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Electro-stimulation of cerebellar fastigial nucleus (FNS) improves axonal regeneration

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1. ABSTRACT

This study focused on the effect of electro-stimulation of fastigial nucleus on the expression of NgR and on axonal regeneration after focal cerebral ischemia-reperfusion in rats. Cerebral ischemia and reperfusion was induced by nylon monofilament. Ninety-six male SD rats were randomly divided into sham group and ischemic insult groups at 12 hours, 24 hours, and 1 to 3 weeks after cerebral ischemia-reperfusion. Reverse transcription-polymerase chain reaction (RT-PCR) was used to determine the changes of NgR mRNA expression. Immunohistochemistry was used to detect the expression of NgR protein and the state of axonal regeneration. Fastigial nucleus stimulation was applied at 2 hours after ischemia for one hour. The results demonstrated that NgR mRNA and protein in the infarcted cortex and hippocampus were significantly increased ($p<0.01$). The axons were grossly damaged at 24h after cerebral ischemia-reperfusion when compared to the sham group. Fastigial nucleus stimulation decreased NgR mRNA and protein levels in the infarcted cortex and hippocampus ($p<0.01$) and improved axonal growth at 24 hours and 2 weeks after ischemia-reperfusion ($p<0.05$). These results suggest that electrostimulation of fastigial nucleus might provide a new strategy to promote CNS axonal regeneration.

2. INTRODUCTION

Ischemic stroke causes cerebral infarction and long term neurological and neurobehavioral dysfunctions (1-7). No effective treatment is available. Electrostimulation of fastigial nucleus is an experimental therapeutic method to promote neurological functional recovery. Several previous studies have demonstrated that cerebellar stimulations or lesions induce diverse changes of resting and reflex control of arterial pressure and heart rate (8-13), profoundly modify cerebral blood flow (CBF) (14-17) and influence sympathetic (18) and vagal nerve activity (19-20). Other studies have reported that cerebellar stimulations influences on respiration and behavior function (21-24). Golanov *et al* discovered that stimulation of the rostral-ventromedial pole of the cerebellar fastigial nucleus profoundly modifies CBF, and, moreover, can provide long-lasting protection against adverse effects of ischemia on the brain (25). In rat, electrical stimulation of the cerebellar fastigial nucleus for 1 hour reduces, by 50%, the infarction volume produced by occlusion of the middle cerebral artery (MCA) (26-30). Stimulation also salvages over 50% of the hippocampal pyramidal neurons of the CA1 region, which undergo delayed degeneration after global cerebral ischemia (31).

After the cerebral ischemia, lesioned axons in the adult brain of mammals are unable to spontaneously

regenerate when compared with peripheral and embryonic nervous systems, thus, resulting in devastating and permanent functional deficits. Axonal growth inhibitors have mainly accounted for such a failure (32). To date, three CNS myelin proteins are capable of inhibiting axonal growth, including Nogo-A, myelin associated glycoprotein (MAG), and oligodendrocyte-myelin glycoprotein (OMgp) (33). In addition, Tanascin-R (TN-R) is also reported to involve in inhibition of axonal outgrowth (34). Nogo-A is mainly expressed in the endoplasmic reticulum and the cell surface of oligodendrocytes producing myelin which appears to have two inhibitory domains comprising an amino terminal domain that acts on many cell types (amino-Nogo) and extracellular domain flanked by two hydrophobic segment, termed Nogo-66 (35-36). NgR, as Nogo-66 receptor, is a glycosylphosphatidylinositol (GPI)-linked, leucine-rich repeat (LRR) protein expressed in cell surface and tranduces inhibitory signal to the cell interior by a transmembrane co-receptor, p75. After CNS injury, Nogo-A binds with Nogo-66 receptor to block axonal regeneration and limits motorial recovery (37-40). Two other axonal outgrowth inhibitors, MAG and OMgp, also bind to NgR despite their lack of similar sequences with Nogo-66 (41-42, 38). These signal pathways have been studied most intensively in traumatic spinal cord injury.

