Exogenous Netrin-1 Inhibits Autophagy of Ischemic Brain Tissues and Hypoxic Neurons via PI3K/mTOR Pathway in Ischemic Stroke

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Background and Objective: Ischemic stroke is a serious disease that endangers human health. How to reduce the damage of neurons in ischemic regions is an urgent problem to be explored. Autophagy is an important pathophysiological process in cerebral ischemia and Netrin-1 is an effective neuroprotective protein. This study aims to investigate the effect of Netrin-1 on autophagy of ischemic brain tissues and hypoxic neurons. *Methods*: We constructed rat persistent middle cerebral artery occlusion model in vivo and constructed the Oxygen Glucose-Deprivation model in vitro. Rats and cells were treated with or without Netrin-1. Western blot analysis was performed to detect autophagy related proteins LC3B, P62 and pathway related proteins PI3K, p-PI3K, mTOR, p-mTOR. CCK-8 assay was performed to detect the viability of hypoxic neurons. We also performed western-blot analysis and qRT-PCR test to detect levels of Netrin-1 protein and mRNA. *Results*: Autophagy enhanced both in ischemic brain tissues and hypoxic neurons. Netrin-1 inhibited autophagy through PI3K/mTOR pathway both in vivo and in vitro. At the same time, we found that exogenous Netrin-1 can promote the secretion of Netrin-1 protein by neurons themselves, which indicated that Netrin-1 can further amplify the neuroprotective effect through the positive feedback mechanism. *Conclusions*: Exogenous Netrin-1 alleviates damage of ischemic brain tissues and enhances viability of hypoxic neurons by inhibiting autophagy via PI3K/mTOR pathway. This effect can be amplified by positive feedback mechanism.

Key Words: Netrin-1—ischemic stroke—autophagy—positive feedback

Introduction

Acute ischemic stroke (AIS), a highly lethal and disabling disease, has become a global health problem.[1] Due to death of neurons, stroke can lead to severe neurological deficits, such as motor dysfunction and aphasia, etc. In severe cases, patients may result in death. Therefore, rescue of the ischemic neurons is the key to improve long-term prognosis of cerebral infarction. Currently, treatment options are rather limited. Tissue plasminogen activator (tPA) and mechanical thrombectomy has emerged as promising therapies, but with strict time window (usually no later than 6-8 hours from onset), reperfusion out of the time window is not helpful for saving ischemic neurons, but can pose risks like cerebral hemorrhage.[2,3] Therefore, it is valuable to explore the pathophysiology processes of neurons under the insults of

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ischemia and deoxygenation, and to find protective agents which might help to reduce neuronal injury.

Netrin-1, a laminin-like protein with a molecular weight of 60-80 kd, was first discovered in caenorhabditis elegans and is involved in the development of nervous and vascular systems.[4] During embryonic development, Netrin-1 has an effect of axonal guidance, which involves inducing axonal growth toward the midline, hippocampal formation, and the development of nerve conduction bundles.[5-8] At the same time, Netrin-1 is also a strong angiogenic factor, which can promote vascular proliferation, migration, as well as strengthen the adhesion of endothelial cells and vascular smooth muscle cells.[9,10] In previous studies, our group revealed that Netrin-1 had a protective effect on ischemic stroke. Overexpression of Netrin-1 remarkably reduced infarct areas and improved motor function.[10,11] In addition, Netrin-1 mediates the proliferation of newly generated vessels in the ischemic penumbra, thus playing a cerebral protective role.[12] However, it is not clear what kind of direct influence Netrin-1 has on ischemic neurons.

Autophagy is a special cellular metabolic process for maintaining cell homeostasis and self-renewal of some organelles.[13,14] Autophagy plays an important role in cell proliferation, differentiation and survival.[15] But its roles in diseases remain controversial. Some authors showed that autophagy had neuroprotective effects in acute spinal cord injury through reducing metabolic rate,[16,17] while others reported excessive autophagy led to cellular death.[18]

By inducing autophagy, tissue energy metabolic demand can be reduced, which is conducive to alleviating tissue injury and promoting functional recovery.[16,17] However, some other scholars reported that excessive autophagy may lead to cell death.[18] Till now, the roles of autophagy in brain ischemia remain unknown.

There is no consensus on the effect of Netrin-1 on autophagy. Bai L et al.[19,20] reported that Netrin-1 could induce autophagy in rat spinal cord injury model. Bouhidel et al.[21] reported that Netrin-1 could inhibit autophagy in mice with acute myocardial infarction. In this study, we detected the effect of Netrin-1 on the autophagy of ischemic brain tissues and hypoxic neurons both in vivo and in vitro in ischemic stroke.

Materials and Methods

Experimental Animals

All animal experiments in this experiment were approved by the Animal Welfare and Use Committee of Shanghai Jiaotong University. Experiment license number: SYXK2013-0106. The SD rats used in this experiment were purchased from Shanghai Slack Laboratory Animals Co., Ltd., and the production license number is SCXK2017-0005.

