Journal Pre-proofs

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PII: S0006-2952(20)30211-2
DOI: https://doi.org/10.1016/j.bcp.2020.113983
Reference: BCP 113983

To appear in: *Biochemical Pharmacology*

Received Date: 17 February 2020
Accepted Date: 15 April 2020

Please cite this article as: Z. Peng, M. Li, X. Tan, P. Xiang, H. Wang, Y. Luo, Y. Yang, H. Huang, Z. Chen, H. Xia, Y. Li, J. Zhang, C. Gu, M. Liu, Q. Wang, M. Chen, J. Yang, miR-211-5p alleviates focal cerebral ischemia-reperfusion injury in rats by down-regulating the expression of COX2, *Biochemical Pharmacology* (2020), doi: https://doi.org/10.1016/j.bcp.2020.113983

miR-211-5p alleviates focal cerebral ischemia-reperfusion injury in rats by down-regulating the expression of COX2

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Abstract

The present study was to investigate the role of microRNA (miR)-211-5p on cerebral ischemia-reperfusion injury (CIRI) and clarify its underlying mechanisms. Middle cerebral artery occlusion/reperfusion (MCAO/R) was operated on male Sprague Dawley (SD) rats, oxygen-glucose deprivation/reperfusion (OGD/R) was conducted on pheochromocytoma-12 (PC12) cells. Here, we found that miR-211-5p and Cyclooxygenase (COX2) expressions were altered in the plasma, cortex and hippocampus of MCAO/R-treated rats, as well as in the OGD/R-treaded PC12 cells. In vivo, overexpression of miR-211-5p resulted in decrease of infarct volumes, neurological deficit scores and histopathological damage. In vitro, miR-211-5p overexpression significantly decreased cell apoptosis and Lactate dehydrogenase (LDH) release rate, increased cell viability. Furthermore, our data showed that miR-211-5p overexpression markedly reduced the expressions of COX2 mRNA and protein, and the contents of Prostaglandin D2 (PGD2), PGE2, Tumor necrosis factor-α

(TNF-α) and Interleukin-1β (IL-1β). In addition, inhibition of COX2 significantly rescued the effects of miR-211-5p inhibitor. At last, dual luciferase experimental data showed that miR-211-5p regulated the mRNA stability of COX2 by directly binding to the 3'-untranslated region (3'-UTR) of COX2. In conclusion, our data suggested the neuroprotective effects of miR-211-5p on CIRI by targeting COX2.

Keywords:COX2, miR-211-5p, stroke, CIRI.

Abbreviations

COX-2	Cyclooxygenase-2
CNS	Central nerve system
CIRI	cerebral ischemia-reperfusion injury
MTT	Thiazolyl Blue Tetrazolium Bromide
LDH	Lactate dehydrogenase
3'-UTR	3'-Untranslated region
TTC	2,3,5-Triphenyltetrazolium chloride
HE	Hematoxylin and eosin
OGD/R	Oxygen and glucose deprivation/reperfusion
MCAO/R	Middle cerebral artery occlusion/reperfusion
miRNA	MicroRNA
SD	Standard deviation
PGs	Prostaglandins

1. Introduction

Stroke is a kind of cerebral blood circulation disorder, which is one of the

leading causes of long-term neurological disability. It has high morbidity, high disability, high mortality [1, 2]. Stroke has been the leading cause of death in recent years in China [3], constituting almost one-third of the total number of deaths from stroke worldwide [4]. Furthermore, the burden of stroke has increased over the past 30 years, especially in rural areas [5].

Ischemic stroke occupies approximately 75%-85% of all strokes [2], which is a pathological condition characterized by an initial restriction of blood supply to brain, The treatment is usually to restore perfusion and reoxygenation in clinically.However, reoxygenation and restoration of blood flow is frequently associated with exacerbation of tissue injury and profound inflammatory response, called cerebral ischemia-reperfusion injury (CIRI) [6]. Due to its mechanism is still unclear, there are great difficulties in the treatment and prognosis of stroke in clinical [7]. Studies have shown that inflammation/immune response plays a key role in the pathogenesis of cerebral ischemia- reperfusion injury, which acts a dual role in promoting tissue damage and repair. Therefore, inhibiting neuroinflammation induced by cerebral ischemic stroke is becoming an attractive strategy in stroke therapy [8]. During the reperfusion, energy depletion, intracellular calcium Ca^{2+} overload, excitotoxicity and inflammation responses in the ischemic area lead to massive cells necrosis [9], which results in the production of a large number of inflammatory factors and inflammatory mediators such as IL-1β, TNF-α, IL-6, IL-10, NF-κB and TGF-β [10]. At the same time, cells necrosis causes a large release of cell membrane phospholipids, resulting in accumulation of arachidonic acid (AA) and activation of the downstream

inflammatory pathway. Cyclooxygenase (COX) metabolic pathway plays a most important role in inflammation, that catalyze prostaglandins (PGs) by AA to aggravate the inflammatory response cycle. It has been reported that the expression of COX precede the appearance of inflammatory factors in neurons and glial cells after brain injury, and further promote the transformation of microglia and the release of a large amount of inflammatory factors and inflammatory mediators [11]. Therefore, activation of COX expression and increased levels of inflammatory factor may be closely related to cerebral ischemia-reperfusion injury.

COX is mainly classified into COX1, COX2 and COX3. *Bela et al* found that COX3 mRNA levels were higher in major brain arteries and microvessels, which indicated that the expression pattern of COX3 mRNA in the central nervous system (CNS) of rats is mainly related to the vascular density in a given area [12]. However, COX1 and COX2 are both constitutively expressed in the CNS. Human brain autopsy found that the expression of COX1 was weak in control brain tissue neurons, but high expression in microglia. In the acute phase of focal ischemic injury, the expression of COX2 was up-regulated in microglia and neurons, and decreased in the subacute phase. These COX2-immunoreactive neurons accumulated in the peri-infarct regions, but were absent from the distant regions [11]. Our last study has shown that COX2 was overexpressed in the cortex of T2DM rat [13]. In addition, we also found that the expression of COX2 in the hippocampus of rats with global cerebral ischemia-reperfusion increased significantly in a time-dependent manner: increased 30 min after reperfusion, peaked on the 7th d, and remained significantly higher than

normal until on the 15th d [14]. In middle artery ischemia/reperfusion (MCAO/R)-treated rats, focal neuronal necrosis and degeneration were more stronger in the core, whereas inflammatory infiltration, INOS, COX2 expressions were richer in the penumbra [9]. These results suggested that COX2 is closely related to neuronal damage caused by stroke. Some studies have indicated that COX2 inhibitors could provide an expanded treatment window for CIRI and protect vulnerable brain tissue from secondary damage caused by reperfusion. *Santos-Galdiano, M., et al* have found that celecoxib which is a specific COX2 inhibitor could reduce selective neuronal loss and extenuate neurologic deficit in rats after MCAO operation [15]. Parecoxib, a specific COX2 inhibitor, has been confirmed attenuating postischemic neuronal apoptosis by phosphorylating Akt and GSK-3β [16]. However, the side effects of COX2 inhibitors limited their clinical application. Therefore, it is necessary to clarify the upstream regulatory mechanisms of endogenous expression of COX2, such as the ability to intervene from the upstream of COX2 endogenous expression [17].

In the past decade, non-coding RNA (nc RNA) has attracted people's attention. These include rRNA, tRNA, snRNA, LncRNA, microRNA and et al. miRNAs are endogenous short (21~25 nucleotides) single-stranded RNAs[18]. Previously, miRNAs have been focused on cancer research and people tried to use miRNA as a specific marker for cancer. In addition to cancer, more and more studies have shown that it plays an important role in the regulation of gene expression in the pathophysiological processes of various organisms [19]. In CNS, various pathological conditions such as stroke, schizophrenia and neurodegernerative diseases significantly

altered cerebral miRNA profiles and affect the disease outcome [20-23]. microRNA-211-5p (miR-211-5p) is available in the miRNA database under the accession number MIMAT0000882, with a total length of only 22 bps. It is conserved and localized on the chromosome 15 of human beings. [24]. Previous study have identified miR-211-5p suppresses tumour cell proliferation, invasion, migration and metastasis in triple-negative breast cancer by directly targeting SETBP1 [25]. In another experiment, miR-211-5p was found to play an important role in Alzheimer's disease (AD) [26]. In this study, we found that miR-211-5p was altered in the brain following transient cerebral ischemia. As mentioned above, inflammation plays an important role in ischemia-reperfusion injury. Therefore, we will investigate the effect of miR-211-5p on neuroinflammation induced by CIRI and explore the potential mechanisms. This study will provide novel therapeutic targets for CIRI in clinical.

2. Material and methods

2.1 Animals and Experimental Groups

Adult male Sprague Dawley rats (SD, 250-280g, 46-54d) were purchased and raised at the Laboratory Animal Center, Chongqing Medical University, China (license number: SYXK YU 2012-0001). The rats were housed in the same temperature and humidity-controlled animal facility with a 12-h light/dark cycle.

For the time course study, SD rats were randomly separated into 6 groups: sham group and MCAO/R groups (The brains of rats were removed at 6h, 12h, 24h, 48h and 72h after MCAO/R) (n=3).

For the mechanism study, SD rats were randomly separated into 6 groups: (1) sham group (n=10), (2) MCAO/R+vehicle group (n=10), (3) MCAO/R+miR-211-5p agomir group (n=10), (4) MCAO/R+agomir negative control group (n=10), (5) MCAO/R+miR-211-5p antagomir group (n=10), (6) MCAO/R+antagomir negative control group (n=10). The brains of rats were removed at 24h after MCAO/R operation.

2.2 Middle Cerebral Artery Occlusion/Reperfusion Model

Ischemic stroke was produced by using the MCAO/R method as previously reported [27, 28]. Rats were deeply anesthetized with intraperitoneal injection of sodium pentobarbital (40 mg/kg). Blunt dissection was performed to expose the right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA). A nylon suture (*Jialing, Guangzhou, China*) was inserted into the CCA until it blocked the origin of the middle cerebral artery. 90 mins after MCAO, the suture was withdrawn to reperfusion. The sham group received the same surgical without occlusion. Laser Doppler flowmetry (*Periflux System 5000, Sweden*) was used to monitor the cerebral blood flow (CBF) in ischemia (regional CBF $\leq$ 80 of baseline) and reperfusion (regional CBF $\geq$ 70 of baseline). Animals dying before the chosen endpoint or no infarction except for sham were eliminated.

