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Sevoflurane enhanced the clearance of Aβ1-40 in hippocampus under surgery *via* up-regulating AQP-4 expression in astrocyte

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ABSTRACT

The neurotoxicity of anesthetics on developing brain has been a focused issue for years. However, controversy exists between human and animal studies and surgery may be a potential reason for this. The discovery of glymphatic system, a pathway eliminating soluble substance from central nervous system (CNS), together with recent evidence that surgery-induced Aβ increase contributes to cognition dysfunction made us rethink about the influence of anesthetics on cognitive function. The function of glymphatic system was proved to be enhanced by sleep and sedation, so we assumed that under clinical situation Aβ1-40 whose accumulation played important role in cognitive dysfunction was increased by surgery and eliminated from CNS by glymphatic system. The function of glymphatic system is facilitated by aquaporin-4 (AQP-4), a water channel expressed in highly polarized manor in astrocytic endfeet, whose transcription is regulated by nuclear factor of activated T cells 5 (NFAT5). Our results suggest that under brief operation and sevoflurane exposure, surgery may be the main cause of Aβ increase and sevoflurane increase the elimination of Aβ by up-regulating AQP-4 which is the key component in glymphatic system.

1. Introduction

Anesthetics and their potential neurotoxicity on developing brain have attracted great attention of medical practitioners and the public for > 15 years. The data from rodents' studies [1] showed that long-lasting or repeated but not brief exposure to anesthetics caused neurological deficits. Since 2015, the results of five major clinical studies, including GAS (General Anesthesia compared to Spinal anesthesia) study [2], the PANDA (Pediatric Anesthesia and Neuro Development Assessment) study [3], two Canadian population-based studies conducted in the provinces of Ontario and Manitoba [4,5], Swedish population-based study [6], showed no or modest association between anesthesia exposure and neurological outcome [7]. But why we got different results from rodent and human studies? Actually, the main difference existed in the condition of these two kinds of researches was: all the human studies received surgeries under anesthesia while most rodent studies were under anesthesia only without surgeries. For further understanding this difference, two questions must be answered: first, whether surgery *per se* has directly harmful effect on brain; second, what is the role of anesthetics exposure in the process of surgery influence on brain function. The peripheral surgery was able to induce cognitive impairment independent of general anesthesia, and that the increase of Aβ in brain was needed for the postoperative cognitive dysfunction (POCD) to occur in old animal [8]. The POCD patients were associated with higher postoperative plasma levels of MDA, higher IL-1β and lower Aβ1–42 levels in preoperative cerebrospinal fluid (CSF) that might predispose the development of POCD in aged patients following total hip-replacement surgery with spinal anesthesia [9]. All those researches reminded that the surgery-induced Aβ increase played an important role in POCD occurrence. In normal condition, the production and clearance rate of Aβ *in vivo* in human CNS are 7.6% per hour and 8.3% per hour respectively [10]. Iliff et al. showed that some plasma Aβ could be transferred into interstitial solution through the para-vascular pathway [11] formed by astroglia cells helping to eliminate the soluble proteins and metabolites from CNS. Though the key role of this clearance system is to keep the normal function of brain, the influence of surgery and sevoflurane treatment on its function is not clear. We set out this research to clarify the changes of neuroanatomy and neurocognition after brief sevoflurane exposure in the presence of surgery and their potential effects on elimination system of CNS.

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2. Materials and methods

2.1. Rat anesthesia and treatment

All procedures were approved by the Animal Care and Use Committee of Fudan University and followed institutional guidelines. Two-week old Sprague Dawley (SD) male rats (SLAC Laboratory animals, Shanghai, China), weighting 26–30 *g* were used in this study. Animals were raised in a temperature controlled (22°–23°) room under a light/dark cycle (12 h light/12 h dark). Food and water were available *ad libitum*. The rats were randomly divided into five groups: control group (n = 28), received 100% oxygen at an identical flow rate in an anesthetizing box for 2 h; pentobarbital sodium group (n = 28), intraperitoneal injection of 3% pentobarbital sodium about 0.3 ml/100 g and received 100% oxygen in the box for 2 h; sevoflurane group (n = 28), received 2.5% sevoflurane delivered by 100% oxygen in the box for 2 h; pentobarbital sodium with splenectomy group (n = 28), underwent splenectomy under 3% pentobarbital sodium about 0.3 ml/100 g and received 100% oxygen with a breathing mask for 2 h; sevoflurane with splenectomy group (n = 28), underwent splenectomy under 2.5% sevoflurane delivered by 100% oxygen with the mask for 2 h. The duration of pentobarbital sodium anesthesia was 120 ± 12 min while sevoflurane anesthesia was ceased by terminating its supply at 2 h. The anesthetic and oxygen concentrations were monitored closely with a gas analyzer (Drager Inc.). The temperature of the anesthetizing box was controlled to maintain a 37° ± 0.5 °C rectal temperature in the mice. The procedure of splenectomy referred to JoVE video magazine. The size of the anesthetizing box was $30 \times 20 \times 22\ cm^3$. As compared with control group, the anesthetized rats did not show significant changes in behavior (*e.g.*, eating, drinking, general activity, and body weight) after the anesthesia (data not show). Mortality rate of rats in these studies was < 1%.

2.2. Primary astrocyte culture and anesthesia

Neonatal SD rat was decapitated in an ice-chilled dish and the brain was removed. After removal of the meninges, the cortex and hippocampus were dissected, cut into pieces, digested with 0.125% trypsin at 37 °C for 15 min and terminated by adding 2 ml serum-supplied medium. Then the tissue was transferred into centrifuge tube (15 ml), added to 6 ml with culture medium (10% FBS + DMEM + Penicillin-Streptomycin) and dissociated 20 times with a 1000ul plastic pipette for 6 times. The supernatant was removed after centrifugation at 1000 rpm for 5 min. The cells were resuspended with pre-warmed medium, seeded into poly-L-lysine-coated six well plate at initial concentration of 1×10^5 cells/ml and placed in an incubator with 5% CO_2 at 37 °C. After 7 days, the plates were agitated on an orbital shaker for 12 h at 200 rpm at 37 °C and the non-adherent oligodendrocytes, microglia cells and neurons were removed. The astrocytes were treated with 4% sevoflurane delivered by 5%CO_2 in 37 °C incubator for 6 h.

