

Effects of regulating miR-132 mediated GSK-3 β on learning and memory function in mice

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Abstract. The aim of this study was to explore effects of miR-132 and glycogen synthase kinase-3 β (GSK-3 β) on learning and memory in mice. miR-132 inhibitor GSK-3 β overexpression agent (sh-GSK-3 β) and normal saline (negative control group) were injected into the hippocampus of adult mice, and healthy adult mice were taken as the unrelated control group. The expression of miR-132 and GSK-3 β in the hippocampus of adult and elderly mice was detected using reverse transcription-quantitative PCR (RT-qPCR) and western blot analysis. Morris water maze test was employed to detect learning and memory function in mice. The dual luciferase reporter was adopted to determine the relationship between miR-132 and GSK-3 β . Compared with the adult group, the expression of miR-132 was significantly downregulated in the hippocampus in the elderly group, while the expression of GSK-3 β was upregulated. Injecting miR-132 inhibitor into the hippocampus of adult mice led to a significant increase in escape latency and a significant decrease in the number of times of crossing platforms. The injection of GSK-3 β overexpression agent into the hippocampus of adult mice resulted in a marked increase in escape latency and a significant decrease in the number of times of crossing platforms in the water maze test. It was also found that downregulation of GSK-3 β reversed the decline in learning and memory in mice caused by downregulation of miR-132 expression. The dual luciferase report identified a targeted regulatory relationship between miR-132 and GSK-3 β . Overexpression of miR-132 can inhibit the expression of GSK-3 β in mouse learning and memory ability, which provides some inspiration for understanding the occurrence of learning and memory disorders and future treatment methods.

Introduction

MicroRNA (miR) is a type of non-coding short-chain RNA of 22nt in length. In eukaryotes, it can regulate the expression of target genes by binding to the downstream target gene 3'UTR, 5'UTR and coding region, thus participating in intercellular signal modulation (1,2). At present, approximately 1,500 genes encoded by miR have been identified in the human genome (3). Along with the continuous exploration of miRs, there is emerging evidence showing that miRs play an important part in synaptic plasticity, learning and memory function (4). Among them, miR-132 is an important member of the miR family, which has been widely studied in a variety of neurological diseases. It has been reported that downregulation of miR-132/21 disrupts the S-nitrosation balance of Alzheimer's disease (AD) and induces tau phosphorylation, thereby promoting the pathogenesis of AD (5). Other studies have pointed out that elevated levels of miR-132 are associated with visual memory dysfunction in patients with depression (6). All the above studies have shown that miR-132 is closely related to learning and memory function and related diseases, however, its specific mechanisms of action remain a subject of investigation.

To explore the pathways in which miR-132 affects learning and memory function, we predicted the presence of a targeted binding site between glycogen synthase kinase-3 β (GSK-3 β) and miR-132 by targetscan, an online biological prediction software. GSK-3 β and GSK-3 α constitute the GSK-3 family, a ubiquitously expressed and highly conserved serine/threonine kinase that was first discovered in 1980 and is implicated in a variety of central intracellular signaling pathways, including glucose metabolism, inflammation and immune response as well as cell biological functions (7). Numerous scholars in the past have found that GSK-3 β is closely related to learning and memory function. For example, it is stated that the activation of GSK-3 β is closely related to aluminum-induced long-term potentiation injury in rats (8). Others have shown that tetramethylpyrazine can protect the memory loss of AD patients by inhibiting GSK-3 β activity (9). In recent years, studies have also found that there is a certain regulatory relationship between GSK-3 β and miR. For example, it is reported that upregulation of miR-26a can promote apoptosis of neonatal cardiomyocytes in hypoxic rats by inhibiting the expression of GSK-3 β protein (10). Still, some others have

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revealed that miR-199a can inhibit the proliferation and survival of renal cancer cells by targeting GSK-3 β (11). All these findings suggest that miR has a regulatory relationship with GSK-3 β and may affect learning and memory function.

Thus, it is hypothesized that miR-132 might affect the learning and memory function by targeting the activity of GSK-3 β , and the present study was conducted.

