

Maternal sevoflurane exposure affects differentiation of hippocampal neural stem cells by regulating microRNA-410-3p and ATN1

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Research

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Abstract

Background Currently, numerous animal studies have shown that exposure to commonly used general anesthetics during pregnancy may cause neurocognitive impairment in the offspring. Reportedly, exposure to sevoflurane during mid-trimester of pregnancy can inhibit proliferation of neural stem cells (NSCs) and lead to early apoptosis. Whether exposure to sevoflurane during pregnancy affects the differentiation of NSCs remains unclear.

Methods In the present study, pregnant rats were exposed to 3% sevoflurane once for 2 h on gestational day 14 (G14) or 3 times for 2 h on G13, G14, and G15. Next, the differentiation of NSCs was measured using neuron marker β -tubulin III and astrocyte marker glial fibrillary acidic protein (GFAP) in fetal brain tissues 24 h and 72 h after anesthesia and in hippocampus on postnatal day 28. Primary cultured rat NSCs were exposed to 4.1% sevoflurane to explore the mechanism.

Results The results showed that during mid-trimester, multiple exposures to sevoflurane can cause premature differentiation of NSCs in developing brains of offspring and lead to long-term neuron reduction and astrocyte proliferation in hippocampus. The data from the present study indicated that repeated exposure to sevoflurane downregulated atrophin-1 (ATN1) expression and caused early differentiation of NSCs. Overexpression of ATN1 via lentivirus transfection attenuated the influence of sevoflurane. Using dual luciferase assay, ATN1 was found to be a target gene of microRNA-410-3p (miR-410-3p). MiR-410-3p suppression via lentivirus transfection recovered the ATN1 expression and differentiation of NSCs.

Conclusions The results from the present study demonstrated that repeated exposure to sevoflurane leads to early differentiation of NSCs and long-term effects via the miR-410-3p/ATN1 pathway.

Background

Numerous animal studies have shown that exposure to anesthesia drugs can lead to long-term neurocognitive impairment in the developing brain. Most research has focused on the newborn period (Xu, Shen, Yu, Sun, & Yan, 2018) (Shen et al., 2018) (Pearn et al., 2018). Because the surgical technologies are constantly advancing, the number of non-obstetric surgeries are continually increasing. Mid-trimester is considered a relatively safe period to perform surgery during pregnancy. However, the mid-trimester is a critical period for fetal brains because neural stem cells (NSCs) undergo high proliferation and differentiation (Meredith, Dawitz, & Kramvis, 2012) (Silbereis, Pochareddy, Zhu, Li, & Sestan, 2016). In our previous studies (Yuan Wang et al., 2018) (X. Li et al., 2017) (Wang, Yin, Zhang, & Zhao, 2018), exposure to sevoflurane in the mid-trimester of pregnancy suppressed NSC proliferation and led to early apoptosis. However, whether sevoflurane affects NSC differentiation remains unclear.

In the present study, the expression level of several genes crucial for NSC maintenance after exposure to sevoflurane was investigated. The results showed that after sevoflurane exposure, the atrophin-1 (ATN1) level was significantly decreased. ATN1 is highly expressed in brain tissues and mutations in the ATN1

gene can cause a rare neurodegenerative disease, Dentatorubral-pallidoluysian atrophy (DRPLA) (Schilling et al., 1999). In most of the previous ATN1 expression studies (Bidollari et al., 2019) (Napoletano et al., 2011), the focus was on DRPLA. Recently, ATN1 was reported to play an important role in NSC maintenance (F. Zhang, Xu, Yuan, Sun, & Xu, 2014), which is in agreement with our results. Thus, in the present study, whether sevoflurane could affect NSC differentiation by changing the expression of ATN1 was investigated.

In numerous studies, microRNAs (miRNAs) have been reported to play significant roles in neural development (Rajman & Schratt, 2017; Shu et al., 2019). Several miRNAs were confirmed to participate in NSC apoptosis and differentiation (Shi et al., 2018) (Morgado, Rodrigues, & Solá, 2016). Abnormal miRNA expression after exposure to anesthesia drugs could cause significant neurocognitive impairments in developing brains (Shao & Xia, 2019) (Cao, Tian, Chen, Zhang, & Zhang, 2015). In the present study, several databases were used to determine if ATN1 is a direct target of miRNA-410-3p (miR-410-3p), which was confirmed using dual-luciferase reporter assay. However, whether miR-410-3p plays a role in NSC differentiation after exposure to sevoflurane remains unknown.

To investigate whether sevoflurane affects NSC differentiation during the early stage of brain development, rat models in mid-trimester of pregnancy and primary cultured NSCs were repeatedly exposed to sevoflurane. We hypothesized that repeated sevoflurane exposure would lead to early NSC differentiation and long-term neuron reduction by regulating the miR-410-3p and ATN1 expression.

Methods

Animals

Adult Sprague-Dawley rats were housed in a room with constant temperature of $24 \pm 1^\circ\text{C}$ under a 12-h light/12-h dark cycle, with free access to water and food. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Shengjing Hospital, China Medical University (No.2016PS028K) and were conducted following the National Institute of Health Guideline for the Care and Use of Laboratory Animals.

Cell culture and differentiation

Rat NSCs were isolated from the hippocampus of fetal Sprague-Dawley rats on G14 or G15. The isolated cells were plated in culture flasks at a density of $1 \times 10^5/\text{mL}$ and maintained in an atmosphere of 5% CO_2 and 95% air. The cells were cultured in serum-free medium of Dulbecco's modified Eagle's medium (DMEM)/F12, HEPES (11330032; Gibco, USA) supplemented with 2% B27 without vitamin A (12587010; Gibco), 20 ng/mL basic fibroblast growth factor (rat bFGF, 3339-FB-025; R&D, USA), 20 ng/mL epidermal growth factor (rat EGF, 3214-EG-100; R&D), 1% penicillin-streptomycin (1512022; Gibco). Half of the culture medium was replaced every other day and the cells were digested with Accutase (A6964; Sigma-Aldrich, USA). All experiments were performed on cells from passages 2–4 to reduce experimental deviations. To induce NSC differentiation, rat NSCs were digested into single cells and plated onto plates pre-coated with 0.25% poly-D-lysine (P6409; Sigma-

Aldrich) using differentiation medium of DMEM/F12 supplemented with 2% B27 without vitamin A, 1% fetal bovine serum (FBS, 10100139; Gibco), and 1% penicillin-streptomycin.

HEK293T cells were cultured in high-glucose DMEM (11965084; Gibco) supplemented with 10% FBS (10100139; Gibco) and antibiotics (1512022; Gibco). Cells were incubated in an atmosphere at 37°C in 5% CO₂ and the culture medium replaced every other day.

Sevoflurane exposure

Two or three adult female Sprague-Dawley rats were caged with one male rat to allow free mating. If sperm or vaginal emboli were detected on the second day, the female rat was marked as G0. For the *in vivo* experiments, pregnant rats were placed into a 30% oxygen plastic chamber and exposed to 3% sevoflurane (1.5 MAC) for 2 h on G14 (SEV×1 group; n = 9/group) or on G13, G14, and G15 (SEV×3 group; n = 9/group). CON group (n = 9/group) consisted of rats placed in the same conditions without exposure to sevoflurane. The fetal brain tissues were obtained after cesarean section at 24 h, 72 h, and postnatal day 28. For the *in vitro* experiments, hippocampal NSCs were obtained as described above. The NSCs were inoculated into 24-well plates and randomly allocated into 8 groups: CON group, 4.1% SEV×1 group, 4.1% SEV×3 group, negative lentivirus group (NC), ATN1-overexpression lentivirus group (LV-410), 4.1% SEV×3 plus ATN1-overexpression lentivirus group (LV-ATN1+SEV×3), miR-410-3p-suppression lentivirus group (LV-410), 4.1% SEV×3 plus miR-410-3p-suppression lentivirus group (LV-410+SEV×3). The plates were placed into an incubator containing a mixture of 5% CO₂ and 95% air and a gas monitor (Dräger, Germany) was used to detect sevoflurane concentration. The SEV×1 group was exposed to 4.1% sevoflurane for 2 h and all SEV×3 groups were exposed to 4.1% sevoflurane for 2 h on 3 consecutive days. All groups that were not exposed to sevoflurane were kept under the same conditions of a mixture of 5% CO₂ and 95% air for the same amount of time.