2.3. NFAT-5 shRNA design, lentivirus construction and transfection with shRNA

In order to explore the relation of NFAT-5 and AQP-4 protein, small interfering RNA(shRNA) specifically targeting NFAT-5 was designed to inhibit NFAT-5 expression efficiently and lentivirus expressing such NFAT-5 shRNA was constructed and prepared as described previously [12] Briefly, shRNA (target sequence: AATGGAAGTGTTGACTTGG) targeting rat NFAT-5 was designed and the negative control was as follows: TTCTCCGAACGTGTCACGT. Then related stem-loop-stem oligonucleotides were synthesized, annealed and inserted into lentiviral vector GV248(GeneChem, Shanghai, China). At last, lentivirus expressing NFAT-5 shRNA or control shRNA were produced through Lentivector Expression System (GeneChem, Shanghai, China).

Astrocytes were used to examine the knockdown efficiency of NFAT-5 shRNA. Cells were cultured as described above and infected with lentivirus expressing NFAT-5 shRNA or scrambled shRNA. Then cells were cultured in an incubator with 5% CO_2 at 37 °C for another 5 days. As before, after being purified, astrocytes were treated with 4% sevoflurane delivered by 5%CO_2 in 37 °C incubator for 6 h. Following these procedures, cells were harvested and total protein and RNA were extracted to determine the knockdown efficiency and verify the relation between NFAT-5 and AQP-4 using western blot and quantitative real-time PCR.

2.4. Morris water maze (MWM)

The MWM test was performed as described in our former study [13]. Ten rats were selected randomly in each group 5 days after splenectomy when wound healed to be tested in the pool four times a day started in each quadrant for 6 days. Rat was placed in each quadrant in random order facing the wall of the pool to search for the platform. Rat that found the platform was allowed to stay on it for 20s and if failed to find the platform within 60s, it was guided to reach the platform and allowed to stay on it for 20s. All the results (escape latency, platform crossing times, swimming speed and time in target quadrant) were recorded and analyzed by a video tracking system motion-detection software specialized for MWM (Noldus Etho Vison XT, Noldus Information Technology Beijing, China). On the seventh day, the platform was removed from the pool and the rat was placed from the chose quadrant and allowed to swim for 60s. After every trail, each rat was placed in a special cage with a heat lamp for 5 min to dry before being returned to its regular cage. The interval time between two trials was > 15 min.

2.5. Brain tissue harvest and astrocyte protein extraction

Immediately after the anesthesia, rats were killed by cardiac perfusion with saline. For western blot, 0.1 g tissue of the harvested hippocampus was separated and homogenized on ice using mixture buffer (RIPA lysis buffer , PMSF, Beyotime Biotechnology)plus protease inhibitor (Beyotime Biotechnology, Shanghai, China) after being oscillated at high speed by grinder (Bertin Technologies, France). Cultured astrocytes were collected by scraping and gone through the same procedure as brain tissue. The lysates were collected, centrifuged at 14,000 rpm for 15 min and quantified for total protein concentration with Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, Shanghai, China).

2.6. Western blot analysis

Aquaporin-4 (AQP-4) antibody (1:1000 dilution, Arigo, Taiwan, China) and Beta-Amyloid (1-40) antibody (1:1000 dilution, Millipore, America) was used to recognize AQP-4 (35 kDa) and Aβ1-40(4 kDa). NFAT-5 antibody (1:1000 dilutions, Abcam, Cambridge, MA) was used to recognize NFAT-5 (170 kDa). β-actin antibody (1:1000 dilutions, Santa Cruz Biotechnology) was used to recognize β-actin. 50 μg of the total protein of each sample was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto nitrocellulose membranes. We used β-actin levels to normalize target proteins levels and control for loading differences in total protein amount. Blots were visualized using Odyssey Imaging System (LI-COR Bioscience). The results were showed as the ratio of interest protein to β-actin and control levels were normalized to 100% for comparison with experimental conditions.

2.7. Immunofluorescence

Rats in control group were anesthetized with pentobarbital sodium and other groups were kept anesthetized as experimental conditions and all of them were perfused transcranial with saline followed by 4%

