MicroRNA-195 rescues the impaired MS-dCA1 neural circuit and spatial memory in rats with chronic cerebral hypoperfusion

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Abstract

Chronic cerebral hypoperfusion (CCH) is a common risk factor for vascular dementia and Alzheimer’s disease (AD). The previous studies have shown that CCH-induced multiple AD-like pathological changes in the hippocampus and cortex through downregulating the microRNA-195 (miR-195) expression. However, whether and how miR-195 participates in the dysfunction of the medial septum (MS)-dorsal hippocampal CA1 (dCA1) neural circuit following CCH is still obscure. In the present study, we found that miR-195 was downregulated in the MS region of CCH rats. Moreover, using electrophysiological recording and immunofluorescence staining technique, we found that the knockdown of miR-195 through the injection of lentiviral-AMO-195 into the MS region led to a pathological change that mimicked the damage to the MS-dCA1 neural circuit in CCH rats, including the decreased input-output (I/O) curve, increased paired-pulse ratio (PPR), decreased numbers of ChAT+ and PV+ neurons in MS, and diminished theta rhythm in the hippocampus. More importantly, exogenously supplemented miR-195 into the MS region rescued the damaged neural circuit in CCH rats by injecting lenti-pre-miR-195. Gain-of-function of miR-195 in the MS region significantly improved the cognitive dysfunction in CCH rats assessed by the Morris water maze test. In conclusion, knockdown of miR-195 in the MS region can impair MS-dCA1 neural circuit function, while upregulation of miR-195 can rescue the impaired function of MS-dCA1 neural circuit and spatial memory ability in CCH rats. This provides a valuable reference for future anti-dementia therapy involving miR-195.

Keywords: Chronic cerebral hypoperfusion; MicroRNA-195; Medial septum-dorsal hippocampal CA1 neural circuit

1. Introduction

With the increasing incidence of cerebrovascular diseases and the aggravation of population aging, vascular dementia (VaD) has become the most common type of dementia after Alzheimer’s disease (AD)[1]. VaD is a clinical syndrome of cognitive decline caused by cerebral hypoperfusion due to various ischemic or hemorrhagic cerebrovascular diseases[2]. It is well known that chronic cerebral hypoperfusion (CCH), which usually
occurs before mild cognitive impairment (MCI), is an early clinical stage shared by both AD and VaD[13]. The previous studies have shown that CCH leads to cognitive decline and induces a series of AD-like pathological changes[14-18], such as Aβ deposition, tau hyperphosphorylation, neuronal death, microglial polarization, and synaptic dysfunction in the hippocampus and cortex[19-24]. Furthermore, our recent study found that CCH also impairs the function of the medial septum (MS)-dorsal hippocampal CA1 (dCA1) (MS-dCA1) neural circuit and reduces hippocampal presynaptic neurotransmitter release in rats[10]. However, the mechanism remains unknown.

There are highly interconnected cholinergic, GABAergic, and a small number of glutamatergic neurons in the MS region of the basal forebrain that can project to glutamatergic pyramidal neurons and GABAergic interneurons in the dCA1 region. This process can modulate the excitability of hippocampal dCA1 neurons[25-27]. The MS-dCA1 neural circuit is essential for various behavioral situations because it controls the rhythm of hippocampal electrical activity by regulating the release of acetylcholine (ACh) in the hippocampus[28]. Importantly, it has been found that the degeneration of nerve fibers in the MS-dCA1 circuit occurs earlier than neuronal death in AD or VaD patients[29]. This phenomenon suggests that activation of MS-dCA1 neural circuit may be a good strategy for treating CCH-induced cognitive impairment.

Clinically, cholinesterase inhibitors are the only class of drugs that treat early AD by blocking hippocampal ACh degradation and provide temporary symptom relief. Finding a drug targeting on multi-targets of AD pathologies has become a consensus. The main characteristic of microRNAs (miRNAs) is that they regulate protein expression at the post-transcriptional level by targeting multiple genes[30-32]. Many miRNAs have been found to participate in AD pathological process by regulating classical amyloid precursor protein (APP) and β-site APP cleaving enzyme 1 (BACE1) expressions[33-35]. Of note, it has been reported that miR-195 is involved in various AD-like pathologies induced by CCH through regulating the expression of multiple proteins, such as APP/BACE1, as well as the N-APP/DR6/caspase pathway and the CX3CL1/ CX3CR1 signaling pathway[36-38], and protected against ApoE4-induced cognitive abnormalities and lysosomal deficiencies in mice[39]. Furthermore, miR-195 attenuates cerebral ischemia-reperfusion injury through the Pten-AKT and KLF5-mediated JNK signaling pathway in rats with ischemic stroke[40,41]. Recently, Cheng et al. reported that miR-195 could also protect against ischemic and hemorrhagic stroke by promoting the neurovasculature and promoting neurogenesis[42]. However, it is unclear whether and how miR-195 modulates the function of MS-dCA1 neural circuit under CCH condition.