Materials and methods

Source of experimental animals and cell lines. Two batches of C57/BL male mice, aged 3-4 months and 24-26 months, respectively, were purchased from Beijing Charles River Laboratory Animal Technology Co., Ltd., and the animals were cultured in an animal room at 21-26°C with relative humidity of 51-57%. They were allowed to eat freely under natural light for 15 days for subsequent experiments. The experiment was conducted in strict accordance with the Guide for the Care and Use of Experimental Animals (12). 293 cells were purchased from ATCC (USA).

Main reagents and instruments. Radio immunoprecipitation assay (RIPA) reagent, bicinchoninic acid (BCA) protein assay kit and electrochemiluminescence (ECL) kit were purchased from Thermo Fisher Scientific, Inc. GSK-3 β (cat. no. MAB2506), β -actin antibody (cat. no. MAB8929), Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum, all from R&D Systems, Inc. Goat anti-rabbit IgG secondary antibody (cat. no. BA1032) from Wuhan Boster Biological Technology, Co., Ltd. TRIzol extraction kit (CDLG-4396; Wuhan Chundu Biotechnology Co., Ltd.). Reverse transcription kit [FP209; Tiangen Biotechnology (Beijing) Co., Ltd.]. Dual luciferase reporter assay kit (D0010; Beijing Solarbio Science and Technology Co., Ltd.). PCR instrument (7500; Applied Biosystems; Thermo Fisher Scientific, Inc.). The primers were designed and synthesized by Shanghai GenePharma Co., Ltd., and the lentiviral vectors were synthesized by Guangzhou Ribo Biotechnology Co., Ltd.

Grouping and treatment of mice. Virus injection: Adult mice were anesthetized with 5% chloral hydrate, and their scalp was fixed and exposed. Then, the hippocampus of the mice was injected with miR-132-inhibitor or GSK-3 β overexpression agent (sh-GSK-3 β) using stereoscopic localization technique, and disinfection was performed after the injection was completed. While the negative control group was injected with normal saline in the same manner, and the unrelated control group was healthy adult mice without any treatment. Follow-up studies were conducted 2 weeks after viral infection.

Cell culture. The cells were placed in DMEM (containing 10% bovine fetal serum) medium at 37°C in a constant temperature incubator with 5% CO₂, and cultured until the cells were adherent to approximately 90%. Then cells were digested and passaged with conventional trypsin. One day before the experiment, DMEM medium was replaced with DMEM medium without bovine fetal serum and further cultured overnight.

Morris water maze test. Ten mice from each group were subjected to Morris water maze test. The mice were placed

in a water-filled cylindrical bucket, which was divided into four quadrants (one of which hid a platform that could hold the mice in the quadrant), and randomly selected one of them as the entry point. After putting the mice into the water, the time of finding the platform was recorded, and the mice were allowed to stay on the platform for 30 sec. The operation was repeated every 1 h until the mice could find the platform in all the four quadrants. After 6 days of continuous training, the mice were allowed to rest for 1 day before spatial probe test. The steps were as follows: The underwater platform was removed and the mouse was placed in the quadrant opposite the platform to record the duration of its stay in the target quadrant and the number of times of crossings the original platform within 90 sec.

Western blot analysis. The hippocampus was isolated from the mouse brain, ground and pulverized in a grinder, and the total protein was extracted by RIPA lysis. Then the protein concentration was adjusted to 4 $\mu\text{g}/\mu\text{l}$ by BCA and separated by 6% SDS-PAGE electrophoresis before transferring to the PVDF membrane and staining in Ponceau S working solution. Followed by washing after immersion in PBST, sealed with 5% skim milk powder for 2 h, then added with GSK-3 β and β -actin primary antibody (1:1,000), and closed in a refrigerator at 4°C overnight. After that, the primary antibody was washed and the horseradish peroxidase-labeled goat anti-rabbit secondary antibody (1:5,000) was added, incubated at 37°C for 1 h, and rinsed 3 times with PBS, 5 min each. Finally, development was carried out in the dark, the excess liquid on the film was blotted with filter paper, and then illuminated by use of an ECL kit and developed. The protein bands were scanned and the gray value was analyzed using Quantity One software, wherein the relative expression level of the protein = the gray value of the target protein band/the gray value of the β -actin protein band.

