Inhibition of Pontine CaMKII Alleviates Sevoflurane-Induced Long-Term Memory Impairment in Rats

Yusheng Liu*, Wei Wang*Δ

SUMMARY General anesthesia-associated cognition impairment has been becoming one of the major issues derived from medical procedure, particularly when it was performed in younger patients. Our previous data noted that early exposure of sevoflurane impaired the adulthood spatial memory function in dose and time-dependent manner. Gamma aminobutyric acid (GABA) is the key inhibitory neurotransmitter in the central nervous system, and functions through mediating Wnt signaling pathway. The pontine GABA takes a special part in keeping awakening. We in this study investigated the role of pontine GABA signaling pathway activation in sevoflurane anesthesia-related long-term cognition in infantile rats via intracranial microinjection of CaMKII inhibitor KN-93 into the pons under different minimum alveolar concentrations of seveflurane. Morris Water Maze (MWM) was used to detect the spatial memory changes after speculated interventions. The results showed that pontile inhibition of GABA-Wnt-CaMKII through blocking CaMKII substantially alleviated sevoflurane-induced long-term special memory, which demonstrated dose- and time-dependent association. These preliminary observations indicated that the pontile GABA signaling plays an essential role in sevoflurane-induced adulthood cognition impairment, and further evaluation is needed on the exact interaction among sevoflurane, GABA, glutamate, receptors, corresponding signaling mediators, and cognition alteration in the pontine region.■

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THE CHILDREN younger than 3 years are more than 35% in all children experienced general anesthesia each year, account for 4-7% of all children and the number is more than 80 million (1). Recent clinical cohort studies and meta-analysis suggested that the infants younger than 3 years exposed to the anesthetic were followed by long-term cognitive function and learning ability markedly reducing (2,3). At the same time, animal studies have found that early life exposure to the anesthetic causes degenerative change of brain neurons and spatial cognitive dysfunction (4,5). But the mechanism of anesthetic impairing learning and cognitive function is not yet clear. Most studies demonstrated that neuronal apoptosis induced by anesthesia is the main reason (6). There is also other research thinking that is because of cerebral energy metabolism disorder (7).The "central inhibition" theory is a new hotspot new, on which some researchers have carried out a series of studies involving ion channels, synaptic plasticity, glial cells inflammatory reaction, relevant signal pathways activation, etc. and have gained some progress (8,9,10,11,12).

Sevoflurane, a new type inhalational general anesthetic, is now the most common agent for pediatric patients due to its sweet-smelling property as well as fast onset plus recovery. Our recent studies found that early-life rats undergone sevoflurane anesthesia possessed long-term learning and spatial cognitive function serious damage. The damage degree was closely related to sevoflurane exposure duration, repetitions, weeks age of rats. The longer sevoflurane exposure duration, the more repetitions, the smaller weeks age, the more serious impairment was (8,13). Through literature review, the general anesthesia mechanism of sevoflurane mainly focused on that sevoflurane activates gamma aminobutyric acid (GABA) neural pathways in hippocampus to lead to unconsciousness (9,10,11,14,15,16). It is known now pontine reticular formation as an important part of the uplink reticular activation system also plays an important role in the process of awakening. But interestingly, pontine reticular formation activates GABA neural pathways to achieve awakening (12,17). Earlier studies have found that sevoflurane inhibits the activity of GABA neural pathways in pontine reticular formation (13,18), so we speculated that the activity inhibition of GABA neural pathways in pontine reticular formation may be one of the mechanisms by which early life undergone sevoflurane anesthesia leads to long-term learning and cognitive function damage.

