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Original Article

The protective role of the miR-25-mediated notch signaling pathway in the memory capacity and brain tissue of mice with central nervous system infections

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Abstract: Objective: The study aimed to explore the role of miR-25 and the notch signaling pathway in the memory capacity and brain tissue of mice with central nervous system (CNS) infections. Methods: A bioinformatics website and the dual-luciferase reporter assay were used to analyze the targeting relationship between miR-25 and Notch1. The mice were randomized into 7 groups (n=10 per group), including the normal group, the model group (lipopolysaccharide at a dose of 500 μg/kg for the model establishment), the NC group, the miR-25 mimic group, the miR-25 inhibitor group, the DAPT group, and the miR-25 inhibitor + DAPT group. qRT-PCR and western blot were used to measure the miR-25, Notch1, and Hes5 expression levels in the hippocampal CA1 region of the mice's brains, along with the cyclo-oxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) levels in the mice's hippocampi. Results: Compared with the normal mice, the model mice had up-regulated miR-25, COX-2, and iNOS expressions and down-regulated Notch1 and Hes5 expressions, lower superoxide dismutase (SOD) levels in the hippocampi, and higher malondialdehyde (MDA) levels. Compared with the model group, the miR-25 mimic and DAPT groups had down-regulated Notch1 and Hes5 expressions, lower learning and memory capacities and SOD levels, higher MDA levels, and up-regulated COX-2 and iNOS expressions. Conclusion: Down-regulating miR-25 may improve the memory capacity in mice with CNS infections by activating the Notch signaling pathway.

Keywords: MiR-25, central nervous system infections, brain injuries, memory capacity, notch signaling pathway

Introduction

An estimated 50 million people worldwide suffer from dementia/Alzheimer's disease. A further 7 million have developed Parkinson's disease and more than 2 million have multiple sclerosis [1]. The incidence of such neurodegenerative diseases will continue to increase as the population ages [2]. Factors other than heredity may also be related to the deterioration of the central nervous system (CNS), hence the rise in neurodegenerative diseases. For decades, viral, bacterial, and eukaryotic parasite infections have been studied as possible triggers for neurodegeneration [3]. However, no infection has been identified as an etiology. One of the obvious consequences of an infection is an increase in the inflammatory reactions and immune responses, which may lead to cognitive impairment [4]. Although more studies are focusing on neuroinflammation and the expressions and responses of cytokines and chemokines affected by resident brain immune cells such as microglia and astrocytes, the mechanisms of these responses affecting brain cells are still unclear [5].

MicroRNA (miRNA) is a small (18 to 22 base pairs) and highly conserved non-coding RNA molecule that controls protein expression and regulates most biological processes after transcription [6, 7]. Recently, increasing evidence has suggested that different miRNAs play key regulatory roles in the pathogenesis and potential treatment of various nervous system diseases. For example, miR-212/132 regulates the incidence of epilepsy, and miR-124 and miR-9 can promote nerve development [8, 9]. Recently, a number of studies on the role of miR-25 in cancer have been published. How-

ever, little is known about the role of miR-25 in nervous system diseases [10].

The Notch pathway regulates the development of most tissues. It can regulate the differentiation, proliferation, and survival of normal and most cancer cells [11]. The output of Notch signal transduction requires a fine-tuning of the Notch target gene expression to make the cells adapt to the changes in the physiological environment. Various conditions, such as hypoxia, radiation, inflammation, and oxidative stress affect Notch activity. However, at the transcriptional level, the mechanism regulating the background-specific Notch target gene expression is still unclear [12]. Some studies have shown that Notch may protect the CNS and improve existing cognitive impairment [13]. γ-secretase is a key enzyme in the Notch signaling pathway, and it promotes the release of the Notch intracellular domain (NICD). Previous studies have documented that the introduction of DAPT, a γ-secretase inhibitor, may effectively inhibit the activity of γ-secretase, keeping it from hydrolyzing the Notch receptor into NICD and therefore block the downstream gene expression [14].

