

The Expression implication of GDNF in ventral horn and associated remote cortex in rhesus monkeys with hemisected spinal cord injury

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ABSTRACT

Objective(s): Glial cell line-derived neurotrophic factor (GDNF) can effectively promote axonal regeneration, limit axonal retraction, and produce a statistically significant improvement in motor recovery after spinal cord injury (SCI). However, the role in primate animals with SCI is not fully cognized.

Materials and Methods: 18 healthy juvenile rhesuses were divided randomly into six groups, observed during the periods of 24 hr, 7 days, 14 days, 1 month, 2 months, and 3 months after T11 hemisectioning. The GDNF localization, changes in the injured region, and the remote associate cortex were detected by immunohistochemical staining.

Results: Immunohistochemical staining showed that GDNF was located in the cytoplasm and the neurite of the neurons. Following SCI, the number of GDNF positive neurons in the ventral horn and the caudal part near the lesion area were apparently reduced at detected time points ($P < 0.05$). Moreover, the number in the rostral part of the ventral horn in 7 day, 14 day, and 1 month groups were fewer than those in the caudal part. Importantly, in the contralateral cortex motor area, the positive neurons decreased sharply after hemi-SCI, while gradually increased and went back to normal in 3 months after hemi-SCI.

Conclusion: To sum up, GDNF disruption in neurons occurred after SCI especially in cortex motor area. Intrinsic GDNF in the spinal cord, plays an essential role in neuroplasticity. Thereafter extrinsic GDNF supplementing may be a useful strategy to promote recovery after SCI.

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Introduction

Spinal cord injury (SCI) is the world's most disastrous disease because up to now there was no effective treatment. What's more, SCI often results in critical neurologic dysfunction and disability. SCI affects more than 2.5 million people worldwide, often leading to severe disability (1). The successful treatment in the minutes and hours following injury depends on the timely institution of therapy. The experimental research on SCI repair has already involved molecule, cell, tissue, gene, drug treatment, and so on. Among them, the ability of glial cell line-derived neurotrophic factor (GDNF) to protect neurons after SCI has drawn much attention.

GDNF, a member of neurotrophic factor family, is widely expressed in the nervous system. Human GDNF gene, which is located in the 5P13.1—P13.3 of the chromosome, has been successfully cloned (2). It is produced from skeletal muscles as a target-derived neurotrophic factor and absorbed in the nerve

terminal, which is reversely transported through motoneuron axon by mediate-receptor (3); also it could come from the posterior root ganglion and transported along cornu posterius medullae spinalis through sensory neuronal axon. GDNF has nutritional support effect on motoneuron, sensory neuron, and dopaminergic neuron, GDNF also plays an essential role in neuron growth and axonal regeneration. However, its precise mechanism is still not understood. According to its role in neuroplasticity, many experiments about SCI and GDNF have been performed recently.

An experiment demonstrated that GDNF transcription in the spinal cord which showed GDNF began to increase within 30 min after an acute contusion injury, GDNF immunoreactivity was mainly present in microglia and macrophages 1 day after injury (4). It was demonstrated that olfactory ensheathing cells genetically secrete GDNF to promote spinal cord repair (5). It also has been reported that intrathecal epigallocatechin gallate (6), theophylline

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(7), valproic acid (8, 9), simvastatin (10), interferon- γ (11), and hyperbaric oxygen (12) treatment can significantly promote functional recovery from SCI-induced motor dysfunction in rats, and this neuroprotective effect may be related to the up-regulation of GDNF. SCI treatments with GDNF and Schwann cells have been widely reported, and have concluded that GDNF-enhanced propriospinal axonal regeneration. In one study, Schwann cell myelination after SCI through grafted SC-seeded guidance channels was mediated mainly through a direct effect of GDNF on neurons (13–15). What is more, there is a report suggesting possible analgesic effect of GDNF on neuropathic pain in spinal cord-injured rats (16).

According to GDNF's role reported previously in neuroplasticity in rats, it may act as a therapeutic promise for neurological disorders. But previous studies on SCI treatment mainly focus on rodents, which are different in anatomic structure and physiological function from humans. Moreover, currently, there is no more information regarding the effects of GDNF on SCI repair available, especially in primates.

In this study, by using primate macaque as the research object, we investigated the possible role of GDNF at various time intervals following hemi-SCI, so as to gain insights into the contribution of endogenous GDNF in spinal neuroplasticity.

