Immunofluorescence laser confocal expression and localization study of rat nerve growth guidance cues Netrin-1 and Slit2 after spinal cord injury

Objective: To observe the expression and distribution of adult rat axon guidance cues Netrin-1 and Slit2 at different time points after spinal cord injury and to investigate the guidance mechanism of regenerated axons.

Methods: Twenty adult Sprague Dawley (SD) rats were divided randomly into five groups with 4 in each. Four groups of them were used to make Allen’s spinal cord punch models and we took materials randomly from one of them on the 2nd, 4th, 7th and 14th day respectively after operation. The left one group was taken as the control group. Immunofluorescence laser confocal scan was used to examine the co-expression and localization of Netrin-1 and Slit2 proteins in the injured site of the spinal cord.

Results: Within two weeks after SCI, the expression of Netrin-1 and Slit2 proteins increased temporarily and there was co-expression of them on the neuron plasma membrane.

Conclusions: Synchronous high expression and co-expression of axon attractant Netrin-1 and repellent Slit2 are found in the adult rat injured spinal cord in the damaged local and vicinity parts, and probably, they act as the key regulators of axon guidance regeneration.

Key words: Axons; Spinal cord injuries; Regeneration; Co-expression

METHODS

Antibodies and reagents
Rabbit anti rat Slit2 polyclonal antibody (purchased from Sigma, USA), goat anti rat Netrin-1 polyclonal antibody (purchased from Santa Cruz, USA), goat anti rabbit and goat anti rat fluorescent double antibody (purchased from Sigma, USA) were employed in this study. The other analytical reagents were all provided by the Molecular Biological Laboratory of Nanjing Medical University, Nanjing, China.

Animal models of SCI and grouping
Twenty clean adult Sprague Dawley (SD) rats without gender limitation (provided by Laboratory of Nanjing Medical University), weighing 200-250 g, were divided randomly into five groups with 4 in each. One group was taken as the blank controls. The other four groups were operated by cutting the vertebral plate T11 with 1 cm spinal cord exposed. Modified Allen’s method was used as animal model. That is to say, one piece of 2.5 mm×3.5 mm plastic pad was placed on the spinal membrane surface. And a steel stick weighing 10 g
and with 2.5 mm in diameter along a glass tube was used to punch the spinal cord with a force of 100 g/cm² till it paralyzed generally. One group was chosen randomly on 2nd, 4th, 7th and 14th day respectively after operation and was perfused with 4% paraformaldehyde. After that, we took the injured part, which was 2 mm far from the injury center, to make 5-µm frozen sections and then let them float in PBS solution to be ready for further experiment.

**Immunofluorescence double-labeled laser confocal scan**

The floating SCI sections were washed twice with pre-cooled PBS solution. Then they were immersed into pre-cooled 4% paraformaldehyde and fixed for 1.5 hours. They were washed for three times with PBS solution for 5 minutes each time. Rupture of membrane was performed for 15 minutes with PBS containing 0.3% Triton X-100. And 3% BSA (bovine serum albumin, PBS diluted) was used to block out for 1 hour at room temperature. Then the blocking solution was drained and 200 µl primary antibody in 1:50 dilution ratio (the diluent of primary antibody was TBS, which contained 3% BSA and 0.02% NaN₃, pH=7.2) was used. It was incubated at 37°C for 1 hour. PBS (containing 0.3% Triton X-100) was used to wash it for three times for 5 minutes each time. Then fluorescein-labeled secondary antibody (1%BSA-PBS 1:1000 diluted) for 200 µl was added and it was incubated at 37°C for 45 minutes. Then it was washed with PBS for three times, 5 minutes each time. Third antibody (rabbit anti rat slit2 polyclonal antibody) for 200 µl (dilution ratio was 1:1000) were added and the above incubation and PBS washing procedures were repeated. We used phenylisothiocyanate (PITC) to label Netrin-1 and tetraethyl rhodamine isothiocyanate (TRITC) to label Slt2. Glycerin was used to seal the sections. And different fluorescence distributions were observed under an inverse fluorescence microscope (×400).

Laser confocal scanning system, Biorad Radiance Plus (Biorad), was used to examine the localization of merged proteins in the cells. EGFR (epidermal growth factor receptor) was stimulated by argon and krypton laser and treated by 570 nm filter.

**Statistical analysis**

We used Image-Pro Plus software (USA) to analyze the immunofluorescence laser co-focusing images. The obtained data were expressed as mean ± SD. Student’s t test was used for inter-group comparison and P < 0.05 was considered statistically significant.

