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Original Research

Expression changes and roles of Sema3A and Nrp1 in cultured rat cortical neurons after oxygen glucose deprivation

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Abstract: To investigate the expression changes and roles of Semaphorin3A (Sema3A) and Neuropilin-1 (Nrp1) in cultured rat cortical neurons after oxygen glucose deprivation (OGD). Cultured cortical neurons of newborn SD rats were divided randomly into control group and OGD treatment group. Western blot was performed to detect the expression of Sema3A and Nrp-1 protein and TUNEL was used to detect apoptosis. With the increase of OGD treatment time, the cells become swollen, the axon disintegrated and death cells increased. After 2 hours of OGD treatment, the expression levels of Sema3A and Nrp1 were increased by 6.86 and 5.92 times of normal control, respectively. After transfection of Sema3A, apoptosis was significantly reduced with OGD treatment for 2 hours. OGD treatment could induce the up-regulation of Sema3A and Nrp1 expression and transfection of Sema3A could reduce apoptosis after OGD treatment. The results suggest that Sema3A plays a protective role for the neuron cell in OGD treatment.

Key words: Cortical neurons; Oxygen glucose deprivation (OGD); Semaphorin3A; Neuropilin-1.

Introduction

Stroke is one of the most important fatal diseases in the world because of its high morbidity, high disability rate, high recurrence rate and high mortality rate (1). It is of great medical significance and socio-economic value to seek a new treatment for stroke. Ischemic stroke accounts for 75%-85% of the total number of stroke patients. Ischemia reperfusion injury can lead to apoptosis or even death of nerve cells through multiple apoptotic signal transduction pathways, resulting in the corresponding neural function defect (2, 3). Therefore, the effective intervention of apoptosis pathway can play a neuroprotective role after cerebral ischemia and hypoxia, and cerebral ischemia injury is closely related to apoptosis (4).

Semaphorin3A (Sema3A) was a secretory protein with the molecular weight of 100ku, and it was first extracted from chicken brain by Luo et al (5). Neuropilin-1 (Nrp1) was originally identified as a receptor for class 3 semaphorins controlling neuronal guidance and axonal growth. As an inhibitory axon guidance factor in the development of nervous system, Sema3A is combined with its receptor NRP1 and through inducing the collapse of the growth cone at the end of the axon to prompted the reversal of the growth direction of the axon (6). Sequentially, it plays the role of directional navigation of the axon and participates in the process of bunching, branching and synapse formation of nerve fibers (7). It has been proved that Sema3A can achieve neuroprotective effect by mediating apoptosis of microglia cells (8) and it also participates in the pathophysiological process of nerve regeneration and repair after nervous system injury (9), but the role it plays is not entirely clear.

At present, the neuronal oxygen glucose deprivation/reoxygenation model is widely used to explore the important means of cerebral ischemia-reperfusion injury in vitro (10). Meanwhile, the research of cerebral cortex neuron oxygen glucose deprivation/reoxygenation is rare in China (11). In this study, we used rat cortical neurons cultured in vitro as a model to observe the changes of Sema3A and Nrp1 expression after treatment with oxygen glucose deprivation (OGD), and to explore the role of exogenous Sema3A protein in neuronal cells with OGD induction in vitro. Thus it provide new targets and ideas for early neuroprotection after stroke.

Materials and Methods

Main reagents and experimental animal

Sprague Dawley rats (SPF grade) on day 18 of gestation (E18) were used. The DMEM culture medium, sugar-free DMEM medium, trypsin, neurobasal medium, B27, N_2 were all purchased from American Gibco company. The fetal calf serum was purchased from Sijiqing company. The L-glutamine was purchased from SIGMA company. The double antibody and β-Actin antibody were purchased from Hyclone company and SAN company, respectively, and the rest of the reagents were homemade analysis pure.

