LABORATORY INVESTIGATION

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Delayed Intraventricular Nogo Receptor Antagonist Promotes Recovery from Stroke by Enhancing Axonal Plasticity

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Objective : After ischemic stroke, partial recovery of function frequently occurs and may depend on the plasticity of axonal connections. Here, we examine whether blockade of the Nogo/NogoReceptor(NgR) pathway might enhance axonal sprouting and thereby recovery after focal brain infarction.

Methods : Adult male Sprague Dawley rats weighing 250~350g were used. Left middle cerebral artery occlusion(MCAO) was induced with a intraluminal filament. An osmotic minipump (Alzet 2ML4, Alza Scientific Products, Palo Alto, CA) for the infusion of NgR-Ecto(310)-Fc to block Nogo/NgR pathway was implanted 1 week after cerebral ischemia. Prior to induction of ischemia, all animals received training in the staircase and rotarod test. Two weeks after biotin dextran amine injection, animals were perfused transcardially with PBS, followed by 4% paraformadehyde/PBS solution. Brain and cervical spinal cord were dissected. Eight coronal sections spaced at 1mm intervals throughout the forebrain of each animal were stained with cresyl violet acetate for determination of infarction size. Images of each section were digitized and the infarct area per section was measured with image analysis software.

Results : Histological examination at 11 weeks post-MCAO demonstrates reproducible stroke lesions and no significant difference in the size of the stroke between the NgR(310)Ecto-Fc protein treated group and the control group. Behavioral recovery is significantly better and more rapid in the NgR-Ecto(310)-Fc treated group. Blockade of NgR enhances axonal sprouting from the uninjured cerebral cortex and improves the return of motor task performance.

Conclusion : Pharmacological interruption of NgR allows a greater degree of axonal plasticity in response to stroke and this is associated with improved functional recovery of complicated motor tasks.

KEY WORDS : Stroke · Nogo receptor · Plasticity.

Introduction

Recovery from ischemic stroke is quite variable but typically follows a time course in which most improvement occurs over a 1~6 month[3]. Partial recovery from the contralateral hemiparesis associated with a stroke involving the primary motor cortex sometimes occurs by the activation of regions in the opposite, undamaged hemisphere[9,16,21]. In other cases, activation of regions ipsilateral to the stroke occurs as new neurons are recruited to perform specific tasks[6,8,20,23]. Similar shifts have been demonstrated during recovery in rodent models[5]. Currently there is no clinically useful pharmacological method to enhance stroke recovery.

CNS myelin is a primary inhibitor of axonal growth in the adult brain, and three proteins, Nogo-A, MAG and OMgp, appear to be responsible for this inhibition of axonal growth. A receptor molecule, Nogo-66 Receptor(NgR), mediates the action of one of two Nogo-A inhibitory domains, as well as the action of MAG and OMgp on axons[7,9,12]. We recently found that a soluble ectodomain fragment of the NgR prevents all three myelin ligands from interacting with the NgR and is more effective in promoting axonal growth and recovery after spinal cord injury[13,14]. In this study we employed pharmacologic methods to assess the role of the Nogo/NgR system in limiting axonal plasticity and behavioral recovery after ischemic stroke.

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Materials and Methods

Permanent middle cerebral artery occlusion(MCAO)

Adult male Sprague Dawley rats (250~350g) were maintained on a 12-h light/dark cycle. The rats were fasted overnight before the day of the experiment but were allowed free access to tap water. Rats were divided into two experimental groups : (1) MCAO plus Rat IgG in PBS (n=16) and (2) MCAO plus NgR-ecto(310)Fc (n=17). Left MCAO was induced with a intraluminal filament[2,17,22]. Anesthesia was induced with 5% isoflurane and maintained with 2% isoflurane in an oxygen/air mixture using Fluovac Scavenger. The rectal temperature was maintained at 37.5°C with a homeothermic blanket. Under an operating microscope, the left 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 ECA, the left external carotid artery(ECA) was coagulated along with the terminal lingual and maxillary artery branches, which were then divided. The left internal carotid arter(ICA) was isolated and the pterygopalatine artery was ligated close to its origin with a silk suture. A microaneurysm clip was placed across both the CCA and the ICA to prevent bleeding during the insertion of the suture. After tying loosely around the mobilized ECA stump, a small incision was made on the ECA stump and a 23-mm length of 4-0 monofilament nylon suture, heat-blunted at the tip and coated with poly-L-lysine, was inserted into the lumen of the ICA. The temporary clip on the ICA was removed and the nylon suture was advanced 18 to 20mm 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. Rats that failed to exhibit neurological abnormalities were excluded from further study.

