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Spatiotemporal expression of Nogo-66 receptor after focal cerebral ischemia

Yue Cao[1, #], Ya-xian Dong[2, #], Jie Xu[3, *], Guo-liang Chu[3], Zhi-hua Yang[2], Yan-ming Liu[4]

1 Department of Medical Technology, Medical College of Shaoguan University, Shaoguan, Guangdong Province, China
2 Department of Neurology, the First Affiliated Hospital of Guangzhou Medical University, Guangzhou, Guangdong Province, China
3 Department of Human Anatomy, Sun Yat-Sen University, Guangzhou, Guangdong Province, China
4 Department of Clinical Laboratory, Yue Bei People's Hospital, Shaoguan, Guangdong Province, China

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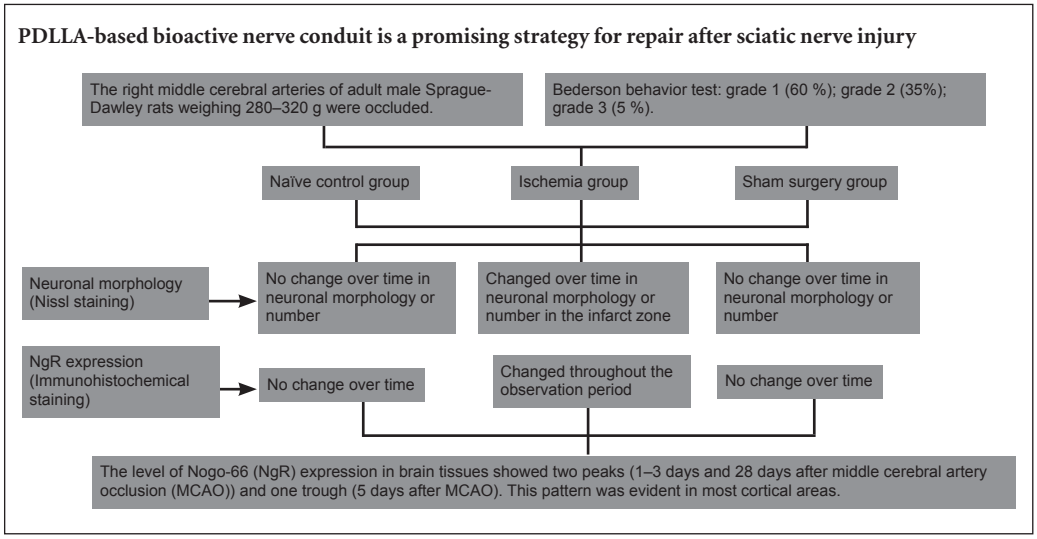
Graphical Abstract



Correspondence to:
Jie Xu, xujie@gzsums.edu.cn.

 # These authors contributed equally to this work.

orcid:
0000-0003-3604-5916 (Jie Xu)

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Abstract

NgR, the receptor for the neurite outgrowth inhibitor Nogo-66, plays a critical role in the plasticity and regeneration of the nervous system after injury such as ischemic stroke. In the present study, we used immunohistochemistry to investigate the regional expression of NgR in rat brain following middle cerebral artery occlusion (MCAO). NgR protein expression was not observed in the center of the lesion, but was elevated in the marginal zone compared with control and sham-operated rats. The cerebral cortex and hippocampus (CA1, CA2, and CA3) showed the greatest expression of NgR. Furthermore, NgR expression was higher in the ipsilesional hemisphere than on the control side in the same coronal section. Although time-dependent changes in NgR expression across brain regions had their own characteristics, the overall trend complied with the following rules: NgR expression changes with time showed two peaks and one trough; the first peak in expression appeared between 1 and 3 days after MCAO; expression declined at 5 days; and the second peak occurred at 28 days.