**RESULTS**

**General status**

No animal died during the entire experiment. There were totally 100 experimental sections. We observed them in three visual fields, i.e., anterior horn, posterior horn and myelocoele of the spinal cord. In all the visual fields, fluorescence intensity met the requirements of statistical analysis.

**Image analysis**

Only low expression (with faint fluorescence spots) of Netrin-1 and Slt2 was observed in the control group (Fig. 1. A-C and Fig. 2. A-C). Distinct expression of Netrin-1 and Slt2 was observed in the injured part on the 2nd day of SCI. Large range of green fluorescence spots and few sporadic red fluorescence were observed in the three visual fields under the microscope (Fig. 1. D-F and Fig. 2. D-F). The expression of Netrin-1 and Slt2 was enhanced obviously on the 4th day and the expression range and the enhanced amplitude of the former were wider and bigger than the latter. The co-expression of both was observed obviously on the neuron plasma membrane. Netrin-1 was distributed equally on different parts, however, Slt2 was distributed unequally, whose expression at the anterior horn was much more distinct than that of the posterior horn (Fig. 1. G-I and Fig. 2. G-I). On the 7th day, the expression of Netrin-1 continued to keep high and the expression of Slt2 also reached peak value. Confocal of Netrin-1 and Slt2 could be observed widely under the microscope and they emitted round orange fluorescence on the neuron plasma membrane. The expression of Slt2 on the posterior horn of the spinal cord also increased obviously (Fig. 2. J-L). After two weeks, the expression of both started to faint with Slt2 more distinct than Netrin-1 (Fig. 2. M-O). The analysis results of fluorescence images indicated that the fluorescence intensity showed significant difference between the control group and any
experimental group \((P<0.05)\). The fluorescence intensities also showed significant difference between different groups at the same time point \((P<0.05)\), which indicated that the expression of Netrin-1 and Slit2 had time-dependent relationship. Netrin-1 fluorescence intensities on different parts of the injured spinal cord at the same time point had no significant difference \((P>0.05)\), however, the fluorescence intensity of Slit2 on the anterior horn was stronger than those in the myelocoele and posterior horn \((P<0.05, \text{Table 1})\).

Fig.1. Double-labeled laser confocal scan images of Netrin-1 and Slit2 in different parts of injured spinal cord at different time points after SCI \((\times 400)\). On the 2nd -14th days, the expression of Netrin-1 and Slit2 at the anterior horn \((D, G, J, M)\), myelocoele \((E, H, K, N)\) and posterior horn of the injured spinal cord \((F, I, L, O)\) is temporarily increased. They reached peak on the 7th day. Arrow indicates the co-expression of both on the neuron plasma membrane and the orange fluorescence that they emitted surrounding the plasma membrane. A-C show low intensity expression of Netrin-1 and Slit2 in normal control group; D-F show a bit enhanced expression of Netrin-1 and Slit2 2 days after SCI; G-I show visibly enhanced expression of Netrin-1 and Slit2 4 days after SCI (Netrin-1 is equally distributed on different parts, while Slit2 is distributed unequally, whose expression at the anterior horn is much more distinct than that at the posterior horn); J-L show continuous higher expression of Netrin-1 and peak value of the expression of Slit2 7 days after SCI; and M-O show fainted expression of Netrin-1 and Slit2, but Slit2 more distinct than Netrin-1 14 days after SCI.

Fig.2. Immunofluorescence and double-labeled confocal scan images of Netrin-1 and Slit2 in different parts of injured spinal cord 7 days after SCI (FITC-labeled Netrin-1 and TRITC-labeled Slit2, \(\times 200\)). A-C: Anterior horn of spinal cord; D-F: Myelocoele and its surrounding; G-I: Posterior horn of spinal cord (arrow indicates the co-focusing of Netrin-1 and Slit2 on the neuron plasma membrane).