Drug administration and axonal tracing

An osmotic minipump (Alzet 2ML4, Alza Scientific Products, Palo Alto, CA) was implanted 1 week after cerebral ischemia. The pump designed to deliver 2.5$\mu\ell$/hr for 28 days was filled with 2ml of a 1.2mg/ml solution of NgR-ecto(310)Fc or Rat IgG in PBS. For pump implantation, all animals were anesthetized with isoflurane and oxygen using a gas anesthesia mask for the stereotaxic instrument and placed in a stereotaxic frame. After the scalp was reopened, a pocket was formed over the neck and scapulae to hold the minipump. A burr hole was drilled in the skull and the cannula (ALZET brain infusion kit II, Alza) was introduced into the right lateral ventricle at stereotaxic coordinates 0.6mm posterior and 1.2mm lateral to bregma and 4.0mm deep to the pial surface. The cannula was held in place with cyanoacrylate and the skin was sutured[10]. Pumps were replaced after 28 days and connected to the same cannula. The soluble NgR(310)Ecto-Fc protein was infused into the lateral ventricle of the rat on the side opposite to the stroke to block NgR pharmacologically.

The continuous infusion was initiated 7 days after the stroke and continued for 2 months. Control animals received rat IgG at the same dose. Strokes were induced in these rats prior to protein infusion by permanent occlusion of the left middle cerebral artery with an intraluminal filament. In order to trace corticofugal axons, a burr hole was made in the skull overlying the right sensorimotor cortex, after removal of the minipump and cannula at 9 weeks post-MCAO. Biotin dextran amine (BDA, MW 10,000 : 10% in PBS, Molecular Probes) was applied at 7 injection sites at the depths of 1.5mm from the cortical surface[9].

Behavioral testing

All animals were housed in individual cages. Prior to induction of ischemia, all animals received training in the staircase and rotarod test, and animals not achieving criteria were excluded from the further study. The Rotarod test was used to examine balance and coordination[11]. Rats were trained on the rotarod on 5 consecutive days for a total 15 sessions before surgery. The rotating drum was accelerated from 4 to 40 rpm over 5 min and the latency in seconds for the animal to fall off the drum was recorded. Each session included three consecutive trials, with a maximum time of 300 sec and the mean fall latency was calculated from the three trials. Animals that did not stay on the rod for an average of at least 1 min at the end of training were excluded from the stroke surgery.

The staircase test was employed to test skilled forepaw use[1,18]. Food was restricted during the pre-stroke training period and 6 days following surgery in order to provide motivation for food rewards. All animals were food restricted to 85 to 90% of their free-feeding weight. Animals were returned to a free feeding schedule for 5 days following surgery to improve postoperative weight and recovery. All animals were fed 12~15g standard laboratory chow at the end of each test. Animals were placed in the staircase apparatus (Lafayette instrument). Each step of the stairs of seven steps was baited with two food pellets of 45mg chocolate flavored purified pellet (Bioserve). Each test session lasted 15 min. The number of pellets retrieved and eaten per side was used as a measure of forelimb reaching ability. Animals were trained for 2 weeks before being subjected to cortical ischemia to establish the baseline performance and animals that did not learn to retrieve 4 pellets from each side at the last training were excluded from the stroke surgery.

Behavioral recovery was assessed the forepaw pellet retrieval task and the rotarod test. Food-restricted ratsare trained to retrieve food pellets from a staircase that positioned the pellets

at increasing distances from the body and allowed only the lesion-affected forepaw access to one set of pellets.

Histology and analysis

Two weeks after BDA injection, animals were perfused transcardially with PBS, followed by 4% paraformadehyde/PBS solution. Brain and cervical spinal cord were dissected, postfixed overnight and embedded in Tissue Freezing Medium for cryostat sectioning. The coronal sections from the brain and transverse sections from the spinal cord were incubated with avidin-biotin-peroxidase complex and the BDA tracer was visualized by nickel-enhanced diaminobenzidine horseradish peroxidase(HRP) reaction. The sections were mounted, dehydrated and coverslipped with mounting medium. Images of each sections at red nucleus level were captured with a 10x objective lens. BDA-stained fiber length in one 0.85×0.68mm area centered on the red nucleus were traced with an image analysis system. For fiber counts in the cervical enlargement of spinal cord, sections were examined with a 40x objective lens. All BDA-positive fibers in the gray matter of one section on the side ipsilateral to the BDA injection were counted.

