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The effect of an NgR1 antagonist on the neuroprotection of cortical axons after cortical infarction in rats

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The effect of an NgR1 antagonist on the neuroprotection of cortical axons after cortical infarction in rats

Running title: The neuroprotection of NgR1 antagonist on cortical axons

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Abstract

We investigated the effect of the soluble Nogo66 receptor (sNgR-Fc) on the protection of cortical axons after cortical infarction in rats. The cortical infarction was induced by photothrombotic cortical injury (PCI) in Sprague Dawley rats, after which sNgR-Fc was injected into the lateral ventricle. The ipsilesional cortices were harvested for analyses using histochemical and transmission-electron microscope techniques. The involved signaling pathways, which include RhoA, JNK, c-JUN and ATF-2, were detected by Western blot. Serious pathologies were found in the brains of the rats after injury, including edemas in the axoplasms of axons that have no medulla sheath and a thickening or shrinkage in the sheath of the axons that have medulla sheathes. However, these pathologies improved after sNgR-Fc treatment. The levels of GTP-RhoA, p-JNK, p-c-JUN and p-ATF-2 in the PCI group were increased when compared with their levels in the sham-operation group ($P < 0.05$), and animals receiving the sNgR-Fc treatment showed lower expression levels of these proteins when compared with the sham-operation group ($P < 0.05$). Our results suggest that sNgR-Fc can alleviate the pathological changes of axons following cortical infarction via decreasing the activation of RhoA/JNK signaling pathways.

Key words: Nogo-66 receptor; Axon; Cortical infarction; Regeneration; Signal pathway

Introduction

A brain infarction (also known as an ischemic stroke) is caused by the blockage of a cerebral artery by a blood clot, which results in the following effects: a critical reduction in blood flow, ischemia, anoxia, and, finally, ischemic necrosis or encephalomalacia in the brain. This condition is characterized clinically by high morbidity, high mortality, high disability rate, high recurrence rates, and it is difficult to treat. Because our population is aging, stroke is becoming a more serious threat and an urgent medical problem. Previous studies have shown that the central nerve system cannot regenerate after injury, as a result of factors that include the insufficiency of neural progenitor cells (NPCs) and a microenvironment that inhibits the regeneration of neurons [1]. Recent studies have demonstrated that the inhibition of axon regeneration after injuries to the central nervous system (CNS) were associated with several CNS myelin proteins, including Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp). These three proteins bind to the Nogo-66 receptor (NgR1), which then leads to the downstream activation of RhoA (Ras homolog gene family, member A) via two transmembrane proteins, LINGO-1 and p75; these proteins inhibit the activity in neurons and their axons [2–4]. Jun N-terminal kinases (JNKs) are intracellular molecules related to stress stimuli. The activation of these molecules mediates many types of injury-induced apoptosis and plays a role in the regeneration of axons. However, the role of the JNK-signaling pathway in the presence of a brain infarction is still unclear. The administration of sNgR-Fc, a recombinant rat soluble NgR-Fc fusion protein [5], has been found to block the interaction of myelin proteins with NgR1 effectively and to promote the regeneration of axons in rodent models of CNS injuries such as spinal cord injury [6–10]. A previous study has shown that anti-Nogo-A antibody promoted the behavioral outcome and

corticospinal plasticity in a rat stroke model [11]. In this study, we established a stroke model in rats and observed the effects of sNgR-Fc on the axonal pathology and the involved RhoA/JNK signaling pathways after cortical infarction.

Experimental Procedure

Animals

The animal experiments were approved by the Committee for the Use of Live Animals in Teaching and Research at the Sun Yat-sen University. All of the experiments were performed in a fully randomized and blinded fashion.

Photothrombotic cortical injury

Photothrombotic ischemia was induced in the rat parietal cortex using previously described methods [9,11,12] with the modifications as outlined below. Twenty seven male SD rats weighing 250 g were anesthetized by an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). Dilute Rose-Bengal in saline (40 mg/kg body weight) was infused into the femoral vein. The portion of the skull exposed at 3 mm posteriorly to the bregma and 3 mm laterally from the midline was illuminated with a cold, white light beam (Volpi Intralux 6000, 150 W; Volpi AG, Schlieven, Switzerland) for 8 min at maximum output via a fiber-optic bundle with a 10 mm aperture. The animals were randomly grouped into sham-operation (operation + Rose-Bengal), PBS (operation + Rose-Bengal + PBS), or sNgR-Fc (operation + Rose-Bengal + sNgR-Fc) groups (n = 9 for each group). A solution of sNgR-Fc (400 μg/kg body weight) in 10μl PBS or equivalent PBS was only once injected into the ipsilateral ventricle using a 26-gauge sterile microsyringe 24 h after photothrombotic cortical injury [11]. The site of injection was at 1 mm posteriorly to the bregma and 1.5 mm

laterally from the midline, and the depth of injection was about 3.5~4.0 mm. For each group, the whole cerebrum of 3 animals was collected 24 h after injury for tissue staining. The ipsilateral cortex of peripheral penumbra at 7 mm posteriorly to the bregma and 3 mm laterally from the midline of rats was collected at 27 d after injury for electron microscopy examination (n = 3 for each group) and for Western blotting (n = 3 for each group).

Triphenyltetrazolium chloride staining

To evaluate the extent of cortical infarction, we used 2, 3, 5-Triphenyltetrazolium chloride (TTC) to assess cerebral injury. At 24 h after PCI, the animals were sacrificed using an overdose of pentobarbital sodium (150 mg/kg, Alcon-Couvreur, Rijksweg, Puurs, Belgium). The brains were removed immediately and then were chilled at −30 °C for 4 min to slightly freeze the tissue. Two-mm coronal sections from the olfactory bulb to the cerebellum were prepared, stained with 1.5% TTC (Genetime) at 37 °C for 30 min, and fixed in 10% buffered formalin solution.

Transmission electron microscopy

Seven days after PCI, the peripheral penumbra at 7 mm posteriorly to the bregma and 3 mm laterally from the midline on the injury side was removed and immediately cut into 0.5 cm^3 cubes at 0 °C, fixed with 4% paraformaldehyde for 4 h, and washed with PBS. Then, the tissue was fixed with 2% osmic acid for 2 h, washed with pure water, dehydrated with a mixture of ethanol, propylene oxide, and resin, embedded in pure resin, and ultramicrocut and stained with uranyl acetate and lead citrate [13, 14]. The sections were observed under an HE-800 transmission electron microscope (Hitachi, Japan).