Rat Middle Cerebral Artery Occlusion Model

Adult male SD rats weighing 280-300 g were used in our study. Rats were randomly divided into sham operation group (sham), model group (MCAO), and Netrin-1 administration group (MCAO+Netrin-1). Persistent middle cerebral artery occlusion (pMCAO) was induced as former reported.[12] Briefly, rats were initially anesthetized with 3.5% halothane and maintained with 1.0%-2.0% halothane in 70% N_2O and 30% O_2 with the use of a face mask. The right common carotid artery (CCA), external carotid artery (ECA), and internal carotid artery (ICA) were carefully exposed. A 4-0 monofilament nylon suture (Guangzhou Jialing Biotechnology Co., Ltd., Guangzhou, China), whose tip is wrapped by silicone, was inserted from CCA into the lumen of ICA to block the origin of the middle cerebral artery (MCA). In the sham group, the same procedure was performed without the insertion of a suture. According to the modified neurological severity score (mNSS) after surgery (Table 1),[22] rats with a score of 7-18 were included in the follow-up experiments.

Lateral Ventricle Injection

Rats that underwent MCAO surgery were treated by intraventricular administration. The first dose was given 1 hour after surgery, and then once a day in following 2 days. Anesthetized rats sufficiently and then fixed the rats on the brain localizer. A hole was drilled 1.5-mm lateral to the sagittal suture and 1-mm posterior to the coronal suture. A 10-ml syringe was inserted into right lateral ventricle 3-mm under the meninges. 5μl of the recombinant human Netrin-1 protein (R&D, cat.6419-n1-025) with a concentration of 100 ng/μl was injected into the lateral ventricle at the rate of 0.2μl/min. In the MCAO group, rats were injected with equal volume of PBS.

Morphometric Analysis of Cerebral Infarcts

Morphometric analysis of cerebral infarcts was performed 4 days after operation. Rats were euthanized and their brains were dissociated. The brains were coronally sliced into 5 serial 2-mm sections. Coronal brain sections of rats in the MCAO and MCAO+Netrin-1 groups were stained with 2, 3, 5-triphenyltetrazolium chloride (TTC). The infarcted areas were stained white.

Rat Primary Cortical Neuron Culture

The protocol is based on the previously reported method[23] and improved according to the conditions of our laboratory. The brain tissue of 1-day old SD rats was digested with 0.25% trypsin. The cells were resuspended in a neurobasal medium containing 2% B27, 200 mM glutamine, 1% penicillin/streptomycin, and then seeded at a density of 50,000/cm^2 in 6 cm culture dishes. Further experimental procedures were carried out after 7 days of culture.

Neuronal Oxygen Glucose-Deprivation (OGD) Treatment

Cells were randomly divided into control group, OGD group and Netrin-1 administration (OGD+Netrin-1) group. In the OGD+Netrin-1 group, recombinant human Netrin-1 protein was added and the final concentration

Table 1. *Modified neurological severity score (mNSS) points*

Motor tests	
Raising rat by tail	0-3
Flexion of forelimb	1
Flexion of hindlimb	1
Head moved >10° To vertical axis within 30 s	1
Placing rat on floor	0-3
Normal walk	0
Inability to walk straight	1
Circling toward paretic side	2
Falls down to paretic side	3
Sensory tests	0-2
Placing tests (visual and tactile test)	1
Proprioceptive tests (deep sensation, pushing paw against table edge to stimulate limb muscles)	1
Beam balance tests	0-6
Balances with steady posture	0
Grasps side of beam	1
Hugs beam and 1 limb falls down from beam	2
Hugs beam and 2 limbs fall down from beam, or spins on beam (>60 s)	3
Attempts to balance but falls off (>40 s)	4
Attempts to balance but falls off (>20 s)	5
Falls off; No attempt to balance or hang on beam (<20 s)	6
Reflex absence and abnormal movements	0-4
Pinna reflex (head shake when auditory meatus is touched)	1
Corneal reflex (eye blink when cornea is lightly touched with cotton)	1
Startle reflex (motor response to a brief noise from snapping a clipboard paper)	1
Seizures, myoclonus, myodystony	1
Maximum points	18

One point is awarded for inability to perform the tasks or for lack of a tested reflex: 13-18, severe injury; 7-12, moderate injury; 1-6, mild injury.

was 250 ng/ml. Equal volume PBS was added to the OGD group. The neurons are gently washed twice with PBS and replaced with a sugar-free medium. The cells were placed in a 37°C incubator containing 95% N_2/5% CO_2 to construct a hypoxic environment.