2.3 Bioinformatics miRNAs Prediction

TargetScan(http://www.targetscan.org),miRBase(http://www.ebi.ac.uk/enright-srv/microcosm), and miRanda (http://www.microRNA.org) were used to target prediction databases to assess which miRNAs target COX2 gene. miRNAs present in all three

databases were used for in vivo screening.

We used bioinformatics (http://www.targetscan.org) to predict the binding score of COX2 and miRNAs and found that miR-211-5p got a higher score than miR-204-5p. Therefore, we chose the miR-211-5p for our further studies.

2.4 The siRNA of COX2, The agomir, antagomir, mimic, inhibitor and relative NC of miR-211-5p

Three candidate siRNA duplexes against COX-2 were synthesized from *Ribobio* (*guangzhou, China*) with the sequences: siRNA-1, sense 5′-GGAUUUGACCAGUAUAAGUTT-3′, antisense 5′-ACUUAUACUGGUCAAAUCCTG-3′; siRNA-2, sense 5′-AGACAGAUCAUAAGCGAGGTT-3′, antisense 5′-CCUCGCUUAUGAUCUGUCUTT-3′; siRNA-3, sense 5′-AACCUCGUCCAGAUGCUAUTT-3′, antisense 5′-AUAGCAUCUGGACGAGGUUTT-3′. The miR-211-5p mimic was a duplex RNA, with the sense sequence (5'-UUCCCUUUGUCAUCCUUUGCCU-3') and the antisense sequence (5'-AGGCAAAGGAUGACAAAGGGAA-3'), the miR-211-5p inhibitor was a single RNA sequence (5'-AGGCAAAGGAUGACAAGGGAA-3') exactly complementary to miR-211-5p. Non-targeting negative control sequence (for mimic: sense, 5'-UUUGUACUACACAAAAGUACUG-3'; antisense, 5'-CAGUACUUUUGUAGUACAAA-3'; for inhibitor, 5'-CAGUACUUUUGUAGUACAAA-3') were used as controls. The inhibitor and mimic were used only in vitro experiments. The miR-211-5p agomir and antagomir were the same sequences with mimic and inhibitor, respectively, but had more affinity in tissue cells. The agomir and antagomir were used in vivo experiments.

2.5 Intracerebroventricular injection

The miR-211-5p agomir, antagomir, agomir NC, antagomir NC were purchased from *Genepharma* (*Shanghai, China*) and dissolved in artificial cerebrospinal fluid (ACSF) (*Dingguo, Beijing, China*). The miR-211-5p agomir and agomir NC were respectively injected into the right brain lateral ventricle with 5 min in total volume of 5 μl at the concentration of 50 μM, while the miR-211-5p antagomir and antagomir NC were injected at the concentration of 100 μM. The entire operation was performed at 1day before MCAO on the stereotaxic apparatus (*RWD, Shenzhen, China*). Stereotactic coordinates were previously described: anteroposterior, 0.8 mm; mediolateral, 1.5 mm; depth, 3.5 mm [29].

2.6 Neurological Scoring

The neurological dysfunction of rats was evaluated at 24 h after reperfusion as previously described. The neurological scores were performed according to the five-point scale: score 0, normal motor function; score 1, failure to extend to the right forelimb; score 2, circling to the contralateral side; score 3, falling to the contralateral side at rest; and score 4, no spontaneous motor activity. Animals that died during the process were not included in the statistics.

2.7 2,3,5-Triphenyltetrazolium Chloride Staining

The brains were sliced into five 2 mm serial coronal sections. All five sections were stained with 2% 2,3,5-Triphenyltetrazolium Chloride Staining (TTC) (sigma, *USA*) at 37° C for 10 min in the dark and then turned over for 10 minutes, followed by overnight immersion in 4% paraformaldehyde. Normal brain tissue was stained red,

while the infarct tissue was unstained (white). The integrated volume was measured by Image J software (*NIH, USA*). The total volumes of infarction were calculated by integrating all five sections as previously described (area of non-ischemic hemisphere - area of non-ischemic tissue in the ischemic hemisphere)/area of non-ischemic hemisphere.

2.8 Histopathological Examination

Hematoxylin and eosin (HE) staining was performed to show pathological histological damage in the cerebral cortex and hippocampus. At 24h after reperfusion, the rats were anesthetized with sodium pentobarbital and perfused with PBS, and then perfusion with 4% paraformaldehyde. After that, the brains were dehydrated in a graded series of alcohols and embedded in paraffin. A series of 5-μm-thick sections were cut from the brain. Finally, the sections were stained with HE reagents for histopathological examination.

2.9 PC12 cell culture and oxygen-glucose deprivation/reperfusion (OGD/R)

The rat pheochromocytoma-12 (PC12) cell line is regarded as an established neuron-like system that can be used as a cell model for neuron. PC12 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (*DMEM, Gibco*) supplemented with 10 % heat-inactivated fetal bovine serum(*FBS, Gibco*), 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere of 5 % CO_2 at 37℃.

The culture media of PC12 cells was replaced with glucose-free DMEM before oxygen-glucose deprivation (OGD) operation. Then the cells were put into the

incubator filled with 1.0% oxygen, 5% CO_2 and 94% N_2 for 2h. After OGD operation, the cells were cultured in normal culture medium with oxygen.

2.10 Real-Time Polymerase Chain Reaction

Total RNA was isolated from the rat cerebral cortex and hippocampus and PC12 cells by Trizol reagent (*Vazyme, Nanjing, China*) according to the manufacturer's protocol. mRNA was subjected to reverse transcription using HiScript Q Select RT SuperMix (*Vazyme, Nanjing, China*), while microRNA was subjected to reverse transcription using miRNA First Strand cDNA Synthesis reagent (*Sangon, Shanghai, China*). To detect the amount of COX2 mRNA and miR-211-5p, SYBR Green II (*Biomake, USA*) incorporation method was applied with β-actin being an internal control of mRNA， and U6 was an internal reference of miRNA. The primer sequences are reported in Table 1.

Table 1. List of primers used in qPCR analysis.

GENE	FORWARD Sequence (5′->3′)	REVERSE Sequence (5′->3′)
U6	CCTGCTTCGGCAGCACA	
miR-204-5p	CGCTTCCCTTTGTCATCCTATGCCT	
miR-211-5p	CGTTCCCTTTGTCATCCTTGCCT	
miR-23a-3p	GCGATCACATTGCCAGGATTTCC	AACGCTTCACGAATTTGCGT
miR-23b-3p	CGGATCACATTGCCAGGGATTACC	(Tailing Reaction)
miR-490-3p	CAACCTGGAGGACTCCATGCTG	
miR-219a-3p	CGCGTGATTGTCCAAACGCAATTCT	
COX2	CTTCCTCCTGTGGCTGATGACTG	GGTCCTCGCTTCTGA TCTGTCTTG
β-actin	GGTCCTCGCTTCTGA TCTGTCTTG	GGTCCTCGCTTCTGA TCTGTCTTG

2.11 Western Blotting

Samples were lysed with RIPA containing 1% phenylmethanesulfonyl fluoride (PMSF) (100 mM). The sample lysis buffer was collected into an EP tube and centrifuged at 12,000 rpm and at 4°C for 15min. Then abandoned the precipitate and detected the

protein concentration using BCA protein assay kit (*Beyotime, China*). We performed Western blotting as described previously. In briefly, proteins were separated and transferred onto apolyvinylidene fluoride (PVDF) membrane (*Millipore, USA*). The membrane was incubated with primary antibodies overnight at 4°C. After washing, the membrane was incubated with horseradish peroxidase-conjugated secondary antibodies (*Proteintech, Wuhan, China*) for 1h at RT. At last, the immunoblots were developed by ECL western blotting detection reagent (*Millipore, USA*). The primary antibodies against COX2 (dilution 1:400, *Proteintech, Wuhan, China*), and β-actin (dilution 1:4000, *Proteintech, Wuhan, China*) were purchased from Abcam.

2.12 Immunofluorescence

At 24h after reperfusion, the rats were anesthetized with sodium pentobarbital and perfused with PBS, and then perfusion with 4% paraformaldehyde. After that, the brains were dehydrated in a graded series of alcohols and embedded in paraffin. A series of 5-μm-thick sections were cut from the brain. Sections were rinsed with PBS and permeabilized with 0.3% Triton X-100 in PBS for 30 min. Specimens were blocked with 10% goat serum for 1h and in-cubated at 4°C overnight with a primary rabbit monoclonal anti-COX2 antibody (1:100, *Proteintech, Wuhan, China*) and a primary mouse monoclonal anti-NEUN antibody (1:100; *Proteintech, Wuhan, China*) (Neun, a marker protein of neuron) followed by an Alexa 488-labeled goat anti-rabbit IgG (1:500, *Beyotime, Wuhan, China)* and an Alexa 568-labeled goat anti-mouse IgG respectively for 2h at 37°C. NEUN was considered a marker protein for neurons and could be used to quantify the number of neurons. DAPI was applied to stain all the

nuclei as a background staining. Imaging was performed on a laser scanning confocal micro-scope (*Eclipse TE2000U; Nikon*) with the Nikon EZ-C1 software.

2.13 Cell Transfection

PC12 cells were cultured in the 96-well (or 6-well) plates at 1×10^5 cells/ml. COX2 siRNA, miR-211-5p mimic, miR-211-5p mimic NC, miR-211-5p inhibitor and miR-211-5p inhibitor NC were transfected by Ribobio transfection kit (*Ribobio, Guangzhou, China*). After transfection for 24 h, OGD/R operation was performed.

2.14 Biochemistry test

The MDA content and SOD activity were measured according to the instruction manual of the kit (*Beyotime, China*). The contents of PGD_2, PGE_2, IL-1β, TNF-α were detected using commercially available enzyme linked immunosorbent (ELISA) assay kit (*Meibiao, Jiangsu, China*) according to the manufactures' instructions.