In this study, we examined the expression changes of NgR mRNA and protein in the infarcted cortex and hippocampus at different times after cerebral ischemia-reperfusion in rat. Furthermore, we determined whether electrostimulation of fastigial nucleus can have an effect on the expression of NgR and thereby promote axonal regeneration after focal cerebral ischemia.

3. MATERIALS AND METHODS

3.1. Animal groups

Ninety-six adult male Sprague-Dawley rats (250gm-260gm) were randomly divided into (n=16 in each group) sham group and ischemia groups at 12 hours, 24 hours, 1 to 3 weeks. In addition, seventy-two male Sprague-Dawley rats were randomly divided into sham, ischemia-reperfusion and ischemia-reperfusion with an electrostimulation of fastigial nucleus treatment groups (n=24 in each group).

3.2. Transient middle cerebral artery occlusion in rats

Right middle cerebral artery occlusion was induced with an intraluminal filament (49-51). Anesthesia was induced with 3.5% Chlora Hydrate (1ml/100mg). The rectal temperature was maintained at 37 ± 0.5ºC with a homeothermic blanket. Under an operating microscope, the right common carotid artery (CCA) was exposed through a midline neck incision and dissected from its bifurcation to the base of the skull. After coagulation of the occipital artery branches of the external carotid artery (ECA), the right ECA was coagulated along with the terminal lingual and maxillary artery branches. The right internal carotid artery (ICA) was isolated, and the pterygopalatine artery was ligated close to its origin with a silk suture. A microaneurysm clip was placed across both CCA and the ICA to prevent bleeding during the insertion of the suture. After the silk suture was tied loosely around the mobilized ECA stump, a small incision was made on the ECA stump, and a 40mm-length monofilament nylon suture, heat-blunted at the tip and coated with meling paraffin wax, was inserted into the lumen of the ICA. The temporary clip on the ICA was removed, and the nylon suture was advanced 18–20 mm from the bifurcation of the CCA until mild resistance was felt. The silk suture around the ECA stump was tightened on the intraluminal nylon suture. After removal of the microaneurysm clip, the neck incision was closed. After 2 hours of occlusion, the animals were reanesthetized and the filament was withdrawn. Animals subjected to sham surgery were treated similarly, except that the filament was not advanced to the origin of the MCA. Rats that failed to exhibit neurological abnormalities at 2h after stroke were excluded from this study.

3.3. Electrical stimulation of cerebellar fastigial nucleus

Rats were placed in a stereotaxic apparatus and the bregma which was used as stereotaxic zero and the region of posterior cerebellar vermis were exposed by epicraniotomy. A dipolar electrode, fabricated from Teflon-insulated stainless steel wire (outer diameter of 150 μm), carried in stainless steel tubing and exposed at the tip for 100 μm, was lowered into the cerebellum to stimulate cerebellar fastigial nucleus. A hole, 1.5-2.0 mm in diameter, was drilled overlying the right skull with a dental drill through the interparietal bone and the area of the fastigial nucleus is located 11.4-11.8 mm posterior, 0.8-1.0 mm lateral, to bregma, and 11.4-11.8 mm deep to the pial surface. The electrode was moved through the cerebellum in steps of 0.2 mm during stimulation with 2.5-s trains of pulses of 0.5-ms duration at 50 Hz and a stimulus current of 50μA for an hour. In the whole procedure rats were light-anesthetized. If the most active site located in cerebellar fastigial nucleus was stimulated, animals exhibit tails-swing and hair- erection following the increasing of respiration, heart rate and blood pressure (26). At the end of the stimulation, we kept animal warm under lamplight.

3.4. Collect samples

At the end of the observed process, animals were anesthetized with an overdose of Chloral Hydrate. The brains were removed and the right infracted cortex and hippocampus were collected and conserved in liquid nitrogen for RT-PCR (n=8). In addition, other animals in the same group were fixed via the left ventricle for morphology as described (1). The animals were first perfused with 200ml Sodium Chloride and with the 300ml of 4% paraformaldehyde (PFA) until the limbs of animals got stiff. The brains were removed and post-fixed in 4□paraformaldehydein for 24h (4ºC). Ten-micron-thick serial sections were prepared from autopsied brains and embedded in paraffin for histology and immunohistochemistry analysis.