Cell Viability Test (CCK-8 Assay)

The cell suspension was seeded into 96-well plate with a density of 50,000/cm^2. After OGD treatment for 12 hours, Netrin-1 was added at a concentration of 0, 50, 100, 150, 200 and 250 ng/ml. OD values at 490 nm-wavelength was detected. 6 replicas were set in each group. The CCK-8 kit was purchased from Shanghai Beyotime co., LTD.

Western-Blotting

Tissue samples were dissected 4 days after operation. Cells proteins were extracted 12 hours after OGD. 50 μg of proteins were loaded onto polyacrylamide gels for electrophoresis. Then the proteins were transferred onto polyvinylidene fluoride membranes. The membranes were then blocked with 5% nonfat milk in Tris-buffered saline, with 0.1% Tween-20 for 1 hour, followed by incubation with primary antibody overnight at 4°C. After washing, the membranes were incubated with secondary antibody, followed by ECL detection (Amersham) and film (GE Healthcare) exposure. GAPDH was used as the internal control.

The primary antibodies were: rabbit anti-Netrin-1 (ab126729, Abcam, 1:2000), rabbit anti-LC3B (ab192890, Abcam, 1:1500), rabbit anti-P62 (39749, CST, 1:1500), mouse anti-GAPDH (AF0006, Beyotime, 1:2000), rabbit anti-mTOR (A11354, Abclonal, 1:1000), rabbit anti-p-mTOR (AP0094, Abclonal, 1:1000), rabbit anti-PI3K (ab191606, Abcam, 1:1000), rabbit anti-p-PI3K (ab182651, Abcam, 1:500).

The secondary antibodies were HRP (horseradish peroxidase)-labeled anti-rabbit IgG (A0208, Beyotime Biotechnology, Shanghai, China) and HRP-labeled anti-mouse IgG (A0216, Beyotime Biotechnology).

Quantitative Real-Time RT-PCR(qRT-PCR)

Cells were collected and RNA was extracted with Trizol and chloroform. Reverse transcription was performed to obtain cDNA according to the instructions of Takara Reverse Transcription Kit. Then real-time quantitative PCR analysis was performed by using Takara SYBR Premix ExTaqTM kit. The primers are shown below:

GAPDH:
F:5′-ACGGCAAGTTCAACGGCACAG-3′;
R:5′-CGACATACTCAGCACCAGCATCAC-3′.
Netrin-1:
F:5′-CCTTCCTCACCGACCTCAACAATC-3′;
R:5′- CTTCTTGCCGAGCGACAGAGTG-3′.

Statistical Analysis

SPSS 13.0 and GraphPad Prim5 were used for statistics and mapping. All data are expressed as mean ± standard error. One-way ANOVA was used for multiple comparisons between groups, and t-test was used for comparison between two groups. $P < .05$ was considered to be statistically significant.

Results

Exogenous Netrin-1 has a Protective Effect Both on Ischemic Brain Tissues and Hypoxic Neurons

We first constructed pMCAO models in rats, and injected the recombinant human Netrin-1 protein intraventicularly. We found that mNSS scores in rats treated with Netrin-1 were significantly decreased compared with untreated rats, indicating an improved neurological deficits (Fig 1A). Meanwhile, TTC staining showed that the infarct volume of Netrin-1-treated rats were significantly smaller than that of untreated rats (Fig 1B). These results are consistent with our previous report.[10]

In order to investigate the effect of Netrin-1 on ischemic neurons in vitro, we detected the viability of OGD neurons by CCK-8 assay. The results showed that the viability of Netrin-1 treated hypoxic neurons was higher than that of non-Netrin-1 treated hypoxic neurons, and the OD value at 490 nm-wavelength was positively correlated with Netrin-1 concentration. The highest neuronal viability was observed when Netrin-1 concentration was 250 ng/ml. Therefore, in the subsequent experiments, 250 ng/ml was used as the treatment concentration (Fig 1C).

Autophagy is Induced by Ischemia and Hypoxia, and Netrin-1 Reverses This Effect

To investigate the effects of hypoxia on autophagy in neurons, we cultured rat cortical neurons and treated them with OGD. The results showed that the LC3B II/I ratio increased with extension of time, while P62 protein decreased (Fig 2A). It is indicated that the longer the duration of hypoxia is, the more obvious the autophagy of neurons will be. Autophagy reached its peak at 12 hours in this experiment.

