptosis levels of experimental groups were presented as a percentage of those of control group. An unpaired two-tailed Student’s t test was used to calculate differences between two groups. Differences among multiple groups were analyzed by one-way ANOVA followed by post-hoc Tukey test. Behavioral evaluations (place trials in the Morris water maze) were analyzed using two-way repeated measures ANOVA followed by post-hoc Tukey test to show interaction between treatment and time and differences between two individual groups. *P < 0.05 was considered to be statistically significant. All statistical analyses were performed using the SPSS 18.0 software. The graphs were plotted with assistance of PRISM 5 software (GraphPad, La Jolla, CA, USA).

3. Results

3.1. Isoflurane induced S6, but not 4EBP1 inhibition in vitro and in vivo

mTOR signaling pathway functions through two major downstream substrates S6 and 4EBP1. To investigate whether isoflurane affected only S6 or both S6 and 4EBP1, we detected

Fig. 2. Effect of isoflurane on the activity of IGF-1 and IGF-1 receptor. Postnatal day 7 rats were treated with 1.5% isoflurane for 6 h and after treatment the hippocampi were harvested for determining level of IGF-1 by ELISA detection and level of p-IGF-1 receptor β by Western blot analysis. (A) Bar graph shows levels of IGF-1 in the control group and isoflurane group, n = 5 for each group. (B) Representative image showing expression level of p-IGF-1 receptor β in both control group and isoflurane group. (C) Bar graph summarizes experiments in (B). All data are presented as mean ± S.E.M., n = 6 for each group. *P < 0.05, ***P < 0.001 versus Ctrl group.
Fig. 3. Effect of IGF-1/MEK/ERK and IGF-1/PI3K/Akt signaling pathways in the isoflurane-induced S6 inhibition. (A) Representative images showing IGF-1-induced S6 activation in a dose-dependent manner. (B) The histogram shows the p-S6/S6 ratio in (A). (C) IGF-1 (200 ng/mL) alone or combined with LY294002 (25 μM) or MK-2206 (1 μM) were added into the neurobasal medium 30 min prior to exposure of neuronal cultures to 1.5% isoflurane for 6 h. The cells were lysed and subjected to Western blot analysis for detecting proteins expression of p-Akt Ser 473, Akt p-S6 and S6. (D) The histogram shows the p-Akt Ser 473/Akt and p-S6/S6 ratio in (C). (E) IGF-1 (200 ng/mL) alone or combined with U0126 (10 μM) or rapamycin (100 nM) were applied to primary hippocampal neurons 30 min before a 6-h exposure to 1.5% isoflurane. The cells were harvested for evaluating proteins expression of p-ERK, ERK, p-S6 and S6. (F) The histogram shows the p-Akt Ser 473/Akt and p-S6/S6 ratio in (E). All data were presented as mean ± S.E.M., n = 6 for each group, **P < 0.01, ***P < 0.001 versus Ctrl group, #P < 0.05, ##P < 0.01, ###P < 0.001 versus Iso group, ^^^P < 0.001 versus Iso + IGF-1 group.
phosphorylated S6 and 4EBP1 respectively. Primary hippocampal neurons (7 DIV) were treated with 1.5% isoflurane for 6 h, 12 h and 24 h. As shown in Fig. 1A, isoflurane induced p-S6/S6 decrease in a time-dependent manner. However, p-4EBP1/4EBP1 was not changed neither for 6 h, 12 h nor 24 h isoflurane treatment. A one-way ANOVA illustrated a significant difference in terms of p-S6/S6 expression between control group and various isoflurane exposure groups ($F=67.154, P<0.001$) (Fig. 1B). A post-hoc Tukey test showed that there were lower levels of p-S6/S6 in groups with 6 h, 12 h and 24 h isoflurane exposure as compared to control group ($P<0.001$) (Fig. 1B). No significant difference was observed in terms of p-4EBP1/4EBP1 expression between control group and isoflurane groups with different exposure levels ($F=1.570, P=0.228$) (Fig. 1B).