Primary culture of neurons

The pregnant SD rats on E18 (SPF grade) were selected. The uterus was cut open after neck removal and the fetal mice were taken out and placed in a culture dish containing cold phosphate-buffered saline (PBS) solution. The fetal rat cerebral cortex was dissected with

microsurgical forceps in aseptic environment under anatomic microscope, and the meninges and vascular tissues were removed. Then placed it in a culture dish containing ice DMEM/HIGH medium and shredded. It was digested with 0.05% trypsin at 27℃ for 20min and terminating digestion with serum-containing medium. Then centrifuged and removed supernatant, dispersed the mixture into cell suspension and filtered. The cells were inoculated in Lysine-coated culture dishes at proper density after the cell count and then cultured in 5% CO_2 incubator at 37℃. After 4 hours, the original medium was replaced by neurobasal+B27+L-glutamine mixed medium, and then the medium was changed every 2-3 days in half volume.

Establishment of Neuron OGD Model

After culturing of the primary cortical neurons for 6 days, the original neurobasal+B27+L-glutamine mixed medium was replaced by the glucose free DMEM medium. After the hypoxia reoxygenation incubator was filled with 95% N_2 and 5% CO_2 for 10min, and the three gas incubator was placed in a constant temperature incubator at 37℃ for 0, 1, 2, 3, 4 hours by anoxic treatment. Then the hypoxia glucose free DMEM medium was removed and the 10% high glucose DMEM medium was added. The medium was cultured by reoxygenation in constant temperature incubator at 37℃, 5% CO_2 and the time of oxygen glucose deprivation was determined 24 hours later through taking photos.

Western blot and immunoblot assay

After 6 days of primary neuron culture, the cells were divided randomly into two groups: one group was used as control group of neurons, namely NC cell group, the other group was treated with OGD, namely NC cell + OGD treatment group. The protein of attached cells for each group was extracted, rinsed, splited and centrifuged. And the protein was quantified by Bicinchoninic acid (BCA) protein quantitative kit and 30μg of the protein sample was loading. The protein was separated by SDS-PAGE and transferred to the PVDF membrane. After the trarsmembrane, the PVDF membrane was stained in Ponceau's staining solution for 2 to 5 minutes to observe the effect of protein transfer. Then the PVDF membrane was transferred to the TBST blocking reagent (with Tween-20 concentration of 0.05%) containing 5% BSA and blocked at room temperature for 1.5 h. For detection of Sema3A, Nrp1 and Cleaved caspase-3 proteins, the extracts were incubated at 4℃ overnight using antibodies raised against Sema3A, Nrp1 and Cleaved caspase-3 with a 1:800, 1:1000 and 1:1000 dilution, respectively. After incubation with appropriate horseradish peroxidase antibody for 1 h, they were reacted in the enhanced chemiluminescence solution for 2-3 min and developed with X-ray film. The data were statistically analyzed by the relative expression of the target protein (Sema3A, Nrp1 and Cleaved caspase-3)/reference protein (β-Actin).

Immunofluorescent staining

Fluorescence immunocytochemistry staining of Sema3A and Nrp1 was performed as follows. Cultured cells were fixed with 4% paraformaldehyde for 30 min at room temperature, incubated with goat polyclonal anti-Sema3A (diluted 1:100, purchased from Abcam) and anti-Nrp1 antibody (diluted 1:200, purchased from Abcam) at 4℃ overnight. Then they were incubated with Alexa-594 donkey rabbit anti-goat (diluted 1:400) for 30min, and mounted in Vectashield mounting medium with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI, purchased from Vector). The immunostained samples were observed and analyzed with a fluorescent inverted microscope system (OLYMPUS IX5). (12, 13).

