MicroRNA-195 Prevents Hippocampal Microglial Polarization Towards The M1 Phenotype Induced By Chronic Brain Hypoperfusion Through Regulating CX3CL1/CX3CR1 Signalling

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Abstract

**Background** Microglial polarization was found respond dynamically to acute brain hypoxia induced by stroke and traumatic brain injury (TBI). However, studies on the process of microglial polarization during chronic cerebral ischaemia (CCI) are limited. Our goal is to investigate the influence of CCI on microglial polarization following chronic brain hypoperfusion (CBH) and exploit potential molecular mechanisms.

**Methods** CBH model was performed by bilateral carotid artery ligation (2VO) in rats. Using stereotaxic injection technique, lenti-pre-\textit{miR-195} and anti-\textit{miR-195} oligonucleotide fragments (lenti-pre-AMO-195) were injected into CA1 region of the hippocampus to construct animal models with high or low expression of \textit{miR-195}. Immunofluorescence staining and flow cytometry were conducted to examine the status of microglial polarization. \textit{In vitro}, Transwell co-culture system was taken to investigate the role of \textit{miR-195} on neuronal-microglial communication through CX3CL1-CX3CR1 signalling. Quantitative real-time PCR was used to detect the level of \textit{miR-195} and inflammatory factors. The protein levels of CX3CL1 and CX3CR1 were evaluated by both western blot and immunofluorescence staining.

**Results** CBH induced by 2VO initiated microglial activation in the rat hippocampus from 1 week to 8 weeks, as evaluated by increased Iba-1 immunofluorescence, that the balance between microglial polarization towards the M1 and M2 phenotypes was shifted towards the M1 phenotype and that the expression of CX3CL1 and CX3CR1 was increased at 8 w following CBH. An \textit{in vitro} study in a Transwell co-culture system demonstrated that transfection of either primary cultured neonatal rat neurons (NRNs) or microglial BV2 cells with AMO-195 induced M1 polarization of BV2 cells and increased CX3CL1 and CX3CR1 expression and that these effects were reversed by \textit{miR-195} mimics. Furthermore, overexpression of \textit{miR-195} induced by lenti-pre-\textit{miR-195} prevented the changes triggered by knockdown of endogenous \textit{miR-195} induced by lentiviral vector-mediated expression of lenti-pre-AMO-195 and 2VO surgery.

**Conclusions** Our findings conclude that downregulation of \textit{miR-195} in the hippocampus is involved in CBH-induced microglial polarization towards M1 phenotype by governing communication between
neurons and microglia through the regulation of CX3CL1 and CX3CR1 signalling. This indicates that 
miR-195 may provide a new strategy for clinical prevention and treatment of CBH.

Background
Microglia play an important role in various neurodegenerative diseases, including Alzheimer’s disease (AD), Parkinson’s disease (PD), Huntington’s disease (HD), and prion diseases [1, 2]. It is well known that AD pathology comprises extracellular Aβ aggregation, tau protein hyperphosphorylation, and neuronal loss [3]. Previous studies have demonstrated that the accumulation of Aβ can lead to a microglial response that aggravates tau hyperphosphorylation and the formation of neurofibrillary tangles. On the other hand, activated microglia respond to Aβ deposition and initial neuronal loss by secreting multiple proinflammatory cytokines, such as IL-1β, tumour necrosis factor α (TNF-α) and reactive oxygen species (ROS) [4]. Chronic brain hypoperfusion (CBH), one of the major pre-clinical phases of AD [5, 6], has been found to cause extracellular Aβ aggregation [7], tau protein hyperphosphorylation [8], neuronal loss [9] and even early astrocytic glial activation [10]. However, the influence of CBH on microglial function is unclear.

Microglia are immune cells that are considered the brain’s first line of defence, and are double-edged swords in responding to pathogens and supporting CNS homeostasis and plasticity [11]. It has been documented that microglia act as guardians of the brain to respond to various types of acute brain injury via activation, termed microglial polarization, to classically activated M1 microglia, which release proinflammatory mediators, and alternatively activated M2 microglia, which release numerous protective/trophic factors [12–14]. Therefore, manipulating the polarization of microglia might be a promising therapeutic strategy for brain repair. Acute severe brain ischaemia, such as that induced by intracerebral haemorrhage (ICH) and traumatic brain injury (TBI), has been found to be associated with the "healthy" M2 phenotype early on, followed by a transition to the "sick" M1 phenotype [15–17]. However, whether CBH can also induce dynamic microglial polarization in the hippocampus is unknown.

Chemokine (C-X3-C motif) ligand 1 (CX3CL1), which is anchored to the neuronal membrane, is an intriguing chemokine that plays a central role in microglial activation by interacting with CX3CR1
expressed by microglia [18, 19]. Interestingly, clinical studies have reported that CX3CL1 expression levels differ significantly during the pathological process of AD, with the levels of CX3CL1 being increased in intermediate AD patients while being considerably lower in late-stage AD patients [20, 21]. Importantly, the temporal dynamics of plasma CX3CL1 have also observed to depend on stroke severity in patients suffering from ischaemic stroke, with a higher level being found in patients with moderate stroke than in severe patients [22]. In addition, CX3CR1 deficiency results in fewer apoptotic neurons, reduced ROS levels, facilitated alternative activation (towards the M2 phenotype) of microglia/macrophages, and attenuated synthesis and release of inflammatory cytokines in a CX3CR1−/− MCAO mouse model [23] and reductions in Aβ deposition and the number of microglia surrounding Aβ deposits in a mouse model of AD [24]. However, whether dysfunction of the CX3CL1/CX3CR1 pathway is also involved in CBH pathology, a kind of chronic mild ischaemia, has not been reported.

Previous studies have reported that CBH induces upregulation of NF-κB, in turn downregulating the expression of microRNA-195 (miR-195), which posttranscriptionally regulates Aβ aggregation, hyperphosphorylation of tau protein and cell death [1, 8, 9, 25]. Another study found that miR-195 protects against focal cerebral ischaemia-induced cell apoptosis by targeting CX3CR1 [26]. Additionally, miR-195 reduces the expression of multiple NF-κB downstream effectors by directly targeting IKKa and TAB3 in hepatocellular carcinoma (HCC) [27] and inhibits an M1-like polarization-induced proinflammatory profile in macrophages [28]. Nevertheless, whether miR-195 is involved in CBH-induced hippocampal microglial polarization through regulating CX3CL1-CX3CR1 signalling has not been studied.

