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Local Nogo-66 administration reduces neuropathic pain after sciatic nerve transection in rat

Li Lihong [a], Qin Huaizhou [a], Shi Weiqi [b], Gao Guodong [a,*]

[a] *Department of Neurosurgery, Tangdu Hospital, Fourth Military Medical University, China*
[b] *Department of Craniofacial Traumas and Plastic Surgery, School of Stomatology, Fourth Military Medical University, China*

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Abstract

Neuropathic pain after periphery nerve injury is frequently accompanied by the regeneration of the injured nerve fibers. We tested in this study whether local administration of Nogo-66, a well-studied axon growth inhibiting peptide in the central nerve system, could reduce the pain related behavior after sciatic nerve transection in rat. Nogo-66 peptide was purified as a GST fusion protein. Its inhibitory function was testified by neurite outgrowth assay of primary cultured neurons, and then it was given directly at the lesion site by a minipump for 2 weeks. Mechanical nociceptive withdrawal responses and heat hyperalgesia responses were assessed during a 4-week period, and autotomy was evaluated during a 6-week period. The results showed that the mechanical allodynia and heat hyperalgesia scores of the rats treated with GST-Nogo-66 were significantly higher than the controls between 7 and 14 days after sciatic nerve transection. The autotomy scores in the GST-Nogo-66 group were significantly lower than the controls from 28 days after surgery. Taken together, the results of our present study suggest that Nogo-66 may be utilized to decrease the neuropathic pain after periphery nerve injury.

Keywords: Nogo-66; Neuropathic pain; Regeneration; Sciatic nerve transection

Up to date, the neuropathic pain related to periphery nerve injury is still a formidable issue for clinic treatment. This annoying pain is frequently accompanied by the regeneration of the injured nerve fibers. The increased excitability of the sprouting fibers changed the electrophysiological property of the upper neurons, thus leading to the development of pain [19]. Researches have been conducted to explore the possible roles of molecules involved in the periphery nerve regeneration in the injury-associated pain. Neurotrophins, which are highly expressed by the Schwann cells after periphery nerve injury and important for the outgrowth of the injured axon, have been demonstrated to be also the important mediators and modulators of the neuropathic pain [5,13]. For example, the expression of nerve growth factor (NGF) is positively related to the periphery nerve injury induced neuropathic pain, and anti-NGF antibody can decrease the painful behavior [15].

On the contrary to PNS, the central nerve system (CNS) is known for its inability to regenerate after injury. One important reason for this difference is that the oligodendrocytes of CNS express plenty of axon growth inhibitors [2]. Nogo-A is such an oligodendrocyte-derived axon growth inhibitor in the CNS, and its major inhibitory domain Nogo-66, a 66-amino extracellular loop of Nogo-A, has been well studied in the regeneration of CNS [1,4,12]. Schwann cell, the counterpart of oligodendrocyte in the periphery nerve does not express Nogo-A in the normal condition. Transgenic mice with Schwann cell expressing Nogo-A showed impaired sciatic nerve regeneration after injury [14]. Nogo-C, an isoform of Nogo-A, which beard the conserved inhibitory domain of Nogo-66, could also delay the regeneration of the injured sciatic nerve when ectopically expressed by Schwann cell [6]. Taken together, these facts raise a hypothesis that whether the axon growth inhibitors, like the neurotrophins, could play some roles in the injury associated neuropathic pain.

The sciatic nerve transection is a good model to study both the regeneration and the neuropathic pain [18]. Both the regeneration process of sciatic nerve and the pain related behavior after sciatic nerve transection have been well studied [8,11]. In the present study, we explored the possible effects of Nogo-66, the conserved inhibitory domain of Nogo-A and Nogo-C on the neuropathic hyperalgesia and autotomy behavior of rat in the

* Corresponding author. Tel.: +86 29 84777435; fax: +86 29 84777435.
E-mail address: gaogd2007@163.com (G. Gao).

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sciatic nerve transection model, showing a novel pain reducing effects of Nogo-66 after sciatic nerve transection.