Western blot

The brain tissues and collected cells were stored at -80°C before use and homogenized to determine protein expression using the western blotting protocol described in our previous study (Wu et al., 2018). The protein concentration was measured using the BCA Protein Assay Kit (P0010; Beyotime, China). Electrophoresis was performed using 10% or 12.5% SDS-polyacrylamide gels, then proteins were electrotransferred to polyvinylidene fluoride membranes (IPVH0010; Millipore, Germany). The membranes were blocked with BSA or 5% non-fat milk and incubated with primary antibodies β-tubulin III (1:1,000, T2200; Sigma-Aldrich), GFAP (1:10,000, ab53554; Abcam, UK), nestin (1:1,000, ab6142; Abcam), ATN1 (1:500, orb213859; Biorbyt, UK), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1,000, 60004-1-Ig; Proteintech, USA) overnight at 4°C. The membranes were incubated with second antibodies the next day for 2 h at room temperature, then photographed using an GE Amersham Imager 600. Number of tissues was 5 per group and number of cells was 3 per group in western blot experiments. Images were analyzed using Image-Pro Plus 6.0 software.

RT-qPCR

The mRNA in each group was extracted using RNAiso Plus (9108; TaKaRa, China) and miRNA was extracted using RNAiso for small RNAs (9753A; TaKaRa, China). The primers were designed and synthesized by Sangon Biotech (China; Table 1). Reverse transcription and reaction conditions were performed according to the instructions in the PrimeScript™ RT reagent Kit with gDNA Eraser (RR047A; TaKaRa) and Mir-X miRNA First-Strand Synthesis Kit (638315; Clontech, USA) using SYBR green (RR420A; TaKaRa, China). Quantitative analysis of gene expression was calculated using $2^{-\Delta\Delta C_t}$ method with GAPDH (B661204; Sangon biotech, China) and U6 as internal references.

Table 1. Primer sequences for RT-qPCR

Gene	Sequence
ATN1	Forward: GTCTTCGTCTCAAGCCGCCTATTC
	Reverse: AGGAGGTGGTGATTGGAGGAACTG
miR-410-3p	cgcgAATATAACACAGATGGCCTGT

Immunofluorescence staining

Brains were immersed in precooled 4% paraformaldehyde (PFA) for 24 h followed by dehydration in an ethanol gradient, then paraffin embedded. Next, the tissues were sliced into sections approximately 3.0- μ m-thick. The sections were deparaffinized and heated in citrate buffer for 7.5 min at 121°C. The cells were plated onto glass coverslips pre-coated with poly-D-lysine, then fixed in 4% PFA for 30 min at room temperature. To reduce background staining and permeabilize membranes, 10% FBS and 0.5% Triton X-100 were added to the sections and coverslips for 40 min at room temperature, and then incubated with primary antibodies overnight at 4°C. The next day, the sections were incubated with secondary antibodies for 2 h and DAPI for 5 min at room temperature. The primary antibodies were the following: nestin (1:250, ab92391; Abcam; 1:300, 4760; Cell Signaling Technology, USA), Sex determining region Y box 2 (SOX2) (1:500, abs131219-50ug; absin), β -tubulin III (1:250, T2200; Sigma-Aldrich), GFAP (1:500, ab53554; Abcam), and ATN1 (1:500 **HPA031619**; Sigma-Aldrich). The nuclei were stained with DAPI. Sections and coverslips were imaged with Nikon C1 microscope by an investigator who was blinded to the experimental interventions. The β -tubulin III and GFAP immunoreactivity was quantified by the ratio of the number of β -tubulin III or GFAP positive cells to the total number of cells. Number of rats per group was 5 (n=5) and number of cell coverslips per group was 3 (n=3).

Lentivirus transfection

ATN1 overexpression lentivirus and miR-410-3p suppression lentivirus were purchased from GeneChem Corporation, China. NSCs in 96-well plates were transfected to detect the optimum multiplicity of infection

(MOI) and experiments performed in 24-well plates. Medium containing lentivirus was replaced 24 h post-transfection with fresh medium. Transfection efficiency was confirmed using RT-qPCR.

Dual-luciferase reporter assay

Dual-luciferase reporter assay was performed in 293T cells using the Dual-luciferase Reporter System (Promega, China). All pmirGLO vectors were purchased from GenePharma, China. The pmirGLO-ATN1-WT or pmirGLO-ATN1-MUT vector was transfected with miR-410-3p mimics or mimics negative control (mimics-NC) into 293T cells using Lipo2000 (Invitrogen, USA). Luciferase activity was detected at 24 h after transfection. The experiments were performed three times independently.

Statistical analysis

All data were analyzed using GraphPad Prism 7.0 software or SPSS 17.0 for Windows. Results were presented as means \pm standard deviation (SD). Student's *t*-test and one-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was used for data analyses. A *p*-value < 0.05 was considered statistically significant.

Results

1. Confirmation and differentiation of NSCs

To investigate the mechanism *in vitro*, primary cultured hippocampal NSCs were used. The NSCs were isolated from fetal hippocampi of Sprague-Dawley rats on embryonic (E) day 14 to E15. Observation under microscope showed that NSCs grew in a typical round shape (Figure 1A), which was confirmed using immunofluorescence with the NSC marker nestin (Figure 1B). For NSC differentiation, NSCs were induced to differentiate for 120 h which was confirmed using immunofluorescence with neuron marker β -tubulin III (Figure 1C) and astrocyte marker glial fibrillary acidic protein (GFAP; Figure 1D).

Figure 1

Confirmation and differentiation of neural stem cells (NSCs). (A) Primary cultured hippocampal NSCs observed under microscope. Scale bar = 100 μ m, 50 μ m. (B) Immunofluorescence staining of NSC marker nestin (red) and Sex determining region Y box 2 (SOX2) (green). Scale bar = 20 μ m. (C) Results of β -tubulin III (red) and nestin (green). Scale bar = 100 μ m. (D) Results of glial fibrillary acidic protein (GFAP) (green) and nestin (red). Scale bar = 100 μ m.

2. Repeated exposure to sevoflurane led to early differentiation of hippocampal NSCs

To determine whether sevoflurane can affect NSC differentiation, the expression of NSC marker nestin, neuron marker β -tubulin III, and astrocyte marker GFAP was examined in fetal brains and primary cultured hippocampal NSCs using western blotting and immunofluorescence at 24 h and 72 h after single or repeated exposure to sevoflurane (Figure 2, 3). After repeated maternal exposure to 3% sevoflurane, the β -tubulin III (Figure 2A, B, D, G) and GFAP levels (Figure 2A, C, E, H) were increased and the nestin level (Figure 2A–C, F) was decreased in fetal brain tissue. However, significant difference was not observed between control (CON) group and single 3% sevoflurane exposure (SEV \times 1) group; Figure 2A–H). Primary cultured NSCs exposed to 4.1% sevoflurane once or 3 times (SEV \times 3) showed β -tubulin III (Figure 3A, B, D, G), GFAP (Figure 3A, C, E, H), and nestin (Figure 3A, C, F) levels corresponded to *in vitro* results. The results showed that repeated exposure to sevoflurane led to early differentiation in hippocampal NSCs.

Figure 2

Repeated maternal exposure to 3% sevoflurane led to early NSC differentiation in fetal brains. (A) Western blotting images of β -tubulin III, GFAP, and nestin. (B) Immunofluorescence images of β -tubulin III (green) and nestin (red). Scale bar = 100 μ m. (C) Immunofluorescence images of GFAP (green) and nestin (red). Scale bar = 100 μ m. (D) Quantitative analysis of β -tubulin III. (E) Quantitative analysis of GFAP. (F) Quantitative analysis of nestin. (G) Quantification of β -tubulin III-positive cells. (H) Quantification of GFAP-positive cells. Values are means \pm SD (n = 5/group). * p < 0.05, ** p < 0.01, *** p < 0.001 compared with control (CON) group; # p < 0.05, ## p < 0.01, ### p < 0.001 compared with sevoflurane (SEV) \times 1 group. One-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was used for data analysis.