The aim of this study is to investigate the influence of CBH on microglial polarization and exploit the potential molecular mechanisms of miR-195 on this process.

Materials And Methods
Experimental design
To investigate how CBH affects microglial activation, we detected the polarization phenotypes of microglia in the hippocampus of rats after bilateral common carotid artery occlusion (2VO) surgery by
immunofluorescence staining, flow cytometry analysis and qRT-PCR. Then, to investigate the role of *miR-195* on hippocampal microglial polarization in rats following CBH, we constructed rats with high or low expression of *miR-195* by stereotaxic injection technique and evaluated the polarization phenotypes of microglia and related proteins in the hippocampus of these rats by immunofluorescence staining, qRT-PCR and western blotting. The potential molecular mechanisms of *miR-195* on hippocampal microglial polarization were examined *in vitro* Transwell co-culture system.

**Animals**

Adult male Sprague Dawley (SD) rats (280–300 g) were supplied by Changsheng biotechnology (Liaoning, Shenyang Province, China) and housed in individual cages under a standard 12-hour light-dark cycle with water and food supplied. All animal handling and surgical procedures were performed in accordance with the guidelines established by the National Institutes of Health for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at Harbin Medical University (China).

**Permanent bilateral occlusion of the common carotid arteries (2VO) in rats**

Bilateral common carotid artery occlusion (2VO) in rats was prepared according to our previous study [1, 29]. In brief, animals were anesthetized with chloral hydrate (300 mg/kg). No. 3–0 silk sutures were place under the separated common carotid artery and tightened up and then the common carotid artery was cut off in between the two ligated silk sutures. The same procedure was performed on the sham group but without the ligation. The brain tissues and slices were harvest from 2VO rats at the time points of 1 w, 2 w, 4 w and 8 w for the subsequent experiments.

**Primary Culture Of Neonatal Rat Hippocampal Neurons (nrns)**

Primary neuron cultures were prepared as previously described [30]. In brief, the hippocampus were removed from postnatal day 0 (P0) male SD rat pups and dissociated cells were maintained in DMEM containing 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA), then seeded into a poly-D-lysine precoated 6-well plate at a density of 1–2 × 10^6 cells/well. After 4 h of incubation, the culture medium was replaced with neurobasal medium (Gibco, USA) with 2% B27 supplement (Invitrogen, USA). Cultures were placed in a 37 °C humidified atmosphere with 5% CO2. Neurons were collected from day 5–7 of in vitro culture were used for the experiments.
Cell Culture Of Bv2 Cells

BV2 microglial cells were purchased from the National Infrastructure of Cell Line Resource and cultured in DMEM (Invitrogen, USA) supplemented with 0.1% penicillin-streptomycin (Solarbio, China) and 10% fetal bovine serum (FBS, HyClone, Logan, UT, USA). Cultures were maintained at 37°C in a 5% CO2 humidified atmosphere for 3 days, which were then collected for further experiments.

Synthesis Of Oligonucleotides And Cell Transfection

MiR-195 mimics, AMO-195 and NC were synthesized by GenePharma Corporation (Suzhou, China) as previously described [9]. Cx3cl1-masking antisense ODNs were synthesized by Sangon Biotech Corporation (Shanghai, China). The sequence of CX3CL1-ODN, which was used to mask the binding sites of miR-195 located in the 240–246 bp region of the Cx3cl1 3’UTR, was 5’—+ C + C + A + G + CCAGCAGCAGAG + G + A + U + U + C—3’. The sequence of CX3CR1-ODN, which was used to mask the binding sites of miR-195 located in the 1236–1242 bp region in the Cx3cr1 3’UTR, was 5’— + C + C + A + C + GCAGCAGCACCU + G + C + A + G + G + C—3’. The nucleotides or deoxynucleotides at both ends of the antisense molecules were locked by a methylene bridge connecting the 2′-O and the 4′-C atoms. These plasmids were transfected into cells using X-treme GENE siRNA transfection reagent (Roche, Switzerland) according to the manufacturer's instructions. Forty-eight hours after transfection, the cells were processed for further experiments.

Co-culture Of Bv2 Cells With Neurons

After NRNs were transfected with miR-195, AMO-195 and CX3CL1-ODN for 48 h, the culture medium was discarded, and the NRNs were gently washed 3 times with PBS. Then, the NRNs were removed from the 6-well plates and placed on the bottom of a Transwell plate (Corning Company, USA). After washing 3 times with PBS, the cultured BV2 cells were placed in the upper chamber of the Transwell plate, which was separated from the NRNs by a semipermeable membrane (pore size of 0.4 µm). DMEM supplemented with 10% foetal bovine serum was placed in the two separate chambers, and the plate was placed in an incubator at 37 °C with saturated humidity and 5% CO2. After 24 h of co-culture, both BV2 cells and neurons were collected for further experiments [30].

Construction Of Lentivirus Vectors

The details of the construction of the lentivirus vectors were described in our previous studies [7, 9].
The synthesis and lentiviral packaging of two double-stranded oligonucleotides, pre-miR-195 and NC, and a single-stranded DNA oligonucleotide, pre-AMO-195, were performed by GeneCopoeia Inc. (Rockville, MD, USA).

**Stereotaxic Injection Of Lentiviral Vectors**

Rats were anaesthetized with chloral hydrate (300 mg/kg) and placed in an animal stereotaxic apparatus (RWB Life Science Co, Ltd., China). The injection coordinates relative to bregma were as follows: anteroposterior, -4.52 mm; mediolateral, ± 3.2 mm; dorsoventral, -3.16 mm below the surface. The coordinates were determined based on the atlas by Paxinos and Watson. A total of 2 µL (10,000 TU/µL) lenti-pre-miR-195 and/or lenti-pre-AMO-195 was injected into the CA1 region of the hippocampus using a 5 µl Hamilton syringe with a 33-gauge needle (Hamilton, Bonaduz, Switzerland).

Subsequent experiments were performed 8 w after virus injection [7, 9].

**Immunofluorescence Staining**

Twenty-micron-thick rat brain slices were incubated in PBS containing Triton X-100 and 10% goat serum for 2 h at room temperature. After blocking, the slices were incubated with an anti-Iba-1 (1:500, Wako, USA), anti-rat-CD68 (1:200, Bio-Rad, UK) or anti-rat-CD206 (1:300, Santa Cruz, USA) primary antibody overnight at 4 °C followed by secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 594 (1:300, Invitrogen, USA) and DAPI the next day.

