

# Activation of Canonical Notch Signaling Pathway Is Involved in the Ischemic Tolerance Induced by Sevoflurane Preconditioning in Mice

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## ABSTRACT

**Background:** A wealth of evidence has demonstrated that sevoflurane preconditioning induces brain ischemic tolerance, but the mechanism remains poorly understood. This study was designed to investigate the role of canonical Notch signaling in the neuroprotection induced by sevoflurane preconditioning in a mouse model.

**Methods:** C57BL/6 mice were pretreated with 1-h sevoflurane exposure at a dose of 2.5% for 5 consecutive days. Twenty-four hours after the last exposure, all mice were subjected to focal cerebral ischemia by right middle cerebral artery occlusion for 60 min. Neurobehavioral scores, brain infarct volumes, and cellular apoptosis were determined at 72 h after reperfusion (n = 10 per group). The activation of Notch signaling was evaluated (n = 5 per group), and its role in ischemic tolerance was assessed by intraperitoneal administration of  $\gamma$ -secretase inhibitor DAPT (100 mg/kg, n = 10 per group) and conditional Notch-RBP-J knockout technique (n = 8 per group).

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Received from the Department of Anesthesiology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi Province, China. Submitted for publication July 21, 2011. Accepted for publication June 29, 2012. Supported by National Science Fund for Distinguished Young Scholars, Beijing, China, grant 30725039 (to Dr. Xiong), the Major Program of National Natural Science Foundation of China, Beijing, grant 30930091 (to Dr. Xiong), and the National Natural Science Foundation of China, Beijing, grant 81072888 (to Dr. Wang). Drs. Yang and Yan contributed equally to this work.

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## What We Already Know about This Topic

- Sevoflurane preconditioning induces brain ischemic tolerance by an unknown mechanism

## What This Article Tells Us That Is New

- Sevoflurane preconditioning-induced protective effects against transient cerebral ischemic injuries are mediated by the activation of canonical Notch signaling pathway in mice

**Results:** Sevoflurane preconditioning reduced brain infarct volumes (42.5%), improved neurologic outcomes ( $P < 0.01$  vs. control), and attenuated neuronal cell apoptosis (cells positive for terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling reduced to 21.2%). The expression of Notch1 intracellular domain (1.35 folds) and the transcriptions of Hes1 (1.95 times) and Hes5 (1.48 times) were up-regulated. DAPT augmented the brain infarcts (1.64-fold) and decreased neurologic scores ( $P = 0.43$  vs. sevoflurane) in sevoflurane-preconditioned mice. Brain infarct volumes, neurobehavioral scores, and apoptotic cell numbers showed no significance between Notch knockout mice with sevoflurane preconditioning and wild-type mice without preconditioning.

**Conclusions:** Sevoflurane preconditioning-induced protective effects against transient cerebral ischemic injuries are mediated by the activation of canonical Notch signaling pathway in mice.

**P**RECONDITIONING with volatile anesthetics induces neuroprotective effects in cerebral ischemic animals<sup>1,2</sup> and prevents neurologic complications such as perioperative stroke in patients.<sup>3</sup> The underlying mechanisms were associated with down-regulation of apoptosis,<sup>4</sup>

◆ This article is accompanied by an Editorial View. Please see: Zuo Z: Novel mechanism for sevoflurane preconditioning-induced neuroprotection. ANESTHESIOLOGY 2012; 117:942–4.

activation of mitochondrial  $K_{ATP}$  channels,<sup>5</sup> reduction of excitatory amino acids,<sup>6</sup> and so forth. However, the mechanism is not fully understood.

Notch signaling pathway assumes a fundamental role in communications between cells in metazoans. It plays a pivotal role in neuronal proliferation, differentiation, and so forth.<sup>7,8</sup> The Notch signaling cascade appears remarkably simple with apparently no second messengers involved. Intramembrane proteolysis of receptor produces Notch receptor intracellular domain (NICD), which translocates to the nucleus and such with a DNA binding protein (RBP-J) to activate downstream target genes. This core transduction cascade is known as the “canonical” Notch pathway.

Recent studies showed the Notch signaling pathway was up-regulated after cerebral ischemia.<sup>9</sup> Ablation or attenuation of Notch receptor expression had a remarkable impact on stroke outcome in rodent models,<sup>9,10</sup> suggesting that Notch signaling may be an important determinant for the pathogenesis of cerebral ischemic injury. Furthermore, Notch signaling has been reported to participate in the protective effects of soybean isoflavone against cerebral ischemic injury in rats,<sup>11</sup> which indicates a prominent role of Notch in neuroprotection. However, the involvement of Notch signaling pathway in the neuroprotection conferred by sevoflurane preconditioning remains unclear.

Therefore, the aim of the current study was to investigate the role of the Notch signaling pathway in the neuroprotection induced by sevoflurane preconditioning in a mouse middle cerebral artery occlusion (MCAO) model, by using both pharmacologic and transgenic approaches. Specifically, we hypothesized that sevoflurane preconditioning would activate the canonical Notch signaling in brain, which could give rise to the protective effects against MCAO-induced brain infarcts, neurologic behavior damage, and neuronal apoptosis in mice.

## Materials and Methods

### Animals and the Preparation of RBP-J Knockout Mice

All animal-related procedures were approved by the Ethics Committee for Animal Experimentation of the Fourth Military Medical University (Xi'an, China), and proceeded in accordance with the guidelines for Animal Experimentation of the University. The male wild-type (WT) C57BL/6 mice (aged 10–12 weeks, weighing 20–25 g) were provided by the Experimental Animal Center of the Fourth Military Medical University.