Figure 3

Repeated exposure to 4.1% sevoflurane led to early differentiation in primary cultured hippocampal NSCs. (A) Western blotting images of β -tubulin III, GFAP, and nestin. (B) Immunofluorescence images of β -tubulin III (green) and nestin (red). Scale bar = 100 μ m. (C) Immunofluorescence images of GFAP (green) and nestin (red). Scale bar = 100 μ m. (D) Quantitative analysis of β -tubulin III. (E) Quantitative analysis of GFAP. (F) Quantitative analysis of nestin. (G) Quantification of β -tubulin III-positive cells. (H) Quantification of GFAP-positive cells. Values are means \pm SD (n = 3/group). * p < 0.05, ** p < 0.01 compared with CON group; # p < 0.05, ## p < 0.01 compared with SEV \times 1 group. One-way ANOVA followed by Tukey's post hoc multiple comparison test was used for data analysis.

3. Repeated exposure to sevoflurane led to long-term neuron reduction and astrocyte proliferation in hippocampus

Our previous study showed that a single maternal 3% sevoflurane exposure does not cause long-term neurocognitive impairment in fetal rats, while repeated exposure of 3% sevoflurane can cause learning and memory impairment in the offspring (Wu et al., 2018). The β -tubulin III and GFAP levels in rat brains were examined on postnatal day 28 (Figure 4) as well as in the cultured NSCs (Figure 5). After repeated exposure to sevoflurane, β -tubulin III protein was reduced in both postnatal fetal hippocampus (Figure 4A,

B) and cultured NSCs (Figure 5A, B) compared with the CON group and SEV×1 group. GFAP expression was upregulated in postnatal fetal hippocampus (Figure 4A, C) and cultured NSCs (Figure 5A, C). Immunofluorescence showed similar results regarding the quantity of neurons and astrocytes in CA1 hippocampal region (Figure 4D-G). Significant difference was not observed between CON group and SEV×1 group.

Figure 4

Effects of sevoflurane exposure on the expression of β -tubulin III and GFAP in fetal hippocampi on postnatal day 28. (A) Western blotting images of β -tubulin III and GFAP. (B) Quantitative analysis of β -tubulin III. (C) Quantitative analysis of GFAP. (D) Immunofluorescence images of β -tubulin III (green) in hippocampal CA1 region. Scale bar = 100 μ m, 50 μ m. (E) Immunofluorescence images of GFAP (green) in hippocampal CA1 region. Scale bar = 100 μ m, 50 μ m. (F) Quantification of β -tubulin III-positive cells. (G) Quantification of GFAP-positive cells. Values are means \pm SD (n = 5). ** p < 0.01, *** p < 0.001 compared with CON group; ## p < 0.01 compared with SEV×1 group. One-way ANOVA followed by Tukey's post hoc multiple comparison test was used for data analysis.

Figure 5

Effects of sevoflurane exposure on β -tubulin III and GFAP expression in cultured NSCs on postnatal day 28. (A) Western blotting images of β -tubulin III and GFAP. (B) Quantitative analysis of β -tubulin III. (C) Quantitative analysis of GFAP. Values are means \pm SD (n = 3). * p < 0.05 compared with CON group; # p < 0.05 compared with SEV×1 group. One-way ANOVA followed by Tukey's post hoc multiple comparison test was used for data analysis.

4. Exposure to sevoflurane affected NSC differentiation by downregulating ATN1 expression

The expression of ATN1 was downregulated in both fetal brain tissues (Supplement 1A, B) and primary cultured NSCs (Figure 6A, E) after repeated exposure to sevoflurane. After transfecting ATN1 overexpression lentivirus into NSCs and exposing to sevoflurane, in the 4.1% SEV×3+ATN1-overexpression lentivirus (LV-ATN1+SEV×3) group, the levels of β -tubulin III (Figure 6A, C, F, K), GFAP (Figure 6A, D, G, L), and nestin (Figure 6A, H) were not significantly different than in the CON group at 24 h and 72 h. In the LV-ATN1+SEV×3 group, β -tubulin III (Figure 6A, C, F, K) and GFAP (Figure 6A, D, G, L) expressions were significantly reduced and nestin (Figure 6A, H) expression was upregulated compared with the SEV×3 group. ATN1 mRNA expression level was detected using reverse transcription quantitative polymerase chain reaction (RT-qPCR; Figure 6B). β -tubulin III (Figure 6A, I) and GFAP (Figure 6A, J) protein levels were rescued in the LV-ATN1+SEV×3 group at day 28. Results confirmed that sevoflurane affected differentiation of hippocampal NSCs by downregulating ATN1 expression.

Figure 6

ATN1 overexpression alleviated sevoflurane-induced early NSC differentiation. (A) Western blotting images of β -tubulin III, GFAP, nestin, and atrophin 1(ATN1). (B) RT-qPCR analysis of ATN1 mRNA

expression. (C) Immunofluorescence images of β -tubulin III (green). Scale bar = 100 μ m. (D) Immunofluorescence images of GFAP (green). Scale bar = 100 μ m. (E) Quantitative analysis of ATN1. (F) Quantitative analysis of β -tubulin III. (G) Quantitative analysis of GFAP. (H) Quantitative analysis of nestin. (I) Quantitative analysis of β -tubulin III on day 28. (J) Quantitative analysis of GFAP on day 28. (K) Quantification of β -tubulin III-positive cells. (L) Quantification of GFAP-positive cells.

Values are means \pm SD (n = 3). * p < 0.05, ** p < 0.01, **** p < 0.0001 compared with CON group; # p < 0.05, ## p < 0.01, #### p < 0.0001 compared with SEV \times 3 group. One-way ANOVA followed by Tukey's post hoc multiple comparison test was used for data analysis.

5. ATN1 is the direct target of miR-410-3p

Whether ATN1 is the direct target of miR-410-3p was investigated using TARGETSCAN database (Agarwal, Bell, Nam, & Bartel, 2015) (Friedman, Farh, Burge, & Bartel, 2009). The analysis showed ATN1 was the target site of miR-410-3p as described in Figure 7A. The site was further verified using the dual-luciferase reporter assay (Figure 7B).

Figure 7

ATN1 is a target gene of miR-410-3p. (A) Target prediction program predicted a specific binding region between the ATN1 gene and miR-410-3p sequence. (B) Relative luciferase activity assay analysis. The experiment was performed three times. Paired Student's t -test was used for data analysis.

6. Exposure to sevoflurane affects NSC differentiation by upregulating miR-410-3p

The miR-410-3p level was upregulated in NSCs repeatedly exposed to sevoflurane. The levels of ATN1 (Figure 8A, F), β -tubulin III (Figure 8A, D, G, L), GFAP (Figure 8A, E, H, M), and nestin (Figure 8A, I) in NSCs treated with miR-410-3p suppression lentivirus and repeatedly exposed to sevoflurane were significantly different than levels in the SEV \times 3 group at 24 h and 72 h. In the 4.1% SEV \times 3+miR-410-3p-suppression lentivirus (LV-410-SEV \times 3) group, β -tubulin III expression (Figure 8A, J) was upregulated and GFAP (Figure 8A, K) expression was downregulated compared with the SEV \times 3 group in cultured NSCs on day 28. RT-qPCR showed that miR-410-3p was suppressed by lentivirus (Figure 8B). ATN1 mRNA level in LV-410+SEV \times 3 group was upregulated compared with the SEV \times 3 group (Figure 8C).