3.5. TTC Staining

Infarct was observed as described earlier using 2,3,5-triphenyl tetrazolium chloride monohydrate (TTC) staining (52-54). For TTC staining, each rat brain was placed in ice-cold saline for 10 min, and then cut into 1-mm coronal slices in a rat brain matrix (Activational Systems,

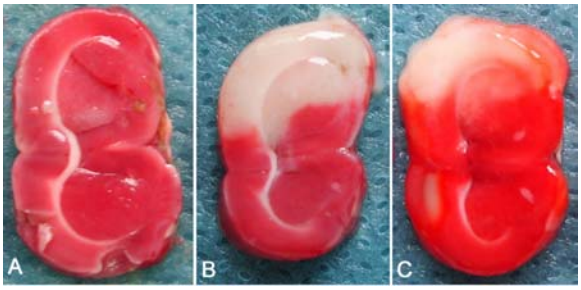

Figure 1. The comparison of infarcted area of each group at 24 h after focal ischemia/reperfusion: Red area represents normal, white area represents infraction (TTC staining). A, sham group, B, ischemia-reperfusion group, C, FNS treatment group.

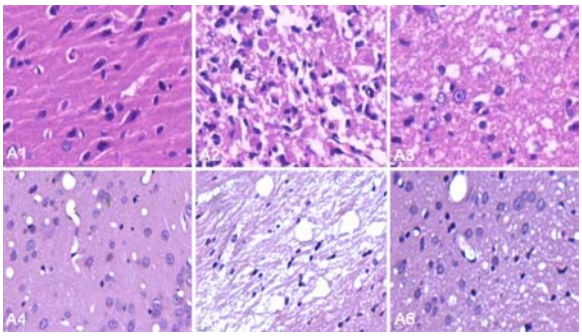

Figure 2. A1-A3 and B1-B3 represent the infarcted cortex and hippocampus from the sham, cerebral ischemia-reperfusion and FNS treatment groups, respectively. Normal neurons were shown in the sham group (A1, B1). Most neurons in the infarcted cortex and hippocampus were damaged at 24 h after cerebral ischemia-reperfusion (A2, B2). A few normal neurons could be observed in FNS treatment group (A3, B3).

Warren, MI, USA). The slices were stained with 2% TTC for 30 min at 37□, taken by a digital camera and the infarct area was observed.

3.6. Histology and immunohistochemistry

Sections were incubated in 3% hydrogen peroxide (H_2O_2) diluted in PBS (10 min) to prevent reaction with endogenous peroxidases, and those sections were used for immunohistochemical staining. After 30 min of normal serum in PBS, the sections were incubated with primary antibodies overnight at 4□ and the following antibodies were used: goat anti-Nogo-R (N-20) (1:100) and mouse anti-Neurofilament 200 (1:100) (Santa Cruz Inc). After rinsing with PBS, the brain sections were incubated with rabbit anti-goat IgG and rabbit anti-mouse IgG as secondary antibody (1:100) for 2 h and then placed in avidin-peroxidase complex solution containing avidin-peroxidase conjugate for 30 min. Peroxade activity was revealed by dipping the sections in a mixture containing 3-3' diaminobenzidine (DAB) and H_2O_2 for 5 min. All the procedures were conducted at room temperature. The brain sections were then mounted, air-dried, dehydrated, coverslipped, and observed under a microscope. The control serum in stead of primary antibody on other sections of the same sections was used for negative control.

3.7. RT-PCR analysis

3.7.1. Primer Design

Primers were designed using GenBank sequences. AF462390 (Nogo receptor as template was optimized using Oligo Version 6.0 software (Molecular Biology Insights, Inc., Cascade, CO)). The primer sequences are designed as following: sense-5'TGC TGG CAT GGG TGT TAT GG-3', anti-sense-5'CGG AAG GTG TTG TCG GGA AG -3', (NgR, product size 493 bp), sense-5'CGT AAA GAC CTC TAT GCC AAC A-3', anti-sense-5'CGG ACT CAT CGT ACT CCT GCT-3' (β-actin, product size 229bp).