2.15 MTT Assay

PC12 cells were cultured in the 96-well plates at 1×10^5 cells/mL and treated as described above. After OGD/R treatment for 24 h, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (*MTT, 20 μL, 5 mg/ml; Sigma, USA*) was added per well and incubated at 37° C for 4 h. Then 150 μL dimethyl sulfoxide (DMSO) was added to each well. After the formazan was dissolved, the optical density (OD) was determined at 490 nm on a multi-function microplate reader (*Thermo Scientific, USA*).

2.16 LDH Release Detection

Cell death was evaluated by the lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (*Jiancheng, nanjing, China*). PC12 cells were cultured in 96-well plates at 1×10^5

cells/ml. After OGD/R treatment for 24 h, the LDH release rate in the cell culture supernatant was detected according to the manufacturer's instruction.

2.17 Flow Cytometry Analysis

PC12 cells were cultured in six-well plates at 1×10^5 cells/mL and treated as described above. After OGD/R treatment for 24 h, cells were trypsinized without EDTA, collected and dispersed in 1 ml PBS. Apoptosis was determined by flow cytometry by the Annexin V-FITC/propidium iodide (annexin V/PI) apoptosis detection kits according to the manufacturer's protocol.

2.18 Dual Luciferase Reported Assay

we constructed luciferase reporter vectors containing the normal COX2 nucleotides, the mutated nucleotides in the first binding site (nucleotides 178-185) and the second binding site (nucleotides 224-230). Plasmids containing either the wild-type or mutant COX-2 3′-UTR were then co-transfected with the miR-211-5p mimic in HEK 293 cells. After 48 h, the firefly and luciferase activity were detected with the dual-luciferase reporter assay system (*Promega*) according to the manufacturer's instructions. Firefly luciferase activity was normalized to Renilla luciferase activity for each transfected well. The experiments were performed in triplicate.

2.19 Statistical Analysis

The values are presented as the mean ± SD. Statistical significance was determined using t test to compare two groups or one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test for multiple comparisons. Statistical analysis was performed by GraphPad Prism 5 (*GraphPad Software, USA*). P value of

less than 0.05 was regarded as statistically significant.

3. Results

3.1 The expressions of miRNAs in the cortex, hippocampus and plasma of MCAO/R-treated rats

To determine whether miRNAs had changed in CIRI, we detected miRNAs expressions in the brain of SD rats by RT-PCR. There was no significant difference between the normal group and the sham group. While compared with the sham group, miR-211-5p markedly decreased in cortex and increased in hippocampus in MCAO/R group (Fig. 1A, 1B). Interestingly, the expression of miR-211-5p markedly decreased in the plasma of MCAO/R-treated rats, while the expressions of COX2 mRNA and protein significantly increased (Fig. 1C-1F).

3.2 The spatio-temporal expression of COX-2 and miR-211-5p in the cortex and hippocampus of MCAO/R-treated rats, as well as in OGD/R-treated PC12 cells

Compared with sham group, COX2 mRNA expression first increased and then decreased in the cortex and hippocampus of MCAO/R-treated rats and OGD-treated PC12 cells, peaked at different time (Fig. 2D, 2E and 2F). miR-211-5p expression significantly decreased in cortex and reached nadir at 12 h (Fig. 2A), while miR-211-5p expression significantly increased in hippocampus and peaked at 48 h (Fig. 2B), miR-211-5p expression significantly increased in OGD-treated PC12 cells and peaked at 24 h (Fig. 2C).

3.3 Effect of miR-211-5p on MCAO/R-treated rats

To determine whether miR-211-5p had effects on CIRI, the TTC assay was used to

detect the brain infarct size. Representative images of TTC-stained brain sections are shown in Fig. 3A. Evidently, the infarct volume was significantly increased in vehicle treated MCAO/R group. Compared with the vehicle treated MCAO/R group, the administration of miR-211-5p agomir significantly decreased the infarct volume and neurological score, miR-211-5p antagomir was significantly increased the infarct volume and neurological score (Fig. 3B, 3C).

To determine whether the brain had histopathological changes, HE staining was conducted. In the sham group, nerve cells were arranged tightly in order, and most cells were round or oval with large cell bodies. In contrast, in the vehicle, agomir NC and antagomir NC groups, nerve cells presented significant nuclear pyknosis, vacuolization and disordered arrangement. Compared with the vehicle treated MCAO/R group, treatment with miR-211-5p agomir reduced the nuclear deep staining of the nucleus, the vacuolization and the mortality of nerve cells. However, treatment with miR-211-5p antagomir aggravated the damage compared with the vehicle group (Fig. 3D-G) (HE 100×, 200×, 400×). In short, miR-211-5p had protective effect on CIRI.

3.4 Effect of miR-211-5p on CIRI via targetting COX2

we examined whether miR-211-5p had effects on COX2 expression. Compared with the sham group, the expressions of COX2 mRNA and protein were significantly increased in the cortex and hippocampus of MCAO/R-treated rats. Compared with the vehicle treated MCAO/R group, treatment with miR-211-5p agomir significantly decreased the expressions of COX2 protein and mRNA, while treatment with

miR-211-5p antagomir significantly increased the expressions of COX2 protein and mRNA (Fig. 4A -4E). In short, our data showed that miR-211-5p overexpression markedly reduced the expressions of COX2 mRNA and protein.

3.5 Effect of miR-211-5p on the expression of COX2 protein in neurons of cortex and hippocampus

In order to determine whether the endogenous expression of COX2 protein had changes on neurons, immunofluorescence was used to detect the COX2 fluosecent intensity in the cortex and hippocampus of rats. Compared with the sham group, the expression of COX2 protein were significantly increased in the neurons of the cortex and hippocampus of rats following MCAO/R injury. Compared with the vehicle treated MCAO/R group, miR-211-5p overexpression markedly decreased the expression of COX2 protein in neurons of cortex and hippocampus in miR-211-5p agomir group, while miR-211-5p inhibition significantly increased the expression of COX2 protein in miR-211-5p antagomir group (Fig. 5A-D). Our immunofluorescence results showed that miR-211-5p overexpression significantly reduced the positive neurons that expressed COX2.

3.6 Effect of miR-211-5p on changes of PGD2, PGE2, TNF-α, IL-1β content in cortex and hippocampus of MCAO/R treated rats

To determine whether related products and inflammatory factors had changed, ELISA assay was used to test PGD2, PGE2, TNF-α, IL-1β content in the rats' cortex and hippocampus. Compared with the sham group, the rat cortical and hippocampal PGD2, PGE2, TNF-α, IL-1β content were significantly increased in the vehicle treated

MCAO/R group. Compared with the vehicle treated MCAO/R group, treatment with miR-211-5p agomir significantly blunted the increase of rat cortical PGE2, TNF-α and IL-1β content and rat hipocampal PGD2, TNF-α and IL-1β content caused by MCAO/R. However, treatment with miR-211-5p antagomir significantly blunted the decrease of rat cortical PGE2, TNF-α and IL-1β content and rat hipocampal PGD2, TNF-α and IL-1β content caused by MCAO/R (Fig. 6A-H).

3.7 Effect of miR-211-5p on OGD/R-treated PC12 cells

To explore whether miR-211-5p had effect on OGD/R-treated PC12 cells, the miR-211-5p mimic or miR-211-5p inhibitor and their corresponding negative control were transfected into OGD/R-treated PC12 cells. As shown in Fig. 7A, 7B and 7C, compared with the normal group, cell viability, LDH release and apoptosis in the OGD/R group were significantly altered. These suggested that treatment with OGD/R significantly damaged cells. At the same time, the results shown that miR-211-5p mimic had significant protective effects on OGD/R-treated PC12 cells, while miR-211-5p significantly aggravated the damage of OGD/R.

Next, we tested changes of some biochemical indicators. Compared with the normal group, the expressions of COX2 protein, COX2 mRNA, PGD2 and PGE2 were significantly increased in the OGD/R group. Treatment with miR-211-5p mimic significantly decreased the expressions of COX2 protein, COX2 mRNA, PGD2 and PGE2, while treatment with miR-211-5p inhibitor significantly increased the expressions of those indicators(Fig. 7D, 7E, 7F and 7G). In short, miR-211-5p had significant protective effects on OGD/R-treated PC12 cells by inhibiting COX2.

3.8 COX2 is a direct target of miR-211-5p

To prove that there is a binding site for miR-211 in the 3′-UTR of COX-2 mRNA, we identified the potential binding site of miR-211-5p by using the starbase program, two potential binding sites in the 3′-UTR of COX2 mRNA were found as being targeted by miR-211-5p (Fig. 8A). To obtain direct evidence that COX2 mRNA was a target of miR-211-5p, we constructed luciferase reporter vectors containing the normal COX2 nucleotides, the mutated nucleotides in the first binding site (nucleotides 178-185) and the second binding site (nucleotides 224-230). Plasmids containing either the wild-type or mutant COX-2 3′-UTR were then co-transfected with the miR-211-5p mimic in HEK 293 cells. The miR-211-5p mimic inhibited luciferase activity in the HEK 293 cells transfected with the wild-type COX-2 3′-UTR, but not in the cells transfected with the mutated COX2 3′-UTR (Fig. 8B). The data suggested both binding sites of 3’-UTR of COX2 are targeted by 211-5p. Moreover, We did a series of reply experiments. First, we designed three siRNA to knockdown the expression of COX2. As we shown in Fig. 8C, COX2 siRNA-3 significantly reduced the expression of COX2. Next, we co-transfected miR-211-5p inhibitor and COX2 siRNA-3 in OGD/R-treated PC12 cells. Compared with the miR-211-5p inhibitor group, cell viability was significantly increased (Fig. 8D) and LDH release rate was significantly decreased (Fig. 8E) in the co-transfection group, moreover, COX2 siRNA-3 significantly reversed the up-regulation of COX2 protein by miR-211-5p inhibitor(Fig. 8F). In short, the data suggested that COX2 siRNA could reverse the damages of miR-211-5p by inhibiting COX2.