Through a bioinformatics analysis, we found that there is a targeted relationship between miR-25 and Notch1, a receptor of Notch. Thus we speculated that miR-25 may down-regulate the expression of Notch-1, thereby inhibiting the Notch signaling pathway, and promoting the development of immune inflammation to cause brain tissue damage. Based on this hypothesis, we studied the role of miR-25 expression and its inhibition of Notch signaling and its subsequent effect in brain tissue protection.

Materials and methods

Experimental grouping and processing

A total of 70 healthy, male C57BL/6 mice (30 to 40 g, 2 weeks old, clean grade) were obtained for this study from the animal center of The Affiliated Hospital of Guizhou Medical University. The experiment was performed in accordance with the ARRIVE guidelines and was approved by the ethics committee of The Affiliated Hospital of Guizhou Medical University.

The mice were randomized into the normal group (n=10) and the model group (n=60). In the model group, lipopolysaccharide at a dose of 500 μg/kg (Beijing Solarbio Science & Technology Co., Ltd., China) was injected into the brain's lateral ventricle of each mouse to establish the mouse model with CNS infection [15]. After we established the model, the mice were divided into 7 groups (n=10 per group), including the normal group (no treatment), the model group (no treatment), the NC group (injected with the NC vector), the miR-25 mimic group (injected with the miR-25 mimic), the miR-25 inhibitor group (injected with the miR-25 inhibitor), the DAPT group (Notch signaling pathway inhibited by injection of the γ-secretase inhibitor DAPT), and the miR-25 inhibitor + DAPT group (injected with the miR-25 inhibitor and DAPT). The miR-25 mimic, the miR-25 inhibitor, and the DAPT were purchased from the MedChemexpress Limited Liability Company.

The mice were anesthetized using 2% pentobarbital acid (Shanghai Hongshun Biotechnology Co., Ltd., China) on the second day of the modeling. With the help of a dental drill, the 3 mM corresponding reagent was injected perpendicularly to the dorsal longitudinal axis of the hippocampal dentate gyrus of the mice in each group once a week, for 3 times in total. The mice were tested in the Morris water maze 1 h after the final injection, and then the mice were sacrificed by amputating their tails. Fresh brain resection samples were used for biochemical analyses, and the hippocampal tissue the of mice in each group was taken for expression analyses. The hippocampal tissue was preserved in liquid nitrogen for future analyses.

Dual-luciferase reporter system assay

A biological prediction website (www.targetscan.org) was used to analyze the binding sites of miR-25 and Notch1. The relationship between miR-25 and Notch1 was verified using a dual-luciferase reporter system assay. PGL3-Notch1 wild-type (the dual-luciferase reporter gene vector of the target gene Notch1) and the PGL3-Notch1 mutant (the dual-luciferase reporter gene vector of miR-25 with the mutation on the binding site), were constructed respectively. A *Rellina* plasmid (Thermo Fisher

Table 1. qRT-PCR primer sequences

Name	Sequences
miR-25	F: 5'-CAGTGTTGAGAGGCGGAGACT-3'
	R: 5'-GCACTGTCAGACCGAGACAAG-3'
Notch1	F: 5'-TGCCAGTATGATGTGGATGAG-3'
	R: 5'-GGTCCCTGTGTAACCTTCTGT-3'
Hes5	F: 5'-AGTCCCAAGGAG AAAAACCGA-3'
	F: 5'-GCTGTGTTTCAGGTAGCTGAC-3'
COX-2	F: 5'-TTCCAATCCATGTCAAAACCGT-3'
	R: 5'-TGCACATTGTAAGTAGGTGGAC-3'
iNOS	F: 5'-GTTCTCAGCCCAACAATACAAGA-3'
	R: 5'-CAGAGGGGTAGGCTTGTCTC-3'
U6	F: 5'-CTCGCTTCGGCAGCACATATACT-3'
	R: 5'-ACGCTTCACGAATTTGCGTGTC-3'
GAPDH	F: 5'-CCAATGTGTCCGTCGTGGATCT-3'
	R: 5'-GTTGAAGTCGCAGGAGACAACC-3'

Note: COX-2: cyclo-oxygenase 2; iNOS: inducible nitric oxide synthase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

Scientific Inc., USA) and two reporter plasmids were co-transfected with the miR-25 plasmid or the NC plasmid into the HEK 293T cells, respectively. The dual-luciferase assay was performed 24 h after the transfection experiment using a dual-luciferase reporter kit (Promega, Beijing, China).

