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Expression of netrin-1 and its receptors, deleted in colorectal cancer and uncoordinated locomotion-5 homolog B, in rat brain following focal cerebral ischemia reperfusion injury

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Abstract

Netrin-1 is currently one of the most highly studied axon guidance factors. Netrin-1 is widely expressed in the embryonic central nervous system, and together with the deleted in colorectal cancer and uncoordinated locomotion-5 homolog B receptors, netrin-1 plays a guiding role in the construction of neural conduction pathways and the directional migration of neuronal cells. In this study, we established a rat middle cerebral artery ischemia reperfusion model using the intraluminal thread technique. Immunofluorescence microscopy showed that the expression of netrin-1 and deleted in colorectal cancer in the ischemic penumbra was upregulated at 1 day after reperfusion, reached a peak at 14 days, and decreased at 21 days. There was no obvious change in the expression of uncoordinated locomotion-5 homolog B during this time period. Double immunofluorescence labeling revealed that netrin-1 was expressed in neuronal cells and around small vessels, but not in astrocytes and microglia, while deleted in colorectal cancer was localized in the cell membranes and protrusions of neurons and astrocytes. Our experimental findings indicate that netrin-1 may be involved in post-ischemic repair and neuronal protection *via* deleted in colorectal cancer receptors.

Key Words

neural regeneration; brain injury; cerebral ischemia and reperfusion; netrin-1; uncoordinated locomotion-5 homolog B; deleted in colorectal cancer; neuron; brain injury; grant-supported paper; photographs-containing paper; neuroregeneration

Research Highlights

(1) Netrin-1 induces the directional migration of nerve cells and plays an important role in the repair of adult central nervous system injuries.
(2) We confirmed the upregulated expression of netrin-1 and its receptor, deleted in colorectal cancer, in the cortex of rats after focal cerebral ischemia and reperfusion injury, both of which reached their peak expression level at 14 days and began to decrease at 21 days.
(3) Netrin-1 expression was observed in neuronal cell bodies by laser confocal microscopy, particularly in neurons at the edges of small vessels, while it was scarcely expressed in astrocytes and microglial cells.

Abbreviations

DCC, deleted in colorectal cancer; UNC5B, uncoordinated locomotion-5 homolog B

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INTRODUCTION

The functions of the nervous system are determined by the precise connections between neurons. Netrin-1 is a soluble axon guidance factor[1] that is widely expressed in the embryonic central nervous system; it plays a guiding role in the development of neural conduction pathways by regulating neuronal migration. Netrin-1 mediates its axon guidance activity in combination with the deleted in colorectal cancer (DCC) receptor family and the uncoordinated locomotion-5 homolog B (UNC5B) receptor family, thus guiding the axonal growth and migration of neural progenitor cells during the development of the central nervous system[2-3]. DCC antibodies can prevent the migration of optic nerve cell axons on netrin-1[4-5], while netrin-1 antibody can interrupt the netrin-1-induced projection of nerve axons from the thalamus to cerebral cortex. In addition, knock out of the netrin-1 gene results in the disordered projection of dorsal thalamus nerve fibers through the ventral telencephalon[6]. Furthermore, many experimental studies have demonstrated that netrin-1 induces directional nerve cell migration[7]. Increasing evidence indicates that netrin-1 plays a significant role in the repair of adult central nervous system injury, and also in tissue repair outside of the nervous system. Hamasaki *et al* [7] found that netrin-1 expression was significantly increased to almost 40 times the basal expression level at 2 weeks after sciatic nerve transection injury and in the process of nerve injury repair. Netrin-1 also inhibits glutamate-mediated neuronal toxicity and reduces neuronal apoptosis[8]. During sciatic nerve autoimmune inflammation, the expression levels of netrin-1 and DCC increased significantly, which contributed to the promotion of cell survival and axon regeneration[8]. Netrin-1 suppresses the migration of inflammatory cells *via* UNC5B receptors[9]. During treatment of myocardial ischemia and reperfusion injury, netrin-1 in combination with the DCC receptor promoted nitric oxide production in endothelial cells and induced angiogenesis[10]. Evidence for the neuroprotective effect of netrin-1 against ischemia and the expression of its receptor after focal cerebral ischemia remain inconclusive. Wu *et al* [11] demonstrated that the expression of UNC5B was upregulated after ischemia, while the expression of DCC remained unchanged. Tsuchiya *et al* [12] proposed that the expression levels of netrin-1 and its receptor, DCC, increased in the peri-infarction site. Hoang *et al* [13] reported that UNC5B expression was unchanged at the peripheral infarct site. Therefore, in this study, we aimed to characterize the expression of netrin-1 and its receptors, DCC and UNC5B, after cerebral ischemia reperfusion in rats.