4. Disscion

Inflammation plays an important role in the occurrence and development of central nervous system diseases [30-32], microglia are the main immune cells of central nervous system (CNS), and its function is similar to macrophage [33]. When there are foreign antigen substances or neuronal cellular debris, microglia could quickly be activated and produce kinds of cytokines to promote the occurrence and development of inflammatory reaction [34]. When cell fragments or antigen substances are removed, microglia will return to the resting state [35]. Although microglia are the primary immune cells in the brain [36], neurons have been found to be closely related to inflammatory injury by some experts, *Sriramula et al* found that the activation of Kinin B1 Receptor II in mouse neonatal primary hypothalamic neuronal cultures upregulated several proinflammatory genes (IL-1β, IL-6, and TNFα), and increased NF-kB p65 DNA binding activity [37]. Meanwhile, neurons play a leading role in the regulation of human body, and the pathological features of many neurological diseases include the injury and loss of neurons[38]. Generally speaking, the sustained damage of neurons contribute to secondary brain ischemic injury and represent the main cause of cerebral injury aggravation [39, 40]. Therefore, We think it is more meaningful to research the inflammatory damage of neurons.

In the present study, we investigated the effects of miR-211-5p on CIRI. Here we showed that miR-211-5p decreased in the plasma of MCAO/R-treated rats as well as in the cortex. According to previous reports, plasma or serum miRNAs have been identified as diagnostic biomarkers for disease [41, 42], it suggested that miR-211-5p

may serve as a biomarker for stroke diagnosis or a potential target for treatment. In our study, we showed the spatio-temporal expressions of COX2 and miR-211-5p in the cortex and hippocampus of MCAO/R-treated rats and OGD/R-treated PC12 cells, the results suggested that there is significant negative correlation between COX2 and miR-211-5p. In our previous experiments, we tried to select primary neurons for in vitro experiments, but the transfection efficiency of primary neurons was so low that we had to give up (data not shown). Because of the neuron-like function, the PC12 cell was widely used in in vitro experiments to simulate neurons [43-45]. Therefore, PC12 cells were selected as experimental cells for in vitro study.

More and more experiments had shown that miRNA is involved in regulating mRNA stability and translation [19]. Small RNA technology can efficiently regulate the expression of specific genes, but their therapeutic application faces numerous challenges because of its off-target mechanism and potency [46]. However, a new era for the field was marked with the US Food and Drug Administration approving in august 2018. Patisiran, the first RNAi-based drug, was used to target a rare condition that can impair heart and nerve function by a lipid nanoparticle (LNP) delivery formulation, which brought light to the research of nucleic acid drugs [47]. Therefore, it is very meaningful to explore small molecule drugs. Our study demonstrated that miR-211-5p had protective effects on CIRI. MiR-211-5p agomir significantly reduced infarct volume and histopathological damage of MCAO/R-treated rats, while miR-211-5p antagomir aggravated the damage. Interestingly, histopathological damage of hippocampus in MCAO/R-treated rats appeared in different positions,

mainly in the CA1 area, and also appeared in the CA2 and CA3 areas. *Junqing Yang et al* also found this phenomenon in delayed brain injury of mice induced by subacute carbon monoxide [48], which may be caused by individual differences. In vitro, miR-211-5p also showed obvious promotion of proliferation and alleviation of apoptosis on OGD/R-treated PC12 cells.

COX2 is a key rate-limiting enzyme in the inflammatory pathway [13]. Previously, most studies on the effect of miRNA on COX2 have been conducted in tumors [49, 50]. MiR-146a affects the prognosis of colon cancer by regulating COX2 expression and cell apoptosis [49], miR-136 inhibits malignant progression of hepatocellular carcinoma cells by targeting COX2 [50]. Furthermore, we showed the spatio-temporal expressions of COX2 and miR-211-5p in the cortex and hippocampus of MCAO/R-treated rats, as well as in OGD/R-treated PC12 cells. The results suggested that there is significant negative correlation between them. Consistent with *Weidong Yu et al*, the expression of COX2 mRNA first increased and then decreased, indicating that the level of inflammation increased first and then decreased in the MCAO/R model [51, 52]. However, miR-211-5p appeared two different trends in the cortex and hippocampus of MCAO/R-treated rats, suggesting that different brain regions may have different tolerance to hypoxic and ischemic injury. *Aijia Shang et al* found the expressions of neuroglobin (NGB) were also consistent in the cortex and hippocampus of global cerebral ischemia-reperfusion models gerbils [53]. At the same time, our immunofluorescence results showed that the proportion of neurons in the cortex and hippocampus was different, which could explain the expressions of

miR-211-5p were consistent in hippocampus and PC12 cells, also suggested that microglia and astrocytes may participate in the development of CIRI through miR-211-5p. Next, we will design experiments to explore the role of microglia and astrocytes on stroke.

In our experiment, we found that miR-211-5p overexpression markedly reduced the endogenous expressions of COX2 mRNA and protein in the cortex and hippocampus of rats, while miR-211-5p inhibition markedly increased the endogenous expressions of COX2 mRNA and protein. Meanwhile, our immunofluorescence results also showed the same results. Therefore, the protective effect of miR-211-5p may be achieved by inhibiting the expression of COX2 in neurons. PGD_2 and PGE_2 were downstream products of COX2, which are abundant prostaglandins in brain [54-56]. It has been reported that PGD_2 and PGE_2 play important roles in multiple brain pathophysiological processes, including neuroinflammation, modulation of synaptic plasticity, and sleep promotion [57]. IL-1β and TNF-α are inflammatory cytokines that represent the level of inflammation [58, 59]. Compared to the normal group, their levels were elevated to varying degrees in the model group, but decreased significantly in the miR-211-5p agomir group. In addition, COX2 siRNA reversed the effects of miR-211-5p inhibitor in the functional recovery experiments. Finally, dual luciferase assay showed that there were two binding sites in the 3′-UTR of COX2 mRNA. Therefore, Our results suggested that miR-211-5p can protect against CIRI by directly targeting COX2. This finding can provide references for the treatment of stroke patients.

In conclusion, the present results suggested that miR-211 had a protective effect on CIRI, and this effect was achieved by targeted reducing endogenous COX2 expression. We expect miRNAs to be used in clinical treatment soon, just like patisiran.

Acknowledgments

This work was supported by the Chongqing science technology commission of China (NO: cstc2017shms-zdyfX0053)

Author contributions

Junqing Yang made substantial contribution to conception, design, and performance of the study. Zhe Peng, Miaomiao Li, Hong Wang, Yin Luo, Yang Yang, Pu Xiang, Haifeng Huang, Xiaodan Tan, Chao Gu, Maozhu Liu, Qiong Wang, Mengyuan Chen took part in all the experiments and carried out the data analysis. Zhe Peng wrote the final manuscript and all authors approved the final manuscript.

Conflicts of Interest

The authors declare that there is no competitive financial interest.

REFERENCES

[1] I.Y. Elgendy, A.N. Mahmoud, H. Mansoor, M.K. Mojadidi, A.A. Bavry, Evolution of acute ischemic stroke therapy from lysis to thrombectomy: Similar or different to acute myocardial infarction?, Int J Cardiol 222 (2016) 441-447.

[2] X. Fu, C. Chu, X. Li, Q. Gao, J. Jia, Cerebral arterial stiffness for predicting functional outcome in acute ischemic stroke, Hypertension research : official journal of the Japanese Society of Hypertension (2019).

[3] L. Liu, D. Wang, K.S. Wong, Y. Wang, Stroke and stroke care in China: huge burden, significant workload, and a national priority, Stroke 42(12) (2011) 3651-4.

[4] V.L. Feigin, R.V. Krishnamurthi, P. Parmar, B. Norrving, G.A. Mensah, D.A. Bennett, S. Barker-Collo, A.E. Moran, R.L. Sacco, T. Truelsen, S. Davis, J.D. Pandian, M. Naghavi, M.H. Forouzanfar, G. Nguyen, C.O. Johnson, T. Vos, A. Meretoja, C.J. Murray, G.A. Roth, Update on the Global Burden of Ischemic and Hemorrhagic Stroke in 1990-2013: The GBD 2013 Study, Neuroepidemiology 45(3) (2015) 161-76.

[5] W. Wang, B. Jiang, H. Sun, X. Ru, D. Sun, L. Wang, L. Wang, Y. Jiang, Y. Li, Y. Wang, Z. Chen, S. Wu, Y. Zhang, D. Wang, Y. Wang, V.L. Feigin, Prevalence, Incidence, and Mortality of Stroke in China: Results from a Nationwide Population-Based Survey of 480 687 Adults, Circulation 135(8) (2017) 759-771.

[6] D.M. Yellon, D.J. Hausenloy, Myocardial reperfusion injury, The New England journal of medicine 357(11) (2007) 1121-35.

[7] V. Hachinski, K. Einhaupl, D. Ganten, S. Alladi, C. Brayne, B.C.M. Stephan, M.D. Sweeney, B. Zlokovic, Y. Iturria-Medina, C. Iadecola, N. Nishimura, C.B. Schaffer, S.N. Whitehead, S.E. Black, L. Ostergaard, J. Wardlaw, S. Greenberg, L. Friberg, B. Norrving, B. Rowe, Y. Joanette, W. Hacke, L. Kuller, M. Dichgans, M. Endres, Z.S. Khachaturian, Preventing dementia by preventing stroke: The Berlin Manifesto,

Alzheimer's & dementia : the journal of the Alzheimer's Association 15(7) (2019) 961-984.

[8] H.K. Eltzschig, T. Eckle, Ischemia and reperfusion--from mechanism to translation, Nat Med 17(11) (2011) 1391-401.

[9] V. Kaushal, L.C. Schlichter, Mechanisms of microglia-mediated neurotoxicity in a new model of the stroke penumbra, The Journal of neuroscience : the official journal of the Society for Neuroscience 28(9) (2008) 2221-30.

[10] X. Wang, S. Chen, J. Ni, J. Cheng, J. Jia, X. Zhen, miRNA-3473b contributes to neuroinflammation following cerebral ischemia, Cell Death Dis 9(1) (2018) 11.

[11] H. Tomimoto, M. Shibata, M. Ihara, I. Akiguchi, R. Ohtani, H. Budka, A comparative study on the expression of cyclooxygenase and 5-lipoxygenase during cerebral ischemia in humans, Acta Neuropathol 104(6) (2002) 601-7.

[12] B. Kis, A. Snipes, F. Bari, D.W. Busija, Regional distribution of cyclooxygenase-3 mRNA in the rat central nervous system, Molecular Brain Research 126(1) (2004) 78-80.