Quantitative reverse transcription PCR (qRT-PCR)

The total RNA of the brain tissue was extracted using Trizol (Thermo Fisher Scientific Inc., USA). cDNA was synthesized using TaqMan MicroRNA Assays with Reverse Transcription Primers. The SYBR® PremixExTaq™ II kit (Guangzhou Peiyu Biological Products Co., Ltd., China) was used to carry out the fluorescence quantitative PCR in a 10 μL system: 2.2 μL of ddH_2O, 0.4 μL of PCR upstream and downstream primers, 5 μL of SYBR® PremixExTaq™ II (2×), 1 μL of ROX Reference Dye (50×), and 1 μL of DNA template. The fluorescence quantitative PCR was conducted in a BIORAD system. The reaction conditions were as follows: pre-denaturation at 95°C for 5 min, denaturation at 95°C for 15 s, annealing at 60°C for 1 min, and extension for 1 min at 60°C, for a total of 40 cycles. U6 was used as an internal reference for miR-25. GAPDH was used as the internal reference for the other genes. $2^{-\Delta\Delta Ct}$ was used to express the relative expression of each target gene (**Table 1**).

Western blot

The total protein was extracted from the RIPA lysate containing PMSF (R0010, Solarbio, Beijing, China). The BCA kit (Thermo Fisher Scientific Inc., USA) was used to measure the protein concentration. The sample was mixed with a sample buffer and incubated in boiling water for 10 min. Protein samples of 30 μg each were added to each well and electrophoresed at 80 V constant current for 2 h. The proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane (ISEQ00010, Millipore, Billerica, MA, USA) at a voltage of 110 V for 2 h. Skim milk (5%; Wuhan Servicebio Technology Co., Ltd., China) was used to block the proteins on the PVDF membrane at 4°C for 2 h. The blocking buffer was discarded, and the membrane washed using Tris-Buffered Saline and Tween 20 (TBST) once. Subsequently, rabbit anti-mouse Notch1 (ab8925, 1:1,000, Abcam, UK), Hes5 (ab194111, 1:10,000, Abcam, UK), cyclo-oxygenase 2 (COX-2; ab15191, 1:1,000, Abcam, UK), inducible nitric oxide synthase (iNOS; ab213987, 1:1,000, Abcam, UK), GAPDH (ab8226, 1:2,000, Abcam, UK) were added and incubated at 4°C overnight. Then HRP-labeled goat anti-rabbit IgG antibody (Beijing Zhongshan Biotechnology Co., Ltd., 1:5,000 dilution) was added and incubated for 2 h. After the TBST rinsing, the membrane was developed using an ECL detection kit (No. BB-3501, Amersham, UK). The Bio-Rad image analysis system (BIORAD, USA) was used for the image acquisition. Image J software was used to analyze the data.

Morris water maze

The Morris water maze is a circular pool of water (150 cm diameter * 60 cm high), kept at a temperature of 20-25°C. The circular pool was divided into four quadrants: lower right, upper right, lower left, and upper left. A platform was installed on the lower right quadrant. After 7 days of training, the mice were put in the water to record the time they took to search for the platform (their escape latency). If the mice found the platform within 2 min, the actual escape latency was recorded. Otherwise, the experimenter led them to the platform and let them stay for 10 s, and the escape latency was recorded as 2 min. On the 5th day, the underwater platform was removed, and a space exploration experiment was carried out: the

entry point was fixed, and the staying time in the original platform quadrant (the target quadrant) and the number of times the mice traveled through the original platform were recorded. Long staying times and a higher number of cycles indicate strong learning and memory abilities in the mice.