RESULTS

Quantitative analysis of experimental animals

In this study, a total of 44 Sprague-Dawley rats were divided into a sham surgery group (n = 8) and a model group (n = 36). The focal cerebral ischemia reperfusion model was established in the model group, in which four rats died of excessive anesthesia-induced respiratory arrest, external carotid artery, cervical vagus nerve injury, or perforated vessel rupture at the carotid artery bifurcation. Ultimately, 8 rats in the sham surgery group and 32 rats in the model group were analyzed.

Netrin-1 expression in rat brain after focal cerebral ischemia reperfusion

Immunofluorescence microscopy demonstrated that netrin-1 was not expressed in the cerebral cortex of sham-operated rats and model rats. At 1 day after focal cerebral ischemia reperfusion, weakly fluorescent netrin-1 positive cells were visible in the cerebral cortex and striatum around the infarct in the model rats, but netrin-1 expression was absent in the center of infarct site. Netrin-1 expression in brain tissue of model rats increased significantly with reperfusion time, with the fluorescence becoming more intense particularly in the cerebral cortex at the peripheral ischemic site. The expression of netrin-1 peaked at 14 days and then gradually decreased. Netrin-1 was localized in the neuronal cell body (Figure 1).

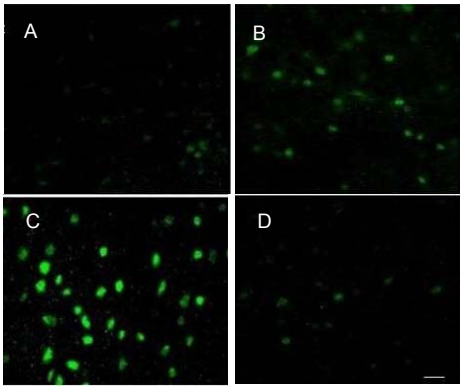

Figure 1 Netrin-1 expression around the ischemia site in the cerebral cortex of model rats (immunofluorescence staining; scale bar: 50 μm).

(A–D) 1, 7, 14 and 21 days after ischemia, respectively. Netrin-1 expression was detected with an Alexa Fluor 488-conjugated secondary antibody (green).

Double immunofluorescence staining showed that

netrin-1 immunoreactive cells and neuronal nuclear antigen-labeled neurons were co-expressed in the brain tissues of model rats after ischemia and reperfusion[14]. The fluorescence intensities of glial fibrillary acidic protein and CD11b, markers of reactive astrocytes and activated microglia[15-16], were significantly enhanced in large cells at the peripheral infarct site. However, glial fibrillary acidic protein and CD11b did not overlap with netrin-1, indicating that netrin-1 was expressed in neurons but not in astrocytes and microglia. In addition, netrin-1 expression was found at the edge of small vessels around the infarct site (Figure 2).

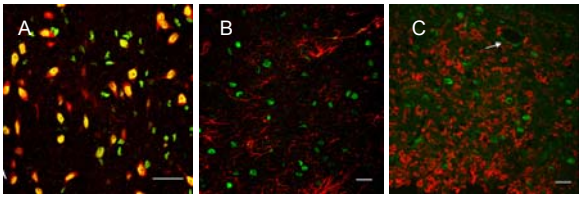

Figure 2 Netrin-1 was co-expressed with NeuN, GFAP and CD11b around the infarct site in the cerebral cortex at 14 days after middle cerebral artery occlusion (immunofluorescence double staining; scale bars: 20 μm).

Netrin-1 positive cells and NeuN labeled neurons were co-expressed (A), but netrin-1 did not co-localize with GFAP labeled astrocytes (B) and CD11b labeled microglia (C). (C) Netrin-1 was expressed around the vessels (arrow). Netrin-1 fluorescence is shown in green.

