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Original Article

Neurotrophin exerts neuroprotective effects after spinal cord injury by inhibiting apoptosis and modulating cytokines



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ABSTRACT

Background/objective: Spinal cord injury (SCI) severely and irreversibly damages the central nervous system. Neurotrophin (NTP), a nonprotein extract obtained from inflamed rabbit skin inoculated with vaccinia virus, is a drug that has been used for more than sixty years to alleviate neuropathic pain. It also reportedly exerts a neuroprotective role in peripheral nerves and in response to various central nervous system diseases, such as brain injury and Alzheimer disease. However, whether NTP promotes SCI recovery remains unknown. This study evaluated NTP's effects after SCI and explored its underlying mechanisms in a rat contusion model of SCI.

Method: NTP was intraperitoneally administered to adult female Wistar rats subjected to contusion-induced SCI. Functional recovery was evaluated with behavioural scores and electrophysiological examinations. Tissue recovery was assessed with magnetic resonance imaging as well as histological staining with haematoxylin and eosin and Luxol Fast Blue. Neuronal survival and gliosis were observed after NeuN and glial fibrillary acidic protein immunofluorescence. Levels of apoptosis were demonstrated with TdT-mediated dUTP nick-end labeling (TUNEL) staining, Caspase-3 and B-cell lymphoma-2 (Bcl-2) Western blot, and Annexin V/propidium iodide flow cytometry. A protein antibody chip analysis was performed to evaluate the expression levels of 67 rat cytokines.

Results: NTP treatment improved the hindlimb locomotor recovery of the injured animals as well as their electrophysiological outcomes after SCI. A dosage of 50 NTP units/kg was found to optimize the efficacy of NTP. Magnetic resonance imaging revealed that lesion sizes decreased after NTP treatment. The haematoxylin and eosin and Luxol Fast Blue staining showed significant increases in the amount of spared tissue. The NeuN and glial fibrillary acidic protein immunofluorescence revealed that NTP treatment increased neuronal survival and reduced gliosis in tissue samples obtained from the lesion's epicentre. That NTP inhibited apoptosis was confirmed by the decreased number of TUNEL-positive cells, level of Caspase-3 expression, and number of Annexin V/propidium iodide-positive cells, as well as the increased level of Bcl-2 expression. The protein array analysis identified 28 differentially expressed proteins in the NTP group, and the gene ontology (GO) analysis showed that the enriched differentially expressed proteins implicate janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling pathways. The expression levels of proinflammatory cytokines such as interleukin 6, thymus chemokine-1 (TCK-1), and lipopolysaccharide-induced CXC chemokine (LIX) decreased after NTP treatment, whereas the levels of prorepair cytokine hepatocyte growth factor and adiponectin increased.

Conclusion: Our research provides evidence that NTP can improve functional outcomes and alleviate secondary injury after SCI by inhibiting apoptosis and modulating cytokines.

The translational potential of this article: The multicomponent NTP might have broad target spectra in SCI pathophysiology and halt the secondary injury cascade. As a safe drug that features sixty years of clinical use as an

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analgesic, translating this demonstrated efficacy of NTP to addressing SCI in human patients may potentially be accelerated.

Introduction

Spinal cord injury (SCI) causes severe damage to the central nervous system that burdens the affected individuals physically, economically, and socially [1,2]. The development of several previously promising candidate drugs for the treatment of SCI has been halted in preclinical and clinical trials owing to safety concerns [3], and the complex mechanisms of the secondary injuries induced by SCI have hitherto obstructed the discovery of novel, effective pharmacotherapies [4]. The reassessment of clinically used drugs used in other diseases for their expanded effectiveness on SCI could speed up the translation [5].

A nonprotein extract obtained from inflamed rabbit skin inoculated with the vaccinia virus, neurotrophin (NTP), is clinically used as an analgesic and antiallergic agent [6,7]. Although NTP was originally reported as an effective agent against oedema in animal models and in clinical application [8,9], an accumulating body of evidence indicates that NTP may exert neuroprotective effects by attenuating local inflammatory responses and inhibiting demyelination of the injured peripheral nerves [10]. *In vitro* research has revealed that NTP inhibits amyloid β -peptide-induced hippocampal neuronal damage by modulating the p38/modulating thymogen-activated protein kinase (p38/MAPK) signalling pathway [11], and an *in vivo* study of an animal model of Alzheimer disease observed that NTP features positive effects in reducing memory impairment and neuroinflammation [12,35]. NTP has further been demonstrated to fulfil neuroprotective roles in a mouse model of hypoxic-ischaemic brain injury by suppressing proinflammatory cytokines [13]. However, the neuroprotective efficacy of NTP in application to SCI is yet to be investigated.

In addition to its potential neuroprotective role, the use of NTP in treating SCI warrants exploration for two reasons. First, the use of NTP in Japanese and Chinese clinical practice for more than sixty years demonstrates its safety. Second, NTP is composed of more than 300 compounds, many of which have been shown to be neuroprotective, such as γ -aminobutyric acid (GABA), adenosine, carnosine, and so on (unpublished data); the presence of multiple neuroprotective substances may broaden the target spectrum and, hence, the therapeutic potential of NTP in the complex pathophysiologic milieu after SCI [14].

The present study thus aimed to investigate the clinical efficacy of NTP in treating SCI and explore the mechanisms underpinning its effects: specifically, to identify the optimal dose of NTP for functional recovery, the underlying mechanisms were elucidated through an analysis of apoptosis and a cytokine protein chip array.

Materials and methods

Animals

Adult female Wistar rats weighing 240 ± 10 g were purchased from the Laboratory Animal Center of the Academy of Military Medical Sciences (Beijing, China). The rats were maintained in a humidity- and temperature-controlled environment with a 12:12 light–dark cycle and allowed free access to food and water. All experiments were approved by the Ethics Committee of the Institute of Radiation Medicine, Chinese Academy of Medical Science & Peking Union Medical College (CAMS & PUMC), as per the guidelines of animal experiments (IRM-DWLL-2018010). A total of 129 rats were used.

Experimental groups

Rats were randomly assigned to five groups: sham group, laminectomy without SCI and saline (0.9% NaCl); injury group, SCI and

saline (0.9% NaCl); 25 NTP units (NU) group, SCI and 25 NU/kg NTP; 50 NU group, SCI and 50 NU/kg NTP; and 100 NU group, SCI and 100 NU/kg NTP.