Construction of Sema3A interference vector

Interference fragment was designed based on accession number NM_017310 of Sema 3A gene in NCBI gene bank with the sequence described as follows (Figure S1A). The synthesized shRNA fragment was a single stranded DNA, which needed to be annealed before connected with the carrier. The complementarities of the designed shRNA fragments were shown in Figure. S1B. The annealing results was detected using 2% agarose gel electrophoresis. Double enzyme digestion was performed using endonuclease BamHI and EcoRI. After annealing, it was connected with dilution of 50 times (10nmol) for the shRNA. Routine transformation was performed using Escherichia coli competence stbl3 (lentivirus plasmid). The vector sequencing primers were used for the polymerase chain reaction (PCR) verification of the bacterial solution. The sequences of primers for the PCR of the pLVshRNA-EGFP(2A)Puro vector were as follows: F: GACTATCATATGCTTACCGTAACT; R:TGCTACTTCCATTTGTCACG. Then bacterial solution sequencing was carried out.

Interference vector screening

Transfections were performed using the liposome of lipofectamine 2000. 293T cells were laid on the day before packaging, so that the degree of cell confluence was about 80% before transfection. The cell culture medium was replaced with Opti-MEM medium before transfection. The plasmid was added to a serum-free Opti-MEM medium and mixed gently. The liposome Lip2000 was dissolved in serum-free Opti-MEM medium, mixed gently and incubated at room temperature for 5 min. The above two solutions were mixed together and incubated at room temperature for 20 min to form liposome-DNA complex. The liposome-DNA complex was added to 293T cell culture dish, mixed gently, cultured in 5% CO_2 cell incubator at 37℃ for 6 h, then the culture medium was replaced by DMEM medium and cultured for 48-72h. The lentivirus supernatant was collected, centrifuged, filtered, subpackaged and stored at -80℃. On the third day of neuron culture, virus original solution thawed in ice bath was added into the 6-well plate, mixed well, cultured in incubator for 12 hours, then replaced with normal culture medium. The efficiency of interference vector was verified by qPCR when the cell appeared fluorescence.

Neuronal cell transfection

Virus titer determination was performed after packaging and concentration. The preliminary experiment was performed after 72 hours of neuron inoculation. Virus dilution solution was added to each well of the plate according to the MOI value of 0, 1, 5, 10, 15. Each titer was added to six wells and there were six groups, in

which the MOI value of 0 was the blank control group with no virus was added, and the culture conditions were the same as before. Total liquid was exchanged every 12 hours. The fluorescence intensity of the cells was observed under fluorescence inverted microscope after 72 hours and the time of infection was recorded everyday. The optimized conditions of lentivirus infection can be roughly known through the preliminary experiment. The formal experiment can be carried out amplify with reference to the results of the pre-experiment, and the amplification principle was to keep the concentration of the virus unchanged.

Detection of apoptosis by TUNEL

TdT-mediated dUTP Nick-End Labeling (TUNEL) assay is one the immunohistochemistry method which is widely used to detect apoptotic or degenerated neurons. In this study, the detected cells were divided into NC cell group, NC cell+OGD treatment group and NC cell+OGD treatment+Sema3A shRNA group. The cultured cells was fixed and incubated away from light with TUNEL detection solution at 37°C for 60 min. After staining and sealing, the cells were observed under fluorescence microscope, in which red indicated the apoptotic cells and blue indicated all cells in the field of view.

Results

Neuronal axonal injury caused by OGD treatment

In normal condition, after 6 days culturing of the primary rat cortical neurons, the neuronal bodies were full, the cytoplasm was clear, the nucleus was large and obvious, the cells mostly presented as long fusiform and individually as cones, the surface of the cells was smooth, the boundary was clear, and the cells had strong refraction. The pellicle and protrusions were obvious, the intercellular synapses were root shaped and intertwined to form a distinct "neural net" (Figure 1A). However, with the increase of oxygen glucose deprivation (OGD) time, the cells showed obvious changes. The main manifestations were the decrease of adherent ability, the enlargement of intercellular space, the decrease of refractivity, the swelling of cells, the disintegration of axon, the destruction of the "neural net", which became incomplete, and the number of dead cells increased obviously (Figure 1B). There were no significant changes found in the morphology of reoxygenated cells (Figure 1C). After 3 hours of oxygen glucose deprivation, most of the cells died, so the time of oxygen glucose deprivation was determined to be 2 hours (Figure 1B). These results demonstrate that OGD treatment induced axonal injury in neuronal.