Figure 8

MiR-410-3p suppression alleviated sevoflurane-induced early NSC differentiation. (A) Western blotting images of β -tubulin III, GFAP, nestin, and ATN1. (B) RT-qPCR analysis of miR-410-3p expression. (C) RT-qPCR analysis of ATN1 expression. (D) Immunofluorescence images of β -tubulin III (green). Scale bar = 100 μ m. (E) Immunofluorescence images of GFAP (green). Scale bar = 100 μ m. (F) Quantitative analysis of ATN1. (G) Quantitative analysis of β -tubulin III. (H) Quantitative analysis of GFAP. (I) Quantitative analysis of nestin. (J) Quantitative analysis of β -tubulin III on day 28. (K) Quantitative analysis of GFAP on day 28. (L) Quantification of β -tubulin III-positive cells. (M) Quantification of GFAP-positive cells.

Values are means \pm SD (n=3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ compared with CON group; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with SEV \times 3 group. One-way ANOVA followed by Tukey's post hoc multiple comparison test was used for data analysis.

Discussion

Numerous animal studies have shown that exposure to anesthesia drugs can lead to long-term neurocognitive impairment in developing brains (Zhong et al., 2018) (Han et al., 2018). The FDA has published a warning that neurodevelopment of children's brains may be affected in children under 3 years of age or pregnant women in the third trimester who undergo anesthesia more than once or for more than 3 h (Olutoye, Baker, Belfort, & Olutoye, 2018). Mid-trimester is the most common time for non-obstetric surgery, however, the developing brain is in a vulnerable window (Pletikos et al., 2014) (Vasung et al., 2019) and minor influences such as drugs and environment may cause severe neurocognitive impairment (Heroux, Horgan, Rosen, & Stanton, 2019) (Kanlikilicer, Zhang, Dragomir, Akay, & Akay, 2017) (Slotkin, Skavicus, Card, Levin, & Seidler, 2016). A safe surgery period does not equate to a safe anesthesia period. In the present study, the mechanism of NSC differentiation after exposure to sevoflurane during mid-trimester was investigated and advice provided for further clinical application.

Cell cycle timing dictates cell fate (Dalton, 2015). Premature NSC differentiation may have negative effects on developing brains. In several studies, premature differentiation of NSCs was shown to cause severe brain developmental disorders (Večeřa et al., 2020). Research showed that aneuploidy causes NSCs to exit the cell cycle and differentiate prematurely, eventually resulting in the formation of microcephalic brains (Gogendeau et al., 2015). In another study, *Nap111* knockdown caused less BrdU incorporation and greater expression of Tuj1⁺ cells (neuronal marker) in embryonic day (E) 13.5 fetal brains, resulting in developmental deficiencies (Qiao et al., 2018). In maternal diabetes model, the levels of circulating metabolite detoxifying enzyme glyoxalase 1 were decreased, which led to premature neurogenesis and adverse long-term influence on the developing brain of offspring (Yang et al., 2016).

Although some study results have shown certain anesthesia drugs may affect NSC differentiation, the findings are conflicting and controversial during different periods of brain development. For example, in a previous study, 4.1% sevoflurane for 6 h repressed neural differentiation by influencing self-renewal of mouse embryonic stem cells (mESCs) (Yi, Cai, Zhang, Wang, & Li, 2016). In another study, 7-day-old rats exposed to ketamine showed significantly reduced number of nestin/BrdU double-stained positive cells and GFAP/BrdU double-stained positive cells and increased number of β -tubulin III/BrdU double-stained positive cells (Huang et al., 2015). Prolonged 2.4% isoflurane significantly suppressed neuronal fate while promoting glial fate in RcNcell CX human neural progenitor cells (Zhao et al., 2013). In the present study, 3% sevoflurane (1.5 minimum alveolar concentration, MAC) was chosen on pregnant rats, a commonly used concentration in clinical non-obstetric surgeries. 4.1% sevoflurane was commonly used in NSCs to explore the neurotoxicity of SEV in vitro in previous studies (Yi et al., 2016) (S. Liu et al., 2018). There are other studies (Y. Zhang et al., 2019) (J. Zhang, Dong, Zhou, Zhang, & Xie, 2015) using 3% SEV in vivo and 4.1% SEV in vitro to investigate the mechanism of SEV. The results showed that during mid-trimester,

multiple exposures to sevoflurane can cause premature differentiation of NSCs in developing brains of offspring. A single exposure to 3% sevoflurane did not significantly influence NSCs after 24 h and 72 h. After 3 times exposure to 3% sevoflurane, the expression of NSC marker nestin was downregulated, however, the expression of neuron marker β -tubulin III and astrocyte marker GFAP was upregulated in the early neurodevelopmental period. The results indicated that multiple exposures to anesthesia can reduce NSCs self-renewal capability and lead NSCs to early differentiation. Over an extended period, β -tubulin III was suppressed while GFAP remained overexpressed. The reduction of β -tubulin III indicates the number of neurons was reduced, which supports the same behavioral test results obtained in our previous research showing multiple exposures to sevoflurane can lead to neurocognitive impairment and learning disability in the offspring (Wu et al., 2018). Assumedly, sevoflurane causes premature differentiation of NSCs which result in neuronal damage in postnatal off-spring. Consequently, due to the reduction of neurons, gliocytes, such as astrocytes, appear to undergo compensatory proliferation. However, the specific mechanism remains unknown.

Because sevoflurane is one of the most common anesthesia drugs used during pregnancy, the effects on neurodevelopment are crucial. In our previous research, results indicated that sevoflurane can affect proliferation of NSCs and leads to early NSC apoptosis in the offspring (Yuan Wang et al., 2018) (X. Li et al., 2017). β -tubulin III stained positive cells and GFAP stained positive cells in proportion to nestin stained positive cells of SEV \times 3 group rates than CON group which confirms sevoflurane can cause early differentiation of NSCs. Although NSCs have proliferation ability, if they differentiate early to neurons, the proliferation process is disrupted and results in early reduction of neurons. The results from the present study indicate the number of neurons is reduced in the developing brain for an extended period of time after exposure to sevoflurane. Gliocytes tend to proliferate to compensate for the reduction of neurons but cannot reverse the effects on neurocognitive impairment.

The normal ATN1 expression is important for NSC maintenance (F. Zhang et al., 2014). Lower ATN1 expression indicates early differentiation of NSCs (F. Zhang et al., 2014). After exposure to sevoflurane, ATN1 expression was significantly reduced simultaneously with early NSC differentiation. To detect whether sevoflurane affects NSC differentiation by downregulating ATN1 expression, ATN1 overexpression lentivirus was transfected into primary rat NSCs. The results showed that ATN1 overexpression can reverse the effects of sevoflurane which confirms sevoflurane affected NSC differentiation by regulating ATN1 expression.

MiRNAs play an important role in brain development and miRNAs were affected after exposure to anesthesia drugs in several studies (Jiang et al., 2018) (Bahmad et al., 2020). To further explore the mechanism of NSC differentiation after exposure to sevoflurane, we predicted several possible binding sites using multiple target prediction programs (Agarwal et al., 2015) (Friedman et al., 2009) and confirmed the sites through experiments. After exposure to sevoflurane, the miR-410-3p expression was significantly upregulated. Dual luciferase reporter assay showed that ATN1 is a direct target of miR-410-3p; miR-410-3p suppression can alleviate the effects of sevoflurane.

In the present study, the focus was on the mid-trimester of pregnancy which is the period when NSCs undergo significant proliferation and differentiation. Despite differences among studies, anesthesia drugs were shown in several reports to cause long-term neural impairment (B. Liu, Ou, Chen, & Zhang, 2019) (G. F. Li, Li, Zhuang, & Li, 2018). In the present study, mid-trimester pregnant rats and primary cultured hippocampal NSCs extracted from the same period were used to investigate the influence of sevoflurane on hippocampal NSC differentiation.

The present study had several limitations. First, due to technical limitations, the *in vivo* mechanism was not investigated. Primary cultured NSCs were used to closely simulate the *in vivo* environment. Second, the density of cultured NSCs at day 28 was too low to perform immunofluorescence, however, western blot analysis was used to detect the expression of β -tubulin III and GFAP. Third, only a single inhalational anesthetic was used instead of a drug combination. Combined use of general anesthetics may cause diverse neurotoxicity outcomes, which may be the subject of our future investigations.