NTP was provided as sterile solutions of 20 NU/mL by Nippon Zoki Pharmaceutical Co Ltd. (Osaka, Japan). NU represents the biological activity of NTP standardized with an analgesic test using SART-stressed mice [15]. NTP was administered by intraperitoneal injection immediately after surgery and once a day thereafter until Day 7. The rats in the sham and injury groups were injected with the same volume of 0.9% NaCl.

Spinal cord injury

The contusion SCI model was established using a modified version of Allen's method. The rats were anaesthetized with pentobarbital by intraperitoneal injection. A 1-cm midline incision was made along the dorsal skin, and a blunt dissection of the muscle layers over the area of the vertebral T10 level was conducted to expose the T10 vertebral laminae. Subsequently, a T10 vertebra dorsal laminectomy was conducted. The impact bar was placed on the spinal cord, and the 10-g node (2.5 mm in diameter) was allowed to fall freely from the height of 2.5 cm, inducing a severe contusion injury to the spinal cord. The muscles and skin were then sutured. Cefuroxime sodium was used for 3 days after surgery to prevent infection. Manual bladder expression was conducted twice a day for 7 days after injury. In the sham group, the rats only underwent laminectomy and showed a normal Basso, Beattie, and Bresnahan (BBB) score after surgery.

Behaviour assessment

BBB and inclined plane tests were performed before the spinal cord surgery and weekly thereafter. All the tests were performed at the same time of day and were graded by the blinded observers. Functional restoration was assessed as per BBB locomotor scores, as previously described [16]. The BBB test score evaluated hindlimb locomotor function on a scale from 0 to 21. Scoring was based on spontaneous hindlimb movement during a 5-min observation period in the open field. The progress of hindlimb function and trunk control was also assessed with the inclined plane test using a board secured at one end. The unsecured end of the board was gradually raised, and the maximum angle at which the rats could stay stable for 5 s was recorded as the test angle. Each test was performed thrice.

Electrophysiology

The rats were anaesthetized at 4 weeks after injury with pentobarbital, and their somatosensory evoked potentials (SEPs) were recorded using electrophysiological devices (YRKJ-G2008; Zhuhai Yiruikeji Co, Ltd, Guangdong, China). A constant stimulator was used to generate a 5.1 Hz square wave of 0.1 ms in duration to stimulate the median nerve along the hindlimbs of the rats. For each rat, a total of 100 SEP responses were counted and averaged. The current intensity was 2 mA for SEP.

Magnetic resonance imaging

The rats were maintained under anaesthesia during imaging. Magnetic resonance imaging (MRI) of the rat spinal cord was performed using a 3.0 T spectrometer (Signa HDX, GE, CT, USA) at 4 weeks after injury. The parameters were as follows: repetition time/echo time (TR/TE), 3000/110 ms; slice thickness, 2 mm; slice gap, 0.5 mm. T2-weighted images were used to calculate the T2 intensity at the lesion site using ImageJ software (National Institutes of Health, US).

Histology

At Week 4 after SCI, the rats were anesthetized and perfused transcranially with 0.9% NaCl, followed by 4% paraformaldehyde. A 1-cm segment of the spinal cord obtained from the focus of the injury site was dissected. The specimen was immersed in 4% paraformaldehyde and stored at 4 °C for 24 h. The samples were embedded in paraffin and sliced into 5- μ m-thick sections. The sections were subjected to haematoxylin and eosin staining and Luxol Fast Blue (LFB) staining as previously described [17].

Immunofluorescence

Spinal cord sections (5- μ m thick) from each specimen were deparaffinized with xylene (2 \times 20 min), incubated in graded concentrations of ethanol (2 \times anhydrous ethanol, 95%, 90%, 80%, and 70%), and washed thrice in phosphate-buffered saline (PBS). The sections were incubated in H₂O₂ for 15 min to block endogenous peroxidase, with a blocking solution (0.1% Triton X-100 in PBS and 10% goat serum) at room temperature for 1 h and finally with the primary antibodies overnight at 4 °C. The primary antibodies used were as follows: mouse anti-NeuN antibody (1:200, ab104225; Abcam, MA, USA) and goat anti-rat glial fibrillary acidic protein (GFAP) antibody (1:400, ab53554; Abcam, MA, USA). The sections were washed thrice with PBS and then incubated with secondary antibodies for 1 h at room temperature. After 3 rinses in PBS, the nuclei were counterstained with 1 g/ml 4',6-diamidino-2-phenylindole (DAPI) (Biosharp, Hefei, China) for 5 min. The anti-fade mounting medium (Solarbio, Beijing, China) was placed on each slide. Immunofluorescence imaging was visualized using an Olympus fluorescence microscope (TH4-200; Olympus, Tokyo, Japan), and quantification was performed using ImageJ software (National Institutes of Health, US).

TUNEL staining

The rats were deeply anaesthetized with 4% chloral hydrate via intraperitoneal injection and perfused with 4% paraformaldehyde. TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. In brief, the sections were rehydrated as previously described in the description of immunofluorescence staining. The samples were then incubated for 30 min at 37 °C with proteinase K (Promega, WI, USA) working solution. After 2 rinses with PBS, Converter-Peroxidase (POD) solution was added. The specimens were then incubated for 30 min at 37 °C, followed by two washes with PBS. The diaminobenzidine (DAB) substrate was added to each sample, all of which were incubated for an additional 10 min. Glycerol was applied before mounting coverslips. The percentage of TUNEL-positive cells was counted as follows: TUNEL/DAPI \times 100%.

Western blot

Samples of injured spinal cord tissue of an approximate length of 0.5 cm were collected from the foci of the lesion sites and homogenized in 300 μ l of lysis buffer containing 20 mM Tris pH 7.4, 50 mM NaCl, 1% Triton X-100, and protease inhibitor. We separated 40 μ g of total tissue lysate on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels by electrophoresis and transferred them to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked at room temperature for 2 h and incubated with primary antibodies at 4 °C overnight. Goat anti-rabbit IgG conjugated with horseradish peroxidase (1:2000; MDL, Shanghai, China) was added for 1.5 h. Immunosignals were detected with the DAB kit. Bands were analyzed using Image J software. The primary antibodies used in Western blot are listed as follows: cleaved caspase-3 (1:500, ab49822; Abcam, MA, USA) and Bcl-2 (1:1000, ab32124; Abcam, MA, USA).