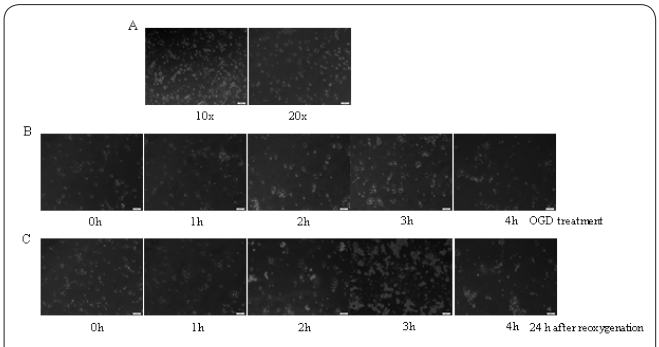

Figure 1. Determination of time of oxygen glucose deprivation of neurons. (A) Cellular morphology of the primary rat cortical neurons after 6 days culturing. (B) State of cells after oxygen glucose deprivation. (C) State of cells after 24 h reoxygenation.

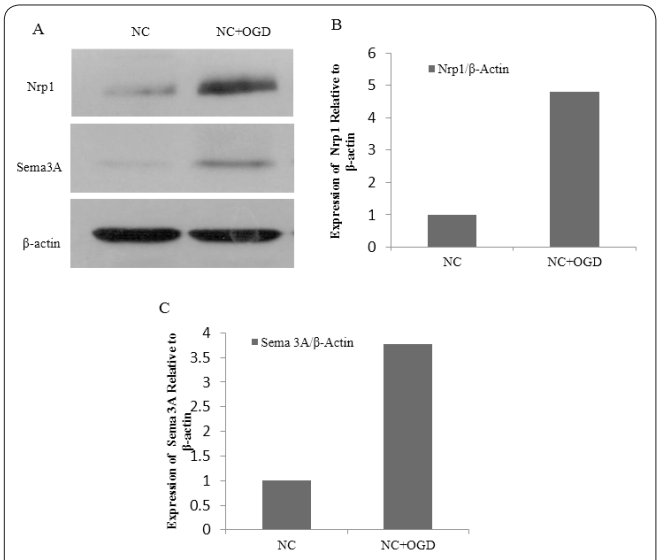

Figure 2. Expression of Sema3A and Nrp1 following OGD treatment in neuron cells. (A) Western blot analyses of Sema3A and Nrp1 in the protein extracted from neuron cells with or without OGD treatment. The blot was probed with antibodies against Sema3A and Nrp1. Antibody of β-Actin has been used as a control for protein arrays. (B) Quantitive analyses of the results of Nrp1 accumulation in the cells with or without OGD treatment by statistical methods. (C) Quantitive analyses of the results of Sema3A accumulation in the cells with or without OGD treatment by statistical methods.

Effects of OGD on the altered expression of Sema3A and Nrp1 in neuron cells

A further study was carried out to investigate the mechanism of neuronal axonal injury caused by OGD treatment. Semaphorins are cell surface and soluble signals that exert an inhibitory control on axon guidance. Sema3A, the vertebrate-secreted semaphorin, binds to Nrp1, which together with plexins constitutes the functional receptor (14). An Nrp1 selective peptide inhibitor provided neuroprotection against oxygen glucose deprivation and Nrp1 was reported to modulate axonal damage and neuronal death in response to cerebral ischemia. NRP-1 activation leads to axonal retraction and neuronal death and inhibition of the signaling pathway may have the potential for protection of both axons and neurons (15). To determine whether Nrp1 and Sema3A was regulated in OGD-induced neuronal axonal injury, we examined the expression levels of Sema3A and Nrp1 in neuron cells by western blot. It was found that the amounts of Sema3A and Nrp1 protein were increased in OGD treated cells (Figure 2). This indicates that Sema3A and Nrp1 is probably involved in OGD-induced neuronal axonal injury.