Conclusions

In conclusion, the results from the present study demonstrated that repeated sevoflurane exposure during mid-trimester pregnancy caused early differentiation of NSCs by regulating miR-410-3p and ATN1 expression in the offspring. The results may provide several novel strategies for future clinical use.

Declarations

Ethics approval and consent to participate

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Shengjing Hospital, China Medical University (No.2016PS028K)

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Author contributions

YZ and PZ designed the experiments. YZ, ZW, and PZ contributed to the conception of the work. YZ performed experiments, collected, and analyzed the data with the help of ZW, XL, YW and YZ. YZ wrote the manuscript with the help of ZW. PZ supervised the study. All authors read and approved the article.

Competing interests

The authors declare the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Acknowledgement

Not applicable.

Abbreviations

NSC Neural stem cell

G Gestational day

GFAP Glial fibrillary acidic protein

ATN1 atrophin 1

MiR MiRNAs

DRPLA Dentatorubral-pallidoluysian atrophy

DMEM Dulbecco's modified Eagle's medium

bFGF Basic fibroblast growth factor

EGF Epidermal growth factor

FBS Fetal bovine serum

SEV Sevoflurane

E Embryonic

ANOVA analysis of variance

CON Control group

SOX2 Sex determining region Y box 2

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Figures

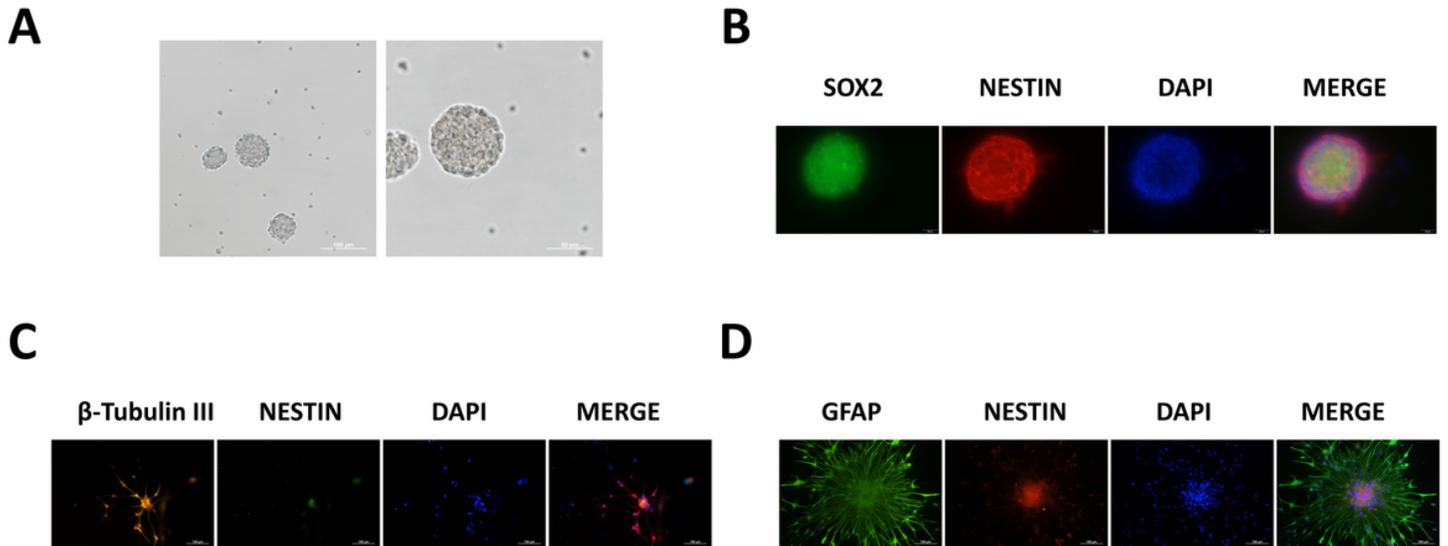


Figure 1

Confirmation and differentiation of neural stem cells (NSCs). (A) Primary cultured hippocampal NSCs observed under microscope. Scale bar = 100 μm , 50 μm . (B) Immunofluorescence staining of NSC marker nestin (red) and Sex determining region Y box 2 (SOX2) (green). Scale bar = 20 μm . (C) Results of β -tubulin III (red) and nestin (green). Scale bar = 100 μm . (D) Results of glial fibrillary acidic protein (GFAP) (green) and nestin (red). Scale bar = 100 μm .

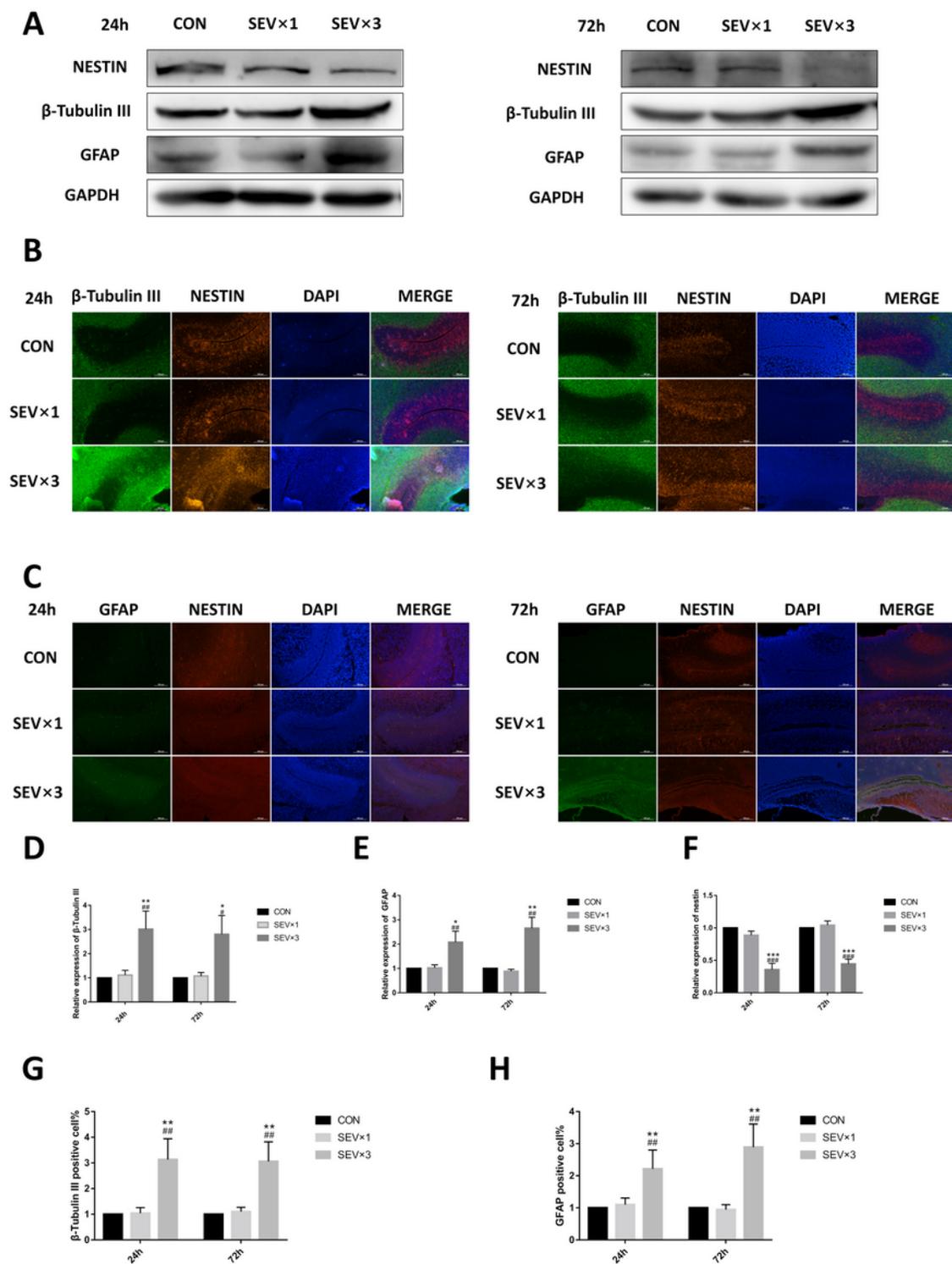


Figure 2

Repeated maternal exposure to 3% sevoflurane led to early NSC differentiation in fetal brains. (A) Western blotting images of β-tubulin III, GFAP, and nestin. (B) Immunofluorescence images of β-tubulin III (green) and nestin (red). Scale bar = 100 μm. (C) Immunofluorescence images of GFAP (green) and nestin (red). Scale bar = 100 μm. (D) Quantitative analysis of β-tubulin III. (E) Quantitative analysis of GFAP. (F) Quantitative analysis of nestin. (G) Quantification of β-tubulin III-positive cells. (H) Quantification of