Materials and Methods

Experimental animals

Healthy juvenile rhesus monkeys aged from 2 to 3 years were obtained from Animal Welfare Committee of Kunming Medical University. The animals were male, weighing 3–5 kg, and randomly divided into six groups. They were separately allowed to acclimatize for 24 hr (control group), 7 days, 14 days, 1 month, 2 months, and 3 months after operation, and sham-operated animals served as controls.

Surgical procedure for spinal cord injury

Rhesus monkeys were anesthetized with ketamine hydrochloride (10 mg/kg, IM). Laminectomies were performed at the T9-T10 levels, leaving the dura intact. All the rhesuses were hemisected at T11 of left spinal cords, then muscles and skin were sutured layer to layer, and the rhesus monkeys placed in warm cages,

food and water were provided *ad libitum*. They were put to death at 24 hr, 7 days, 14 days, 1 month, 2 months, and 3 months after hemi-SCI, respectively. All these procedures were executed in accordance with the Guide for Care and Use of Laboratory Animals published by the National Institute of Health Guide (1996). The animals were housed individually in clear steel cages under standard conditions of humidity and temperature with 12 hr light/dark cycle and given *ad libitum* access to food and water, as approved by the Laboratory Animal Care Assessment Association.

Histological procedures

At 5 mm rostral and caudal to the lesion and contralateral cortex, motor tissue was collected to detect the expression of GDNF using the immunohistochemical SP method (details are shown in the Immunohistochemical procedure section).

Characterization of antibodies

To localize the GDNF protein in the spinal cord, an affinity-purified rabbit polyclonal antibody was used in this study. The specificity of the antibody for GDNF was confirmed by Western blots using rhesus spinal cord homogenates and rat spinal cord homogenates (there are no specific antibodies for rhesus). As little as 10 ng of each neurotrophin can be visualized by just using an appropriate antiserum. To prove the specificity of these antisera, immunostaining was attempted by omitting the primary antibodies or pre-absorbing them with the appropriate immunogen. Detailedly, the spinal cords were harvested from normal rhesuses and rats, and then homogenized on ice in RIPA Lysis Buffer (Beyotime, Jiangsu, China). The productions were washed with 0.1 M PBS and centrifuged at 4°C, 3000 g for 5 min. The supernatants were obtained and stored in the freezer (-80°C). BrBlford was used to assay the protein concentration. 80 μ g of total protein was resolved in 15% SDS-PAGE and then electrophoresed at constant 120 V for 2.5 hr. The proteins were separated and transferred to PVDF membranes at 24 V for 435 min. The membrane was blocked by TBST, containing 5% nonfat milk for 1 hr at room temperature. Then the membrane was rinsed with PBST and incubated with the primary antibody (Table 1 GDNF, rabbit, Santa, 1:1000) for several signals (4°C, overnight). Then the membranes were washed with TBSB 4 times and incubated with secondary antibody ((goat

Table 1. The antibody information of the immunohistochemical staining

Primary antibody	Species	Dilution	Company	Secondary antibody	Dilution	Company
GDNF (D-20)	Rabbit	1:300	Santa Cruz	biotinylated goat-anti-rabbit immunoglobulin G(IgG)	1:200	Vector Labs
NeuN	Mouse	1:100	ZSGB-BIO	biotinylated goat-anti-mouse immunoglobulin G(IgG)	1:200	Vector Labs

anti-rabbit, ZSGB-BIO, 1:5000) (room temperature, 2 hr). At last, the membranes were washed again and developed in Alpha Innotech with ECI.

Tissue preparation

The animals in each group were forced on open heart and perfused with 4% paraformaldehyde in PBS (pH 7.4) after an overdose of ketamine hydrochloride anesthesia. At 5 mm rostral and caudal to the lesion and contralateral cortex including the right contralateral cortex, motor area was removed and postfixed in the same fixative overnight, stored in 20% sucrose in PBS at 4 °C, and embedded in OCT, then frozen and sectioned at 25 µm thickness in a freezing micro-tome (Lecia CM1900, Germany) and one of ten sections was processed for immunohistochemical stain demonstration of GDNF.