Flow cytometry apoptosis analysis

The Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (Sanjian Biotechnology, Tianjin, China) was used to determine apoptotic cell death. The rats were sacrificed, and injured spinal cord epicentres of 1 cm in length were collected and dispersed in Hank's Balanced Salt Solution (Biyun Biotechnology, Shanghai, China). After the specimens were filtered through a 70- μ m cell strainer, the cell concentration was adjusted to 2 \times 10⁶/ml. We then added 5 μ l of Annexin V and 5 μ l of PI to the samples at room temperature in the dark. The samples were assessed using a flow cytometer (Accuri C6; BD Biosciences, California, USA). The Annexin V-FITC⁺/PI⁺ cell populations were considered apoptotic.

Protein array

Spinal cord tissues were screened using a rat cytokine array (67 cytokines, G-series rat cytokine array, RayBiotech, Norcross, GA, USA) according to the manufacturer's instructions. Chemiluminescence signals were detected using a ChemiDocMP System (Bio-Rad, Hercules, California, USA) and analyzed using ImageLab software (NIH, USA).

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5 software (San Diego, CA, USA). Comparisons among multiple groups were performed using a one-way analysis of variance followed by a Bonferroni correction. Significant differences in behavioural results were analyzed with a two-way repeated measures analysis of variance and Tukey's post hoc test. All data were presented as the mean \pm standard error of mean. The P-values of <0.05 indicate statistically significant differences.

Results

NTP promotes functional recovery after SCI

To detect the efficacy of NTP in addressing SCI and find the optimal dose for promoting this efficacy, different doses of NTP (25, 50, and 100 NU/kg) were intraperitoneally administered on seven consecutive days after injury. Motor function recovery after SCI was quantified as the hindlimb BBB score across the 8-week period after injury (Fig. 1A). We found a significant difference in the mean BBB score between the injury and NTP-treated groups: 13.17 \pm 1.28 and 9.28 \pm 1.07 for the 50 NU and injury groups at 8 weeks after injury, respectively. As 50 NU/kg is the optimal dose shown by the BBB score, 50 NU/kg NTP was used for the following experiments; this regimen effected a significant improvement in the inclined plane test from the 1st to the 8th week (Fig. 1B).

To evaluate the electrophysiological outcomes of NTP treatment, SEPs were recorded at 4 weeks after injury (Fig. 1C), and the latency and amplitude of N1 in each group were analyzed. In the injury group, the latency of SEP was significantly prolonged, and the amplitude was apparently dropped, indicating damaged axonal conduction owing to the induced contusion injury. Relative to the data obtained from the injury group, the latency of the SEP was shortened, and the amplitude was recovered in the 50 NU group, indicating enhanced axonal conduction after SCI.

NTP reduced tissue damage after SCI

To determine whether the functional improvement in NTP-treated rats was associated with reduced secondary tissue damage after SCI, the spinal cord lesions of the live animals were assessed by MRI. As T2-weighted MRI hyperintense areas correspond to areas of oedema, inflammation, demyelination, axon loss, and astrogliosis [18,19], the relative diminishment of T2 enhancement observed in the 50 NU group (Fig. 2A) indicates that NTP administration decreases the spinal cord