GFAP-positive cells. Values are means \pm SD (n = 5/group). *p < 0.05, **p < 0.01, ***p < 0.001 compared with control (CON) group; # p < 0.05, ## p < 0.01, ### p < 0.001 compared with sevoflurane (SEV) \times 1 group. One-way analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison test was used for data analysis.

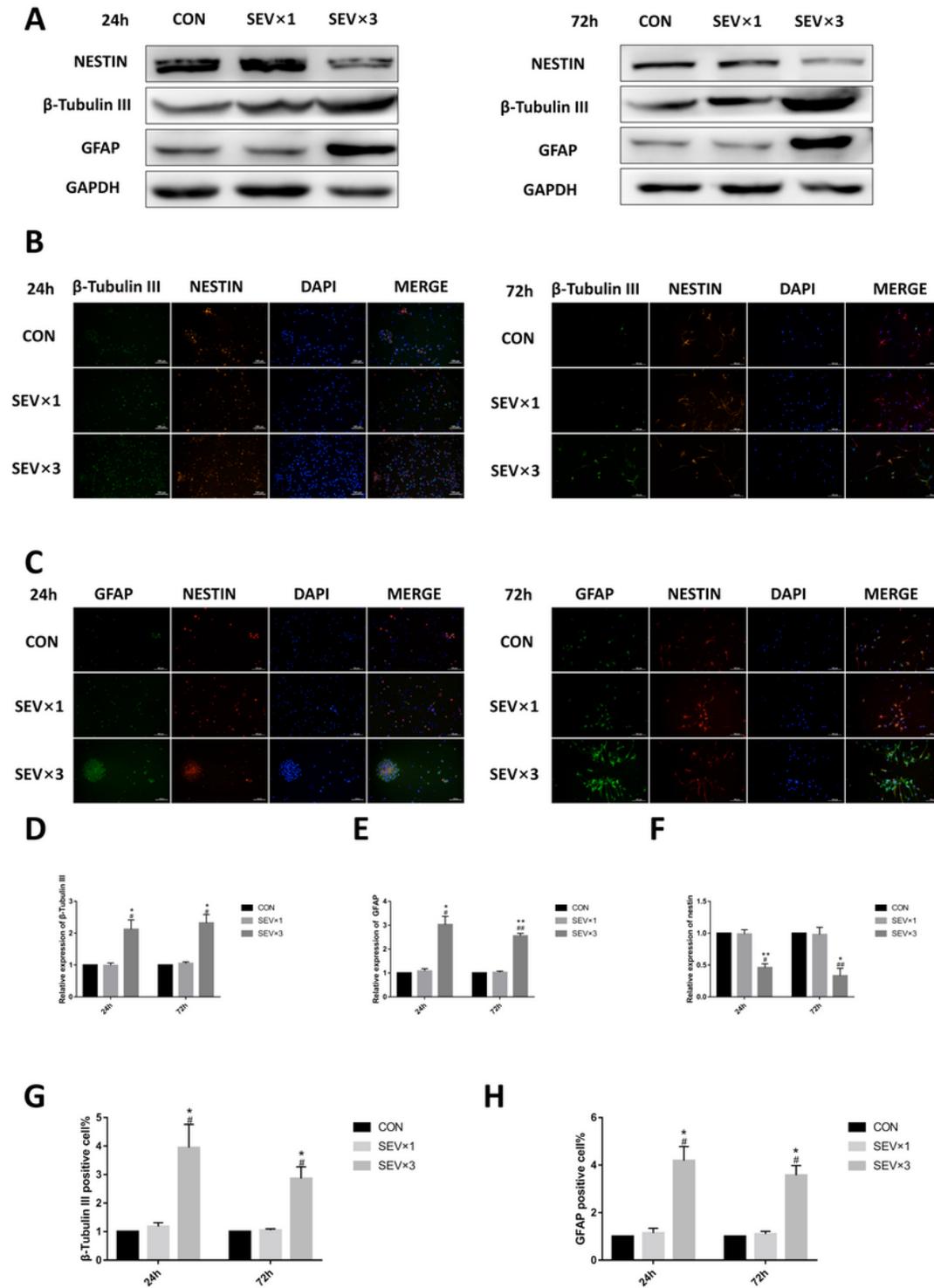


Figure 3

Repeated exposure to 4.1% sevoflurane led to early differentiation in primary cultured hippocampal NSCs. (A) Western blotting images of β -tubulin III, GFAP, and nestin. (B) Immunofluorescence images of β -tubulin III (green) and nestin (red). Scale bar = 100 μ m. (C) Immunofluorescence images of GFAP (green) and nestin (red). Scale bar = 100 μ m. (D) Quantitative analysis of β -tubulin III. (E) Quantitative analysis of GFAP. (F) Quantitative analysis of nestin. (G) Quantification of β -tubulin III-positive cells. (H) Quantification of GFAP-positive cells. Values are means \pm SD (n = 3/group). *p < 0.05, **p < 0.01 compared with CON group; #p < 0.05, ##p < 0.01 compared with SEV \times 1 group. One-way ANOVA followed by Tukey's post hoc multiple comparison test was used for data analysis.

