Overexpression of miR-133b protects against isoflurane-induced learning and memory impairment

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Abstract. A number of microRNAs (miRs) have been identified as being involved in the regulation of anesthesia-induced cognitive impairment. The aim of the present study was to investigated the role and potential mechanism of miR-133b in isoflurane-induced learning and memory impairment. An animal model of isoflurane exposure was established using neonatal Sprague-Dawley rats. The rats were trained for Morris water maze (MWM) testing to assess their spatial learning and memory ability. Reverse transcription-quantitative polymerase chain reaction was used for the measurement of miR-133b expression in hippocampal tissues and primary hippocampal neuron cultures. Cell viability was assessed using a Cell Counting Kit-8 assay, and flow cytometric analysis was used to determine the rate of apoptosis. The MWM test results indicated that during the training period, the time required to locate the platform was significantly increased for rats exposed to isoflurane, and this increased time was reduced by the overexpression of miR-133b. The results of a probe trial indicated that isoflurane exposure increased escape latency and decreased the time spent in the platform area for isoflurane-treated rats; however, these effects were reversed by the injection of miR-133b agomir. The *in vitro* experiments demonstrated that the overexpression of miR-133b attenuated the reduction of neuronal cell viability induced by isoflurane, and inhibited the isoflurane-induced apoptosis of hippocampal neurons. In conclusion, the present study revealed that the overexpression of miR-133b attenuated isoflurane-induced learning and memory impairment in rats. Furthermore, miR-133b overexpression promoted the viability of hippocampal neurons and their resistance to apoptosis when exposed to isoflurane.

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Introduction

Numerous newborn babies are required to receive anesthesia for diagnostic or surgical purposes (1). The application of anesthetics has been suggested to be harmful to brain function, leading to impairments of learning and cognitive functions (2,3). Isoflurane is one of the most commonly used inhaled anesthetics, and has been reported to contribute to long-term memory deficits (4). Anesthesia-associated neuronal apoptosis is considered to be an important mechanism involved in the neurological impairment induced by anesthesia (5,6). Notably, exposure of the developing brain to isoflurane can cause severe damage to neurological functions, which may result in persistent learning deficits and cognitive impairment (3,7). Previous studies performed in developing rodent models have demonstrated that almost all anesthetics, including isoflurane, induce widespread neuronal cell death followed by long-term memory and learning disabilities (8,9). These findings have raised serious concerns about the safety of anesthetic use in pregnant women and young children. However, the underlying mechanisms of anesthesia-induced learning and cognitive dysfunction remain to be clarified.

MicroRNAs (miRNAs) are a class of short non-coding RNA molecules, which function as negative regulators of target genes by binding to their 3'-untranslated regions. miRNAs have been widely suggested to play important roles in various biological processes, including cell proliferation, apoptosis and differentiation, and neuronal inflammation (10,11). The abnormal expression of miRNAs has been reported to have a regulatory effect on learning and memory function (12,13). For example, in one study, miR-132 expression was shown to be downregulated in the hippocampus of elderly mice compared with younger mice, and *in vivo* experiments suggested that overexpression of miR-132 may reverse the decline in learning and memory (12). Furthermore, a number of miRNAs have been identified as being involved in the regulation of anesthesia-induced cognitive impairment, including miR-24, miR-124 and miR-214 (3,14,15). The role of miR-133b, a muscle-specific miRNA, in neuronal development and dysfunction has also been investigated (16,17). In a rat model of depression, miR-133b was found to be expressed at low levels in hippocampal tissues, and the overexpression of miR-133b inhibited the apoptosis of hippocampal neurons (16). In addition, a study by Takeuchi *et al* (18) reported that miR-133b was downregulated in the plasma of sevoflurane-anesthetized

rats. Therefore, the potential involvement of miR-133b in the underlying mechanism of isoflurane-induced learning and memory impairment is a subject of interest.

The present study aimed to investigate changes in the expression of miR-133b in isoflurane-treated rats. Additionally, the role and potential mechanism of miR-133b in isoflurane-induced learning and memory impairment were further explored.