Nogo-66 gene was cloned in the pGEX-4T-2 vector as molecular cloning described. The plasmid was transfected into the DH5α strain of *E. coli* and induced to express by 0.5 mM IPTG for 4 h at 37 °C. The bacterial was then collected and ultrasonically lysed. The precipitate was washed three times with 0.5% Triton X-100, and the remaining inclusion bodies were further lysed by the denaturing solution which contained 4 M urea. The denatured protein was then refolded overnight at 4 °C in refolding solution containing 2 M urea. The refolding solution was exchanged out gradually by dialysis until the refolded protein was in PBS, which was then purified through the Glutathione Sepharose™ 4B column and confirmed by Western-blotting using antibody against GST. Purified GST-Nogo-66 and GST protein were diluted to the concentration of 50 μg/ml to coat the same 35 mm cell culture dishes in half, respectively. The cerebellum granular cells from 7 days old rats were cultured on the dishes pre-coated with GST-Nogo-66 and GST protein in Neurobasal medium plus B27. Axon growth was observed under a phase-contrast microscopy 24 h later.

The following experiment was conducted in accordance with the guidelines of the International Association for the Study of Pain. In this study, surgical preparation and experimental protocols were approved to the Animal Care and Use Committee of the Fourth Military Medical University. Adult male SD rats (240–260 g) were housed in the experimental animal laboratory of our university and had unlimited access to food and water throughout the duration of the experiments. Under ketamine anesthesia (1–2 mg subcutaneously), all rats had right common peroneal nerve (CPN) scalpel transection, 10 mm distal to the bifurcation of the sciatic nerve following mobilization of the nerve. A 12-mm segment of the CPN distal to the transection was then removed. Animals were divided as follows with each group consisting six rats, (1) sciatic nerve transection without any treatment, (2) transection with GST treatment and (3) transection with GST-Nogo-66 treatment. An osmotic minipump (delivering 0.5 μl/h for 14 days, Alzet Model; Alza, CA, USA) was implanted subcutaneously in the back, and connected by a silicon tube. The other end of the tube (Silascon; Kaneka Medix, Osaka, Japan) was placed along the proximal side of the right sciatic nerve transection site. GST-Nogo-66 and GST protein were infused for 14 days at 1 μg per day into the transection site via the silicon tube.

The mechanical nociceptive withdrawal response was measured by the application of calibrated von Frey fibers (North Coast Medical, San Jose, CA) over the medial dorsum of the hind paw. Each fiber was tested three consecutive times, pushing down on the hind paw until the rat withdrew its paw or the fiber bowed. Four different fibers were used in graduating sequence (10, 23, 57 and 85 g), for a total of 12 consecutive fiber applications. The withdrawal threshold was the smallest fiber that evoked at least two withdrawal responses during three consecutive applications with the same fiber [7,9].

Heat nociceptive thresholds were determined from the mean of three consecutive withdrawal thresholds to a Peltier device (4.4 cm surface, CP1: 4-127-06L, Melcor, Trenton, NJ) applied to the hind paw. A linearly ramped temperature (1 °C/s, starting at 40 °C and with a cut-off of 52 °C) was used as previously described [7]. The examiner controlled the Peltier using a foot pedal switch. Heat thresholds were tested over the medial dorsum surface of the hind paw.

Animals were tested weekly, and before baseline measurements were taken the animals were trained with two sessions of Peltier and von Frey testing. The testing procedure always followed the same sequence, first measuring the von Frey thresholds, and then the Peltier testing. The testing room was dimly lit and the room temperature was maintained between 24 and 26 °C. The rats were gently held during the nociceptive testing and the test was performed only when the rats were quietly resting in the investigator's hand. The investigator performing the measurements was blinded to the treatment.

