Inhibition of SPRY2 expression protects sevoflurane-induced nerve injury via ERK signaling pathway

Lin Hu¹, Jianan Du¹, Heye Zhu¹, Xia Xu¹, Zemei Mao²*
¹Department of Anesthesiology, Sanya Central Hospital (Hainan Third People’s Hospital), Sanya, Hainan Province, 572000,
²Department of Anesthesiology, Wuhan Children’s Hospital (Wuhan Maternal and Child Healthcare Hospital), Tongji Medical
College, Huazhong University of Science & Technology, Wuhan, Hubei Province 430016, China

*For correspondence: Email: zm_mao@163.com; Tel: +86-02782433292

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Abstract

Purpose: To investigate the effect of Sprouty2 (SPRY2) on sevoflurane (SEV) induced nerve injury in rats and its potential signaling pathway.

Methods: Male Sprague-Dawley rats were divided into sham and SEV groups containing six rats per group. Neurological injury assessment and H & E staining were performed to evaluate the degree of nerve injury in the rats, while quantitative polymerase chain reaction (qPCR) and immunoblot assays were performed to confirm the expression levels of SPRY2 in hippocampus tissues. Morris water maze tests were performed to determine the degree of cognitive deficit in rats. TUNEL and immunoblot assays were performed to evaluate the effects of SPRY2 on the apoptosis of hippocampus tissues.

Results: The SPRY2 expression was elevated in sevoflurane-induced hippocampus injury (p < 0.001). Ablation of SPRY2 inhibited sevoflurane-induced hippocampal neuron apoptosis (p < 0.001). In addition, depletion of SPRY2 promoted hippocampal neuron activity and decreased apoptosis (p < 0.001). Knockdown of SPRY2 promoted ERK signaling pathway, thereby protecting against sevoflurane-induced nerve injury and cognitive deficit in the rats (p < 0.001).

Conclusion: Sevoflurane induces cognitive dysfunction and upregulates SPRY2 expression in brain tissues in rats. The SPRY2 knockdown improves SEV-induced neural injuries and cognitive deficits, inhibits hippocampal neuron apoptosis, and enhances its activity. Meanwhile, SPRY2 depletion protects SEV-induced nerve injury via the ERK pathway. Thus, Sprouty2 could serve as a promising drug target for the treatment of SEV-induced cognitive dysfunctions.

Keywords: Sevoflurane, Sprouty2 (SPRY2), Nerve injury, Apoptosis, ERK signaling pathway

INTRODUCTION

Repeated exposure to anesthesia could lead to learning disabilities [1,2]. Sevoflurane (SEV) is a volatile anesthetic agent that is often used as an inhalant because of its early-wake properties [3]. Sevoflurane has the characteristics of low blood gas distribution coefficient, aromatic odor, quick action, and low airway irritation. It is one of the most commonly used volatile anesthetics to stimulate and maintain general anesthesia during surgery [4]. Some studies have shown that SEV easily crossed the blood-brain barrier, causing neurodegeneration of the central nervous system...
and long-term neurocognitive dysfunction [5]. Therefore, it is necessary to identify effective strategies to protect neurons from SEV-induced cell damage.

Sprouty (SPRY) protein is a negative feedback inhibitor of receptor tyrosine kinase signaling [6]. In vertebrates, SPRY2 is one of the dominant subtypes, while SPRY3 has a low level of detection in the brain [7]. SPRY1 and SPRY2 are strongly expressed in the early neural plate and could mediate cortical proliferation and differentiation [8]. SPRY2 and APRY4 mRNA decrease gradually during differentiation but are present in the adult hippocampus and cortex [9]. SPRY2 limits intestinal tuft and goblet cell numbers [10]. More recently, it has been reported that reduction of SPRY2 has promoted axon growth, as well as astrocyte proliferation and hippocampal neuroprotection in a mouse model of erythrocyamine-induced epilepsy [11]. Other studies have found that siRNA-mediated SPRY2/4 downregulation reduced ischemic brain injury and stimulated injury-induced astrocyte proliferation, thereby limiting neuronal cell death and lesion sizes [9]. This study indicated that SPRY2 expression was of great significance in brain injury. However, there is no study on the effect of SPRY2 on SEV-induced nerve injury. In the present study, the effect of SPRY2 expression on SEV-induced cognitive dysfunction in rats was investigated.