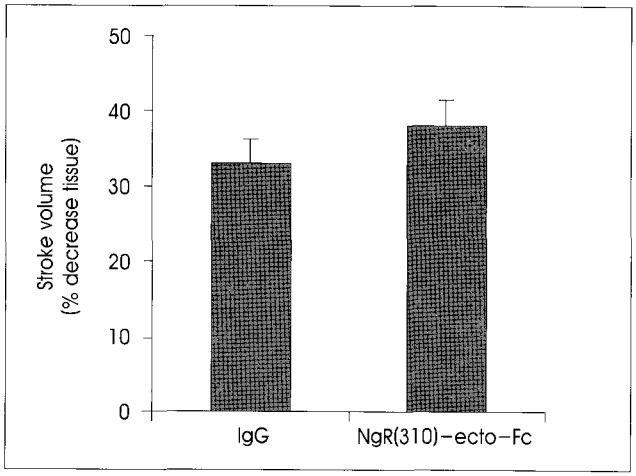

Fig. 1. Stroke volume. There is no significant effect of NgR(310)ecto-Fc on infarct volume.

Quantification of stroke volume

Eight coronal sections spaced at 1mm intervals throughout the forebrain of each animal were stained with cresyl violet acetate for determination of infarction size. The infracted area was fully bracketed by this set of sections. Images of each section were digitized and the infarct area per section was measured with image analysis software. The area of intact tissue in the left hemisphere was subtracted from the area of the contralateral hemisphere. Volume was interpolated across the eight sections.

Statistical analysis

The data was analyzed using SPSS 10.0 for windows (SPSS inc, Chicago, IL, USA). For evaluating the difference of the behavioral test between two groups, 2-way ANOVA was used at 95% and 99% significance levels. For the BDA stained axon, Student's t test was used.

Results

Histological examination at 11 weeks post-MCAO demonstrated reproducible stroke lesions and no significant difference in the size of the stroke between the NgR(310)Ecto-Fc protein treated group and the control group (Fig. 1). Thus, any improvement in behavioral recovery after this treatment could not be attributed to neuroprotection.

In control rats, food pellet retrieval with the affected right forepaw droped from 8.5/session in pre-stroke training to 1/session at one week post-stroke and slowly recovers to 4 pellets/session by 6 weeks post-stroke. Recovery was significantly better ($p<0.01$, MANOVA) and more rapid in the NgR-