Here, using the in vivo electrophysiological recording and neurotracing technique, we demonstrated for the 1st time that CCH induced the downregulation of miR-195 expression in the MS region and inhibited hippocampal rhythmogenesis in the dCA1 region by reducing the release of ACh. The mechanism is that miR-195 leads to the loss of cholinergic and GABAergic neurons in the MS region. Upregulation of miR-195 expression can rescue MS-dCA1 neural circuit damage and decline of spatial memory in CCH rats. Collectivity, the present data provide further evidence that miR-195 may be a valuable candidate for the miRNA-based treatment of dementia.

2. Materials and methods

2.1. Animals

Adult male Sprague-Dawley (SD) rats (250–280 g) were bought from the Animal Center of the Second Affiliated Hospital of Harbin Medical University, Harbin, Heilongjiang Province, China. The rats were accommodated in a rearing room at 24°C, 55% humidity, and a 12-h dark/light cycle with adequate food and water. All animal experiments were approved by the Ethics Committee of Harbin Medical University (Approval No: IRB3199620). All procedures conformed to Directive 2010/63/EU of the European Parliament.

2.2. Permanent bilateral common carotid artery occlusion

The surgical procedure of 2VO rats, which was conducted according to our published work, is briefly described as follows: Rats were anesthetized with chloral hydrate (300 mg/kg) and maintained with isoflurane (0.5–1.0%). The bilateral common carotid arteries of rats were ligated with 5-0 sterile silk thread, and the arteries were cut with ophthalmic scissors 5 min later. The incision was sutured and disinfected at the end of the procedure. After the rats were fully awake, they were put into home cages and kept for another 8 weeks for all subsequent experiments[7].

2.3. Construction of lentivirus vectors

The procedure for constructing lentivirus vectors has also been described in our previous work[7]. Lenti-pre-miR-195, lenti-AMO-195, and lenti-NC were created by GeneCopoeia Inc. (Rockville, MD, USA).

2.4. Stereotactic brain injection procedure

The rats were intraperitoneally injected with 10% choral hydrate after fasting for 8 h. T-shaped incision was made in the rat scalp, and the periosteum was peeled off to
expose herringbone and sagittal suture. The coordinate of the injection site located in MS was referenced by the rat brain atlas as follows: Anterior-posterior (AP), 0.6 mm; mediolateral (ML), 0.1 mm; and dorsal-ventral (DV), 6.0 mm. After drilling a hole in the skull of MS, microsyringes containing lenti-pre-miR-195, lenti-AMO-195, and lenti-NC were fixed to the stereotaxic instrument and injected into the MS region of rats. The injection rate was set to 1.5 μL/min. The needle was pulled out slowly after 5 min to prevent the leakage of lentivirus from tissue. The holes in the skull were filled with bone wax. Then, the scalp incisions of the rats were sutured. After surgery, the rats were placed in home cages and irradiated with a warm light source until they were awake.

### 2.5. Neuro-retrotracing technique

Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate. Then, holes were drilled in the dCA1 (AP, −3.84 mm; ML, ±2.1 mm; DV, 2.7 mm) and DG (AP, −3.84 mm; ML, ±2.1 mm; DV, 3.3 mm) areas of the hippocampus bilaterally according to the rat brain atlas. The green retrobeads were injected at a rate of 0.25 μL/min in each injection site. The needle was pulled out slowly after 5 min to prevent the green retrobeads from spreading. Next, the holes in the skull were filled with bone wax and the scalp incisions were closed with 5-0 medical sutures. The subsequent experiments were performed 7 days later.