NeuN, GFAP and CD11b were detected with Alexa Fluor 594-conjugated secondary antibodies (red). NeuN: Neuronal nuclear antigen; GFAP: glial fibrillary acidic protein.

Expression of the Netrin-1 receptor, DCC, was upregulated in rat brain after focal cerebral ischemia

Immunofluorescence microscopy demonstrated that DCC was not expressed in the cerebral cortex of sham-operated rats and model rats, while few DCC positive cells were visible in the subcortical white matter and the hippocampus. At 1 day after reperfusion, the number of DCC positive cells in the cerebral cortex around the infarct in model rats increased significantly, with the DCC fluorescence intensity and the number of positive cells peaking at 14 days and then gradually decreasing (Figure 3). DCC expression was localized in the cell membrane and the protrusions. Double immunofluorescence staining detected the co-expression of DCC, neuronal nuclear antigen and glial fibrillary acidic protein in the cerebral cortex around the infarct site, which mainly localized to neurons, and to a lesser extent astrocyte protrusions (Figure 4).

Focal cerebral ischemia did not affect the expression of the netrin-1 receptor, UNC5B, in rat brain

Immunofluorescence microscopy demonstrated that UNC5B was expressed in the cerebral cortex of sham-operated rats, and in the cerebral cortex and basal ganglia of model rats. The number of UNC5B positive cells and their staining intensity in the model rats were similar to those in the control group. UNC5B was expressed at the cell membrane and the protrusions, similar to DCC (Figure 5).

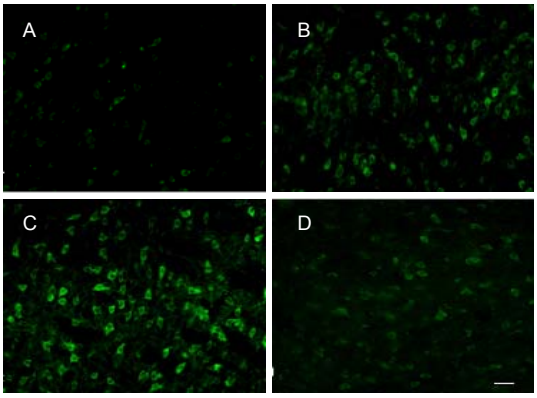

Figure 3 Expression of deleted in colorectal cancer at the peripheral infarct site in the cerebral cortex of model rats (immunofluorescence staining; scale bar: 50 μm).

(A–D) 1, 7, 14 and 21 days after middle cerebral artery occlusion, respectively. Deleted in colorectal cancer expression was detected with an Alexa Fluor 488-conjugated secondary antibody (green).

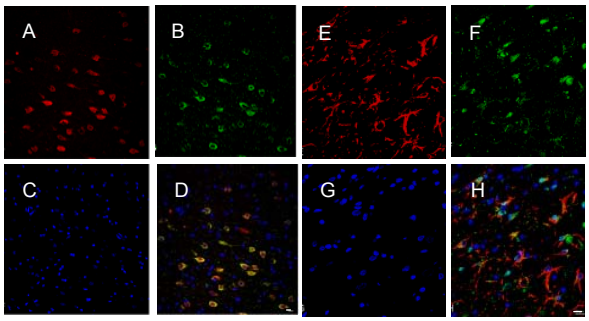

Figure 4 The deleted in colorectal cancer (DCC) receptor was co-expressed with NeuN and GFAP around the infarct site in the cerebral cortex at 14 days after middle cerebral artery occlusion (immunofluorescence double staining; scale bars: 20 μm).

The DCC positive signals are distributed in the cell membrane and protrusions, and are co-expressed with NeuN labeled neurons and GFAP labeled astrocytes. DCC fluorescence is shown in green. NeuN and GFAP were detected with Alexa Fluor 594-conjugated secondary antibodies (red). DAPI labeled nuclei are shown in blue.

(A) NeuN; (B) DCC; (C) DAPI; (D) $DCC^+/NeuN^+/DAPI$; (E) GFAP; (F) DCC; (G) DAPI; (H) $DCC^+/GFAP^+/DAPI$. (A–D) The same field of view; (E–H) the same field of view. NeuN: Neuronal nuclear antigen; GFAP: glial fibrillary acidic protein.