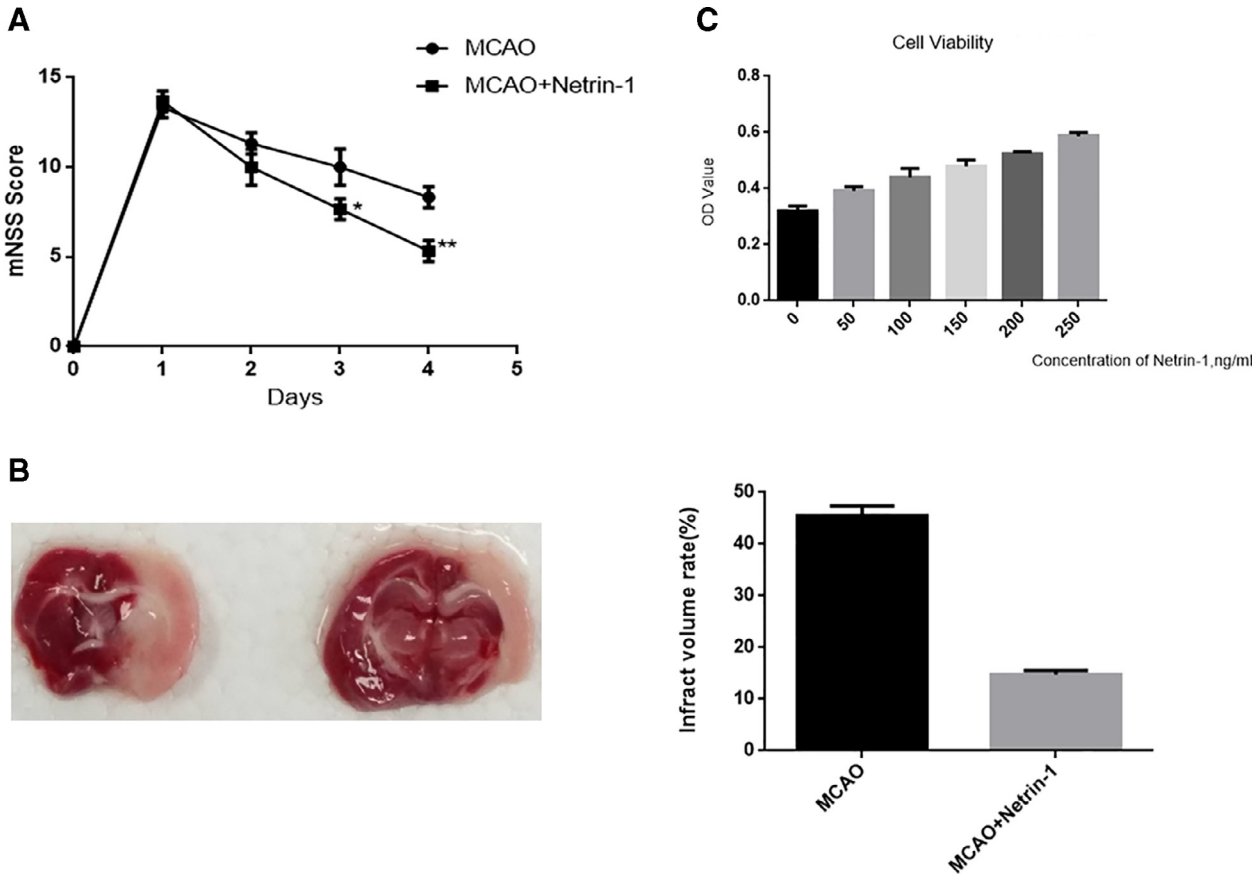

Figure 1. *Exogenous Netrin-1 has a protective effect on ischemic brain tissues and hypoxic neurons. (A). Exogenous Netrin-1 treatment improved neurological deficits after MCAO, and mNSS scores significantly decreased on the 3rd day (*, P < .05) and the 4th day (**, P < .01). N = 6. (B). TTC staining indicated that exogenous Netrin-1 treatment significantly reduced the infarct volume, P < .01. N = 3. (C). Netrin-1 enhanced the viability of hypoxic neurons. The viability of neurons was detected by CCK-8 assay at different Netrin-1 concentrations. The results suggested that addition of exogenous Nerin-1 can improve the viability of neurons after OGD treatment, and the higher the exogenous Netrin-1 concentration, the higher the OD value at 490 nm-wavelength. N = 6. Abbreviations: OGD, Oxygen Glucose-Deprivation; mNSS, modified neurological severity score; TTC, 2, 3, 5-triphenyltetrazolium chloride.*

Therefore, we took 12 hours as the duration of OGD treatment in subsequent experiments (Fig 2A).

After revealing that OGD treatment enhanced neuronal autophagy and Netrin-1 increased the viability of hypoxic neurons, we proposed a hypothesis that Netrin-1 might have an effect on autophagy in neurons under OGD condition. The results showed that, when compared with control, P62 in OGD group significantly decreased ($P < .01$), while LC3B II/I ratio significantly increased ($P < .01$). However, in the OGD+Netrin-1 group, P62 significantly increased ($P < .01$) and LC3B II/I ratio significantly decreased ($P < .05$) compared to OGD group. It was suggested that Netrin-1 could inhibit autophagy in OGD neurons (Fig 2B).