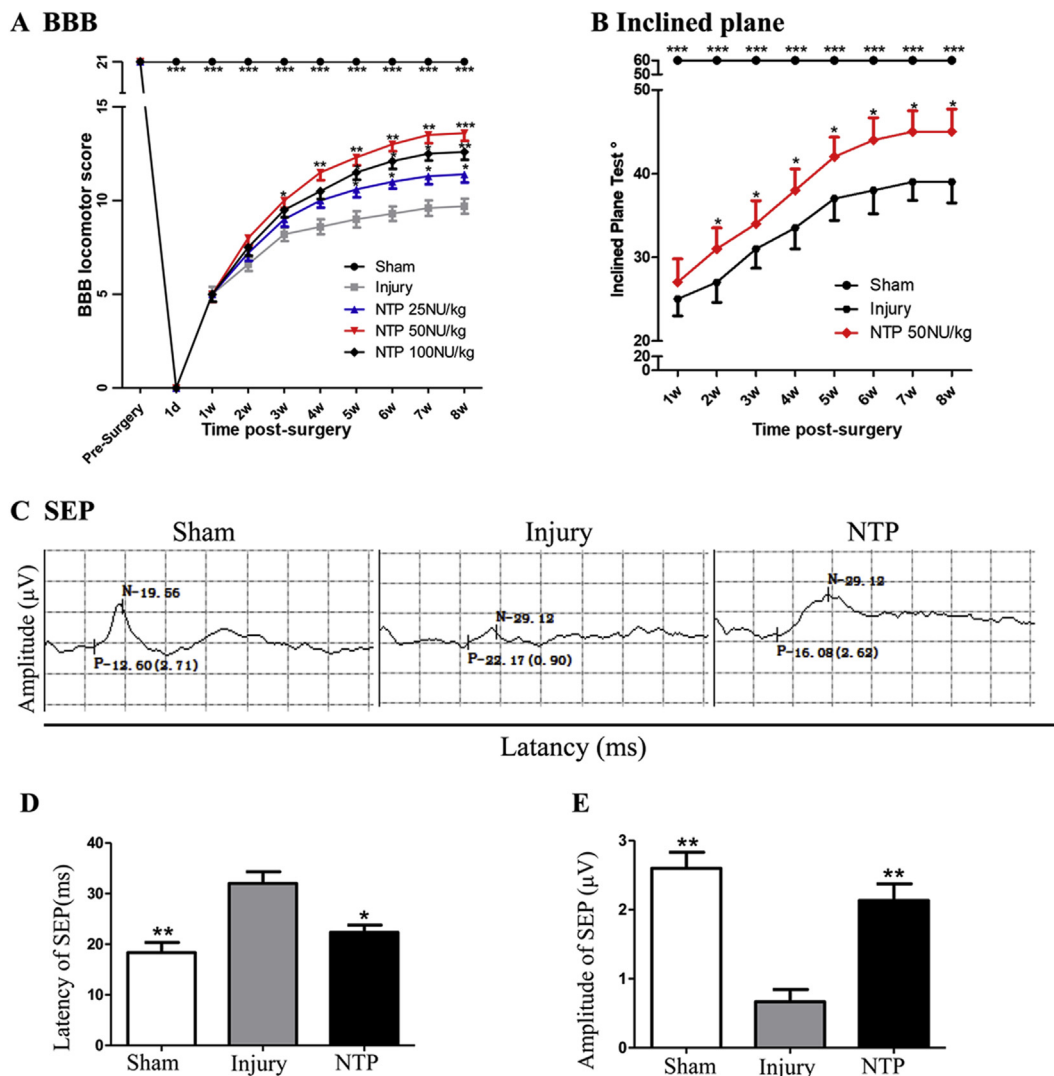


Figure 1. NTP enhanced functional recovery after SCI. (A) Functional restoration was assessed with the BBB locomotor scores of hindlimbs obtained from 1 day to 8 weeks after surgery. All data are expressed as mean ± SEM. Data were analyzed using a two-way ANOVA and Tukey's post hoc test. *P < 0.05, **P < 0.01, ***P < 0.001 vs. the injury group, n = 8 animals per group. (B) The inclined plane test was performed from 1 week to 8 weeks after surgery. The NTP dose was 50 NU/kg (*P < 0.05, **P < 0.01 vs. the injury group, n = 5). (C–E) Representative image of SEP (average of 100 responses) after treatment with 50 NU/kg NTP. *P < 0.05, **P < 0.01 vs. the injury group, n = 5. ANOVA = analysis of variance; BBB = Basso, Beattie, and Bresnahan; NTP = neurotrophin; NU = NTP units; SCI = spinal cord injury; SEM = standard error of mean; SEP = somatosensory evoked potential.

lesion volume.

Consistent with the MRI assessment, the sections obtained from the injury epicentre and stained with haematoxylin and eosin at Week 4 after injury showed more tissue sparing in NTP groups relative to the injury group (Fig. 2B). LFB staining revealed enhanced myelin sparing in the NTP-treated rats when compared with the untreated animals (Fig. 2C). These results indicate that NTP prevents myelin loss. Collectively considered, our results indicate that NTP treatment markedly reduced secondary injury after SCI as evinced by the increased sparing of tissue and myelin.

NTP treatment increased neuronal survival and reduced gliosis after SCI

To identify the role of NTP in different neuronal cell types, we conducted fluorescent immunostaining of NeuN and GFAP on SCI epicentre sections. NeuN staining was used to explore the neuronal protective effects of NTP after SCI (Fig. 3A). We found more NeuN-positive cells in the NTP group than in the injury group at 4 weeks after SCI in the ventral horn at the site of motor neurons (Fig. 3B). These results indicate that NTP protected neurons after SCI.

We also detected the expression of GFAP, a major component of scar matrices (Fig. 4A), to study the gliosis process after NTP treatment. At 4 weeks after SCI, the number of GFAP-positive astrocytes in the 50 NU group was lower than that in the injury group (Fig. 4B), suggesting that NTP treatment reduces gliosis after SCI.

NTP attenuates apoptosis in the injured spinal cord

To determine whether NTP can attenuate apoptosis after SCI, TUNEL staining was conducted at 1 week after injury, the peak of apoptotic processes (Fig. 5A and B). NTP significantly reduced the number of TUNEL-positive cells relative to that observed in the injury group. The expression of two apoptosis-related factors was detected by Western blot (Fig. 5C–E). NTP treatment reduced the expression of proapoptosis factor cleaved caspase-3 and upregulated the level of antiapoptosis factor Bcl-2 (p < 0.05). For further confirmation, Annexin V-FITC/PI double staining flow cytometry showed that NTP significantly decreased the number of Annexin V-FITC/PI double-positive cells, indicating reduced apoptosis (Fig. 5F). Taken together, these results demonstrate that NTP elicited neuroprotective effects against SCI by attenuating apoptosis in the spinal cord.