### 2.6. Electrophysiological recording in vivo

Following urethane anesthesia (1.2 g/kg) treatments, the rats were put on the stereotaxic frame device (DW-2000, Chengdu Taimeng Software Company Limited, China) for the implantation of electrodes. The rats were put on a heating pad to keep their body temperature at 37°C. Bipolar stimulating electrodes (stainless steel, 0.5 mm) were implanted into the MS area (AP, 0.6 mm; ML, 0.1 mm; and DV, 6.0 mm). The recording electrodes stuffed by 3 mol/L of NaCl were implanted into the pyramidal cell layer of the dCA1 (AP, 3.8 mm; ML, 2.4 mm; and DV, 2.7 mm). To get the best results, specific positions of the stimulating and recording electrodes were varied[28,29]. MS was activated by constant controlled pulses from the BL-420S stimulus generator after an electrode implantation recovery period. A ME-1 preamplifier was used to amplify the field excitatory post-synaptic potential (fEPSP) obtained in hippocampal dCA1 area. The signals were recorded by the BL-420S device after being filtered at 1 Hz–1 kHz and digitalized at 20 kHz. To set up the input-output (I/O) curves, electric stimulation with intensity ranging from 1 to 20 V was applied in the MS. The amplitude and slope of fEPSP were expressed as the average of two responses under each stimulation intensity.

The paired-pulse ratio (PPR) was recorded using the stimulation intensity matching to the 50% peak amplitude of fEPSP. A 5 ms step was used to set the interstimulus interval. The interstimulus interval was set between 20 and 70 ms. The amplitude of the second pulse was divided by the first pulse to calculate the PPR.

### 2.7. Local field potential (LFP) recording

The LFP of hippocampal dCA1 was recorded as the previous studies[30,31]. In brief, rats were anesthetized by intraperitoneally injecting 20% urethane solution to the rats placed on a stereotaxic frame. The animals were maintained at a level of anesthesia at which spontaneous theta rhythm was not presented but could be elicited by a tail pinch. LFPs were recorded by implanting a monopolar tungsten electrode into the hippocampal dCA1 pyramidal cell layer (AP, 3.8 mm; ML, 2.4 mm; and DV, 2.7 mm). The reference electrode was placed on the skull 2 mm away from the recording electrode. The ground electrode was clamped to the scalp. The LFP of hippocampal dCA1 was amplified by a ME-1 preamplifier (Chengdu Taimeng Software Company Limited, China), and then, the signals were filtered at 0–20 Hz and recorded by BL-420S system. After the LFP waveform became stable, the basic spontaneous LFP was recorded for 2 min. Subsequently, the vicinity of tail base was gently pinched with a metal plastic clamp for 1 min to induce theta rhythm. After recording procedure, the rats were executed for brain tissue or perfused for brain extraction.

### 2.8. Real-time polymerase chain reaction (PCR)

Utilizing the Trizol reagent to separate the total RNA from the basal forebrain regions, TaqMan MicroRNA reverse transcription kit was used to perform reverse transcription (Applied Biosystems, Carlsbad, CA, USA). The SYBR green core reagent kit’s instructions for real-time PCR were followed (Applied Biosystems). Settings for the reactions were 95°C for 10 min, 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s, for a total of 40 cycles. In this experiment, all quantitative PCR data were used to calculate the relative content of target genes by the 2-ΔΔCt method. ΔCt = each group (Ct target gene-Ct housekeeping gene), ΔΔCt = experimental group ΔCt - control group ΔCt.

### 2.9. Western blot

Total hippocampal proteins were extracted, and the protein content was determined by the BCA Protein Assay Kit. Protein samples fractionated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred onto nitrocellulose membranes...
and incubated with the primary antibodies anti-choline acetyltransferase (ChAT; Catalog. #297013, 1:1,000, Synaptic Systems) and β-actin (Catalog. #8432, 1:1,000, Santa Cruz). The membranes were then incubated with fluorescent secondary antibody. The protein bands were captured using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA) and quantified using the Odyssey V3.0 software.

2.10. Assessment of ACh concentration

The guidelines were followed to produce the dorsal hippocampus samples, which were then utilized for the ELISA assay. To obtain the supernatant, frozen tissues were homogenized in pre-chilled phosphate-buffered saline (PBS) and centrifuged at 3000 rpm/min for about 20 min. Following extraction, the concentrations of ACh were assessed using the ACh ELISA Kit (Catalog #MB5774123, MyBioSource) in accordance with the manufacturer’s instructions. Then, the standard curve’s linear regression equation was fitted using the concentration of the standard sample and the associated optical density (OD) value, and ACh concentration of the test sample was computed using this equation (y=aln(x)+b).