Detection of the expression of Sema3A and Nrp-1 through immunofluorescent staining

To further investigate Sema3A and Nrp1 expression in neuron cells during OGD, primary cultured neurons were analyzed by immunofluorescent staining with anti-Sema3A and anti-Nrp1 antibody. After staining, the

cells were observed under fluorescence microscope, in which blue fluorescence represents all the nucleus, and red fluorescence represents Sema3A and Nrp-1. Similarly, as shown in Figure 3, enhanced fluorescent signals for Sema3A and Nrp1 were observed in OGD treated neuron cells. Collectively, these results suggested that OGD treatment induced an enhanced expression of Sema3A and Nrp1.

Transfection with Sema3A for the neuronal cells

The synthesized shRNA fragment was a single strand DNA and annealing was needed before connected with the carrier. As shown in figure S1C, there was obvious nucleic acid band in the 50bp, which was the target shRNA. The empty vector amplification should be used as control when PCR for the bacteria solution was carried out. The results of PCR for the verification of vector construction were detected by 2% agarose gel electrophoresis (120V, 40min) and three results were observed as shown in Figure S1D. High fluorescence intensity of cells indicated the successful packaging of virus (Figure S2). Based on the expression of GFP in the following fluorescence images (Figure S3), two cells with fluorescence were observed in the well containing 10-5μL virus solution, indicating that at least two viral particles were infected the cells. Then the titer of the virus is equal to the number of cells with fluorescence divided by the amount of the virus's original solution. In this example, the titer is $2/10^{-5}$, which is equal to 2E+8TU/mL. After viral packaging and neuronal infection, total RNA was extracted to verify the efficiency

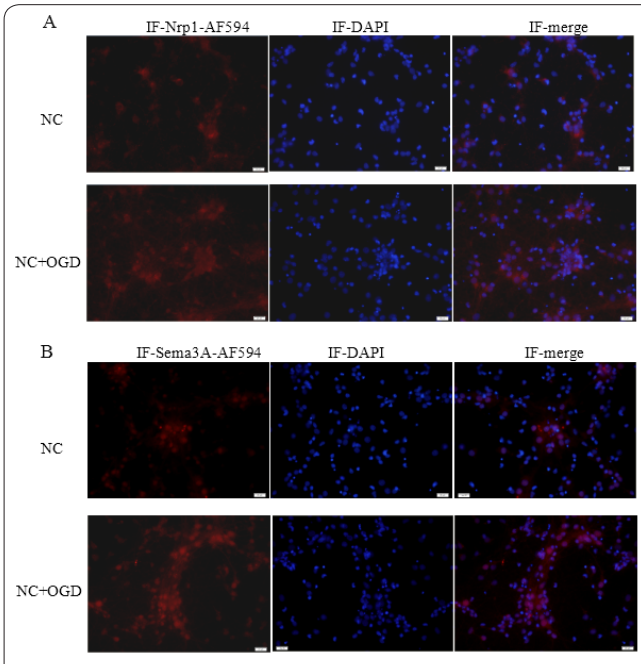

Figure 3. Immunofluorescence detection of Sema3A and Nrp1 expression using anti-Sema3A and anti-Nrp1 antibody. (A) Images of Nrp1 fluorescence in the representative neuron cells. Blue fluorescence represents the nucleus, which were stained with DAPI, and red fluorescence represents Nrp1, which was stained by incubating with Alexa Fluor 594 donkey-rabbit IgG. Merge of the two images shows colocalization in neurons. (B) Images of Sema3A fluorescence in the representative neuron cells. Blue fluorescence represents the nucleus, which were stained with DAPI, and red fluorescence represents Sema3A, which was stained by incubating with Alexa Fluor 594 donkey-rabbit IgG. Merge of the two images shows colocalization in neurons.

