

# Endogenous neurotrophin-3 promotes neuronal sprouting from dorsal root ganglia

Xu-yang Wang<sup>1,2,3</sup>, Pei-yuan Gu<sup>1</sup>, Shi-wen Chen<sup>2</sup>, Wen-wei Gao<sup>2</sup>, Heng-li Tian<sup>2,\*</sup>, Xiang-he Lu<sup>3</sup>, Wei-ming Zheng<sup>3</sup>, Qi-chuan Zhuge<sup>3</sup>, Wei-xing Hu<sup>1,\*</sup>

1 Department of Neurosurgery, First Affiliated Hospital of Nanjing Medical University, Nanjing, Jiangsu Province, China

2 Department of Neurosurgery, Shanghai Jiao Tong University Affiliated 6<sup>th</sup> People's Hospital, Shanghai, China

3 Department of Neurosurgery, First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang Province, China

## Abstract

In the present study, we investigated the role of endogenous neurotrophin-3 in nerve terminal sprouting 2 months after spinal cord dorsal root rhizotomy. The left L<sub>1-5</sub> and L<sub>7</sub>-S<sub>2</sub> dorsal root ganglia in adult cats were exposed and removed, preserving the L<sub>6</sub> dorsal root ganglia. Neurotrophin-3 was mainly expressed in large neurons in the dorsal root ganglia and in some neurons in spinal lamina II. Two months after rhizotomy, the number of neurotrophin-3-positive neurons in the spared dorsal root ganglia and the density of neurite sprouts emerging from these ganglia were increased. Intraperitoneal injection of an antibody against neurotrophin-3 decreased the density of neurite sprouts. These findings suggest that endogenous neurotrophin-3 is involved in spinal cord plasticity and regeneration, and that it promotes axonal sprouting from the dorsal root ganglia after spinal cord dorsal root rhizotomy.

**Key Words:** nerve regeneration; neurotrophin-3; sensory neurons; dorsal root ganglion; cats; nerve terminal; neural regeneration

**Funding:** This study was supported by grants from the Shanghai Municipal Commission of Health and Family Planning, No. 20114351, the Traditional Chinese Medicine Science Funding of Zhejiang Province of China, No. 2010ZB091 and the Natural Science Foundation of Zhejiang Province of China, No. Y2090864.

Wang XY, Gu PY, Chen SW, Gao WW, Tian HL, Lu XH, Zheng WM, Zhuge QC, Hu WX (2015) Endogenous neurotrophin-3 promotes neuronal sprouting from dorsal root ganglia. *Neural Regen Res* 10(11):1865-1868.

## \*Correspondence to:

Wei-xing Hu, M.D., Ph.D. or  
Heng-li Tian, M.D.,  
weixinghu@njmu.edu.cn or  
htianhengli@163.com.

## orcid:

0000-0001-7463-4493 (Wei-xing Hu)  
0000-0002-1219-4952 (Heng-li Tian)

doi:10.4103/1673-5374.170318

http://www.nrronline.org/

Accepted: 2015-07-21

## Introduction

Neurotrophic factors are endogenous signaling proteins that promote the survival, differentiation and function of neurons. Neurotrophic factors are produced by various cell types, including target neurons and muscle cells, as well as microglia and Schwann cells (Ekester, 2004). Among the various neurotrophic factors that are involved in spinal cord regeneration (Li et al., 2007, 2008), neurotrophin-3 (NT-3) has been particularly well studied (Blits et al., 2003; Liu et al., 2012; Tuinstra et al., 2012; Wang et al., 2013). NT-3 plays important roles in regulating the growth of muscle sensory neurons and in maintaining proprioceptive sensory organs (Chen et al., 2002; Gorokhova et al., 2009). NT-3 contributes to the survival of muscle spindle sensory afferent fibers. NT-3 also promotes the elaboration of terminal projections to motor neurons during the late stages of development, and in addition, potentiates group Ia synaptic projections to motor neurons. Delivering NT-3 to peripherally axotomized afferent fibers promotes the growth of axons. The monosynaptic projections from spindle afferent fibers to motor neurons also exhibit acute potentiation when exposed to NT-3 in the isolated spinal cord. NT-3 also enhances the mechanical sensitivity of the neuroma of spindle afferents that have been axotomized.