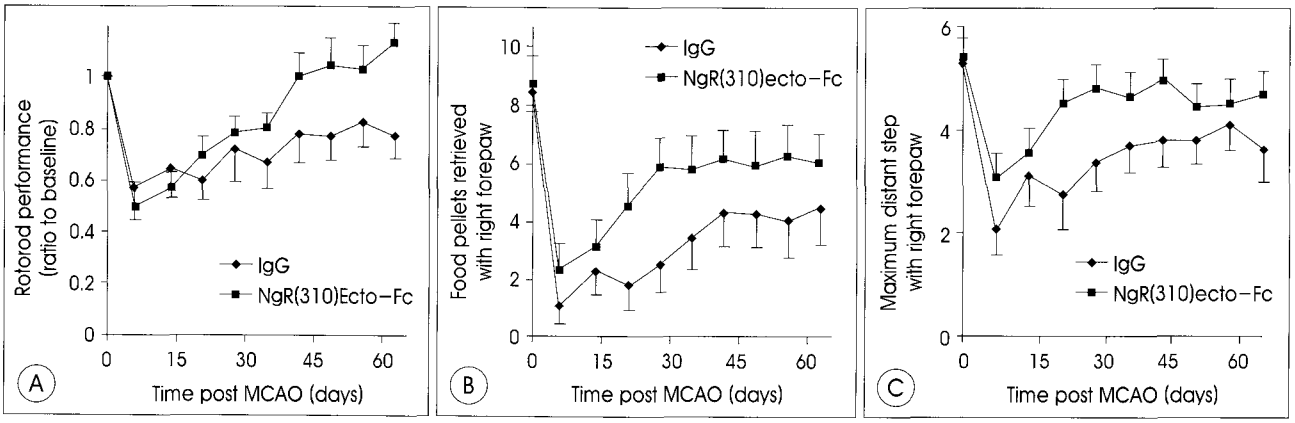

Fig. 2. A : Rotarod test. Postoperative performance ratio to baseline was significantly better in rats treated with NgR(310)ecto-Fc. ($p<0.001$, Two-way parametric analysis of variance). B, C : Staircase test of skilled forelimb reaching ability. The number of pellets retrieved with the contralateral forepaw and the lowest step with <2 pellets remaining(numbered 1-7 from the top) are significantly increased in NgR(310)ecto-Fc group compared to the control group. ($p<0.01$, Two-way parametric analysis of variance). Data are shown as mean±SEM. n=16 rats for IgG and n=17 rats for NgR(310)Ecto-Fc rats in A-C.

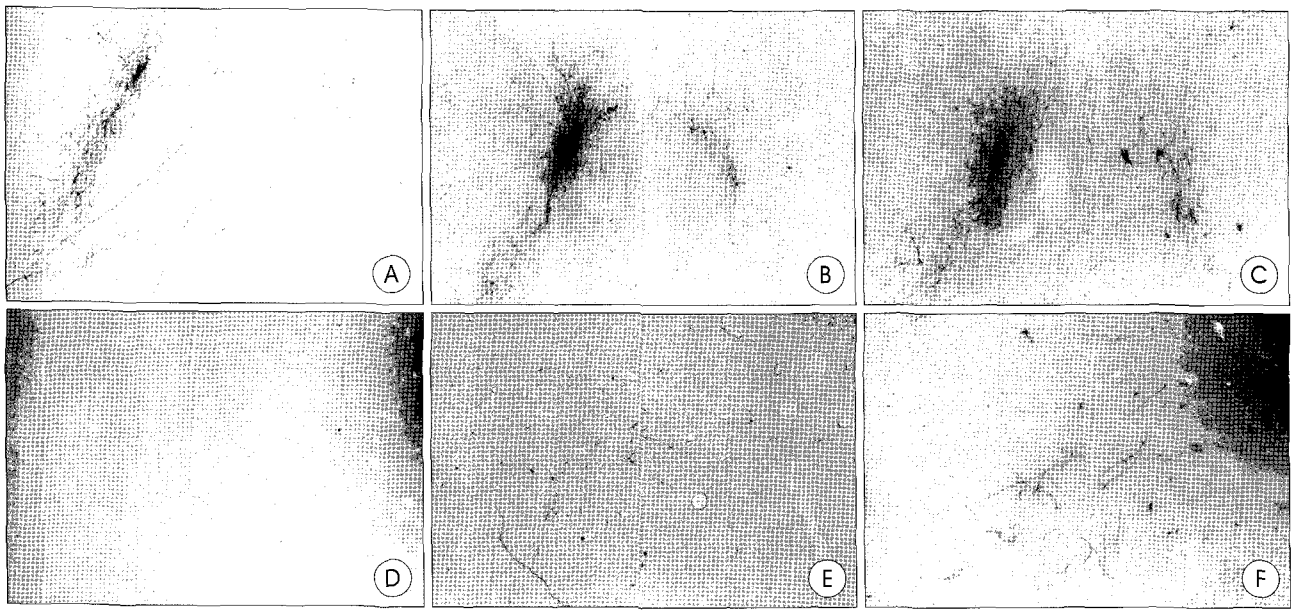

Fig. 3. Biotin dextran amine(BDA)-labeled axons traced from the uninjured cerebral cortex are illustrated at the level of the red nucleus (A-C, horseradish peroxidase stain, ×10) and cervical spinal cord (D-F, horseradish peroxidase stain, ×40) from different rats. Control rats without stroke exhibit very few BDA-labeled fibers in the red nucleus contralateral to the tracer injection or in the spinal gray matter ipsilateral to the injection (A, D). After stroke, many BDA-labeled fibers sprouting into the territory normally innervated by the infarcted cortex are present (B, E). This is more prominent in rats treated with NgR(310)Ecto-Fc than in control IgG rats (C, F).