Western blotting

The cortex from the injury side was collected at 7 days after injury and homogenized in a lysis buffer (in mM: Tris, 10, pH 7.4; NaCl, 150; EDTA, 1; EGTA, 1; 10% protease inhibitor, 1% phosphatase inhibitor), and incubated on ice for 30 min. The protein lysate was then centrifuged at 13,500 rpm for 30 min at 4 ℃. The supernatant was aliquoted and stored at −80 °C until its use. The supernatant was measured for its protein concentration using a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, CA, USA). A 40 μg aliquot of protein from each sample was subjected to 5–12% SDS–polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. Each membrane was blocked with 5% non-fat milk and 2% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature and then probed with rabbit anti-p-JNK (Cell Signaling Technology, MA, USA), anti-p-c-JUN (Cell Signaling Technology), p-ATF-2 (Cell Signaling Technology), total-JNK (Cell Signaling Technology) and total-RhoA antibodies at 4 °C overnight. As a gel loading control, β-actin was used. After washing, the membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2000; DakoCytomation, Denmark). Its immunoreactivity was visualized using the Amersham enhanced chemiluminescence kit (Amersham, Piscataway, NJ, USA). The protein loading was controlled using the goat antibody against β-actin (C-11; Santa Cruz Biotechnology). The quantification of the results was performed by densitometry, and the results were analyzed as total integrated densitometric values (arbitrary units). The protein GTP-RhoA was pulled down using a RhoA activation assay kit, according to the manufacturer's instruction (Cytoskeleton, Denver, CO, USA) and assayed by Western blotting using an antibody against RhoA (Cytoskeleton). All experiments for Western blotting were performed with 3 animals in each group, and the samples were run on the gels as individual animals.

Data and statistical analysis

The values are presented as the mean ± SD. The statistical analyses was performed using Student's t-test for comparisons between two groups or the one-way analysis of variance (ANOVA) followed by post-hoc tests (Student-Neuman-Keuls) for comparisons of more than two groups. The data were analyzed statistically with the software SPSS 12.0. The significance level was set at $P < 0.05$.

Results

Measurement of brain infarct volume and identification of blood brain barrier integrity

After TTC staining, the rats in the sham group did not appear to have any infarctions (Fig. 1b). However, the right parietal lobes of the rats 24 h after PCI presented pale infarctions that were bowl-shaped, with the bottom pointing to the lateral ventricle; these infarctions affected the entire cortex. The total infarct volumes were 297.1 ± 23.4 μl in the PBS group and 285.4 ± 19.7 μl for the sNgR-Fc group respectively before the treatment. The locations of the infarctions were stable, and the infarctions showed similar volumes to those in the rats (Fig. 1).

Pathological changes of axons

The morphology of ischemic penumbra on the injury side was evaluated using TEM at 7 days after PCI. Compared with the normal axons observed in the sham group (Fig. 2a), more serious pathologies were observed in the PCI group (Fig. 2b). For the axons without a medulla sheath, many edemas, dilutions of cytoplasmic matrices, and axonal fusions or dissolutions were found. Occasionally, we observed that oligodendrocytes appeared to "swallow" the axons (Fig. 2b). For axons with a medulla sheath, compared with the sham

group, thickening or shrinking was more in the axoplasm of the fibers, and amyloidosis and swollen axons were observed at a higher rate. In addition, the axolemma were found to be separated from the inner layers of the myelin (Fig. 2e). In addition, there were only minor pathological changes, including slight nuclear edemas with complete nucleoli, slightly swollen mitochondria, and slightly widened endoplasmic reticula with complete ribosomes and Golgi bodies. In unmyelinated axons, there were some axonal edemas, axonal deformities, and dilutions of cytoplasmic matrices; in addition, there were no axonal fusions or cytophagic effects of oligodendrocytes (Fig. 2c). In myelinated axons, the morphology of axons was almost normal. There was little demyelination and no amyloidosis of the myelin sheath was found (Fig. 2f). Compared with normal synapses (Fig. 2g), the synaptic cleft widened, and there was edema in the PBS group (Fig. 2h). However, no clear edema or widening of the synaptic cleft was observed in the group that received the sNgR-Fc treatment (Fig. 2i).

sNgR-Fc inhibited RhoA signal pathway

The level of GTP-RhoA in the PBS group was significantly higher than that in the sham-operation group (P <0.05). After treatment with sNgR-Fc, the level of GTP-RhoA was significantly decreased. Moreover, the level of Total-RhoA was not different among the three groups (Fig.3, Table 1). These data indicate that sNgR-Fc could decrease the activation of RhoA.

sNgR-Fc inhibit the activation of SAPK/JNK signal pathway

The levels of p-JNK1, 2 and its downstream targets, p-c-JUN and p-ATF-2, in the cortex around the infarcts in the PCI group were significantly higher compared with those in the sham operation group (P < 0.05). In contrast, the cortex in the sNgR-Fc-treated group showed,

on average, reductions in the levels of p-JNK1, 2, p-c-JUN and p-ATF-2 to their basal levels ($P < 0.05$). The levels of total JNK1, 2 were not different among the three groups ($P > 0.05$). These results demonstrate that the cortical infarction injury activates the SAPK/JNK signal pathway and that treatment with sNgR-Fc has the potential to decrease or prevent this activation (Fig.3, Table 1).

Discussion

Watson et al reported a stroke model induced by a photochemical method in 1985 [12]. The principle underlying this model was based on a photochemical reaction. Following the injection of a potent photosensitive agent into rats, ischemic lesions were formed by irradiating the left parietal convexities of the exposed skulls under a light with a specific wavelength. Free radicals were released, damaging the endothelium of the cerebral vessels and inducing platelet aggregation. Furthermore, this pathology causes thrombosis in cerebral parenchymal vessels and results in irreversible hypoxic-ischemic brain damage (HIBD). Because of the advantages of a stable location and infarction volume, a low mortality rate, and a good replication rate, the stroke model is widely used in current studies of brain injury. In this study, we successfully induced a cortical ischemic infarction model in rats in which the BBB was also damaged. The primary pathological features of stroke were induced, and the volumes of infarction were consistent. No infarction and no burn were observed in the sham-group, indicating that the light stimulation alone did not induce the infarction and that it acts as a good control.