Determination of the superoxide dismutase (SOD) and malondialdehyde (MDA) levels in the hippocampal tissue

The SOD levels in the hippocampal tissue were measured using WST-1 assays, and the MDA level was measured using thiobarbituric acid assays. The hippocampal tissue was cut into small pieces of about 1 mm^3, and then digested with trypsin (Wuhan Servicebio Technology Co., Ltd., China) at 37°C for 30 min. PBS was used to terminate the digestion. A nylon 300-micron mesh screen was used for the filtration. The cells were collected by centrifugation at 1,000 rpm for 10 min. The estimation of the SOD (A001-3-2, Nanjing Jiancheng Biology Engineering Institute) and MDA (A003-1-2, Nanjing Jiancheng Biology Engineering Institute) levels was carried out in strict accordance with the kit instructions.

Statistical analysis

All the data were analyzed using SPSS 21.0 statistical software. The measurement data were represented as the mean ± standard deviation ($\bar{x}$ ± sd). The multiple group comparisons were performed using one-way analyses of variance, followed by Tukey's post-hoc test for the comparison of the pairwise mean values. $P<0.05$ showed that the differences were statistically significant.

Results

miR-25 targeting negatively regulates notch1 gene

A biological prediction website (www.targetscan.org) was used to predict the specific miR-25 and Notch1 binding sites (**Figure 1A**). The luciferase activity of the Wt-Notch1 co-transfection in the miR-25 mimic group was significantly lower than it was in the NC mimic group ($P<0.05$), but there was no significant change in the luciferase activity of the mutant Mut-Notch1 plasmid between two groups ($P>0.05$; **Figure 1B**). Therefore, miR-25 can negatively regulate the expression of the Notch1 gene.

The expressions of the related factors in the hippocampi of the mice in each group were measured to further clarify the regulatory relationship between miR-25 and the Notch signaling pathway. Compared with the normal group, the miR-25 expression levels in the mice hippocampi in the other groups were significantly lower, and the Notch1 and Hes5 mRNA and protein expressions were significantly increased ($P<0.05$). There was no significant difference in both the Notch1 and Hes5 gene expressions between the NC group and the model group ($P>0.05$). However, compared with the model group, the miR-25 mimic group had a significantly increased expression level of miR-25, and the miR-25 inhibitor group showed the opposite. Compared with the model group, the miR-25 mimic group and the DAPT group had significantly decreased Notch1 and Hes5 expression levels, but the miR-25 inhibitor group had significantly higher Notch1 and Hes5 expression levels ($P<0.05$). Compared to the miR-25 inhibitor + DAPT group, the Notch1 and Hes5 mRNA expression levels in the miR-25 inhibitor group were significantly higher, but those in the DAPT group were significantly lower ($P<0.05$; **Figure 1C-E**).

Mouse learning and memory capacity

The results of the Morris water maze test (**Figure 2**) showed that compared with the normal group, the latency times of the mice in the other groups were significantly longer, and the number of times the mice traversed the platform was significantly less ($P<0.05$). Compared to the model group, the NC group and the miR-25 inhibitor + DAPT group showed no significant differences ($P>0.05$). Compared to the model group, the latency times were significantly longer, and the number of total platform traverses of the platform was significantly less in the miR-25 mimic group or the DAPT group ($P<0.05$), but the opposite results were seen in the miR-25 inhibitor group ($P<0.05$). Compared with the miR-25 inhibitor group, the latency times were longer in the miR-25 inhibitor + DAPT group but the traversing number was less. However, the latency times were significantly shorter along with a higher traversing

A

	Predicted consequential pairing of target region (top) and miRNA(bottom)	Site type	Context++ score
Position 1576-1582 of NOTCH1 3'UTR	5' ...UUUUGUUAACUAAUUUGCAAUAA...	7mer-A1	-0.16
mmu-miR-25-3p	3' AGUCUGGCUCUGUUCACGUUAC		