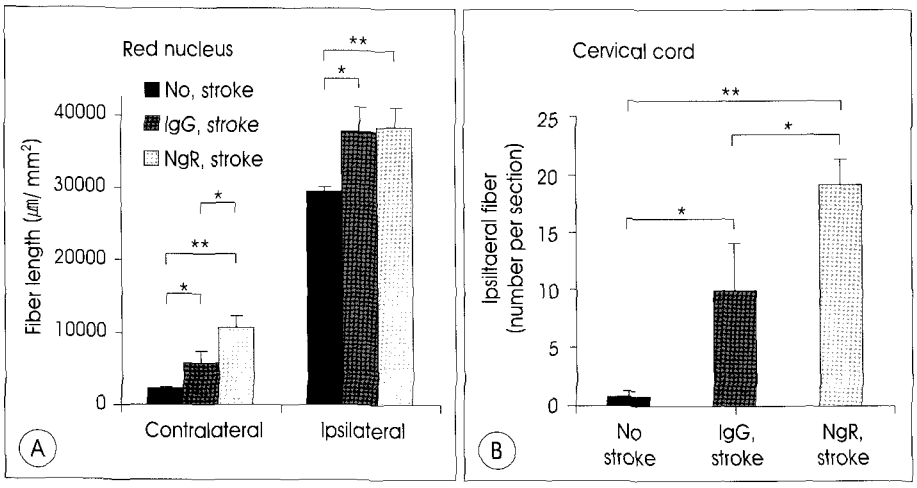

Fig. 4. A : The total length of BDA axon centered on the red nucleus is plotted. B : The total number of BDA-positive axons in the gray matter ipsilateral to the injection site per transverse section of the cervical spinal cord is plotted. Data are shown as mean ± SEM. *, $p<0.05$; **, $p<0.01$ (Student's *t* test).

Ecto(310)-Fc treated group, achieving a level of 6 pellets/session by 4 weeks post-stroke. A related parameter from the staircase test, the most distant step reached per session, showed a nearly identical pattern of greater improvement in the NgR(310)Ecto-Fc treated group ($p<0.01$, MANOVA). Similarly, the NgR-Ecto treated rats exhibited increased recovery of motor performance after stroke on the rotarod test of coordination. Recovery as a percentage of baseline value was significantly ($p<0.001$, MANOVA) greater in the NgR-Ecto treated rats (Fig. 2). In fact, no deficit was detectable at 9 weeks post-stroke in the treated rats, while a persistent deficit was present in the control group.

Because the stroke volumes were identical in the NgR-Ecto treated rats but recovery was greater, we searched for evidence of increased axonal plasticity in the treated animals. The axonal tracer biotin dextran amine was injected into the intact (non-stroke) motor cortex and then fibers were traced into the midbrain at the level of the red nucleus. Prior to a stroke, the pattern of labeling in treatment and control group was identical with a predominance of innervation confined to the side ipsilateral to the cortical injection and the number of crossed corticorubral fibers was low in normal control animals (Fig. 3A). At the level of the red nucleus, the stroke induced a significant increase in fiber length on the side contralateral to the tracer injection (ipsilateral to the stroke); this reflected a level of endogenous axonal plasticity that was likely to contribute to motor recovery. There was a moderate increase of both ipsilateral and contralateral fiber sprouting in control IgG rats (Fig. 3B). NgR(310)Ecto-Fc protein treatment during the period from 1~9 weeks after MCAO resulted in significantly more contralateral fiber length at this level, which was 5-fold above the number seen in normal animals, and an additional 2-fold over the level seen in injured

animals receiving IgG (Fig. 3C, Fig. 4A).

The pattern of axons descending from the intact motor cortex to the ipsilateral cervical cord gray matter was also examined. The vast majority of corticofugal axons from the uninjured right cortex had crossed to the left side of the spinal cord and form a tight bundle as the dorsal corticospinal tract. Very few fibers (0.8 ± 0.4) were detectable in animals with no stroke (Fig. 3D). The stroke significantly increased the numbers of such fibers (10.1 ± 4.2) in IgG-treated animals (Fig. 3E). In contrast, NgR(310)Ecto-Fc protein had a profound effect, increasing the number of crossed axons to 19.5 ± 2.2 (difference from IgG-treated controls, $p < 0.05$, Student's t test) (Fig. 3F, Fig. 4B).

