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Inhibiting Rho kinase 2 reduces memory dysfunction in adult rats exposed to sevoflurane at postnatal days 7-9

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Abstract. The aim of the present study was to investigate the roles of Rho protein A (RhoA) and Rho kinases 2 (ROCK2) in the memory dysfunction of adult rats exposed to sevoflurane at postnatal days 7-9 (P7-9). One-week-old Sprague-Dawley rats were divided into four groups known as C, S1, S3 and F. Rats in the S1 (2 h at P7) and S3 groups (2 h/day at P7-9) were exposed to sevoflurane. The rats in the F group were treated with the ROCK2 inhibitor and subsequent sevoflurane exposure (2 h/day at P7-9). The rats in the C group received no sevoflurane. The protein levels of RhoA, ROCK2 and cleaved caspase-3 (Cl-Csp3) in the adult hippocampus were assessed by western blot analysis. Learning and memory of rats at postnatal 45-50 days (P45-50) were detected by the Morris water maze (MWM) test. During the training of MWM, the latency and distance of rats in the S3 group were significantly longer than that of the C group ($P<0.05$, respectively). In the probe test, the percentages of time and distance in the target quadrant for the S3 group were evidently less than that of the C group ($P<0.05$). There was no significant difference in the behaviors between the C and S1 groups ($P>0.05$, respectively). Corresponding to the behavioral changes, the levels of RhoA, ROCK2 and Cl-Csp3 in the hippocampus of the S3 group significantly increased, compared to that of the C and S1 groups ($P<0.05$). Additionally, the ROCK2 inhibitor clearly decreased ROCK2 and Cl-Csp3 expression and shortened the latency during the training ($P<0.05$, P46-49 respectively) and probe test ($P<0.05$) in the F group, compared to that of the S3 group. Compared to the C group, the expression of RhoA, ROCK2 and Cl-Csp3 in the hippocampus of the S1 group had no significant difference ($P>0.05$). Multiple inhalation of sevoflurane can induce neurotoxicity and memory dysfunction. RhoA and ROCK2 played important roles in the impairment of learning and memory of adults rats exposed to sevoflurane at the postnatal early stage.

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Introduction

Pediatric patients may be at risk for later learning and behavioral impairment when exposed to general anesthesia and surgery (1-4). This is consistent with studies in preclinical experiments by Yon *et al* (5). In the study, rats were exposed at postnatal day 7 to compound anesthetic agents (midazolam, isoflurane and nitrous oxide) and identified neuronal apoptosis in the brain and persistent defects of memory and learning when the rats were juvenile. In addition, these rats also showed the impairment of spatial reference and working memory as they grew up. Similar impairment of learning and memory induced by neonatal anesthesia was also reported for adult patients and other animals (1-4). However, the exact mechanism underlying the neonatal anesthesia remains unclear.

Rho kinase 2 (ROCK2), one of the members of the family of serine/threonine protein kinase, is a downstream effector of Rho protein A (RhoA) (6). RhoA regulates the reorganization of cytoskeleton protein actin by controlling ROCK2, affecting cell migration, apoptosis, gene transcription, nerve regeneration and other biological processes (7,8). RhoA/Rho-kinase is an important pathway underlying neuronal injury in rats of experimental spinal cord injury (9). In addition, the Rho/Rho-kinase pathway is associated with the modulation of *N*-methyl-D-aspartate (NMDA) receptor function and the NMDA receptor is associated with learning and memory processing (10). In a previous study, Lemkuil *et al* (11) demonstrated that isoflurane induced neurotoxicity of mouse neurons by activating $p75^{NTR}$-RhoA, and inhibiting activation of RhoA attenuated isoflurane-induced impairment. Pearn *et al* (12) also reported that propofol-induced apoptosis is involved in $p75^{NTR}$ and RhoA kinase activation in developing neurons *in vivo* and *in vitro*. These suggested that RhoA played important roles in the neurotoxicity of anesthetics. However, it remains unclear whether ROCK2, the key downstream molecule of the RhoA signals, mediates the neurotoxicity of anesthetics cognitive impairment of adult rats with neonatal exposure of sevoflurane.