Materials and methods

Experimental animals and grouping. A total of 80 male or female Sprague-Dawley (SD) rat pups (age, 7 days; weight, 12-15 g) were purchased from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. All rats were raised in standard conditions, under 50-60% atmospheric humidity, on a 12-h light/dark cycle, at a room temperature of 24-26˚C and with free access to food and water. All procedures conducted in this study were approved by the Ethics Committee of the Experimental Animal Center of Jining No. 1 People's Hospital.

The rats were randomly divided into four groups, each comprising 20 rats, as follows: i) Control group, treated with regular air; ii) isoflurane group, anesthetized with 1.5% isoflurane for 6 h; iii) isoflurane + agomir NC group, treated with 2 nmol agomir NC (sequence: 5'-UUCUCCGAACGUGUC ACGUTT-3'; Guangzhou RiboBio Co., Ltd.) by lateral cerebroventricular injection and anesthetized 30 min later with 1.5% isoflurane for 6 h; iv) isoflurane + miR-agomir group, treated with 2 nmol miR-133b agomir (sequence: 5'-UUUGGU CCCCUUCAACCAGCUA-3'; Guangzhou RiboBio Co., Ltd.) by lateral cerebroventricular injection and anesthetized 30 min later with 1.5% isoflurane for 6 h. Following the air or isoflurane treatment, 10 rats from each group were euthanized by cervical dislocation in accordance with the procedure approved by the Ethics Committee of the Experimental Animal Center of Jining No. 1 People's Hospital in accordance with AVMA Guidelines, 2020 edition (19) and the hippocampal tissues were collected for RNA extraction. The remaining 10 rats in each group were used for subsequent Morris water maze (MWM) tests.

MWM tests. One week after the various treatments were administered, rats (postnatal day 14) were trained for a MWM test to assess spatial learning and memory ability. The MWM test was performed on 5 consecutive days. The swimming route was observed using VideoMot software version 2.4.50923 (TSE Systems GmbH). The time taken for the rats to locate a submerged platform (latency, using a cut-off time point of 120 sec) and the swimming speed were recorded on the first 4 days. On day 5, a probe trial was performed without the platform. The escape latency, meaning the time taken for the rat to swim to the former location of the platform, was recorded. The percentage of time that each rat spent in the quadrant that previously contained the platform in a 120-sec period was determined.

Cell culture and transfection. Hippocampal cells were prepared as described in a previous study (20). Three newborn rats (0-24 h old) were purchased from Shanghai Laboratory Animal Center of the Chinese Academy of Sciences. Hippocampal tissues were collected from the newborn rats within 24 h of birth. Hippocampal neuronal cells were cultured in Neurobasal™ Medium supplemented with 2% B27, 0.3% glucose, 1 mM glutamine, and 5% FBS (Invitrogen; Thermo Fisher Scientific, Inc.) in a humidified incubator with 5% CO_2 at 37˚C. The cells were seeded in 96-well plates at a density of $1x10^5$ cells/well and cultured for 24 h. miR-133b mimic (50 nM; sequence: 5'-UUUGGUCCCCUUCAACCA GCUA-3') and negative control (50 nM; miR-NC, sequence: 5'-UUCUCCGAACGUGUCACGUTT-3') were provided by RiboBio Co., Ltd. Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) was used for cell transfection according to the manufacturer's protocols. In addition to control group, cells in other groups were cultured in a 37˚C incubator with 5% CO_2 and 1.5% isoflurane for 6 h, then transfected with miR-mimic or miR-NC at 37˚C. At 24 h post transfection, cells in different groups were collected for futher experiment.

The cells were divided into four groups as follows: i) Negative control group (control), untreated cells; ii) isoflurane group, cells treated with 1.5% isoflurane for 6 h; iii) isoflurane + miR-NC group, cells treated with 1.5% isoflurane for 6 h and transfected with miR-NC; iv) isoflurane + miR-mimic group, cells treated with 1.5% isoflurane for 6 h and transfected with miR-133b mimic.

RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from hippocampal tissues and cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The miScript Reverse Transcription Kit (Qiagen GmbH) was used to transcribe the total RNA into cDNA. The reverse transcription conditions were as follows: 37˚C for 15 min, followed by 5 sec at 85˚C for RT inactivation. Thereafter, qPCR was conducted using a SYBR Green Master Mix kit (Invitrogen; Thermo Fisher Scientific, Inc.) to detect the expression level of miR-133b. The following thermocycling conditions were used for the PCR: Initial denaturation at 95˚C for 5 min; 30 cycles of 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 20 sec; and a final extension at 72˚C for 10 min. The relative expression of miR-133b was evaluated using the $2^{-\Delta\Delta Cq}$ method with U6 as the reference gene (21). The primer sequences were as follows: miR-133b forward, 5'-AAAGGACCCCAACAACCAGCAA-3 and reverse, 5'-TTGCTGGTTGTTGGGGTCCTTT-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACATATACT-3 and reverse, 5'-ACGCTTCACGAATTTGCGTGTC-3.

Cell viability. Cell viability was assessed using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.) assay. Cells from the different groups were inoculated into 96-well plates at a density of $5x10^4$ cells per well and cultured continuously for 3 days. At 0, 24, 48 and 72 h after the initiation of incubation, 10 ml CCK-8 reagent was added to each well and incubated for 2 h. The absorbance of each well was then measured at 450 nm.

Flow cytometric assay. A FITC Annexin V Apoptosis Detection kit (BD Biosciences) was used to measure cell

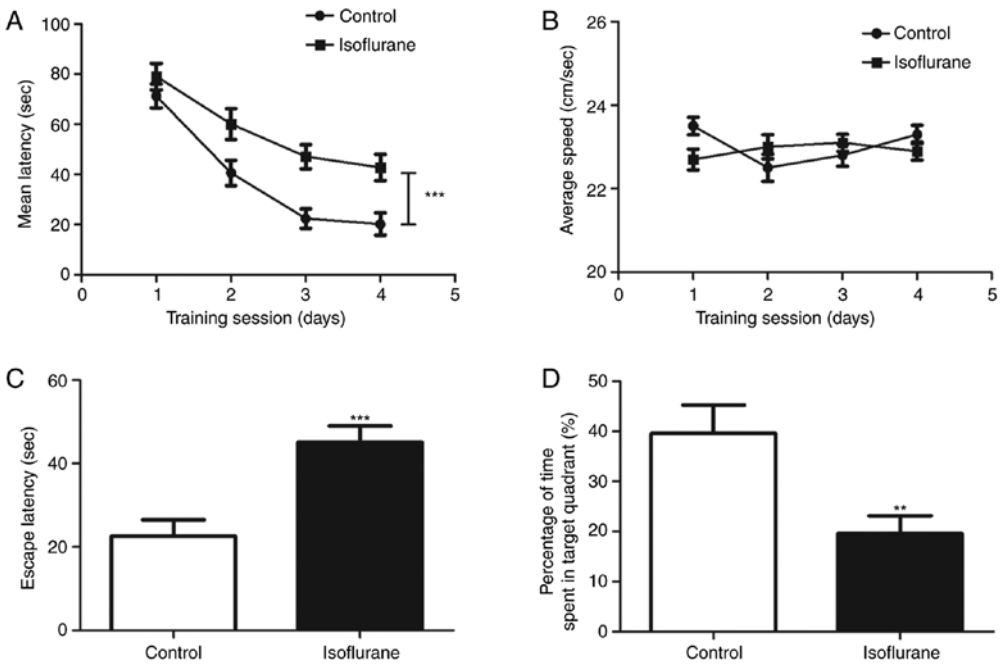

Figure 1. Isoflurane affects the learning and memory functions of rats. (A) During the training session, the time required for the rats to locate the platform was significantly increased after treatment with isoflurane. (B) No significant difference was detected in the swimming speed of rats according to whether or not they were treated with isoflurane. (C) A probe trial indicated that the isoflurane-treated rats exhibited a significantly longer escape latency than the control rats. (D) For rats in the isoflurane group, the time spent in the quadrant that previously contained the platform was significantly shorter than that of the control group. **P<0.01, ***P<0.001.

apoptosis. Cells ($5x10^5$) were collected from each group, fixed with 3.7% formaldehyde for 15 min at room temperature and then permeabilized with 0.1% Triton X-100 for 5 min at 37˚C. After washing, the cells were mixed with 5 μl Annexin V-FITC and propidium iodide, and incubated at room temperature for 10 min. The apoptotic rates of the cells were detected and recorded using a FACScan™ flow cytometer in combination with CellQuest Pro v5.1.1 software (BD Biosciences).