3.7.2. RT-PCR reaction

All amplifications were performed in a MJ Research PTC 200 DNA Engine thermocycler (MJ Research, Watertown, MA). For the first-round reaction, 5μl total RNA was reverse transcribed with AMV Reverse Transcriptase and the obtained cDNA was amplified for PCR reaction. Secondly, each 50 μl reaction contained 5μl 10×buffer, 3μl $MgC1_2$ (25mM), 0.5μl Taq mix DNA polymerase, 1μl cDNA, 37.5μl ddH2O, 1μl forward and reverse primers (10pmol). The cDNA was amplified at 94°C for 3min followed by 35 cycles: 94°C for 30s, 54°C for 30s, 72°C for 36s, and a final extension step was performed at 72°C for 5 min.

3.7.3. Gel Electrophoresis

5μl of the PCR product was electrophoresed on a 1.5% agarose gel (E-Gel, Invitrogen, Carlsbad, CA). The gel was photographed under UV transillumination with the AlphaImager system (AlphaInnotech, San Leandro, CA).

3.8 Statistical analysis

All of these data in this study were expressed with means ± standard deviation (SD). Statistical difference between the control and each group of focal ischemia, with or without FNS treatment, were compared by using one-way analysis of variance (ANOVA). A value of $P<0.05$ was considered statistical significant.

4. RESULTS

4.1. Infarct area observation

Figure 1 demonstrates the infract area of representative samples from the cerebral cortex and hippocampus of the control, focal cerebral ischemia, and FNS treatment groups at 24 h after ischemia-reperfusion. FNS treatment reduced ischemic area.

4.2. HE staining

In Figure 2 A1-A3 and B1-B3 exhibit the HE staining of the infracted cortex and hippocampus from sham, 24 h after cerebral ischemia and FNS treatment groups. Normal neurons were shown in the sham group. At the 24 h after cerebral ischemia-reperfusion, the neurons in the infracted cortex and hippocampus were damaged, demonstrating pyknosis, stroma edema, neuronal necrosis

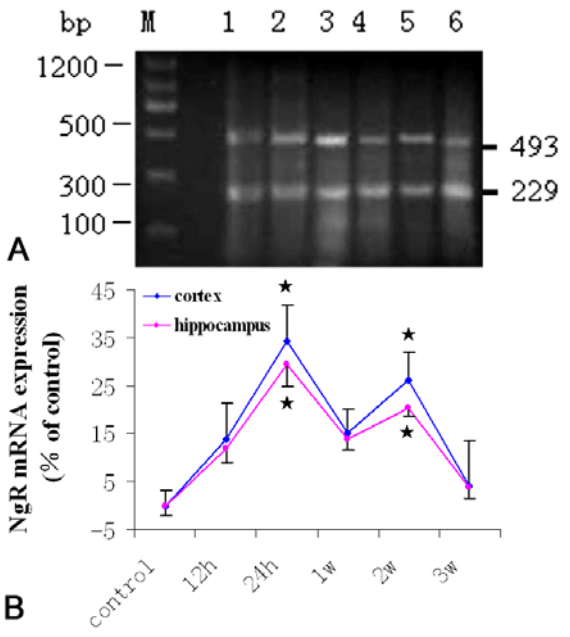

Figure 3. RT-PCR analysis demonstrates the expressions of NgR mRNA in the infarcted cortex and hippocampus from rats suffering from ischemia-reperfusion at different times. Representative bands from 12 h to 3 weeks as well as corresponding β-actin are shown (A). The right panel shows the change curve of NgR mRNA expression in the infarcted cortex and hippocampus respectively. The expression of NgR mRNA is significantly increased at 24 h and peaked again at 2 weeks. (* $p<0.01$ vs. control group in the same region, one-way ANOVA).

and inflammatory cells infiltration around the infarct region. However, in FNS treatment group neurons were only damaged lightly and a few normal neurons could be observed.

4.3. Expression of NgR mRNA: RT-PCR Analysis

A slight increasing of NgR mRNA expression in the infracted cortex and hippocampus were observed at 12 h and remained gradually elevated tendency to the peak at 24 h after cerebral ischemia-reperfusion, then gradually decreased at 1 week after ischemia. The expression of NgR mRNA significantly increased again at 2 weeks which compared to the sham group ($P<0.01$). At 3 weeks after focal cerebral ischemia, NgR mRNA dropped to the normal level (Figure 3). FNS treatment significantly suppressed the level of NgR mRNA at 24 h after stroke (Figure 4).