**EXPERIMENTAL**

**Animals and groups**

Male Sprague-Dawley rats (six rats per group, 8 – 10 weeks of age, 260 – 280 g) were obtained from Weitong Lihua (Beijing, China). All animal experiments were approved by the Medical Ethics Committee of Hainan Third People’s Hospital for the use of animals (approval no. LLKY211151). The research project followed the principles in the Guide for the Care and Use of Laboratory Animals [12]. The rats (divided into Sham and SEV groups) were maintained in a sterilized and 50 % humidity room at 24 ± 2 °C with free access to a standard chow and drinking water. The rats were anesthetized with 3 % SEV for 2 h to induce nerve injury. For SPRY2 knockdown, AAV targeting SPRY2 shRNA purchased from Hanbio Biotechnology was injected into rats.

**Evaluation of neurological injury**

The Modified Neurological Severity Score (MNSS) was used for the evaluation of neurological injury using rat motion, sensation and response, abnormal behavior, vision, touch, and balance. The score range of the MNSS test is 0 to 18. Among the scores, a score ranging from 13 to 18 means severe injury, a score of 7 to 12 means moderate injury, and a score of 1 to 6 means mild injury. A score of 0 reflects a normal state.

**H and E staining**

The brain tissues isolated from each group were sectioned into slices. The slices were then dehydrated with absolute alcohol and rehydrated with grading alcohol. Slides were incubated with hematoxylin for 4 min, rinsed, differentiated in 70 % alcohol, counterstained with eosin, and cleared in xylene followed by mounting.

**Quantitative real time-polymerase chain reaction (qRT-PCR)**

Cells were harvested and mRNAs were isolated with Total RNA Kit (Tiangen, Beijing, China). After the extraction of the RNA, it was used for reverse transcription to generate cDNA after the measurement of RNA concentration and purity. Real-time PCR was conducted with the use of a SYBR-Green Master Mix (RoPLCE1, USA) and the respective primers. The primers are listed in Table 1. The relative expression level was calculated through 2ΔΔCt.

**Table 1: The qPCR primer sequence**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
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<tbody>
<tr>
<td>IL-1β</td>
<td>CCGTTGCTGA</td>
<td>ACTTGCCACAGTC</td>
</tr>
<tr>
<td>β-actin</td>
<td>GAGATTACTG</td>
<td>GCAGGACTCCTC</td>
</tr>
</tbody>
</table>

**Immunofluorescence staining**

The brains were dehydrated, fixed, and embedded before being cut into 10 μm slices. The slices were then incubated with goat serum for 1 hour. Slices were further incubated with SPRY2 antibody (1:200 dilution; Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. After washing, the sections were incubated with fluorescent secondary antibody at room temperature for 1 hour. Finally, the slices were mounted with anti-quenching agents and then observed with a confocal fluorescence microscope.

**Western blotting**

Cell lysates were collected using RIPA buffer. After centrifugation, the protein concentration was measured with a BCA kit (Beyotime
Biotechnology, Shanghai, China). The proteins were then subjected to 10 % SDS-PAGE, then transferred onto polyvinylidene difluoride membranes. Then, the membranes were incubated with 5 % bovine serum albumin, followed by incubation in primary antibodies against SPRY2 (1:1,000; Cell Signaling Technology), Bcl-2 (1:1,000; Abcam, Cambridge, UK), Bax (1:1,000; Abcam), ERK1/2 (1:1,000; Cell Signaling Technology), p-ERK1/2 (1:1,000; Cell Signaling Technology), and β-actin (1:1,000; Abcam). The membranes were then incubated in horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:1,000 for 2 h after rinsing in TBST for 15 min. The blots were detected with an ECL detection kit (Invitrogen, Carlsbad, CA, USA).

Morris water maze (MWM) tests

To analyze neurological defects, MWM tests were performed in a swimming pool with four quadrants. The rats were trained for 5 days before the experiment, to allow the rats to become familiar with the platform location. The time to find the platform was then measured. Afterward, the platform was removed and the number of rats that reached the platform was recorded and the data analyzed.