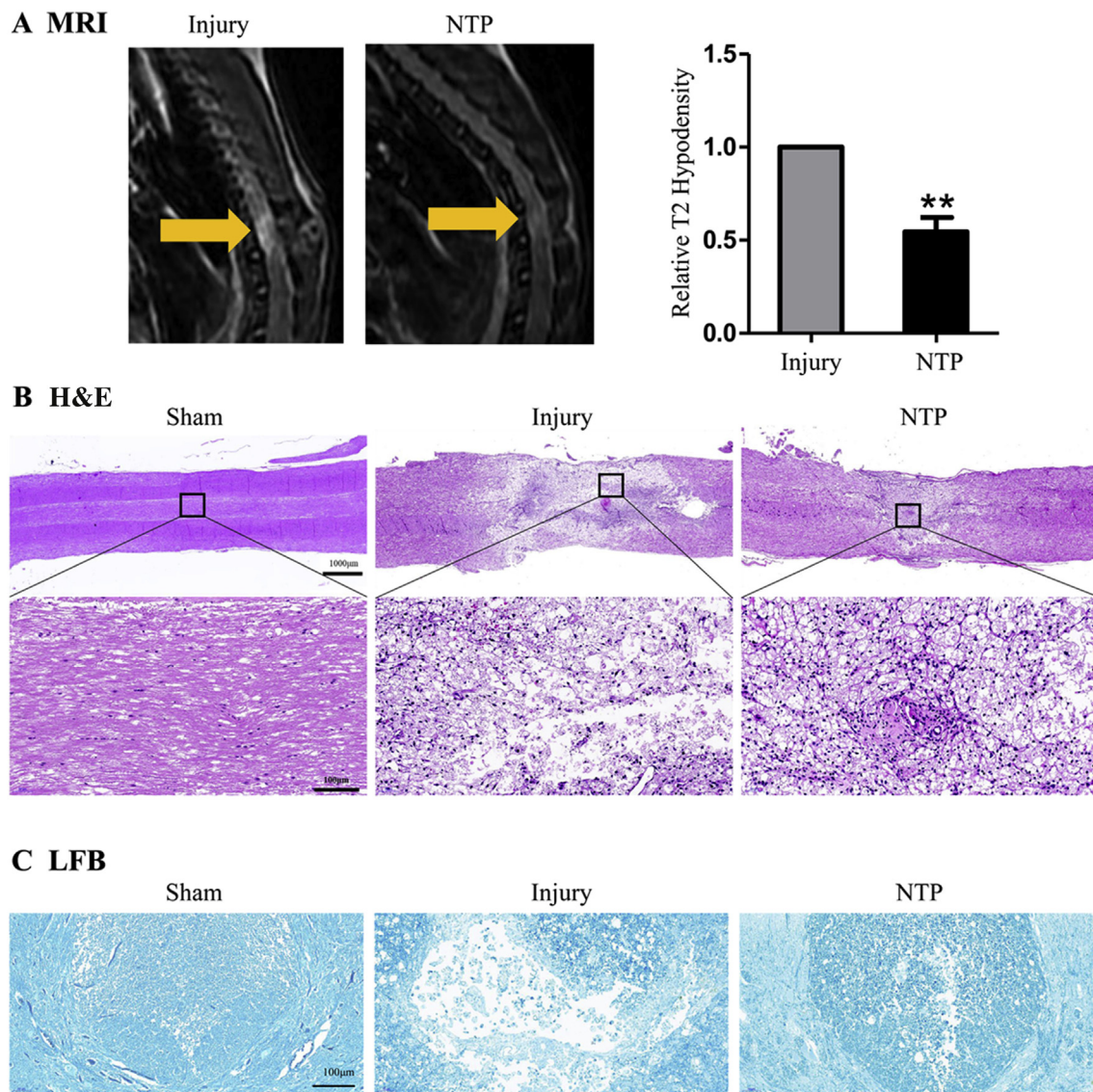


Figure 2. NTP reduced tissue damage after SCI. (A) Representative MRI T2 image of injury and NTP (50 NU/kg) treatment groups. The T2 intensity was semi-quantified using ImageJ software as the lesion size. $**P < 0.01$ vs. the injury group, $n = 5$. (B) Representative H&E staining of spinal cord sections from the injury and NTP (50 NU/kg) groups at 4 weeks after surgery. Scale bar = 100 μm ; $n = 3$ animals per group. (C) Representative micrograph showing myelin sparing at the injury epicentre in sections stained with LFB at 4 weeks after surgery; $n = 3$ animals per group. H&E = haematoxylin and eosin; LFB = Luxol Fast Blue; MRI = magnetic resonance imaging; NTP = neurotrophin; NU = NTP units; SCI = spinal cord injury.

NTP regulates the expression of cytokines and signalling pathway after SCI

To obtain insight into the mechanisms by which NTP achieved the aforementioned effects, we screened the cytokine chips as a means of identifying the key proteins and pathways for these processes. The protein array was used to detect 67 proteins (including inflammatory proteins and neurotrophic factors) at 3 days after injury. We identified 28 differentially expressed proteins (DEPs) in the NTP-treated and injury groups. A clustering analysis (Fig. 6A) and GO analysis were subsequently performed. With reference to the specific biological process (Fig. 6B), DEP enrichment indicated the cytokine-mediated signalling pathway, regulation of lymphocyte activation, and regulation of leucocyte cell–cell adhesion; from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, we found that DEP enrichment indicated cytokine–cytokine receptor interaction, the JAK-STAT signalling pathway, and the interleukin (IL) 17 signalling pathway (Fig. 6C). The levels of IL-6, TCK-1, and LIX were particularly decreased after NTP treatment, whereas the levels of hepatocyte growth factor and

adiponectin had notably increased (Fig. 6D).

NTP is reported to influence the NF- κ B and JNK pathway. Whether it modulates the same pathway in SCI is unknown, and we evaluated the spinal cord tissue 3 days after injury. In the injured group, the p-65:t-p65 ratio significantly increased as previously reported and indicates that the NF- κ B signalling pathway is upregulated. On NTP 50 NU/kg treatment, the p-65 expression dramatically decreased, whereas t-p65 expression remains the same, indicating the NF- κ B pathway is downregulated (Fig. 7A and B). The JNK pathway is upregulated on injury and downregulated after NTP treatment, as shown by Western blot of p-JNK and JNK expression (Fig. 7C and D). The results showed that NTP may have suppressed the NF- κ B and JNK pathway to modulate the cytokines.

Discussion

This study showed that NTP promotes recovery in a rat contusion model of SCI. In addition to improving the behavioural and the electrophysiologic outcomes, NTP treatment decreased lesion size and gliosis

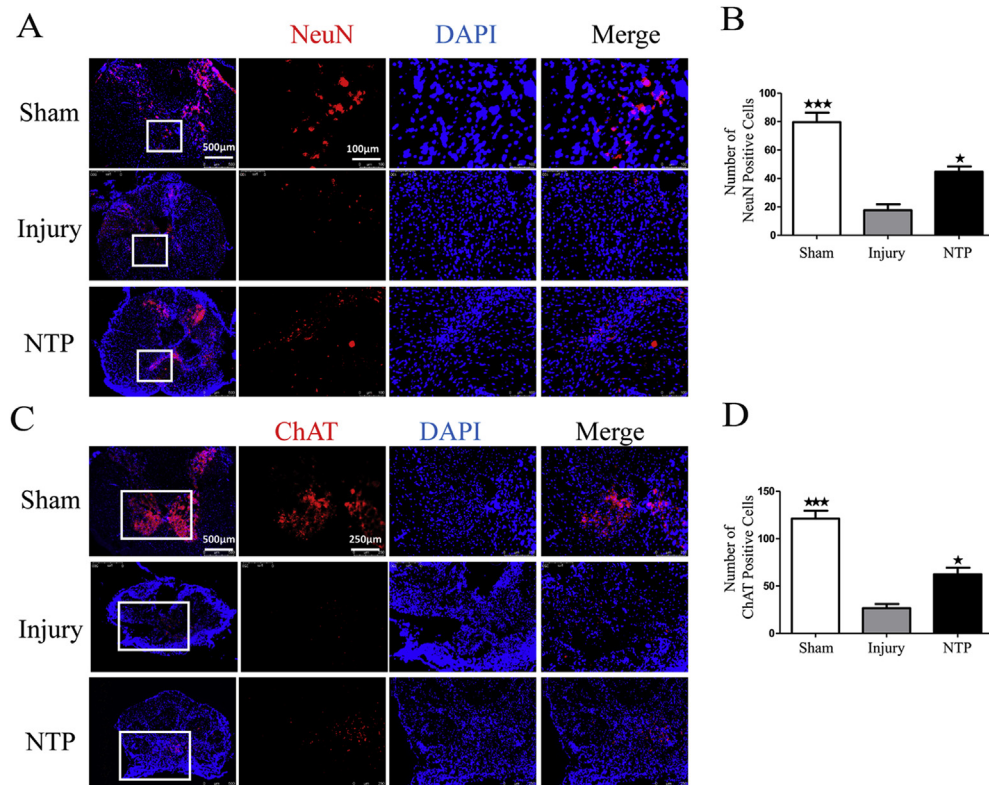


Figure 3. NeuN immunofluorescence staining in the ventral horn of the injury epicentre. (A) Representative photo of NeuN (in red) and DAPI (in blue) staining at 4 weeks after SCI in different groups. Scale bar = 500 μ m, in the first row in the left and in the three rows in the right. (B) Semi-quantification of the NeuN-positive cells. Quantification was performed using ImageJ software. (C) Representative photo of CHAT (in red) and DAPI (in blue) staining at 4 weeks after SCI in different groups. Scale bar = 500 μ m in the first row in the left and in the three rows in the right. (D) Semi-quantification of the CHAT-positive cells. Quantification was performed using ImageJ software. All data are expressed as mean \pm SEM and analyzed using a one-way ANOVA followed by Bonferroni post hoc tests. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. the injury group, $n = 3$ animals per group. ANOVA = analysis of variance; NTP = neurotropin; SEM = standard error of mean.

and increased neuronal survival. Subsequent protein analyses revealed that the beneficial effects of NTP administration in acute SCI might be due to its antiapoptotic and cytokine modulatory mechanisms.