2.11. Immunofluorescence detection

Rats were infused with 0.9% sodium chloride (NaCl) and 4% buffered paraformaldehyde after the electrophysiological and LFP recordings. The brain tissue was preserved in 4% paraformaldehyde for 2 days before being sliced into 30 μm slices with a Leica oscillating microtome. The brain slices were sealed with goat serum for 2 h and then incubated with the primary antibodies (ChAT, #GTX163114, Genetex; GAD67, #MAB5406, Millipore; PV, #MCA3C9, Encor) at 4°C for 12 h. On the next day, the brain slices were taken out of the freezer and rinsed 3 times in PBS for 15 min followed by secondary antibodies conjugated to Alexa Fluor 594 (1:200) and DAPI (1:50). Then, brain samples were examined under bright-field microscopy using a Zeiss Axio Scope A1 microscope with ×5, ×20, and ×40 objective.

2.12. Morris water maze

The Morris water maze consists of a 2.0 m diameter pool with a black bottom. As hints, circles, triangles, and squares are draped from a black curtain around the pool. In the middle of the first quadrant, there is a hidden escape platform with a 20 cm diameter and a top surface that was 2 cm just under the water. The procedure for preparing 2VO rats is outlined in previous works[7,32]. At 8.00 am, rats were placed in the pool, facing the wall, in the second, third, and quadrants (3-trial per day for 5 days), and they had 120 s to reach the platform. If not, they were led to the platform and allowed to relax for at least 20 s. On day 6, the platform was taken out of the water, and each rat participated in a single 120-s swim probe session. Next, the following parameters were tracked: escape latency, length of swim path, swim speed, frequency of crossing, and percent of swimming distance in target quadrant of overall distance.

2.13. Statistical analysis

The data are presented as mean ± standard error of mean (SEM). MatLab 7.0 was used to examine the power spectra of LFPs. P < 0.05 was regarded as statistically significant when comparing two groups using t-test. Our statistical analysis was performed using SAS 9.1 software (Institute Incorporation, serial number: 989155).

3. Results

3.1. Downregulation of basal forebrain mir-195 expression impairs the electrical activity of the MS-dCA1 neural circuits

It has been reported that CCH can induce downregulation of mir-195 expression in the hippocampi and cortices of rats, which can regulate Aβ deposition, tau hyperphosphorylation, neuronal death, and microglial polarization[7,10]. Here, we found that mir-195 levels in the MS region were also significantly reduced in 2VO rats (Figure 1A). Our recent study found that CCH can also impair the function of the MS-dCA1 neural circuits in rats[8]. To investigate whether mir-195 is involved in CCH-induced impairment of MS-dCA1 neural circuit, we stereotaxically injected lenti-AMO-195 into the MS region of rats to establish a rat model of mir-195 knockdown (Figure 1D). Eight weeks later, the qRT-PCR result showed that compared with lenti-NC group, lenti-AMO-195 injection induced a decrease in the expression of mir-195 in MS, which was reversed by coinjecting lenti-pre-mir-195 (Figure 1B).

Subsequently, we assessed the basic electrophysiological properties of the MS-dCA1 neural circuit in vivo (Figure 1C and E). Compared with lenti-NC-injected rats, higher stimulation intensity was required to elicit the responsiveness of the MS-dCA1 circuit in lenti-AMO-195-injected rats (Figures 1F). When applying a 1-V-step stimulation to MS, we found that the amplitude of fEPSPs increased with the enhancement of stimulus intensity among lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-mir-195-treated rats. However, the fEPSP amplitude in lenti-AMO-195-injected rats was significantly lower than that in lenti-NC-injected rats, which was rescued by coinjection of lenti-pre-mir-195 in the MS (Figure 1G-I).

To further assess the presynaptic function of the MS-dCA1 neural circuit, we monitored the paired-pulse
facilitation (PPF) of the MS-dCA1 circuit using an 8 V paired-pulse stimulus (Figure 1I). PPF is commonly used to evaluate presynaptic plasticity and can be expressed as PPR\(^{33}\). An increase of PPR indicates a decreased probability of presynaptic neurotransmitter release\(^{34}\). Compared with lenti-NC-injected rats, the PPR value was significantly increased in the lenti-AMO-195-injected rats under paired-pulse stimulus using 20–70 ms interval, which was rescued by coinjection of lenti-pre-miR-195 into MS of rats (Figure 1K and L). Collectively, the knockdown of septal miR-195 impairs the basic neurotransmission and presynaptic plasticity of the MS-dCA1 neural circuit.