MATERIALS AND METHODS

Animal care and ethics

After approval by the Institutional Committee of Animal Care and Use, male Sprague-Dawley rats aged 3 days (postnatal 3 days, P3D) were used for pontine injection and sevoflurane intervention and behavioral tests. The pups (P3D) were housed with maternal rats to a same plastic cage with soft bedding on a reverse 12:12 h light/dark cycle with lights on at 8:00 AM and maintained in climate with 23±1 ℃ housing temperature and free access to food and water. The pups were returned to their mother cage after sevoflurane anesthesia. Three weeks after birth, the offspring were separated from maternal rats and housed one animal to one cage until the completion of behavior tests. The littermates were randomly assigned to each testing group. The random numbers were generated by means of the QuickCalcs (GraphPad Software Inc, La Jolla, San Diego, CA; Online Calculators for Scientists, available at http:// www.graphpad.com/quickcalcs/RandMenu.cfm. Last accessed April 03, 2014.). Anesthesia and pontine injection were conducted during the light phase between 08:00 AM and 05:00 PM in a quiet room maintained at 22–24 ℃. Each animal was used for the same anesthesia and pontine injection regimen and was euthanized after completion of the water maze behavioral experiment by administering a lethal dose of pentobarbital.

Pontine medicine injection procedures

This methods of pontine medicine injection used at the present study referenced to the methods by David I. et al. (19), and were modified somewhere accordingly. Before sevoflurane exposure procedures, the rat's head was secured in a stereotactic frame. The rat's head position was adjusted within the stereotactic frame to facilitate the appropriate trajectory for cannula insertion into the target site within the pontine tegmentum. The place was selected as the target site because of its relatively large size, the predominance of surrounding white matter, and the absence of cranial nerve nuclei or major ascending and descending fiber tracts. To obtain the appropriate trajectory for this target, the anterior portion of the animal's head was elevated 3 mm above a horizontal plane, and the skull target point was located within the bregma area at 0.6 mm to the right of the sagittal suture and 0.4 mm anterior to the lambdoid suture. After antisepsis with 2% iodine solution, a 25-gauge needle was used to puncture through the skine and the dura mater under the skull target point gently to make a channel for cannula inserting. The infusion cannula apparatus was secured in the clamping device of the stereotactic frame and inserted to its target depth in the brain (2.6 mm below the skull) though the channel.

The infusion cannulas were prepared by inserting 33-gauge internal cannulas into 26-gauge guide cannulas with a 1.5-mm projection of the internal cannula tip. The space between the distal end of the guide cannula and the projecting portion of the infusion cannula was sealed by applying methyl-2-cyanoacrylate to prevent backflow of infusate into the space between the guide cannula and the internal cannula. The sealed guide