RT-qPCR detection. The total RNA was extracted from the hippocampal tissues of mice with TRIzol kit, whose purity, concentration and integrity were detected by UV spectrophotometer and agarose gel electrophoresis. Then RNA was reversely transcribed into cDNA according to the instructions of the reverse transcription kit (one-step method), followed by PCR amplification, with the PCR reaction system as follows: 2xTalent qPCR PreMix: 10 μl , upstream and downstream primers each 1.25 μl , cDNA: 100 ng, and water was added to reach 20 μl . PCR reaction conditions: Pre-denaturation at 95°C for 3 min, denaturation at 95°C for 5 sec, and annealing at 60°C for 15 sec, totaling for 40 cycles. With U6 as the internal parameter of miR-132, the upstream of miR-132 was 5'-TAACAGTCTACAGCCATGGTCG-3', and the downstream was 5'-CTTCTTGCTGGTCTTGCCATTCC-3', while the upstream of U6 was 5'-GCTTCGGCAGCACATATACTAAAAT-3', and the downstream was 5'-CGCTTCACGAATTTGCGTGCAT-3'. The data was analyzed using 2^{- $\Delta\Delta\text{Cq}$} (13).

Double luciferase report. The downstream target genes of miR-132 were predicted by Targetscan 7.2. Lipofectamine™ 2000 kit was used to transfect GSK-3 β -3'UTR wild-type (Wt) and GSK-3 β -3'UTR mutant (Mut) and miR-132-inhibitor, miR-NC into target cells. Luciferase activity was determined

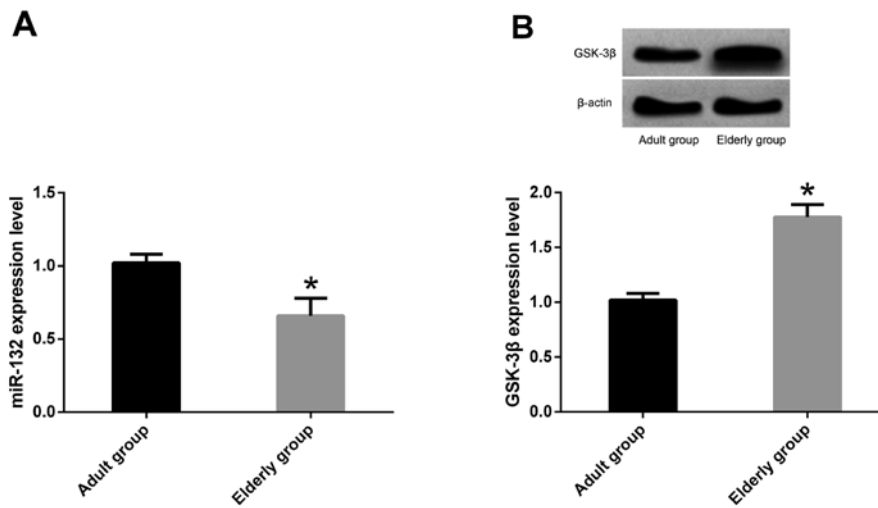


Figure 1. Expression of miR-132 and GSK-3 β in mice of different ages. (A) miR-132 was downregulated in the hippocampus of the elderly group compared with that of the adult group. (B) GSK-3 β was upregulated in the hippocampus of the elderly group compared with that of the adult group. * P <0.05. GSK-3 β , glycogen synthase kinase-3 β .