The experiment revealed that there was gradually enhanced synchronous high expression and co-expression of neuroaxon attractant Netrin-1-1 and repellent Slit2 on the injured spinal cord of adult rats, which reached peak value at 7 days and subsequently trailed off like parabola. Netrin-1-1 boosted up relatively early and declined relatively late, while Slit2 boosted up relatively late and declined relatively early, for about 1 day late. Netrin-1 distributed in equilibria, while Slit2 did not distribute in equality, with the former corner expressing more visibly than the back corner. At 2 days after SCI, both Netrin-1 and Slit2 expressed in gliocytes, Netrin-1 expressed in microglia, and Slit2 expressed in astrocytes. At 4 days after spinal cord injury, Netrin-1 and Slit2 expressed in the cell bodies of neurons and in the growth cone in the cacumen of axons, and they reached peak value at 7 days. Netrin-1 distributed in equilibria, while Slit2 appeared more in motor neurons in the former corner. Faintly hemorrhage occurred in the cinerea after 1 day of SCI, fragmentation in the center sections, axon rupture and demyelination were also found. Neuroma and nervous gray inanition occurred gradually, and gliocytes decreased at 3 days after SCI. Neuron decrease and gliocyte hyperplasia were found at 5-7 days. The staining of Nissl body in cytolymph of
neurons liked tiger speckle, which showed that physiological function gradually came back at 14 days after SCI. Regeneration process of axons was consistent with the synchronous high expression of Netrin-1 and Slit2, which showed that Netrin-1 and Slit2 promoted axon regeneration and chose the projectile precise direction through integration of action in attractant and repulsion.

**DISCUSSION**

Axon regeneration after SCI is a complicated molecular affair, in which many kinds of cells and chemical factors participate and they react on each other and mix together at different time points and change variably along with the time. Experiments in recent years have proven that many nerve nutrition factors such as NGF, NT-3, bFGF, IGF, BDNF and GDNF (glia cell-derived neurotrophic factor) all had temporary expression and enhanced axon growth in different degrees after SCI. However, the regenerated axons are all very short and cannot build correct connections with the injured far terminals, therefore they often form neuromas. So people consider that the accurate direction function of nerve growth guidance cues is possibly one of the key factors affecting axon regeneration.

The current guidance cues are classified into two types: attractants, i.e., Netrin family, and repellents, which include Slit, Sema and Ephrin families. With collagen gel incubation method in 1988, Tessier-Lavigne et al found that Netrin is a kind of factor secreted by soleplate, which have peptide of 550-800 aa chain and attractive receptors DCC (Deleted colorectal cancer). During the development phase of embryo spinal cord, Netrin highly expresses in the soleplate cells and attracts commissural axons of the neuron in the long distance to grow into the ventral median line. However, when the axon reaches and passes the median line, it loses the sensitivity to Netrin. This indicates that there are some other factors involved in the projection regulation. Kidd et al and Brose et al verified that this factor was Slit and it was also a large molecular protein with molecular weight of 170-190 u, secreted by median line soleplate glial cells during the development phase of embryo spinal cord (at 11-13 days for rat embryo and 23-30 weeks for human being embryo). It causes special short-distance repelling effect to the axons. Its receptor is Roundabout (Robo). Bashaw et al made transgenic experiments to verify that the external cell fields of DCC and Robo were only in charge of recognizing ligands and their internal cell fields decided the growth direction of spinal cord axons. The combination of external cell fields with its respective ligands causes the configuration change of internal cell fields. Then it causes rearrangement of cell framework by pathway of complicated molecular transfer.

This study verified that during the acute phase (within two weeks) of adult rat SCI, the expression of Netrin and Slit proteins increased temporarily and synchronously. We considered that this should be one of the early reactions of astrocyte multiplication. When compared with Slit2, the high expression of Netrin-1 was earlier and wider in the injured spinal cord, which implied the early phase appearance of its attractive effect in the long distance on the grey matter of regenerated axons in the whole scope. However, the following increase of Slit2 expression indicated that the sequent appearance of repelling effect in the short distance was to realize the accurate guidance to different kinds of neuron axons. The unequal distribution of Slit2 at the anterior horn, myelocoele and posterior horn of the injured spinal cord implied that its repelling guidance to the regenerated axons appeared firstly at the motor column of the anterior horn. But 7 days after injury, the enhanced expression of Slit2 at the poste-

**Table 1. Expression and fluorescence intensity of Netrin-1 and Slit2 in anterior horn, posterior horn and myelocoele of spinal cord at different time points after SCI in adult rats (X±s, V)**