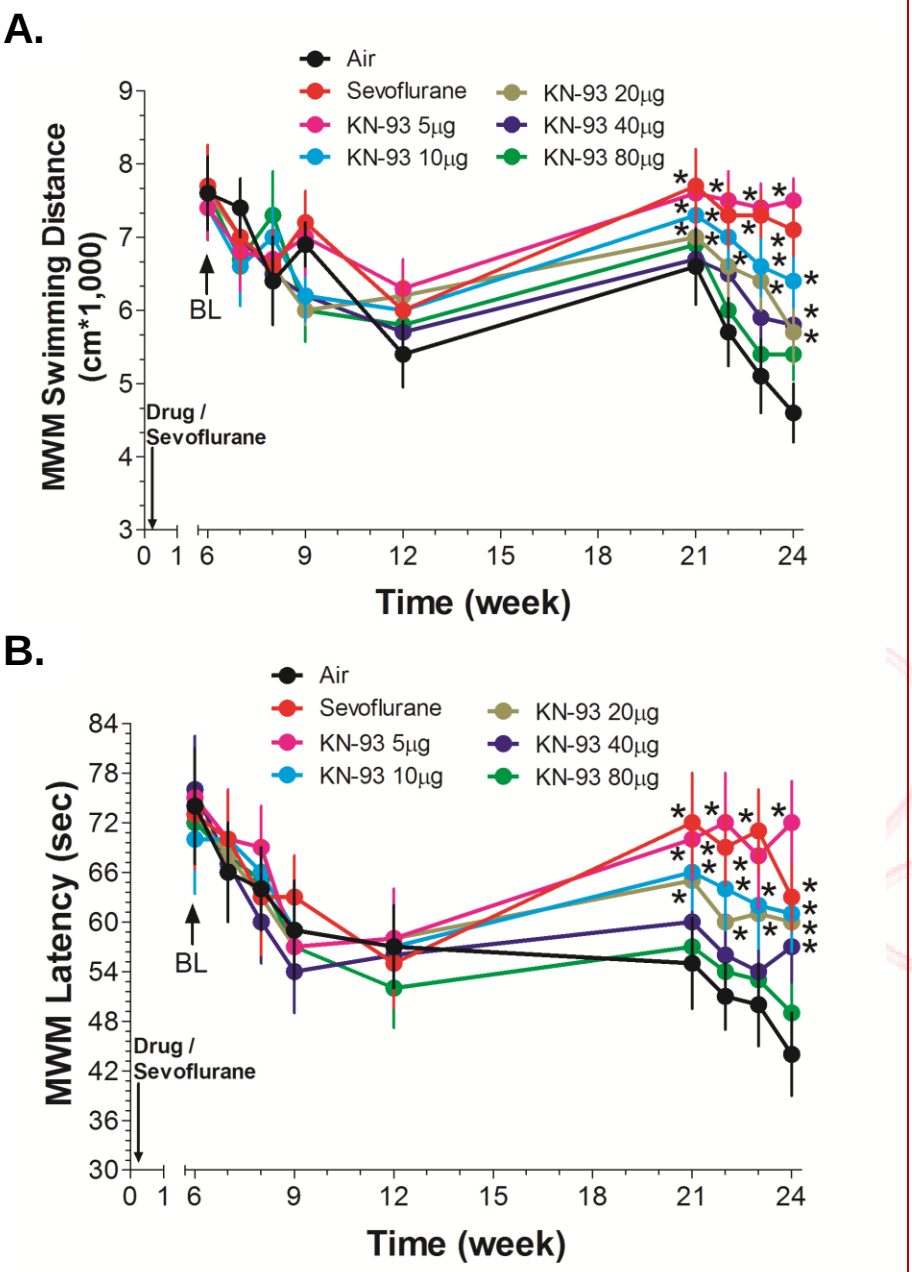

Figure 1. The effect of pontine injection with different doses of CaMKII inhibitor KN-93 on the long-term spatial memory impairment induced by sevoflurane exposure. The p3d rats were undergone 2% sevoflurane exposure for thirty minutes once after pontine medicine injection of different doses of CaMKII inhibitor KN-93 respectively 5, 10, 20, 40, 80μg / 5μl or no pontine medicine injection, and trained with MWM at the forth week after birth followed by spatial memory detection from the 6th to 24th week after birth. The pontine KN-93 injection improved the impaired long-term spatial memory induced by sevoflurane exposure dependently on the doses of KN-93, i.e. the larger dose, the more obvious the improvement was, including the swimming distance (A) and latency (B) of the rats. Data are depicted as mean±SEM, n = 8. Compared with Air Group, *P < 0.05. Air Group only breathed air without sevoflurane exposure.

cannula and the internal cannula were attached to the connector assembly, which consisted of a polyethylene supply tube encased in tough vinyl tubing, and the free end of the tubing was attached to a 10-μl syringe. The infusion system was sterilized before use and preloaded with the infuscate corresponding to the experimental design to eliminate air bubbles before injection.

All infusions were administered at a constant rate of 0.3 μl/min via a syringe pump attached to the connector assembly. The duration of the infusions was 5 min. In rats receiving infusions, cannulas were removed 5 minutes following completion of the infusion. Burr hole site bleeding was controlled by applying gentle pressure with a cotton-tipped applicator, then coated with erythromycin eye ointment to protect from infection. The animal was then removed from the stereotactic frame and put into its cage, and observed thirty minutes. If its behavior was normal, the animal continued to participate in the subsequent experiment.

Sevoflurane exposure procedures

Animals were exposed to sevoflurane (Abbott Laboratories, Animal Health Division, IL, USA) through a specific vaporizer (Penlon Sigma Delta Sevoflurane Vaporizer; Kent Scientific Co., Torrington, CT, USA). A total of 150 rats aged P3D were used in different exposure sessions. Sevoflurane was given through conical tubes with apropriate size to the pups under 100% oxygen.