Previous studies have shown that rhizotomy can signifi-

cantly increase the expression of NT-3 in the spared dorsal root ganglion (DRG) (Wang et al., 2002) and in neurons and glial cells in lamina II of the afferent segments (L<sub>5</sub> and L<sub>7</sub>) in cats (Zhou et al., 2002). In addition, DRG cells from cats with spinal cord injury produce significantly more neuronal spheres and longer axonal projections than those from normal control cats (Zhang et al., 2004). However, whether endogenous NT-3 secreted by the DRG is involved in neuroplasticity after rhizotomy remains unclear. In this study, we examined the function of NT-3 at an extended period (2 months) after rhizotomy in cats.

## Materials and Methods

### Establishment of spinal cord dorsal root rhizotomy model

A total of 25 adult male outbred cats (1 year old, clean grade, weighing 3–3.5 kg) were provided by the Laboratory Animal Center of Nanjing Medical University (Nanjing, Jiangsu Province, China). The cats were individually housed in a vivarium with a 12-hour light/dark cycle for at least 3 days before surgery, with free access to food and water. The experimental procedures used in this study were approved by the Ethics Committee of Nanjing Medical University Affiliated First Hospital (Nanjing, Jiangsu Province, China). The cats were randomly assigned to the following three

pentobarbital solution (3.5%, 1.3 mL/kg), the lumbar laminae and part of the sacral vertebrae were removed. The dura was incised to expose the L<sub>1-5</sub> and L<sub>7</sub>-S<sub>2</sub> DRGs. The DRGs along with 2–3-mm segments of the associated dorsal roots were then removed at the intervertebral foramina on the left side, leaving the L<sub>6</sub> DRG and its associated dorsal root intact (**Figure 1**).

### NT-3 blocking

NT-3 blocking in the cats was performed as previously described (Liu et al., 2009). Briefly, after the cats were anesthetized by intraperitoneal injection of sodium pentobarbital solution (3.5%, 1.3 mL/kg), the L<sub>5-6</sub> processes and vertebral arches were resected, exposing the lumbar subarachnoid space. A catheter was inserted into the subarachnoid space and fixed by suturing the muscles and skin. NT-3-specific antibody (1:1,500; 50 µL; rabbit anti-cat; Santa Cruz Biotechnology, Dallas, TX, USA) was injected through the catheter once every week starting on postoperative day 1 during the first month, then every 2 weeks during the second month.

### Immunohistochemical staining

All cats were sacrificed 2 months post-surgery and transcardially perfused. Five animals from each of the normal control and rhizotomy groups were used for immunohistochemical staining. The L<sub>6</sub> DRG and spinal cord segment were harvested and fixed in 4% paraformaldehyde. The tissues were dehydrated in 20% sucrose solution overnight and cryosectioned into 30-µm sections for the spinal cord and 15-µm sections for the DRG. For unbiased sampling of data, a systematic sampling method was used, and the 10<sup>th</sup>, 20<sup>th</sup>, 30<sup>th</sup>, 40<sup>th</sup> and 50<sup>th</sup> sections were selected for analysis. Immunohistochemical staining was performed using rabbit anti-cat NT-3 monoclonal antibody (1:1,500; Santa Cruz Biotechnology), with a 48-hour incubation at 4°C. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:200; Santa Cruz Biotechnology) was added at 37°C for 1.5 hours. Sections were then incubated with 3,3'-diaminobenzidine for staining. PBS instead of primary antibody was used for the negative control. NT-3-positive neurons in the L<sub>6</sub> DRG and spinal cord lamina II were counted in a randomly selected square of 1.5 × 1.5 µm<sup>2</sup> under a microscope (X51, Olympus, Shanghai, China). The results of all sections were averaged.