[13] Y. Yang, Q. Chen, Q. Zhao, Y. Luo, Y. Xu, W. Du, H. Wang, H. Li, L. Yang, C. Hu, J. Zhang, Y. Li, H. Xia, Z. Chen, J. Ma, X. Tian, J. Yang, Inhibition of COX2/PGD2-Related Autophagy Is Involved in the Mechanism of Brain Injury in T2DM Rat, Front Cell Neurosci 13 (2019) 68-68.

[14] L. Yu, B. Yang, J. Wang, L. Zhao, W. Luo, Q. Jiang, J. Yang, Time course change of COX2-PGI2/TXA2 following global cerebral ischemia reperfusion injury in rat hippocampus, Behavioral and brain functions : BBF 10 (2014) 42.

[15] M. Santos-Galdiano, D. Perez-Rodriguez, B. Anuncibay-Soto, E. Font-Belmonte, I.F. Ugidos, C.C. Perez-Garcia, A. Fernandez-Lopez, Celecoxib Treatment Improves Neurologic Deficit and Reduces Selective Neuronal Loss and Glial Response in Rats after Transient Middle Cerebral Artery Occlusion, The Journal of pharmacology and experimental therapeutics 367(3) (2018) 528-542.

[16] Z. Ye, N. Wang, P. Xia, E. Wang, Y. Yuan, Q. Guo, Delayed administration of parecoxib, a specific COX-2 inhibitor, attenuated postischemic neuronal apoptosis by phosphorylation Akt and GSK-3beta, Neurochem Res 37(2) (2012) 321-9.

[17] K.I. Strauss, COX2 inhibitors for acquired brain injuries: is the time ripe?, Critical care medicine 38(2) (2010) 723-4.

[18] M. Esteller, Non-coding RNAs in human disease, Nat Rev Genet 12(12) (2011) 861-74.

[19] R.E. Crossland, J. Norden, M. Kralj Juric, K.F. Pearce, C. Lendrem, L.A. Bibby, M. Collin, H.T. Greinix, A.M. Dickinson, Serum and Extracellular Vesicle MicroRNAs miR-423, miR-199, and miR-93* As Biomarkers for Acute Graft-versus-Host Disease, Frontiers in immunology 8 (2017) 1446.

[20] B.H. Miller, Z. Zeier, L. Xi, T.A. Lanz, S. Deng, J. Strathmann, D. Willoughby, P.J. Kenny, J.D. Elsworth, M.S. Lawrence, R.H. Roth, D. Edbauer, R.J. Kleiman, C. Wahlestedt, MicroRNA-132 dysregulation in schizophrenia has implications for both neurodevelopment and adult brain function, Proceedings of the National Academy of Sciences of the United States of America 109(8) (2012) 3125-30.

[21] R. El Fatimy, S. Li, Z. Chen, T. Mushannen, S. Gongala, Z. Wei, D.T. Balu, R. Rabinovsky, A. Cantlon, A. Elkhal, D.J. Selkoe, K.C. Sonntag, D.M. Walsh, A.M. Krichevsky, MicroRNA-132 provides neuroprotection for tauopathies via multiple signaling pathways, Acta Neuropathol 136(4) (2018) 537-555.

[22] S. Huang, J. Zhao, D. Huang, L. Zhuo, S. Liao, Z. Jiang, Serum miR-132 is a risk marker of post-stroke cognitive impairment, Neuroscience letters 615 (2016) 102-6.

[23] X. Zuo, J. Lu, A. Manaenko, X. Qi, J. Tang, Q. Mei, Y. Xia, Q. Hu, MicroRNA-132 attenuates cerebral injury by protecting blood-brain-barrier in MCAO mice, Experimental neurology 316 (2019) 12-19.

[24] S.E. Linsen, E. de Wit, E. de Bruijn, E. Cuppen, Small RNA expression and strain specificity in the rat, BMC genomics 11 (2010) 249.

[25] L.L. Chen, Z.J. Zhang, Z.B. Yi, J.J. Li, MicroRNA-211-5p suppresses tumour cell proliferation, invasion, migration and metastasis in triple-negative breast cancer by directly targeting SETBP1, British journal of cancer 117(1) (2017) 78-88.

[26] C. Fan, Q. Wu, X. Ye, H. Luo, D. Yan, Y. Xiong, H. Zhu, Y. Diao, W. Zhang, J. Wan, Role of miR-211 in Neuronal Differentiation and Viability: Implications to Pathogenesis of Alzheimer's Disease, Frontiers in aging neuroscience 8 (2016) 166.

[27] Z. Feng, X. Yu, M. Jiang, L. Zhu, Y. Zhang, W. Yang, W. Xi, G. Li, J. Qian, Excretable IR-820 for in vivo NIR-II fluorescence cerebrovascular imaging and photothermal therapy of subcutaneous tumor, Theranostics 9(19) (2019) 5706-5719.

[28] W. Liang, C. Lin, L. Yuan, L. Chen, P. Guo, P. Li, W. Wang, X. Zhang, Preactivation of Notch1 in remote ischemic preconditioning reduces cerebral

ischemia-reperfusion injury through crosstalk with the NF-kappaB pathway, Journal of neuroinflammation 16(1) (2019) 181.

[29] J. Tao, W. Liu, G. Shang, Y. Zheng, J. Huang, R. Lin, L. Chen, MiR-207/352 regulate lysosomal-associated membrane proteins and enzymes following ischemic stroke, Neuroscience 305 (2015) 1-14.

[30] J.A. Contreras, V. Aslanyan, M.D. Sweeney, L.M.J. Sanders, A.P. Sagare, B.V. Zlokovic, A.W. Toga, S.D. Han, J.C. Morris, A. Fagan, P. Massoumzadeh, T.L. Benzinger, J. Pa, Functional connectivity among brain regions affected in Alzheimer's disease is associated with CSF TNF-alpha in APOE4 carriers, Neurobiology of aging (2019).

[31] J.R. Godin, P. Roy, M. Quadri, D. Bagdas, W. Toma, R. Narendrula-Kotha, O.A. Kishta, M.I. Damaj, N.A. Horenstein, R.L. Papke, A.R. Simard, A silent agonist of alpha7 nicotinic acetylcholine receptors modulates inflammation ex vivo and attenuates EAE, Brain, behavior, and immunity (2019).

[32] A. Serhan, J.L. Aerts, E. Boddeke, R. Kooijman, Neuroprotection by Insulin-like Growth Factor-1 in Rats with Ischemic Stroke is Associated with Microglial Changes and a Reduction in Neuroinflammation, Neuroscience (2019).

[33] W.J. Streit, Microglia as neuroprotective, immunocompetent cells of the CNS, Glia 40(2) 133-139.

[34] H. Wilms, P. Rosenstiel, J, G. Deuschl, L. Zecca, R. Lucius, Activation of microglia by human neuromelanin is NF-kappaB dependent and involves p38

mitogen-activated protein kinase: implications for Parkinson's disease, 17(3) (2003) 500.

[35] M. Greter, M. Merad, Regulation of microglia development and homeostasis, Glia 61(1) (2013) 121-127.

[36] L. Kodama, E. Guzman, J.I. Etchegaray, Y. Li, F.A. Sayed, L. Zhou, Y. Zhou, L. Zhan, D. Le, J.C. Udeochu, C.D. Clelland, Z. Cheng, G. Yu, Q. Li, K.S. Kosik, L. Gan, Microglial microRNAs mediate sex-specific responses to tau pathology, Nature neuroscience (2019).

[37] R.U. Parekh, J. Robidoux, S. Sriramula, Kinin B1 Receptor Blockade Prevents Angiotensin II-induced Neuroinflammation and Oxidative Stress in Primary Hypothalamic Neurons, Cell Mol Neurobiol (2019).

[38] Y. Konishi, L.B. Yang, P. He, K. Lindholm, Y. Shen, Deficiency of GDNF Receptor GFR alpha 1 in Alzheimer's Neurons Results in Neuronal Death, Journal of Neuroscience the Official Journal of the Society for Neuroscience 34(39) (2014) 13127-13138.

[39] E. Caballero-Garrido, J.C. Pena-Philippides, T. Lordkipanidze, D. Bragin, Y. Yang, E.B. Erhardt, T. Roitbak, In Vivo Inhibition of miR-155 Promotes Recovery after Experimental Mouse Stroke, Journal of Neuroscience the Official Journal of the Society for Neuroscience 35(36) 12446-12464.

[40] J. Huang, U.M. Upadhyay, R.J. Tamargo, Inflammation in stroke and focal cerebral ischemia, Surgical neurology 66(3) (2006) 232-45.

[41] S. Huang, M. Chen, L. Li, M. He, D. Hu, X. Zhang, J. Li, R.M. Tanguay, J. Feng, L. Cheng, H. Zeng, X. Dai, Q. Deng, F.B. Hu, T. Wu, Circulating MicroRNAs and the occurrence of acute myocardial infarction in Chinese populations, Circulation. Cardiovascular genetics 7(2) (2014) 189-98.

[42] A. Zampetaki, S. Kiechl, I. Drozdov, P. Willeit, U. Mayr, M. Prokopi, A. Mayr, S. Weger, F. Oberhollenzer, E. Bonora, A. Shah, J. Willeit, M. Mayr, Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes, Circulation research 107(6) (2010) 810-7.

[43] Y. Chen, H.Q. Xie, R. Sha, T. Xu, S. Zhang, H. Fu, Y. Xia, Y. Liu, L. Xu, B. Zhao, 2,3,7,8-Tetrachlorodibenzo-p-dioxin and up-regulation of neurofilament expression in neuronal cells: Evaluation of AhR and MAPK pathways, Environment international 134 (2020) 105193.

[44] D.P. Bhattarai, T.I. Hwang, J.I. Kim, J.H. Lee, S. Chun, B.S. Kim, C.H. Park, C.S. Kim, Synthesis of polypyrrole nanorods via sacrificial removal of aluminum oxide nanopore template: A study on cell viability, electrical stimulation and neuronal differentiation of PC12cells, Materials science & engineering. C, Materials for biological applications 107 (2020) 110325.