Next, we intended to test whether similar performance occurred in the animal model. Postnatal day 7 rat pups were inhaled with 1.5% isoflurane for 6 h. Similar to in vitro study, isoflurane induced p-S6/S6 decrease, but not p-4EBP1/4EBP1 (Fig. 1C). The effect of isoflurane on S6 inhibition lasted almost 24 h. A one-way ANOVA showed that p-S6/S6 expression displayed a great difference between control group and various post-isoflurane groups ($F=40.265, P<0.001$) (Fig. 1D). A post-hoc Tukey test showed that lower levels of p-S6/S6 were observed at 0 h, 3 h, 6 h, 12 h, but not 24 h after isoflurane exposure comparing with control group ($P<0.001$) (Fig. 1D). The p-4EBP1/4EBP1 expressions in various isoflurane groups were similar to that of control group ($F=0.306, P=0.906$) (Fig. 1D). To further demonstrate whether isoflurane induced S6 inhibition in the neuron of hippocampus, brains were fixed after isoflurane treatment and double-stained with phospho-S6 and β-tubulin-III by immunofluorescence. As shown in Fig. 1E and F, phospho-S6 and β-tubulin III co-located in the neuron of hippocampus in both control group and isoflurane group. Moreover, S6 inhibition in the isoflurane group diffusely occurred within CA1, CA3 and DG areas of hippocampus.

![Fig. 4](image-url)

**Fig. 4.** Effect of S6 inhibition on the isoflurane-induced neuroapoptosis. (A) IGF-1 (200 ng/mL) or PF-4708671 (5 μM) was applied to neuronal cultures (7 DIV) 30 min prior to exposure to 1.5% isoflurane for 6 h. After treatment, neuronal cultures were trypsined and incubated with FITC Annexin V and propidium iodide for detecting apoptosis by flow cytometry. FITC Annexin V positive and PI negative cells were considered to be apoptotic. (B) The histogram represents the number of apoptotic cells in (A), $n=4$ for each group. (C) After treatment with isoflurane, cell lysates were prepared for probing expression levels of cleaved caspase-3, Bad and Bcl-xL. (D) Bar graph shows experiments in (C), $n=6$ for each group. All data are presented as mean ± S.E.M., ***$P<0.001$ versus Ctrl group, *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus Iso group.
3.2. Isoflurane suppressed activation of IGF-1 and IGF-1 receptor

Insulin-like growth factor 1 (IGF-1) and IGF-1 receptors are extensively expressed in the developing brain and both are critical for brain development (Bondy et al., 1992; Niblock et al., 2000). To evaluate the effect of isoflurane on the activity of IGF-1 and IGF-1 receptor, postnatal day 7 rat pups were treated with 1.5% isoflurane for 6 h. As shown in Fig. 2A, IGF-1 expression level in the isoflurane group significantly decreased comparing with control group ($P = 0.0247$, Student’s $t$ test). Furthermore, isoflurane treatment reduced expression level of phosphorylated IGF-1 receptor (Fig. 2B). A Student’s $t$ test showed that there was a great difference between control group and isoflurane group ($P < 0.001$) (Fig. 2C). These results suggested that isoflurane suppressed IGF-1 activation.

3.3. Isoflurane inhibited S6 activity through IGF-1/PI3K/Akt and MEK/ERK pathways.

As shown in Figs. 1 and 2 above, S6 inhibition correlated with decrease of IGF-1 after exposure to 1.5% isoflurane for 6 h. The result in Fig. 3A further showed that supplementary IGF-1 could...
dose-dependently rescue the decreased phosphorylated S6. A one-way ANOVA showed that there were great differences between Ctrl group, Iso group, Iso + 50 ng/mL IGF-1 group, Iso + 100 ng/mL IGF-1 group and Iso + 200 ng/mL IGF-1 group (F = 26.938, P < 0.001) (Fig. 3B). A post-hoc Tukey test showed that p-S6/S6 expressions in Iso + 100 ng/mL IGF-1 group and Iso + 200 ng/mL IGF-1 were comparable respectively with that of Ctrl group (P = 0.678 and P = 0.855 respectively) (Fig. 3B). These results indicated that isoflurane inhibited S6 activity via suppressing IGF-1 activation. IGF-1 acts upstream signal of numerous signaling kinases, especially PI3K/Akt and MEK/ERK MAP kinases. Thus, we investigated the possible role of PI3K/Akt and MEK/ERK pathways in the isoflurane-induced S6 inhibition. As shown in Fig. 3C, p-S6/S6 decrease was accompanied by p-Akt/Akt reduction after isoflurane treatment, but returned to normal level when adding Akt agonist IGF-1 (200 ng/mL). However, the effect of IGF-1 was offset by PI3K antagonist LY294002 (25 μM) and Akt inhibitor MK-2206 (1 μM) (Fig. 3C). There were significant differences among these groups (F = 155.779, P < 0.001, one-way ANOVA) (Fig. 3D). A post-hoc Tukey test showed that p-S6/S6 level in Iso + IGF-1 group was higher than that of Iso group (P = 0.001), Iso + IGF-1 + LY294002 group (P < 0.001) and Iso + IGF-1 + MK-2206 group (P = 0.001) (Fig. 3D). To evaluate the effect of MEK/ERK pathway in the isoflurane-induced S6 inhibition, MEK inhibitor U0126 (10 μM) was co-treated with isoflurane and IGF-1. As shown in Fig. 3E, S6 activation by IGF-1 could be reversed by U0126. The p-S6/S6 level in Iso + IGF-1 + U0126 group decreased significantly as compared with Iso + IGF-1 group (P < 0.001, post-hoc Tukey test) (Fig. 3F). These results together suggested that IGF-1/PI3K/Akt and IGF-1/MEK/ERK pathways contributed to isoflurane-induced S6 inhibition. In addition, we examined the effect of mTOR in the S6 inhibition caused by isoflurane exposure. Treatment with rapamycin (100 nM) significantly decreased p-S6/S6 expression, indicating that mTOR was probably involved (Fig. 3E). Interesting, we found that p-ERK/ERK significantly increased after adding rapamycin, which was an mTOR inhibitor (Fig. 3E).