Immunohistochemical procedure

Free-floating sections of macaque tissues were washed three times in 0.1 M PBS, 5 min each time. They were incubated at room temperature in 3% hydrogen peroxide for 30 min in the dark box to block the action of any endogenous peroxidase. Next, they were immersed in 0.1 M PBS 15 min containing 0.3% Triton X-100 and 5% normal goat serum at 37°C. Subsequently, the sections were incubated for 48 hr at 4°C in a primary antibody solution containing 2% normal goat serum and 0.3% Triton X-100 (the primary antibody was substituted with 0.1 M PBS in the negative control group at this procedure). Subsequently, three washed in 0.1 M PBS for 5 min each, the sections were incubated with secondary antibody solution for 2 hr at room temperature. Then the sections were incubated with an avidin-biotin-peroxidase reagent (1:300 dilution, ABC Elite; Vector Labs) after washing three times with 0.1 M PBS for 5 min each, next the sections were immersed in the buffer Tris-HCL for 15 min at 37 °C. Subsequently, processed sections were visualized by immersion in DAB staining solution containing 0.04% 3, 3'-diaminobenzidine, 0.06% nickel sulfate and 0.06% hydrogen peroxide for 20 min. At last, they were dehydrated, coverslipped, mounted, and observed under a light microscope (Olympus, Japan). The details of the antibodies are shown in Table 1.

Statistical analysis

The above experimental data were analyzed with SPSS 11.0 software package. One way ANOVA was used to compare the identical spinal cord and right cerebral cortex premotor area, the paired samples T test was used for analyzing data of the left and right side in the spinal cord. The statistical significance was defined as P -value < 0.05.

Results

Specificity of the antibody

The antibodies recognized a single band at approximately 20 KDa representing GDNF in the rhesus Spinal

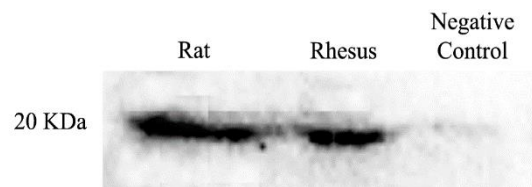


Figure 1. The specificity of the GDNF antibody

The antibody recognized a single band at approximately 20 Kda in rats and rhesuses, which coincided with the molecular weights of the neurotrophic factors in the specification and disappeared in the negative control, representing GDNF in the rhesus spinal cord homogenates

cord homogenates and the rat spinal cord homogenates (Figure 1). Pre-absorption of the corresponding primary antibodies with 0.43 microgram per milliliter rhGDNF abolished the bands in the lanes of rhesus tissue homogenates (Figure 1). These indicated that although there were no specific antibodies for rhesuses, the expression of GDNF in the rhesus spinal cord could be detected especially by using D-20 (Santa Cruz Biotechnology).

GDNF-like immune or microgram activity

Immunopositive neurons with varying intensities were scattered throughout the gray matter of the cortical regions examined, and were relatively intense in layers III and V/VI. Based upon their somatodendritic morphology, GDNF-positive neurons were identified as pyramidal and stellate neurons. Cells observed as pyramidal neurons were characterized by a triangular or elliptical cell body that gave rise to a prominent apical dendrite and a spray of dendrites emanating from the base of their soma. Stellate neurons had dendrites that either radiated from all aspects of the cell body or arose from clusters emanating from the apical and basal somatic poles. Furthermore, the cell bodies of stellate neurons most often were round or fusiform. The GDNF IR observed was mainly in the cytoplasm, although some weak nuclear staining was also seen along with some positive punctuate products accumulated around the nuclei (Figures 2 a-b, e).

The number of GDNF immunoreactive neurons

GDNF immunoreactive neurons appeared as brown and were located in the cytoplasm of both the ventral horn of spinal cord (Figure 2 a-d) and right cerebral cortex pre-motor area (Figure 2 e); there was no obvious change in any other group except for the neuron body shrinking and gap of tissue edema appearing at 7 days post-operation, as shown in (Figure 2 c-d). There was an obvious decrease in the number of GDNF immunoreactive neurons in the ventral horn of spinal cord and right cerebral cortex pre-motor area following SCI at 7 days (Figure 3 a-e), 14 days (Figure 3 f-j), 1 month (Figure 3 k-o), 2 months (Figure 3 p-t), and 3 months (Figure 3 u-y, w) post trauma compared with the 24 hr group. In the ventral horn, changes of GDNF showed ascent tendency till 2 months after descent post-trauma lasted 2 weeks, then decreased again to