Key Words: *nerve regeneration; focal cerebral ischemia; cerebral cortex; hippocampus; NgR; Nogo-A; immunohistochemistry; neural regeneration*

Introduction

The Nogo-66 receptor (NgR) was first identified as a receptor for neurite outgrowth inhibitor (Nogo) (Fournier et al., 2001) and later also for oligodendrocyte-myelin glycoprotein (Wang et al., 2002a; Rosochowicz et al., 2015) and myelin-associated glycoprotein (Domeniconi et al., 2002; Liu et al., 2002). These three myelin-derived growth inhibitory proteins converge at the level of NgR, which is thought to offer an important target for enhancing plasticity and regeneration in the nervous system. Expression of NgR and Nogo-A is juxtaposed in the uninjured mouse brain (Wang et al., 2002b). However, whereas NgR protein is found throughout axons in the adult central nervous system, it is not detected in oligodendrocytes, where Nogo-A is expressed (Chen et al., 2000; GrandPré et al., 2000). Under normal circumstances, NgR stabilizes the cerebral cortex, hippocampus and other areas and allows plasticity in these areas (O'Neill et al., 2004; Endo et al., 2007). In the present study, we observed time-dependent NgR changes in rat brain after focal cerebral ischemia.

Materials and Methods

Establishment of models of focal cerebral ischemia

Clean adult male Sprague-Dawley rats weighing 280–320 g were provided by the Animal Care Center of the Sun Yat-Set University in China (License No. 0036808). Animals were housed in a temperature- and humidity-controlled room on a 12/12-hour light/dark cycle. All tests were performed during the light phase. All animal experiments were approved by the Ethics Committee, Zhongshan University, China. A total of 192 rats were equally and randomly allocated to three experimental groups: ischemia, sham surgery, and naïve control.

In the ischemia group, we used cauterization to occlude the right middle cerebral artery 2 mm proximal to the olfactory tract and extending to the inferior cerebral vein (Wang et al., 2002b). Animals were anesthetized with 10% chloral hydrate (0.35 mL/100 g, intraperitoneally). A 1.5-cm incision was made between the right eye and the right ear, and the temporalis muscle was retracted inferiorly to avoid compression of the orbital contents. Under an operating microscope (Institute of Optics and Electronics, Chinese Academy of Sciences, Sichuan Province, China), a burr hole was made to expose the middle cerebral artery transcranially without damage to the zygomatic bone. The middle cerebral artery was permanently occluded by monopolar coagulation and then transected with microscissors. The temporalis muscle and skin were then closed in layers, and the rats were warmed under a heat lamp until they awoke. Rats in the sham surgery group underwent the same procedures as those in the ischemia group but without occlusion of the middle cerebral artery. Control rats did not undergo any procedures.

Bederson behavior test

The neurological status of each rat was carefully evaluated 24 hours after surgery to assess the effects of occlusion, using the following grading scale of 0–3: 0, no observable deficit; 1, forelimb flexion; 2, decreased resistance to lateral push (and forelimb flexion) without circling; 3, as 2 but with circling. The tests were conducted sequentially; if a rat exhibited the behavior at one step but not at the subsequent step, it was graded as the former (Bederson et al., 1986).

Nissl staining

Two rats from each group were chosen at random for Nissl staining. Routine Nissl staining using 0.3% cresyl violet was performed on frozen brain sections (35-μm thick) and viewed under a light microscope (Shanghai Medical Instruments Company, Shanghai, China).