To verify these results in vivo, rats were randomly divided into sham, MCAO, and MCAO+ Netrin-1 group. Rats were euthanized 4 days after surgery, and brain tissue proteins were extracted for western-blot detection. The results reflected the trends that in Netrin-1 treated group, LC3B II/I ratio was downregulated compared with the untreated group ($P < .01$), while P62 was upregulated ($P < .01$) (Fig 2C). These results are consistent with the in vitro data, further confirming the role of Netrin-1 in inhibiting autophagy.

Inhibition of Autophagy by Netrin-1 Depends on PI3K/mTOR Pathway

In previous literatures, some researchers reported that autophagy is regulated by PI3K/mTOR pathway in tumors.[24-26] In this study, in order to verify the involvement of PI3K/mTOR pathway ischemic stroke, we further detected the changes of PI3K, p-PI3K, mTOR and p-mTOR proteins by western-blotting.

The in vitro results showed that p-PI3K /PI3K ($P < .01$) and p-mTOR/mTOR ($P < .01$) in OGD group were significantly lowered than that in control group. In OGD+ Netrin-1 group, p-PI3K /PI3K ($P < .05$) and p-mTOR/

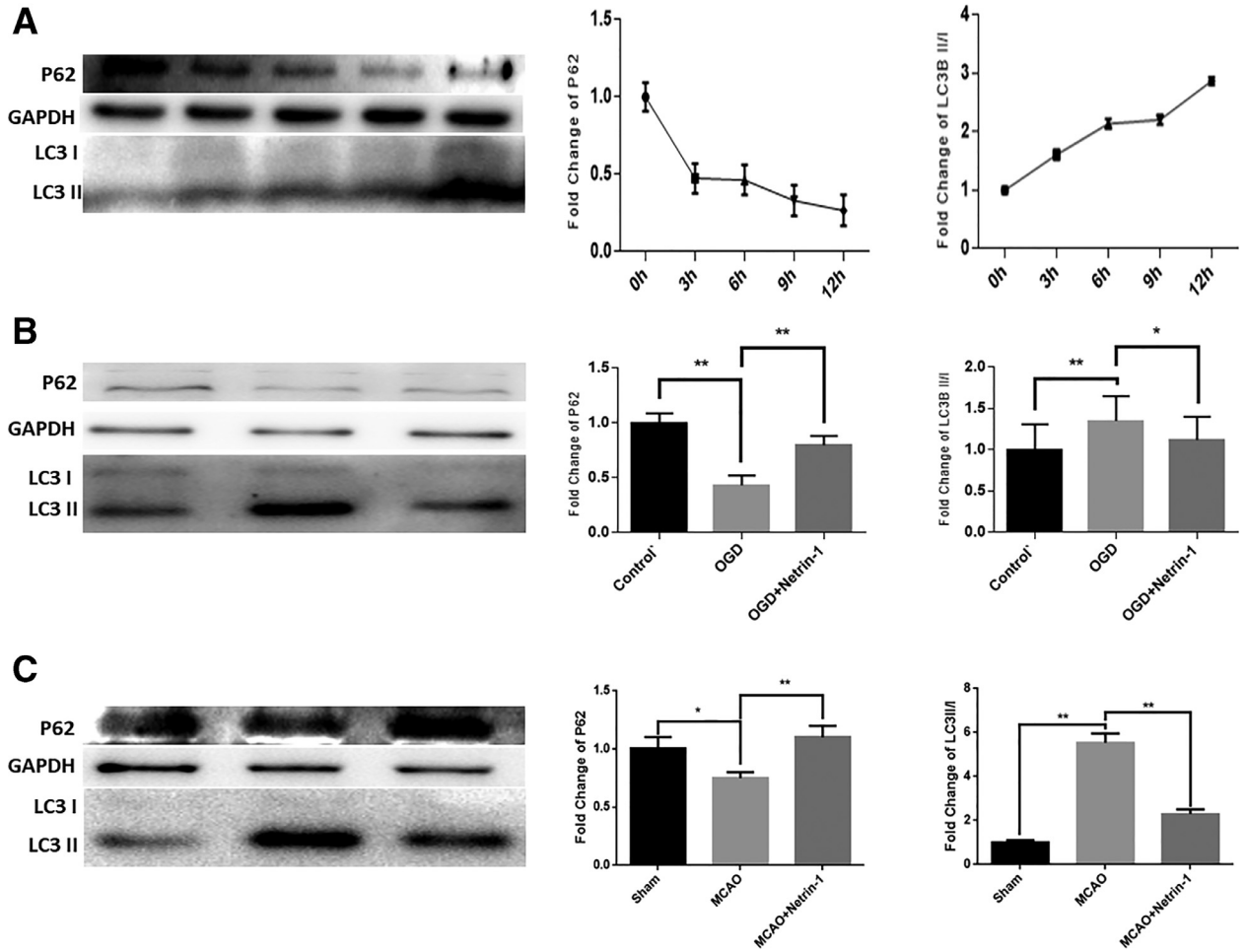

Figure 2. *Ischemia and hypoxia induce autophagy in brain tissues and neurons, while exogenous Netrin-1 inhibits autophagy. (A). After OGD treatment, autophagy enhanced in neurons. P62 decreased with the extension of hypoxia time and LC3B II/I ration increased with the extension of hypoxia time. N = 3. (B). In in vitro experiments, the P62 protein in OGD treatment group was significantly decreased (**, P < .01) compared with control group, while LC3B II/I was significantly increased (**, P < .01). Compared with the OGD treatment group, the P62 protein in the OGD+Netrin-1 group was significantly increased (**, P < .01), while LC3B II/I was significantly decreased (*, P < .05). N = 3. (C). In in vivo experiments, the P62 protein in MCAO group was significantly decreased (*, P < .05) compared with sham group, while LC3B II/I was significantly increased (**, P < .01). Compared with the MCAO group, the P62 protein in the MCAO+Netrin-1 group was significantly increased (**, P < .01), while LC3B II/I was significantly decreased (**, P < .01). N = 3. Abbreviations: OGD, Oxygen Glucose-Deprivation; MCAO, middle cerebral artery occlusion.*