TUNEL assay

Cell apoptosis was measured using a TUNEL assay (Roche Diagnostics, Basel, Switzerland). Paraffin-embedded sections were permeabilized and stained with 50 µL TUNEL reaction solution at 37 °C, followed by incubation in a dark box for 1 h. The sections were then counterstained with 4µm-diamidino-2-phenylindole and analyzed using a fluorescence microscope.

Cell culture

The H19-7 cells were incubated with DMEM with 10 % fetal bovine serum, 50 U/mL penicillin, and 50 mg/mL streptomycin. For SPRY2 depletion, an annealed oligonucleotide targeting SPRY2 (shRNA target: GCAGGTACATGTCTTGTCT) was inserted into pGLTR-puro plasmids and transfected into H19-7 cells.

Statistical analysis

Data are shown as the mean ± standard deviation (SD). Evaluation of statistical significance was performed using GraphPad (San Diego, CA, USA). Significance was assessed using the analysis of variance. A value of $p < 0.05$ were considered statistically significant.

RESULTS

SPRY2 was enhanced in the hippocampus of SEV-induced rats

After construction of a neural injury model in rats, the MNSS scores in SEV rats were measured. Sevoflurane treatment resulted in severe injury to rats (Figure 1 A) and morphological changes in the hippocampus after hematoxylin & eosin staining. Sevoflurane also resulted in a sparse distribution of neurons and a dense cytoplasm and nucleus (Figure 1 B). Moreover, SPRY2 was upregulated in the hippocampus of rats after SEV treatment, as assessed through qPCR and immunofluorescence analyses (Figure 1 C and D). Together, the results showed that SPRY2 was upregulated in the hippocampus of SEV-treated rats.

Figure 1: SPRY2 is enhanced in the hippocampus of SEV-induced rat model, (A) The MNSS score in the sham and SEV groups; (B) histological analyses in the hippocampus of rats in each group (C, D). The mRNA and protein levels of SPRY2 in each group as assessed through qPCR and immunofluorescence, respectively. ***$P < 0.001$ vs. sham

SPRY2 knockdown reduced SEV-induced neural impairment

To determine the effect of SPRY2 after SEV-induced brain damage, SPRY2 was ablated by injection of plasmid-containing AAV virus. SPRY2 was downregulated after AAV injection (Figure 2 A). The MNSS scores in SEV rats were recovered by knockdown of SPRY2 (Figure 2 B). Moreover, the histological lesions induced by SEV were inhibited by SPRY2 ablation (Figure 2 C). Together, these results indicated that the impaired neural defects were at least partially inhibited by SPRY2 depletion.
SPRY2 depletion ameliorated SEV-induced apoptosis

Elevated cell apoptosis was present in SEV-induced cognitively impaired rats. After SPRY2 depletion, cell apoptosis was significantly inhibited (Figure 4 A). The level of Bax was enhanced, while Bcl-2 was inhibited in the SEV-treated group (Figure 4 B). However, in SPRY2-depleted rats, Bax was reduced and Bcl-2 was enhanced when compared with the SEV-treated group (Figure 4 B). SPRY2 depletion ameliorated SEV-induced apoptosis.

SPRY2 depletion promoted cell viability and reduced cell apoptosis in SEV-induced H19-7 cells

To further confirm the role of SPRY2 in SEV-induced neuronal injury, an in vitro assay was performed. An increase of SPRY2 in SEV-induced cells was found in H19-7 cells (Figure 5 A), and SEV treatment reduced cell viability in cells, but SPRY2 depletion rescued the cell viability (Figure 5 B). Elevated cell apoptosis was also found in SEV-induced H19-7 cells, but after SPRY2 depletion, cell apoptosis was significantly inhibited (Figure 5 C). Furthermore, the level of Bax was enhanced and Bcl-2 was inhibited in SEV-treated cells (Figure 5 D). However, in SPRY2-depleted cells, Bax was reduced and Bcl-2 was enhanced when compared with SEV-treated cells (Figure 5 D). Together, the results showed that SPRY2 depletion promoted cell viability and reduced cell apoptosis in SEV-treated H19-7 cells.