4.4 Expression of NgR protein: immunohistochemistry

Positive immunoreactive cells were mainly distributed in the ischemic region compared to sham group. In the normal cortex and hippocampus, NgR protein was distributed on the membrane of the neurons and their processes and a few NgR-positive immunoreactive cells were detected in the sham group. After ischemia-reperfusion NgR positive cells were slightly increased at 12 h, peaked at 24 h and started to decline at 1 week. A second peak appeared at 2 weeks and then gradually decreased to normal level. FNS treatment reduced the number of NgR positive cells significantly even though some positive cells remained (Figure 5).

4.5. Visualization of axonal growth: immunohistochemistry

Figure 6 demonstrated the growth state of axons from sham, ischemia-reperfusion and FNS treatment groups. Axons emerged on most neurons which were observed in the sham group. In contrast, the number of axons in the infracted cortex and hippocampus were grossly decreases at 24 h and 2 weeks after cerebral ischemia-reperfusion. After the FNS treatment, more axons emerged on neurons when compared to 24 h and 2 weeks after cerebral ischemia-reperfusion. These results suggested that FNS treatment has the potential to affect directly axonal growth.

Imaging-Pro-Plus was used to determine the mean optical density of immun-positive axons from each group. Figure 6 showed the change of mean optical density of immun-positive axons in the infracted cortex and hippocampus from each group. At 24 h after cerebral ischemia-reperfusion, the mean optical density of axons were significantly lower than the sham group ($p<0.05$). After the FNS treatment, the mean optical density of axons was higher than that of the ischemia untreated groups ($P<0.05$). The similar result emerged at 2w after cerebral ischemia-reperfusion with FNS treatment. These results suggest that FNS treatment has the potential to affect directly axonal growth.

5. DISCUSSION

We studied the expression of NgR mRNA and protein, morphology of neurons and the state of axonal growth in the infarcted cortex and hippocampus at different times after cerebral ischemia. The expression of NgR mRNA and protein in the infarcted cortex and hippocampus significantly increased at 24 h and 2 weeks. In addition, neurons were damaged and the number of axons was decreased at 24 h, similar to a previous study that NgR protein peaked in the ischemic cerebral cortex around 24 h after global ischemia (55). A feature of the present study is that NgR mRNA increased at 12 h, peaked at 24 h after ischemia and was increased by 38% than the sham group. This observation may indicate that the mechanism for axonal plasticity begins at the acute stage after brain injury. Indeed, the binding of NgR and Nogo-A play an important role in preventing axonal regeneration immediately after CNS injury before the scar forms (56). The Nogo-A antibody, IN-1, applied within 24 h improved the regeneration of axons (56). An enhanced activity of Nogo-A/NgR might interfere with CNS plasticity and hamper the recovery of neurological function. Early application of FN stimulation (24 h after global ischemia) promoted cerebral blood flow and decreased cell death which might partially contribute to the improvement of neurological function (57-59). Furthermore, some investigators have manifested that FN electrostimulation after stroke can evoke obvious neuroprotection, especially 1-3 days after ischemia (60). Taken together, it seems that FNS treatment improved