The present study investigated i) the effects of sevoflurane exposure of rats at postnatal days 7-9 on their learning and memory at adulthood; and ii) the association of the cognitive dysfunction induced by sevoflurane to expression changes of RhoA and ROCK2. Sevoflurane exposure at postnatal days 7-9 impaired the learning and memory of rats after they grew up. Corresponding to the behavioral changes of rats, hippocampal RhoA and ROCK2 increased; and inhibiting ROCK2

expression reversed the cognitive dysfunction induced by sevoflurane exposure.

Materials and methods

Animals. Fifty-six Sprague-Dawley rats (7 days old) were obtained from the Experimental Animal Center of The Third Xiangya Hospital of Central South University (Changsha, China). The institutional guidelines regarding animal safety were strictly followed and authorized by the Institutional Animal Care. The room temperature was maintained at 22-24˚C under suitable humidity. The rats were divided into four groups (S1, S3, F and C) randomly, with 14 in each group. The rats in group S1 inhaled 2% sevoflurane and 80% oxygen for 2 h. Rats in the S3 group were exposed to sevoflurane three times from postnatal day 7 (2 h/time/day). The F group rats were pretreated with ROCK2 inhibitor fasudil hydrochloride (10 mg/kg, HY-10341; MedChem Express LLC, Princeton, NJ, USA) and 2 h later were exposed to sevoflurane three times from postnatal day 7 (2 h/time/day). The rats in group C inhaled 80% oxygen. All the rats were placed in a self-made organic glass box (30x40x30 cm) with soda lime in the bottom. The multi-function monitor (Datex-Ohmeda, Helsinki, Finland) was used to monitor the concentration of sevoflurane, O_2 and CO_2. When experimental models were completed for 3 h, rats were sacrificed by decapitation and the hippocampus was removed to assess the expression of RhoA, ROCK2 and cleaved caspase-3 (Cl-Csp3) by western blotting. The neurobehavioral tests of the Morris water maze (MWM) at P45-50 were used to examine the memory function. Latency to locate the target platform, swimming distance and speed in target zone were determined and compared in all the groups.

Western blotting. The protein concentration of samples was determined using a bicinchoninic acid protein assay kit (Wellbio, Changsha, China) according to the manufacturer's instructions. Equal amounts of protein samples (/lane) were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 10% skimmed milk in phosphate-buffered saline with Tween-20 buffer for 2 h and subsequently incubated with primary antibodies [rabbit anti-RhoA polyclonal antibody (1:600; cat. no. 10749-1-AP; Proteintech, Wuhan, Hubei, China), rabbit anti-ROCK2 polyclonal antibody, 1:200; cat. no. sc5561; rabbit anti-cleaved caspase3 polyclonal antibody (1:400; cat. no. sc22139; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin (1:2,000; cat. no. KGAA001-4; KeyGen BrioTech, Nanjing, China) overnight at 4˚C. After three washes, membranes were incubated with the secondary antibody [goat anti-rabbit IgG (H+L) (1:2,000; cat. no. KGAA35; KeyGen BioTech)] at room temperature for 2 h. Finally, visualization of the proteins was accomplished by ECL detection reagents (Advansta Corp., Menlo Park, CA, USA). The images were developed on luminescent image analyzer (ImageQuant 350 Capture; GE Healthcare Life Sciences, Shanghai, China) and quantified by densitometry (Beckman Coulter, Inc., Pasadena, CA, USA). Relative expression levels of protein were normalized by the ratio of the target protein (ROCK2, RhoA and Cl-Csp3) to β-actin.

MWM test. The MWM test was used to evaluate the learning and memory of rats. A computerized video track system (Logitech, Suzhou, China) was used to record the movement of the rats in the water maze by following a previous method (13). Briefly, a transparent circular platform was placed below the water surface of the southeast quadrant in a circular black pool. During the training, rats were first placed on the platform for 30 sec, and subsequently were put into the water facing the tank wall. The maximum trial time was 60 sec, following a relaxation of 20 sec on the platform. When a rat could not locate the platform within 60 sec, it was guided to the platform and remained there for 30 sec. All the rats were trained for 5 days with three trials/day. Following training, the memory of the rats was evaluated by the percentage of searching time and distance in the targeted area.