Immunohistochemical staining

At 1, 2, 3, 5, 7, 14, 28, and 56 days after surgery, NgR expression was examined in the cerebral cortex and several white matter regions of rats from the three experimental groups. Four rats were randomly selected from each group at each time point. Rats were euthanized and perfused transcardially with 0.9% saline, followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and fixed in 4% paraformaldehyde for 4 hours at 4°C, and immersed in 0.1 M phosphate buffer overnight in 10%, overnight in 20% and overnight in 30% sucrose at 4°C. The front and back of the brain were discarded, and serial sections were cut coronally from the remaining part (bregma +1.60 mm to −3.30 mm), to examine the following areas of interest: motor and cingulate cortices, glomerular layer of the olfactory bulb, dysgranular insular cortex, primary and secondary somatosensory cortices, piriform cortex, and hippocampal CA1, CA2 and CA3 regions (Paxinos and Watson, 2005). Sections were cut at 35 μm on a rapid sectioning cryostat (Leica, CM1900; Meyer Instruments, Inc., Houston, TX, USA). Free-floating sections were treated with a 1:50 mix of 30% H_2O_2 and methanol for 30 minutes, and then incubated in a blocking solution of 5% bovine serum albumin for 20 minutes. Sections were then incubated in a rabbit anti-rat NgR monoclonal antibody (1:100; Abcam, Cambridge, UK) overnight at 4°C. Negative control sections were incubated with 0.01 M PBS instead of primary antibody. Sections were rinsed thoroughly in PBS and incubated in mouse anti-rabbit IgG (1:100; Boster, Wuhan, Hubei Province, China) at room temperature for 20 minutes. After a further PBS rinse, sections were incubated in streptavidin-biotin complex for 20 minutes, and rinsed again in PBS, and the proteins were visualized using 3,3′-diaminobenzidine (Boster). Finally, sections were mounted from PBS onto slides and coverslipped with neutral balsam aqueous mounting medium. NgR expression was analyzed using the HMIAS-2000 system (Champion Image, Wuhan, China).

Statistical analysis

Data are expressed as the mean ± SD and were compared by one-way analysis of variance (SPSS 15.0, SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Behavioral changes in rat models of focal cerebral ischemia

Twenty-four hours after surgery, control and sham-operated rats showed normal behavior with no observable deficits, whereas rats in the ischemia group presented with various behavioral changes. About 60% (38 rats) of the ischemia group consistently flexed the forelimb contralateral to the injured hemisphere (grade 1); 35% (22) had forelimb flexion with decreased resistance to lateral push (grade 2); and 5% (4) showed the above symptoms in addition to circling behavior (grade 3).

Neuronal morphology in rat brain after focal cerebral ischemia

Nissl staining (**Figure 1**) revealed intact neurons in all non-affected brain regions, such as the motor cortex, in the ischemia group, and throughout the brain in naïve controls and sham-operated rats, with no change over time in neuronal morphology or number. However, in the infarct zone of rats in the ischemia group, the shape and quantity of neurons changed. In the center of the infarct zone, some neurons had