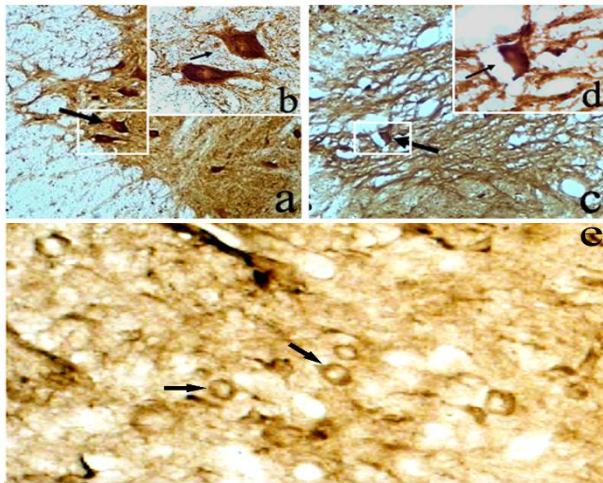


Figure 2. GDNF immunoreactivity-like cells were observed in the ventral horn of T11 spinal cord and the right cortex motor area a. Immunoreactivity positive motor neurons in the ventral horn at 24 hr after operation group (arrows, 4×10) b. Immunoreactivity mainly happened in the cytoplasm with negative nuclei (arrows, 4×50) c. The soma of immunoreactivity positive motor neurons in the ventral horn of intact side of the spinal cord for the upside of the lesion after hemi-SCI in the 7 day group is atrophic, and the tissue is dropsical (arrows, 4×10) d. A higher-magnification image from c, showing that the cell body of immunoreactivity positive motor neurons is atrophic, and the tissue is dropsical (arrows, 4×50) e. Shows GDNF immunoreactivity-like neurons in the right cortex motor area of the 24 hr group (arrows 4×50) and mainly located in the cytoplasm with negative nuclei.

the lowest level at 3 months; however, the number of GDNF immunoreactive neurons went up to the peak at 1 month in the intact and inferior of trauma segment of the spinal cord, but the superior of the trauma segment reached high peak at 2 months; on the whole, the number of GDNF immunoreactive neurons in the traumatic side were more than that of the intact side of

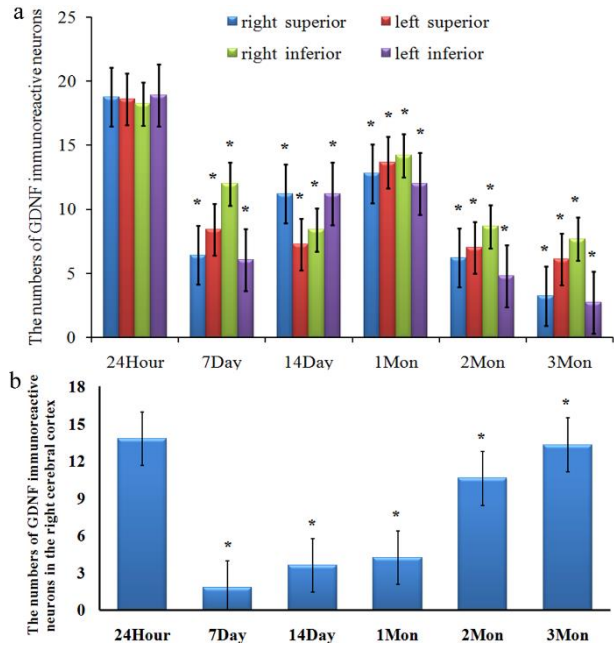


Figure 4. The change of GDNF immunoreactive neurons in the ventral horn of spinal cord and the right cerebral cortex pre-motor area in macaque following hemi-SCI

a. Bar graph of the number of GDNF immunoreactive neurons in the ventral horn of the spinal cord in macaque compared with each group (n=3), treat 24 hr group as the control group, Statistical significance, $P < 0.05$ is denoted by a single asterisk (*), all data were compared with other data. b. Bar graph of the number of GDNF immunoreactive neurons in the right cerebral cortex pre-motor area of macaque of each group following SCI. Mon means month.

the spinal cord (Figure 4 a). However within these times, the number of GDNF was in disagreement with the right cerebral cortex pre-motor area, which rapidly declined to the lowest level post-operation, then slowly went up to the level of 24 hr at about 3 months after operation (Figure 4 b).