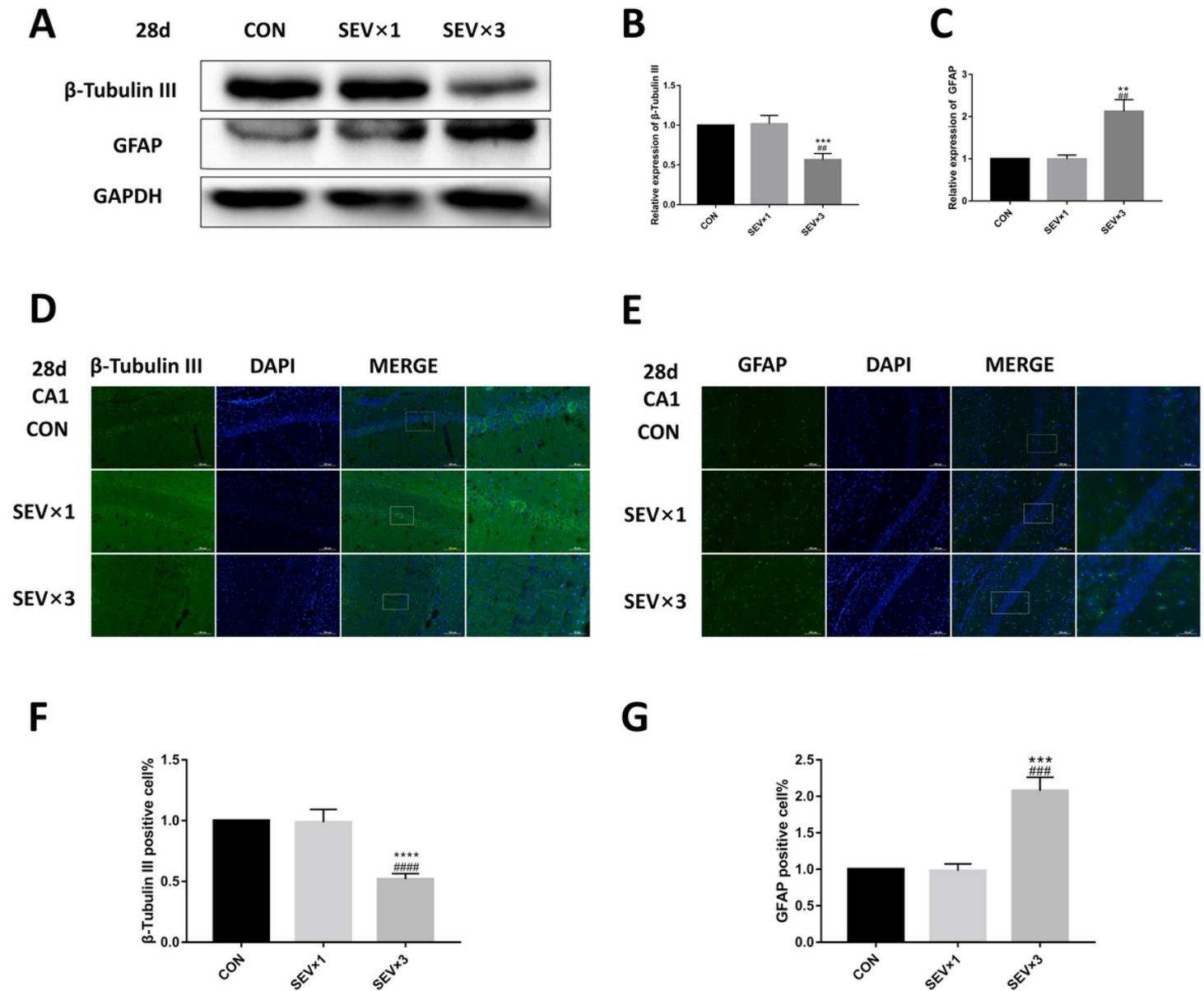


Figure 4

Effects of sevoflurane exposure on the expression of β -tubulin III and GFAP in fetal hippocampi on postnatal day 28. (A) Western blotting images of β -tubulin III and GFAP. (B) Quantitative analysis of β -tubulin III. (C) Quantitative analysis of GFAP. (D) Immunofluorescence images of β -tubulin III (green) in

hippocampal CA1 region. Scale bar = 100 μ m, 50 μ m. (E) Immunofluorescence images of GFAP (green) in hippocampal CA1 region. Scale bar = 100 μ m, 50 μ m. (F) Quantification of β -tubulin III-positive cells. (G) Quantification of GFAP-positive cells. Values are means \pm SD (n = 5). **p < 0.01, ***p < 0.001 compared with CON group; ##p < 0.01 compared with SEV \times 1 group. One-way ANOVA followed by Tukey's post hoc multiple comparison test was used for data analysis.

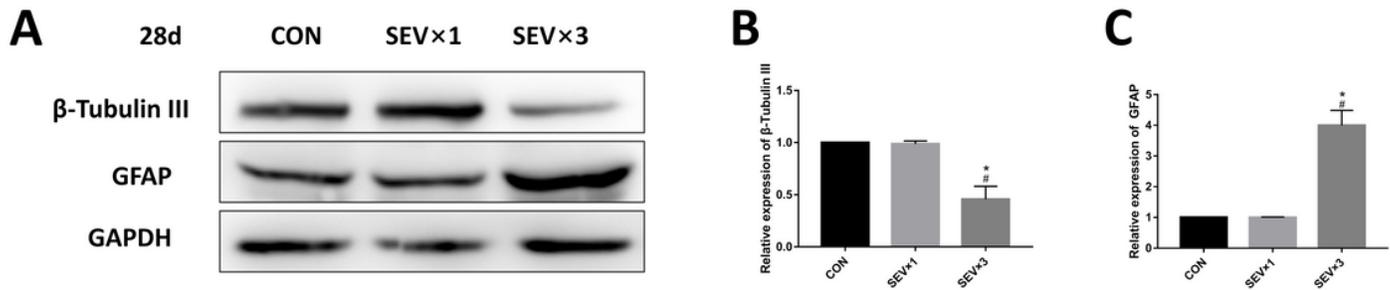


Figure 5

Effects of sevoflurane exposure on β -tubulin III and GFAP expression in cultured NSCs on postnatal day 28. (A) Western blotting images of β -tubulin III and GFAP. (B) Quantitative analysis of β -tubulin III. (C) Quantitative analysis of GFAP. Values are means \pm SD (n = 3). *p < 0.05 compared with CON group; #p < 0.05 compared with SEV \times 1 group. One-way ANOVA followed by Tukey's post hoc multiple comparison test was used for data analysis.

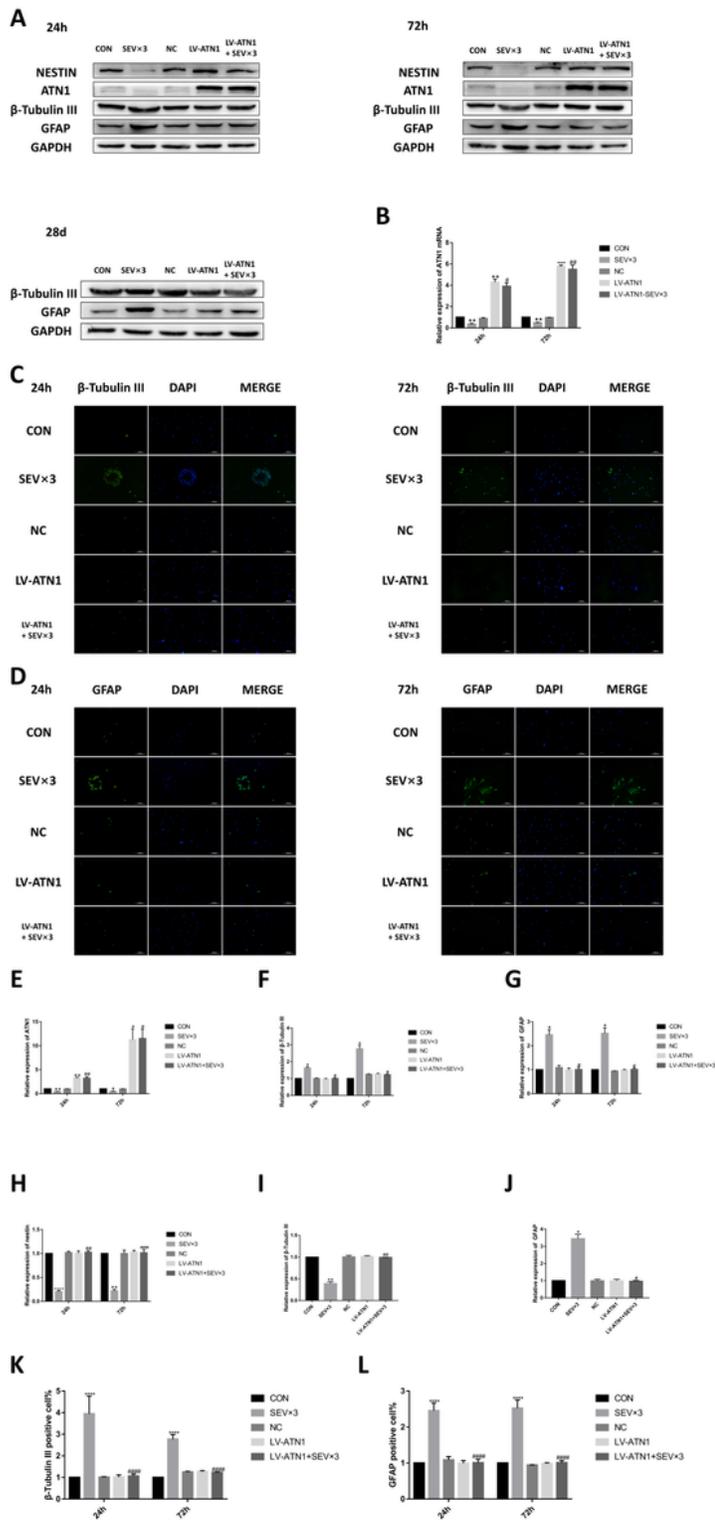


Figure 6

ATN1 overexpression alleviated sevoflurane-induced early NSC differentiation. (A) Western blotting images of β -tubulin III, GFAP, nestin, and atrophin 1(ATN1). (B) RT-qPCR analysis of ATN1 mRNA expression. (C) Immunofluorescence images of β -tubulin III (green). Scale bar = 100 μ m. (D) Immunofluorescence images of GFAP (green). Scale bar = 100 μ m. (E) Quantitative analysis of ATN1. (F) Quantitative analysis of β -tubulin III. (G) Quantitative analysis of GFAP. (H) Quantitative analysis of