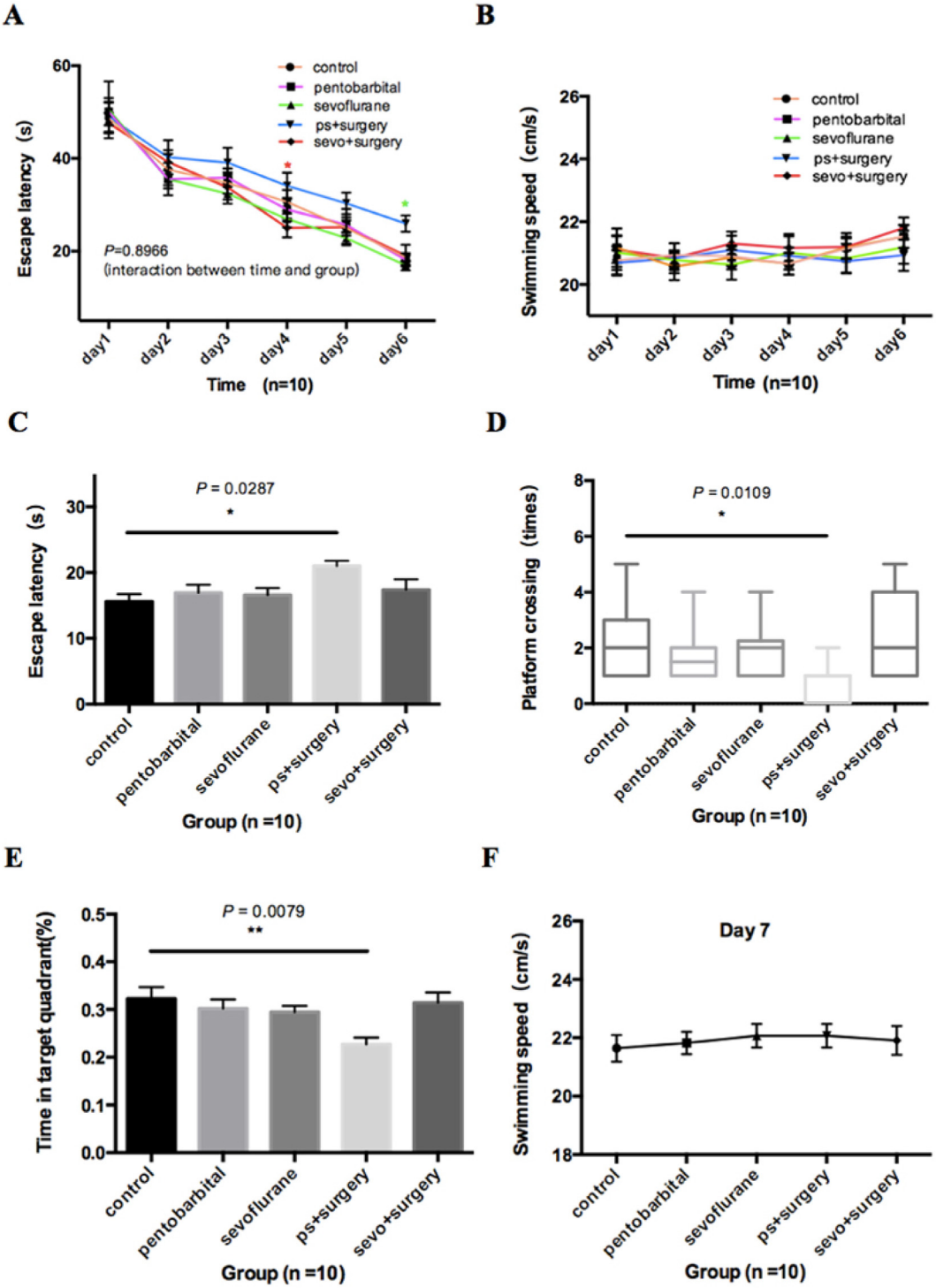

Fig. 1. Anesthesia with 2.5% sevoflurane for 2 h does not induce cognitive impairment under surgery situation. (A) the Escape latency time of each group and rats received splenectomy under pentobarbital sodium (ps + surgery) anesthesia had a longer escape latency swimming in MWM as compared with the rats under sevoflurane anesthesia with surgery (sevo + surgery) (*) or not (*) on the day 4 and the day 6($P < 0.05$, n = 10). (B) Swimming speed of each group had no significantly difference (time factor: $F_{(5,225)} = 1.525$, $P = 0.1828$; group factor: $F_{(4,45)} = 0.3758$, $P = 0.8247$; interaction: $F_{(20,225)} = 0.2997$, $P = 0.9987$, two-way ANOVA with repeated measurement). (C) In spatial probe test, the rats received surgery with pentobarbital sodium anesthesia ($n = 10$) had longer escape latency as compared to control condition (One-way ANOVA, $F_{(4,45)} = 2.984$, $P = 0.0287$). (D) In spatial probe test, the rats received surgery with pentobarbital sodium anesthesia (n = 10) had decreased platform crossing times as compared to control condition (One-way ANOVA, $F_{(4,45)} = 3.705$, $P = 0.0109$). (E) In spatial probe test, the rats received surgery with pentobarbital sodium anesthesia (n = 10) had shorter time in target quadrant (One-way ANOVA, $F_{(4,45)} = 3.943$, $P = 0.0079$). (F) There was no significant difference in rat swimming speed among each group (n = 10) (One-way ANOVA, $F_{(1.821,16.39)} = 0.1898$, $P = 0.8094$).