SPRY2 depletion relieved SEV-induced cognitive injury by modulating the ERK pathway

For depicting the potential mechanism of SPRY2-mediated cognitive damage, the role of the ERK pathway was analyzed in each group. The expression level of p-ERK was repressed in
SEV rats and H19-7 cells, and SPRY2 ablation reversed the downregulation of p-ERK both in rats and H19-7 cells (Figure 6 A and B). Together, these results showed that SPRY2 depletion mitigated the neurotoxicity induced by SEV via the ERK pathway.

**Figure 5:** SPRY2 depletion promoted cell viability and reduced cell apoptosis in sevoflurane (SEV)-induced H19-7 cells, (A) The protein level of SPRY2 in each group, (B) The cell viability in shNC, shSPRY2, and SEV-treated shNC, shSPRY2 cells, (C) Cell apoptosis in shNC, shSPRY2, and SEV-induced shNC, shSPRY2 cells, (D) The levels of Bax and Bcl-2 in different groups. *P < 0.05, **p < 0.01, and ***p < 0.001

**Figure 6:** SPRY2 depletion alleviates sevoflurane (SEV)-induced cognitive injury by modulating the ERK pathway (A, B). The expression levels of ERK1/2 and p-ERK1/2 in shNC, shSPRY2, and SEV-induced shNC, shSPRY2 rats and cells. *P < 0.05, **p < 0.01, and ***p < 0.001

**DISCUSSION**

General anesthetics inhibit brain development as a result of their neurotoxicities [4]. Because of its early-wake properties, SEV is considered a volatile anesthetic. Studies have shown that SEV induced nerve injury [5]. Previous studies have also shown that severe stress is one of the important mechanisms of nerve damage. In addition, other studies have shown that SEV easily crossed the blood-brain barrier and caused neurodegeneration of the central nervous system. Sevoflurane can induce apoptosis of HT22 cells by inducing endoplasmic reticulum stress, so it is important to further study its pathogenesis and identify its key targets. In the present study, SPRY2 depletion was shown to inhibit SEV-induced nerve injury.

In vivo assays using immunohistochemical, qPCR, and Morris water maze assays showed that SPRY2 depletion inhibited nerve injury and cognitive deficits induced by SEV treatment in rats, which showed that SPRY2 was a critical protein in regulating SEV-induced nerve injury. SPRY2 was highly expressed in the early neural plate and was involved in cortical proliferation and differentiation [13]. Ablation of SPRY2 promotes axon growth and nerve regeneration in the peripheral nervous system [14]. In addition, ablation of SPRY2 reduces ischemic brain injury and stimulates injury-induced astrocyte proliferation, thereby limiting neuronal cell death and lesion sizes [15]. Overall, these studies confirmed that SPRY2 was involved in the regulation of nerve cells. However, the precise mechanism needs further study.

Transient inhibition of ERK phosphorylation in neonatal mice by intraperitoneal injection of SL327 has been reported to lead to apoptosis of brain cells, with profound long-term effects on brain function, such as long-term enhancement and reduction, impaired memory, and social deficits [16]. These results suggest that ERK phosphorylation plays an important role in neuronal development during the neonatal period. Effects induced by SL327 are similar to those induced by neonatal exposure [17]. Studies have also shown that metformin may reduce SEV-induced neuronal apoptosis and that it may play a neuroprotective role by activating erK1/2 phosphorylation. SPRYs act as a signal inhibitor by specifically interfering with processes upstream of ERK [18]. However, depletion of SPRY2 protected SEV-induced nerve injury via the ERK pathway, so the ERK pathway may be a possible target for nerve injury treatment.

**CONCLUSION**

Sevoflurane induces cognitive dysfunction and upregulates SPRY2 expression in brain tissues in rats. The SPRY2 knockdown improves SEV-induced neural injuries and cognitive deficits, inhibits hippocampal neuron apoptosis, and
enhances its activity. Moreover, SPRY2 depletion protects SEV-induced nerve injury via the ERK pathway. Therefore, SPRY2 might be as a promising drug target for the treatment of SEV-induced cognitive dysfunctions.

DECLARATIONS

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Ethical approval

Approval for this study was obtained from the Medical Ethics Committee of Hainan Third People’s Hospital for the use of animals (approval no. LLKY211151).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Lin Hu and Jianan Du designed the study and conducted the experiments, Heye Zhu supervised the data collection, and analyzed and interpreted the data; Xia Xu and Zemei Mao wrote the manuscript and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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