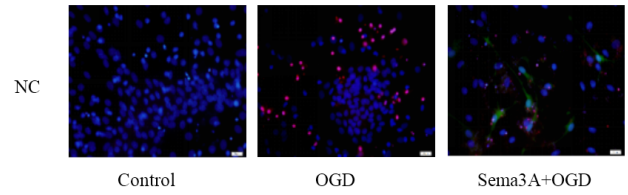

Figure 4. Detection of apoptosis by TUNEL. After staining and sealing, the cells were observed under fluorescence microscope. Red indicated the apoptotic cells and blue indicated all cells in the field of view.

of interference vector. Transfection was performed after virus packaging, concentration and titer determination. As shown in figure S4, on the fifth day of lentivirus infection, the number of GFP positive cells were the most when multiplicity of infection was 10 and 15. But the state of cells with mol=10 was significantly better than that with mol=15, so the multiplicity of neuronal infection was determined to be 10.

Protective effect of Sema3A on neuronal cells during OGD treatment

The experiment was divided into three groups: NC cell group, NC cell + OGD group, NC cell + OGD + Sema3A group. OGD treatment was carried out after the target cells was infected by the lentivirus. The TUNEL technique was performed to detect and quantitate cell death. As shown in Figure 4, after the neuronal cells were transfected with Sema3A, the apoptosis of neurons was significantly reduced with OGD treatment for 2 hours.

To further determine whether reduced apoptosis of neurons was affected by Sema3A, western blot analysis was performed to determine the Sema3A and Nrp1 expression using the anti-Sema3A and anti-Nrp1 antibody in the three groups described above under OGD induction. Transfection of neuronal cells with Sema3A led to down-regulation of Sema3A and Nrp1 at protein level upon OGD treatment (Figure 5 A-C). To check whether OGD treatment affects the expression of apoptosis-associated protein, the protein levels of Cleaved caspase-3 was examined. It was found that the expression of Cleaved caspase-3 protein increased in OGD treated neurons and this increase was reduced by transfecting with Sema3A. It was concluded that Sema3A played a protective role in OGD treatment and could reduce apoptosis.

Discussion

In the brain injury caused by stroke, intracellular calcium overload leads to a series of harmful cascade reactions, resulting in neuronal damage and death (16). In the acute phase of cerebral ischemia, neuronal death occurs in the central region of ischemia, which is mainly performed as necrosis. While secondary death occurs in the penumbra after ischemia, which is mainly performed as apoptosis. There are penumbra in the cortex during cerebral ischemia injury, and the location of the penumbra is relatively constant (17). Therefore, the OGD model in this study was constructed by hypoxia-glucose deprivation in rat cortical neurons.

Previous studies have shown that in the process of inducing neuronal death in vitro, it was first appeared