Delayed neuron death (DND) is an important pathology of cerebral ischemia and cerebral degenerative disease. Cell apoptosis was the primary DND pattern after an acute

HIBD, which aggravated the injury of sub-acute or chronic cerebral infarction [15, 16]. In our study, axonal pathologies were observed via the TEM at 7 days after PCI, including many edemas in the axoplasm of axons that have no medulla sheath, and thickening or shrinkage in the sheaths of the axons that have a medulla sheath. However, these conditions improved after sNgR-Fc treatment. The administration of the soluble NgR1 fusion protein (sNgR-Fc) effectively blocked the interaction of myelin molecules with NgR1 and promoted axon sprouting and functional recovery after spinal cord injuries[7–9], or dorsal root rhizotomy[10]. A previous study found that Nogo-A is involved in the secondary axonal degeneration of the thalamus with cerebral infarction in hypertensive rats [17] and after a focal ischemic stroke [18]. Anti–Nogo-A antibody improved behavioral outcome and corticospinal plasticity but did not decrease the infarct volume after experimental stroke [11]. In this study, sNgR-Fc did not cause any difference on infarct volume, but still provided protection of axons as observed in TEM.

Nogo-A, MAG and OMgp were considered to be the inhibitory factors of axonal regeneration in the CNS. NgR1 mediates their inhibitory effect via two co-receptors, p75 and Lingo-1, and transduces the signals via RhoA and its downstream pathways [19, 20]. In this study, the level of the GTP-RhoA protein in the model group was significantly higher than that of the sham-operation group. With the treatment of sNgR-Fc, the level of the GTP-RhoA protein was significantly decreased, suggesting that sNgR-Fc may promote axonal recovery by preventing the activation of RhoA after cerebral infarction.

The stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signaling pathway is a powerful signaling pathway under the stresses of ultraviolet light, radiation and inflammation [21–23]. In publications on neuronal apoptosis, some of the proteins in the

mitogen-activated protein kinases (MAPK) signaling pathways have been well-studied in recent years. The MAPK signaling pathway is a family of serine/threonine kinases that include three members: the extracellular signal-regulated protein kinases (ERK), JNK and p38 [24]. The JNK/SAPK and p38 are induced by cellular stress and some of the cytokines. The JNK protein can be activated by many stress stimulators, such as lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), interleukin (IL)-1 or ultraviolet light. Activation of the JNK signaling pathway is related to cell apoptosis [24]. Following its activation, JNKs may phosphorylate specific sites of some of the transcription factors, such as ATF-2, SAP-1 and Elk-1, and increase the expression of genes, subsequently increasing the synthesis of proteins that may participate in the apoptosis pathway and cause cell death [25]. We found high levels of p-JNK，p-c-JUN and p-ATF-2 after stroke injury, indicating that cortical infarction activates the SAPK/JNK signal pathway and leads to the pathology of tissues and cells. The administration of sNgR-Fc decreased the activation of those proteins, suggesting that sNgR-Fc has the potential to prevent or decrease the activation of the SAPK/JNK signal pathway. We previously identified that sNgR-Fc binds to Nogo66, OMgp, and MAG [9] and inhibited the Nogo66-NgR interaction [6]. sNgR-Fc may protect axons by inhibiting the interaction of Nogo-A-NgR1 after stroke injury, decreasing the activation of RhoA and JNK. A schematic diagram illustrating the proposed mechanism for sNgR-Fc on axons in stroke is shown in Figure 4: In normal conditions, the cortex expresses low levels of Nogo-A and NgR1. However, cortical infarction under stroke injury increases the expression of Nogo-A and NgR1. Nogo-A activates RhoA and JNK signaling pathways via interacting with NgR1 and then results in pathological changes on axons. sNgR-Fc perturbs the interaction of Nogo-A and NgR1, decreases the activation of RhoA and JNK signaling pathways and

relieving the pathological changes of axons.

RhoA regulates the intracellular microfilament associated with cell proliferation through a series of complex mechanisms [26]. Previous studies have observed that RhoA stimulates Jun expression through the Rho-associated kinase (ROCK), which phosphorylates c-Jun and ATF2 after binding to the c-Jun promoter [27]. In this study, we observed that changes in the expression of p-JNK, p-c-Jun and p-ATF-2 were consistent with changes in the GTP-RhoA level following both the cerebral infarction and the sNgR-Fc treatment. These findings suggest that sNgR-Fc can relieve the axonal pathology partially by decreasing the activation of the SAPK/JNK signal pathways.

In summary, pathological changes in axons were induced by the cerebral hypoxia-ischemia for an extended period after the cortical infarction. This phenomenon may be associated with the activation of RhoA/ROCK/JNK/c-Jun signaling pathways. We determined that an NgR1 antagonist, sNgR-Fc, alleviated axonal pathology changes and prevented the activation of RhoA/ROCK/JNK/c-Jun signaling pathways. Further studies should be performed before this treatment is used in a clinical study.

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Disclosure of potential conflicts of interest

The authors indicate no potential conflicts of interest.

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Figure Legends

Figure 1. The morphology and characteristics of the rat brain after a stroke induced by photothrombotic cortical injury. (a) Representative photographs of the coronal brain sections stained with 2,3,5-triphenyltetrazolium chloride in rats, (b) sham-operated rats, (c) and photothrombotic cortical injury (PCI) rats. White indicates the infarct area (arrow).

Figure 2. The protective effect of sNgR-Fc on axon neurons under transmission electron microscope after stroke injury. Ultrastructural appearances of morphology of axons in sham-operated, PBS and sNgR-Fc groups: (a) Normal unmyelinated axons in sham-operated group; (b) Extensive edema, axon deformity, dilution of cytoplasmic matrices and axonal fusion in stroke model; (c) Slight edema and dilution of cytoplasmic matrices with the treatment of sNgR-Fc in stroke model; (d) Normal myelinated axons in sham-operated group; (e) Thickening, shrinking or amyloidosis was more in stroke model; (f) Little demyelination and no amyloidosis of the myelin sheath were found with the treatment of sNgR-Fc in stroke model; (g) Normal synapses in the sham-operated group; (h) Widened and swollen synaptic cleft in stroke model; (i) Approximately normal synapses with the treatment of sNgR-Fc in stroke model. Arrows show the changes in axons.

Figure 3. The RhoA, SAPK/JNK signaling pathways after stroke injury and with the treatment of sNgR-Fc under Western blotting. The levels of p-JNK， p-c-JUN, p-ATF-2 and GTP-RhoA increased after stroke injury. Treatment with sNgR-Fc decreased the levels of p-JNK, p-c-JUN, p-ATF-2 and GTP-RhoA to normal levels.