### Retrograde tracing

Retrograde tracing was performed on axons of DRG neurons using a previously described protocol (Liu et al., 2009). Briefly, 5 days before the animals were sacrificed, the cats were given general anesthesia and the bilateral lumbosacral trunks were isolated. Cholera toxin B subunit conjugated to horseradish peroxidase (CB-HRP) 15 µL (30%; Sigma-Aldrich, St. Louis, MO, USA) was injected into the bilateral lumbosacral trunks for retrograde labeling of DRG neurons. Staining was performed using tetramethylbenzidine (TMB). Briefly, the L<sub>6</sub> DRG and spinal cord segment were fixed in 4% paraformaldehyde and stained with TMB (1%; Sigma-Aldrich). PBS instead of CB-HRP was used for the negative control. The area of the spinal dorsal horn was measured.

CB-HRP-positive nerve fibers were counted under a microscope (X51, Olympus) to calculate their density in the dorsal horn. The final measurements were the average of the five selected sections from each cat.

### Statistical analysis

All data are expressed as the mean ± SD. Inter-group comparisons were performed with unpaired *t*-test or one-way analysis of variance using SPSS 10.0 software (SPSS, Chicago, IL, USA). A *P*-value < 0.05 was considered statistically significant.

## Results

### Quantitation of NT-3-positive neurons in the L<sub>6</sub> DRG and lamina II after rhizotomy

Immunohistochemical staining showed that 2 months after rhizotomy, the number of NT-3-positive neurons in the L<sub>6</sub> DRG was significantly increased compared with the normal control group (*P* < 0.05). However, no significant difference in the number of NT-3-positive neurons in the L<sub>6</sub> spinal cord lamina II was found between the rhizotomy and normal control groups (*P* > 0.05; **Figure 2**).

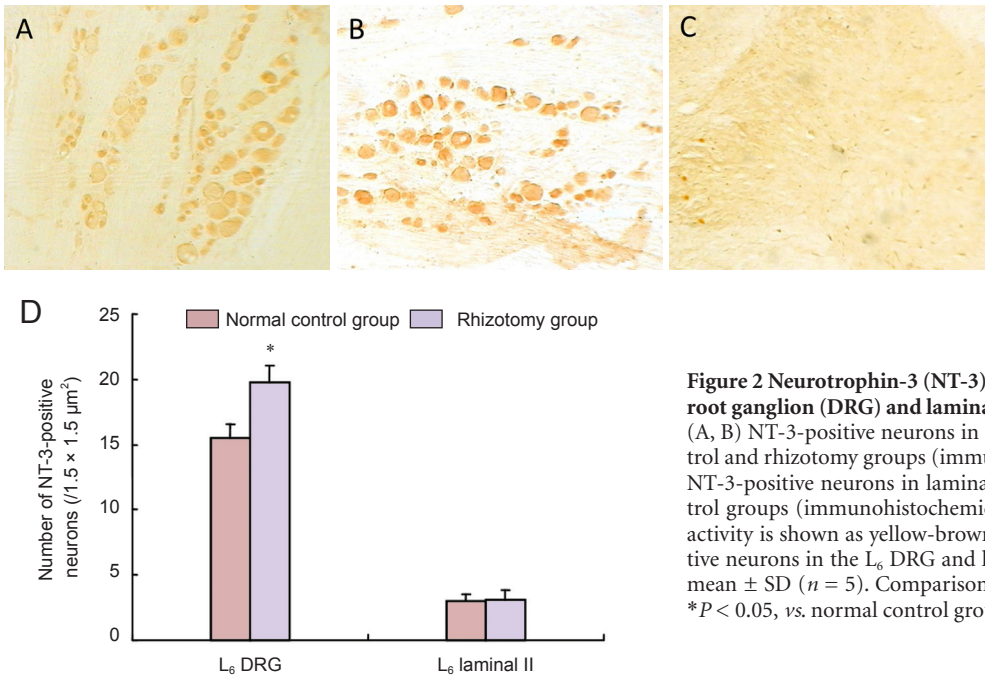
### Density of CB-HRP-labeled afferent fibers in the spinal dorsal horn after rhizotomy