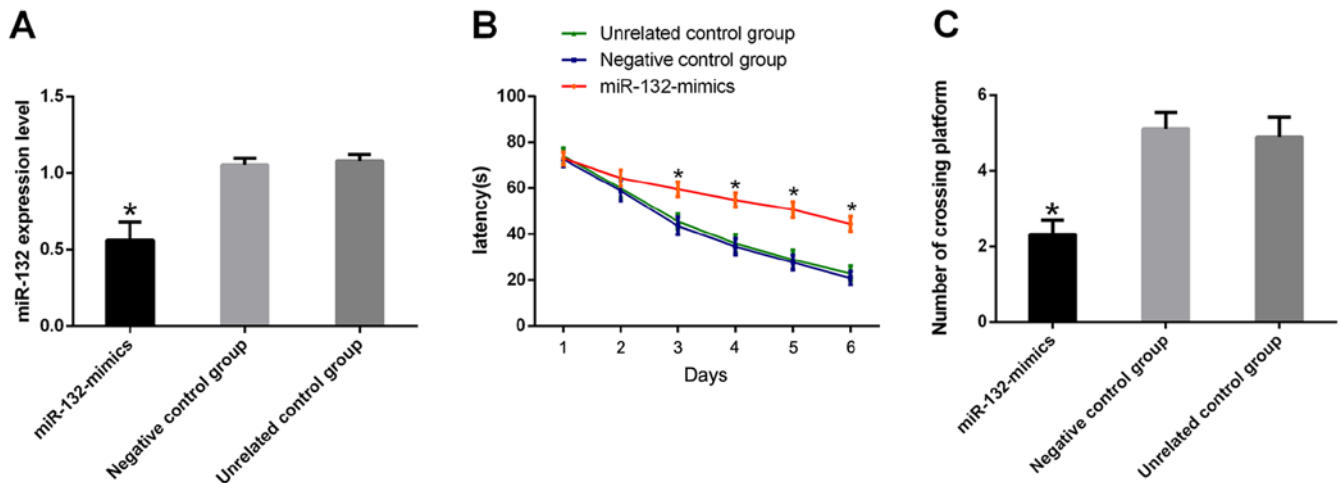


Figure 2. Effects of downregulated miR-132 on learning and memory in mice. (A) The expression of miR-132 in the hippocampus of the miR-132-inhibitor group was significantly increased compared with that of the two control groups. (B) Compared with the two control groups, the escape latency of the miR-132-inhibitor group was significantly increased. (C) Compared with the two control groups, the number of times of crossing platforms in the miR-132-inhibitor group was significantly reduced. * P <0.05.

using a dual luciferase reporter assay kit 2 days after transfection.

Statistical analysis. The data processing of the experiments was analyzed by SPSS 22.0 (IBM Corp.), and the illustrations were plotted using the GraphPad 7. Student's *t*-test was employed for pairwise comparison of the mean, while one-way ANOVA was applied for multi-group comparison of the mean. Dunnett's test was adopted for post-hoc pairwise comparison. P <0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-132 and GSK-3 β in mice of different ages. RT-qPCR showed that the expression of miR-132 in hippocampus of elderly mice was downregulated compared

with that of adult mice (P <0.05). While WB revealed that the expression of GSK-3 β in hippocampus of elderly mice was upregulated compared with that of adult mice (P <0.05) (Fig. 1).

Downregulation of miR-132 impairs learning and memory in mice. The expression of miR-132 in the hippocampus of the miR-132-inhibitor group was significantly higher than that of the two control groups (P <0.05). Morris water maze test results showed that compared with the two control groups, the miR-132-inhibitor group had a significant increase in escape latency and a significant decrease in the number of times of crossing platforms (P <0.05) (Fig. 2).

Upregulation of GSK-3 β impairs learning and memory in mice. It was found that the expression of GSK-3 β in hippocampus of sh-GSK-3 β group was significantly higher