The cultured cells were fixed in 4% paraformaldehyde for 30 min and then incubated with PBS containing Triton X-100 and 10% goat serum for 1 h at room temperature. After blocking, they were incubated with a rat anti-CX3CL1 primary antibody (1:300, R&D system, USA) to detect neurons and an anti-lba-1 (1:1000, Wako, USA), anti-mouse CD68 primary antibody (1:300, AbDserotec, Oxford, UK) or anti-mouse CD206 primary antibody (1:300 AbDserotec, Oxford, UK) to detect BV2 cells overnight at 4 °C followed by secondary antibodies conjugated to Alexa Fluor 488 and Alexa Fluor 594 (1:500, Invitrogen, USA) and DAPI the next day.

The fluorescence signals were detected using a fluorescence microscope (Zeiss, Scope A1). The number of cells in the dentate gyrus (DG) or CA1 region of the hippocampus in each animal was calculated by ImageJ software (NIH, MD, USA). Iba-1⁺ cells, CD68⁺/lba-1⁺, and CD206⁺/lba-1⁺ cells
were counted in a blinded manner. The mean values were calculated from 3 randomly selected microscopic fields from each section for each animal. A total of 3 animals per group were analysed. The data are expressed as the mean number of cells per square millimetre.

Isolation And Purification Of Microglial Cells

After anaesthetisation with chloral hydrate (300 mg/kg), the rats were transcardially perfused with phosphate-buffered saline (PBS). Microglial cells were extracted and isolated from the hippocampus according to a protocol published on Bio-protocol [31]. Briefly, the homogenized tissues were digested to form cell suspensions, which were then filtered through a 70-µm nylon filter and then centrifuged at 300 × g for 10 min. The cells pellets were resuspended in 20 ml of 30% isotonic Percoll solution (GE Healthcare, Uppsala, Sweden). Then, we transferred 10 ml of the resuspended cell pellets to a 50 ml centrifugation tube and carefully added 10 ml of 80% sotonic Percoll solution to the bottom of the centrifugation tube using a serological pipette with the help of gravity. Finally, the rest of the cell pellet was gradually added to the upper layer of the centrifugation tube, which was then centrifuged at 1050 × g for 40 min at room temperature. The microglia were then collected from the interphase between the 80% and 30% Percoll layers. The cells were washed and resuspended in sterile HBSS and used for flow cytometry.

Flow Cytometry Analysis Of Immunostained Cells

Flow cytometry analysis of immunostained cells was performed following standard cell protocols. Prior to antibody labelling, the cell suspensions were incubated with anti-murine CD16/CD32 FC-Receptor blocking reagent at 4 °C for 10 min (eBioscience, CA, USA). After blocking, the microglia were stained with FITC-conjugated mouse anti-rat CD11b (BD Biosciences, USA) and PerCP/Cy5.5-conjugated anti-rat CD45 (Biolegend, CA, USA). The microglia were then fixed and permeabilized with BD Fixation/Permeabilization buffer for 20 min. The microglia were washed with BD Perm/Wash buffer, resuspended in BD Perm/Wash buffer, and incubated with anti-iNOS (Abcam, UK) and rabbit mAb anti-arginase-1 (Arg-1) (Cell Signaling Technology, USA) primary antibodies for 30 min followed by a PE-conjugated anti-rabbit IgG (H + L) secondary antibody (Cell Signaling Technology, USA). The cells were analysed using a CytoFLEX instrument (Beckman Coulter Biotechnology, SuZhou). The results
were analysed using CytExpert software (Beckman Coulter Biotechnology, SuZhou).

Western Blot Analysis
Total protein was extracted from the hippocampus of rats or primary cultured neurons for immunoblotting analysis. The protein concentrations of all extracted samples were measured using the Bio-Rad Protein Assay (BioRad, Hercules, CA) and bovine serum albumin standards. Fifty micrograms of the protein samples was separated by SDS-PAGE and then transferred to PVDF membranes, which were then incubated with primary antibodies at 4 °C overnight followed by fluorescent secondary antibodies (LICOR Biosciences, Lincoln, NE, USA). Anti-CX3CL1 (1:1000, ab25088, Abcam, USA) and anti-CX3CR1 (1:1000, ab8021, Abcam, USA) were used as the primary antibodies. β-Actin (1:1000, G8795, Sigma, St. Louis, MO, USA) was used as an internal control. The bands on the blot were detected with the Odyssey infrared imaging system (LICOR Biosciences, Lincoln, USA). The signal intensities were analysed using Odyssey v. 1.2 software and normalized to the intensity of the loading control, β-actin.

Real-time Pcr
Total RNA was extracted from the rat hippocampus or neurons using TRIzol (Invitrogen, USA) according to the manufacturer's protocol. RNA was reverse transcribed into cDNA using a ReverTra AceqPCR RT Kit (Tobyo Co., Osaka, Japan). qPCR reactions were run in a volume of 20 µl using FastStart Universal SYBR Green Master (Roche, Switzerland) in an Applied Biosystems machine (Thermo Fisher Scientific, USA). The protocol was (1) 10 min at 95 °C, (2) 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C for 40 cycles, and (3) melt curve analysis. The qPCR primer sequences were as follows: miR-195: forward (F), GGGGTAGCAGCAGAAAT and reverse (R), TCCAGTGCCTGTCGTTGA; U6: F, GCTTCGGCAGCACATATACTAAAAT and R, CGCTTCACGAATTTCGTGTCAT; IL-1β: F, GGCAACTGTCCCTGAACT and R, TCCACAGCCACAATGAGT; TNF-α: F, GACCCTCACACTCAGATCATCTTCT and R, TGCTAGACGGGCTGCTACG; TGF-β: F, TGGCCAGATCCTGTCGTTGA and R, GTTGTACAAAGCGAGCACCG; and GAPDH: F, CTGGCATTGTCTCAGATCATCTTCT and R, CTTGCTGATTATCCGCTGGCTG. Gene expression was normalized to the expression of the internal control.

Statistical analysis
The data are presented as the mean ± S.E.M. Student’s t-test was used for statistical analysis of differences between two groups. One-way ANOVA was performed for comparisons among multiple groups, and post hoc analyses were performed using Fisher’s PLSD test when a significant main effect was found. All statistical analyses were performed using SPSS11. P < 0.05 was considered statistically significant, and graphs were generated using GraphPad Prism 8.0 software (La Jolla, CA, USA).