### 3.2. Septal loss-of-function of miR-195 reduced the dCA1-projecting neurons in MS and impaired hippocampal theta rhythmogenesis

The MS-dCA1 neural circuit includes excitatory cholinergic and inhibitory GABAergic projections, in which 66% are excitatory cholinergic neurons which are the major source of ACh release in the hippocampus\(^{35}\). In addition, the majority of the GABAergic neurons in the MS-dCA1 neural circuit are parvalbumin (PV)-positive neurons, which are the coordinator regulating the activity of cholinergic neurons in the hippocampus. Therefore, the next question is to clarify whether the cholinergic and GABAergic neurons in MS are involved in the loss-of-function of miR-195-induced impairment of MS-dCA1 neural circuits. To this end, we injected green retrobeads, a retrotracer dye, into the dCA1 of rats (Figure S1A and S1B). The retrobeads can track back from the synaptic terminal to the cell body by axoplasmic transport, thus labeling dCA1-projecting neurons from MS (Figure S1C, S1D, and S1E). One week later (Figure 2A), we compared the number of ChAT\(^{+}\), GAD67\(^{+}\), and PV\(^{+}\) neurons that were labeled by retrobeads in MS of rats administered with lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-miR-195 (Figure 2B, E, and H). The quantification analysis showed that the numbers of total (Figure 2C, F, and I) and retrobeads-labeled (Figure 2D, G, and J) ChAT\(^{+}\), GAD67\(^{+}\), and PV\(^{+}\) neurons were lower in
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The hippocampal theta rhythmogenesis is controlled by the MS-dCA1 neural circuit depending on the synergistic action of cholinergic and GABAergic neurons. In this process, sufficient ACh release can increase the power of relatively low-frequency theta oscillation. Meanwhile, the activation of PV+ GABAergic neurons in MS can elicit high-frequency firing, which cooperates with cholinergic neurons to maintain normal hippocampal theta rhythm.

To further identify the effect of miR-195 on dCA1-projecting neurons, lentivirus-mediated knockdown of miR-195 was performed. Knockdown of miR-195 significantly reduced the number of dCA1-projecting neurons in the MS region of CBH rats, as shown in Figure 2. The numbers of neurons in the MS region were significantly lower in the lenti-AMO-195 + lenti-pre-miR-195 group than in the lenti-NC group. Moreover, the decreased neurons were all reversed by lenti-pre-miR-195 coinjection. These results suggest that MiR-195 negatively regulates MS-dCA1 neural circuits in CBH rats.

**Figure 2.** Knockdown of miR-195 reduces the dCA1-projecting neurons in MS. (A) Schematic illustration of the timeline for the injection of green retrobeads. (B) Representative confocal images of cholinergic neurons (ChAT+) in MS region of rat administered with lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-miR-195. (C) Quantitative analysis of the number of neurons double labeled by ChAT and retrobeads. (D) Quantitative analysis of the percentage of retrobead-labeled neuron of the ChAT-marked neurons. (E) Representative confocal images of cholinergic neurons (GAD67+) in MS region of rat administered with lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-miR-195. (F) Quantitative analysis of the number of neurons double labeled by GAD67 and retrobeads. (G) Quantitative analysis of the percentage of retrobead-labeled neuron of the GAD67+ -marked neurons. (H) Representative confocal images of cholinergic neurons (PV+) in MS region of rat administered with lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-miR-195. (I) Quantitative analysis of the number of neurons double labeled by PV and retrobeads. (J) Quantitative analysis of the percentage of retrobead-labeled neuron of the PV+ -marked neurons. n = 6. *P < 0.05 versus NC rats. P < 0.05 versus lenti-AMO-195 rats.
MS neurons, we recorded hippocampal LFP in the pyramidal cell layer of the dCA1 region of rats (Figure 3A and B). The data showed that the hippocampal LFP changed from delta to theta rhythm following a 1-min tail squeeze in all three group rats (Figure 3C and D). Importantly, we found that the peak frequency and duration of hippocampal theta rhythm were significantly lower in lenti-AMO-195-injected rats than in lenti-NC-injected rats, which were rescued by coinjection of lenti-pre-miR-195 in vivo (Figure 3E and F). Taken together, knockdown of septal miR-195 expression impairs the hippocampal theta rhythmogenesis, which is associated with decreased cholinergic and GABAergic neurons in MS that projects to dCA1 region.