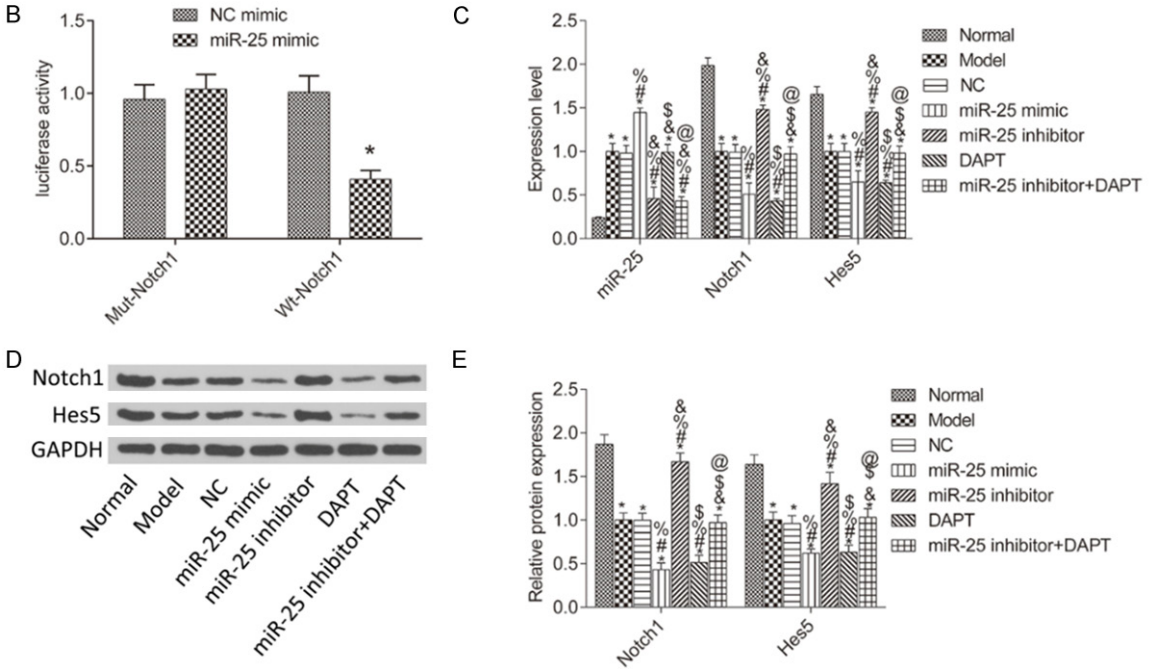

Figure 1. miR-25 inhibits the Notch signaling pathway in mice's hippocampi. A: The sequence of the 3'-UTR region of miR-25 binding to Notch1; B: A dual-luciferase assay detecting luciferase activity; *P<0.05, compared with the NC mimic group; C: A statistical map of the miR-25, Notch1, and Hes5 mRNA levels in the mice's hippocampi; D: A protein band map of Notch1 and Hes5 in the mouse hippocampus; E: A protein level map of Notch1 and Hes5 in the mice's hippocampi; *P<0.05, compared with the normal group, #P<0.05, compared with the model group, %P<0.05 compared with the NC group; &P<0.05, compared with the miR-25 mimic group; $P<0.05 compared with the miR-25 inhibitor group; @P<0.05, compared with the DAPT group, n=5. NC: negative control; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

number in the miR-25 inhibitor + DAPT group compared to the DAPT group (P<0.05).