**Results**

**CBH induces microglial activation and polarization in the rat hippocampus**

To investigate how CBH affects microglial activation in the rat hippocampus, we generated an animal model of CBH through permanent bilateral occlusion of the common carotid arteries (2VO) and evaluated microglial polarization in the hippocampus 1, 2, 4, and 8 w after 2VO surgery. As illustrated in Fig. 1A - E, immunofluorescence staining showed that the number of cells positive for the microglial marker Iba (Iba-1⁺ cells) in the dentate gyrus (DG) region was significantly increased at 1 w but gradually decreased to the same level as that in the sham group by 8 w. However, the percentage of activated microglia gradually increased from 2 w to 8 w (Fig. 1F). Since activated microglia can be polarized to the cytotoxic M1 phenotype and the pro-repair M2 phenotype [31], we next analysed the polarization phenotypes of microglia using immunofluorescence staining to analyse the co-localization of the M1-associated marker CD68 or the M2-associated marker CD206 with Iba-1. The data showed that, although the percentages of both types of microglial cells gradually increased in the DG region of CBH rats from 2 w to 8 w (Fig. 1A-D, G & H), the level to which the percentage of CD68-positive (CD68⁺) microglial cells increased was much higher than that to which CD206-positive (CD206⁺) microglial cells increased in the rats at 4 w and 8 w but not at 1 w and 2 w after 2VO surgery (Fig. 1G & H). The ratio of CD68/CD206 was then analysed to better understand the dynamic changes in microglial polarization. The ratio of CD68/CD206 higher than 1 indicates that microglial polarization towards to the M1 phenotype. The data showed that the ratio of CD68/CD206 was the same between 2VO and sham rats at 1 w and 2 w; however, it was increased in 2VO rats starting at 4 w and reached the highest value, which was 1.76 ± 0.13-fold greater than that in the sham rats at 8 w (Fig. 1I).

Similar to what was observed in the DG, the number of Iba-1⁺ cells in the CA1 region of 2VO rats was
also significantly increased at 1 w and progressively decreased to the same level as that in the sham group by 8 w (Fig. 2A - E); meanwhile, the number of activated microglial cells increased gradually (Fig. 2F). Interestingly, although the number of CD68\(^+\) microglial cells was also higher than the number of CD206\(^+\) microglial cells in the CA1 region of 2VO rats at 8 w (Fig. 2G & H), the ratio of CD68/CD206 in the CA1 region (1.39 ± 0.11) was lower than that in the DG region (Fig. 1I & 2I); this suggests that CBH-induced microglial polarization is more sensitive in the DG region. Taken together, these results suggested that CBH can result in the activation of microglial cells in the hippocampus from the early stage (1 w) through 8 w. However, polarization transitioned to a more detrimental M1 phenotype at 8 w after being balanced between the M1 and M2 phenotypes at 1 w and 2 w after 2VO surgery.

To further verify that microglial polarization tended to be associated with the M1 phenotype for 8 w following 2VO surgery, we performed flow cytometry analysis to assess the status of the M1 and M2 phenotypes using a variety of markers of microglial cells. First, we sorted microglia using CD11b\(^+\)/CD45\(^\text{low}\) as a marker (Fig. 3A) [33, 34]. Then, the mean fluorescence intensities (MFI) of the M1 marker iNOS and the M2 marker Arginase-1 (Arg-1) were detected. The data revealed a much higher percentage of iNOS expression in microglial cells of 2VO rats than in microglial cells of sham rats (Fig. 3B & D). However, the level of Arg-1 was only slightly increased in microglial cells from 2VO rats compared to sham rats (Fig. 3C & D). This phenomenon was further verified by the higher ratio of iNOS between 2VO and sham groups than Arg-1 (Fig. 3E). Currently, proinflammatory cytokines (TNF-\(\alpha\) and IL-1\(\beta\)) are thought to be produced by M1 microglia, while M2 microglia can secrete anti-inflammatory cytokines and trophic factors, such as TGF-\(\beta\) [35, 36]. QRT-PCR analysis showed that the mRNA levels of the proinflammatory cytokines TNF-\(\alpha\) and IL-1\(\beta\) were elevated in the hippocampus of 2VO rats compared with sham rats (Fig. 3F & G). However, the mRNA level of the anti-inflammatory cytokine TGF-\(\beta\) was only slightly increased (Fig. 3H). All these data suggest that microglial polarization is skewed towards the M1 phenotype in the hippocampus of 2VO rats at week 8.

Knockdown of miR-195 polarizes microglia towards the detrimental M1 phenotype
Previous studies have reported that microRNA-195 ([miR-195]) reduces M1-like macrophage polarization and inhibits the inflammatory pathway in the peripheral nervous system [27, 28]. In addition, our previous study found that [miR-195] is downregulated in the hippocampus of CBH rats [7, 9]. Therefore, we speculated that [miR-195] might have the potential to polarize microglia in the hippocampus. To test this hypothesis, [miR-195] oligonucleotide fragments (lenti-pre-[miR-195]) and an anti-[miR-195] oligonucleotide fragments (lenti-pre-AMO-195) packaged in a lentivirus vector were delivered directly into the bilateral hippocampal CA1 region of rats to examine the role of [miR-195] in microglial polarization. The successful delivery of lenti-pre-AMO-195 and lenti-pre-[miR-195] was verified by the detection of [miR-195] levels by qRT-PCR (Fig. 4A). Immunofluorescence analysis showed that lenti-pre-AMO-195 application resulted in an increased number of CD68+ microglia in both the DG and CA1 region compared with that in the sham group and that this effect was prevented by co-injection of lenti-pre-[miR-195] (Fig. 4B, E & F). Interestingly, injection of lenti-pre-AMO-195 did not significantly affect the number of CD206+ microglial cells, but there was a decreasing trend in the number of these cells in the DG and CA1 regions (Fig. 4C, E & F). Accordingly, the ratio of CD68/CD206 was markedly increased after lenti-pre-AMO-195 was injected into the hippocampus, and this effect was reversed by co-injection of lenti-pre-[miR-195] (Fig. 4D). In addition, we observed that knockdown of [miR-195] induced significant increases in the levels of TNF-α and IL-1β, which were blocked by [miR-195] gain of function (Fig. 4G & H). However, downregulation of [miR-195] had no effect on TGF-β and this effect was not influenced by upregulation of [miR-195] (Fig. 4I). These data suggested that knockdown of [miR-195] can drive microglial polarization towards the M1 phenotype in the rat hippocampus.