Neuron-specific RBP-J knockout mice were generated by the Cre/loxP system. Conditional RBP-J allele (RBP-J floxed) mice were a generous gift from Professor Hua Han, M.D., Ph.D. (Department of Medical Genetics and Developmental Biology, Fourth Military Medical University). The CamKII $\alpha$ -Cre mouse, in which Cre expression is controlled by the forebrain-specific calcium- or calmodulin-dependent kinase II (CamKII) promoter in neuronal cells,<sup>12</sup>

was presented by Gunther Schutz, M.D. (Professor, German Cancer Research Center, Heidelberg, Germany). By mating RBP-J-floxed mice with CamKII $\alpha$ -Cre transgenic mice, we obtained bitransgenic mice CamKII $\alpha$ -Cre-RBP-J<sup>flox/-</sup>, which were then mated with each other to generate RBP-J knockout mice (CamKII $\alpha$ -Cre-RBP-J<sup>flox/flox</sup>). The genotypes of generated mice were identified by polymerase chain reaction (PCR) analysis with genomic DNA extracted from mouse tail biopsies. RBP-J floxed mice, CamKII $\alpha$ -Cre transgenic mice, and RBP-J knockout mice were viable and healthy and showed no macroscopic abnormalities by gross observations. Male mice were used in all experiments. WT and heterozygous (CamKII $\alpha$ -Cre-RBP-J<sup>flox/-</sup>) littermates were used in the studies as controls. All mice were maintained in specific pathogen-free condition with a light–dark cycle (12:12 hr), temperature at 21° ± 2°C, and humidity of 60–70%. Food and water were available *ad libitum*.

### Experiment Protocols

**Experiment 1.** To assess the neuroprotective effect of sevoflurane preconditioning, WT mice were randomly assigned to control, vehicle, and sevoflurane preconditioning (Sevo) groups. Mice in the control group were subjected only to MCAO, whereas mice in the vehicle and Sevo groups were subjected to MCAO at 24 h after oxygen or sevoflurane preconditioning. Neurologic function was scored at 72 h after MCAO, and mice were then decapitated to calculate the brain infarct volume.

**Experiment 2.** To study the effect of sevoflurane preconditioning on Notch signaling pathway activity, sevoflurane-preconditioned mice were compared with the naive mice. Protein expression of NICD in brain tissue was determined by western blot. Transcription levels of target genes (Hes1 and Hes5) were evaluated by real-time PCR at 0, 2, and 24 h after preconditioning. The cellular localization of NICD was examined by double immunofluorescence staining with neuronal nuclei at 2 h after preconditioning.

**Experiment 3.** To evaluate the role of the Notch signaling pathway in sevoflurane preconditioning,  $\gamma$ -secretase inhibitor DAPT (EMD Biosciences, San Diego, CA) was used. Mice were randomly assigned to naive, vehicle, DAPT, Sevo, and Sevo+DAPT groups. Animals in the DAPT and vehicle groups were injected intraperitoneally with DAPT (100 mg/kg) or its vehicle dimethyl sulfoxide, respectively, 2 h before oxygen preconditioning. DAPT, a potent inhibitor of activation of Notch receptor, was dissolved in 30% dimethyl sulfoxide and 70% saline. Sevo and Sevo+DAPT groups were pretreated with 1-h sevoflurane preconditioning per day, for 5 consecutive days. Animals in all groups were then subjected to MCAO 24 h after preconditioning. NICD protein concentrations in the brain were assessed by western blot 2 h after preconditioning. Neurologic scores and brain infarct volumes were evaluated 72 h after reperfusion.

The dosage and administration protocols used in this experiment were based on previous studies.<sup>13,14</sup>

**Experiment 4.** To further elucidate the role of the Notch signaling pathway in the ischemic brain injury, we used a conditional RBP-J-knockout approach. WT, RBP-J knockouts (RBP-J<sup>-/-</sup>), and heterozygous littermates (RBP-J<sup>±</sup>) were used. A total of six groups were studied, three with vehicle (oxygen) preconditioning (WT, RBP-J<sup>±</sup>, and RBP-J<sup>-/-</sup>) and three with sevoflurane preconditioning (Sevo, Sevo+RBP-J<sup>±</sup>, and Sevo+RBP-J<sup>-/-</sup>). All mice were subjected to MCAO 24 h after the last preconditioning. Neurologic scores and brain infarct volumes were determined 72 h after MCAO. A separate cohort of animals was used in the experiment to measure apoptotic cell death 24 h after MCAO.

### Sevoflurane Preconditioning

The protocol of sevoflurane preconditioning was based on our previous publication.<sup>2</sup> In brief, mice were placed in a temperature-controlled, transparent chamber comprised of an air-tight box (30 × 20 × 30 cm<sup>3</sup>) with a gas inlet and an outlet port. During preconditioning, the box was flushed with 2.5% sevoflurane (sevoflurane preconditioning) in 100% oxygen. Inspired and expired fraction of sevoflurane, oxygen, and carbon dioxide were monitored continuously (MP-60, Phillips Medical Systems, Best, The Netherlands). Soda lime (Molecular Products Limited, Essex, United Kingdom) was placed at the bottom of the container to clear the carbon dioxide.

### Focal Cerebral Ischemia and Reperfusion

The animals were fasted overnight but had free access to drinking water before surgery. MCAO was induced as described previously.<sup>15,16</sup> Briefly, C57BL/6 mice were anesthetized with 60 mg/kg pentobarbital sodium (intraperitoneal injection) and allowed to breathe spontaneously. After the right common

carotid artery was ligated, a 6–0 nylon monofilament with a rounded tip was inserted from the right common carotid artery to the right middle cerebral artery. Sixty minutes after brain ischemia, the filament was withdrawn to allow reperfusion of the right middle cerebral artery territory. The cerebral blood flow was monitored by laser Doppler flowmetry (PeriFlux 5000, Perimed AB, Järfälla, Sweden). During occlusion, a reduction in cerebral blood flow of more than 75% baseline (preischemia) was confirmed as effective occlusion; otherwise, animals were excluded. Throughout surgery, the temporal temperature was maintained at 37°C by a thermostatic blanket and a lamp. All animals undergoing MCAO had comparable exposure times (10.0 ± 1 min).

### Arterial Blood Gas Determination

Left femoral arterial catheters were placed in separate groups of control, vehicle and Sevo pretreatment (n = 5 in each group) to determine the arterial blood gas. Approximately 0.2 ml blood was taken *via* femoral artery of each mouse at the end of the last exposure, the onset of MCAO, and reperfusion, and the same volume of warm saline was injected simultaneously into the caudal vein. After the final blood sample was taken, the mice were euthanized, and blood gas testing was done immediately (Rapidlab 1260, Bayer HealthCare, Uxbridge, United Kingdom).