The SOD and MDA levels in the mice's hippocampi

Compared to the normal group, the SOD levels in the hippocampal CA1 region of the mice in the other groups were lower, but the MDA levels were higher (P<0.05; **Figure 3**). Compared with the model group, however, neither the NC group nor the miR-25 inhibitor + DAPT group had significant differences in their SOD and MDA levels (P>0.05). When compared with the model group, the SOD level was significantly lower but the MDA level was significantly higher in the miR-25 mimic group and the DAPT group (P< 0.05), but the SOD and MDA levels were significantly higher and lower, respectively, in the miR-25 inhibitor group (P<0.05). The miR-25 inhibitor + DAPT group showed a significantly lower SOD level but a significantly higher MDA level than the miR-25 inhibitor group. The SOD level was significantly higher, but the MDA level was significantly lower in the miR-25 inhibitor + DAPT group compared to the DAPT group (P<0.05).

The COX-2 and iNOS mRNA and protein expressions in the hippocampal CA1 region of the mice

The COX-2 and iNOS mRNA and protein expression levels in the hippocampal CA1 region of the mice in the other groups were significantly higher than they were in the normal group

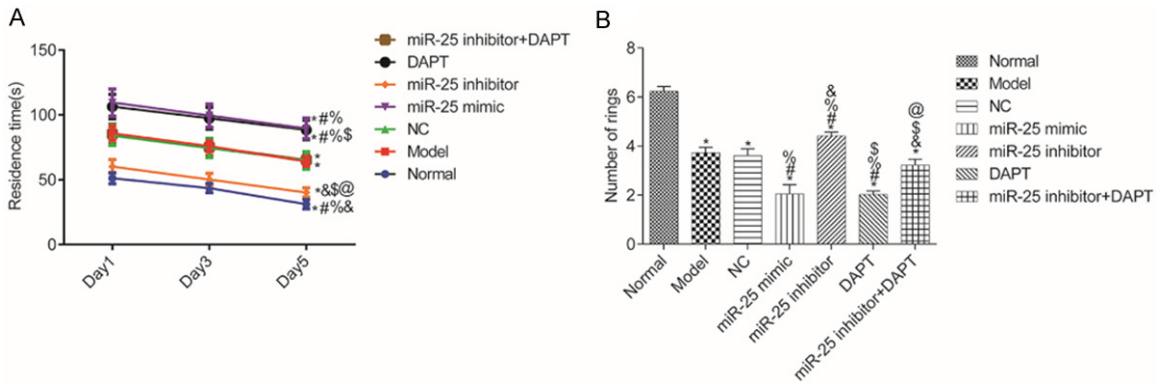

Figure 2. Mouse learning and memory capacities. A: Latency times of the mice in the Morris water maze test; B: The traversing number of the mice in the Morris water maze test. Compared with the normal group, *P<0.05; compared with the model group, #P<0.05; compared with the NC group, %P<0.05; compared with the miR-25 mimic group, &P<0.05; compared with the miR-25 inhibitor group, $P<0.05; compared with the DAPT group, @P<0.05 (n=10). NC: negative control.