To further confirm this observation, we transfected [miR-195] mimics and the [miR-195] antisense oligonucleotide AMO-195 directly into BV2 microglial cells. As illustrated in Fig. 5A & B, transfection with [miR-195] mimics did not affect the percentage of CD68+ microglia cells, but the antisense oligonucleotide AMO-195 induced a marked increase in the number of CD68+ microglia cells, which was prevented by co-transfection with [miR-195] mimics. However, transfection with the [miR-195]
mimics or AMO-195 and co-transfection with the miR-195 mimics and AMO-195 did not affect the number of CD206+ microglial cells (Fig. 5A & C). Furthermore, it has been reported that microglia can be primed towards the M1 phenotype by LPS [36]. Thus, we added LPS (100 µg·mL−1) to the cultured BV2 cells for 24 h and observed that the number of CD68+ microglial cells but not that of CD206+ microglial cells was increased (Fig. 5B & C); additionally, this effect was prevented by co-administration of the miR-195 mimics. By analysing the ratio of CD68/CD206, we found that AMO-195 administration had the same effect as LPS on M1 microglial polarization and that the effects of both these agents were blocked by co-transfection with the miR-195 mimics (Fig. 5D).

CX3CL1-CX3CR1 signalling involving miR-195 regulated microglial polarization
CX3CL1-CX3CR1 signalling plays an important role in neuroinflammatory diseases of the CNS [37]. A previous study demonstrated that the expression of CX3CL1 and CX3CR1 is increased in rats early after ischaemic stroke and that reducing the expression of CX3CL1 and CX3CR1 is beneficial for the recovery of neurological function [38]. Importantly, it has been found that CX3CL1/CX3CR1-mediated microglial activation can promote the generation of TNF-α and IL-1β, inducing a detrimental effect in the brains of ischaemic mice in the early stage [39]. Since M1 microglia typically release destructive proinflammatory mediators such as TNF-α and IL-1β [35], the levels of TNF-α and IL-1β expression were significantly increased in 2VO rats compared with sham rats (Fig. 3). We hence hypothesized that CX3CL1-CX3CR1 signalling might be involved in microglial polarization in CBH rats. As predicted, the immunofluorescence signals of CX3CL1 and CX3CR1 in the hippocampus of 2VO rats at 8 w were much higher than those in sham rats (Fig. 6A & B). Western blotting analysis also verified the increase in the protein expression of CX3CL1 and CX3CR1 in the hippocampus of 2VO rats compared with sham rats (Fig. 6C).

It has been reported that miR-195 protects against focal acute ischaemic stroke by targeting CX3CR1 but not CX3CL1 in mice, although both CX3CL1 and CX3CR1 are direct targets of miR-195 [26]. However, the effect of miR-195 on microglial polarization in the rat hippocampus following chronic mild brain ischaemia is unknown. We first evaluated the effect of miR-195 on CX3CR1 and CX3CL1 expression. We found that knockdown of miR-195 induced by injection of lenti-pre-AMO-195 induced a
significant increase in CX3CL1 (Fig. 6D) and CX3CR1 (Fig. 6E) expression and that this change was inhibited by co-injection of lenti-pre-miR-195.

The CX3CL1-CX3CR1 pathway is a critical signalling pathway for cellular communication between neurons and microglia [1, 40]. To further clarify whether the CX3CL1-CX3CR1 signalling pathway participates in miR-195 knockdown-induced microglial polarization, we established a neuronal-microglial co-cultured model. In this co-culture system (Fig. 7A), we first transfected primary cultured neonatal rat neurons (NRNs) with miR-195 mimics and AMO-195 for 48 h and co-cultured microglia with NRNs for 24 h. The western blot results showed that AMO-195 upregulated the expression of CX3CL1 on NRNs and that this effect was prevented by co-transfection with the miR-195 mimics (Fig. 7B). To further clarify the direct effect of miR-195 on CX3CL1 expression, we designed a miRNA-masking antisense oligodeoxynucleotides (ODN) of the Cx3cl1 gene to mask the miR-195 binding site of the Cx3cl1 gene. The data showed that co-transfection of NRNs with CX3CL1-ODN (240–246 bp region of the 3’UTR) and miR-195 blocked the inhibitory effects of miR-195 on CX3CL1 expression (Fig. 7B). The results were consistent with immunofluorescence analysis (Fig. 7C). Communication between neurons and microglia is achieved via the binding of the chemotactic factor CX3CL1, which is released from neurons, to CX3CR1, which is located on microglial cells, to elicit the activation of microglia [41, 42]. Therefore, we monitored the expression of CX3CL1 in co-cultured BV2 microglial cells. We found that the miR-195 mimics inhibited the expression of CX3CL1 while AMO-195 increased CX3CL1 expression and that these changes did not occur when NRNs were co-transfected with the miR-195 mimics and AMO-195 or CX3CL1-ODN (Fig. 7D). However, the expression of CX3CR1 on co-cultured BV2 cells was not changed in these groups (Fig. 7E). Importantly, by analysing the microglial polarization of co-cultured BV2 cells, we found that AMO-195 transfection induced a marked increase in the CD68/CD206 ratio and that this effect was prevented by the miR-195 mimics and Cx3cl1-ODN (Fig. 7F). This phenomenon suggests that blocking the release of CX3CL1 from NRNs can prevent the M1 polarization of microglia.

Next, we directly transfected BV2 microglial cells with miR-195 and AMO-195 to observe the regulatory effect of miR-195 on microglial polarization and the levels of CX3CR1, which has been
reported to be expressed on microglial cells [41]. Similar to the effect of miR-195 on CX3CL1 expressed on NRNs, miR-195 inhibited CX3CR1 expression on BV2 microglial cells, and this effect was reversed by AMO-195 and CX3CR1-ODN (Fig. 8A); this suggests that miR-195 regulates CX3CR1 expression by targeting the 3′UTR (the 1236–1242 bp region of the 3′UTR) of the Cx3cr1 gene. Furthermore, we observed transfecting AMO-195 into BV2 cells induced the M1 microglial polarization, which was prevented by the miR-195 mimics and CX3CR1-ODN (Fig. 8B).