nestin. (I) Quantitative analysis of β -tubulin III on day 28. (J) Quantitative analysis of GFAP on day 28. (K) Quantification of β -tubulin III-positive cells. (L) Quantification of GFAP-positive cells. Values are means \pm SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.0001 compared with CON group; #p < 0.05, ##p < 0.01, ###p < 0.0001 compared with SEV \times 3 group. One-way ANOVA followed by Tukey's post hoc multiple comparison test was used for data analysis.

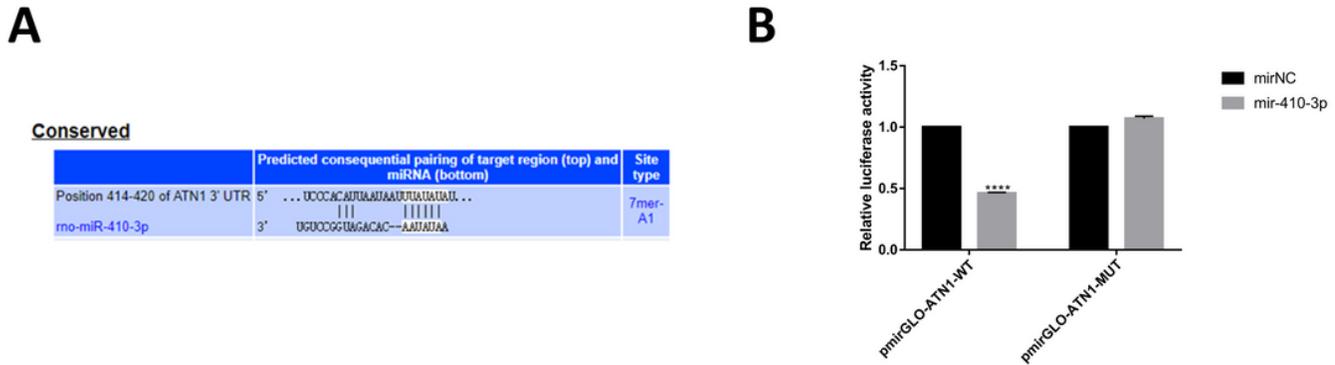


Figure 7

ATN1 is a target gene of miR-410-3p. (A) Target prediction program predicted a specific binding region between the ATN1 gene and miR-410-3p sequence. (B) Relative luciferase activity assay analysis. The experiment was performed three times. Paired Student's t-test was used for data analysis.

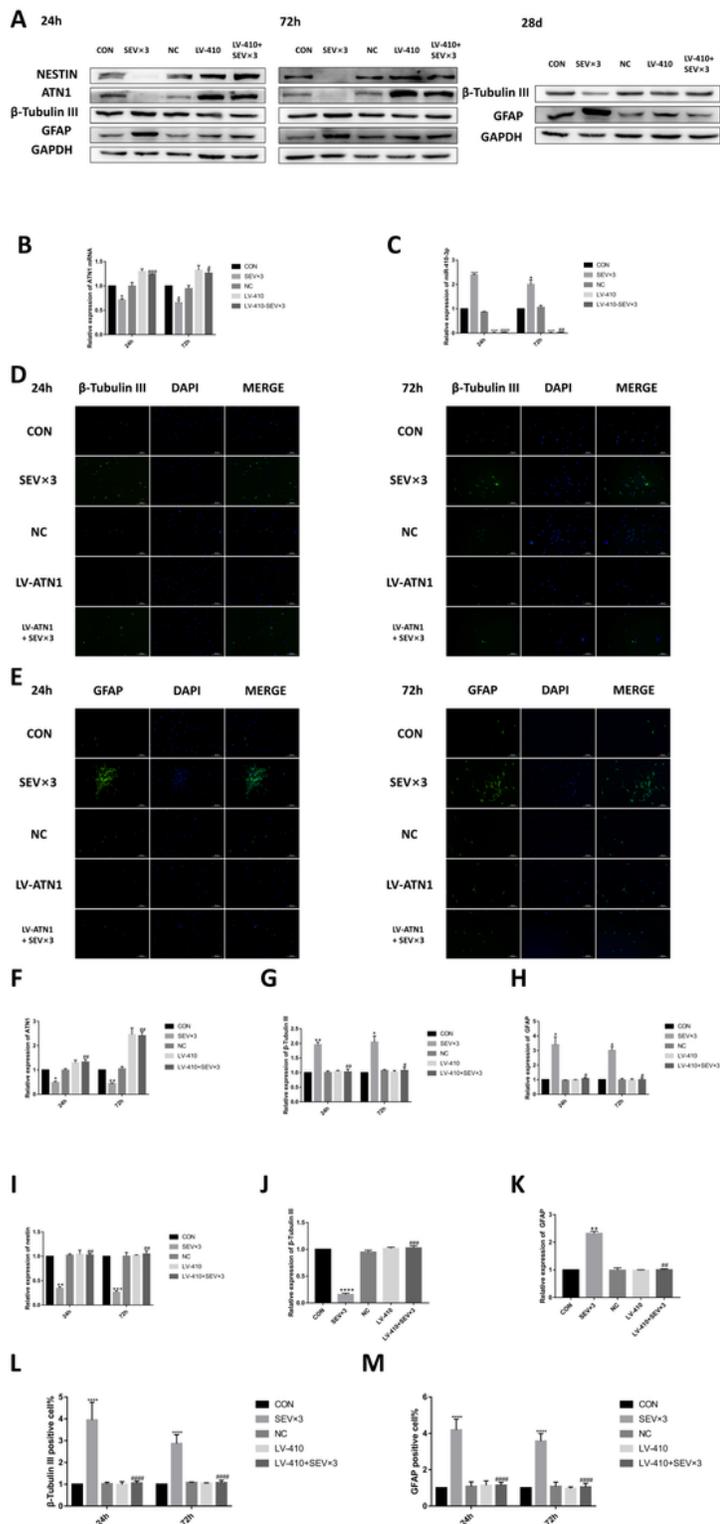


Figure 8

MiR-410-3p suppression alleviated sevoflurane-induced early NSC differentiation. (A) Western blotting images of β -tubulin III, GFAP, nestin, and ATN1. (B) RT-qPCR analysis of miR-410-3p expression. (C) RT-qPCR analysis of ATN1 expression. (D) Immunofluorescence images of β -tubulin III (green). Scale bar = 100 μ m. (E) Immunofluorescence images of GFAP (green). Scale bar = 100 μ m. (F) Quantitative analysis of ATN1. (G) Quantitative analysis of β -tubulin III. (H) Quantitative analysis of GFAP. (I) Quantitative

analysis of nestin. (J) Quantitative analysis of β -tubulin III on day 28. (K) Quantitative analysis of GFAP on day 28. (L) Quantification of β -tubulin III-positive cells. (M) Quantification of GFAP-positive cells. Values are means \pm SD (n=3). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared with CON group; #p < 0.05, ##p < 0.01, ###p < 0.001 compared with SEV \times 3 group. One-way ANOVA followed by Tukey's post hoc multiple comparison test was used for data analysis.

Supplementary Files

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