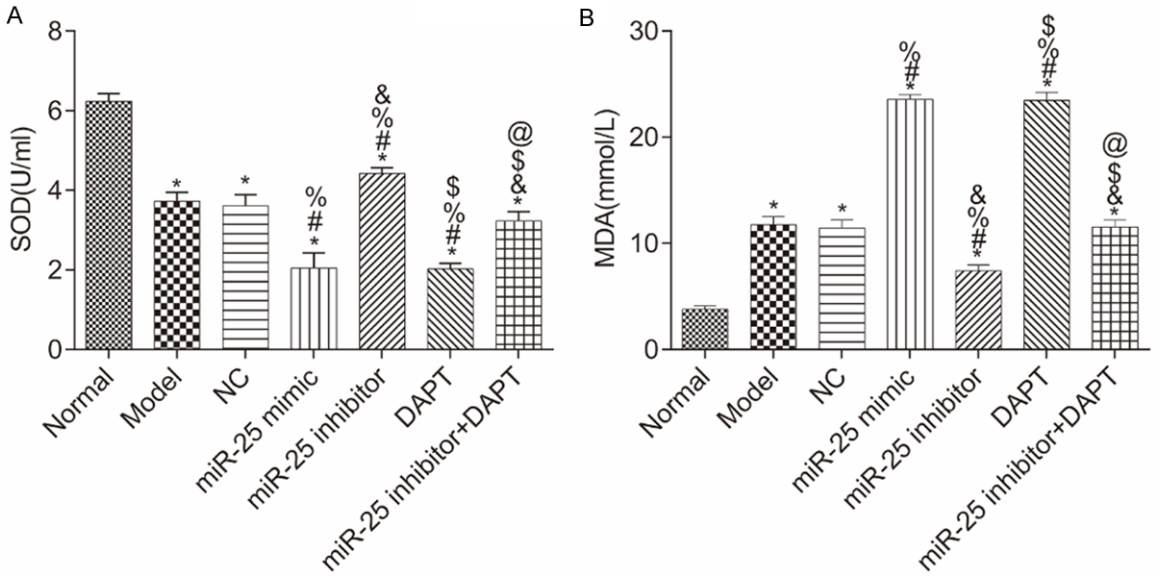

Figure 3. The mouse learning and memory capacity levels of SOD and MDA in the CA1 region of the hippocampi in the mice. A: SOD in the CA1 region of hippocampus; B: MDA in the CA1 region of the hippocampus. Compared with the normal group, *P<0.05; compared with the model group, #P<0.05; compared with the NC group, %P<0.05; compared with the miR-25 mimic group, &P<0.05; compared with the miR-25 inhibitor group, $P<0.05; compared with the DAPT group, @P<0.05 (n=10). NC: negative control; SOD: superoxide dismutase; MDA: malondialdehyde.

(P<0.05; **Figure 4**). There was no significant difference in the COX-2 or iNOS levels between the NC group and the model group, nor was there a significant difference between the miR-25 inhibitor + DAPT groups and the model group (P>0.05). Compared with the model group, the mRNA and COX-2 and iNOS protein expression levels were significantly higher in the miR-25 mimic and the DAPT groups (P< 0.05), but they were significantly lower in the miR-25 inhibitor group (P<0.05). The COX-2 and iNOS mRNA and protein expression levels in the miR-25 inhibitor + DAPT group were significantly higher than they were in the miR-25 inhibitor group, but their expressions in the miR-25 inhibitor + DAPT group were significantly lower than they were in the DAPT group (P<0.05).

Discussion

CNS infection lurks in the occurrence of neurodegenerative diseases, and its development is associated with the symptoms of Alzheimer's