3.4. S6 inhibition was associated with isoflurane-induced neuroapoptosis

To investigate the role of S6 inhibition in the isoflurane-induced neuroapoptosis, S6 agonist IGF-1 (200 ng/mL) and S6 antagonist PF-4708671 (5 μM) were added into the medium 30 min prior to exposure of primary hippocampal neurons to 1.5% isoflurane for 6 h. As shown in Fig. 4A, treatment with isoflurane increased level of apoptotic cells assayed by FITC Annexin V detection and this level further increased when adding S6 antagonist PF-4708671, but significantly decreased after activating S6 by IGF-1. Significant differences were observed among Ctrl group, Iso group, Iso + PF-4708671 group and Iso + IGF-1 group (F = 146.355, P < 0.001, one-way ANOVA) (Fig. 4B). There were higher levels of apoptotic neurons in Iso + PF-4708671 group (P < 0.001, post-hoc Tukey test), but lower level in Iso + IGF-1 group (P < 0.001) as compared to Iso group (Fig. 4B). Similarly, a considerable increase in cleaved caspase-3 expression was found after isoflurane exposure, and this situation worsened when PF-4708671 was combined with isoflurane, however, the cleaved caspase-3 decrease could be reversed by IGF-1 (Fig. 4C). A one-way ANOVA showed there were great differences in terms of cleaved caspase-3 level among Ctrl group, Iso group, Iso + PF-4708671 group and Iso + IGF-1 group (F = 29.087, P < 0.001) (Fig. 4D). A post-hoc Tukey test indicated that cleaved caspase-3 level in Iso group was significantly lower than that of Iso + PF-4708671 group (P < 0.001), but higher than that of Ctrl group (P > 0.001) and Iso + IGF-1 group (P = 0.045) (Fig. 4D). Next, we investigated the mechanism by which S6 inhibition induced apoptosis. As shown in Fig. 4C, decrease of ratio of Bcl-xL to Bad corresponded well to S6 inhibition after isoflurane treatment, significantly decreased or increased when further inhibiting or activating S6 respectively. There were great differences in terms of ratio of Bcl-xL to Bad among Ctrl group, Iso group, Iso + PF-4708671 group and Iso + IGF-1 group (F = 40.843, P < 0.001, one-way ANOVA) (Fig. 4D). There were lower levels of ratio of Bcl-xL to Bad in Iso group than that of Ctrl group (P < 0.001, post-hoc Tukey test) and Iso + PF-4708671 group (P = 0.001), but higher levels in Iso + IGF-1 group (P = 0.031) (Fig. 4D). These results suggested that isoflurane triggered neuroapoptosis through inhibiting S6 and accordingly reducing ratio of Bcl-xL to Bad.