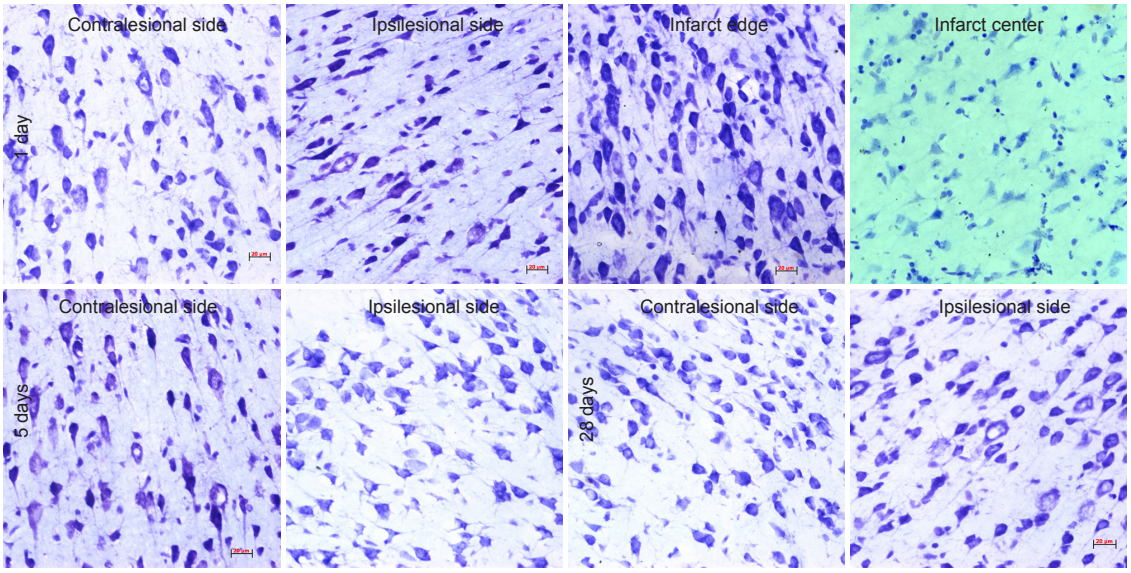

Figure 1 Neuronal morphology in rat brain after focal cerebral ischemia (Nissl staining, × 400).
Major neuronal loss was observed in the central region. Dark and intensive staining was observed at the edge of the infarct zone.

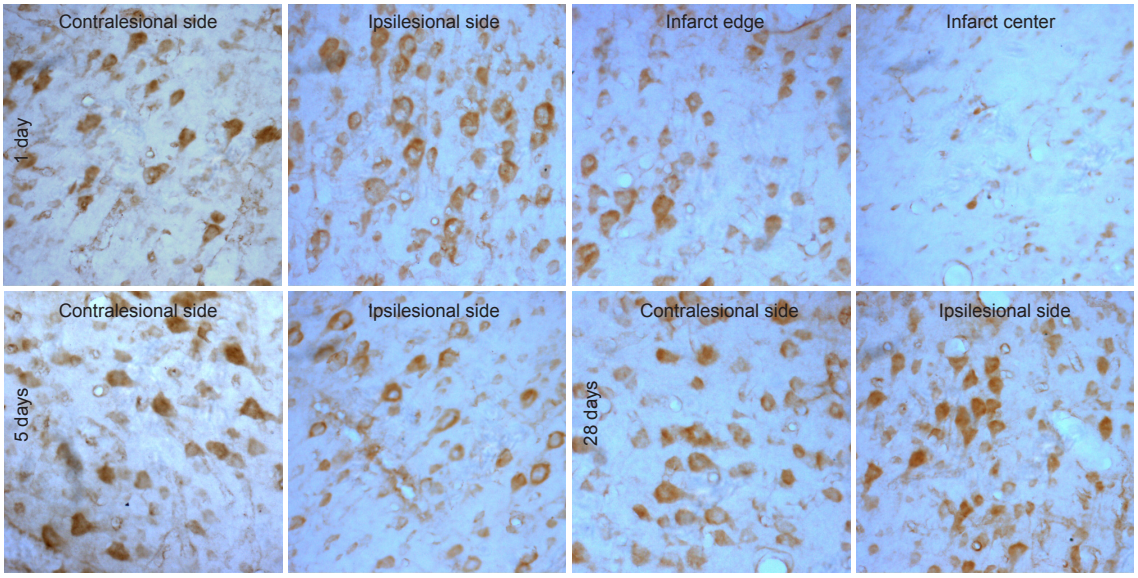

Figure 2 NgR expression in rat brain after focal cerebral ischemia (immunohistochemical staining, × 400).
NgR was not expressed in the infarct center, but there was an increase in NgR expression at the edge of the infarct zone. NgR expression was higher on the ipsilesional side than on the contralesional side. NgR-positive cells appeared brown. NgR: Nogo-66 receptor.

disappeared, and at its edge neurons showed dark staining.

NgR expression in rat brain after focal cerebral ischemia

There was no significant difference in NgR expression between control and sham-operated rats. However, in the ischemia group, brain NgR expression changed throughout the observation period. After middle cerebral artery occlusion, the level of NgR expression in brain tissues showed two peaks; the first appeared 1–3 days postoperatively, and the second at 28 days. Interestingly, 5 days after surgery, NgR expression decreased to below control levels. At the end of the observation period, 56 days after surgery, NgR expression was slightly lower than at day 28, but still higher than at day 1. This pattern was evident in most cortical areas. NgR expression was observed in the marginal zone but not in the center of the lesion. In most cortical regions, NgR expression was greater in the ipsilesional hemisphere than in the control side (**Figures 2**, **3**).