MAC determination and survival rate

The minimum alveolar concentration (MAC) of sevoflurane for P3D rats have been measured in our previous studies (13). The results were referenced in the present study. The MACs displayed an exposure time-dependent decrease, exposure, duration 1, 2, 3, 4 hours, the MACs corresponding to about 8%, 6.5%, 4%, 3.5%. When exposed for 4 hours at 1 MAC, the P3D rats survival rate was 40%. In our study, we set the exposure-duration 30 minutes each time at 2% concentration of sevoflurane, so the survival rate was 100% for P3D rats.

Session 1: The effect of pontine

injection with different doses of CaMKII inhibitor KN-93 on the long-term spatial memory impairment induced by sevoflurane exposure

A total of fifty-six P3D rats were randomly divided into seven groups consisting of eight animals each (n = 8). The rats in negative control group weren't undergone sevoflurane exposure and pontine medicine injection (Air Group). The rats in positive control group were undergone 2% sevoflurane exposure for thirty minutes only once without pontine medicine injection (Sevoflurane Group). The rats in the other groups (KN-93 5, 10, 20, 40, 80 Group) were administrated pontine medicine injection with different doses of CaMKII inhibitor KN-93 (Sigma) respectively 5, 10, 20, 40, 80μg in volume 5μl by the different group, after thirty minutes, undergone sevoflurane exposure as Sevoflurane Group. After sevoflurane anesthesia, the animals were returned to maternal cages for three weeks and then separated into individual cages until the water maze test was performed at the 24th week after birth. The Morris Water Maze (MWM) training was given once a day for three consecutive days at the forth week after birth and followed by measurement at 1, 2, 3, 4, 7, 16, 17, 18, and 15 weeks after training (Fig. 1).

Session 2: The effect of pontine injection with 30μg KN-93 on the long-term spatial memory impairment induced by sevoflurane exposure

In session 1, we found that there was the tendency of improvement in KN-93 20 Group, and more obvious improvement in KN-93 40 Group at the 21, 22, 23th week after birth compared with Sevoflurane Group about the impaired long-term spatial memory induced by sevoflurane exposure in the rats. In this session, we wondered how about the pontine injection with 30μg KN-93.

A total of twenty-eight P3D rats were randomly divided into 4 groups consisting of eight animals each (n = 8). The rats in negative control group weren't undergone sevoflurane exposure and pontine medicine injection (Air Group). The rats in positive control group were undergone 2% sevoflurane exposure for thirty