[45] L. Vucicevic, M. Misirkic, D. Ciric, T. Martinovic, M. Jovanovic, A. Isakovic, I. Markovic, J. Saponjic, M. Foretz, Y. Rabanal-Ruiz, V.I. Korolchuk, V. Trajkovic, Transcriptional block of AMPK-induced autophagy promotes glutamate excitotoxicity in nutrient-deprived SH-SY5Y neuroblastoma cells, Cellular and molecular life sciences : CMLS (2019).

[46] R.L. Setten, J.J. Rossi, S.P. Han, Publisher Correction: The current state and future directions of RNAi-based therapeutics, Nature reviews. Drug discovery (2019).

[47] H. Ledford, Gene-silencing technology gets first drug approval after 20-year wait, Nature 560(7718) (2018) 291-292.

[48] J. Yang, Q. Zhou, Establishment of a model of delayed brain injury in mice induced by subacute carbon monoxide Chong qing yi ke da xue xue bao (04) (1998) 332-335.

[49] W. Zhang, J. Xiao, X. Lu, T. Liu, X. Jin, Y. Xiao, X. He, PVT1 (rs13281615) and miR-146a (rs2910164) polymorphisms affect the prognosis of colon cancer by regulating COX2 expression and cell apoptosis, Journal of cellular physiology 234(10) (2019) 17538-17548.

[50] H. Jia, H. Wang, Y. Yao, C. Wang, P. Li, miR-136 Inhibits Malignant Progression of Hepatocellular Carcinoma Cells by Targeting Cyclooxygenase 2, Oncology research 26(6) (2018) 967-976.

[51] S. Yotsuyanagi, O. Yokoyama, K. Komatsu, K. Kodama, Y. Nagasaka, M. Namiki, Role of cyclooxygenase-2 in the development of bladder overactivity after cerebral infarction in the rat, The Journal of urology 174(1) (2005) 365-9.

[52] W. Yu, F. Jie, J. Yang, H. Hua, Expression and significance of cyclooxygenase-2 in focal cerebral ischemia-reperfusion injury in rats, Zhong Guo Xian Dai Yi Xue Za Zhi (16) (2007) 1934-1937+1940.

[53] A. Shang, D. Zhou, L. Wang, Y. Gao, M. Fan, X. Wang, R. Zhou, C. Zhang, Increased neuroglobin levels in the cerebral cortex and serum after ischemia-reperfusion insults, Brain Res 1078(1) (2006) 219-26.

[54] M.S. Abdel-Halim, I. Lunden, G. Cseh, E. Anggard, Prostaglandin profiles in nervous tissue and blood vessels of the brain of various animals, Prostaglandins 19(2) (1980) 249-58.

[55] E.K. Chiu, J.S. Richardson, Behavioral and neurochemical aspects of prostaglandins in brain function, General pharmacology 16(3) (1985) 163-75.

[56] D.F. Legler, M. Bruckner, E. Uetz-von Allmen, P. Krause, Prostaglandin E2 at new glance: novel insights in functional diversity offer therapeutic chances, The international journal of biochemistry & cell biology 42(2) (2010) 198-201.

[57] M. Tachikawa, K. Hosoya, T. Terasaki, Pharmacological significance of prostaglandin E2 and D2 transport at the brain barriers, Advances in pharmacology (San Diego, Calif.) 71 (2014) 337-60.

[58] O.T. Somade, B.O. Ajayi, O.A. Safiriyu, O.S. Oyabunmi, A.J. Akamo, Renal and testicular up-regulation of pro-inflammatory chemokines (RANTES and CCL2) and cytokines (TNF-alpha, IL-1beta, IL-6) following acute edible camphor administration is through activation of NF-kB in rats, Toxicology reports 6 (2019) 759-767.

[59] S.E. Owumi, U.J. Dim, Manganese suppresses oxidative stress, inflammation and caspase-3 activation in rats exposed to chlorpyrifos, Toxicology reports 6 (2019) 202-209.

Figure Legends

Fig.1 The relative expression of miRNAs in the cortex, hippocampus and plasma of MCAO/R-treated rats. (**A**) The expression of miRNAs in the cortex (n=3); (**B**) The expression of miRNAs in the hippocamus (n=3); (**C**) The expression of miR-211-5p in plasma (n=4); (**D**) The expression of COX2 mRNA in plasma (n=4). (**E,F**) Relative COX2 protein expressions in plasma (n=4). Data are presented as the mean ± SD. *P<0.05, **P<0.01 campared with the sham group.

Fig.2 The spatio-temporal expression of COX-2 and miR-211-5p in the cortex and hippocampus of SD rats and PC12 cells.(A) miR-211-5p expression in cortex; (B) miR-211-5p expression in hippocampus; (C) miR-211-5p expression in PC12 cells; (D) COX2 mRNA expression in cortex; (E) COX2 mRNA expression in hippocampus; (F) COX2 mRNA expression in PC12 cells. Data are presented as the mean ± SD in the four individual experiments .*P<0.05, **P<0.01 and ***P<0.001 compared with the sham group in vivo, as well as the normal group in vitro.

Fig.3 Effect of miR-211-5p on MCAO/R-treated rats. (**A**) Representative images of TTC staining of infarct size in brain sections (n=3); (**B**) Quantification of infarct volume by TTC staining after 24 h of reperfusion (n=3); (**C**) Neurological score were estimated after 24h of reperfusion (n=10); (**D,E**) Representative images of histopathological assay of rats in cortex (n=3, scale bar= 50 μm); (**F,G**) Representative images of histopathological assay of rats in hippocampus (n=3, scale bar= 50 μm). Data are presented as the mean ± SD *P<0.05, **P<0.01 and ***P<0.001 compared with the sham group; $^{@}$P<0.05 and $^{@@@}$P<0.001 compared with the miR-211-5p agomir group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with the miR-211-5p antagomir group.

Fig.4 Effect of miR-211-5p on CIRI via targetting COX2. (**A,B**) Relative COX2

mRNA expressions in cortex and hippocampus after 24h of reperfusion (n=4); (**C-E**) Relative COX2 protein expressions in cortex and hippocampus after 24h of reperfusion. Data are presented as the mean ± SD. ***P<0.001 compared with the sham group; $^{@@}$P<0.05 and $^{@@@}$P<0.001 compared with the miR-211-5p agomir group; #P<0.05, $^{###}$P<0.001 compared with the miR-211-5p antagomir group.

Fig.5 Effect of miR-211-5p agomir and antagomir on the expression of COX2 protein in neurons of brain(200×, 400×). (**A,B**) Representative images of immunofluorescence staining for COX2 (green), Neun (red, a marker protein of neuron) in cortex (n=3, scale bar= 50 μm); (**C,D**) Representative images of immunofluorescence staining for COX2 (green), Neun (red) in hippocampus (n=3, scale bar= 50 μm). Data are presented as the mean ± SD. ***P<0.001 compared with the sham group; $^{@@}$P<0.01 compared with the miR-211-5p agomir group; $^{##}$P<0.01 and $^{###}$P<0.001 compared with the miR-211-5p antagomir group.

Fig.6 Effect of miR-211-5p on changes of PGD2, PGE2, TNF-α and IL-1β in cortex and hippocampus of MCAO/R-treated rats. (A-D) Changes of PGD2, PGE2, TNF-α, IL-1β content in cortex (n=4); **(E-H)** Changes of PGD2, PGE2, TNF-α, IL-1β content in hippocampus (n=4). Data are presented as the mean ± SD. *P<0.05 and **P<0.01 compared with the sham group; $^{@}$P<0.05 compared with the miR-211-5p agomir group; $^{#}$P<0.05 and $^{##}$P<0.01 compared with the miR-211-5p antagomir group.

Fig.7 Effect of miR-211-5p on OGD/R-treated PC12 cells. (A) Cell viability was determined by MTT assay (n=6); (**B**) LDH release in PC12 cells ;(n=6) (**C**) Apoptosis rate was determined by flow cytometry analysis (n=3); (**D**) Relative COX2 protein expression in PC12 cells after 24h of reperfusion (n=4); (**E**) Relative COX2 mRNA expression in PC12 cells after 24h of reperfusion (n=4); (**F-G)** Changes of PGD2, PGE2 content in PC12 cells (n=5). Data are presented as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 compared with the normal group; $^{@}$P<0.05 and

$^{@@@}$P<0.01 compared with the miR-211-5p mimic group; $^{\#}$P<0.05 and $^{\#\#\#}$P<0.001 compared with the miR-211-5p inhibitor group.

Fig.8 COX2 is a direct target of miR-211-5p. (A) Predicted binding site of miR-211-5p in the 3′-UTR of COX2; (**B**) Dual luciferase assay co-transfected with the miR-211-5p mimic and reporter vectors containing either the wild-type or mutated 3'-UTR of COX2. The Renilla luciferase activity was normalized to the Firefly luciferase activity. WT: the normal COX2 nucleotides; MT1: the construct with the mutated nucleotides in the first binding site between 178-185; MT2: the construct with the mutated nucleotides in the second binding site between 224-230 (n =5); (**C**) siRNA candidates against COX-2 were evaluated (n =4) ; (**D**) Cell viability was determined by MTT assay in different groups (n =6); (**E)** LDH release in differrent groups (n =6) ; (**F**) Relative COX2 protein expression in different groups (n =4). Data are presented as the mean ± SD. **P < 0.05, ***P < 0.01, $^{@}$P, $^{@@}$P, $^{\#}$P, $^{\#\#}$P, $^{\#\#\#}$P compared between the indicated groups.