Two months after rhizotomy, TMB staining revealed labeled neurons and nerve fibers in the L<sub>6</sub> DRG in both the normal control and rhizotomy groups (**Figure 3A, B**), suggesting that the CB-HRP retrograde tracing was successful. In the normal control group, the stained L<sub>6</sub> DRG neurons projected into laminae III, IV and V, but not lamina II (**Figure 3C**). In the rhizotomy and rhizotomy plus NT-3 blocking groups, the projections of CB-HRP-labeled nerve fibers appeared similar to that in the normal control group (**Figure 3D, E**). However, the density of CB-HRP-labeled nerve fibers was significantly increased in the rhizotomy group compared with the normal control group (*P* < 0.05; **Figure 3F**). This suggests that rhizotomy stimulates neurite sprouting from the spared L<sub>6</sub> DRG neurons into the dorsal horn. The neurites mainly projected into laminae III, IV and V. In the rhizotomy plus NT-3 blocking group, there was a significant decrease in the density of CB-HRP-labeled nerve fibers compared with the rhizotomy group (*P* < 0.05; **Figure 3F**). Our finding suggests that the NT-3 antibody inhibits neurite growth from DRG neurons.

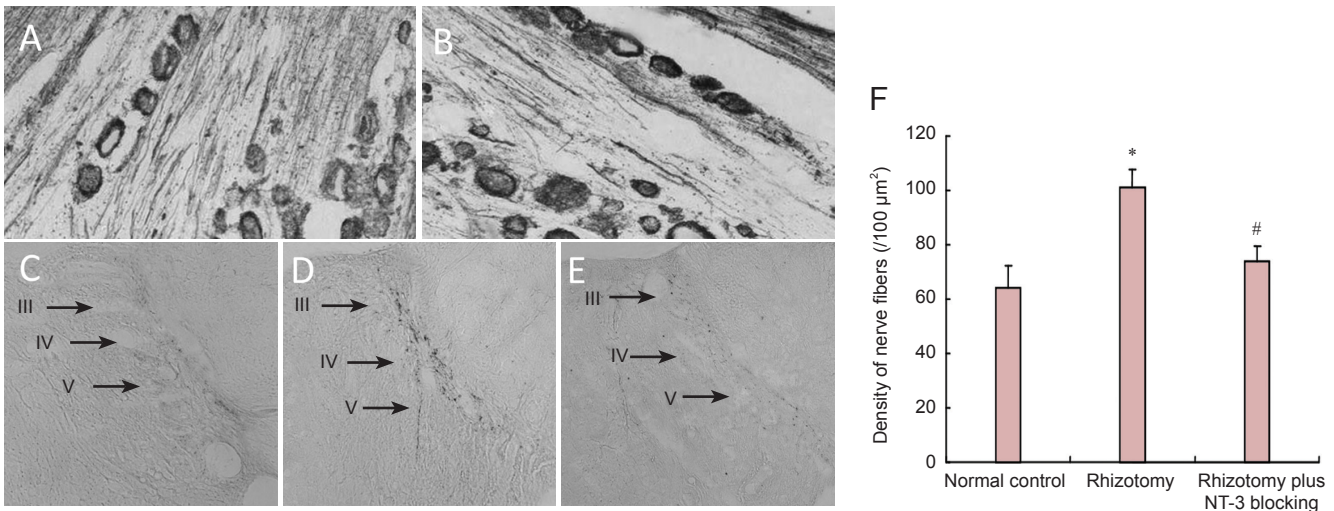
## Discussion

In this study, we found that rhizotomy significantly increased the number of NT-3-positive neurons in the spared L<sub>6</sub> DRG in cats 2 months after rhizotomy. This effect was inhibited by an NT-3 antibody, suggesting that NT-3 plays an important role in neural regeneration after rhizotomy.