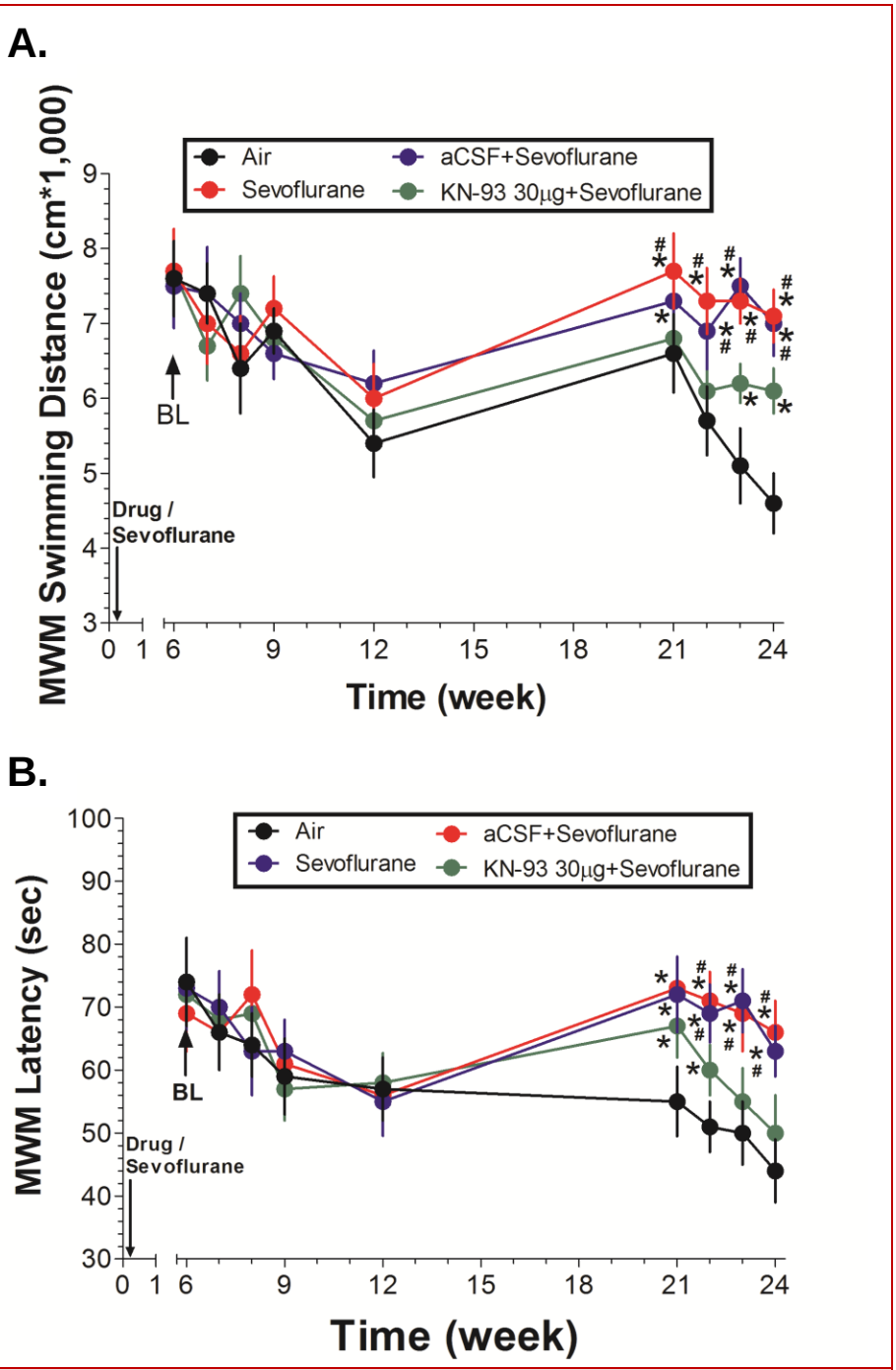

Figure 2. The effect of pontine injection with 30μg KN-93 on the long-term spatial memory impairment induced by sevoflurane exposure. The P3D rats were administrated pontine injection with 30μg KN-93 or artificial cerebrospinal fluid before exposed to 2% of sevoflurane for thirty minutes, and trained with MWM at the forth week after birth, then measured their spatial memory from the 6th to 24th week after birth. The pontine injection with 30μg KN-93 also improved the impaired long-term spatial memory induced by sevoflurane exposure, including the swimming distance (A) and latency (B) of the rats. Data are depicted as mean ± SEM, n = 8. Compared with the Air Group, $^{*}P < 0.05$. Compared with the KN-93-30 Group, $^{\#}P < 0.05$. Air Group only breathed air without sevoflurane exposure.