MiR-195 prevents microglial polarization towards the M1 phenotype induced by CBH

We next assessed whether miR-195 indeed plays a beneficial role in 2VO-induced microglial polarization. To address this issue, lenti-pre-miR-195 was injected into the hippocampal CA1 region of 2VO rats. Using real-time PCR analysis, we found that lenti-pre-miR-195 injection increased the level of miR-195 in the hippocampus of 2VO rats, suggesting a successful delivery of miR-195 in 2VO rats (Fig. 9A). As predicted, injection of lenti-pre-miR-195 into the hippocampus of 2VO rats effectively reversed 2VO-induced a high number of CD68+ microglia and an increase in the ratio of CD68/CD206 in both the DG and CA1 region (Fig. 9B, D, E & F), but did not significantly affect the number of CD206+ microglial cells (Fig. 9C, E & F). Consistent with these results, injection of lenti-pre-miR-195 prevented an increase in the mRNA levels of TNF-α and IL-1β (Fig. 9G & H) and increased the TGF-β level slightly (Fig. 9I). Accordingly, lenti-pre-miR-195 significantly inhibited the elevation of CX3CL1 and CX3CR1 expression in the hippocampus of 2VO rats (Fig. 9).

Discussion

Microglia play an important role in neurodegenerative diseases [1, 2]. Meanwhile, CBH has been found to be a preclinical phase of AD and VaD [5, 6]. However, how CBH influences the neuroimmunity process is unknown. Here, we first reported that CBH initiates microglial activation in the rat hippocampus from 1 w to 8 w after 2VO surgery. The balance of microglial polarization towards the M1 and M2 phenotypes was shifted towards the M1 phenotype at 8 w following CBH. Further study demonstrated that CBH downregulated the expression of miR-195, which promoted M1 microglial activation through posttranscriptional inhibition of CX3CL1 and CX3CR1 expression. This study provides evidence that miR-195 treatment may be a good strategy for preventing CBH-induced
polarization towards the detrimental M1 phenotype.

Microglia can be activated and polarized towards the proinflammatory M1 phenotype or the anti-inflammatory M2 phenotype [11, 36]. Previous studies have reported that microglia/macrophages respond dynamically to stroke and TBI, exhibiting an early “healthy” M2 phenotype, followed by a transition to a “sick” M1 phenotype [15, 17]. However, studies on the process of microglial polarization during chronic cerebral ischemia are limited. In the current study, we demonstrated that, like acute and severe brain injury, mild chronic brain ischemia induced by CBH also elicited dynamic microglial responses. However, in contrast to what has been observed in severe brain injury, we found that the ratio of CD68/CD206 in Iba-1+ cells in the hippocampus of 2VO rats was approximately 1.0 at 1 w and 2 w after surgery but gradually increased by 4 w and 8 w. The results demonstrated that there was a balance between microglial polarization towards the M1 and M2 phenotypes in the early stage of mild brain ischemia followed by a switch towards the detrimental M1 phenotype. This phenomenon was further proven by significant increase in the levels of M1 markers, such as iNOS, TNF-α and IL-1β, a minor elevation in the expression of M2 markers, such as Arg-1 and TGF-β at 8 w.

Our previous studies reported that CBH lasting for 8 w results in multiple AD-like phenotypes, including Aβ aggregation [7], tau hyperphosphorylation [8], inactivation of protein phosphatase-2A (PP2A) [25] and cell death [9] in rats. Interestingly, all these pathological changes can be regulated by a single microRNA, miR-195. It has been reported that miR-195 targets the inflammatory protein IL-1β in macrophages [42]. Additionally, it reduces M1-like macrophage polarization [28]. In the present study, using the BV2 microglial cell line, we found that blocking endogenous miR-195 by transfection with the antisense oligonucleotide AMO-195, which was similar to LPS, resulted in M1-type microglial polarization. Transfection with the miR-195 mimics inhibited the increase in the percentage of CD68+ cells that were induced by both AMO-195 and LPS administration. Furthermore, we demonstrated that knockdown of miR-195 induced by injection of lenti-pre-AMO-195 into the hippocampus elicited a marked increase in CD68 expression in Iba-1+ cells but had no effect on CD206 expression,
suggesting that miR-195 loss of function can prime detrimental M1 microglial polarization and that supplementation of miR-195 by lenti-pre-miR-195 injection into the hippocampus not only blocks the effects of lenti-pre-AMO-195 but also prevents 2VO-induced M1 microglial polarization.

The CX3CL1/CX3CR1 signalling pathway plays a key role in the process of microglial polarization [18]. However, its function in ischaemic brain injury is controversial [21–23, 39, 44]. Consistent with a previous study in ischaemic stroke mice [26], we found that the expression of both CX3CL1 and CX3CR1 increased significantly in the hippocampus of rats and that this effect was mimicked by knockdown of miR-195. As we predicted, upregulation of miR-195 by lenti-pre-miR-195 injection directly into the CA1 region reversed the elevation of CX3CL1 and CX3CR1 expression in 2VO rats.

CX3CL1 is expressed and secreted from neurons and binds to its receptor, CX3CR1, on the surface of microglia to further regulate microglial polarization [2, 37]. To clarify the role of the CX3CL1 and CX3CR1 proteins in miR-195-mediated M1 polarization in CBH rats, we established a neuronal-microglial co-cultured model. We found that transfecting NRNs with AMO-195 significantly upregulated the expression of CX3CL1 on NRNs without affecting CX3CR1 levels in co-cultured BV2 cells but significantly increased the percentage of Iba-1+ cells that were CD68+; these effects were prevented by co-transfection with miR-195 mimics and CX3CL1-ODN. This result suggest that CX3CL1 is the direct target of miR-195 and mediates miR-195-mediated microglial polarization. To further evaluate the effect of CX3CR1 on miR-195-mediated microglial polarization, we delivered AMO-195 directly to BV2 microglia and observed that the expression of CX3CR1 on BV2 cells was upregulated and that there was an increased number of Iba-1+ cells that were CD68+; these effects were prevented by co-transfection with miR-195 mimics and CX3CR1-ODN. These data suggest that miR-195 controls microglial polarization by governing CX3CL1-CX3CR1 signalling through the direct regulation of CX3CL1 and CX3CR1 expression.

Conclusions

In summary, our study provides convincing evidence that downregulation of miR-195 is involved in CBH-induced microglial polarization towards the M1 phenotype through the activation of CX3CL1-CX3CR1 signalling between neurons and microglia (Fig. 10). The results suggest that increasing miR-
Expression in the brain is a strategy for preventing the CBH-induced neuroimmune response and subsequent brain damage, such as cell death.