Statistical analysis. GraphPad Prism 5.0 software (GraphPad Software, Inc.) was used for data analysis. Differences between two groups were compared using the Student's t-test, while differences among multiple groups were evaluated using one-way analysis of variance followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

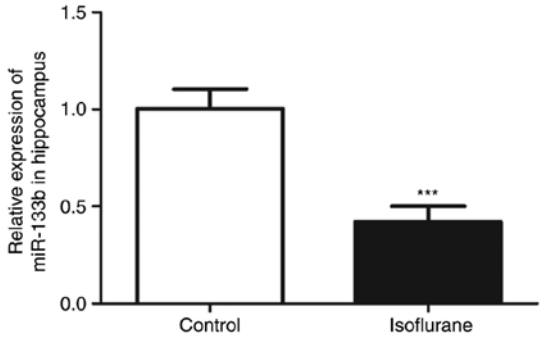

Figure 2. Reverse transcription-quantitative polymerase chain reaction was applied for the measurement of miR-133b expression in rat hippocampal tissues. The level of miR-133b in the hippocampal tissues of rats after treatment with isoflurane was significantly decreased compared with that in the control rats. ***P<0.001. miR, microRNA.

Results

Effects of isoflurane on learning and memory in rats. MWM tests were conducted to determine whether isoflurane affects the learning and memory functions of neonatal rats. As shown in Fig. 1A, during the training session, the time taken for the isoflurane-treated rats to locate the platform was significantly increased compared with that of the control rats (P<0.001). However, no significant difference in swimming speed was observed between the isoflurane-treated and control rats (P>0.05, Fig. 1B). A probe trial was then performed to assess the effect of isoflurane on memory. It was found that the rats treated with isoflurane exhibited a significantly higher escape latency than the control group (P<0.001; Fig. 1C). Additionally, the rats in the isoflurane group spent significantly less time than the control rats in the quadrant that previously contained the platform (P<0.01; Fig. 1D). These results indicate that isoflurane exposure affected the learning and memory functions of the rats, and the isoflurane-induced learning and memory impaired rat model was established successfully.

miR-133b expression is reduced in rats anesthetized with isoflurane. RT-qPCR was used to measure the hippocampal expression levels of miR-133b in the isoflurane-treated and control rats. The hippocampal expression of miR-133b in the rats treated with isoflurane was significantly decreased compared with that of the control rats (Fig. 2; P<0.001).

Effect of miR-133b on isoflurane-induced learning and memory impairment in rats. To further investigate the role of