Statistical analysis. Water maze data are presented as mean ± standard error of the mean and were analyzed using analysis of variance for repeated measures followed by the least significant difference test. Western blotting data are presented as mean ± standard deviation and were analyzed using a Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Sevoflurane exposure at postnatal day 7-9 impairs the learning and memory of rats after they grew up. In the training, the latency and distance of rats in the S3 group at postnatal days 45-50 (P45-50) were significantly longer than that of the C group ($P<0.05$, respectively). There was no significant difference of the latency and distance between the C and S1 groups ($P>0.05$, respectively) (Fig. 1A and B). In all the groups, the swimming speed did not show a significant difference on all the days ($P>0.05$) (Fig. 1C). In the probe trial test, the percentage of searching time and distance in target quadrant in the S3 group was evidently less than that of the C ($P<0.05$) and S1 groups ($P<0.05$). The percentage of searching time and distance in target quadrant in the S1 group were not different from that of the C group ($P>0.05$) (Fig. 1D-F). These suggested that sevoflurane exposure at postnatal days 7-9 dose-dependently impaired the learning and memory of rats after they grew up.

RhoA and ROCK2 upregulation of rat hippocampus is involved in memory dysfunction induced by postnatal exposure of sevoflurane. Corresponding to the behavioral changes of the S3 group, the levels of RhoA, ROCK2 and Cl-Csp3 in the hippocampus of the S3 group significantly increased, compared to that of the C and S1 groups ($P<0.05$) (Fig. 2 A-C). The ROCK2 inhibitor, fasudil hydrochloride, in the F group clearly decreased the expression of ROCK2 and Cl-Csp3, and shortened the latency in the training ($P<0.05$, P46-49 respectively) and probe test ($P<0.05$), compared to that of the S3 group. In addition, there was no significant difference in the expression of ROCK2 and Cl-Csp3, and the latency in the place navigation test and probe test between the C and F groups ($P>0.05$). These suggested that the increasing expression of RhoA and ROCK2 in the hippocampus was an important mechanism underlying the cognitive dysfunctions induced by sevoflurane exposure.