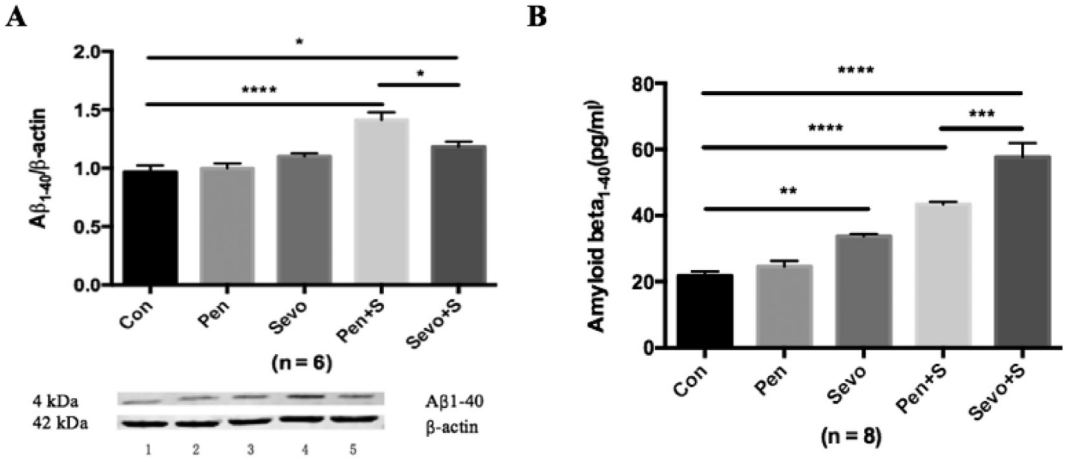

Fig. 2. The sevoflurane treatment enhances the clearance of surgery induced accumulation of Aβ1-40 from hippocampus to superior vena cava blood. (A) Compared with control group, Aβ1-40 in hippocampus increased in two surgery groups (Pen + S = pentobarbital sodium + surgery, Sevo + S = sevoflurane + surgery). Aβ1-40 in sevoflurane anesthesia group was significantly lower than pentobarbital sodium anesthesia group (one-way ANOVA, *post hoc*, $F_{(4,25)} = 13.44$, $P < 0.05$, n = 6) (1 = control, 2 = pentobarbital sodium, 3 = sevoflurane, 4 = pentobarbital sodium + surgery, 5 = sevoflurane + surgery). (B) Sevoflurane (Sevo) treatment increased Aβ1-40 in superior vena cava blood. After surgery, sevoflurane + surgery group showed a higher concentration of Aβ1-40 in superior vena cava blood compared with pentobarbital sodium + surgery group (one-way ANOVA, *post hoc,* $F_{(4,35)} = 45.29$, $P < 0.05$, n = 8).

paraformaldehyde 2 h after anesthesia. Then rat brain tissue was removed, kept at 4 °C in 4% paraformaldehyde overnight and placed sequentially in 20% and 30% sucrose at 4 °C until completely sank. Cryosection was used for coronal sections (25 μm) and then kept in preservation solution. The sections were blocked with 10% goat serum and 0.3% Triton-X-100, incubated with AQP-4 antibody (ARG22361, 1:200, Arigo), glial fibrillary acidic protein (GFAP) antibody (#3670, 1:250, Cell Signaling Technology) and secondary antibody (A-21206/A-21203, Alexa Flour 488/594, 1:1000/1:500, Invitrogen). Then the sections were incubated with DAPI solution (D9542, 1:10000, Sigma) for 5 min. Images of AQP-4, GFAP and DAPI were acquired using Leixa fluorescence microscope.

Primary astrocyte cultured in 24 well plate with small round glass slide were fixed with 4% paraformaldehyde at room temperature for 30 min and blocked with 10% goat serum. The rest procedures were as same as brain sections.

2.8. Real-time quantitative polymerase chain reaction (RT-qPCR)

RNA was reverse transcribed to cDNA using EZ-press Cell to Ct Kit (B0001, ZScience Biotechnology) following manufacturer's instructions. RT-qPCR was performed using qPCR SYBR Green Master Mix Kit (B0001, A0001, ZScience Biotechnology). Changes in gene expression of NFAT-5 were determined using the ΔΔCt method [14] and β-actin was used as a reference gene for loading control. Expression of NFAT-5 gene in experimental group was defined relative to control group in which the expression was defined as 100%. The primer sequences of NFAT-5 and β-actin were as follows: NFAT-5, forward 5′-GGGTCAAA CGACGAGATTG TG-3′; reverse 5′-TGCCTTTGTTGTCCGTGGTA-3′; β-actin, forward 5′-CCTCTA TGCCAACACAGT-3′; reverse 5′-AGCCACCA ATCCACACAG-3′.

2.9. Enzyme linked immunosorbent assay (ELISA)

Superior vena cava blood was collected from each rat 2 h after anesthesia and serum was extracted using serum separated tube (SST) tube and applied for concentration determination of Aβ1-40 using Beta-Amyloid (1-40) ELISA Kit (ARG80980, Arigo) following manufacturer's instructions.

2.10. Statistical analysis

All the data was expressed as mean ± SEM. GraphPad Prism 6 was used to analyze the data. One-way ANOVA was used to compare the difference about escape latency, swimming speed, platform crossing time and time in target quadrant in spatial probe test of MWM. Interaction between time and group factors in two-way ANOVA with repeated measurements was used to analyze the difference of learning curves which was based on escape latency between control and anesthesia groups in navigation test of MWM. The *P* value was corrected using Bonferroni test in multiple comparisons. The Student two sample *t*-test was used to analyze the difference of AQP-4 and MFAT-5 expression in primary cultured astrocyte between control and sevoflurane group. One-way ANOVA was also used in the comparison between control and anesthesia groups in levels of AQP-4, NFAT-5, Aβ1-40 and β-actin. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Short time (2 h) exposure to sevoflurane do not induce cognitive impairment in young rat whether under surgery or not

To study whether short time exposure to anesthesia induces cognitive impairment under surgery situation, we established an animal model in which young rats exposed to 2.5% sevoflurane for 2 h or underwent splenectomy at the same time.

Pentobarbital sodium is the most commonly used anesthesia drug for animal experiment so that we set up pentobarbital sodium group and pentobarbital sodium with splenectomy group to separate the anesthesia effect of sevoflurane on the cognition. Rats treated with splenectomy under 2.5% sevoflurane or pentobarbital sodium anesthesia, exposed to 2.5% sevoflurane or pentobarbital sodium anesthesia without surgery and the rats without any treatment were used as control. Rats in each group were tested in MWM 5 days after surgery or anesthesia. In navigation test (day1–6), results showed that there was no significantly difference in escape latency time in MWM among rats of control condition, sevoflurane anesthesia and pentobarbital sodium anesthesia groups (Fig. 1A). The *post hoc* Bonferroni test showed that rats received surgery under pentobarbital sodium anesthesia had longer escape latency than the rats under sevoflurane anesthesia with surgery or not on day 4 and day 6(time factor: $F_{(5,225)} = 99.18$, $P < 0.05$; group factor: $F_{(4,45)} = 1.66{,}1$ $P = 0.176$; interaction: $F_{(20,225)} = 0.62$,