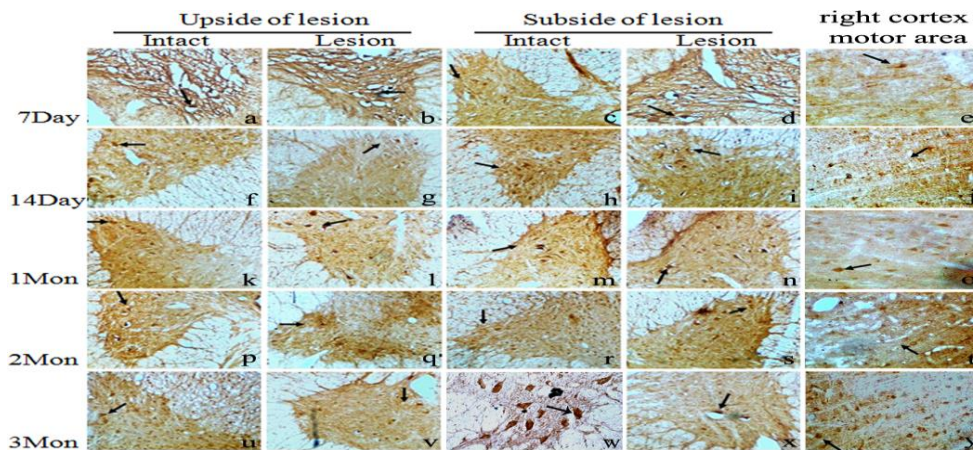


Figure 3. GDNF immunoreactivity-like cells observed in the ventral horn of the spinal cord and the right cerebral cortex pre-motor area of 7 day, 14 day, 1 month, 2 month, and 3 month groups

a, f,k,p,u. GDNF immunoreactivity-like motor neurons in the ventral horn of intact side of the spinal cord for the upside of the lesion in 7 day, 14 day, 1 month, 2 month, and 3 month groups (arrows 4×10). b,g,l,q,v. GDNF immunoreactivity-positive motor neurons in the lesioned side of the spinal cord for the upside of the lesion of 7 day, 14 day, 1 month, 2 month, and 3 month groups (arrows 4×10). c,h,m,r,w. GDNF immunoreactivity-positive motor neurons in the lesioned side of the spinal cord for subside of the lesion in 7 day, 14 day, 1 month, 2 month, and 3 month groups (arrows 4×10). d,i,n,s,x. GDNF immunoreactivity-positive motor neurons in the intact side of the spinal cord for subside of the lesion in 7 day, 14 day, 1 month, 2 month, and 3 month groups (arrows 4×10). e,j,o,t,y. GDNF immunoreactivity-like cells in the right cerebral cortex pre-motor area of 7 day, 14 day, 1 month, 2 month, and 3 month groups (arrows 4×10). Mon means month

Discussion

Until now, no effective treatment for patients suffering from SCI has been discovered because the injury mechanism is very complicated. Furthermore no matter, given timely handling or not, the outcome of SCI is not satisfactory. Thus, it's urgent to find effective treatment methods for SCI, and many experiments have been done covering many aspects, such as molecule, cell, gene, drug treatment, and so on.

In this study, we have discovered that GDNF immunoreactive products were found in the cytoplasm of neurons from both the ventral horn of the spinal cord and the right cerebral cortex pre-motor area. As is reported (3) previously, GDNF was mainly produced from skeletal muscles as a target-derived neurotrophic factor and reverse transported through motoneuron axon, then absorbed in nerve terminal; also it could be synthesized in dorsal root ganglion sensory neurons (17) as GDNF-containing dense-cored vesicles in the neuronal somata and the vesicles transported anterogradely to peripheral or central axon terminals. Researchers have already demonstrated that GDNF was localized mostly in the Golgi complex, secretogranin II, and Rab3A-positive vesicles (18). Thus, the previous reports, together with the present study, support that GDNF mRNA is mainly located in the cytoplasm of neurons in the ventral horn of spinal cord or in the right cerebral cortex pre-motor area.

In our experiment, the number of GDNF positive neurons at different time points was detected. In the ventral horn, after hemisected at T11 of left spinal cords, GDNF descended at first, 2 weeks later it appeared with ascent tendency for 2 months, then decreased again, and got to the lowest level at 3 months; on the whole, numbers of the GDNF immunoreactive neurons in the traumatic side were more than those of the intact side. Although, we did not quantitatively test the GDNF gene in this experiment, many present studies could support that the changes of GDNF after CNS injury were both at mRNA and protein expression levels. Researchers (19) examined the expression of GDNF mRNA in rats subjected to transient forebrain ischemia; they found that GDNF was increased slightly from 3 to 24 hr after ischemia and subsequently declined to below the baseline level. They found that those ischemia-induced reactive astrocytes, as well as surviving neurons, produced GDNF in 3–7 days after the ischemia, and suggested that expression of GDNF mRNA is regulated in part via ischemia-induced neuronal degeneration. They also suggest that ischemia-induced reactive astrocytes may produce GDNF to protect against neuronal death. Tokumine *et al* (20) have also characterized spinal GDNF changes after ischemia and its localization in the tissue. GDNF was increased towards two peaks after ischemia. Those results suggest its necessity for GDNF to be increased

to protect against ischemic damage, in addition, activated astrocytes may play an important role in reversing the level of GDNF.