Autotomy behavior following sciatic nerve transection was measured weekly, using the method described by Wall et al. [20]. A score of 1 was given for the removal of one or more nails. An additional score of 1 was added for each distal half-digit attacked. A further score of 1 was added for each proximal half digit attacked. Thus, if all nails and all parts of all toes were attacked in one hind paw, a maximal score of 11 would be achieved. During this investigation autotomy behavior was only observed in the insensate digits. At the end of the experiment, the sciatic nerves were removed and the diameter of the neuroma measured under a microscopy after removing the surrounding fibrous tissue.

All the studies were repeated three times. All the behavioral data was analyzed by SPSS software. A repeated-measures analysis of variance (ANOVA) was performed for each test date, comparing treatment groups, where the repeated measure was time. A Fisher PLSD test was used to determine the source of differences among groups. A Wilcoxon signed-ranks test was used to compare the source of differences for the von Frey fiber thresholds. All data are presented as the mean ± S.D. and differences are considered significant at a P-value less than 0.05.

The purity of GST-Nogo-66 was checked by Western-blotting using anti-GST monoclonal antibody. The result showed a single band of about 32 kDa for GST-Nogo-66, comparing with a 26 kDa of purified GST protein (Fig. 1A). The cerebellum

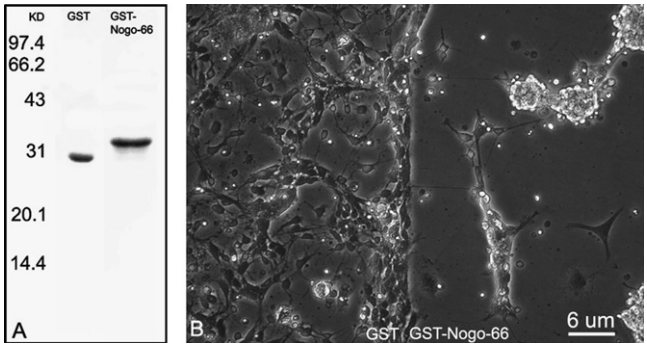

Fig. 1. The purity and function of GST-Nogo-66 in vitro. (A) Western-blotting. Lane 1, purified GST protein. Lane 2, purified GST-Nogo-66 protein. (B) Axon growth inhibition assay of cerebellum neuron. Neurons on the side of the dish coated by GST-Nogo-66 had fewer and shorter neuritis than on the side coated with GST.

granular neuron was cultured on dishes coated with the purified GST-Nogo-66 and GST in half to testify its inhibitory effects on axon growth. Twenty-four hours after cell seeding, observation was performed under a phase-contrast microscopy. Only a few neurons with poorly developed neuritis were found on the side of dish coated with GST-Nogo-66, while the neurons with multiple neuritis grew well on the other side of the same dish coated with GST (Fig. 1B). Forty-eight hours after cell seeding, most of the neuritis on the GST-Nogo-66 retracted to the cell bodies, while the neuritis grew longer on the GST protein (data not shown). No cell detachment was found during this period.

After demonstrating the axon growth inhibition effects of the GST-Nogo-66, we delivered the peptide through a minipump directly into the proximal end of the injured sciatic nerve to observe its effects on the pain behavior of rat. Three days after surgery, the mechanical withdrawal thresholds in all three groups remained unchanged in comparison with the baselines before surgery. From 7 days after surgery, a significant decrease in mechanical withdrawal thresholds was observed in all three groups. No significant difference in withdrawal responses existed between the normal control and GST treated group at all the time points after surgery ($P > 0.05$). Between 7 and 14 days after surgery, the withdrawal thresholds of the GST-Nogo-66 group were significantly higher than those of the control groups ($P < 0.05$). From 21 days after injury, the withdrawal thresholds in the GST-Nogo-66 group dropped gradually to the similar levels of the control groups (Fig. 2).

On the profile of heat thresholds, rats treated with GST protein showed similar thresholds at all time points with the normal control, which increased at 3 days and decreased markedly from 7 days after surgery and lasted to the end of observation. In comparison with the two control groups, the heat thresholds in the GST-Nogo-66 group increased significantly from 7 to 14 days after surgery ($P < 0.05$), and then reduced gradually to the similar levels of the control groups (Fig. 3).