3.5. S6 inhibition contributed to isoflurane-induced synaptogenesis impairment

Except for inducing neuron damages, isoflurane may disturb synaptogenesis during brain growth spurt period. To investigate the effect of isoflurane on synaptogenesis, the expression levels of postsynaptic marker of inhibitory synapses Gephyrin and maker of excitatory synapses PSD95 were assessed by Western blot in vitro and in vivo. Primary hippocampal neurons (10 DIV) were treated with 1.5% isoflurane for 6 h, 12 h and 24 h respectively. As a result, PSD95 expression significantly decreased at all timepoints comparing with control group (Fig. 5A). However, Gephyrin expression did not change no matter the exposure to isoflurane for 6 h, 12 h or 24 h (Fig. 5A). A one-way ANOVA showed a significant difference in terms of PSD95 expression among Ctrl group, Iso 6 h group, Iso 12 h group and Iso 24 h group (F = 57.672, P < 0.001) (Fig. 5B). A post-hoc Tukey test showed that lower levels of PSD95 expression were observed in groups with 6 h, 12 h and 24 h isoflurane exposure as compared to control group (P < 0.001) (Fig. 5B). There was no significant difference in Gephyrin expression between control group and isoflurane groups with different exposure levels (F = 1.650, P = 0.210, one-way ANOVA) (Fig. 5B). In model of postnatal day 7 rats, the PSD95 expression in the hippocampi significantly decreased after inhalation with 1.5% isoflurane for 6 h and returned to normal level 24 h after isoflurane exposure (Fig. 5C). A one-way ANOVA showed there was a great difference between control group and various post-isoflurane groups (F = 22.831, P < 0.001) (Fig. 5D). A post-hoc Tukey test showed that lower levels of PSD95 were observed at 0 h (P < 0.001), 3 h (P < 0.001), 6 h (P < 0.001), 12 h (P = 0.049), but not 24 h after isoflurane exposure as compared to control group (Fig. 5D). These results in vitro and in vivo together showed that isoflurane impaired formation of excitatory synapses, not inhibitory synapses.

To investigate the role of S6 inhibition in the isoflurane-induced synaptogenesis impairment, S6 agonist IGF-1 (200 ng/mL) and S6 antagonist PF-4708671 (5 μM) were applied to primary hippocampal neurons 30 min before a 6-h exposure to 1.5% isoflurane. As shown in Fig. 5E, isoflurane treatment induced a significant decrease in PSD95 expression and S6 antagonist PF-4708671 enhanced this situation. Strikingly, activation of S6 with IGF-1 completely abolished the suppression of PSD95 expression by isoflurane treatment (Fig. 5E). There were significant differences in PSD95 level among Ctrl group, Iso group, Iso + PF-4708671 group and Iso + IGF-1 group (F = 41.338, P < 0.001, one-way ANOVA) (Fig. 5F). PSD95 level in Iso group was significantly higher than that of Iso + PF-4708671 group (P = 0.001, post-hoc Tukey test), but lower than that of Ctrl group (P < 0.001) and Iso + IGF-1 group (P = 0.001) (Fig. 5F). Isoflurane did not affect Gephyrin expression and activation or inhibition of S6 had no impact on the level of Gephyrin (Fig. 5E). In summary, S6 inhibition was
involved in the isoflurane-induced impairment of excitatory synapses formation.

3.6. **S6 inhibition was involved in the isoflurane-induced decline of learning and memory**

To investigate the role of S6 inhibition in the isoflurane-induced decline of learning and memory, postnatal day 7 rats were randomly allocated into four groups (Ctrl group, Iso group, Iso + IGF-1 group and Iso + PF-4708671 group respectively). Schematic of experimental procedure designed in the behavior study was showed in Fig. 6A. No significant difference was observed among four groups in terms of number of line crossings in the open-field test \((F=0.9997, P=0.4041, \text{one-way ANOVA})\) (Fig. 6B), suggesting that isoflurane, IGF-1 and PF-4708671 did not influence spontaneous locomotor activity of rats. In the place trials of Morris water maze, only young rats of Iso + PF-4708671 group had a significantly increased swimming speed at trial day 3 \((P=0.006, \text{post-hoc Tukey test})\), day 4 \((P=0.004)\) and day 5 \((P=0.040)\) as compared to Ctrl group (Fig. 6C). The time to find submerged platform in Iso group was longer comparing with Ctrl group at trial day 2 \((P=0.032, \text{post-hoc Tukey test})\) and trial day 3 \((P=0.013)\) (Fig. 6D). This situation worsened in Iso + PF-4708671 group \((P=0.016, P<0.001, P=0.008, P<0.001 \text{ at trial day } 2, 3, 4 \text{ and 5 respectively})\) (Fig. 6D). In the probe test of Morris water maze, significant differences existed among four groups \((F=12.792, P<0.001, \text{one-way ANOVA})\) (Fig. 6E). The number of target crossings in Iso group decreased (comparing with Ctrl group, \(P=0.017, \text{post-hoc Tukey test}\)) and further decreased when co-treatment with S6 inhibitor PF-4708671 (Iso + PF-4708671 group versus Iso group, \(P=0.024\)), but could be rescued by S6 agonist IGF-1 (Iso + IGF-1 group versus Iso group, \(P=0.041\)) (Fig. 6E). The results above suggested that postnatal isoflurane exposure probably impaired spatial learning and memory via S6 inhibition.