Neurons and oligodendrocytes are particularly vulnerable to SCI, and the survival of neurons plays a critical role in functional recovery after SCI

[20]. NeuN staining revealed that more neurons survived at the lesion site after treatment with NTP. As the neurons at the ventral horn are primarily motor neurons, the improvements of functional recovery as quantified by hindlimb locomotor scores may be attributable to an enhanced preservation of motor neurons. LFB staining showed more preserved

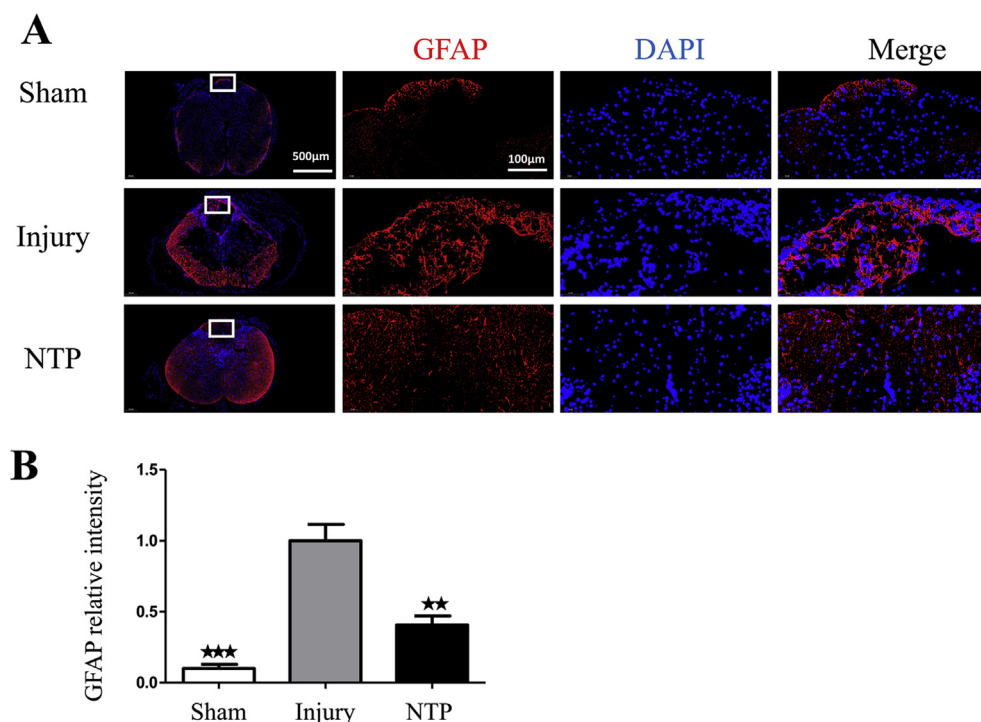


Figure 4. GFAP immunofluorescence staining at the injury epicentre. (A) Representative photo of GFAP (in red) and DAPI (in blue) staining at 4 weeks after SCI in different groups. Scale bar = 50 μ m. (B) Semi-quantification of the GFAP relative intensity. Quantification was performed using ImageJ software. All data are expressed as mean \pm SEM and analyzed using a one-way ANOVA followed by Bonferroni post hoc tests. ** $P < 0.01$, *** $P < 0.001$ NTP vs. the injury group, $n = 3$ animals per group. ANOVA = analysis of variance; GFAP = glial fibrillary acidic protein; NTP = neurotropin; SCI = spinal cord injury; SEM = standard error of mean; DAPI = 4',6-Diamidine-2'-phenylindole dihydrochloride.