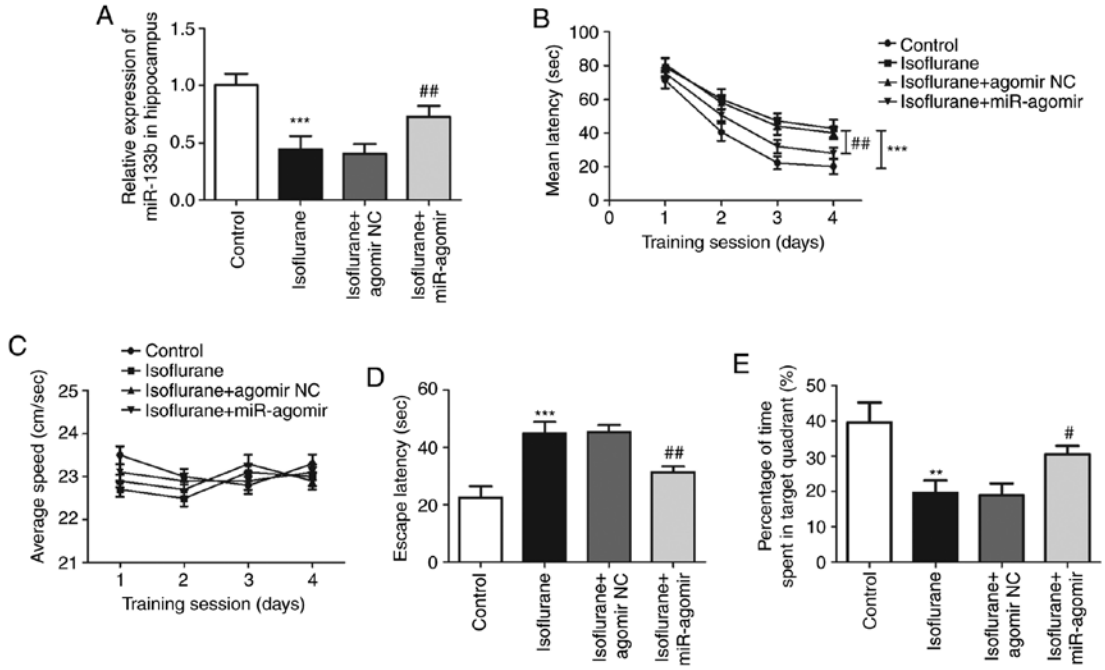

Figure 3. Overexpression of miR-133b attenuates isoflurane-induced learning and memory impairment in rats. (A) Reverse transcription-quantitative polymerase chain reaction results indicated that the injection miR-133b agomir significantly inhibited the isoflurane-induced reduction of miR-133b expression. During the MWM training session, (B) the significant increase in latency time induced by isoflurane treatment was significantly reversed by the overexpression of miR-133b and (C) no significant difference in swimming speed was observed between isoflurane-treated rats with or without miR-133b agomir injection. MWM probe trial test results indicated that the overexpression of miR-133b reversed the effect of isoflurane on (D) escape latency and (E) the time spent in the target quadrant. **P<0.01, ***P<0.001 vs. the control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. the isoflurane group. miR, microRNA; MWM, Morris water maze; NC, negative control.

miR-133b in isoflurane-induced learning and memory impairment *in vivo*, the expression level of miR-133b was regulated by the injection of miR-133b agomir. As shown in Fig. 3A, RT-qPCR analysis demonstrated that the injection of miR-133b agomir significantly increased the expression of miR-133b, counteracting the reduction of miR-133b expression induced by isoflurane treatment (P<0.01; Fig. 3A). In the MWM test, during the training session, the latency time for location of the submerged platform was significantly increased by isoflurane treatment (P<0.001), and this increase was significantly attenuated by the overexpression of miR-133b (P<0.01, Fig. 3B). The swimming speeds of the isoflurane-treated rats during the training session were not observed to differ significantly according to whether or not miR-133b agomir was administered (P>0.0.05, Fig. 3C). Additionally, the probe trial test results indicated that the overexpression of miR-133b reversed the effect of isoflurane on the escape latency (P<0.01) and time spent in the target quadrant (P<0.05) (Fig. 3D and E). These data suggest that the overexpression of miR-133b attenuated the isoflurane-induced learning and memory impairment of the rats.

Effect of miR-133b on hippocampal neuron viability and apoptosis. Rat hippocampal neurons were used *in vitro* to further explore the effect of miR-133b on neuron viability and apoptosis. The expression of miR-133b was regulated by transfection, and it was found that transfection of the neurons with miR-133b mimic significantly increased the level of miR-133b compared with that in the control group (P<0.01, Fig. 4A). RT-qPCR results also revealed that treatment with isoflurane significantly reduced the expression level of miR-133b in neurons *in vitro* (P<0.001), and this reduction was attenuated by transfection with miR-133b mimic (P<0.05, Fig. 4B). The results of the CCK-8 assay indicated that isoflurane treatment reduced the viability of the neurons (P<0.01), and this reduced viability was significantly attenuated by the upregulation of miR-133b (P<0.05, Fig. 4C). Additionally, flow cytometric analysis demonstrated that isoflurane induced neuronal apoptosis (P<0.001), and the overexpression of miR-133b significantly attenuated the isoflurane-induced apoptosis (P<0.001, Fig. 4D).