Fig.1

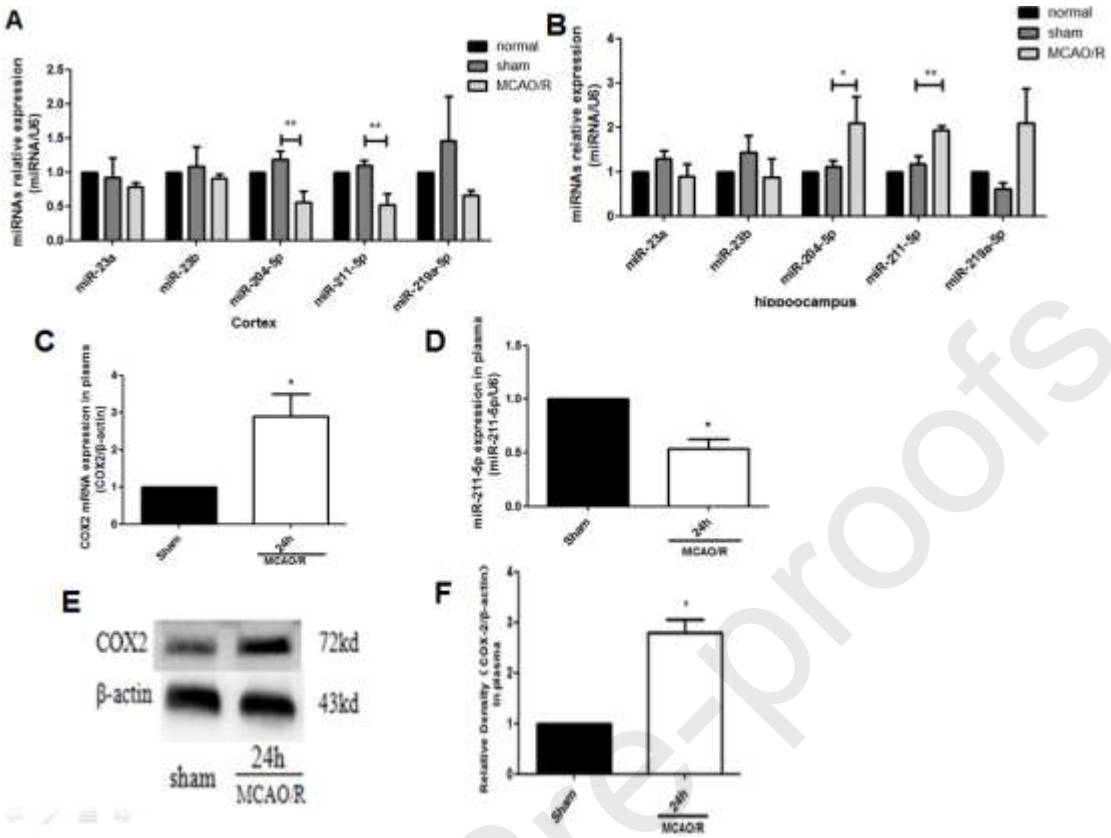

Fig.2

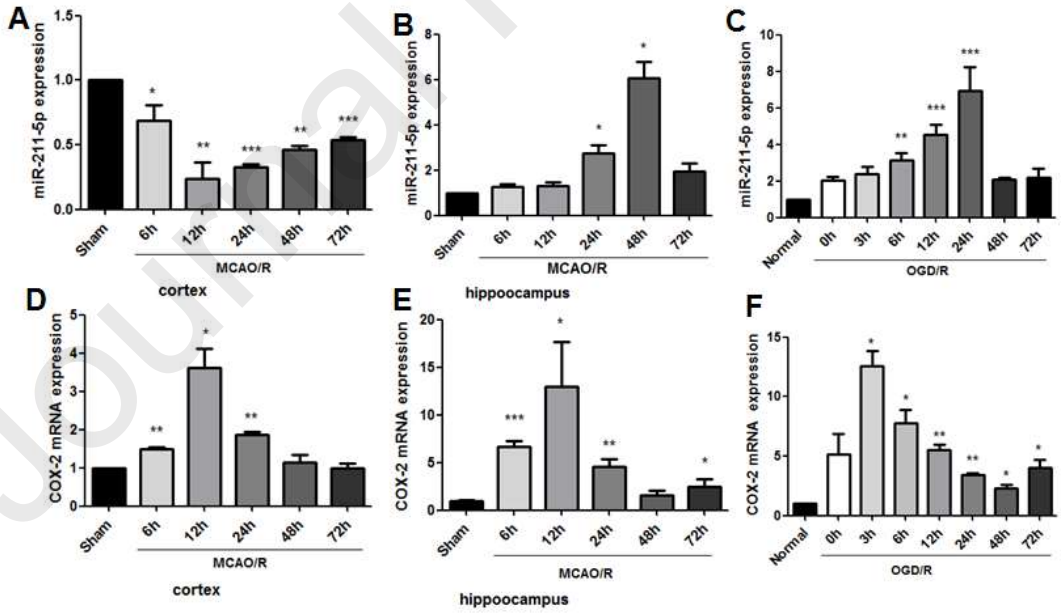

Fig.3

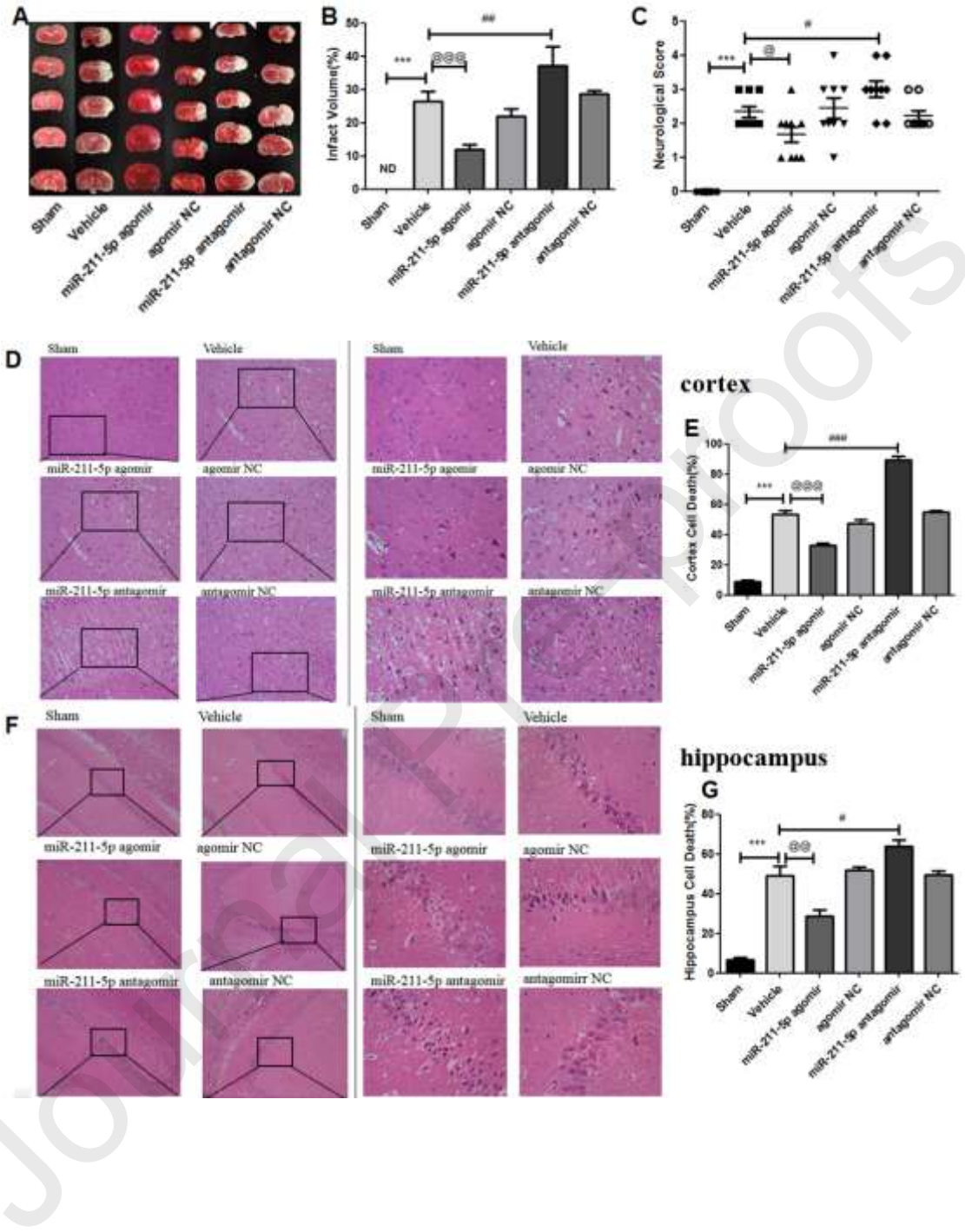

Fig.4