Discussion

Among the ligands of NgR, Nogo-A has been suggested to play an important role in limiting axonal growth (Wang et al., 2012). Many recent studies have focused on the expression and distribution of the NgR, and on the mechanisms underlying the role of the NgR and its ligands in neurite inhibition (Xiao et al., 2012; Benneter et al., 2014; Kumari and Thakur,

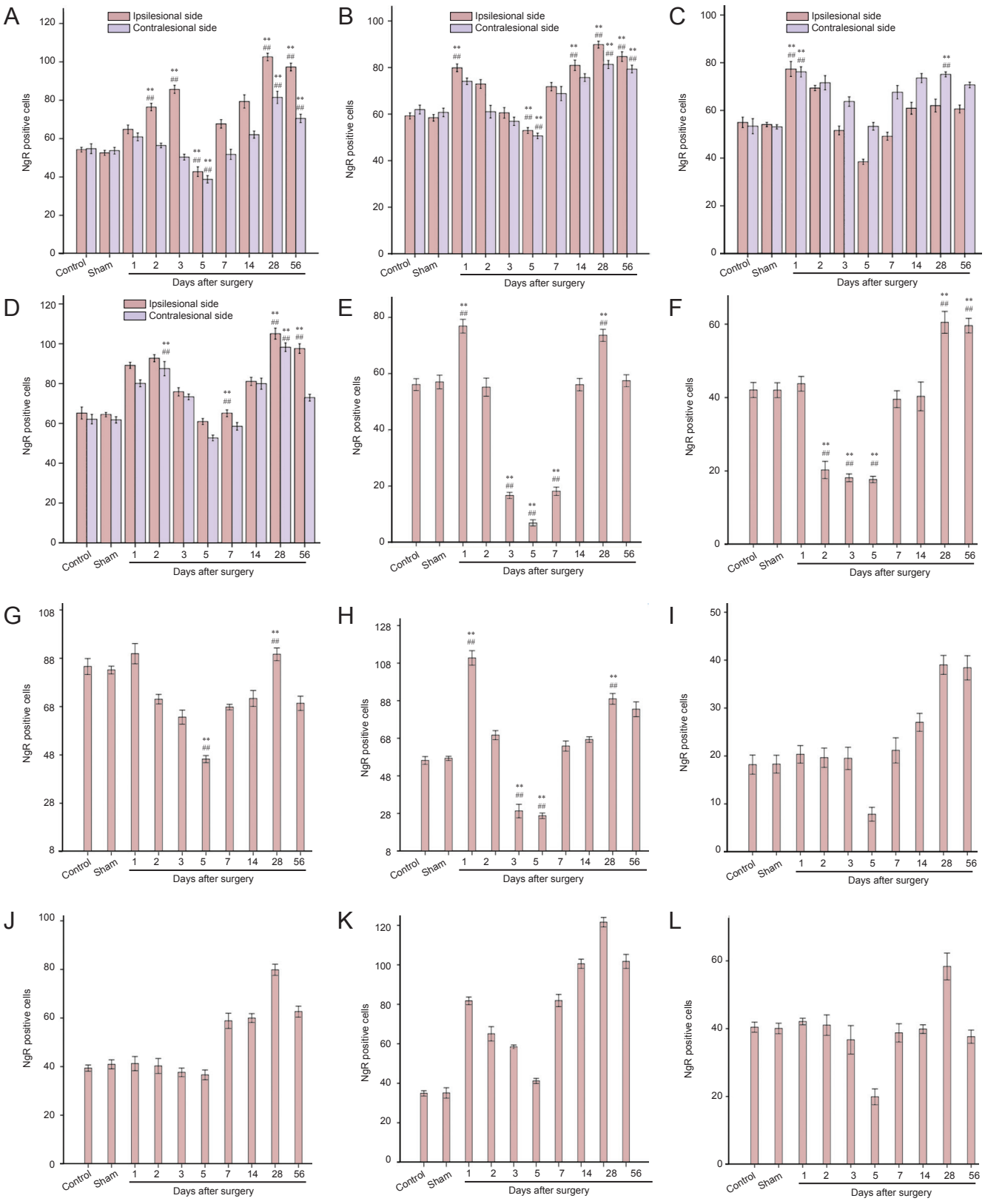

Figure 3 NgR expression in different brain regions in a rat model of focal cerebral ischemia.
Number of NgR-immunoreactive cells per 400× field of vision in the motor cortex (A), hippocampal CA1 (B), CA2 (C) and CA3 (D) on the ipsi- and contralesional sides, and in the cingulate cortex (E), glomerular layer of the olfactory bulb/dysgranular insular cortex (F), piriform cortex (G), primary/secondary somatosensory cortices (H), cingulum (cg) (I), AuDAu1AuV (J), RSGb (K) and Cpu (L) on the contralesional side. Data are expressed as the mean ± SD and compared using one-way analysis of variance. $^{**}P < 0.01$, *vs.* control group; $^{\#\#}P < 0.01$, *vs.* sham-surgery (sham) group. NgR: Nogo-66 receptor. AuD/Au1/AuV: Au1 primary auditory cortex/AuD secondary auditory cortex/AuV secondary auditory cortex; RSGb: retrosplenial granular cortex, b region; Cpu: caudate putamen (striatum).

2014; Pula et al., 2014; Sepe et al., 2014; Zagrebelsky and Korte, 2014). Wang et al. (2002b) showed that Nogo-A and NgR proteins are coexpressed in the healthy adult mouse brain, both widely distributed in the nervous system, across several layers of the cerebral cortex, in the caudate putamen, and in pyramidal and granule cells of the hippocampus. Our present findings are consistent with these observations. Furthermore, Nogo-A expression was reported to be elevated 28 days after stroke in all cortical areas (Cheatwood et al., 2008), also consistent with our findings. Together, these results indicate that the protein expression of both the receptor and its ligand peaked at the same time. They also suggest that NgR and Nogo-A are involved in axonal remodeling after central nervous system injuries such as ischemic stroke.