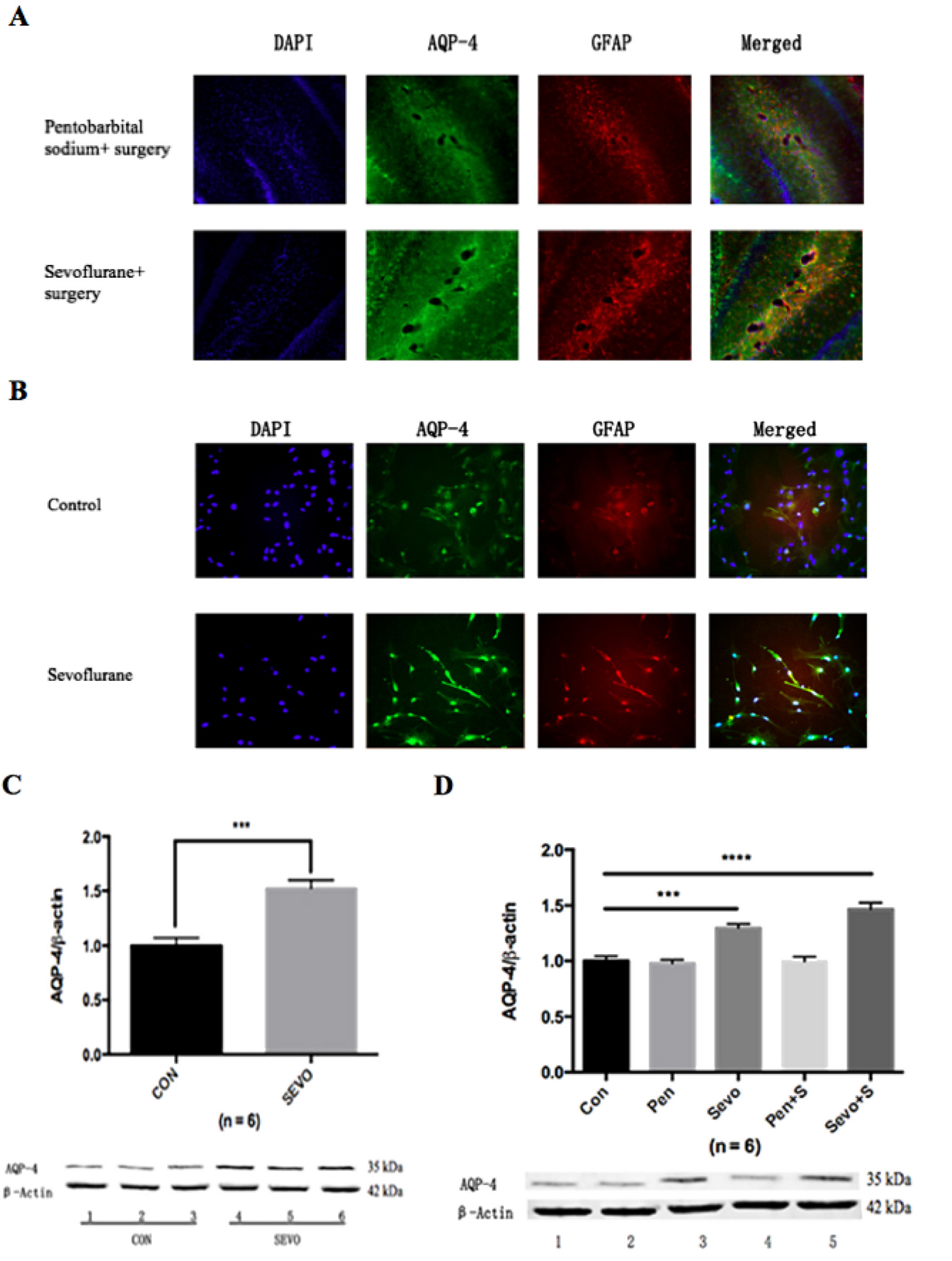

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Fig. 3. Anesthesia with sevoflurane for 2 h upregulated AQP-4 expression in hippocampus and primary astrocyte. (A) In brain slice, the fluorescence signal of AQP-4 enhanced around microvessel in sevoflurane + surgery group compared with pentobarbital sodium + surgery. (B) Compared to control group, AQP-4 fluorescence signal was obviously increased in sevoflurane group in primary astrocyte. (C) Sevoflurane treated astrocyte showed an increased expression of AQP-4(The student two sample t-test, $P < 0.05$, n = 6) (CON = Control, SEVO = Sevoflurane). (D) Compared with control group, the expression of AQP-4 increased in two sevoflurane exposure groups (one-way ANOVA, *post hoc,* $F_{(4,25)} = 25.34$, $P < 0.05$, n = 6) (1 = control, 2 = pentobarbital sodium, 3 = sevoflurane, 4 = pentobarbital sodium + surgery, 5 = sevoflurane + surgery).

$P = 0.8966$, two-way ANOVA with repeated measurement). The swimming speed of each group had no significant difference (Fig. 1B) (time factor: $F_{(5,225)} = 1.525$, $P = 0.1828$; group factor: $F_{(4,45)} = 0.3758$, $P = 0.8247$; interaction: $F_{(20,225)} = 0.2997$, $P = 0.9987$, two-way ANOVA with repeated measurement).