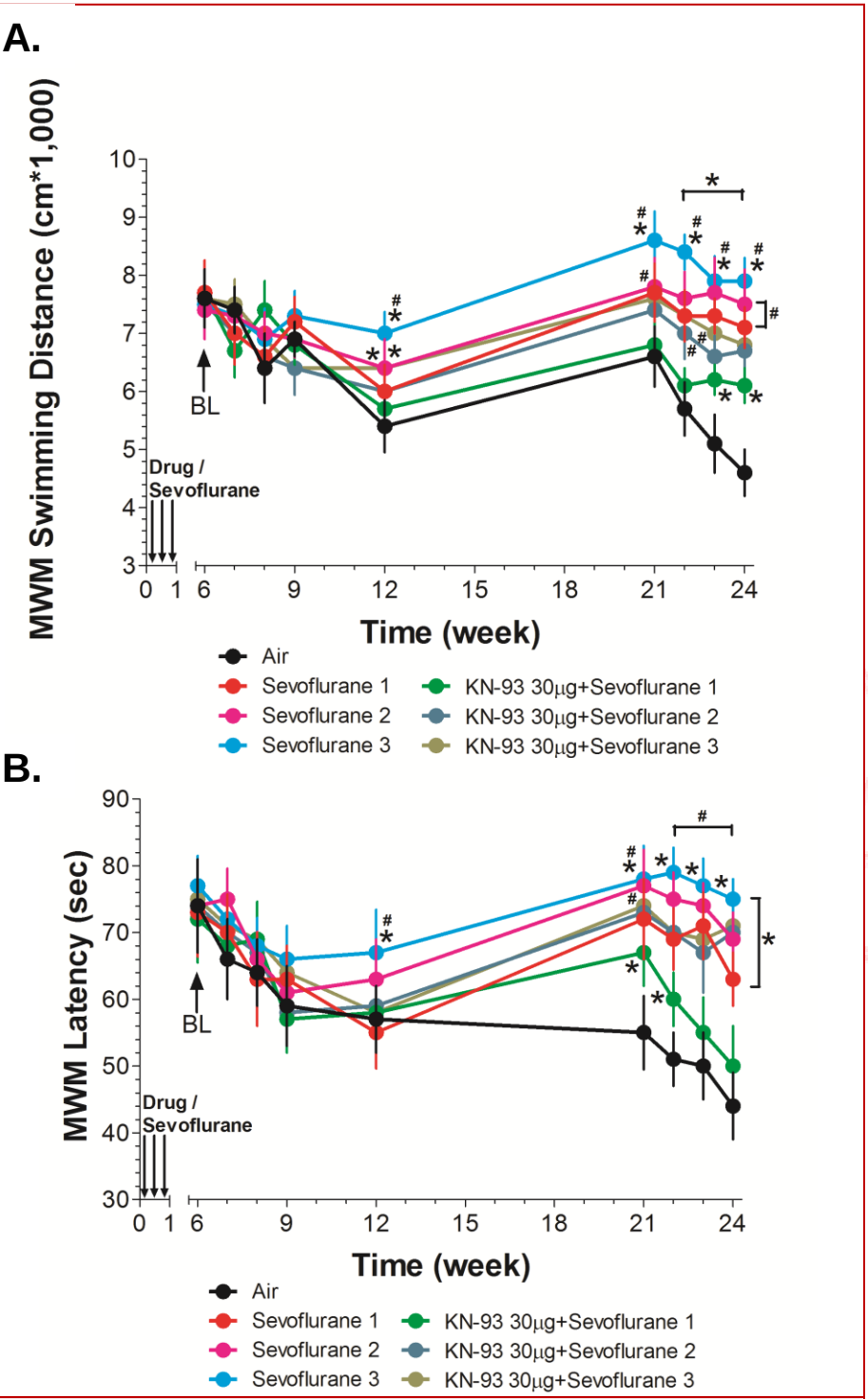

Figure 3. The effect of pontine injection with 30μg KN-93 on the long-term spatial memory impairment induced by repeats sevoflurane exposure. The P3D rats were administrated pontine injection with 30μg KN-93, then exposed repeatedly to 2% of sevoflurane for thirty minutes, and trained with MWM at the forth week after birth, then measured their spatial memory from the 6th to 24th week after birth. The protection of pontine injection with 30μg KN-93 for the damaged spatial memory induced by once sevoflurane exposure was better than repeats sevoflurane exposure, including the swimming distance (A) and latency (B) of the rats. Data are depicted as mean ± SEM, n = 8. Compared with the Air Group, $^{*}P < 0.05$. Compared with the KN-93 30μg + Sevoflurane 1 Group, $^{\#}P < 0.05$. Air Group only breathed air without sevoflurane exposure. 1, 2, 3 means sevoflurane exposure times. BL means base line.

minutes only once without pontine medicine injection (Sevoflurane Group). The rats in the rest groups, were administrated pontine medicine injection with 5μl artificial cerebrospinal fluid (aCSF + sevoflurane Group), or 30μg KN-93 in volume 5μl (KN-93 30μg + sevoflurane Group) by different group, after thirty minutes, undergone sevoflurane exposure as Sevoflurane Group. Then the animals were managed as mentioned above in Session 1.