Several studies described have the distribution of NgR protein in the nervous system. Shi et al. (2008) explored dynamic changes in NgR expression in the hippocampus after chronic cerebral ischemia in rats. NgR protein expression peaked 30 days after permanent occlusion of bilateral common carotid arteries. However, another group showed that although NgR and Nogo-A mRNA expression peaked after 4 weeks of focal cerebral infarction, by 8 weeks, Nogo-A expression had decreased and was not significantly different from that in the control group, whereas NgR had only slightly decreased and remained higher than that in the control group (Ge et al., 2007).

Nevertheless, reports of NgR protein and mRNA expression are generally consistent across studies. Slight differences may arise from the models and/or analytical methods used. In the present study, we sought to identify the time course of NgR expression in more detail, selecting eight time points between 1 and 56 days after middle cerebral artery occlusion. The highest level of NgR expression appeared 28 days after surgery, not at 30 days as previously described, and the lowest level occurred at 5, not 3, days. This minimum point may offer a good therapeutic window. There is also a peak between 1 and 3 days, which is lower than that at 28 days, but higher than the levels observed in naïve control rats.

In conclusion, we have provided an integrated outline of NgR protein expression in middle cerebral artery occlusion models. The dynamic state of NgR mRNA expression over time after focal cerebral ischemia warrants further investigation.

Author contributions: *YC provided data and ensured the integrity of the data, participated in study conception and design. YXD, JX, GLC and ZHY were in charge of paper authorization, provided technical and data supports, and served as a principle investigator. YC and YML ensured the integrity of the data, participated in statistical analysis and wrote the manuscript. All authors approved the final version of the paper.*
Conflicts of interest: *None declared.*
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