Figure 4. The diagram of the signaling pathways involved in the sNgR-Fc on axons after stroke injury. In the normal condition, there is low level of Nogo-A and NgR1 in the cortex. However, the expression of Nogo-A and NgR1 is increased under stroke injury. The increased Nogo-A binds with NgR1 to activate the downstream pathways of RhoA and JNK, and then results in axonal pathology. The treatment of sNgR-Fc inhibits the interaction of Nogo-A and NgR1, decreases the activation of RhoA and JNK signaling pathways and then relieves axonal pathology.

The effect of an NgR1 antagonist on the neuroprotection of cortical axons after cortical infarction in rats

Running title: The neuroprotection of NgR1 antagonist on cortical axons

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Abstract

We investigated the effect of the soluble Nogo66 receptor (sNgR-Fc) on the protection of cortical axons after cortical infarction in rats. The cortical infarction was induced by photothrombotic cortical injury (PCI) in Sprague Dawley rats, after which sNgR-Fc was injected into the lateral ventricle. The ipsilesional cortices were harvested for analyses using histochemical and transmission-electron microscope techniques. The involved signaling pathways, which include RhoA, JNK, c-JUN and ATF-2, were detected by Western blot. Serious pathologies were found in the brains of the rats after injury, including edemas in the axoplasms of axons that have no medulla sheath and a thickening or shrinkage in the sheath of the axons that have medulla sheathes. However, these pathologies improved after sNgR-Fc treatment. The levels of GTP-RhoA, p-JNK, p-c-JUN and p-ATF-2 in the PCI group were increased when compared with their levels in the sham-operation group ($P < 0.05$), and animals receiving the sNgR-Fc treatment showed lower expression levels of these proteins when compared with the sham-operation group ($P < 0.05$). Our results suggest that sNgR-Fc can alleviate the pathological changes of axons following cortical infarction via decreasing the activation of RhoA/JNK signaling pathways.

Key words: Nogo-66 receptor; Axon; Cortical infarction; Regeneration; Signal pathway

Introduction

A brain infarction (also known as an ischemic stroke) is caused by the blockage of a cerebral artery by a blood clot, which results in the following effects: a critical reduction in blood flow, ischemia, anoxia, and, finally, ischemic necrosis or encephalomalacia in the brain. This condition is characterized clinically by high morbidity, high mortality, high disability rate, high recurrence rates, and it is difficult to treat. Because our population is aging, stroke is becoming a more serious threat and an urgent medical problem. Previous studies have shown that the central nerve system cannot regenerate after injury, as a result of factors that include the insufficiency of neural progenitor cells (NPCs) and a microenvironment that inhibits the regeneration of neurons [1]. Recent studies have demonstrated that the inhibition of axon regeneration after injuries to the central nervous system (CNS) were associated with several CNS myelin proteins, including Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp). These three proteins bind to the Nogo-66 receptor (NgR1), which then leads to the downstream activation of RhoA (Ras homolog gene family, member A) via two transmembrane proteins, LINGO-1 and p75; these proteins inhibit the activity in neurons and their axons [2–4]. Jun N-terminal kinases (JNKs) are intracellular molecules related to stress stimuli. The activation of these molecules mediates many types of injury-induced apoptosis and plays a role in the regeneration of axons. However, the role of the JNK-signaling pathway in the presence of a brain infarction is still unclear. The administration of sNgR-Fc, a recombinant rat soluble NgR-Fc fusion protein [5], has been found to block the interaction of myelin proteins with NgR1 effectively and to promote the regeneration of axons in rodent models of CNS injuries such as spinal cord injury [6–10]. A previous study has shown that anti-Nogo-A antibody promoted the behavioral outcome and corticospinal plasticity in a rat stroke model [11]. In this study, we established a stroke model

in rats and observed the effects of sNgR-Fc on the axonal pathology and the involved RhoA/JNK signaling pathways after cortical infarction.

Experimental Procedure

Animals

The animal experiments were approved by the Committee for the Use of Live Animals in Teaching and Research at the Sun Yat-sen University. All of the experiments were performed in a fully randomized and blinded fashion.

Photothrombotic cortical injury

Photothrombotic ischemia was induced in the rat parietal cortex using previously described methods [9,11,12] with the modifications as outlined below. Twenty seven male SD rats weighing 250 g were anesthetized by an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (8 mg/kg). Dilute Rose-Bengal in saline (40 mg/kg body weight) was infused into the femoral vein. The portion of the skull exposed at 3 mm posteriorly to the bregma and 3 mm laterally from the midline was illuminated with a cold, white light beam (Volpi Intralux 6000, 150 W; Volpi AG, Schlieven, Switzerland) for 8 min at maximum output via a fiber-optic bundle with a 10 mm aperture. The animals were randomly grouped into sham-operation (operation + Rose-Bengal), PBS (operation + Rose-Bengal + PBS), or sNgR-Fc (operation + Rose-Bengal + sNgR-Fc) groups (n = 9 for each group). A solution of sNgR-Fc (400 μg/kg body weight) in 10μl PBS or equivalent PBS was only once injected into the ipsilateral ventricle using a 26-gauge sterile microsyringe 24 h after photothrombotic cortical injury [11]. The site of injection was at 1 mm posteriorly to the bregma and 1.5 mm laterally from the midline, and the depth of injection was about 3.5~4.0 mm. For each group, the whole cerebrum of 3 animals was collected 24 h after injury for tissue staining. The

ipsilateral cortex of peripheral penumbra at 7 mm posteriorly to the bregma and 3 mm laterally from the midline of rats was collected at 27 d after injury for electron microscopy examination (n = 3 for each group) and for Western blotting (n = 3 for each group).

Triphenyltetrazolium chloride staining

To evaluate the extent of cortical infarction, we used 2, 3, 5-Triphenyltetrazolium chloride (TTC) to assess cerebral injury. At 24 h after PCI, the animals were sacrificed using an overdose of pentobarbital sodium (150 mg/kg, Alcon-Couvreur, Rijksweg, Puurs, Belgium). The brains were removed immediately and then were chilled at −30 °C for 4 min to slightly freeze the tissue. Two-mm coronal sections from the olfactory bulb to the cerebellum were prepared, stained with 1.5% TTC (Genetime) at 37 °C for 30 min, and fixed in 10% buffered formalin solution.