### Neurobehavioral Evaluation and Infarct Assessment

According to the method of Garcia *et al.*,<sup>17</sup> neurologic behaviors were assessed 72 h after MCAO by an observer who was blind to the animal groups and expressed as median (interquartile range). Animals were decapitated at 72 h after reperfusion, and 1-mm coronal sections throughout the brain were stained with 2,3,5-triphenyltetrazolium chloride (1%; TTC, Sigma-Aldrich, St. Louis, MO) to evaluate the infarct volume.<sup>18,19</sup> The infarct

**Table 1.** Physiologic Parameters

—	T (°C)	pH	Pao <sub>2</sub> (mmHg)	Paco <sub>2</sub> (mmHg)
End of APC				
Control	37.1 ± 0.13	7.40 ± 0.03	103.2 ± 0.5	37.2 ± 0.3
Vehicle	37.2 ± 0.22	7.41 ± 0.02	203.8 ± 1.2	34.4 ± 0.2
Sevo	37.0 ± 0.14	7.37 ± 0.03	201.5 ± 2.6	35.5 ± 0.3
Onset of MCAO				
Control	36.9 ± 0.13	7.38 ± 0.02	190.1 ± 0.5	38.3 ± 0.5
Vehicle	37.0 ± 0.12	7.39 ± 0.03	196.4 ± 0.3	39.2 ± 0.7
Sevo	36.9 ± 0.21	7.37 ± 0.05	193.7 ± 1.1	38.2 ± 1.2
Onset of reperfusion				
Control	36.8 ± 0.20	7.38 ± 0.04	187.1 ± 0.6	38.4 ± 0.8
Vehicle	37.0 ± 0.16	7.38 ± 0.02	192.0 ± 0.7	37.8 ± 0.9
Sevo	37.0 ± 0.23	7.37 ± 0.02	189.9 ± 1.2	37.9 ± 0.8

Data are mean ± SD. Control animals received MCAO only; vehicle animals received 100% oxygen vehicle 1 h per day for 5 days; Sevo animals received sevoflurane (2.5%) in 100% oxygen 1 h per day for 5 days.

APC = anesthetics preconditioning; MCAO = middle cerebral artery occlusion; Pao<sub>2</sub> = arterial oxygen tension; Paco<sub>2</sub> = arterial carbon dioxide tension; Sevo = sevoflurane preconditioning.

volume percentages were calculated by Swanson's method to correct for edema:  $100 \times (\text{contralateral hemisphere volume} - \text{nonlesioned ipsilateral hemisphere volume}) / \text{contralateral hemisphere volume}$ ,<sup>20</sup> and presented as mean  $\pm$  SD.

### Immunohistochemistry Staining

After being fixed and frozen, mouse brains were cut on a cryostat into 10- $\mu$ m coronal sections, approximately 1.3 mm rostral to bregma. In experiment 4, 24 h after MCAO apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL, Roche Diagnostics, Mannheim, Germany) and cleaved caspase-3 labeling. For TUNEL staining, sections were incubated with terminal deoxynucleotidyl transferase for 120 min and processed following the manufacturer's instructions. Positive DNaseI (Sigma-Aldrich) pretreated sections and terminal deoxynucleotidyl transferase incubation-omitted sections were used as positive and negative controls, respectively. Cleaved caspase-3 labeling was performed by incubating sections with primary rabbit anticlaved caspase-3 polyclonal antibody (1:50 dilution, Abcam, Cambridge, MA) overnight at 4°C, followed by goat antirabbit immunoglobulin G (1:400 dilution, Sigma-Aldrich). Immunoreactive products were visualized by the traditional diaminobenzidine methods. TUNEL and cleaved caspase-3-labeled cells were counted from images, acquired by using a 40 $\times$  objective lens from three random areas in penumbra (defined in previous report<sup>21</sup>), and expressed as cells per millimeter squared.

In experiment 2, for double-fluorescent staining, sections were incubated with rat antineuronal nuclei antibody (1:2,000, Chemicon, Billerica, MA) and rabbit anti-NICD antibody (1:400, Abcam) for 12 h at 4°C. After the sections were washed three times with phosphate buffered saline, they were incubated with TRITC-labeled goat anti-rat and FITC-labeled goat antirabbit secondary antibodies (1:800 dilutions for both, Vector Laboratories, Burlingame, CA). Sections were observed by using a fluorescence microscope (BX51, Olympus, Tokyo, Japan), and the images were captured.