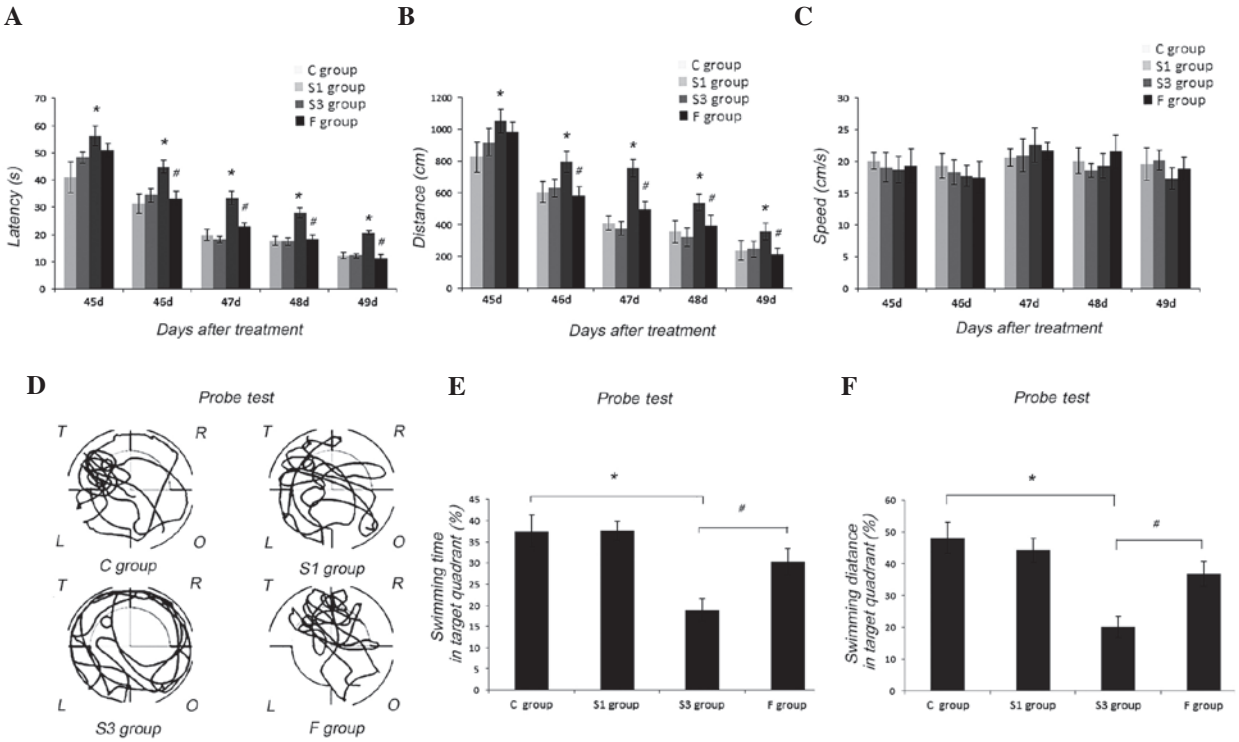

Figure 1. Sevoflurane exposure at postnatal days 7-9 impaired the learning and memory of P45-50 rats in the Morris water maze (MWM) test. (A) In the place navigation test, the latency of the S3 group was longer than that of the C and F groups (*P<0.05, C vs. S3 group at P45-49; #P<0.05, S3 vs. F group at P46-49). There was no significant difference of latency between the C and S1 groups (P>0.05, respectively). (B) The distance of the S3 group was longer than that of the C and F groups (*P<0.05, C vs. S3 group at days 45-49; #P<0.05, S3 vs. F group at P46-49). There was no significant difference of the distance between the C and S1 groups (P>0.05, respectively). (C) The swimming speed had no difference in all the groups (P>0.05). (D) Representative routes of the C, S1, S3 and F groups in the probe test on P50. T: target quadrant; R, O, L quadrants: right, opposite or left of the target quadrant. (E) In the probe trial test, the percentage of searching time in the target quadrant of the S3 group was clearly less than that of the C and F groups (*P<0.05, C vs. S3 group; #P<0.05, S3 vs. F group). The percentage of searching time in the target quadrant of the S1 group was not different from that of the C group (P>0.05). (F) In the probe trial test, the percentage of searching distance in the target quadrant of the S3 group was clearly less than that of the C and F groups (*P<0.05, C vs. S3 group; #P<0.05, S3 vs. F group). The percentage of searching distance in the target quadrant of the S1 group was nott different from that of the C group (P>0.05). Data are expressed as mean ± standard error of the mean.