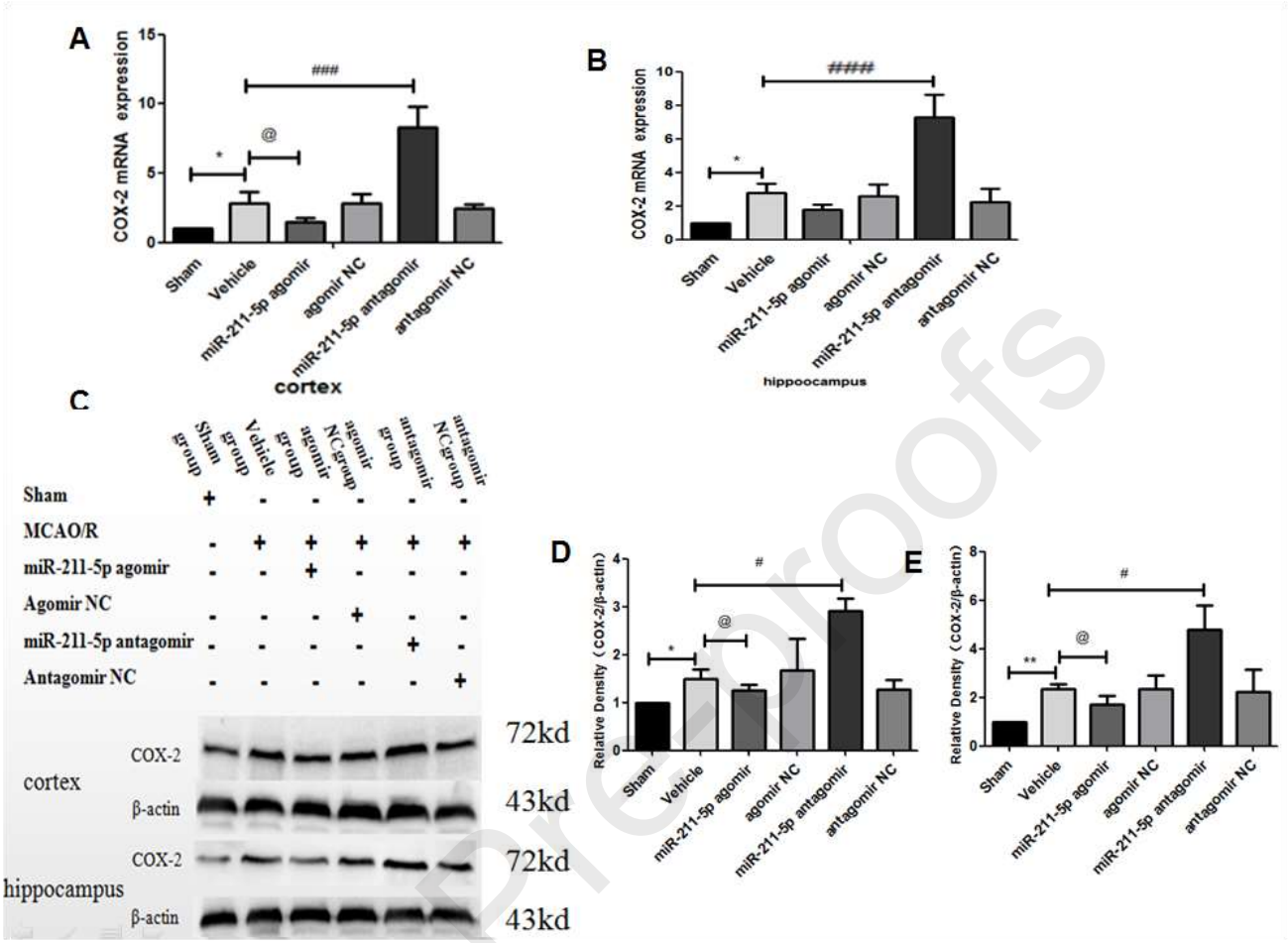

Fig.5

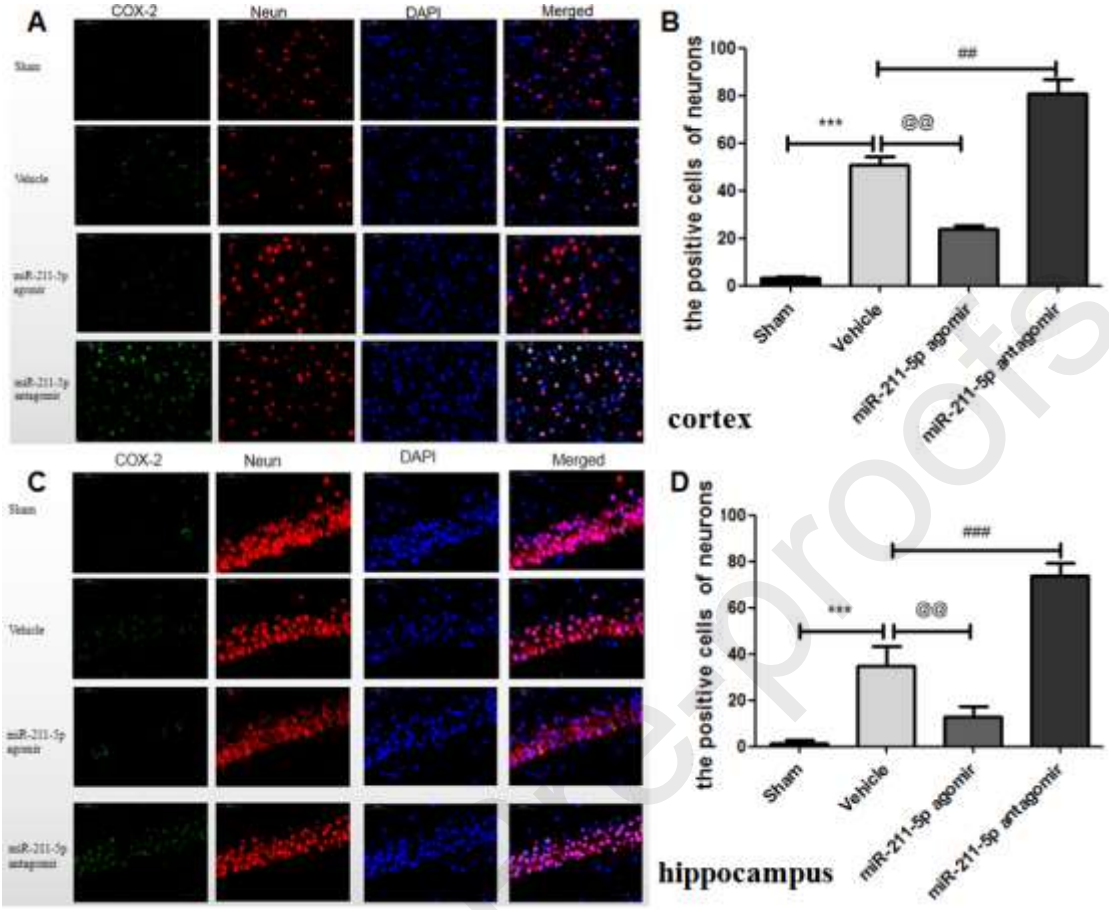

Fig.6

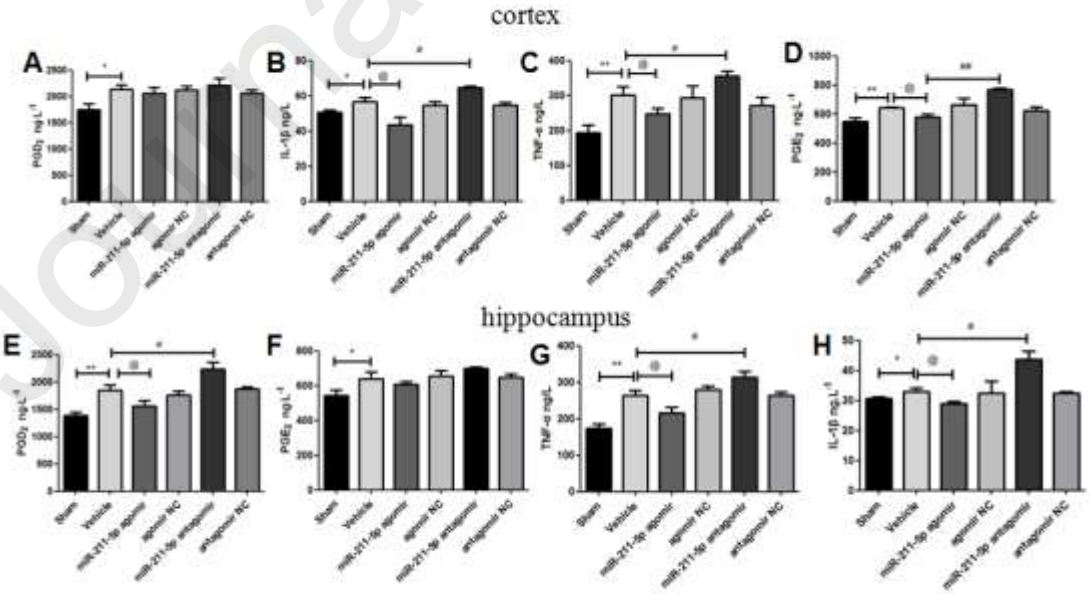

Fig.7

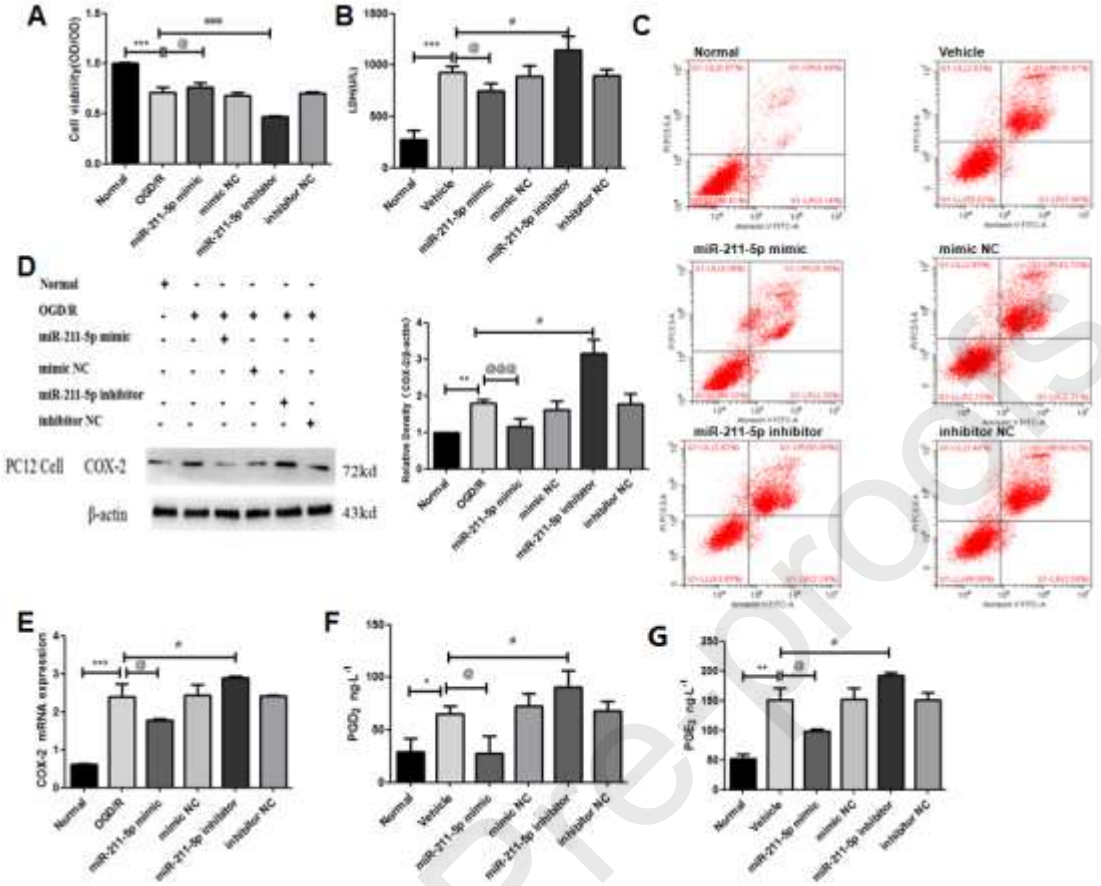

Fig.8