Transmission electron microscopy

Seven days after PCI, the peripheral penumbra at 7 mm posteriorly to the bregma and 3 mm laterally from the midline on the injury side was removed and immediately cut into 0.5 cm^3 cubes at 0 °C, fixed with 4% paraformaldehyde for 4 h, and washed with PBS. Then, the tissue was fixed with 2% osmic acid for 2 h, washed with pure water, dehydrated with a mixture of ethanol, propylene oxide, and resin, embedded in pure resin, and ultramicrocut and stained with uranyl acetate and lead citrate [13, 14]. The sections were observed under an HE-800 transmission electron microscope (Hitachi, Japan).

Western blotting

The cortex from the injury side was collected at 7 days after injury and homogenized in a lysis buffer (in mM: Tris, 10, pH 7.4; NaCl, 150; EDTA, 1; EGTA, 1; 10% protease inhibitor, 1% phosphatase inhibitor), and incubated on ice for 30 min. The protein lysate was then

centrifuged at 13,500 rpm for 30 min at 4 ℃. The supernatant was aliquoted and stored at −80 °C until its use. The supernatant was measured for its protein concentration using a Bio-Rad DC Protein Assay Kit (Bio-Rad Laboratories, CA, USA). A 40 μg aliquot of protein from each sample was subjected to 5–12% SDS–polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. Each membrane was blocked with 5% non-fat milk and 2% bovine serum albumin in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature and then probed with rabbit anti-p-JNK (Cell Signaling Technology, MA, USA), anti-p-c-JUN (Cell Signaling Technology), p-ATF-2 (Cell Signaling Technology), total-JNK (Cell Signaling Technology) and total-RhoA antibodies at 4 °C overnight. As a gel loading control, β-actin was used. After washing, the membranes were incubated for 2 h with horseradish peroxidase-conjugated goat anti-rabbit antibody (1:2000; DakoCytomation, Denmark). Its immunoreactivity was visualized using the Amersham enhanced chemiluminescence kit (Amersham, Piscataway, NJ, USA). The protein loading was controlled using the goat antibody against β-actin (C-11; Santa Cruz Biotechnology). The quantification of the results was performed by densitometry, and the results were analyzed as total integrated densitometric values (arbitrary units). The protein GTP-RhoA was pulled down using a RhoA activation assay kit, according to the manufacturer's instruction (Cytoskeleton, Denver, CO, USA) and assayed by Western blotting using an antibody against RhoA (Cytoskeleton). All experiments for Western blotting were performed with 3 animals in each group, and the samples were run on the gels as individual animals.

Data and statistical analysis

The values are presented as the mean ± SD. The statistical analyses was performed using Student’s t-test for comparisons between two groups or the one-way analysis of variance (ANOVA) followed by post-hoc tests (Student-Neuman-Keuls) for comparisons of

more than two groups. The data were analyzed statistically with the software SPSS 12.0. The significance level was set at $P < 0.05$.

Results

Measurement of brain infarct volume and identification of blood brain barrier integrity

After TTC staining, the rats in the sham group did not appear to have any infarctions (Fig. 1b). However, the right parietal lobes of the rats 24 h after PCI presented pale infarctions that were bowl-shaped, with the bottom pointing to the lateral ventricle; these infarctions affected the entire cortex. The total infarct volumes were 297.1 ± 23.4 μl in the PBS group and 285.4 ± 19.7 μl for the sNgR-Fc group respectively before the treatment. The locations of the infarctions were stable, and the infarctions showed similar volumes to those in the rats (Fig. 1).

Pathological changes of axons

The morphology of ischemic penumbra on the injury side was evaluated using TEM at 7 days after PCI. Compared with the normal axons observed in the sham group (Fig. 2a), more serious pathologies were observed in the PCI group (Fig. 2b). For the axons without a medulla sheath, many edemas, dilutions of cytoplasmic matrices, and axonal fusions or dissolutions were found. Occasionally, we observed that oligodendrocytes appeared to "swallow" the axons (Fig. 2b). For axons with a medulla sheath, compared with the sham group, thickening or shrinking was more in the axoplasm of the fibers, and amyloidosis and swollen axons were observed at a higher rate. In addition, the axolemma were found to be separated from the inner layers of the myelin (Fig. 2e). In addition, there were only minor pathological changes, including slight nuclear edemas with complete nucleoli, slightly swollen mitochondria, and slightly widened endoplasmic reticula with complete ribosomes

and Golgi bodies. In unmyelinated axons, there were some axonal edemas, axonal deformities, and dilutions of cytoplasmic matrices; in addition, there were no axonal fusions or cytophagic effects of oligodendrocytes (Fig. 2c). In myelinated axons, the morphology of axons was almost normal. There was little demyelination and no amyloidosis of the myelin sheath was found (Fig. 2f). Compared with normal synapses (Fig. 2g), the synaptic cleft widened, and there was edema in the PBS group (Fig. 2h). However, no clear edema or widening of the synaptic cleft was observed in the group that received the sNgR-Fc treatment (Fig. 2i).

sNgR-Fc inhibited RhoA signal pathway

The level of GTP-RhoA in the PBS group was significantly higher than that in the sham-operation group (P <0.05). After treatment with sNgR-Fc, the level of GTP-RhoA was significantly decreased. Moreover, the level of Total-RhoA was not different among the three groups (Fig.3, Table 1). These data indicate that sNgR-Fc could decrease the activation of RhoA.

sNgR-Fc inhibit the activation of SAPK/JNK signal pathway

The levels of p-JNK1, 2 and its downstream targets, p-c-JUN and p-ATF-2, in the cortex around the infarcts in the PCI group were significantly higher compared with those in the sham operation group (P < 0.05). In contrast, the cortex in the sNgR-Fc-treated group showed, on average, reductions in the levels of p-JNK1, 2, p-c-JUN and p-ATF-2 to their basal levels (P < 0.05). The levels of total JNK1, 2 were not different among the three groups (P > 0.05). These results demonstrate that the cortical infarction injury activates the SAPK/JNK signal pathway and that treatment with sNgR-Fc has the potential to decrease or prevent this activation (Fig.3, Table 1).

Discussion

Watson et al reported a stroke model induced by a photochemical method in 1985 [12]. The principle underlying this model was based on a photochemical reaction. Following the injection of a potent photosensitive agent into rats, ischemic lesions were formed by irradiating the left parietal convexities of the exposed skulls under a light with a specific wavelength. Free radicals were released, damaging the endothelium of the cerebral vessels and inducing platelet aggregation. Furthermore, this pathology causes thrombosis in cerebral parenchymal vessels and results in irreversible hypoxic-ischemic brain damage (HIBD). Because of the advantages of a stable location and infarction volume, a low mortality rate, and a good replication rate, the stroke model is widely used in current studies of brain injury. In this study, we successfully induced a cortical ischemic infarction model in rats in which the BBB was also damaged. The primary pathological features of stroke were induced, and the volumes of infarction were consistent. No infarction and no burn were observed in the sham-group, indicating that the light stimulation alone did not induce the infarction and that it acts as a good control.