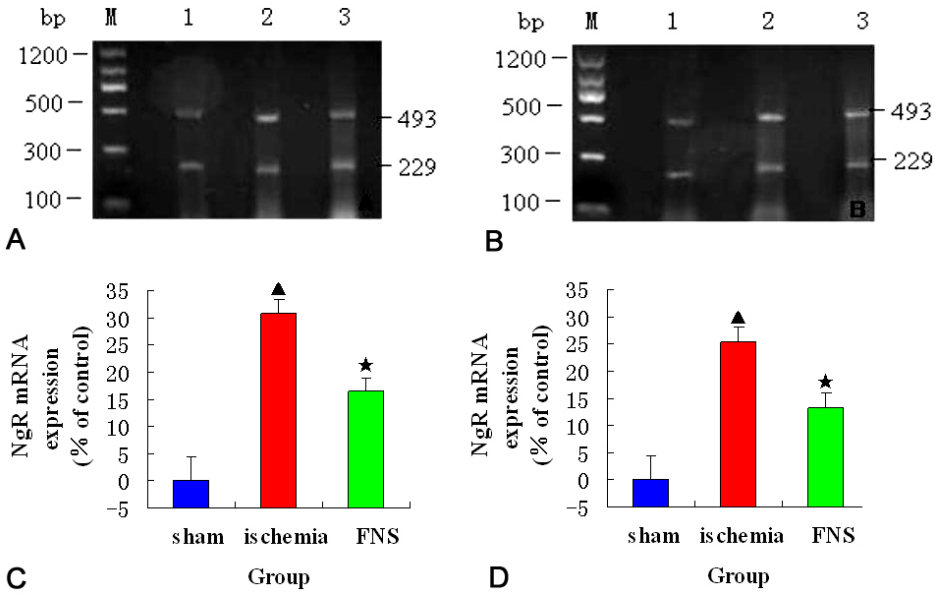

Figure 4. RT-PCR analysis demonstrates the expressions of NgR mRNA in the infarcted cortex and hippocampus from sham (control), cerebral ischemia-reperfusion and FNS treatment groups, respectively. Representative bands for every group as well as corresponding β-actin are shown (A, B). The right panels show the change of NgR mRNA expressions in each group. Bars represent mean ± SD of n = 8 rats per group. The expression of NgR mRNA is significantly increased at 24 h after cerebral ischemia-reperfusion. FNS treatment significantly decreases the level of NgR mRNA expression (▲ P<0.01 vs. control, * P<0.05 vs. ischemia-reperfusion). M: marker,1 indicates sham group, 2 indicates 24 h after cerebral ischemia-reperfusion group, 3 indicates FNS treatment group.