Obvious and similar autotomy was observed from 28 days after surgery in the control groups. The scores of the GST-Nogo-66 group were lower by about two points than those of the control groups at the 28, 35, and 42 days after surgery (Fig. 4). Because autotomy was thought to be associated with the formation of neuroma [21], which was formed mainly by improperly and irregularly regenerating nerve fibers, we compared the diameter of the end of sciatic nerve section where neuroma formed in most cases. The results showed that the diameter of the neuroma in the GST-Nogo-66 group was 0.43 ± 0.11 mm, significantly shorter than the diameter of both the GST and normal control groups (0.72 ± 0.13 and 0.70 ± 0.14 mm, respectively, $P < 0.01$) (Fig. 5).

To explore whether Nogo-66, an axon growth inhibiting peptide in the CNS had some roles in the neuropathic pain after periphery nerve injury, we expressed GST-Nogo-66 protein by *E. coli*. GST-Nogo-66 was expressed in the form of inclusion body, a common problem for researchers to get functional protein [17]. We used a moderate concentration of urea to resolve the inclusion body, thus ensuring more protein in their natural structure and easy to be refolded. The single band of GST-Nogo-66 in Western-blot suggested that we got the right purified protein. Through the neurite outgrowth assay we demonstrated that the

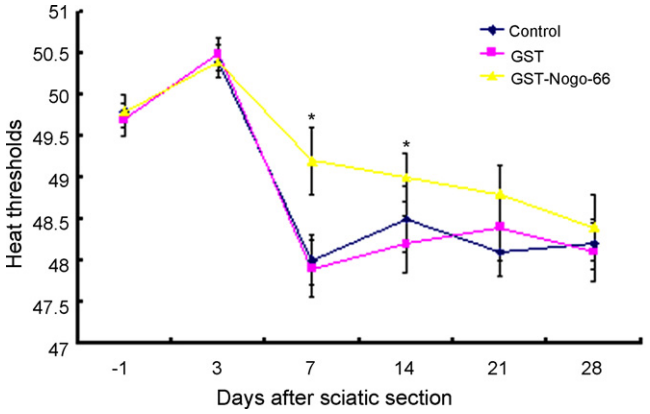

Fig. 3. Effects of Nogo-66 on the heat thresholds of rat after sciatic nerve transection. The thresholds of the two control groups were similar at all time points, while the thresholds in the GST-Nogo-66 treated group were higher than the control groups between 7 and 14 days after surgery (* $P < 0.05$).

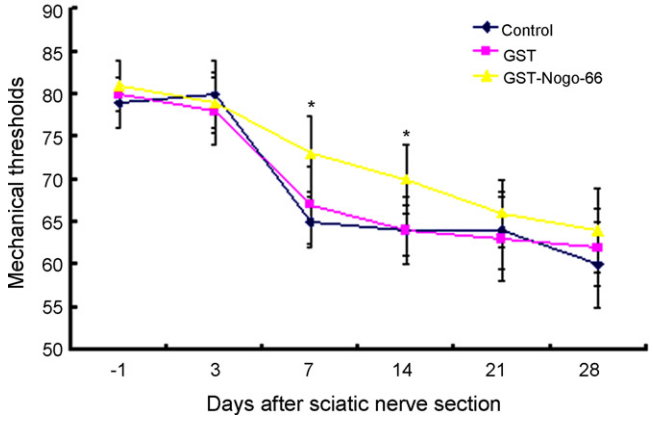

Fig. 2. Effects of Nogo-66 on the mechanical thresholds of rat after sciatic nerve transection. The thresholds of the two control groups were similar at all time points, while the thresholds in the GST-Nogo-66 group were significantly higher than the control groups between 7 and 14 days after surgery (* $P < 0.05$).