Session 3: The effect of pontine injection with 30μg KN-93 on the long-term spatial memory impairment induced by repeats sevoflurane exposure

A total of fifty-six P3D rats were randomly divided into seven groups consisting of eight animals each (n = 8). The rats in negative control group weren't undergone sevoflurane exposure and pontine medicine injection (Air Group). The rats in three positive control groups were undergone 2 % sevoflurane exposure for thirty minutes, repeated respectively once, twice, thrice by the different group once a day for three days, withour pontine medicine injection (Sevoflurane 1, 2, 3 Group). The rats in the rest three groups were administrated pontine medicine injection with 30μg KN-93 in volume 5μl, thirty minutes later, undergone sevoflurane exposure as the three positive control groups (KN-93 30μg + Sevoflurane 1, 2, 3 Group). Then all the animals were managed as the mentioned above in Session 1.

Spatial memory detection

The Morris Water Maze (Shanghai Jiliang Software Technology Co. Ltd., China) was used as we have previously reported. In brief, the MWM was performed to detect sevoflurane-induced changes in animals' spatial memory and cognitive function after different interventional procedures. A circular tank 100 cm in diameter and 30-cm deep was filled with water to a depth of 25 cm. A transparent round platform 10 cm in diameter was

placed at 0.5 cm below the surface of the water. During the test of spatial memory, the animals learned to use distinctive distal visual cues surrounding the pool to navigate a direct path to the hidden platform. The platform remained in a constant location during the acquisition phase. Animals were placed on the platform for 30 s preceding the start of each training session. Animal training took place during a 3-day acquisition phase with three massed trials administered each day. Rats were allowed to swim freely for 90 s or until the platform was reached. If the platform was not located within the 90 s, the rats were guided to the platform and allowed to remain there for 30 s. After completion of three consecutive trials, the rats were placed in their home cages under a heat lamp for 10 min. A video camera mounted to the ceiling directly above the center of the maze was used in conjunction with a computerized animal tracking system to monitor latency to the platform and distance swam.

Statistical analysis

The MWM data are presented as the means ± standard error of the means (SEM), and were compared with two-way ANOVA. The ANOVA tests were followed by Bonferroni *post hoc* tests if applicable. GraphPad Prism v5.0 (GraphPad Software Inc., San Diego, CA, USA) and PASW Statistics v18.0 (IBM Co., Armonk, NY, USA) were used for data analyses. All reported P values are two-sided and a P value of less than 0.05 is accepted for statistical significance.

RESULTS

The effect of pontine injection with different doses of KN-93 on the long-term spatial memory impairment induced by sevoflurane exposure

We exposed P3D rats to 2% of sevoflurane for thirty minutes after pontine injection with different doses KN-93, and trained them with MWM at the forth week after birth, then measured their spatial memory from the 6th to 24th week after birth. We found that pontine KN-93 injection improved the impaired long-term spatial memory induced by sevoflurane exposure dependently on the doses of KN-93, i.e. the larger dose, the more obvious the improvement was. The swimming latency and distance of the rats in KN-93 40, 80 Groups were both shorter significantly than that in Sevoflurane Group($P < 0.05$), and similar to that in Air Group ($P > 0.05$) at the 21, 22, 23th week after birth, even at 24th week so were that in KN-93 80 Group. There was also the tendency of the improvement in KN-93 10, 20 Groups at the 21, 22, 23th week after birth compared with Sevoflurane Group (Fig. 1).