### 3.3. MiR-195 rescues the basic electrophysiological properties and MS projection neurons of MS-dCA1 neural circuit in CCH rats

The next issue we explored was to determine whether exogenously supplemented miR-195 in MS could reverse CCH-induced MS-dCA1 neural circuit impairment. With regard to this issue, we injected lenti-pre-miR-195 into MS region of 2VO rats. The lenti-pre-miR-195 injection effectively elevated miR-195 levels in 2VO rats that were close to sham rats (Figure 4B). We then performed the fEPSP recording in vivo as before (Figure 4A). We found that upregulation of miR-195 expression reduced the increased minimum stimulus intensity that was necessary to record fEPSP in hippocampal dCA1 regions of rats (Figure 4C). In comparison to sham rats, the fEPSP amplitude was noticeably lower in 2VO rats, which was rescued by the upregulation of miR-195 expression following the injection of lenti-pre-miR-195 (Figure 4D-F). As expected, upregulation of septal miR-195 expression successfully inhibited the increased PPR in 2VO rats (Figure 4G-I). Together, upregulation of miR-195 expression in MS region can reverse CCH-induced electrophysiological function impairment of MS-dCA1 neural circuit.

To clarify whether miR-195 is involved in the loss-of-function of CCH-induced impairment of MS-dCA1 neural circuit, we injected green retrobeads into dorsal hippocampi of rats at 7th week after 2VO surgery (Figure 5A). Subsequently, we assessed the alteration of specific MS-dCA1 projecting neurons by costaining ChAT, GAD67, and PV with green retrobeads among sham, 2VO and 2VO + lenti-pre-miR-195 rats (Figure 5B, E, and H). Compared with 2VO rats, exogenous supplementation of miR-195 by injecting lenti-pre-miR-195 into MS of 2VO rats rescued the decreased total number of ChAT+, GAD67+, and PV+ neurons (Figure 5C, F, and I) and elevated the percentage of retrobeads-co-labeled neurons (Figure 5D, G, and J). At the 8th week after lenti-pre-miR-195 injection into the MS of 2VO rats, we recorded hippocampal dCA1 theta rhythm (Figure 6A). As predicted, the peak theta frequency and duration of 2VO rats administrated by lenti-pre-miR-195 were improved compared with 2VO control rats (Figure 6B-E). Since ACh release could increase the septal network excitability and highly correlated with the appearance and maintenance of hippocampal theta rhythm37,38, we next detected ChAT expression and ACh concentration in the dorsal hippocampus of rats. We found that the expression of ChAT protein and the levels of ACh

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**Figure 3.** Loss-of-function of septal miR-195 impairs the theta rhythmogenesis in dCA1 of hippocampus. (A and B) Schematic picture showing theta rhythm recording technique in the hippocampal dCA1 area. (C) Frequency spectrum analysis of LFP in the dCA1 of hippocampus in rats. (D) Comparison of LFP of the dCA1 from rats administered with lenti-NC, lenti-AMO-195, and lenti-AMO-195 + lenti-pre-miR-195. Upper panel: Samples of dCA1 LFP traces. Lower panel: Heatmaps analysis of LFP in the dCA1. (E and F) The analysis of the theta rhythmis peak frequency and duration among three groups. n =6. *P < 0.05 versus NC rats. †P < 0.05 versus lenti-AMO-195 rats.
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Figure 4. MiR-195 improves the basic neurotransmission process and presynaptic function of MS-dCA1 neural circuit in CCH rats. (A) Schedule of the experiment plan. (B) The miR-195 level in the basal forebrain of 2VO rats treated with lenti-pre-miR-195. (C) Differences of minimum stimulus intensity for triggering a measurable response of MS-dCA1 circuit in rats among sham, 2VO, and 2VO + lenti-pre-miR-195 rats. (D) Sample trace of fEPSP in the hippocampal dCA1 with 8 and 20 V stimulations. (E) Comparison of input-output curves in the dCA1 region among three groups of rats. (F) Differences of the fEPSP amplitude with 8- and 20-V stimulations in each group. (G) Samples of MS-dCA1 fEPSP trace after paired pulses stimuli with 20, 40, and 70 ms intervals between in rats from different groups. (H) The changes of PPR induced by lenti-pre-miR-195 injection. (I) The bar graph shows the largest PPR in rats from different groups. n = 6. *P < 0.05 versus sham rats. #P < 0.05 versus 2VO rats.

in the hippocampi of 2VO rats were significantly decreased, which were recovered by lenti-pre-miR-195 injection (Figure 6F and G, Figure S2). These results suggest that upregulation of miR-195 expression can improve the function of MS-dCA1 neural circuit of 2VO rats and prevent the death of ChAT+, GAD67+, and PV+ neuron.