**Abbreviations**

AD
Alzheimer’s disease; Arg-1:Arginase-1; CBH:Chronic brain hypoperfusion; CCI:Chronic cerebral ischaemia; HCC:Hepatocellular carcinoma; HD:Huntingdon’s disease; ICH:Intracerebral haemorrhage; *miR-195*: microRNA-195; MFI:Mean fluorescence intensity; NRNs:Neurons; ODN:Oligodeoxynucleotides; PD:Parkinson’s disease; PP2A:protein phosphatase-2A; ROS:Reactive oxygen species; SD rats:Sprague Dawley rats; TBI:Traumatic brain injury; TNF-α:Tumor necrosis factor α; 2VO:Bilateral common carotid artery occlusion.

** Declarations**

**Author’s contributions**

J.A. and M.M. designed the research; M.M., X.Y.Z. analyzed data; M.M., L.Y., Y.X., X.B.A. performed research; J.A. wrote the manuscript; Y.Q. and Y.N.C. provided experimental technical support. Y.R.W. and T.T.L. made revisions to the manuscript; and all authors read and approved the final manuscript.

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**Availability of data and materials**

All data used during the current study available from the corresponding author on reasonable request.

**Ethics approval**

All animal protocols were approved by the Use Committee at Harbin Medical University (No.HMUIRB-2008-06)

**Consent for publication**

Not applicable.

**Competing interests**
The authors declare that they have no competing interests.

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Figures
Figure 1

Microglia activation and polarization in the dentate gyrus (DG) region of 2VO rats. (A&B) Representative images of CD68 expression in Iba-1+cells in hippocampal DG region of sham and 2VO rats at 1W (A) and 8W (B). (C&D) Representative images of CD206 expression in Iba-1+cells in hippocampal DG region of sham and 2VO rats at 1W (C) and 8W (D) by immunofluorescence staining. Scale: 40 μm. (E) Quantification of the Iba-1+cell number in hippocampal DG region. (F) Quantification of the activated microglia number in hippocampal DG region. (G) Quantification of the ratio of CD68 in Iba-1+cells in hippocampal DG region. (H) Quantification of the ratio of CD206 in Iba-1+cells in hippocampal DG region. (I) Quantification of the ratio of CD68 and CD206 in hippocampal DG region. Values are mean ± S.E.M., n= 9 slices from 3 animals per group, *P< 0.05 vs sham group, Student’s t-test.
Figure 2
Microglia activation and polarization in the CA1 region of 2VO rats. (A&B) Representative images of CD68 expression in Iba-1+cells in hippocampal CA1 region 2VO rats at 1W (A) and 8W (B) by immunofluorescence staining. (C&D) Representative images of CD206 expression in Iba-1+cells in hippocampal CA1 region 2VO rats at 1W (C) and 8W (D) by immunofluorescence staining. Scale: 40 μm. (E) Quantification of the Iba-1+cell number in hippocampal CA1 region. (F) Quantification of the activated microglia number in hippocampal CA1 region. (G) Quantification of the ratio of CD68 in Iba-1+cells in hippocampal CA1 region. (H) Quantification of the ratio of CD206 in Iba-1+cells in hippocampal CA1 region. (I) Quantification of the ratio of CD68 and CD206 in hippocampal CA1 region. Values are mean ± S.E.M., n= 9 slices from 3 animals per group, *P< 0.05 vs sham group, Student’s t-test.
CBH induces microglia polarization toward M1 phenotype and inflammatory responses in 2VO rats after 8 weeks. (A) Representative dot plots of CD11b/CD45 cells detected by flow cytometry. Microglia cells within the gate were used for iNOS/Arg-1 analysis. (B) Representative histograms of the mean fluorescent intensity (MFI) for iNOS in sham (red) and 2VO (blue) rats. (C) Representative histograms of the MFI for Arg-1 in sham and 2VO rats. (D) Quantification of MFI for iNOS/Arg-1 from sham and 2VO rats. Bars represent the mean± S.E.M. n=3, cells was pooled from 3 rats per group for a total of 3 separate experiments for each time point. *P < 0.05 vs sham group, Student’s t-test. (E) The MFI ratio between 2VO and sham rats for the expression of iNOS/Arg-1. Bars represent the mean ± S.E.M. n=3, cells was pooled from 3 rats per group for a total of 3 separate experiments for each time point. *P < 0.05 vs iNOS, Student’s t-test. (F) The mRNA expression of TNF-α in the hippocampus of 2VO rats. (G) The mRNA expression of IL-1β in the hippocampus of 2VO rats. (H) The mRNA expression of TGF-β in the hippocampus of 2VO rats. Bars represent the mean± S.E.M. n=6. *P < 0.05 vs sham group, Student’s t-test.
Knockdown of miR-195 prime microglial polarization to M1 phenotype in rats. (A) MiR-195 expression was detected by qRT-PCR in the hippocampus of rats following the stereotaxic injection of lenti-pre-AMO-195 and/or lenti-pre-miR-195 into CA1 region. Bars represent the mean ±S.E.M. n=6. *P < 0.05 vs sham group, #P < 0.05 vs AMO-195. (B) Changes of miR-195 affect the percentage of CD68 in the Iba-1+cells in hippocampal DG, CA1 region of rats. (C) Changes of miR-195 has no effects on the percentage of CD206 in the Iba-1+cells in
hippocampal DG, CA1 region of rats. (D) Changes of miR-195 regulate the ratio of CD68/CD206 in the hippocampal DG and CA1 regions. Bars represent the mean ±S.E.M., n=9 slices from 3 animals per group, *P< 0.05 vs sham group, #P < 0.05 vs AMO-195. (E& F) Representative images of CD68 and CD206 expression in the Iba-1+cells of rat hippocampal DG (E) and CA1 (F) region following the stereotaxic injection of lenti-pre-AMO-195 and/or lenti-pre-miR-195 into CA1 region by immunofluorescence staining. The scale bar was 40 μm. (G) The mRNA expression of TNF-α in the hippocampus of rats following the stereotaxic injection of lenti-pre-AMO-195 and/or lenti-pre-miR-195 into CA1 region. (H) The mRNA expression of IL-1β in the hippocampus of rats following the stereotaxic injection of lenti-pre-AMO-195 and/or lenti-pre-miR-195 into CA1 region. (I) The mRNA expression of TGF-β in the hippocampus of rats following the stereotaxic injection of lenti-pre-AMO-195 and/or lenti-pre-miR-195 into CA1 region. Bars represent the mean ±S.E.M. n=6. *P < 0.05 vs sham group, #P < 0.05 vs AMO-195. All data were analyzed using one-way ANOVA followed by Fisher’s PLSD test.
Figure 5