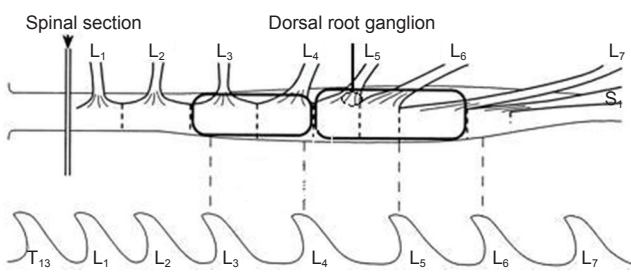
NT-3 is an important neurotrophic factor that plays important roles in nervous system development and synaptic plasticity (Maisonpierre et al., 1990; Schnell et al., 1994; Liu et al., 2009). NT-3 is expressed in motor neurons of the spinal ventral horn, in axons, in the spinal dorsal horn, and in glial cells (Ernfors et al., 1990; Li et al., 2007). In addition,



**Figure 2** Neurotrophin-3 (NT-3)-positive neurons in the L<sub>6</sub> dorsal root ganglion (DRG) and lamina II 2 months after rhizotomy in cats. (A, B) NT-3-positive neurons in the spared L<sub>6</sub> DRG in the normal control and rhizotomy groups (immunohistochemical staining, × 100). (C) NT-3-positive neurons in lamina II in the rhizotomy and normal control groups (immunohistochemical staining, × 200). NT-3 immunoreactivity is shown as yellow-brown staining. (D) Number of NT-3-positive neurons in the L<sub>6</sub> DRG and lamina II. All data are presented as the mean ± SD (*n* = 5). Comparison was performed using unpaired *t*-test. \**P* < 0.05, vs. normal control group.



**Figure 3** Labeling of afferent fibers in the spinal dorsal horn with cholera toxin B subunit conjugated to horseradish peroxidase (CB-HRP) 2 months after rhizotomy. (A) TMB staining of the L<sub>6</sub> DRG in the normal control group (× 200). (B) TMB staining of the L<sub>6</sub> DRG in the rhizotomy group (× 200). (C) TMB staining of the spinal cord in the normal control group (× 200). (D) TMB staining of the spinal cord in the rhizotomy group (× 200). (E) TMB staining of the spinal cord in the rhizotomy plus NT-3 blocking group (× 200). (F) Density of CB-HRP-labeled afferent fibers in the spinal dorsal horn 2 months after rhizotomy. Data are presented as the mean ± SD (*n* = 5). Comparisons were performed using one-way analysis of variance. \**P* < 0.05, vs. normal control group; #*P* < 0.05, vs. rhizotomy group. III, IV and V: Spinal cord lamina III, IV and V, respectively; TMB: tetramethylbenzidine; DRG: dorsal root ganglion.



**Figure 1** Establishment of spinal cord dorsal root rhizotomy model.

groups: normal control group (*n* = 10), rhizotomy group (*n* = 10; given unilateral spinal cord dorsal root rhizotomy), and rhizotomy plus NT-3 blocking group (*n* = 5; given unilateral spinal cord dorsal root rhizotomy and NT-3 blocking). Five animals from each of the normal control and rhizotomy groups were used for immunohistochemical staining and retrograde labeling. The rhizotomy plus NT-3 blocking group was only used for retrograde staining.