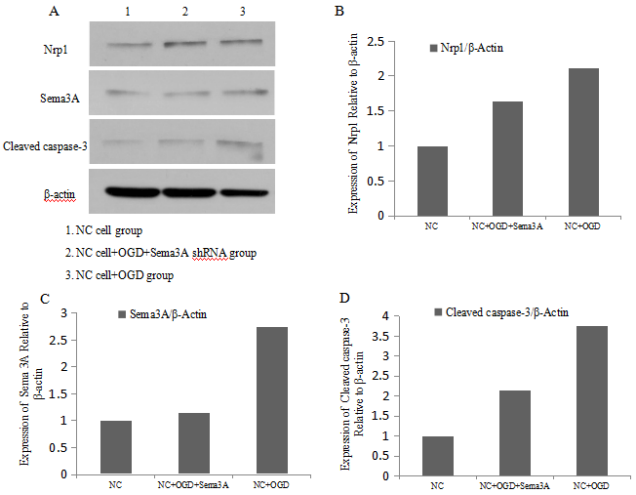

Figure 5. Expression of Sema3A, Nrp1 and Cleaved caspase-3 following OGD treatment in neuron cells transfection with Sema3A. (A) Western blot analyses of Sema3A, Nrp1 and Cleaved caspase-3 in the protein extracted from the following three groups: NC cell group, NC cell+OGD treatment+Sema3A shRNA group and NC cell+OGD treatment group. The blot was probed with antibodies against Sema3A, Nrp1 and Cleaved caspase-3. Antibody of β-Actin has been used as a control for protein arrays. (B) Quantitive analyses of the results of Nrp1 accumulation by statistical methods. (C) Quantitive analyses of the results of Sema3A accumulation by statistical methods. (D) Quantitive analyses of the results of Cleaved caspase-3 accumulation by statistical methods.

axonal retreat and disintegration, then typical morphological changes of neuronal death occur (18). In this study, with the increase of oxygen glucose deprivation time, cell swelled, axon disintegrated, "neural net" was destroyed, and the number of dead cells increased. After treated with OGD for 3 h, most of the neurons were found to be dead irreversible, but the morphology of reoxygenated cells remained unchanged. The results suggest that OGD treatment can induce neuronal axon net disintegration, which can lead to irreversible injury or even death of neurons. Combined with related literature (16), it is speculated that axonal injury may be an early inducing factor for the whole neuron death.

Sema3A is one of the most studied factor currently in secretory signaling family Semaphorins, which binds to its receptor Nrp1 and participates in the processes of axon orientation, cell migration, tumor growth, angiogenesis and immune regulation (19). Previous studies have shown long-term up-regulation of Sema3A expression in infarcted areas and surrounding tissues after cerebral ischemia (20). And the expression of its receptor Nrp1 is also up-regulated during the cerebral ischemia (21, 22), suggesting that Sema3A and Nrp1 may play an important role in the chronic repair of ischemic infarct and surrounding injuried tissues. The present study focused on the acute phase after cerebral ischemia and observed that the expression of Sema3A and Nrp1 was significantly up-regulated after 2 hours of OGD treatment compared with the control group. It is indicated that both Sema3A and Nrp1 may be involved in the pathophysiological process of acute cascade reaction after ischemic brain injury, and the mechanism may be correlated to the changes of some RhoGTP enzymes related to axonal orientation, such as RhoA, Rac1 and cdc42 (23).

It has been demonstrated that the up-regulated expression of Sema3A and its receptor Nrp1 in injured brain tissue may play a role in inducing neuronal axonal contraction and even death and inhibiting axon regeneration after axonal injury (18, 24, 25). But the molecular mechanism is not fully understood. However, Sema3A may also play another role because of the extensive expression of it and its receptor Nrp1 and the multiplicity of their roles. For example, Sema3A secreted by stressed neurons can protect itself by up-regulating Nrp1/PlexinA1 to promote the apoptosis of microglial cell (8). Sema3A can also play a dual role of inhibition and attraction, which depends on the level of cGMP in neurons (26, 27). In this study, after the neuronal cells were transfected with Sema3A, apoptosis was significantly decreased at 2 h after OGD treatment. The results showed that Sema3A had protective effect on neuronal cells during OGD treatment, and could significantly reduce apoptosis. However, the related mechanism need to be further explored. At present, how to inhibit apoptosis after ischemic neuron injury is a hot topic of brain protection (28). To further elucidate the up-regulation mechanism of Sema3A and its receptor Nrp-1 expression after OGD treatment and explore the protective effect of Sema3A on neuronal cells in OGD induction will provide new targets and insights for early neuroprotection after stroke.

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Interest conflict

There is no conflict of interest to be declared by the author.

Author's contribution

All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Ya He; Ying Yang, Ruili Wang, Hui Wang, Qiaoya Ma, Songsheng Chen, Daiying Shi collected and analysed the data; Ya He wrote the text and all authors have read and approved the text prior to publication.

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