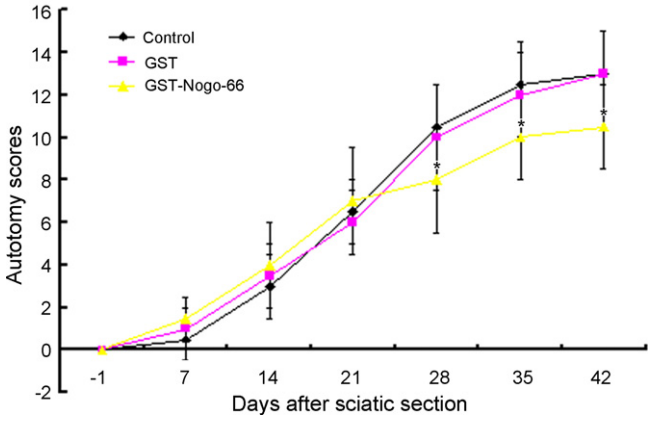

Fig. 4. Effects of Nogo-66 on the autotomy behavior of rat after sciatic nerve transection. The thresholds of the two control groups were similar at all time points, while the thresholds in the GST-Nogo-66 group were lower than the control groups from 28 days after surgery to the end of experiment (* $P < 0.05$).

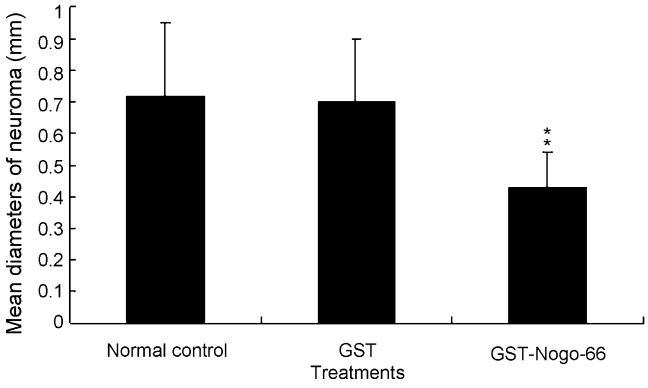

Fig. 5. Effects of Nogo-66 on the diameter of neuroma. The mean diameter of neuroma in the Nogo-66 group was significantly smaller than in the control groups (** $P < 0.01$).

GST-Nogo-66 protein got by this way was functional effective and qualified for further functional study.

The behavior results of present study showed that, in comparison with the GST and normal control, GST-Nogo-66 reduced the threshold of mechanical allodynia and thermal hyperalgesia between 7 and 14 days after sciatic nerve section and decreased the autotomy from 28 days after sciatic nerve section. This temporary pain reduction indicated Nogo-66 could delay the upset of pain rather than alleviate the severity of pain. The underlying mechanism remains to be further studied. One possibility is that the pain reducing effects of Nogo-66 may be due to its axon growth inhibition effects. The sprouting axons are highly excitable and pain initiating. Nogo-66 decreased the size of neuroma, which was formed mainly by improperly regenerating nerve fibers. In addition, transgenic mice with Schwann cell expressing Nogo-A and Nogo-C, both containing Nogo-66, showed an abolishment of the sciatic nerve regeneration [14,6]. Similar pain reducing effects have also been reported for another axon growth repelling factor, Semaphorin 3A, which could inhibit the NGF induced nociceptive fibers sprouting in adult spinal cord [16]. We do not role out other explanations for the pain reducing effects of Nogo-66 in the periphery nerve injury model. The receptor for Nogo-66 was reportedly expressed by the activated macrophages in the lesion site of sciatic nerve injury to mediate the clearance of macrophage from the lesion site [3]. Depletion of macrophage from the lesion site could reduce the neuropathic pain after sciatic nerve injury [10]. Therefore, it is possible that local Nogo-66 administration might promote the migration of macrophage from the lesion site, thus reducing the pain behavior. The detailed mechanism for our observation remains to be further explored.

In short, our study, directly through the observation of painful behavior, showed a novel and beneficial utilization of Nogo-66 in the periphery nerve injury, indicating a new strategy for reducing the injury related pain in the PNS.

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