3.4. Upregulation of miR-195 expression in the MS improves the spatial learning and memory ability of CCH rats

Our previous study found that exogenous supplementation of miR-195 in the hippocampus could prevent 2VO-induced cognitive decline[7]. Therefore, we would like to evaluate whether upregulation of miR-195 expression in MS has the similar effect using the Morris water maze test. First, we compared the swimming speed among the three groups to exclude the influence of locomotor abnormalities on the experimental results. The data showed that there was no difference among the three groups (Figure 7A). On the 1st day of the cued learning trial, basal forebrain injection of lenti-pre-miR-195 reduced the latency to the platform of 2VO rats in the third and fourth quadrants (Figure 7B). Exogenous supplementation of miR-195 also reduced the time spent by 2VO rats to find the hidden platform on the 2nd–5th days after being released into the tank in all of the three non-target quadrants of the localization navigation test (Figure 7C–E). On the 6th day of the spatial exploration test, we found that injection of lenti-pre-miR-195 reversed the number of platform crossings (Figure 7F) and the percentage of swimming time in the target quadrant in 2VO rats (Figure 7G). The above results showed that upregulation of miR-195 expression can attenuate the impairment of spatial learning memory ability in CCH rats (Figure 7H).

4. Discussion

With the aging of population and the prevalence of cerebrovascular diseases, the incidence of VaD is increasing year by year. However, the development of therapeutic drugs has not progressed much[89]. At present, many scientists recognize that improving the function of neural circuits is a potential therapeutic approach to treat neurodegenerative diseases[40,41]. Here, we found that loss-of-function of miR-195 impaired the MS-dCA1 neural circuit in rats, while exogenous supplementation of miR-195 rescued the dysfunction of the MS-dCA1 neural circuit and declined spatial memory in CCH rats. We thus present in this paper a candidate strategy for alleviating the impaired septohippocampal neural circuit during the pathogenesis of dementia.

As the largest cerebral cholinergic nucleus and theta rhythm “pacemaker,” the basal forebrain forms a neural
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In parallel, the dysfunctions of the MS-dCA1 neural circuit are thought to be one of the early pathological features of dementia\textsuperscript{[17,30,40]}. In fact, abnormalities in neural circuit function have repeatedly been recorded before any neurodegeneration or plaque deposition in the brain of transgenic mice overexpressing APP, triple transgenic mice (3 × Tg), and wild-type mice injected with Aβ\textsubscript{42-44}. CCH, a preclinical stage of MCI, has also been shown to be involved in impaired hippocampal theta rhythm and MS-dCA1 neural circuit\textsuperscript{[12,43]}. Previous morphological evidence suggests that the neuronal projection from the MS to the dCA1 pyramidal cells is mainly composed by cholinergic and...
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GABAergic components\textsuperscript{[35]}. Cholinergic projections from the MS region coordinate hippocampal pre- and post-synaptic activity and temporally modulate the synaptic plasticity\textsuperscript{[46,47]}. GABAergic afferents from the MS mostly innervate PV-positive interneurons in the hippocampus, and these interneurons inhibit the activity of peripheral pyramidal neurons\textsuperscript{[48,49]}. Accordingly, rhythmically activated GABAergic neurons in MS may eventually transform this activation into the rhythmic disinhibition of hippocampal pyramidal neurons and provide the synchronization between hippocampus and MS\textsuperscript{[14,50]}. Sufficient ACh production in the hippocampus increases the power of low-frequency theta rhythm but suppresses the opposing non-theta activities\textsuperscript{[36]}. While GABAergic projection in the MS-dCA1 neural circuit provides the driving force for theta rhythm in the hippocampus\textsuperscript{[14]}. Thus, combined with the decreased I-O curve, the increased PPR of the MS-dCA1 neural circuit, and the diminished hippocampal theta rhythm, we speculate that the CCH-induced injury may be the result of neuronal loss in the MS-dCA1 neural circuit. The previous studies have reported the decreased cholinergic and GABAergic functions in septal-related neural circuits in AD transgenic models\textsuperscript{[17,47,51]}. Our previous study demonstrated that CCH could induce neuronal loss in the hippocampi of CCH rats by targeting the gene of DR6 protein\textsuperscript{[8]}. In the present study, we found that CCH can also induce neuronal loss in MS region, which was assessed by costaining the signal of ChAT/GAD67/PV antibody and retrotracer dye from dCA1. The results suggested that the neuronal loss in MS also participates in CCH-induced cognitive decline.