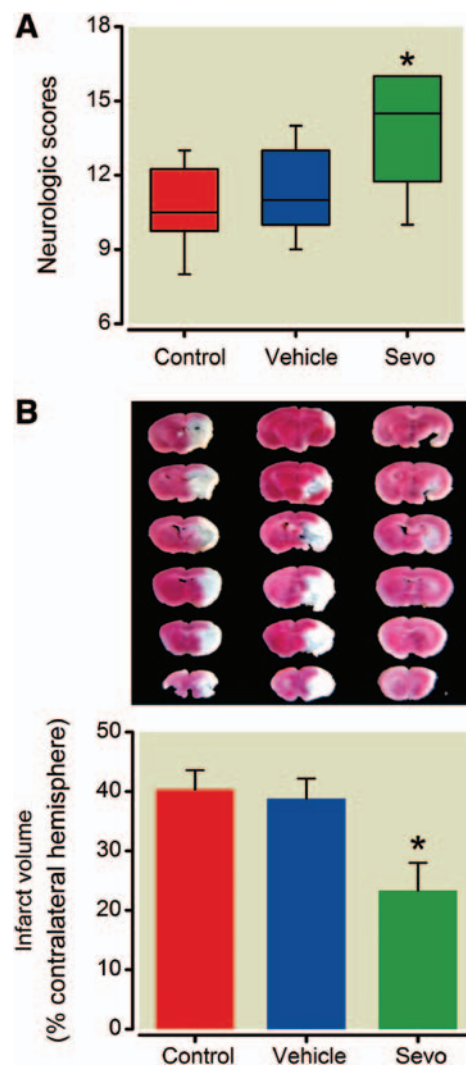
### Western Blot

The brain tissues for western blot and real-time PCR analysis were dissected in the same way as those of ischemic penumbra.<sup>21</sup> The tissues were homogenized in RIPA lysis buffer (Beyotime, Nantong, China) with 1 mM phenylmethanesulfonyl fluoride on ice. The western blot was performed by using the following primary antibodies: rabbit anti-Notch1 NICD antibody (1:800, Abcam, Cambridge, United Kingdom) and rabbit anti-Hes1 and anti-Hes5 antibodies (1:1,000 for both, Santa Cruz Biotechnology, Santa Cruz, CA). Appropriate secondary horseradish peroxidase-conjugated goat antirabbit antibody (1:5,000, Pierce, Rockford, IL) was used.

### Real-time PCR

The total RNA of ipsilateral hemispheres was extracted using RNAiso Plus (TaKaRa, Dalian, China) as described

in a prior protocol.<sup>22</sup> Quantitative PCR was completed with the Bio-Rad iQ5 Gradient Real-Time PCR system (Bio-Rad Laboratories, Hercules, CA). Quantified values of RNA were normalized with those of glyceraldehyde 3-phosphate dehydrogenase and shown by the average with an error bar. The following primers were used in the current study: Hes1 forward: AAA GAC GGC CTC TGA GCA C; Hes1 reverse: GGT GCT TCA CAG TCA TTT CCA; Hes5 forward: GAA GGC CGA CAT CCT GGA GA Hes5 reverse: ACC AGG AGT AGC CCT CGC TGT A; glyceraldehyde 3-phosphate dehydrogenase forward: TCG ACA GTC AGC CGC ATC TTC TT; glyceraldehyde 3-phosphate dehydrogenase reverse: GCG CCC AAT ACG ACC AAA TCC.



**Fig. 1.** Effects of sevoflurane preconditioning on ischemia-induced brain damage. Sevoflurane preconditioning (2.5%, 1 h/day, 5 days) improved neurologic scores (A) and reduced brain infarct volumes (B) in the mice with 60-min middle cerebral artery occlusion. Neurologic behavior scores represent the median (interquartile range); the percentages of infarct volume are expressed as mean  $\pm$  SD ( $n = 10$  per group). \* $P < 0.05$  compared with control group and vehicle group. Sevo = sevoflurane preconditioning.



### Statistical Analysis

For statistical analyses, SPSS 12.0 for Windows (SPSS Inc., Chicago, IL) was used. All values, except for neurologic scores, were expressed as mean  $\pm$  SD (SD) and analyzed by ANOVA. Between-group differences were detected with Tukey *post hoc* test. The neurologic scores, presented as median [interquartile range], were analyzed by a nonparametric method (Kruskal-Wallis test) followed by the Mann-Whitney U test with Bonferroni correction. Two-tailed values of  $P < 0.05$  were considered statistically significant.

## Results

### Physiologic Parameters

Physiologic variables at the end of preconditioning and the different time points of brain ischemia are summarized in table 1. No differences of pH value, temporal temperature ( $^{\circ}\text{C}$ ), or partial pressure of carbon dioxide ( $\text{PCO}_2$ ) were observed among groups.

### Sevoflurane Preconditioning Induced Brain Ischemic Tolerance

Neurologic scores of mice were evaluated 72 h after reperfusion. As shown in figure 1A, scores of the Sevo group (14.5 [11.75, 16.0]) were higher than those of the

oxygen vehicle group (11.0 [10.0, 13.0],  $P < 0.01$ ), but the control group did not show a neurologic effect in mice with MCAO.

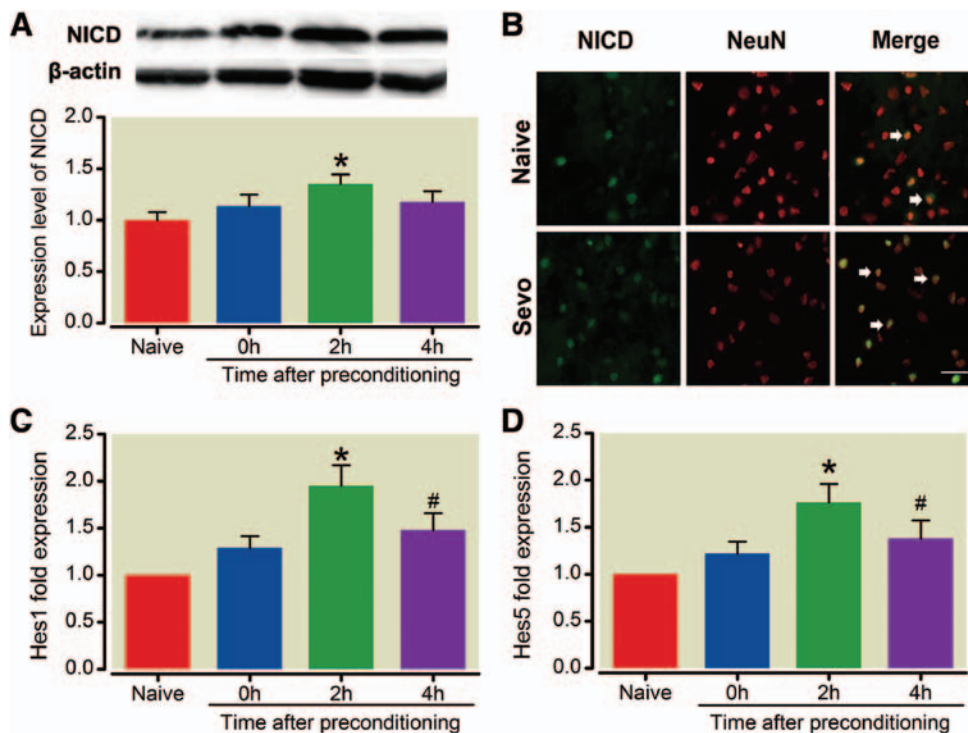
Lesion size was reduced in line with improved neurologic function. Brain infarct volume was reduced by 42.5% in the Sevo group at 72 h after reperfusion compared with the control group. No statistical significance of infarct percentages was detectable between the control and vehicle groups (fig. 1B).