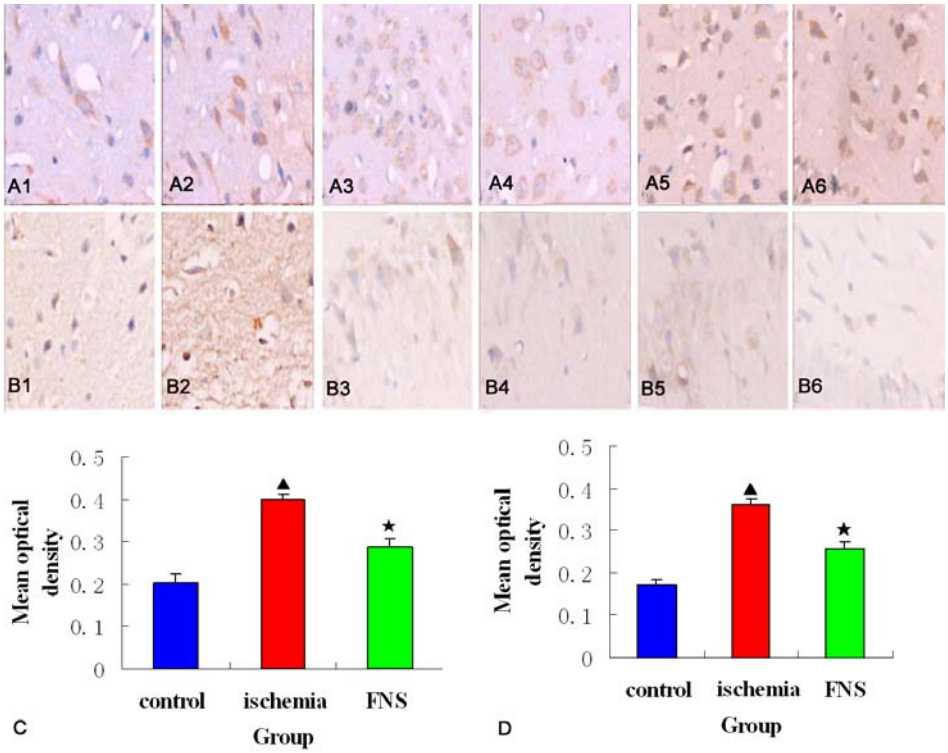

Figure 5. In the sham group a few NgR-positive immunoreactive cells are detected. NgR protein expresses on the membrane of the neurons and their processes in cortex and hippocampus. After ischemia-reperfusion NgR positive cells were slightly increased at 12 h, peaked at 24 h and peaked again at 2 weeks before gradually decreased to normal level. FNS treatment reduced the number of NgR positive cells significantly compared to ischemia-reperfusion group. ▲ P<0.01 vs. control group, * P<0.05 vs. ischemia-reperfusion group. A1-A6:cortex; B1-B6:hippocampus.