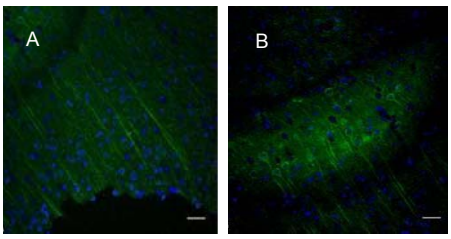

Figure 5 Expression of the netrin-1 receptor, uncoordinated locomotion-5 homolog B, in the rat cerebral cortex. Uncoordinated locomotion-5 homolog B expression was detected with an Alexa Fluor 488-conjugated secondary antibody (green). DAPI labeled nuclei are shown in blue. Scale bars: 50 μm.

(A) Cerebral cortex in the sham operation group.

(B) Peripheral ischemia cerebral cortex at 14 days after reperfusion.

DISCUSSION

In this study, we demonstrated that the expression of both netrin-1 and DCC increased in peripheral ischemia cerebral cortex at 1 day after reperfusion, reached their peak expression level at 14 days and then began to decrease at 21 days. Meanwhile, no changes in the expression level of UNC5B were obvious. These results were consistent with those reported by Tsuchiya *et al* [12] and Hoang *et al* [13]. The expression levels of netrin-1 and DCC were consistent with the time course of axonal regeneration, suggesting that netrin-1 may promote axonal growth after ischemia and reperfusion *via* DCC receptors. Furthermore, immunohistochemical double staining showed that netrin-1 was expressed in neurons, but not in astrocytes and microglia. DCC expression was apparent in the cell membranes and protrusions of neurons and astroglia. These observations suggest that endogenous netrin-1 may be involved in neuroprotection against ischemia reperfusion injury and reconstruction of the glia network *via* DCC receptors.

Netrin-1 plays a role in neuroprotection and can inhibit neuronal apoptosis during ischemia reperfusion injury[11, 17-20]. Netrin-1 overexpression can promote local artery-like neovascularization in adult brain[21]. Furthermore, netrin-1 overexpression significantly improved the neovascularization density at the ischemic penumbra and induced immature neurons to migrate into the ischemic penumbra[22]. Exogenous netrin-1 also suppresses the apoptosis of vascular endothelial cells *via* a vascular endothelial cell growth factor-like effect that promotes vascular regeneration[23]. Here, we found that netrin-1 was not only expressed in peripheral ischemia neurons but also in tissues with vascular morphologies, thus suggesting that netrin-1 may participate in vascular reconstruction after injury.

During central nervous system development, netrin-1 promotes axonal growth, determines axon guidance pathways, and induces the directional migration and aggregation of $A2B5^+$ glial progenitor cells[24]. Netrin-1 overexpression can inhibit neuronal apoptosis and improve the neurological behavior score after middle cerebral artery occlusion[25]. Following cerebral ischemia reperfusion injury, we observed consistent changes in netrin-1 and DCC expression, while the expression of UNC5B was unchanged. These results may suggest that the DCC receptor is involved in the tissue repair process after ischemic nerve injury. In addition, we also observed the expression of the DCC in the membranes and protrusions of neurons and astrocytes, which indicated that netrin-1 in combination with the DCC receptor may play important roles in neurite outgrowth after cerebral ischemia, glial cell network reconstruction, neuroprotection and vascular regeneration.

MATERIALS AND METHODS

Design

A randomized controlled animal experiment.

Time and setting

Experiments were performed between July 2010 and January 2012 in the Central Laboratory of Suzhou Kowloon Hospital, Shanghai Jiao Tong University School of Medicine, China.

Materials

Healthy adult male Sprague-Dawley rats of clean grade, aged 9 weeks, and weighing 250–280 g, were provided by the Experimental Animal Center of Soochow University, China, under license No. SYXK (Su) 2007-0035. The animals were housed at 23 ± 2°C with 40–70% relative humidity under a natural light-dark cycle, and allowed free food and water. Prior to experiments, all rats were adapted to the environment for 1 week. Experimental rats were preoperatively fasted for 12 hours. Experimental animals were in accordance with the *Guidance Suggestions for the Care and Use of Laboratory Animals*, issued by the Ministry of Science and Technology of China[26].