Delayed neuron death (DND) is an important pathology of cerebral ischemia and cerebral degenerative disease. Cell apoptosis was the primary DND pattern after an acute HIBD, which aggravated the injury of sub-acute or chronic cerebral infarction [15, 16]. In our study, axonal pathologies were observed via the TEM at 7 days after PCI, including many edemas in the axoplasm of axons that have no medulla sheath, and thickening or shrinkage in the sheaths of the axons that have a medulla sheath. However, these conditions improved after sNgR-Fc treatment. The administration of the soluble NgR1 fusion protein (sNgR-Fc) effectively blocked the interaction of myelin molecules with NgR1 and promoted axon sprouting and functional recovery after spinal cord injuries[7–9], or dorsal root rhizotomy[10].

A previous study found that Nogo-A is involved in the secondary axonal degeneration of the thalamus with cerebral infarction in hypertensive rats [17] and after a focal ischemic stroke [18]. Anti–Nogo-A antibody improved behavioral outcome and corticospinal plasticity but did not decrease the infarct volume after experimental stroke [11]. In this study, sNgR-Fc did not cause any difference on infarct volume, but still provided protection of axons as observed in TEM.

Nogo-A, MAG and OMgp were considered to be the inhibitory factors of axonal regeneration in the CNS. NgR1 mediates their inhibitory effect via two co-receptors, p75 and Lingo-1, and transduces the signals via RhoA and its downstream pathways [19, 20]. In this study, the level of the GTP-RhoA protein in the model group was significantly higher than that of the sham-operation group. With the treatment of sNgR-Fc, the level of the GTP-RhoA protein was significantly decreased, suggesting that sNgR-Fc may promote axonal recovery by preventing the activation of RhoA after cerebral infarction.

The stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) signaling pathway is a powerful signaling pathway under the stresses of ultraviolet light, radiation and inflammation [21–23]. In publications on neuronal apoptosis, some of the proteins in the mitogen-activated protein kinases (MAPK) signaling pathways have been well-studied in recent years. The MAPK signaling pathway is a family of serine/threonine kinases that include three members: the extracellular signal-regulated protein kinases (ERK), JNK and p38 [24]. The JNK/SAPK and p38 are induced by cellular stress and some of the cytokines. The JNK protein can be activated by many stress stimulators, such as lipopolysaccharide (LPS), tumor necrosis factor-α (TNF-α), interleukin (IL)-1 or ultraviolet light. Activation of the JNK signaling pathway is related to cell apoptosis [24]. Following its activation, JNKs may phosphorylate specific sites of some of the transcription factors, such as ATF-2, SAP-1

and Elk-1, and increase the expression of genes, subsequently increasing the synthesis of proteins that may participate in the apoptosis pathway and cause cell death [25]. We found high levels of p-JNK，p-c-JUN and p-ATF-2 after stroke injury, indicating that cortical infarction activates the SAPK/JNK signal pathway and leads to the pathology of tissues and cells. The administration of sNgR-Fc decreased the activation of those proteins, suggesting that sNgR-Fc has the potential to prevent or decrease the activation of the SAPK/JNK signal pathway. We previously identified that sNgR-Fc binds to Nogo66, OMgp, and MAG [9] and inhibited the Nogo66-NgR interaction [6]. sNgR-Fc may protect axons by inhibiting the interaction of Nogo-A-NgR1 after stroke injury, decreasing the activation of RhoA and JNK. A schematic diagram illustrating the proposed mechanism for sNgR-Fc on axons in stroke is shown in Figure 4: In normal conditions, the cortex expresses low levels of Nogo-A and NgR1. However, cortical infarction under stroke injury increases the expression of Nogo-A and NgR1. Nogo-A activates RhoA and JNK signaling pathways via interacting with NgR1 and then results in pathological changes on axons. sNgR-Fc perturbs the interaction of Nogo-A and NgR1, decreases the activation of RhoA and JNK signaling pathways and relieving the pathological changes of axons.

RhoA regulates the intracellular microfilament associated with cell proliferation through a series of complex mechanisms [26]. Previous studies have observed that RhoA stimulates Jun expression through the Rho-associated kinase (ROCK), which phosphorylates c-Jun and ATF2 after binding to the c-Jun promoter [27]. In this study, we observed that changes in the expression of p-JNK, p-c-Jun and p-ATF-2 were consistent with changes in the GTP-RhoA level following both the cerebral infarction and the sNgR-Fc treatment. These findings suggest that sNgR-Fc can relieve the axonal pathology partially by decreasing the activation of the SAPK/JNK signal pathways.

In summary, pathological changes in axons were induced by the cerebral hypoxia-ischemia for an extended period after the cortical infarction. This phenomenon may be associated with the activation of RhoA/ROCK/JNK/c-Jun signaling pathways. We determined that an NgR1 antagonist, sNgR-Fc, alleviated axonal pathology changes and prevented the activation of RhoA/ROCK/JNK/c-Jun signaling pathways. Further studies should be performed before this treatment is used in a clinical study.

Acknowledgements

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Disclosure of potential conflicts of interest

The authors indicate no potential conflicts of interest.

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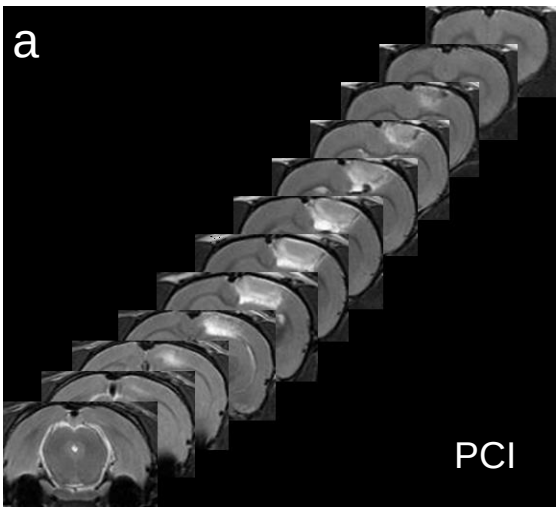
Figure Legends

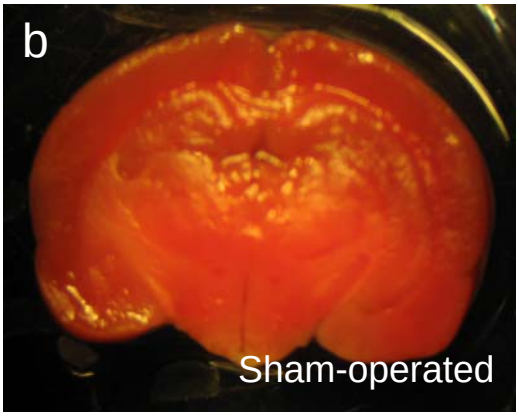
Figure 1. The morphology and characteristics of the rat brain after a stroke induced by photothrombotic cortical injury. (a) Representative photographs of the coronal brain sections stained with 2,3,5-triphenyltetrazolium chloride in rats, (b) sham-operated rats, (c) and photothrombotic cortical injury (PCI) rats. White indicates the infarct area (arrow).