Spinal cord dorsal root rhizotomy was performed as previously described (Liu et al., 2009). Briefly, after the cats were anesthetized by intraperitoneal injection of sodium

NT-3 is expressed in DRG neurons and their axons (Ni et al., 2001; Wang et al., 2002, 2007, 2009; Zhou et al., 2002). However, the role of NT-3 in regeneration following rhizotomy remained unclear.

In the present study, we found that rhizotomy in cats significantly increased the number of NT-3-positive neurons in the DRG, suggesting that NT-3 in the spared DRG might be involved in regeneration following spinal cord injury. This is consistent with a previous finding that neurons in the spared DRG extend longer axonal projections than neurons in the normal DRG (Zhang et al., 2004). These results suggest that the increased number of NT-3-positive neurons in the spared DRG is associated with axonal regrowth from sensory neurons. Therefore, NT-3 may promote the growth of sensory nerve fibers after rhizotomy.

We also found that there was a significant increase in the number of CB-HRP-labeled fibers 2 months after rhizotomy in cats. This might be attributed to increased NT-3 expression in DRG neurons. We speculate that NT-3 secreted by DRG neurons may help in the formation of a microenvironment conducive to neurite sprouting from sensory neurons in the spinal cord. This may ultimately enhance repair after rhizotomy and promote neuronal plasticity. Previous studies have shown that during the early stage of repair after rhizotomy, NT-3-expressing neurons in the spared DRG were mostly medium and small-sized (Liu et al., 2009). However, in this study, the NT-3-expressing neurons in the spared DRG 2 months after rhizotomy were mostly large-sized. We speculate that NT-3 is expressed by different groups of neurons in the DRG after rhizotomy. Changes in NT-3 expression in the DRG might also alter NT-3 levels in the spinal cord, which may further enhance repair following rhizotomy. To evaluate the function of NT-3 in the spared DRG after rhizotomy in cats, we used an NT-3-specific antibody to block its function. We found that the number of NT-3-positive fibers was significantly decreased by this antibody in rhizotomized cats. This is consistent with our previous finding that blocking NT-3 inhibits axonal growth from DRG neurons *in vitro* (Zhang et al., 2004).

In conclusion, we show that the number of NT-3-positive large neurons in the spared DRG is increased after rhizotomy in cats, which may promote neurite sprouting and repair after rhizotomy.

**Author contributions:** WXH and HLT designed the study. XYW, PYG, SWC, XHL and WWG established the animal models and performed the experiments. WMZ and QCZG performed the statistical analysis. XYW drafted the paper. All the authors approved the final version of the paper.

**Conflicts of interest:** None declared.

**Plagiarism check:** This paper was screened twice using Cross-Check to verify originality before publication.

**Peer review:** This paper was double-blinded, stringently reviewed by international expert reviewers.

## References

Blits B, Oudega M, Boer GJ, Bartlett Bunge M, Verhaagen J (2003) Adeno-associated viral vector-mediated neurotrophin gene transfer in the injured adult rat spinal cord improves hind-limb function. *Neuroscience* 118:271-281.