mTOR ($P < .01$) were significantly up-regulated compared with that in OGD group (Fig 3A).

The in vivo results showed that p-PI3K /PI3K ($P < .01$) and p-mTOR /mTOR ($P < .01$) in MCAO group were significantly lowered than that in sham group. In MCAO+ Netrin-1 group, p-PI3K /PI3K ($P < .01$) and p-mTOR /mTOR ($P < .05$) were significantly up-regulated compared with that in MCAO group (Fig 3B).

These results suggested that PI3K/mTOR pathway was involved in the inhibition of autophagy by Netrin-1 in hypoxic neurons and ischemic brain tissues.

Neuronal Netrin-1 Secretion is Promoted by Exogenous Netrin-1

Moore et al reported that in the development of the central nervous system, Netrin-1 secretion can be enhanced by Netrin-1 itself and further promoted chemoattraction, indicating a positive feedback mechanism in Netrin-1 regulatory processes.[27] Therefore, we proposed a hypothesis that exogenous Netrin-1 could induce hypoxic neurons to further secrete Netrin-1 through a positive feedback mechanism, thereby amplifying the protective effect of hypoxic neurons. Western blotting and qRT-PCR test were performed to verify this hypothesis.

Western blotting showed that the expression of Netrin-1 protein in the OGD group was lower than that in the control group ($P < .01$). In the OGD+Netrin-1 group, the expression level of neuronal Netrin-1 protein was higher than that of the OGD group ($P < .05$) (Fig 4A).

In addition, PCR showed that OGD treatment enhanced the level of Netrin-1 mRNA ($P < .01$).The addition of exogenous Netrin-1 to hypoxic neurons further enhanced the level of Netrin-1 mRNA ($P < .01$) (Fig 4B). The above data suggested that exogenous Netrin-1 can promote its expression through the positive feedback mechanism.

Discussion

Autophagy is a metabolic process of cells, and its marker is the formation of autophagosome, in which LC3 protein is the marker protein.[28] In the process of autophagosome formation, LC3 protein changes from LC3 I to LC3 II, so the increase of LC3 II/I ratio indicates up-regulation of autophagy.[29] At the same time, P62 protein interacts with LC3 protein and degrades in the formation of autophagosome.[30] Therefore, variation trends of LC3 and P62 in the process of autophagy is reciprocal.

The role of autophagy in brain ischemia is not clear. Some authors support the protective effects of