Discussion

Isoflurane is a widely used anesthetic in pediatric and obstetric patients; millions of infants and children are exposed to general anesthetic agents during surgical or treatment procedures, which may influence their neuronal functions and brain development (22,23). Therefore, interest in the biological mechanisms underlying anesthesia-induced learning and cognitive dysfunction has been stimulated. A number of *in vivo* studies have suggested that isoflurane leads to spatial learning and memory impairment, and causes cognitive dysfunction (24-26). In order to investigate this, the present study constructed animal models of isoflurane exposure using neonatal SD rats. The results of MWM tests indicated that isoflurane exposure influenced the learning ability of the rats, which was reflected by an increase in the time required to locate a submerged platform during the training session. Additionally, probe trial test results indicated that isoflurane

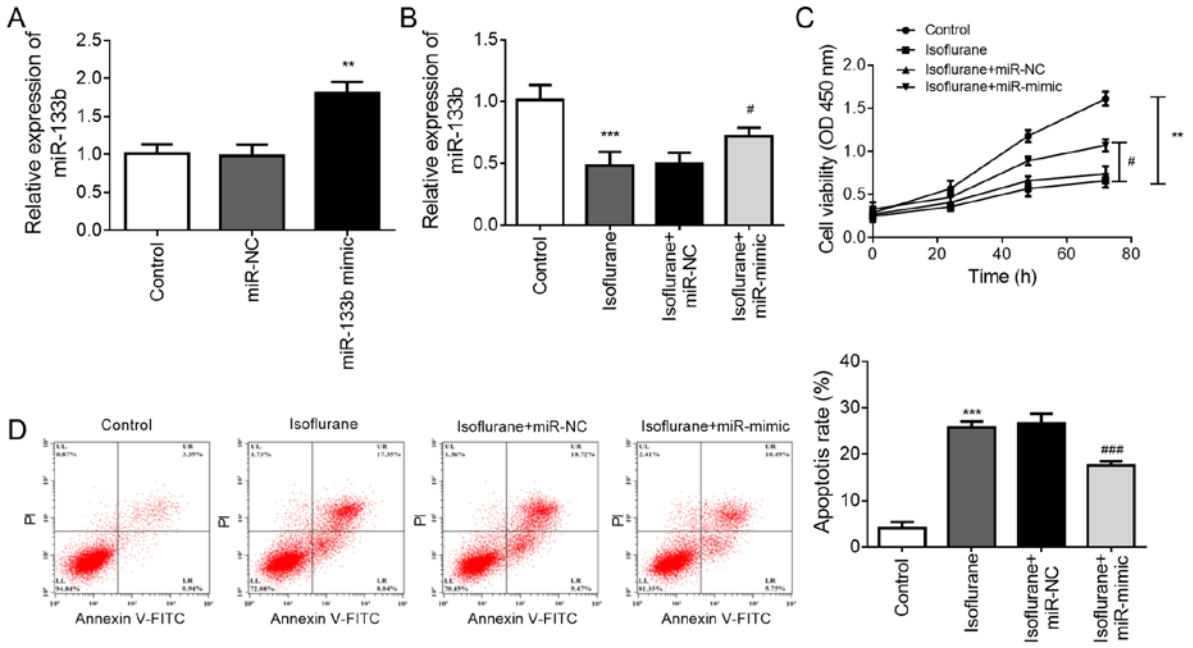

Figure 4. Effect of miR-133b on hippocampal neuron viability and apoptosis. (A) Transfection with miR-133b mimic significantly increased the expression level of miR-133b compared with that in the control group. (B) Isoflurane treatment significantly decreased the expression level of miR-133b *in vitro*, and transfection with miR-133b mimic attenuated the reduction in miR-133b levels. (C) Cell Counting Kit-8 results indicated that isoflurane reduced the viability of neurons, and the overexpression of miR-133b attenuated the isoflurane-induced reduction in viability. (D) Flow cytometry results revealed that isoflurane promoted cell apoptosis, and the overexpression of miR-133b significantly inhibited the isoflurane-induced cell apoptosis. **P<0.01, ***P<0.001 vs. the control group; $^{\#}$P<0.05, $^{\#\#\#}$P<0.001 vs. the isoflurane group. miR, microRNA; NC, negative control.

exposure increased the escape latency of the neonatal rats, and reduced their time spent in the area where the platform was previously located. These data suggest that exposure to isoflurane influenced the learning and memory function of the rats, which is consistent with previous studies (24,25,26) and indicates that the rat model of isoflurane-induced learning and memory impairment was established successfully.

miR-133b is a muscle-specific miRNA that exhibits an anesthesia-regulated expression pattern and participates in various pathological processes (18,27). In a study of the sevoflurane-associated dynamics of circulating miRNAs, the plasma levels of miR-133b were found to be downregulated in sevoflurane-anesthetized rats, which is consistent with the findings of the present study (18). In the present study, the results indicated that in the rat model of isoflurane exposure, miR-133b was expressed at lower levels in the hippocampal tissue of rats exposed to isoflurane than in those of control rats, which is supported by previous studies (18,27). These results suggest that miR-133b potentially serves a role in isoflurane-induced learning and memory impairment.