### Sevoflurane Preconditioning Activated Notch Signaling Pathway

As shown in figure 2A, the expression of Notch1-NICD protein at 2 h after the last sevoflurane exposure was increased 1.35-fold compared with the naive group. However, marked elevation of NICD expression was not found immediately after preconditioning or 24 h after preconditioning.

Double immunofluorescence with neuronal nuclei (neuronal marker, red) at 2 h after preconditioning demonstrated that increased Notch1-NICD proteins (green) were colocalized mainly in neurons in brain (arrowheads), as shown in figure 2B.

The messenger RNA level of Hes1 gene at 2 h after the preconditioning was 1.95 times greater than that of the naive group and 1.48 times greater at 24 h after the



**Fig. 2.** Up-regulation of neuronal Notch signaling pathway in the mice brains after sevoflurane preconditioning. Expression level of Notch receptor intracellular domain (NICD) protein was increased at 2 h after sevoflurane preconditioning,  $n = 5$  per group (A). Double immunofluorescence staining for NICD receptor (green) and neuronal nuclei (NeuN; red) in brain. The NICD levels are increased and colocalized in the neurons (arrowheads) at 2 h after the last sevoflurane exposure. Scale bars = 20  $\mu\text{m}$ ,  $n = 3$  per group (B). The messenger RNA levels of Hes1 (C) and Hes5 (D) were significantly up-regulated at 2 h after sevoflurane preconditioning,  $n = 5$  per group. In A, C, and D, data are expressed as mean  $\pm$  SD. \* $P < 0.05$  versus naive and expression level at 0 h. # $P < 0.05$  versus expression level at 2 h. Sevo = sevoflurane preconditioning.

preconditioning. The Hes1 transcriptional level at 2 h was also higher than those at 0 and 24 h after the preconditioning ( $P < 0.01$ , for both, fig. 2C).

The Hes5 messenger RNA level was increased 1.76-fold at 2 h and 1.38-fold at 24 h after the preconditioning but markedly decreased at 24 h in comparison with that of 2 h after preconditioning ( $P < 0.01$ , fig. 2D).

### Sevoflurane Preconditioning-induced Neuroprotection Was Attenuated by DAPT

The inhibitory effects of DAPT were evaluated by measuring the NICD expression level in mouse brains. Compared with the naive group, NICD expression in the DAPT group was decreased 40.8%, which also was less than that in the vehicle group ( $P < 0.01$ ). The increased NICD in the Sevo group was attenuated by DAPT administration ( $P = 0.43$ , Sevo *vs.* Sevo+DAPT, fig. 3A).

When given alone, DAPT had no effect on neurologic outcomes but reversed the beneficial effects of sevoflurane preconditioning when given before each preconditioning (fig. 3B). Neurologic scores in the Sevo+DAPT group (10.5 [9.0, 12.25]) were less than those in the Sevo group (14.50 [11.75, 16.0],  $P = 0.41$ ). There was no statistical difference in the neurologic scores among the vehicle, DAPT, and Sevo+DAPT groups.

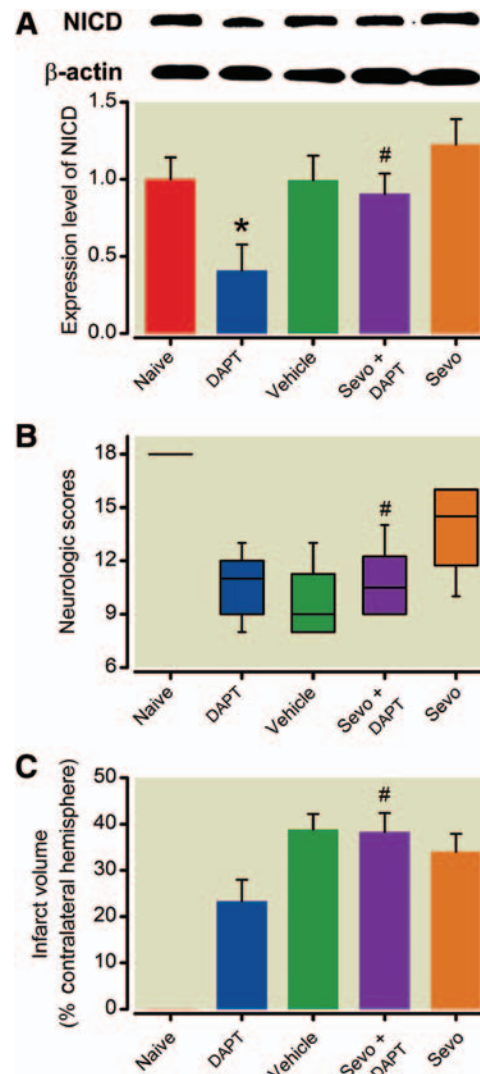
Seventy-two hours after reperfusion, brain infarct volumes of the Sevo+DAPT group were increased 1.64-fold compared with those of the Sevo group, whereas the infarct volume percentage in the DAPT group was similar to that in the vehicle group (fig. 3C).

### Neuroprotective Effects of Sevoflurane Preconditioning Were Absent in RBP-J Knockout Mice

As shown in the images of immunohistochemistry staining in figure 4A-L, protein concentrations of Notch downstream effectors Hes1 and Hes5 but not NICD were diminished in both the cortex and hippocampus in knockout mice, which indicates the ablation of RBP-J.

After MCAO, as shown in figure 4M, RBP-J<sup>-/-</sup> (14 [12.5, 15]) had higher neurologic scores than did the WT (10.5 [9.75, 12.25],  $P = 0.033$ ) and RBP-J<sup>+/+</sup> groups (11 [10, 13],  $P = 0.036$ ). But with sevoflurane preconditioning, RBP-J<sup>-/-</sup> mice (11.5 [9.75, 12.25]) showed worse neurobehavioral performance than did Sevo (14.5 [12, 15.25],  $P = 0.048$ ) and Sevo+RBP-J<sup>+/+</sup> mice (13.5 [11.75, 15],  $P = 0.049$ ) at 72 h after reperfusion; there was no statistically significant difference with WT mice.