The spatial probe test was performed at the seventh day. The rats received surgery with pentobarbital sodium anesthesia ($n = 10$) had longer escape latency ($F_{(4,45)} = 2.984$, $P = 0.0287$, one-way ANOVA), decreased platform crossing times ($F_{(4,45)} = 3.705$, $P = 0.0109$, one-way ANOVA) and shorter time in target quadrant ($F_{(4,45)} = 3.943$, $P = 0.0079$, one-way ANOVA) as compared with control group (n = 10) (Fig. 1C–E). There was no significant difference in swimming speed among each group (n = 10) (Fig. 1F) ($F_{(1.821,16.39)} = 0.1898$, $P = 0.8094$, one-way ANOVA). These results suggest that short time exposure to sevoflurane may not induce cognitive impairment in young rat and surgery may be the main reason causes the change of learning and memory ability. More important, the use of sevoflurane can reverse the learning and memory ability after splenectomy operation while pentobarbital sodium has no this function.

3.2. βeta Amyloid 1-40 (Aβ1-40) slightly accumulated in hippocampus of rats exposure to splenectomy and sevoflurane treatment enhanced the clearance of surgery induced accumulation of Aβ1-40 from hippocampus to superior vena cava blood

βeta Amyloid 1-40 (Aβ1-40) exists in healthy brain during normal physiological conditions and may affect cognitive function when accumulates in pathological situation. To evaluate the influence of Aβ1-40 on the learning and memory ability after surgery, we detected the content of Aβ1-40 in hippocampus in each group by western blot analysis. Compared with control group, Aβ1-40 in hippocampus obviously increased in two surgery groups. However, the increase degree of Aβ1-40 in sevoflurane anesthesia group was significantly lower than pentobarbital sodium anesthesia group (one-way ANOVA, *post hoc*, $F_{(4,25)} = 13.44$, $P < 0.05$, n = 6) (Fig. 2A). These results suggest that surgery may induce the accumulation of Aβ1-40 in hippocampus and sevoflurane treatment can decrease the accumulation of Aβ1-40 in hippocampus induced by surgery. In order to figure out the reason of sevoflurane induced decrease of Aβ1-40 in hippocampus after surgery, we further test the concentration of Aβ1-40 in superior vena cava blood, which partly represent the clearance of Aβ1-40 to systemic circulation from brain. Results showed that sevoflurane treatment without surgery could increase the concentration of Aβ1-40 compare with control and pentobarbital groups. After surgery, compared with pentobarbital + surgery group, sevoflurane + surgery group showed a higher concentration of Aβ1-40 in superior vena cava blood after surgery (Fig. 2B) (One-way ANOVA, *post hoc,* $F_{(4,35)} = 45.29$, $P < 0.05$, n = 8). These results reminded that sevoflurane could enhance the clearance of Aβ1-40 from brain and decrease the accumulation of Aβ1-40 in hippocampus induced by surgery.

3.3. AQP-4 expression were upregulated in hippocampus and primary astrocyte following sevoflurane anesthesia

AQP-4 water channels on the vascular endfeet of astrocytes [15] facilitate convective flow out of the para-arterial space and into the interstitial space [16] to clear waste metabolites, include amyloid-β [17]. To evaluate the role of AQP-4 in sevoflurane anesthesia enhanced clearance of Aβ1-40 from brain, the AQP-4 immunoreactivity on perivascular astrocyte was observed in brain slice. In sevoflurane + surgery group, enhanced fluorescent signal of AQP-4 was seen around microvessel that suggest increased expression and higher polarization of AQP-4 in hippocampus compared with pentobarbital sodium + surgery group (Fig. 3A). To more directly confirm this result, the effect of sevoflurane on the expression of AQP-4 was observed in primary cultured astrocyte. AQP-4 fluorescent signal was obviously increased in sevoflurane treated group compared to control group (Fig. 3B) and western blot analysis showed an increased expression of AQP-4 after sevoflurane exposure both *in vitro* and *in vivo* (Fig. 3C, D) (C: The student two sample *t*-test, $P < 0.05$, n = 6; D: one-way ANOVA, *post hoc,* $F_{(4,25)} = 25.34$, $P < 0.05$, n = 6).

3.4. Sevoflurane promotes AQP-4 expression via up-regulating NFAT-5 expression

Since sevoflurane exposure increased AQP-4 expression, we further explore the up-stream regulator of AQP-4. The essential transcription factor for AQP-4 is Nuclear Factor of Activated T cells 5(NFAT-5) which plays key role in water homeostasis in CNS. Its expression was increased both in sevoflurane treated rat hippocampus (One-way ANOVA, *post hoc*, $F_{(4,25)} = 20.98$, $P < 0.05$, n = 6) and primary astrocyte (The student two sample *t*-test, $P < 0.05$, n = 6) (Fig. 4A, B). In order to verify that AQP-4 is a target gene of NFAT-5 transcriptional activity under sevoflurane exposure, we silenced NFAT-5 with RNAi method in primary astrocyte. The silence effect was quantified using RT-qPCR. Results showed the increase of NFAT-5 mRNA in sevoflurane treated groups could be effectively reversed (One-way ANOVA, *post hoc*, $F_{(3,20)} = 38.97$, $P < 0.05$, n = 6). Consistent with RT-qPCR results, western blot analysis showed that the protein expressions of NAFT-5 (One-way ANOVA, *post hoc*, $F_{(3,28)} = 136.1$, $P < 0.05$, n = 8) and AQP-4(One-way ANOVA, *post hoc*, $F_{(3,28)} = 62.32$, $P < 0.05$, n = 8) decreased after silencing NFAT-5 mRNA (Fig. 4C–E). These data suggest that sevoflurane promotes AQP-4 expression *via* up-regulating NFAT-5.

4. Discussion

In this study, we compared the effects of sevoflurane and pentobarbital sodium, a commonly used anesthetic in non-anesthesia-related animal research, on brain function in the presence and absence of surgery respectively, to address the effect of sevoflurane on the long-term cognitive deficits under surgery situation. We found that surgery *per se* caused the increase of toxic protein (Aβ) in brain while sevoflurane enhanced the clearance of Aβ by up-regulating AQP4 expression in astrocytes. These results were consistent with previous studies from Xie et al. [18] that the clearance of Aβ1-40 was twofold faster in sleeping and anesthetized mice compared with the awake mice through glymphatic system.