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Control</th>
<th>2 d</th>
<th>4 d</th>
<th>7 d</th>
<th>14 d</th>
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<td></td>
<td></td>
<td>Netrin-1</td>
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<td></td>
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<td>Slit2</td>
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<td></td>
<td></td>
<td>Anterior horn</td>
<td>Myelocoele</td>
<td>Posterior horn</td>
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<tr>
<td>Control</td>
<td>4</td>
<td>27.06±5.33</td>
<td>17.11±6.46</td>
<td>77.45±5.54</td>
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<td>9.38±3.58</td>
<td>3.71±4.14</td>
<td>70.46±5.37</td>
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<tr>
<td>2 d</td>
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<td>36.55±3.34</td>
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<td>17.33±5.34</td>
<td>19.56±5.33</td>
<td>77.08±6.14</td>
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<td>4 d</td>
<td>4</td>
<td>76.37±3.26</td>
<td>79.73±5.35</td>
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<td>47.57±2.91</td>
<td>31.26±1.28</td>
<td>70.28±3.39</td>
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<td>7 d</td>
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<td>81.54±2.46</td>
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<td>14 d</td>
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This study verified that during the acute phase (within two weeks) of adult rat SCI, the expression of Netrin and Slit proteins increased temporarily and synchronously. We considered that this should be one of the early reactions of astrocyte multiplication. When compared with Slit2, the high expression of Netrin-1 was earlier and wider in the injured spinal cord, which implied the early phase appearance of its attractive effect in the long distance on the grey matter of regenerated axons in the whole scope. However, the following increase of Slit2 expression indicated that the sequent appearance of repelling effect in the short distance was to realize the accurate guidance to different kinds of neuron axons. The unequal distribution of Slit2 at the anterior horn, myelocoele and posterior horn of the injured spinal cord implied that its repelling guidance to the regenerated axons appeared firstly at the motor column of the anterior horn. But 7 days after injury, the enhanced expression of Slit2 at the poste-
rior horn of spinal cord implied that the repelling effect in this field was enhanced. This may relate to the repelling motive neuron growth at the posterior horn in this phase. The co-expression of Netrin-1 and Slit2 on the neuron plasma membrane verified that both of them could affect the regenerated axon plasma membrane after SCI. So we presumed that during the axon regeneration course, the growing spinal cord needed to obtain attractive and repelling signals from the surrounding microenvironment of projection paths continuously and integrate these signals through the expression of its plasma membrane receptors (DCC or Robo) in order to decide its projection direction.

In the process of this experiment, we observed that the extending pace of the growth cone of axons was consistent with the expression intensity of Netrin-1 and Slit2 over time after SCI. The extending pace was furthest fast at 7 days, subsequently descended, but it did not come into integral axon all the time. So through integration action in attractants and repulsion, Netrin-1 and Slit2 promoted axon regeneration and chose the projectile precise direction. However, neither the expression intensity of Netrin-1 and Slit2 nor the chronergy was sufficient for adjusting and controlling neurons to form axons with integrity synapse, which was the researchful direction ever since.

Then how do Netrin and Slit perform synergistic action? From studies on drosophila, people found that the expression of Robo was regulated and controlled by factor Commissureless (Comm).18 Comm existed in the small vesicles of axon cytoplasm. When the axon reached the median line, it could bind with Robo to form Comm-Robo complex and endocytosed Robo into cytoplasm endosome by encytosis to prohibit the expression of Robo on the growing spinal cord surface. This made Slit lose the opportunity of binding receptors so the axon could pass the median line smoothly. Comm was closed by some unclear mechanism and Robo could express on the axon plasma membrane. Meanwhile CC1 area in Robo plasma coupled with p3 area in DCC plasma to form DCC-Robo heterogeneous dimmer, which made the attraction effect of axon to Netrin produce “resting reaction.”19 In consequence, the axon continued to extend in the opposite gelatinous fiber framework and did not return to the median line or repeat spanning. Although no homologous compounds of Comm were found in vertebrates, the similar mechanism should have existed. From the co-expression of Netrin and Slit on neuron plasma membrane in this experiment, we could presume that the restoration of injured spinal cord after SCI repeated at least part of the above course.

Recently people found that there might be an oligodendroblast network in CNS and these cells could gather in the injured part of the amylination.20 Seven days after SCI, the number of these cells started to increase and at two weeks it started to decrease gradually and maintained for several weeks. New oligodendroglia might be produced and remyelinated to provide supporter for regenerated axons. Combining with our experiment, we can infer that a self restoration course does exist in the spinal cord of mature mammals after SCI. However, when compared with the time needed for restoration of the injured spinal cord, the temporary expression of guidance cues is far adequate. Also along with the further proliferation of astrocytes, gelatine scars form and occupy the hollow space in the spinal cord. They produce some repressive molecules such as Nogo, MAG, CS-PGs, to further repress the self restoration course. Therefore, how to facilitate the continual expression of Netrin and Slit and reform the microenvironment of repressing axon regeneration is likely to be the key of facilitating the restoration of the injured spinal cord.

REFERENCES