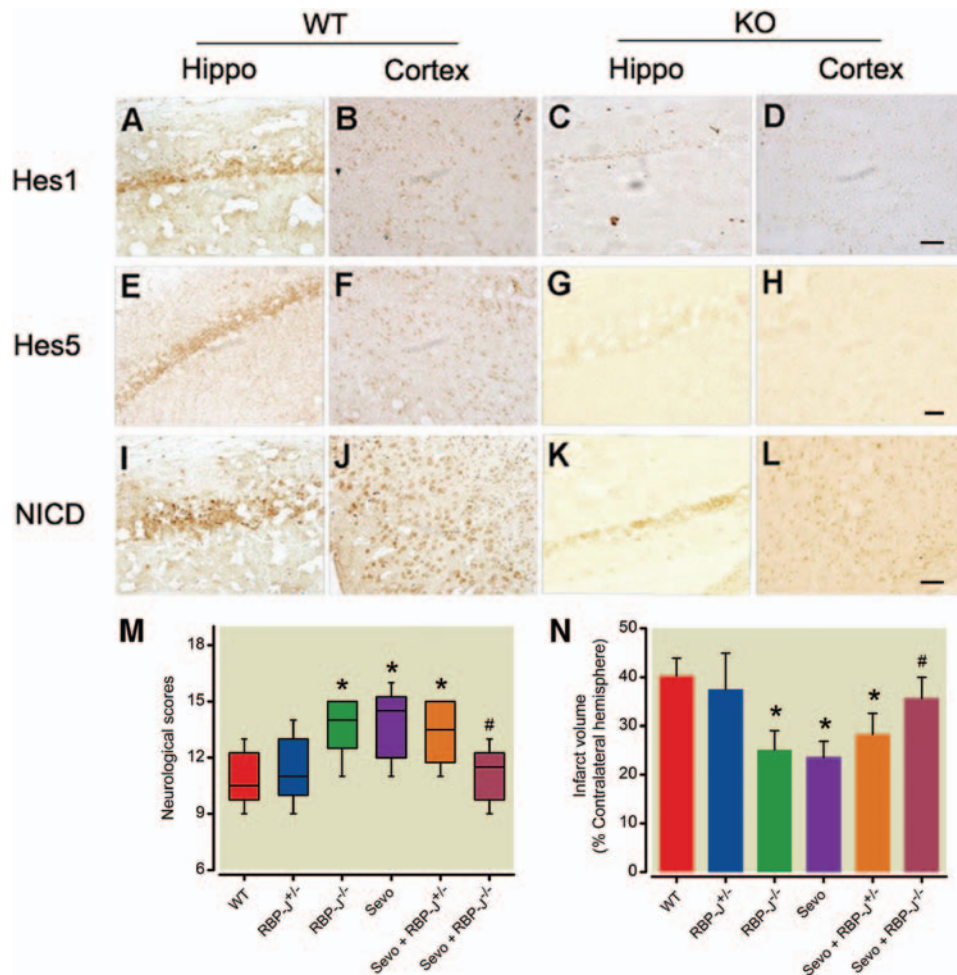
Without preconditioning, RBP-J<sup>-/-</sup> mice ( $25.05 \pm 3.92\%$ ) showed a smaller infarct volume than did WT ( $40.28 \pm 3.61\%$ ,  $P < 0.01$ ) and RBP-J<sup>+/+</sup> ( $37.46 \pm 3.44\%$ ,  $P < 0.01$ ) mice. When preconditioned with sevoflurane, RBP-J<sup>-/-</sup> mice ( $35.69 \pm 4.23\%$ ) had a larger brain infarct volume than did Sevo ( $23.54 \pm 4.27\%$ ,  $P = 0.047$ ) and Sevo+RBP-J<sup>+/+</sup> ( $28.29 \pm 4.26\%$ ,  $P = 0.049$ ) mice but difference compared with the WT mice (fig. 4N).



**Fig. 3.** Notch proteolysis inhibitor DAPT inhibited the expression of Notch receptor intracellular domain (NICD) in mice brains ( $n = 5$  per group) before ischemia (A) and attenuated the protective effects of sevoflurane preconditioning by reducing neurologic behavior scores ( $n = 10$  per group; B) and augmenting infarct volumes ( $n = 10$  per group; C). Data in A and C are expressed as mean  $\pm$  SD and in B represent the median (interquartile range). \* $P < 0.05$  versus naive and vehicle. # $P < 0.05$  versus Sevo. Sevo = sevoflurane preconditioning.

### Cellular Apoptosis Increased in Knockout Mice Preconditioned with Sevoflurane

Neuronal cell apoptosis within the ischemic penumbra is shown in figure 5. At 24 h after reperfusion, the number of TUNEL-positive cells in the Sevo group was reduced significantly to 21.2%, and the caspase-3-positive cells were decreased to 22.58% in comparison with the concentrations in the WT group. No statistical difference was found between the Sevo and RBP-J<sup>-/-</sup> groups. However, with sevoflurane preconditioning, the number of TUNEL-positive cells in the Sevo+RBP-J<sup>-/-</sup> group was increased 2.1-fold compared with that in Sevo group, and the number of caspase-3-positive cells was increased threefold.



**Fig. 4.** RBP-J ablation inhibited sevoflurane preconditioning-induced neuroprotection. Notch receptor intracellular domain (NICD), Hes1, and Hes5 were stained by immunohistochemistry (A–L). Hes1 and Hes5 were decreased significantly in the hippocampus (HIPPO) and cortex of knockout (KO) mice (C, D, G, H) but not NICD (K, L). Sevoflurane preconditioning significantly improved the neurobehavioral outcomes (M) and reduced the brain infarct volumes (N) in wild-type (WT) mice, whereas no protective effect was seen in the RBP-J knockout mice. Neurologic behavior scores are expressed as median (interquartile range), and the infarct volume percentages are presented as mean  $\pm$  SD ( $n = 8$  per group). \* $P < 0.05$  versus WT and RBP-J<sup>-/-</sup>, # $P < 0.05$  versus RBP-J<sup>-/-</sup>, Sevo, and Sevo+RBP-J<sup>-/-</sup>. Scale bars = 20  $\mu$ m. Sevo = sevoflurane preconditioning.