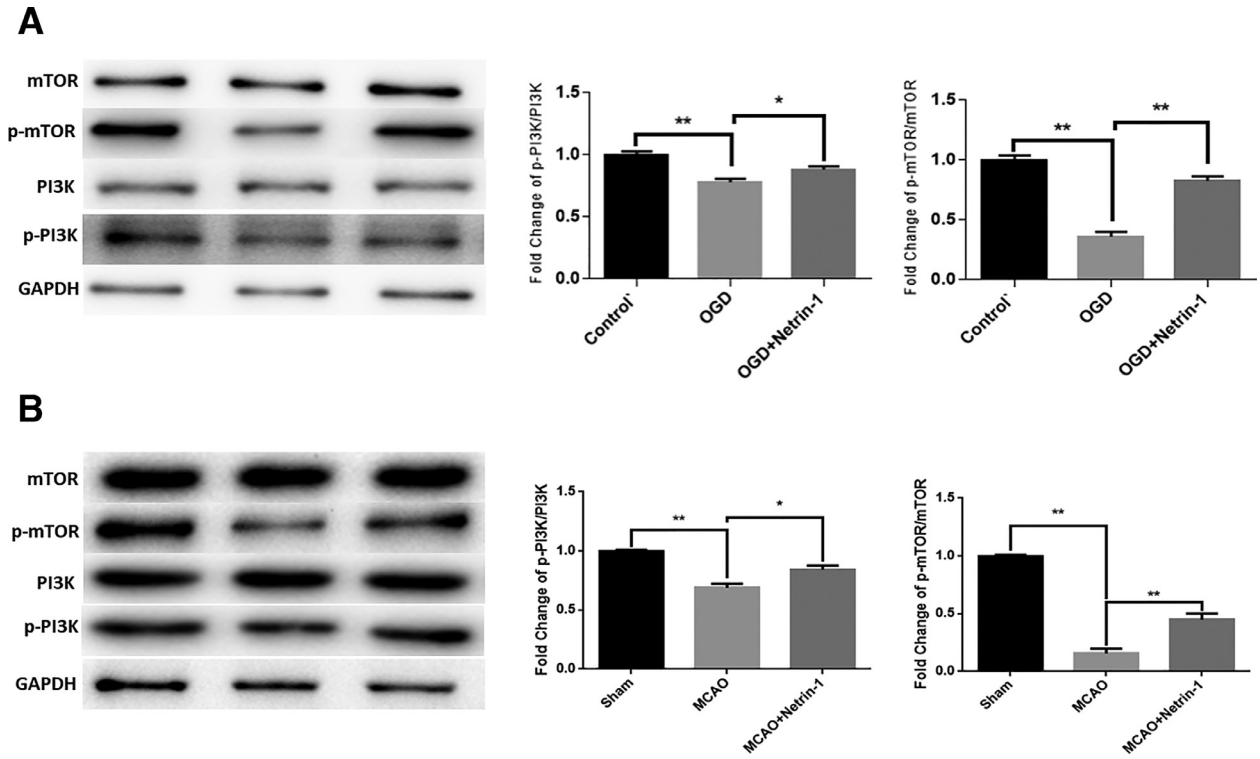

Figure 3. *Inhibition of autophagy by Netrin-1 is mediated by PI3K/mTOR pathway. (A). In in vitro experiments, compared with the control group, p-PI3K /PI3K (**, P < 0.01) and p-mTOR /mTOR was down-regulated (**, P < .01) in the OGD treatment group, indicating that the PI3K/mTOR pathway was inhibited. Compared with the OGD treatment group, p- PI3K /PI3K (**, P < .01) and p-mTOR /mTOR was up-regulated (*, P < .05) in the OGD+Netrin-1 group, indicating that the PI3K/mTOR pathway was activated. N = 3. (B). In in vivo experiments, compared with the sham group, p-PI3K /PI3K (**, P < .01) and p-mTOR /mTOR was down-regulated (**, P < .01) in the MCAO group, indicating that the PI3K/mTOR pathway was inhibited. Compared with the MCAO group, p- PI3K /PI3K (*, P < .05) and p-mTOR/mTOR was up-regulated (**, P < .01) in the MCAO+Netrin-1 group, indicating that the PI3K/mTOR pathway was activated. N = 3. Abbreviations: OGD, Oxygen Glucose-Deprivation; MCAO, middle cerebral artery occlusion.*