Figure 2. The protective effect of sNgR-Fc on axon neurons under transmission electron microscope after stroke injury. Ultrastructural appearances of morphology of axons in sham-operated, PBS and sNgR-Fc groups: (a) Normal unmyelinated axons in sham-operated group; (b) Extensive edema, axon deformity, dilution of cytoplasmic matrices and axonal fusion in stroke model; (c) Slight edema and dilution of cytoplasmic matrices with the treatment of sNgR-Fc in stroke model; (d) Normal myelinated axons in sham-operated group; (e) Thickening, shrinking or amyloidosis was more in stroke model; (f) Little demyelination and no amyloidosis of the myelin sheath were found with the treatment of sNgR-Fc in stroke model; (g) Normal synapses in the sham-operated group; (h) Widened and swollen synaptic cleft in stroke model; (i) Approximately normal synapses with the treatment of sNgR-Fc in stroke model. Arrows show the changes in axons.

Figure 3. The RhoA, SAPK/JNK signaling pathways after stroke injury and with the treatment of sNgR-Fc under Western blotting. The levels of p-JNK， p-c-JUN, p-ATF-2 and GTP-RhoA increased after stroke injury. Treatment with sNgR-Fc decreased the levels of p-JNK, p-c-JUN, p-ATF-2 and GTP-RhoA to normal levels.

Figure 4. The diagram of the signaling pathways involved in the sNgR-Fc on axons after stroke injury. In the normal condition, there is low level of Nogo-A and NgR1 in the cortex. However, the expression of Nogo-A and NgR1 is increased under stroke injury. The increased Nogo-A binds with NgR1 to activate the downstream pathways of RhoA and JNK, and then results in axonal pathology. The treatment of sNgR-Fc inhibits the interaction of Nogo-A and NgR1, decreases the activation of RhoA and JNK signaling pathways and then relieves axonal pathology.