Methods

Establishment of the focal cerebral ischemia model

A middle cerebral artery occlusion model was established in rats according to the suture method of Longa *et al* [27]. The model rats were anesthetized with 10% chloral hydrate and disinfected with iodophor, a median incision was made in the median skin of the cervical part, and then the right common carotid artery and external carotid artery were ligated. The distal end of the common carotid artery was clamped and another incision was made at 2 mm lateral to the common carotid artery bifurcation, following which a nylon line of 0.2 mm in diameter and 25 mm in length was inserted until resistance was encountered. The suture was removed 90 minutes after ischemia and reperfusion was then performed. The sham surgery group underwent the separation and exposure of blood vessels only, without a suture being inserted.

Criteria for a successful model

At 24 hours after reperfusion, the model animals that were scored 1–3 according to the neurological function scale of Longa *et al* [27] were included in the model group. Of the animals in the model group, one rat was killed by decapitation at 24 hours after reperfusion and its brain was frozen at –20°C. Coronal serial slices were then cut from the frozen tissue and stained in PBS containing 1% TTC for 20 minutes at 37°C in the dark. Normal brain tissue was stained dark red while ischemic brain tissue was stained white, thus confirming that the cerebral ischemia model was successfully established.

Frozen section preparation

Rats were fixed with 4% paraformaldehyde and killed by pulling the skull. Brain tissues were isolated and fixed in 4% paraformaldehyde for 6 hours and successively dehydrated in 20% and 30% sucrose solutions overnight. Continuous coronal frozen sections were cut at a thickness of 8 μm, and one out of every six sections was mounted on a poly-L-lysine (Sigma, St. Louis, MO, USA)-coated glass slide and stored at –20°C.

Immunofluorescence analysis of the expression of netrin-1 and its receptors, DCC and UNC5B, in brain tissues from ischemic and reperfusion rats

Brain slices from the same layers were used for immunofluorescence staining[28]. Frozen sections were equilibrated at room temperature for 10 minutes, hydrated in 0.01 M PBS for 10 minutes, and blocked with 10% donkey serum (Jackson Immunoresearch, West Grove, PA, USA) for 1 hour at room temperature. After discarding the blocking solution, the sections were incubated with rabbit anti-netrin-1 polyclonal antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-DCC polyclonal antibody (1:100; Santa Cruz Biotechnology), goat anti-UNC5B polyclonal antibody (1:100; Santa Cruz Biotechnology), mouse anti-rat neuronal nuclear antigen monoclonal antibody (1:100; Chemicon, Billerica, MA, USA), mouse anti-rat glial fibrillary acidic protein monoclonal antibody (1:200; Sigma) or mouse anti-rat CD11b monoclonal antibody (1:200; Sigma) diluted in 5% donkey serum at 4°C overnight. After washing three times with PBS for 10 minutes each, the slices were incubated with donkey anti-rabbit IgG-Alexa Fluor 488, or donkey anti-goat IgG-Alexa Fluor 488 and donkey anti-mouse IgG-Alexa Fluor 594 secondary antibodies (1:200; Invitrogen, Eugene, OR, USA) for 1 hour in the dark. The slices were then washed a further three times with PBS for 10 minutes each, mounted in DAPI-containing quenching sealing agent (Santa Cruz Biotechnology), and stored at 4°C. Negative control slices were incubated with PBS instead of antibodies. Sections were analyzed by confocal laser scanning microscopy (Nikon A1, Tokyo, Japan) to determine the localization of immuno-positive cells at the infarct site and in the cerebral cortex, and also the co-localization of neurons, astrocytes and microglial cells.

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Author contributions: Xiaoping Wang conceived, designed and validated the study. Jinming Xu supervised the study and was in charge of the funding. Xiaodan Wang participated in the data integration and analysis, and drafted the manuscript. Jieqin Gong established the animal model. Hui Shen performed the confocal laser scanning microscopy. All authors approved the final version of the paper.

Conflicts of interest: None declared.

Ethical approval: The experiment was approval by the Experimental Animal Ethics Committee of Shanghai Jiao Tong University School of Medicine, China.

Author statements: The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations.

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