To further elucidate the role of miR-133b in isoflurane-induced learning and memory impairment *in vivo*, the expression level of miR-133b was modified by the injection of miR-133b agomir. The MWM test results indicated that the overexpression of miR-133b attenuated the isoflurane-induced learning and memory impairment of the rats. The present results are supported by previous studies that have focused on the role of miR-133b in neuronal development and neurological disease (17,28). In a study of spinal cord regeneration, the overexpression of miR-133b was demonstrated to promote neurite outgrowth by targeting RhoA (29). Another study suggested that miR-133b promoted neural plasticity and functional recovery in a mouse model of stroke (30). All this evidence supports the present results indicating that miR-133b may be involved in the regulation of isoflurane-induced learning and memory impairment.

Isoflurane is considered to influence neuronal cell viability and apoptosis, which are involved in the regulation of learning and cognitive functions (26,31). To gain further insight into the role of miR-133b in isoflurane-induced neuronal injury, a cell model of isoflurane exposure was established using primary hippocampal neurons and the expression of miR-133b was regulated by transfection. It was observed that the overexpression of miR-133b attenuated the effect of isoflurane on neuronal cell viability and apoptosis. Consistent with these findings, the neuroprotective effect of miR-133b has been demonstrated in a number of previous studies. For example, in a rat model of depression, miR-133b expression was identified to be downregulated, and the elevation of miR-133b expression inhibited the apoptosis of hippocampal neurons, thereby providing a protective effect against neural injury in depressed rats (16). Another study, concerning the pathogenesis of Parkinson's disease (PD), indicated that the expression of miR-133b was decreased in the brain tissues of a mouse model of PD, and was involved in the mechanism by which long non-coding RNA SNHG14 contributes to dopaminergic neuron injury (17). However, the pathological changes and viability of cells in rat brain tissues were not investigated in the present study, which is a limitation of the study. In future studies, such indicators should be analyzed using *in vivo* experiments to better reflect the effect of miR-133b on neuronal cell viability.

In a previous study on the role of isoflurane in dopaminergic neurons, isoflurane was demonstrated to inhibit synaptic transmission in rat midbrain dopaminergic (mDA)

neurons (32). mDA neurons have been reported to be involved in the regulation of learning and memory function (33,34). Sanchez-Simon *et al* (35) used zebrafish embryos as a model to investigate the role of morphine in neuronal development, and found that miR-133b expression was downregulated by morphine treatment, demonstrating that miR-133b has a regulatory role in neuronal development, mediated via the regulation of dopaminergic neuron differentiation. In the present study, low expression levels of miR-133b were detected in the hippocampal tissues of rats treated with isoflurane, and the *in vivo* experiments suggested that the overexpression of miR-133b attenuated isoflurane-induced learning and memory impairment in rats. Considering the crucial role of mDA neurons in learning and memory function, we hypothesize that miR-133b may participate in isoflurane-induced neurological impairment via the regulation of mDA neurons. However, further studies are required to explore the exact mechanism by which miR-133b participates in isoflurane-induced learning and memory impairment. Additionally, the present results in developing rats raise questions about the role of miR-133b in isoflurane-induced cognitive impairment in aged rats, which is an important topic for further exploration.

In conclusion, the results of the present study indicate that the overexpression of miR-133b attenuated isoflurane-induced learning and memory impairment in rats. During exposure to isoflurane *in vitro*, the viability and apoptosis resistance of hippocampal neurons were promoted by the overexpression of miR-133b. These data provide a theoretical basis for the prevention and treatment of the neurological deficits induced by isoflurane exposure.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ, JL, CX and PW participated in the design and interpretation of the study, analysis of the data and review of the manuscript. YZ and JL conducted the experiments. PW wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures of this study were approved by the Ethics Committee of the Experimental Animal Center of Jining No. 1 People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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