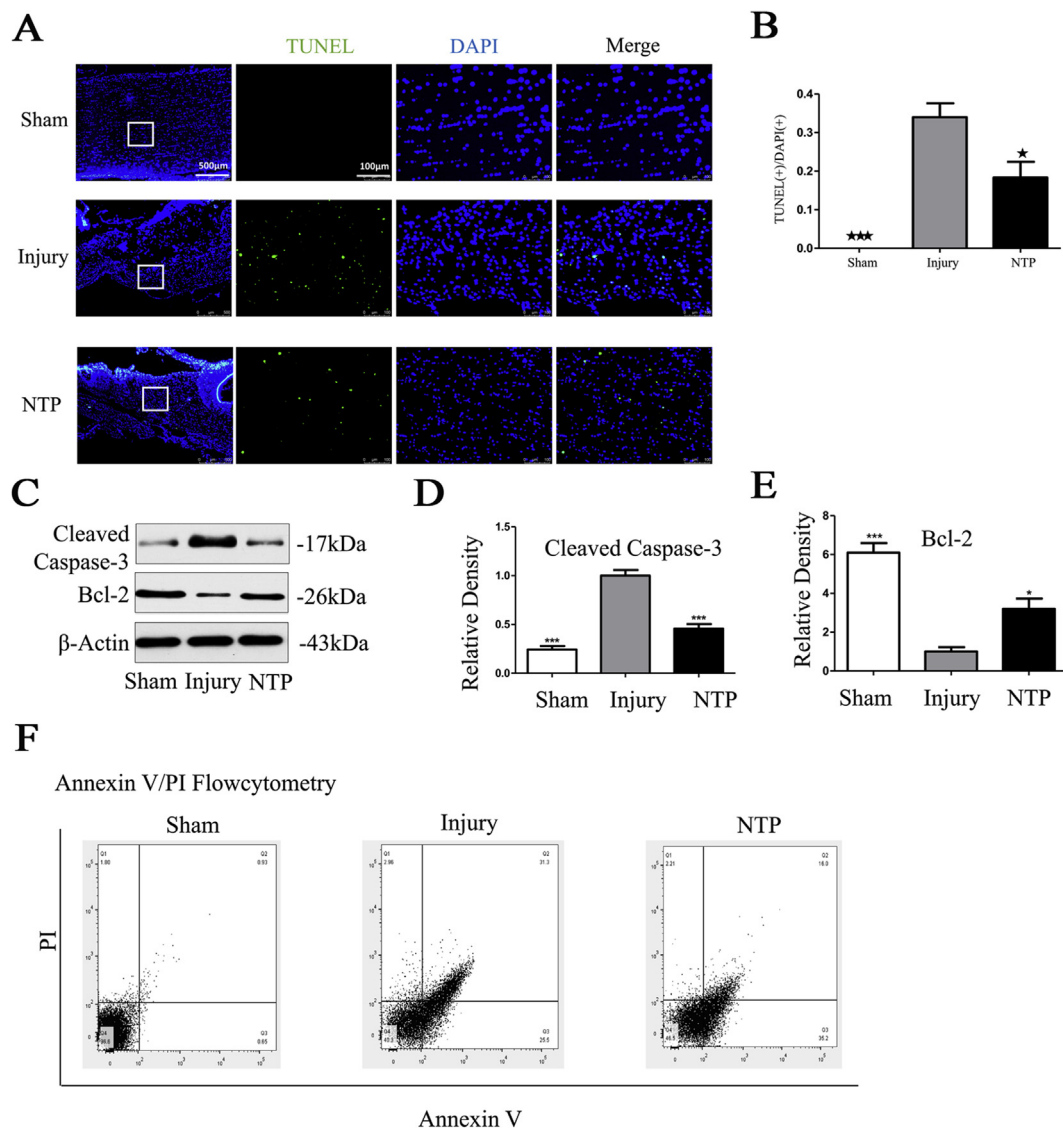


Figure 5. NTP protects the injured spinal cord from apoptosis. (A) Representative TUNEL-positive cells (in green) in spinal cord sections at 1 week after SCI. Nuclei were stained with DAPI (in blue). Scale bar = 50 μ m. (B) Semiquantification of the TUNEL/DAPI. Quantification was performed using ImageJ software. All data are expressed as mean \pm SEM and analyzed using a one-way ANOVA followed by Bonferroni post hoc tests. * $P < 0.05$ NTP vs. the injury group, $n = 3$ animals per group. (C) Representative Western blot of cleaved caspase-3 and Bcl-2 expression at 1 week after surgery. (D and E) Semiquantification of the protein levels of cleaved caspase-3 and Bcl-2, normalized to β -actin; $n = 3$ animals per group. Data are expressed as mean \pm SEM and analyzed using a one-way ANOVA followed by Bonferroni post hoc tests. * $P < 0.05$, *** $P < 0.001$ vs. the injury group. (F) Flow cytometry of PI/Annexin V double labelling. ANOVA = analysis of variance; NTP = neurotrophin; PI = propidium iodide; SCI = spinal cord injury; SEM = standard error of mean; DAPI = 4',6-Diamidino-2-phenylindole dihydrochloride.

oligodendrocytes. In the context of the observed decreases in the latency of the SEP after NTP treatment, the increased transduction speed of electrophysiological signals may reflect the retention of oligodendrocytes.

Cell death is a major issue during the subacute phase of SCI and is a therapeutic target for treatment [21,22]. The number of TUNEL-positive cells decreased in NTP group compared with the SCI group. This finding was consistent with the decreased number of Annexin V/PI double-positive cells and the expression of caspase-3. Considered along with the increased levels of Bcl-2, our results indicate that NTP interferes with apoptosis.

NTP is composed of a mixture of more than 300 small molecule compounds (lower than 500 Da), many of which are neurotrophic such as GABA, adenosine, and carnosine. Although our cytokine chip array failed to identify the effective components of NTP, it did suggest that NTP elicits neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), in neuronal cell lines. Specifically, we observed that hepatocyte growth factor and adiponectin, the neurotrophic effects of which are well

established [23–26], were upregulated at 3 days after SCI and NTP treatment. This upregulation may enhance neuronal survival rather than enhance axon growth; indeed, we found that NTP could not promote neurite outgrowth on primary cortical neurons (data not shown). This finding agrees with the observations of Taneda et al. [27], who demonstrated that NTP could not promote the outgrowth of axons in dorsal root ganglion neurons. Although Fukuda et al. [28] showed that NTP could enhance neurite outgrowth in PC12 cell lines, this seeming inconsistency may be attributable to differences in the primary neurons and cell lines. Isonaka et al. [29] showed that NTP could inhibit axonal transport, which could explain NTP's possible analgesic mechanism. Hence, our study supports the hypothesis that neurons could be protected from the harmful environment after SCI by the elevation of the expression of neurotrophic factors.

Previous research on other disease models has reported that NTP exerts antiinflammatory effects [30]. Our experiments also showed that NTP downregulated inflammatory cytokine expression, such as IL-6, LIX,