- Chen HH, Tourtellotte WG, Frank E (2002) Muscle spindle-derived neurotrophin 3 regulates synaptic connectivity between muscle sensory and motor neurons. *J Neurosci* 22:3512-3519.
- Ekester E (2004) Neurotrophic factors and amyotrophic lateral sclerosis. *Neurodegener Dis* 1:88-100.
- Ernfors P, Ibáñez CF, Ebendal T, Olson L, Persson H (1990) Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. *Proc Natl Acad Sci U S A* 87:5454-5458.
- Gorokhova S, Gaillard S, Gascon E (2009) Spindle-derived NT3 in sensorimotor connections: principal role at later stages. *J Neurosci* 29:10181-10183.
- Li XL, Zhang W, Zhou X, Wang XY, Zhang HT, Qin DX, Zhang H, Li Q, Li M, Wang TH (2007) Temporal changes in the expression of some neurotrophins in spinal cord transected adult rats. *Neuropeptides* 41:135-143.
- Li XL, Liu J, Wang XY, Li LY, Ni W, Zheng RY, Yang HJ, Lu YC, Qi JG, Wang TH (2008) Temporal changes in the expression of TGF-beta 1 and EGF in the ventral horn of the spinal cord and associated precentral gyrus in adult Rhesus monkeys subjected to cord hemisection. *J Neurol Sci* 268:163-171.
- Liu F, Zhang JM, Zhang C, Wang XY, Liao M, Ren YH, Tu WZ, Lei YN (2009) Effect of endogenous NT-3 on spinal cord injury treated with electro-acupuncture. *Jiefangjun Yixue Zazhi* 34:1000-1008.
- Liu T, Xu J, Chan BP, Chew SY (2012) Sustained release of neurotrophin-3 and chondroitinase ABC from electrospun collagen nanofiber scaffold for spinal cord injury repair. *J Biomed Mater Res A* 100:236-242.
- Maisonpierre PC, Belluscio L, Squinto S, Ip NY, Furth ME, Lindsay RM, Yancopoulos GD (1990) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. *Science* 247:1446-1451.
- Ni S, Wang T, Chen Y (2001) The expression of BDNF and NT-3 in dorsal root ganglion following peripheral and central axotomy--an immunohistochemical study. *Hua Xi Yi Ke Da Xue Xue Bao* 32:350-352.
- Schnell L, Schneider R, Kolbeck R, Barde YA, Schwab ME (1994) Neurotrophin-3 enhances sprouting of corticospinal tract during development and after adult spinal cord lesion. *Nature* 367:170-173.
- Tuinstra HM, Aviles MO, Shin S, Holland SJ, Zelivyanskaya ML, Fast AG, Ko SY, Margul DJ, Bartels AK, Boehler RM, Cummings BJ, Anderson AJ, Shea LD (2012) Multifunctional, multichannel bridges that deliver neurotrophin encoding lentivirus for regeneration following spinal cord injury. *Biomaterials* 33:1618-1626.
- Wang TH, Wang XY, Li XL, Chen HM, Wu LF (2007) Effect of electroacupuncture on neurotrophin expression in cat spinal cord after partial dorsal rhizotomy. *Neurochem Res* 32:1415-1422.
- Wang TH, Wu LF, Liao DY, Zhou X, Chen YH, Liu S, A T (2002) The change of NT-3 and its mRNA in spared dorsal root ganglion and spinal dorsal horn lamina following partial dorsal rhizotomy. *Shen-jing Jiepo Xue Zazhi* 18:59-62.
- Wang X, Li Y, Gao Y, Chen X, Yao J, Lin W, Chen Y, Liu J, Yang Y, Wang X (2013) Combined use of spinal cord-mimicking partition type scaffold architecture and neurotrophin-3 for surgical repair of completely transected spinal cord in rats. *J Biomater Sci Polym Ed* 24:927-939.
- Wang XY, Li XL, Hong SQ, Xi-Yang YB, Wang TH (2009) Electroacupuncture induced spinal plasticity is linked to multiple gene expressions in dorsal root deafferented rats. *J Mol Neurosci* 37:97-110.
- Zhang W, Zhou X, Wang TH, Wang TW, Liu S, Chen SX, Ou KQ (2004) The neurotrophic effect of endogenous NT-3 from adult cat spared dorsal root ganglion on ganglionic neurons. *Sichuan Da Xue Xue Bao Yi Xue Ban* 35:25-28.
- Zhou X, Wu L, Chen H, Wang T, Chen L, Bao T, Liao D (2001) The spatiotemporal change of neurotrophin family and their mRNA expression in spinal cord of cats after partial rhizotomy. *Hua Xi Yi Ke Da Xue Xue Bao* 33:165-168, 191.

Copyedited by Patel B, Robens J, Yu J, Yang Y, Li CH, Song LP, Zhao M