Discussion

The coordination of skilled forelimb movements in the normal rat is dependent on several cortico-efferent pathways thar primarily include the croticospinal and rubrospinal tracts. When the cortical lesions including the sensorimotor cortex in the adult rat significantly affect the performance of skilled motor performance in the forelimb contralateral to the lesion, resulting in permanent impairments with limited spontaneous recovery. Multiple factors contributes to the lack of spontaneous regeneration in the CNS, including glial scars, lack of neurotrophins, and growth-inhibitory molecules, such as proteoglycans and myelin-associated proteins. Of the multiple inhibitory components identified in CNS myelin, the most extensively studied is Nogo-A. This protein is primarily expressed on the surface of oligodendrocytes and their product myelin[4,7,9,12,19].

We have employed NgR(310)Ecto-Fc treatment in rats with MCAO to explore the role of the Nogo/NgR system in stroke recovery. NgR(310)Ecto-Fc treatment induces significant axonal reorganization in the rat brain after MCAO and helps restore cortical control of the denervated forelimb. The blockade of NgR function improves motor task performance, in some cases to pre-stroke levels. In all animals suffering a stroke, the axonal pathway undergoes significant sprouting from the intact side to the stroke-denervated side in the midbrain and the cervical spinal cord. This sprouting is enhanced by blockade of NgR. These data demonstrate that pharmacological interruption of Nogo-NgR pathway allows a greater degree of axonal plasticity in response to stroke and this is associated with improved functional recovery of complicated motor tasks. This is not due to tissue preservation or protection. NgR antagonism promotes stroke recovery in the subacute period after injury.

In this study, we assessed fibers emanating from the uninjured motor cortex and focused on two sites of descending projections, the red nucleus in the midbrain and the cervical enlargement of the spinal cord. These sites are innervated in a predominantly unilateral pattern in uninjured rodents. After stroke in control animals, significant bilateral innervation occurs. Some axonal reorganization doses indeed occur spontaneously in the corticospinal and corticorubral tracts after stroke. This natural plasticity of uninjured corticofugal fibers adjacent to denervated areas may underlie at least a portion of stroke recovery and has been seen in previous studies[23,24]. The increased bilaterality of corticofugal projections to the red nucleus and the spinal cord is unlikely to be the only axonal plasticity responsible for improved function. Further studies may reveal additional pathways adaptively regulated by stroke injury. The effect of Nogo-NgR antagonism is not to create new or different pathways, but is to significantly enhance endogenous anatomical plasticity. This implies that myelin-based inhibition of axonal sprouting plays a role in limiting axonal plasticity in response to the stroke injury. These data do not define a mechanism by which the neurons in the contralateral uninjured cortex respond to ischemic damage for initiating a sprouting response. Corticocortical connections may monitor the activity in the contralateral injured hemisphere directly[23], and further studies should be necessary to verify in the future.

The present NgR(310)Ecto-Fc treatment protocol delivered the protein intracerebroventricularly. A similar approach may prove technically difficult in some cases of clinical stroke. However, there are indications that NgR antagonism might be delivered systemically to the injured CNS[14]. After stroke, the blood brain barrier is disrupted for several weeks and this provides the potential for access to the CNS. Alternatively, the identification of small molecule NgR antagonists with CNS access to promote stroke recovery.

Current pharmacological stroke therapy focuses on preventive strategies to reduce risk and on immediate acute thrombolytic and neuroprotective strategies to reduce damage to the brain. The potential for enhancing recovery from subacute stroke provides a new modality for pharmaceutical intervention; this potential is currently addressed only by physical therapy[21,25]. It is clear that there is a significant degree of stroke-induced plasticity of axonal connectivity without treatment[3,5,6,15,20,23], but that interruption of the Nogo/NgR pathway enhances this adaptive recovery mechanism.

Conclusion

In this study we employed Nogo receptor antagonist to explore the role of the Nogo/NgR system in stroke recovery. Rat with MCAO treated by Nogo receptor antagonist recover complex motor function after stroke more completely than control animals. In all animals suffering a stroke, the axonal pathway undergoes significant sprouting from the intact side

to the stroke-denervated side in the midbrain and the cervical spinal cord. These axonal connections from the undamaged motor cortex is augmented by inracerebroventricular administration of a function-blocking NgR fragment. The present data demonstrate that delayed pharmacologic blockade of NgR enhances axonal sprouting from the uninjured cerebral cortex and improves the motor task performance.

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