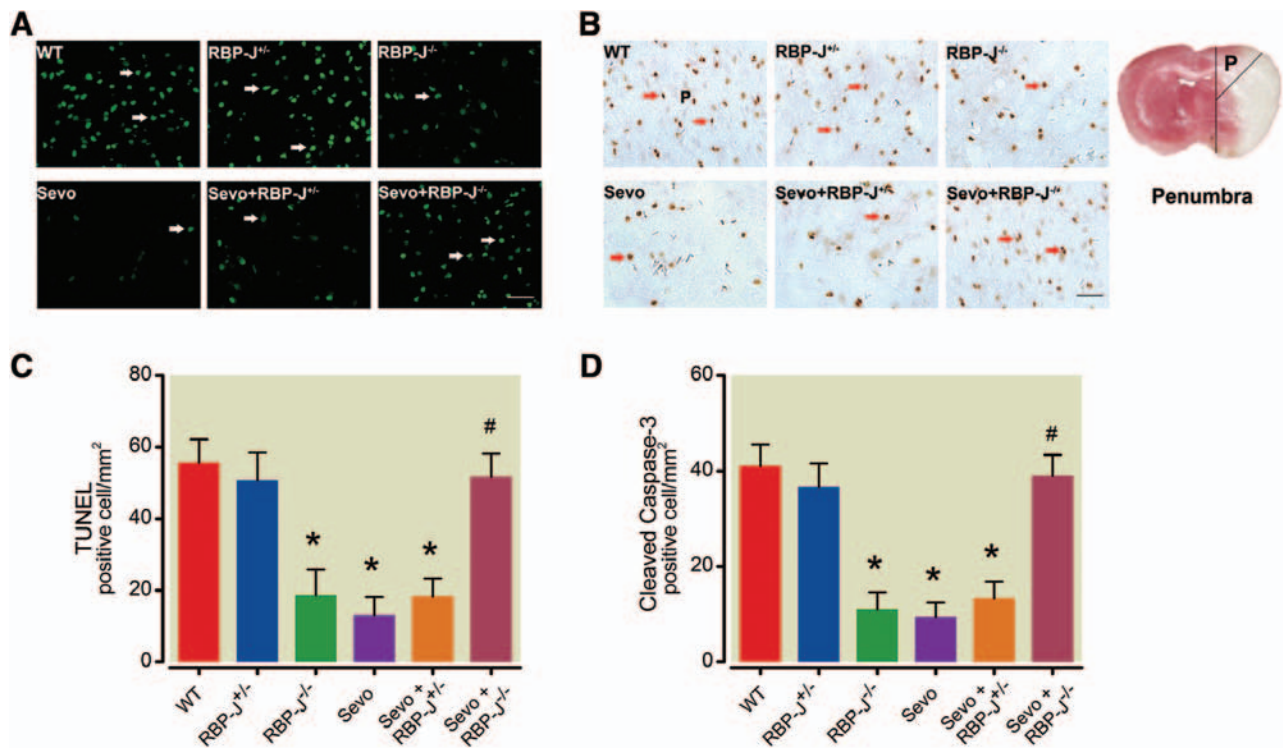
## Discussion

Using an *in vivo* model of transient focal cerebral ischemia in mice, the current study demonstrates that preconditioning with sevoflurane induced neuroprotective effects and activated the Notch signaling pathway as indicated by the expression of neuronal NICD and the transcriptional levels of Hes1 and Hes5 in brain tissue. The neuroprotective effects of sevoflurane were attenuated by the Notch inhibitor DAPT. Furthermore, in the neuron-specific Notch RBP-J knockout mice, sevoflurane preconditioning did not exert any neuroprotective effect as evaluated by infarct volumes, neurologic behavior scores, or neuronal cellular apoptosis after ischemia. These results suggest that sevoflurane preconditioning activated the canonical Notch signaling pathway, which elicited protective effects against transient cerebral ischemia by inhibiting neuronal cell apoptosis in mice.

Tolerance to brain ischemia induced by sevoflurane preconditioning has been observed in both *in vivo* and *in vitro* models.<sup>1,4,23</sup> This study further verified that pretreatment with sevoflurane (2.5%) could induce effective neuroprotection in mice as evidenced by infarct volumes and neurologic behavior scores.

Notch signaling has critical roles in the proliferation, differentiation, and maintenance in the developing brain and the adult brain.<sup>24,25</sup> In mammals, four Notch receptors have been identified, Notch 1–4, with overlapping but nonidentical expression patterns.<sup>10,26</sup> Recently, Notch1 signaling has been reported to participate in the mechanisms of cerebral ischemic injuries<sup>9</sup> and ischemia-induced neurogenesis.<sup>27,28</sup> However, little is known about the involvement of Notch signaling in the mechanisms of preconditioning-induced neuroprotection. In the current study, we have investigated





**Fig. 5.** The cellular apoptosis at 24 h after reperfusion in RBP-J knockout mice with sevoflurane preconditioning. Terminal deoxynucleotidyl transferase-mediated 2'-deoxyuridine 5'-triphosphate nick-end labeling (TUNEL) staining in the ischemic penumbra and quantitative analysis of positive cells. The white arrowheads indicate the TUNEL-positive cells. Scale bars = 20 μm (A). Cleaved caspase-3 staining and quantitative analysis of positive cells. The red arrowheads indicate the caspase-3-positive cells. Scale bars = 20 μm. Ischemic penumbra was identified as the adjoining image of the coronal section (B). Quantitative data in both C and D are presented as mean ± SD (n = 5 per group). \**P* < 0.05 versus wild-type (WT) and RBP-J<sup>+/+</sup>. #*P* < 0.05 versus RBP-J<sup>-/-</sup>, Sevo, and Sevo+RBP-J<sup>+/+</sup>. Sevo = sevoflurane preconditioning; P = penumbra.

the role of the canonical Notch pathway in the ischemic tolerance elicited by sevoflurane preconditioning, using Notch1 as an entry point.