MiR-195 prevents LPS induced activation of microglia towards M1 profile of cultured BV2 cells. (A) Representative images of CD68 or CD206 expression in Iba-1+ BV2 cells by immunofluorescence staining after transfection of NC, miR-195, AMO-195, miR-195+AMO-195, LPS or LPS+miR-195. Scale bar: 40 μm. (B&C) Quantification of the percentage of CD68 (B) or CD206 (C) in Iba-1+ BV2 cells. (D) Quantification of the ratio of CD68/CD206 in BV2 cells. Bars represent the mean ±S.E.M.; n= 9 from 3 batches of cell culture. *P<0.05 vs NC; #P<0.05 vs AMO-195; $P<0.05 vs LPS. All data were analyzed using one-way ANOVA followed by Fisher’s PLSD test.
Figure 6
Upregulation of the expression of CX3CL1 and CX3CR1 in the hippocampus of 2VO and miR-195 loss-of-function rats. (A&B) Expression of CX3CL1 (red) and CX3CR1 (green) in the hippocampus of sham and 2VO rats was shown by immunofluorescence staining. The scale bar is 40um. (C) Expression of CX3CL1 and CX3CR1 in the hippocampus of sham and 2VO rats was detected by western blot technique. Bars represent the mean ±S.E.M, n=6, *P<0.05 vs sham group, Student’s t-test. (D) Lenti-pre-AMO-195 upregulated the expression of CX3CL1 in rat hippocampus. Mean ± S.E.M., n=6, *P<0.05 vs sham; #P<0.05 vs lenti-pre-AMO-195. Data were analyzed using one-way ANOVA followed by Fisher’s PLSD test. (E) Lenti-pre-AMO-195 upregulated the expression of CX3CR1 in rat hippocampus. Mean ± S.E.M., n=6, *P<0.05 vs sham; #P<0.05 vs lenti-pre-AMO-195. Data were analyzed using one-way ANOVA followed by Fisher’s PLSD test.
Figure 7
Knockdown of miR-195 induced microglia polarized toward M1 phenotype dependent on the CX3CL1-CX3CR1 signaling pathway in vitro. (A) Schematic diagram of the neuron-microglia cells co-culture system. Neurons were transfected with miR-195, AMO-195, miR-195+AMO-195, Cx3cl1-ODN or NC for 48 h. Then BV2 cells were seeded in the top compartment of the transwell with the NRNs were cultured in the bottom compartment. Subsequently, BV2 cells were co-cultured with NRNs for 24 h. (B& C) The effects of miR-195 on endogenous CX3CL1 expression in NRNs by western blotting (B) and immunofluorescence staining (C) after the NRNs were transfected with miR-195, AMO-195, miR-195+AMO-195, miR-195+ Cx3cl1-ODN or NC. (D) MiR-195 downregulated CX3CL1 expression in co-cultured BV2 cells assessed by immunofluorescence staining and western blotting. (E) MiR-195 did not affect CX3CR1 expression in co-cultured BV2 cells evaluated assessed by immunofluorescence staining and western blotting.(F) Downregulating miR-195 increased the ratio of CD68/CD206 in Iba-1+ cells in co-cultured BV2 cells. Bars represent the mean ±S.E.M, n=3 batches of cell culture. *P<0.05 vs NC; #P<0.05 vs AMO-195. Scale bar: 40 μm. All data were analyzed using one-way ANOVA followed by Fisher’s PLSD test.
Knockdown of miR-195 directly in BV2 cells induced microglia polarized toward M1 phenotype dependent on the CX3CR1 expression. (A) The effects of miR-195 on endogenous CX3CR1 expression in BV2 cells by immunofluorescence staining and western blotting after the BV2 cells were transfected with miR-195, AMO-195, miR-195+AMO-195, miR-195+Cx3cr1-ODN or NC. (B) Downregulating miR-195 directly in the BV2 cells increased the ratio of CD68/CD206 in Iba-1+ cells. Bars represent the mean ±S.E.M, n=3 batches of cell culture. *P<0.05 vs NC; #P<0.05 vs miR-195. Scale bar: 40 μm. All data were analyzed using one-way ANOVA followed by Fisher’s PLSD test.
MiR-195 prevented the microglia polarization to M1 phenotype induced by 2VO surgery. (A) MiR-195 expression in the hippocampus of 2VO rats at 8W with or without lenti-pre-miR-195 treatment was detected by qRT-PCR. Bars represent the mean ± S.E.M. n=6. *P < 0.05 vs sham group, #P < 0.05 vs 2VO. (B) MiR-195 decreased the percentage of CD68 in the Iba-1+cells in hippocampal DG and CA1 region of 2VO rats. (C) MiR-195 did not affect the percentage of CD206 in the Iba-1+cells either in hippocampal DG region or CA1 region of
2VO rats. (D) MiR-195 reversed the increased ratio of CD68/CD206 in the hippocampal DG and CA1 regions of 2VO rats. Bars represent the mean ± S.E.M., n= 9 slices from 3 animals per group. *P < 0.05 vs sham group, #P < 0.05 vs 2VO. (E& F) Representative images of CD68 and CD206 expression in the Iba-1+ cells of hippocampal DG (E) and CA1 (F) region of 2VO rats following the stereotaxic injection of lenti-pre-miR-195 into CA1 region. The scale bar was 40μm. (G) Lenti-pre-miR-195 injection reversed the increased mRNA level of TNF-α in the hippocampus of 2VO rats. (H) Lenti-pre-miR-195 injection reversed the increased mRNA level of IL-1β in the hippocampus of 2VO rats. (I) Lenti-pre-miR-195 injection did not affect TGF-β level in the hippocampus of 2VO rats. (J) Lenti-pre-miR-195 injection inhibited the increased CX3CL1 and CX3CR1 in the hippocampus of 2VO rats. Bars represent the mean± S.E.M., n= 6. *P < 0.05 vs sham group, #P < 0.05 vs 2VO. All data were analyzed using one-way ANOVA followed by Fisher’s PLSD test.
Schematic for the mechanisms of CBH induces M1 phenotype of microglial polarization. CBH downregulates miR-195 and may induce microglial polarization toward to M1 phenotype in two ways: 1) Downregulated miR-195 upregulates the expression of CX3CL1 by binding with the 3’UTR of Cx3cl1 gene in neuron that were subsequently released and bound to its receptor CX3CR1 in microglia, which further results in the microglial polarization toward M1 phenotype; 2) Downregulated miR-195 upregulates the expression of CX3CR1 by binding with the 3’UTR of Cx3cr1 gene in microglia and results in the microglial polarization toward M1 phenotype directly.