The acute effects of surgery were proved to be associated with the effects of peripheral inflammatory response, such as Tumor Necrosis Factor-α (TNFα) [19,20], IL-6 and Interleukin 1-beta (IL-1β) [21,22] and such effects lasted only a week. In our study, intraperitoneal injection of pentobarbital sodium did not cause neurocognitive deficits in the absence of splenectomy. While under the surgery condition with pentobarbital sodium anesthesia, the neurocognitive deficits were observed in MWM experiment. These data indicated that surgeries instead of anesthetics may contribute to the neurotoxicity. Our result was

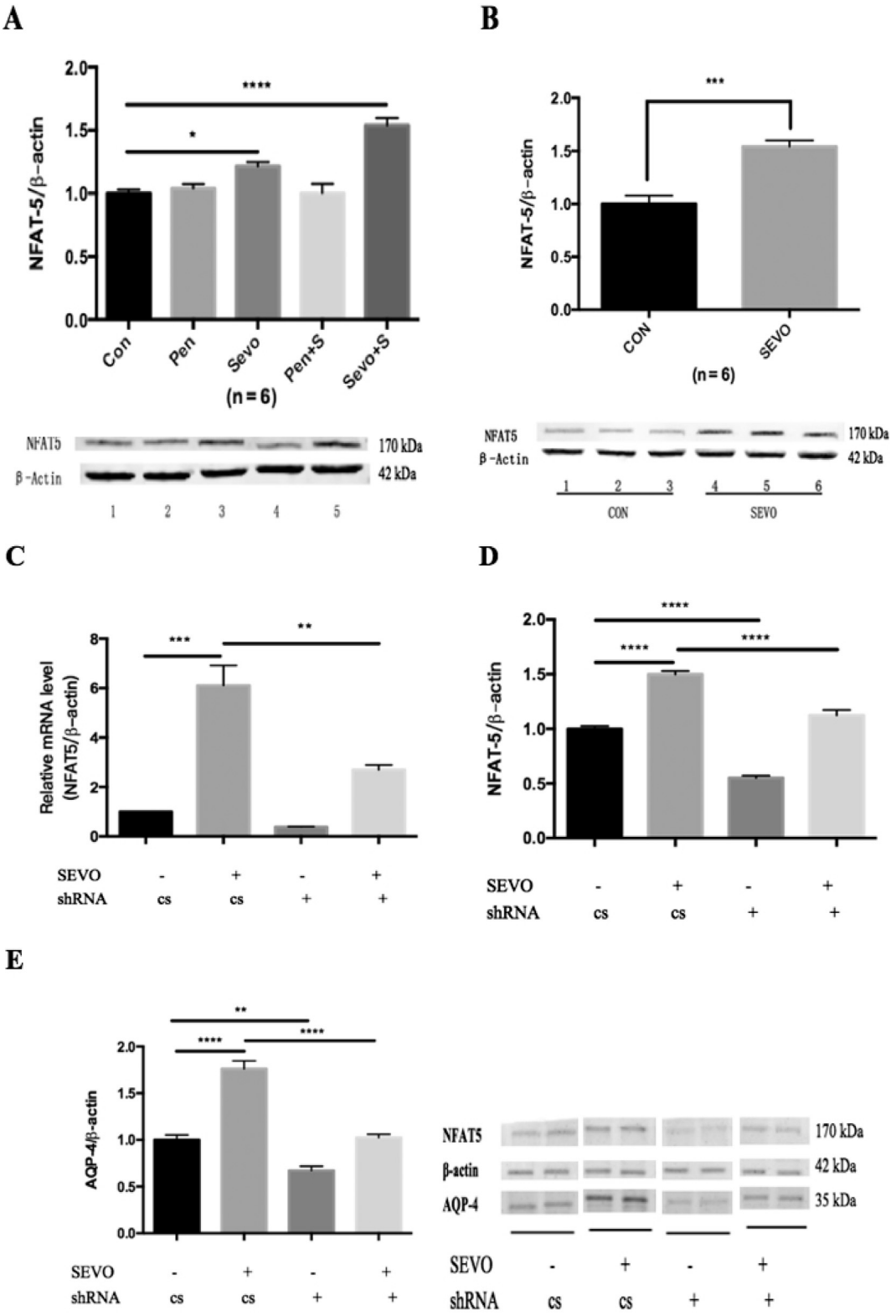

Fig. 4. NFAT-5 expression was increased after sevoflurane exposure and it was the up-stream regulator of AQP-4. (A) Compared to control group, the expression of NFAT-5 increased in sevoflurane anesthesia rat hippocampus (one-way ANOVA, *post hoc*, $F_{(4,25)} = 20.98$, $P < 0.05$, n = 6) (1 = control, 2 = pentobarbital sodium, 3 = sevoflurane, 4 = pentobarbital sodium + surgery, 5 = sevoflurane + surgery). (B) The expression of NFAT-5 was higher in sevoflurane treated primary astrocyte (The student two sample *t*-test, $P < 0.05$, n = 6) (CON = Control, SEVO = Sevoflurane). (C) The mRNA level of NFAT-5 increased in sevoflurane treated primary astrocyte and decreased after RNAi method using RT-qPCR (One-way ANOVA, *post hoc*, $F_{(3,20)} = 38.97$, $P < 0.05$, n = 6) (SEVO = sevoflurane, cs = control shRNA). (D) The protein expression of NFAT-5 decreased after silence of NFAT-5 mRNA (One-way ANOVA, *post hoc*, $F_{(3,28)} = 136.1$, $P < 0.05$, n = 8). (E) The protein expression of AQP-4 decreased after silence of NFAT-5 mRNA (One-way ANOVA, *post hoc*, $F_{(3,28)} = 62.32$, $P < 0.05$, n = 8).

consistent with the study from Wan et al. [23]. They found that orthopedic surgery in young wild type mice under an intravenous anesthetic produced significant post-operative cognitive effects while the anesthetic alone did not.