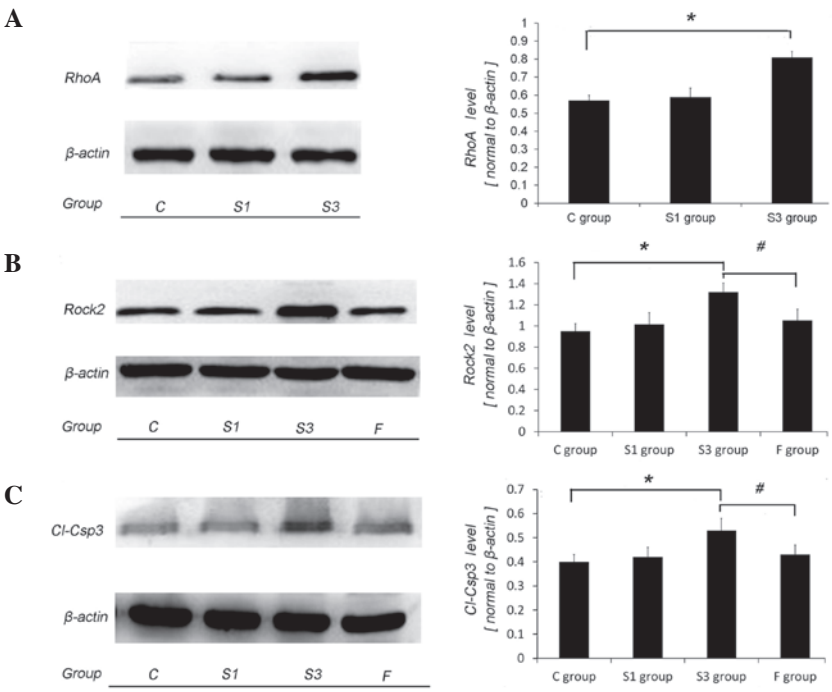

Figure 2. RhoA and ROCK2 upregulation in the rat hippocampus was involved in memory dysfunction induced by postnatal exposure of sevoflurane. The expression of RhoA, ROCK2 and cleaved caspase-3 (Cl-Csp3) in the hippocampus of the S3 group significantly increased, compared to that of the C group (P<0.05). There was no difference of the level of RhoA, ROCK2 and Cl-Csp3 between the C and S1 groups (P>0.05). ROCK2 inhibitor fasudil hydrochloride in the F group clearly decreased the expression of ROCK2 and Cl-Csp3 (P<0.05), compared to that of the S3 group (*P<0.05, C vs. S3 group; #P<0.05, S3 vs. F group). Data are mean ± standard deviation.

Discussion

The aim of the present study was to investigate whether ROCK2 played roles in the cognitive dysfunction of adult rats exposed to sevoflurane during the postnatal early stage. Sevoflurane exposure during the postnatal early stage dose-dependently impaired the cognitive function of rats after they grew up. Corresponding to the cognitive dysfunction of rats, the level of RhoA, ROCK2 and Cl-Csp3 in the hippocampus in the sevoflurane-treated group significantly increased, compared to the control group. Additionally, ROCK2 inhibitor fasudil hydrochloride decreased the level of ROCK2 and Cl-Csp3 in the hippocampus and partly reversed the cognitive dysfunction in the sevoflurane-treated rats. These showed that ROCK2 was closely involved in the neurotoxicity of sevoflurane.

Neurotoxicity of anesthetics was well reported in aged animals (13-15), natal animals (15) and cultured neurons (16). Yan *et al* (17) showed that the isoflurane-induced decrease of nNOS is closely correlated with the cognitive impairment in aged rats. In another study, Zheng *et al* (18) found that sevoflurane use during pregnancy may produce adverse effects on fetal and postnatal rats. In the present study, 2% sevoflurane for 2 h at postnatal day 7 did not impair the cognitive function of rats when they grew up. By contrast, 2% sevoflurane for 2 h at postnatal days 7-9 (2 h sevoflurane/day) significantly impaired the cognitive function of rats when they grew up. These results showed the dose-dependent neurotoxicity of sevoflurane to the neurons. Consistent with our data, Feng *et al* (19) found that inhaling 2.3% sevoflurane for 6 h induced nerve cell death in newborn rats. Shen *et al* (20) found that rats in the growing stage (6 days of age) that inhaled 3% sevoflurane for 2 h at a time did not show cognitive impairment and nerve inflammation, while those that inhaled 3% sevoflurane for 2 h for three days showed cognitive impairment and nerve inflammation. Of note in the present study, corresponding to dose-dependent neurotoxicity of sevoflurane to rats, 2% sevoflurane of 2 h at postnatal day 7 did not induce clear expression changes of hippocampal RhoA, ROCK2 and Cl-Csp3, but 2% sevoflurane of 2 h at postnatal days 7-9 (2 h sevoflurane/day) significantly increased the expression of hippocampal RhoA, ROCK2 and Cl-Csp3. Additionally, when the expression of ROCK2 was inhibited by fasudil hydrochloride, the expression of rat hippocampal ROCK2 and Cl-Csp3 decreased and rat cognitive dysfunction induced by sevoflurane exposure also partly reversed. These showed the key roles of RhoA and ROCK2 in sevoflurane neurotoxicity to natal rats. A previous study showed that RhoA and ROCK2 regulated synaptic plasticity (6,21). Therefore, in the present study, we speculate that sevoflurane may upregulate RhoA and ROCK2, damage synaptic plasticity, and subsequently, induce impairment of learning and memory performance of rats after they grew up. However, its specific mechanism remains to be proved.

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