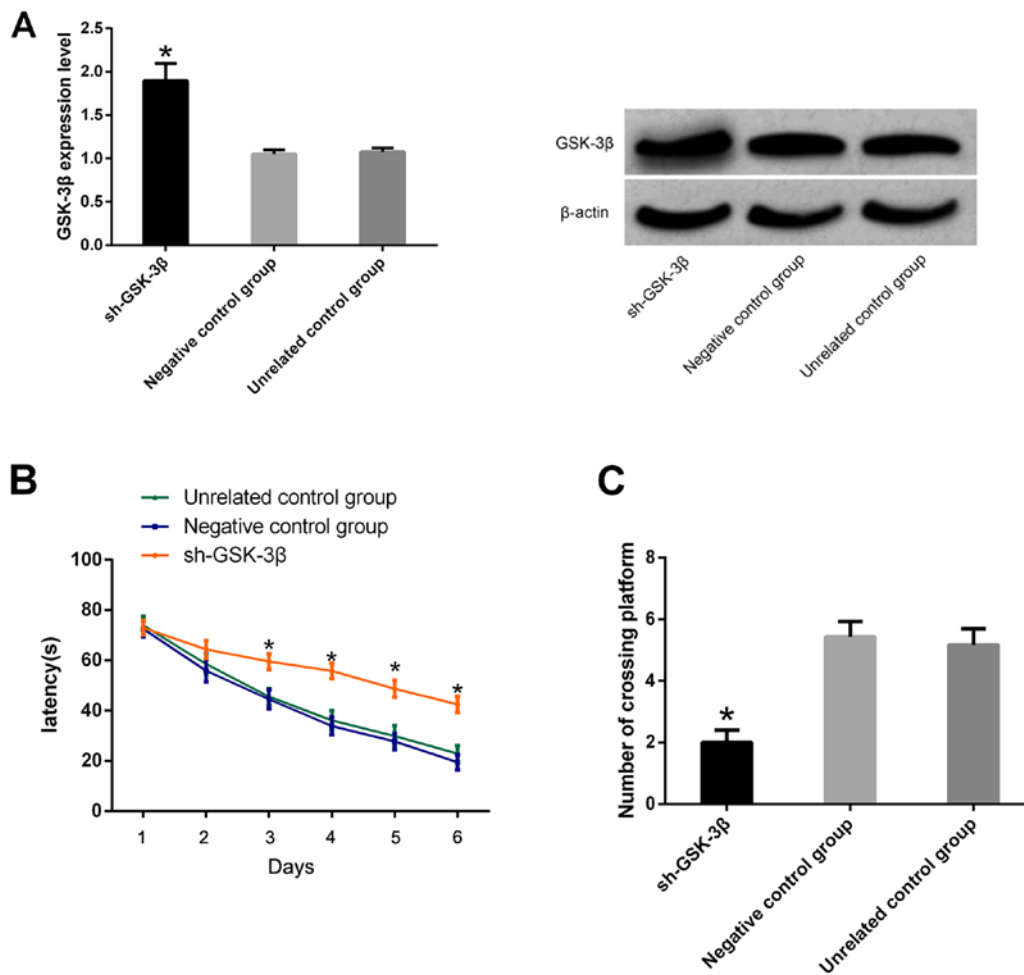


Figure 3. Effects of upregulated GSK-3 β on learning and memory in mice. (A) The expression of GSK-3 β in the hippocampus of the sh-GSK-3 β group was significantly increased compared with that of the two control groups. (B) Compared with the two control groups, the escape latency of mice in the sh-GSK-3 β group increased significantly. (C) Compared with the two control groups, the number of times of crossing platforms in the sh-GSK-3 β group was significantly reduced. *P<0.05. GSK-3 β , glycogen synthase kinase-3 β .