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**Fig. 6.** Effect of S6 inhibition on the decline of learning and memory in the adulthood caused by isoflurane exposure during postnatal period. (A) Schematic of experimental procedure designed in the behavior study. (B) The measurement of number of line crossings in the open-field test was used to represent locomotive activity of rats. The bar graph shows the locomotive activity of young rats in each group. (C and D) In the place trials of Morris water maze, the escape latency and swimming speed of young rats in each group was calculated. (E) In the probe test of Morris water maze, the number of target crossings in each group was recorded. All data are presented as mean ± S.E.M., \(n=10–12\) for each group, \(*P<0.05, **P<0.01, ***P<0.01\) versus Ctrl group, \(#P<0.05\) versus Iso group.
4. Discussion

The period of synaptogenesis, also called brain growth spurt period, matches with two weeks after birth for rodents and correspondingly continues from mid-gestation to the first two or three postnatal years for human beings (Dobbing and Sands, 1979). It has been demonstrated that immature neurons are sensitive to exogenous toxicants during this period (Olney et al., 2002). The present study also confirmed this phenomenon and showed that isoflurane, a commonly used anesthetic, could trigger neuroapoptosis and synaptogenesis impairment, therefore bringing out decline of learning and memory when grown up. Most importantly, our study for the first time discovered that S6 inhibition was responsible for the damages above and further indicated that upstream IGF-1/MAPK/ERK and IGF-1/PI3K/Akt signaling pathways contributed to isoflurane-induced S6 inhibition.

mTOR directly phosphorylates two major substrates: S6 and eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4EBP1) (Laplante and Sabatini, 2012). However, in the present study, treatment of developing brain with isoflurane resulted in S6 inhibition, but not 4EBP1, hinting that they are differentially regulated. Consistent with previous study, rapamycin treatment and knockdown of raptor, surprisingly inhibiting S6 but not 4EBP1, destroy differentiation of glutamatergic neurons from mouse embryonic stem cells (Wang et al., 2012). Furthermore, McNeill et al. (2008) have found that only S6 activation was required for neuronal growth and differentiation of photoreceptor in the Drosophila retina (McNeill et al., 2008). These findings can be partly illustrated by the fact that S6 is highly expressed in the developing neurons and 4EBP1 predominantly maintains in the other tissues except brain (Nishimura et al., 2011). The imbalanced distribution between S6 and 4EBP1 probably contributes to critical role of S6 in the neural differentiation and neurite growth in the course of brain development.

IGF-1, when binding to IGF-1 receptors, activates phosphoinositide 3-kinase (PI3K) and recruits PDK1 to the membrane to activate PKB/Akt followed by stimulation of protein synthesis via phosphorylation of downstream S6 (Fernandez and Torres-Aleman, 2012). Also, IGF-1 activates S6 and regulates cell proliferation, cell differentiation and cell survival through MEK/ERK pathway.

![Fig. 7](image.png)

**Fig. 7.** Proposed model illustrating the mechanism of isoflurane neurotoxicity in the developing brain. Exposure of developing brain to isoflurane reduces IGF-1 level and suppresses activation of IGF-1 receptor, sequentially inhibiting PI3 K/Akt and MEK/ERK signaling pathways and S6 activity. S6 inhibition leads to decrease of Bcl-xL and increase of Bad and thereby induces neuroapoptosis. Furthermore, S6 inhibition contributes decrease of PSD95 expression and destroys formation of excitatory synapses. Loss of cell number and synaptogenesis impairment together results in decline of spatial learning and memory when grown up.
Iso seconds showed that inhibition of S6 with PF-4708671 or MCF-7 breast cancer cells (Choi et al., 2013; Moon et al., 2005).

inhibits downstream PI3K/Akt and MAPK/ERK signaling pathways, suppresses activation of IGF-1 and IGF-1 receptor and thereby be cleaved by caspase-3 in the model of DNA-damaging agent (Chen et al., 2010).

Dhar et al. (2009) demonstrated that S6 must also reported that S6 inhibition induces apoptosis via down-regulating anti-apoptotic proteins Mcl-1 and survivin (Choi et al., 2009). What is more, Dhar et al. (2009) demonstrated that S6 inhibition contributed to the iso.

For the development of therapies, it is essential to understand the how S6 inhibition impacts synapses. The mechanism by which S6 inhibition impacts PSD95 expression and synaptogenesis itself during the rapid growth of synapses. As shown in our study, iso...