Unlike pentobarbital sodium, sevoflurane exposure, whether in the presence of surgery or not, did not change the neurocognitive function according to the results of MWM in our study. This was consistent with clinical studies that general anesthesia showed no or modest association with neurocognitive deficits. At the meantime, animal studies also indicated that brief exposure to general anesthetics did not change neurocognitive behavior in developing brains [24–27].

More interesting, we found that under the condition of surgeries, the rats exposed to pentobarbital sodium showed impaired cognitive function while rats under sevoflurane anesthesia did not. We speculated that sevoflurane might have some neuroprotective effects under surgery circumstances. The peripheral surgery was able to induce cognitive impairment independent of general anesthesia, and that the increase of Aβ in brain was needed for the POCD to occur in old animal [8]. In our present study, the expression of Aβ1-40 in the hippocampus of rats was identified using western blot right after recovery from anesthesia. The results showed significant increased Aβ1-40 level in rats exposed to pentobarbital sodium in the presence of splenectomy, which was consistent with the impaired behavior performance. This result showed that postoperative cognitive deficits were in accordance with increased expression of Aβ1-40 in hippocampus and surgeries may contributed more to the neurotoxicity than anesthesia did. High level of Aβ1-40 in developing brain may due to the effects of peripheral inflammatory response, such as Tumor Necrosis Factor-α (TNFα), IL-6 and Interleukin 1- beta (IL-1β) [19–22]. In consistent with behavior study, the level of Aβ1-40 in hippocampus is significantly higher in pentobarbital sodium group than that in sevoflurane group under surgery condition in this study. To explore the reason of lowered Aβ1-40 level in the hippocampus of sevoflurane, the blood Aβ1-40 concentration in the superior vena cava of rats were identified by ELISA. Previous analysis showed that as much as 65% of exogenously delivered Aβ is cleared by the glymphatic system [11], and Aβ was cleared twofold faster in the sleeping/anesthetized mice as compared with the awake rats [18]. By glymphatic system, waste may ultimately be cleared from the brain by draining into cervical lymphatics, dispersing into the subarachnoid CSF, or crossing the vasculature to enter the bloodstream [11,28,29]. Additionally, lymphatic vessels lining the dural sinuses have recently been identified [30]. These meningeal vessels may play an important role in draining glymphatic fluid and solutes to deep cervical lymph nodes and other peripheral structures [31]. At last, all the lymphatic fluids return to superior vena cava through right lymphatic duct and thoracic duct. Results showed that in the presence of surgery, the blood Aβ1-40 level in superior vena cava was significantly higher in sevoflurane group than that in pentobarbital sodium group, indicated an increased clearance of Aβ1-40 by sevoflurane exposure.

In glymphatic clearance system, AQP4-dependent astroglial water fluxes couple para-arterial CSF influx to para-venous ISF plays an important role. In young animals, AQP4 is localized to astrocytic endfeet and plays a central role in facilitating CSF-ISF exchange along periarterial influx pathways as well as interstitial solute clearance through perivascular drainage pathways [11]. The genetic deletion of AQP4 had been shown to impair CSF-ISF exchange by 65% and reduce the clearance of β–amyloid by 55% [11]. Results of *in vivo* experiments showed that sevoflurane but not pentobarbital sodium significantly increased the expression of AQP4 regardless of surgery. Similar to our results, Chang et al. [32] also showed that in patients performed gliomas resection, sevoflurane showed its neuroprotective effects over propofol by increasing AQP4 expression. Our data of Immunofluorescence staining of brain slices also showed a more polarized distribution of AQP4 on the endfeet of astrocytes (GFAP+) around the blood vessels after sevoflurane exposure.

Nuclear factor of activated T cells 5 (NFAT5) is a recently identified member of the Rel family of transcription factors [33,34] and it is necessary for the transcriptional regulation of AQP4 expression [35]. Consistent with the increased expression of AQP4, the *in vivo* study showed a simultaneously increased NFAT5 after sevoflurane exposure. To further identify the effect of sevoflurane on NFAT5-AQP4 pathway, primary cultured astrocytes were used. Our results showed that sevoflurane did increase the expression of both NFAT5 and AQP4 in primary astrocytes. The increased AQP4 by sevoflurane could be reversed by NFAT-5 shRNA. These data suggest that sevoflurane increased AQP4 through modulating the expression of NFAT5.

Recent study from Gauberti et al. [36] showed that anesthesia especially 3% isoflurane impaired parenchymal CSF circulation in mice using magnetic resonance imaging (MRI) and near-infrared fluorescence imaging (NIRF). But Benveniste et al. [37], using similar methods, showed that anesthetics (low concentration of isoflurane + dexmedetomidine) enhanced the glymphatic transport not simply by inducing unconsciousness but also by repression of norepinephrine release. Neither of these researches was done under surgeries and sevoflurane was not used in their studies.

To figure out the effects of anesthetics on glymphatic system under clinical situation, further studies should be done using imaging methods with different commonly used anesthetics and different anesthesia strategy.

5. Conclusion

Our study provided a new sight into the neurocognitive function disorder in developing brains after anesthesia and surgery in clinical situation and suggested a new clearance mechanism of excessive Aβ1-40 caused by surgery though increasing AQP4 expression induced by sevoflurane exposure.

Author contributions

F.F. designed the study and write part of the manuscript. X.G. planned and carried out the experiments, analyzed the data, generated the figures, and wrote the manuscript. M.J., SW.L. carried out the experiments and analyzed the data. B.L. and J.C. provided editorial assistance in preparing the manuscript.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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