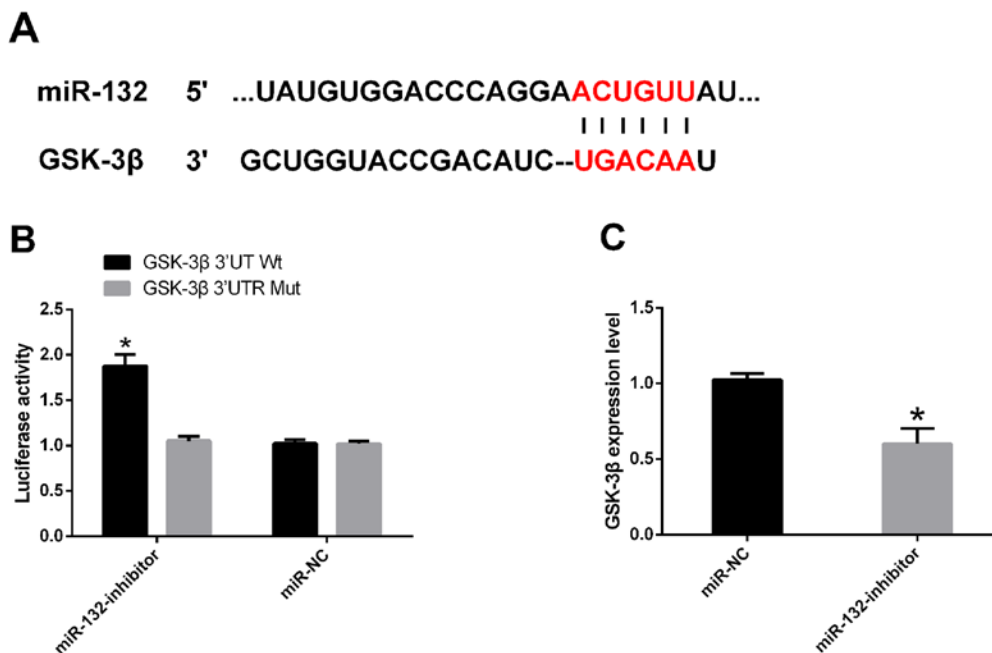


Figure 4. Detection of dual luciferase activity. (A) GSK-3 β has targeted binding sites with miR-132. (B) Inhibition of miR-132 significantly increased the luciferase activity of GSK-3 β 3'UTR Wt, but had no effect on that of GSK-3 β 3'UTR Mut. (C) Compared with cells transfected with miR-NC, GSK-3 β expression was significantly decreased in cells transfected with miR-132-inhibitor. *P<0.05. GSK-3 β , glycogen synthase kinase-3 β .

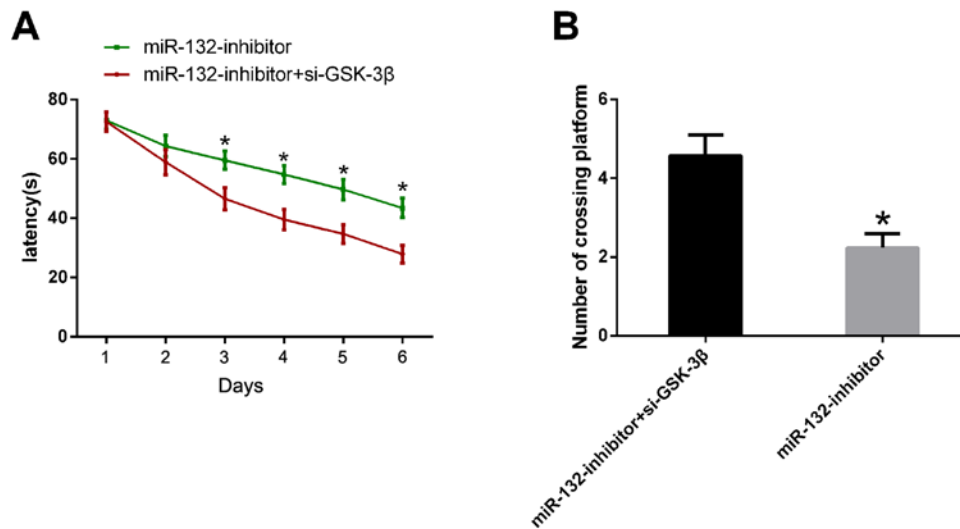


Figure 5. Downregulation of GSK-3 β reverses the decline in learning and memory in mice caused by downregulation of miR-132 expression. (A) Compared with the miR-132-inhibitor group, the escape latency of mice in the miR-132-inhibitor+si-GSK-3 β group was significantly shortened. (B) Compared with the miR-132-inhibitor group, the number of times of crossing the platform in the miR-132-inhibitor+si-GSK-3 β group increased significantly. *P<0.05. GSK-3 β , glycogen synthase kinase-3 β .

than that of the two control groups (P<0.05). Morris water maze test results indicated that compared with the two control groups, the escape latency of sh-GSK-3 β mice increased significantly and the number of times of crossing platforms declined significantly (P<0.05) (Fig. 3).

Double luciferase report. The downstream target gene of miR-132 was predicted by Targetscan 7.2, and GSK-3 β was found to have a target binding site with miR-132. The double luciferase activity test revealed that the luciferase activity of GSK-3 β 3'UT Wt was markedly elevated after miR-132 was inhibited (P<0.05), while had no effect on GSK-3 β 3'UTR Mut luciferase activity (P>0.05). WB assay showed that the expression of GSK-3 β was significantly decreased in cells transfected with miR-132-inhibitor compared with those transfected with miR-NC (P<0.05) (Fig. 4).

Downregulation of GSK-3 β reverses the decline in learning and memory in mice caused by downregulation of miR-132 expression. Adult mice were also taken as the miR-132-inhibitor+si-GSK-3 β group. After injecting miR-132 inhibitor into the hippocampus for 2 days, GSK-3 β inhibitor was further injected into mice before conducting the Morris water maze test. The test results demonstrated that the escape latency of mice in the miR-132-inhibitor+si-GSK-3 β group was significantly shortened and the number of times of crossing the platform was significantly increased compared with those in the Mi-132-inhibitor group (P<0.05) (Fig. 5).