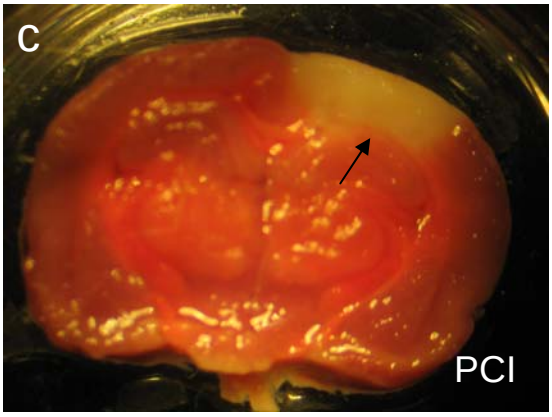


Figure 1

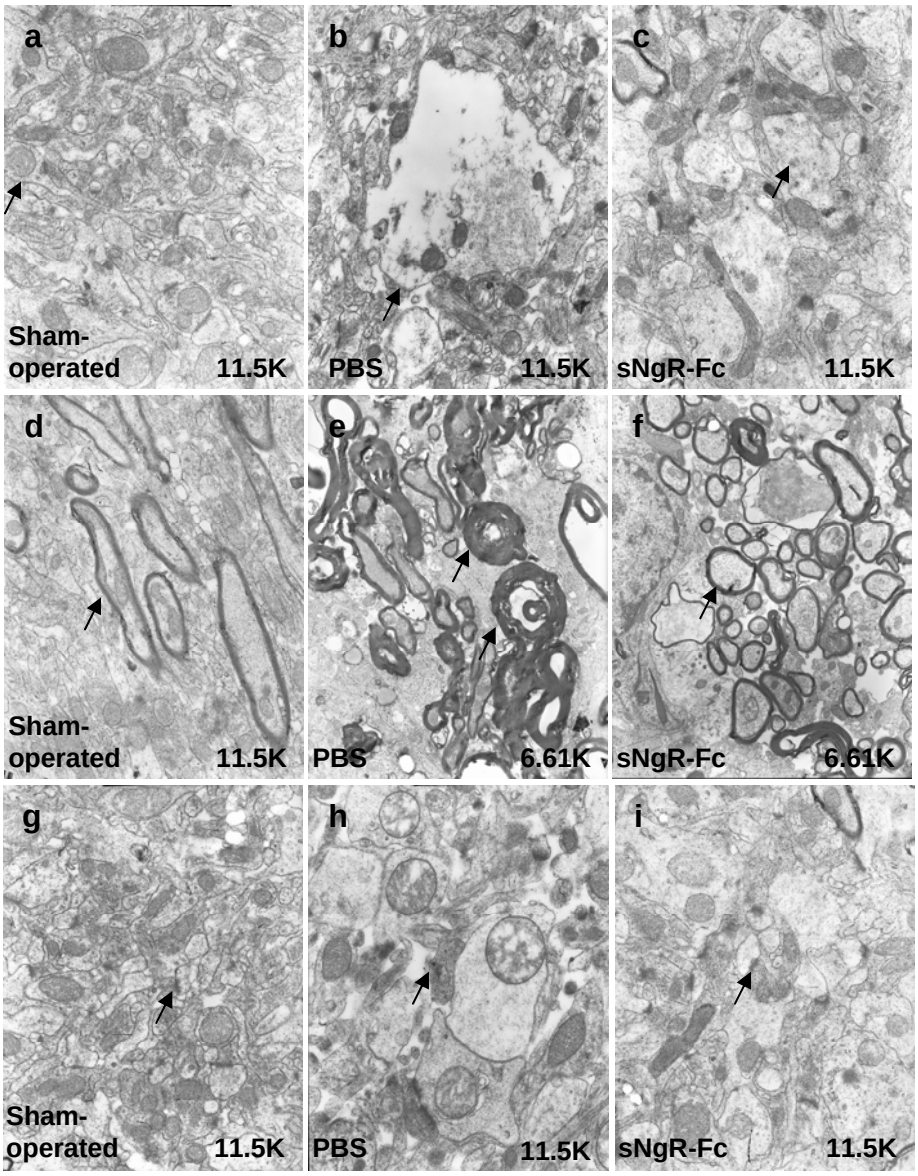

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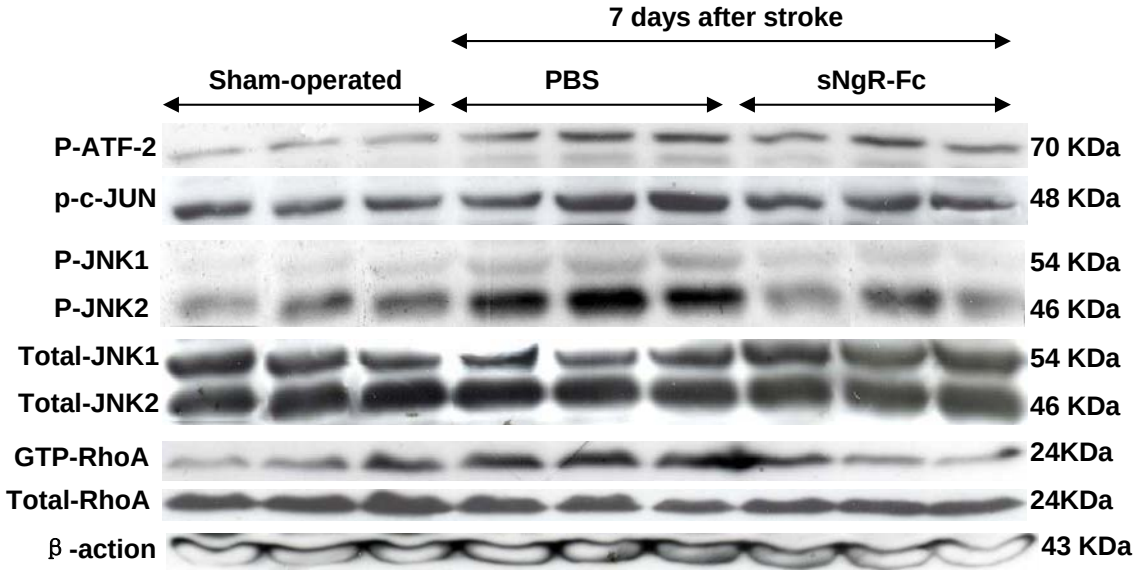
Figure 2


7 days after stroke
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p-c-JUN
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P-JNK2
46 KDa
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Figure 3

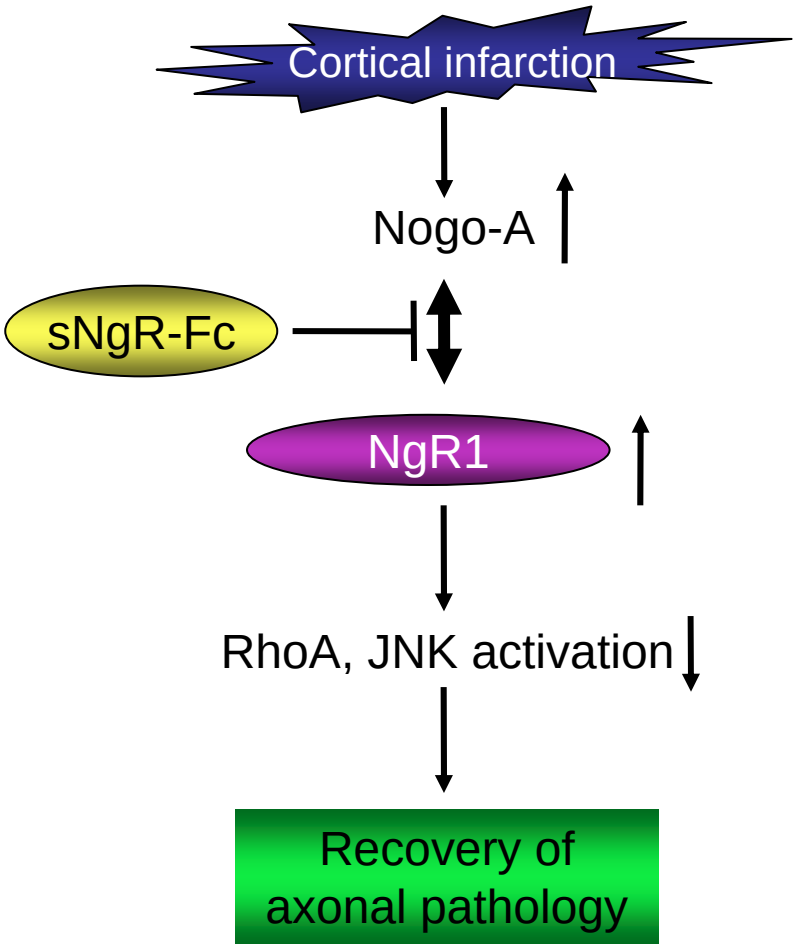
Cortical infarction
Nogo-A
sNgR-Fc
NgR1
RhoA, JNK activation
Recovery of
axonal pathology

Figure 4

Table
Click here to download Table: Table 1.doc

Table 1 The density of the key proteins of RhoA and SAPK/JNK signal pathway

	Sham-operated	PBS	sNgR.Fc
GTP-RhoA	1.0±0. 14	1.73±0. 16▲	1.10±0. 13▲■
Total-RhoA	1.0±0. 09	0.97±0. 11	0.98±0. 10
p-ATF-2	1.0±0. 12	2.19±0. 24▲	1.53±0. 22▲■
p-c-JUN	1.0±0. 062	1.39±0. 139▲	1.12±0. 058▲■
p-JNK1	1.0±0. 29	2.24±0. 27▲	1.30±0. 31▲■
p-JNK2	1.0±0. 14	1.73±0. 12▲	1.02±0. 15▲■
Total-JNK1	1.0±0. 08	1.01±0. 09	1.01±0. 08
Total-JNK2	1±0. 09	0. 99±0. 11	0. 98±0. 12

▲P<0.05 vs Sham-operated group; ■P<0.05 vs PBS group, Mean ±SD, n = 3）