We investigated the effect of sevoflurane preconditioning on Notch signaling pathway activity. In addition to the up-regulation of NICD, the transduction of Hes1 and Hes5, the most overriding downstream target genes of canonical Notch pathway in the brain, was increased after sevoflurane preconditioning, suggesting a potential role of Notch in the neuroprotective effects of sevoflurane in mice brain. In addition, the activation of NICD after sevoflurane preconditioning was observed to colocalize in neurons, which indicates an association between Notch signaling and the neuronal effects of sevoflurane preconditioning. In addition to being expressed in adult neurons, Notch1 was reported to express in the progenitors of the subventricular zone of the cerebral hemispheres and subgranular zone of the dentate gyrus after stroke,<sup>27</sup> where it induced progenitor proliferation.

Compound DAPT [N-[N-(3, 5-Difluorophenacetyl-L-alanyl)]-S-phenylglycine t-butyl ester], a functional γ-secretase inhibitor, suppresses the proteolysis of Notch receptor. We found NICD concentrations with a peak inhibition of 50% in normal brain occurred at 3 h, and a reduction of more than 30% was observed as long as

24 h after DAPT administration (data not shown). This is in line with the study by Lanz *et al.*,<sup>14</sup> in which the peak DAPT concentration was achieved in the brain 3 h after administration. In the current study, DAPT reversed the neuroprotection of sevoflurane preconditioning, which suggests that the γ-secretase-dependent Notch pathway could have an important role in mediating the ischemic tolerance induced by sevoflurane preconditioning.

To elucidate the exact role of Notch signaling in the ischemic tolerance elicited by sevoflurane, we used neuronal Notch-RBP-J knockout mice. We confirmed the inactivation of Notch signaling in RBP-J<sup>-/-</sup> mice owing to the absence of the downstream effector Hes1 and Hes5 protein expression in mice brain. As shown in the current study, sevoflurane-induced neuroprotective effects were abrogated in homozygous Notch-RBP-J knockout mice (RBP-J<sup>-/-</sup>), whereas the heterozygous littermates (RBP-J<sup>+/+</sup>) maintained the ischemic tolerance. In addition, RBP-J<sup>-/-</sup> mice were found to experience less damage from brain ischemia, which is in accordance with previous reports,<sup>9,11</sup> indicating Notch-RBP-J knockout mice do not have reduced ischemic tolerance at baseline. Thus, these data suggest that the activation of the RBP-J-dependent canonical Notch pathway in advance of ischemia makes a positive contribution to the neuronal



protective effects of sevoflurane preconditioning, the mechanisms of which are related to the down-regulation of the apoptosis in neurons after stroke. These results indicated that Notch signaling could be involved in the polyhedral effects after preconditioning and ischemia. We postulate the underlying pattern as follows:

Volatile anesthetic preconditioning is well known as a nonlethal treatment in the brain. In the current study, sevoflurane-induced Notch signaling could be a potential nonlethal inducer, activating the endogenous neuroprotective components and reducing the ischemic brain injury at the early phase after stroke. This could be similar to the mechanisms of reactive oxygen species involved in the volatile anesthetics preconditioning.<sup>29,30</sup> Free radicals were considered to arouse the endogenous antioxidant system after preconditioning, which then decreased the oxidative stress after brain ischemia. Pählman *et al.*<sup>31</sup> demonstrated that Notch signaling is sensitive to oxygen tension and can be activated by oxidative stress,<sup>32</sup> showing the underlying crosstalk between Notch signaling and endogenous antioxidation mechanism.

In addition, it is of note that the results showing the combination of sevoflurane preconditioning and the RBP-J knockout made the ischemic brain injuries worse. Volatile anesthetics are considered pleiotropic agents in the nervous system and to have comprehensive impact on signaling pathways. Sevoflurane may impose a deleterious effect to neurons *via* some signaling pathways while inducing neuroprotection *via* others. When Notch is knocked out during sevoflurane preconditioning, the effects of detrimental pathways could be significant. Data in the current study show that Notch is an important neuroprotective pathway, but it is unlikely to be the only one contributing to neuroprotection. Indeed, many more details about the mechanisms for Notch need to be clarified.

Some limitations in this study need to be noted. Although there are four paralogs of Notch in mammals, we chose only Notch1 as the entry point to investigate the role of canonical Notch pathway, mainly because Notch1 has been well studied and is thought to contribute a lot to neural formation and maintenance. However, we cannot preclude the effects of other Notch receptors in sevoflurane-induced neuroprotection. Actually, Notch3, the expression of which is predominantly in vascular smooth muscle cells in adults, has been reported possibly to define a key determinant of stroke damage through regulation of vascular smooth muscle cell function.<sup>10</sup> All this evidence reconfirms the different functions of Notch paralogs. Therefore, for future studies, we will need several parallel experiments to examine the activation and roles of Notch 2–4 pathways after sevoflurane preconditioning in brain.

In summary, the current study demonstrated for the first time that sevoflurane preconditioning activated the canonical Notch1 signaling pathway, which elicited protective effects against transient cerebral ischemia by decreasing neuronal apoptosis in mice. The Notch signaling pathway may represent a new and potential therapeutic target for neuroprotective mechanisms of sevoflurane preconditioning.

The authors thank Gunther Schutz, M.D. (Professor, German Cancer Research Center, Heidelberg, Germany), for the generous gift of the CamKIIa-Cre mice, and Hua Han, M.D., Ph.D. (Professor, Department of Medical Genetics and Developmental Biology, Fourth Military Medical University, Xi'an, Shaanxi Province, China), for the RBP-J floxed mice. The authors also thank Bairen Wang, M.D., Ph.D. (Professor, Institute of Neuroscience, Fourth Military Medical University), for reviewing the manuscript.

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