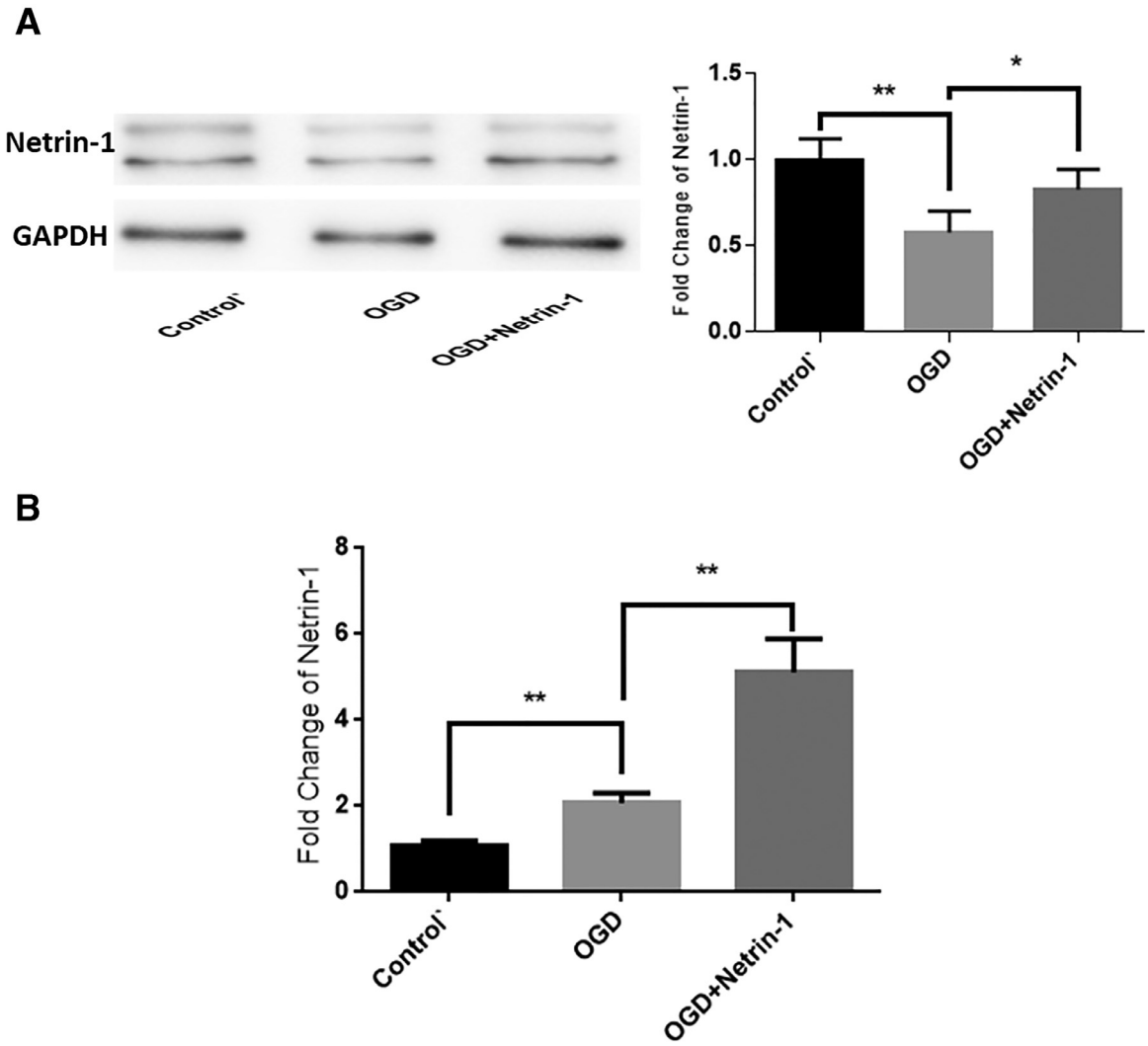

Figure 4. *Exogenous Netrin-1 can promote the secretion of Netrin-1 by neurons themselves, suggesting that Netrin-1 has a positive feedback mechanism in the process of protecting hypoxia neurons. (A). The expression of Netrin-1 protein in cells was detected by western-blot, and the results indicated that Netrin-1 decreased after OGD treatment (**, P < .01), while exogenous addition of Netrin-1 could induce up-regulation of Netrin-1 produced by neurons themselves (*, P < .05), N = 3. (B). The changes of Netrin-1 mRNA level was detected by qRT-PCR. The results indicated that hypoxia can induce up-regulation of Netrin-1 mRNA level (**, P < .01). In the Netrin-1 treatment group, the Netrin-1 mRNA level was further increased (**, P < .01). N = 3. Abbreviations: OGD, Oxygen Glucose-Deprivation.*

autophagy[31] while others object.[30] In our study, we found that ischemia/hypoxia induced excessive autophagy, which implies that autophagy is a hazardous process in ischemic stroke, and inhibition of neuronal autophagy could be a potential treatment target for ischemic stroke.

We further proved that exogenous Netrin-1 inhibits autophagy with ischemic/hypoxic insults. Meanwhile, Netrin-1 promoted the viability of ischemic neurons. The results indicated that Netrin-1's neuroprotective effect is related to its inhibition of autophagy.

PI3K/mTOR pathway is a signal pathway that regulates cell proliferation, growth and death, and is widely involved in the process of ischemic diseases. PI3K/mTOR pathway has negative regulatory effect on autophagy, and activation of PI3K/mTOR pathway can alleviate ischemic injury of cardiac and nervous cells.[32,33] Based on this, we speculated that PI3K/mTOR pathway might mediate the Netrin-1's inhibition of autophagy in ischemic stroke. Our results verified this speculation, and indicated that PI3K/mTOR could be a target for treatment of AIS.

It is found that Netrin-1 takes part in the development of the central nervous system via a positive feedback mechanism.[27] Our results further showed that exogenous Netrin-1 can promote the expression of Netrin-1 in ischemic neurons. It suggests that neuropotective role of exogenous Netrin-1 performs may be amplified by positive feedback mechanism.

In summary, we found that exogenous Netrin-1 can inhibit autophagy through PI3K/mTOR pathway. This effect may be amplified by positive feedback mechanism. However, the hazardous effect of autophagy in ischemic

stroke needs to be further confirmed by using autophagy regulators. The relationship between Netrin-1 and autophagy also need to be further elucidated. In our future work, we plan to modulate autophagy by using autophagy enhancers and inhibitors in MCAO and OGD models, combining with administration of Netrin-1. If the relationship between Netrin-1 and autophagy is fully elucidated, exogenous Netrin-1 can be expected to become a new substance for treatment of ischemic stroke, especially in patients out of the time window.

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