As we know, GDNF plays an important role in neural, function maintenance, and nerve repair post-trauma and it is extensively disposed of in the CNS of animals (21). GDNF played an important role in neural, function maintenance, and nerve repair post-trauma. Some results (22, 23) indicated GDNF could significantly promote the motor neuron survival cultured *in vitro*, which was 75, 650, and 2500 times more than BDNF, CNTF, and LIF, respectively. Studies also indicated GDNF had a protective effect on spinal cord neurons (24, 25), brain nerve (25), and peripheral nerve (26), *in vivo*. The neuroprotection of GDNF was also proved by an experiment in Uppsala University (27). Overexpression of GDNF by transgenesis could improve function after SCI (28). Their results suggested that neurotrophins in combination have potential therapeutic value for the treatment of SCI in clinical situations. Hence, the number of GDNF immunoreactive neurons in the traumatic side is much larger than that of the intact side, indicating the potential role of the high-level neurotrophic factors for the treatment of SCI and function recovery. In addition, in the neonatal rat model, increased GDNF expression was restricted to microglia, suggesting anti-inflammatory property of GDNF (29). This indicates that GDNF neuroprotection mechanism was multiple.

In this study, the changes of GDNF immunoreactivity can be observed in the right cerebral cortex pre-motor area. The decreased degree of the GDNF immunoreactivity in the right cerebral cortex pre-motor area was much larger than the ventral horn's, within 7 days post injury. It hints GDNF has different reactions according to different trauma positions. In addition, GDNF decreased sharply in the cortex pre-motor area, it will gain superior treatment effectiveness if we enhance the expression of GDNF in the cortex per-motor area. Thereafter, the ascending degree of GDNF is extremely slow and stays low level on the whole from post-trauma to 1 month; it hints the treatment time needs to be fairly long, to allow for making the exogenous GDNF to satisfy the regeneration repair request. Also, it illustrates that expressing and obtaining GDNF are not sensitive enough with regard to the corticospinal tract motor neuron following SCI.

Transient forebrain ischemia (19) induced GDNF mRNA expression in cerebral cortex from 3 hr to 3 days after the ischemic episode, with peak expression at 6 hr. The change of GDNF proteins during functional recovery in rats after cerebral infarctions was studied (30). The results showed that the expression of GDNF was significantly increased in the temporal cortex until 7 days on the infarction side. On an equal footing, the functional recovery of paralysis was stable until 7 days post-infarction, and then the improvement was mild.

So we can conclude that the expression of GDNF protein might have some relationship with the functional recovery. It hints that GDNF was partly expressed by some neurons that do not express GDNF under the common conditions, and shows that change of nutrition factor has an important role in remodeling in CNS.

This study indicated that GDNF has been extensively expressed in brain, suggesting that GDNF played an important role in the development of the brain and the regeneration repairs (5) following SCI. Because GDNF has strong nutritional effects in the CNS, the changes of GDNF immunoreactive expressed and absorbed in the trauma corticospinal spinal neurons, which will affect the neuron's survival, regeneration, and function recovery following SCI. Moreover, the change of GDNF immunoreactive in the right cerebral cortex premotor area is not only the reaction of the corticospinal tract after axonal transacted but also the partial appearance of CNS on the whole following SCI. Following SCI, the network of connection was blocked between the brain and the spinal cord, the cerebrospinal fluid circulation destroyed, and the inflammatory cells produced a harmful effect on the microenvironment of neurons in the CNS. The above causes invariably affect GDNF application following SCI, but the mechanism still needs more study.

Conclusion

This study provided evidence supporting possible GDNF contribution to the functional recovery following SCI in the earlier stages. The changes of GDNF immunoreactive in the ventral horn of spinal cord was not consistent with cerebral cortex premotor area, it hints that GDNF has various expressions in different positions under identical conditions. At the same time, the changes of GDNF immunoreactive were under-regulated in both traumatic and intact sides of the spinal cord, but the regulation under traumatic conditions was not as accurate as normal conditions after SCI. It identified a possible treatment for SCI by providing exogenous GDNF to stimulate regrowth of the injured neurons to promote the function recovery.

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Conflict of interest

No competing financial interests exist.

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