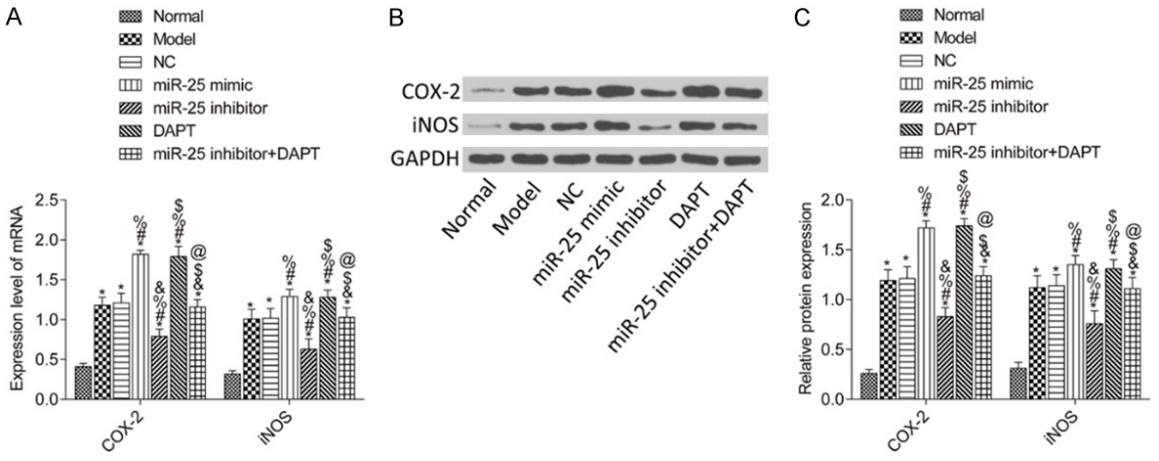

Figure 4. The COX-2 and iNOS mRNA and protein expressions in the hippocampal CA1 region of the mice. A: The mRNA COX-2 and iNOS mRNA expression levels in the hippocampal CA1 region of the mice; B: The protein band map of COX-2, iNOS, mRNA, and GAPDH in the hippocampal CA1 region of the mice; C: The protein expression levels of COX-2 and iNOS mRNA in the hippocampal CA1 region of the mice. Compared with the normal group, *P<0.05; compared with the model group, $^{\#}$P<0.05; compared with the NC group, $^{\%}$P<0.05; compared with the miR-25 mimic group, $^{\&}$P<0.05; compared with the miR-25 inhibitor group, $^{\$}$P<0.05; compared with the DAPT group, $^{@}$P<0.05 (n=10). NC: negative control; COX: cyclooxygenase; iNOS: inducible nitric oxide synthase; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

disease, Parkinson's disease, multiple sclerosis, and steady-state aging diseases [16-18]. Neuroinflammation is a biological response to physiological disturbances, and CNS coordinates a series of immune responses in order to restore homeostasis [19]. However, these inflammatory responses persist in neurodegenerative diseases, leading to uncontrolled oxidative stress, which is harmful to the tissue, and thereby, promotes the destruction of the blood-brain barrier and induces subsequent brain tissue injury [20-24]. At present, the molecular protection mechanisms operating in the brain tissue in CNS infections still remain unclear.

Some studies have suggested that the expression of Notch1 decreased when there was cerebral ischemic injury [25]. DAPT, an inhibitor of the Notch signaling pathway, was used to treat healthy male C57BL/6 mice in this study. We found that the inhibition of the Notch signaling pathway can aggravate brain injuries in mice with CNS immune inflammation associated with cognitive impairment. To further explore the regulatory mechanisms operating upstream of the Notch signaling pathway, a bioinformatics analysis was conducted and miR-25 was screened out. A dual-luciferase assay also confirmed that miR-25 negatively regulated the Notch1 expression. Then, in our study, the mice models were injected with an miR-25 mimic, an miR-25 inhibitor, and an miR-25 inhibitor + DAPT, respectively. The results showed that an overexpression of miR-25 could aggravate brain injuries in mice with CNS immune inflammation associated with cognitive impairment. Therefore, we speculated that miR-25 inhibited the Notch signaling pathway by targeting the expression of the Notch1 gene, thereby downregulating the expression of Hes5 and decreasing learning ability and memory capacity.

Previous studies on miR-25 did not involve CNS infections. Thus, importantly, miR-25 was evaluated for nervous system disease in this study. Our results showed that the SOD level decreased, but the MDA level increased and the COX-2 and iNOS expression levels were increased. This indicates that the inhibition of the Notch signaling pathway blocks the protective effect of miR-25 silencing on brain injury. These observations are consistent with previous studies, which underline the protective effect of the Notch signaling pathway on the cognitive function in mice with brain injuries as well as on oxidative damage, indicating that the Notch signaling pathway may also be a key factor in regulating oxidative stress injuries [26-30].

However, the relationship between miR-25 and CNS diseases has not been fully elucidated as

of yet. The molecular mechanism of miR-25 inhibition on Notch1 has also not been clearly explored. Moreover, to establish the targeted regulatory network of miR-25 in CNS diseases, there is still a long way to go, and more clinical studies are required to supplement this data and to further confirm the above results.

The present study demonstrated that miR-25 inhibits the recovery of brain injury in a mice model by targeting the Notch1 gene-mediated Notch signaling pathway. It further clarified the mechanism of CNS disease development and laid a theoretical foundation for the treatment of clinical CNS diseases.

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Disclosure of conflict of interest

None.

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