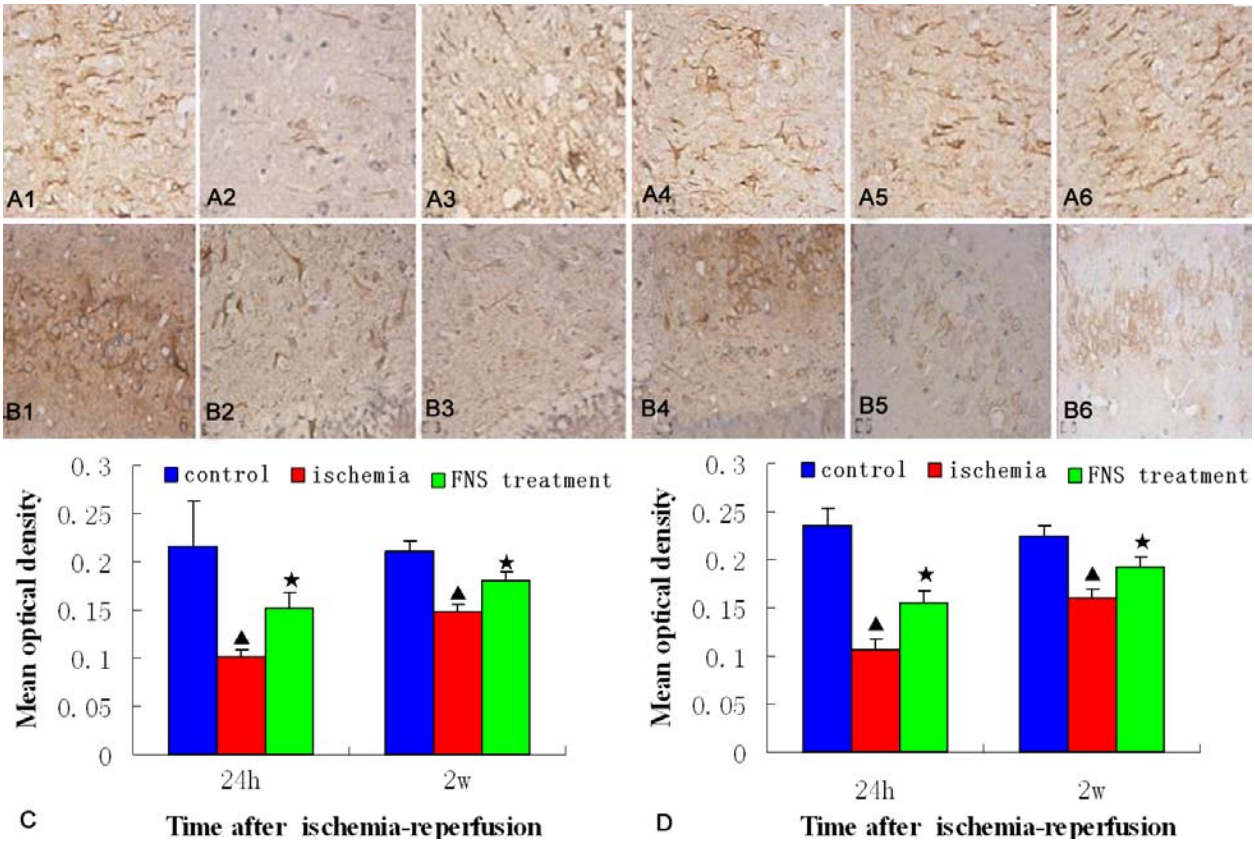

Figure 6. Axonal growth was shown in the infarcted cortex and hippocampus from the sham, 24 h and 2 weeks after cerebral ischemia-reperfusion and FNS treatment groups, respectively, in A1-A6 and B1-B6 (A1-A6: cortex, B1-B6: hippocampus). C and D show the changes of mean optical density of axons in each group. The number and mean optical density of axons were reduced at 24 h and 2 weeks after cerebral ischemia-reperfusion in comparison with the sham group. FNS treatment improved axonal growth (A3-A6,B3-B6).▲ $P<0.05$ vs. sham group, * $P<0.05$ vs. ischemia-reperfusion group. A1-A6:cortex; B1-B6:hippocampus.

growth state of axons in the infarcted cortex and hippocampus which may be mediated by reduced expression of NgR mRNA and protein.

Electrostimulation of cerebellar fastigial nucleus has powerful effects on systemic and cerebral circulation. Excitation of fibers of passage increases systemic arterial pressure and CBF independently which is also independent of brain metabolism and probably mediated by intrinsic neuronal circuitry. Accompanying pronounced changes in systemic and cerebral circulation excitation of FN neurons considerably diminishes brain damage resulting from global or focal ischemia. The neuroprotection initiated by FNS treatment with relatively short period of stimulation for 1 h is long-lasting and protects the brain for up to three weeks. Some available data suggest that salvage is biphasic. The early phase, which develops during FN stimulation, might involve the increase of neuronal tolerance to excitatory stimuli mediated by the opening of potassium channels. Second delayed phase fully develops within 3 days after the FN stimulation and involves suppression of inflammatory and apoptotic cascades, which probably results from changes in genes expression (25). Thus, it is conceivable that FN stimulation might affect the expression of NgR as the inhibitory protein and the recovery of neurological function after stroke.

However, the detailed pathway and molecular mechanisms that FNS treatment can down regulate the expression of NgR are unknown. Except NgR reduction, other factors or mechanisms may be involved. For example, heat shock protein is reported to suppress the expression of Nuclear factor-kappa (NF-κB) that binds to the promoters of exons in many cell factors in the infarcted region by FN stimulation (61). The transcription factors NF-κB play a key role in regulating a diverse array of genes involved in cell growth, differentiation, and adaptive responses to environmental factors that are cell- and stimulus-specific (62). In the central nervous system, NF-κB proteins are ubiquitously expressed in neurons and glia (63-64), in addition to regulating physiological processes, they participate in pathological events associated with neurodegeneration (64-65). Increased NF-κB levels have been observed in the dying neurons of brains exposed to trauma and ischemia (66-69). Many studies demonstrated the anti-apoptotic effects of NF-κB in cultured neurons (71-74), suggesting that evidence has emerged from experimental models of pathological conditions affecting

adult neurons. Some investigators reported that NF-κB mediates the neuroprotection elicited by the tumor necrosis factor in hippocampal cells and promotes neuronal resistance to excitotoxicity (74-76). Other studies showed that the activation of NF-κB triggers neuronal degeneration after cerebral ischemia and induces cell death (67,69,77-78). When the tissue suffers from ischemic damage, NF-κB is activated and expressed increasingly, inducing the transcriptive expression of most cell factors and resulting in the elevated expression of NgR. Some studies indicated that the treatment of FNS reduces the expression of NF-κB after stroke, especially at 24h after cerebral ischemia-reperfusion. In addition, in the infarcted hippocampus FN stimulation also suppresses the expression of NF-κB, which leads to the loss of necrotic neurons and promotes the recovery of function after CNS injury (61). Therefore, FN stimulation might inhibit the expression of NgR mRNA by suppressing the expression of NF-κB, resulting in outgrowth of axons. FN stimulation might affect other factors involved in Nogo-A/NgR pathway comprising P75 and RhoA (79-80) which contribute to the improvement of axonal outgrowth.

In conclusion, this study demonstrates that electro-stimulating cerebellar fastigial nucleus promotes axonal regeneration by down regulating the expression of NgR, which provides an effective therapy for the recovery of CNS injury.

6. ACKNOWLEDGEMENT

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