A large number of studies reported that many miRNAs, which act as crucial modulators, participate in the AD pathological process by regulating APP and BACE1 expressions\textsuperscript{[20-23]}. Our previous studies reported that CCH led to the reduction of miR-195 expression in the hippocampus, and downregulation of miR-195 expression in hippocampal region impaired the spatial cognitive ability by inducing Aβ deposition, tau hyperphosphorylation, microglial polarization, synaptic dysfunction, and neuronal death\textsuperscript{[7,10]}. Here, we also found that CCH could downregulate miR-195 expression in MS region. Similar to 2VO rats, by injecting lentivirus-mediated antisense nucleotide of miR-195, into the MS region but not the hippocampus of normal rats and...
induced the loss of Chat⁺, GAD67⁺, or PV⁺ neurons in MS. Next, we injected lenti-pre-miR-195 specific into the MS region but not the hippocampus of 2VO rats and found that upregulation of miR-195 in MS region but not hippocampus prevented the loss of neurons in the MS following CCH. Taken together, the downregulation of septal miR-195 expression could impair the function of MS-dCA1 neural circuit that was associated with the neuronal losses in the MS area.

Recent studies using both cellular and animal models demonstrated that miR-195 has positive effects on anti-apoptosis in injured neurons by suppressing Sema3A/Cdc42/JNK signaling, promotes neural regeneration by promoting neural stem cell proliferation and migration, and has anti-inflammation action by blocking the NF-κB pathway[27]. Furthermore, the cerebral stroke damage of rats was improved by intravenously injecting miR-195 in the acute stage[27]. These studies suggest that miR-195 may have the potential in the treatment of cerebral ischemic diseases. Our previous studies found that upregulation of miR-195 expression in hippocampal and cortical regions rescued the spatial cognitive ability in 8-week 2VO rats by inhibiting Aβ deposition, tau hyperphosphorylation, microglial polarization, synaptic dysfunction, and neuronal death[17-10]. In the present study, we demonstrated that upregulation of miR-195 expression through the injection of lenti-pre-miR-195 into MS region successfully reversed the impaired function of MS-dCA1 neural circuit as well as neuronal loss, and even attenuate the declined spatial learning memory ability induced by CCH. All these studies provide solid evidence that miR-195 may have the potential to be a biomarker and/or therapeutic drug for early AD or VaD. However, the specific molecular mechanism of miR-195 modulating MS-dCA1 neural circuit function needs to be further investigated.

5. Conclusions
In our study, we found that knockdown of miR-195 in the MS region can impair MS-dCA1 neural circuit function, while upregulation of miR-195 can rescue the impaired function of MS-dCA1 neural circuit and spatial memory ability in CCH rats. This provides a valuable reference for future anti-dementia therapy involving miR-195.

Acknowledgments
None.

Funding
This work was supported by the National Science and Technology Innovation 2030 - Major program of "Brain Science and Brain-Like Research" 2022ZD0211804, the National Natural Science Foundation of China (81870849 to J.A.), the Key Research and Development Program of Heilongjiang Province (GA21C009 to J.A.), and Heilongjiang Touyan Innovation Team Program.

Conflict of interest
The authors declare that they have no conflicts of interest to report.

Author contributions
Conceptualization: Jing Ai

Figure 7. Upregulation of septal miR-195 expression improves the spatial learning and memory ability of CCH rats. (A) Comparison of swimming speed among sham, 2VO, and 2VO + lenti-pre-miR-195 rats. (B) Comparison of the latency to hidden platform in the second, third, and fourth quadrant on the 1st day from rats of the three groups. (C-E) Comparison of the latency to hidden platform in the second, third, and fourth quadrant during the training phase from rats of the three groups. (F and G) Differences of the platform crossing (F) and the percentage of swimming in the goal quadrant (G) during the investigation trials from rats of the three groups. (H) Samples of swimming traces of the rats from the three groups in probe trial. n = 6. *P < 0.05 versus sham rats. *P < 0.05 versus 2VO rats.
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