Discussion

Learning and memory is an essential basic function of human survival. Many diseases related to neurological function can lead to the loss of learning and memory function, which leads to serious social burden. Therefore, understanding the relevant mechanism of learning and memory has been the research hotspot in the field of neuroscience. As a class of short

non-coding RNA, miRs participate in the biological functions of cell growth, proliferation and apoptosis (13). In recent years, extensive studies have demonstrated that miR is involved in neuroplasticity and memory formation, and that miR dysregulation is part of the pathophysiology of neurological diseases and neurodevelopmental disorders (14-16). Among them, miR-132 is a frequent visitor in neurological related research and has been shown to be closely related to neurodevelopment and neurosy-related diseases (17-19). Yet, how miR-132 affects learning and memory function has not been elucidated.

As miR-132 is highly expressed in hippocampal tissue (20), our study selected hippocampus as the very place to examine the expression of miR-132. It is well known that the learning and memory ability of animals gradually declines with aging, as was validated in the current study that miR-132 was down-regulated in the hippocampus of the elderly group compared with that of the adult group. Subsequently, in order to explore the role of miR-132 in learning and memory, we observed the performance of adult mice in Morris water maze test after injecting miR-132-inhibitor. The results showed that compared with the two control groups, the miR-132-inhibitor group had a significant increase in escape latency and a significant decrease in the number of times of crossing platforms, indicating that the expression level of miR-132 was closely related to the learning and memory function of mice. Previous studies have explored the relationship between miR-132 and learning and memory function. For example, studies have revealed that repeated anesthesia with propofol in neonatal rats can lead to a significant decrease in miR-132 expression and a decrease in the number of dendritic spines in the hippocampus of rats, resulting in learning and memory disorders in rats (21). Other studies have shown that knockout of miR-132 expression can significantly reduce spatial memory and learning ability in mice (22). Although here we validated that miR-132 expression disorder can cause changes in learning and memory ability, its mechanisms of action remains a mystery.

GSK-3 β was found to have target binding sites with miR-132 when predicting the downstream target gene of miR132 by TargetsCan7.2. GSK-3 β , is a multifunctional protein kinase implicated in a variety of cellular processes (23). Previous studies have shown that GSK-3 β can affect the memory learning ability of animals. For example, some studies have found that memory retrieval in the passive avoidance task activates the activity of GSK-3 β in the hippocampus of mice, and they also found that injection of GSK-3 β inhibitor into mice can impair the memory capacity, while it is without impact on memory reconstruction (24). Other studies have exhibited that inhibition of GSK-3 β activity can significantly improve cognitive dysfunction in mice caused by chronic unpredictable stress (25). While in our study, the expression of GSK-3 β was upregulated in hippocampus of elderly mice, and the decrease of GSK-3 β level could significantly increase the escape latency of mice and decrease the number of times of crossing the platform in the maze test, suggesting that the expression of GSK-3 β was closely related to the learning and memory ability of mice. Subsequently, we injected the hippocampus of mice with miR-132 inhibitor, and then with GSK-3 β inhibitor to explore the relationship between miR-132 and GSK-3 β . The results indicated that the downregulation of GSK-3 β could reverse the decline in learning and memory ability of mice caused by the downregulation of miR-132 expression, indicating a certain link between miR-132 and GSK-3 β . Therefore, double luciferase report was further performed to verify the relationship between miR-132 and GSK-3 β , and the results showed that the luciferase activity of GSK-3 β 3'UT Wt was significantly decreased after overexpression of miR-132, while without influence on the luciferase activity of GSK-3 β 3'UTR Mut, and GSK-3 β expression was significantly decreased in cells overexpressed by miR-132. From the above experimental results, we can rudimentarily prove that miR-132 overexpression can inhibit the expression of GSK-3 β and affect the learning and memory ability of mice.

In summary, this study preliminarily demonstrated that miR-132 overexpression can inhibit the expression of GSK-3 β and affect the learning and memory ability of mice, which provides some enlightenment for the learning and understanding of learning and memory disorders.

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Availability of data and materials

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

Authors' contributions

GL wrote the manuscript. GL and FY conceived and designed the study. CZ and YZ were responsible for the collection and analysis of the experimental data. CZ and XL interpreted the data and drafted the manuscript. YZ and YL revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of the First Affiliated Hospital of Bengbu Medical College (Bengbu, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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