The effect of pontine injection with 30μg KN-93 on the long-term spatial memory impairment induced by sevoflurane exposure

We administrated pontine injection with 30μg KN-93 or artificial cerebrospinal fluid to the P3D rats before exposed to 2% of sevoflurane for thirty minutes, and trained them with MWM at the forth week after birth, then measured their spatial memory from the 6th to 24th week after birth. We found that pontine injection with 30μg KN-93 also improved the impaired long-term spatial memory induced by sevoflurane exposure. The swimming latency and distance of the rats in the KN-93 30μg Group were both shorter significantly than that in Sevoflurane Group at the 21, 22, 23, 24th week after birth ($P < 0.05$) or than that in aCSF Group at the 22, 23, 24th week after birth ($P < 0.05$), and the swimming distance in the KN-93 30μg Group was similar to that in Air Group at the 21, 22nd week after birth ($P > 0.05$), the swimming latency was also similar to that in Air Group at the 23, 24th week after birth ($P > 0.05$) (Fig. 2.).

The effect of pontine injection with 30μg KN-93 on the long-term spatial memory impairment induced by repeats sevoflurane exposure

We administrated pontine injection with 30μg KN-93 to the P3D rats, thirty minutes later, repeatedly by the different group exposed them to 2% of sevoflurane for thirty minutes once a day for three days , and trained them with MWM at the forth week after birth, then measured their spatial memory from the 6th to 24th week after birth. We found that the protection of pontine injection with 30μg KN-93 for the damaged spatial memory induced by once sevoflurane exposure was better than repeats sevoflurane exposure. The swimming latency and distance of the rats in the KN-93 30μg + Sevoflurane 1 Group were both shorter significantly than that in Sevoflurane 1 Group ($P < 0.05$), but there were significant difference neither between that in the KN-93 30μg + Sevoflurane 2 and Sevoflurane 2 Group ($P > 0.05$), nor between that in the KN-93 30μg + Sevoflurane 3 and Sevoflurane 3 ($P > 0.05$), at the 21, 22, 23, 24th week after birth. and the swimming latency in the KN-93 30μg + Sevoflurane 1 Group was similar to that in Air Group at the 23, 24th week after birth ($P > 0.05$), the swimming distance in the KN-93 30μg + Sevoflurane 1 Group was also similar to that in Air Group at the 22, 23, 24th week after birth ($P > 0.05$) (Fig. 3.).

DISCUSSION

GABA neural pathways action is mediated by Wnt signaling pathways (14,15,20,21). And there are three different ways to activate Wnt signaling pathways, i.e. the classic pathway by upregulating beta-catenin in cytoplasm, the Wnt/PCP pathway by activating c-Jun N-terminal kinase (JNK) , and the Wnt/Ca^{2+} pathway depending on the activation of calmodulin protein kinase II (CaMKII) and protein kinase c (PKC) (16,22). Our previous studies showed CaMKII inhibitor KN-93 pontine injection in early-life rats significant-

ly inhibited the expression of CaMKII and $GABA_A$ receptor ($GABA_A$ R) in pontine reticular formation, shortened the anesthesia induction time of 1 MAC (minimum alveolar concentration effectively) sevoflurane, but β-Catenin inhibitor FH535, JNK inhibitor TAT-TI-JIP, PKC inhibitor Gö 6976 did not produce the similar effect as KN-93. So we speculated that sevoflurane works on GABA-Wnt-CaMKII signaling pathway in pontine reticular formation to produce general anesthesia effect. Based on these findings, the present studies observed the effects of sevoflurane exposure of young rats injected KN-93 to pons on the long-term spatial memory by MWM testing.

In sum, the data showed that pontile inhibition of GABA-Wnt-CaMKII through blocking CaMKII substantially alleviated sevoflurane-induced long-term special memory, which demonstrated dose- and time-dependent association. These preliminary observations indicated that the pontile GABA signaling plays an essential role in sevoflurane-induced adulthood cognition impairment, and further evaluation is needed on the exact interaction among sevoflurane, GABA, glutamate, receptors, corresponding signaling mediators, and cognition alteration in the pontine region.■

Conflict of Interests

None

References

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