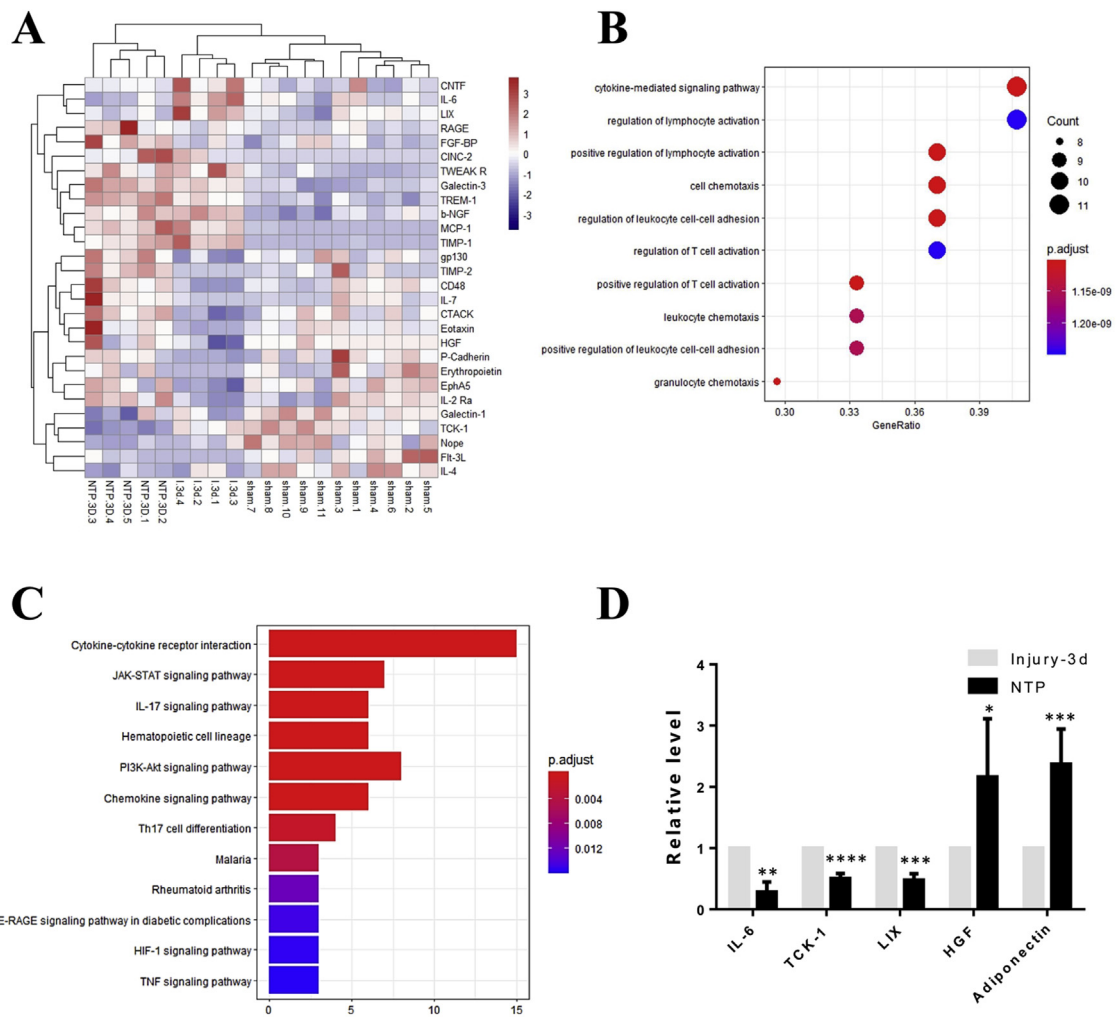


Figure 6. NTP regulates inflammatory proteins and neurotrophic factors. Protein array analysis. (A) The cluster heat map of different proteins at 3 days after injury. The figure was made using the “heatmap” of R (vision 3.5.3, <https://www.r-project.org/>). (B) The biological process results of the GO analysis. (C) The KEGG analysis. (D) The expression of IL-6, TCK-1, LIX, HGF, and adiponectin in the injury and NTP groups. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 vs. the injury group, n = 11 animals for the sham group, n = 4 for the injury group, n = 5 for the NTP group. HGF = hepatocyte growth factor; IL = interleukin; NTP = neurotrophin; GO = Gene ontology; KEGG = Kyoto Encyclopedia of Genes and Genomes.

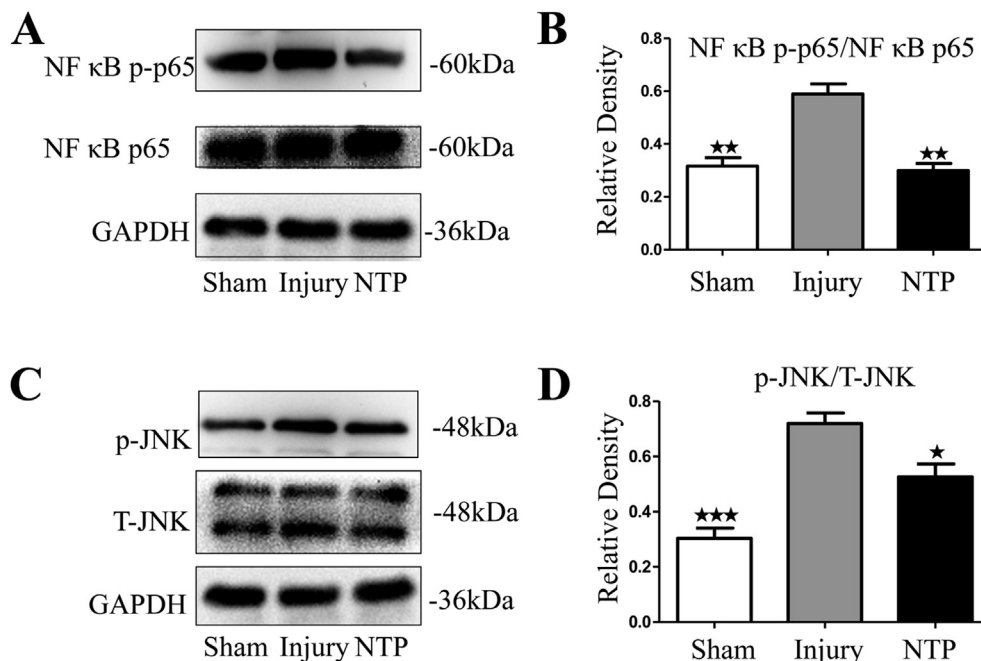


Figure 7. NTP modulates the inflammation signalling pathway. (A) Representative Western blot of phospho-p65 (p-p65) and total-p65 (t-p65) expression at 3 days after surgery. (B) Semi-quantification of the protein levels of p-p65/t-p65; n = 3 animals per group. (C) Representative Western blot of phospho-JNK (p-JNK) and total-JNK (t-JNK) expression at 3 days after surgery. (D) Semi-quantification of the protein levels of p-JNK/t-JNK; n = 3 animals per group. Data are expressed as mean ± SEM and analyzed using a one-way ANOVA followed by Bonferroni post hoc tests. *P < 0.05, ***P < 0.001 vs. the injury group. (F) ANOVA = analysis of variance; NTP = neurotrophin; SEM = standard error of mean.

and TCK-1. The inflammatory response plays an essential role in tissue defence and in responding to damage induced by secondary injury after SCI. Reportedly promotive of neuronal apoptosis after SCI, the proinflammatory tumor necrosis factor- α (TNF- α), IL-1 β , and IL-6 cytokines have been detected in neurons and microglia cells from 5 to 6 h after injury [31]. IL-6 specifically plays an important role after SCI as a proinflammatory cytokine by regulating the inflammatory response and selectively inducing neural stem/progenitor cells to undergo astrocytic differentiation; this action obstructs central nervous system repair after SCI. The results of this present study demonstrated that NTP could reduce the expression of IL-6. An *in vitro* study reported NTP could suppress NF- κ B and MAPK signalling pathways to alleviate neuroinflammation [32]. Furthermore, a study on Alzheimer disease reported that NTP could decrease the expression of IL-1 β and IL-6 to suppress neuroinflammation [12]. LIX C-X-C motif chemokine ligand 5 (CXCL5) is a neutrophil-specific chemokine that promotes neutrophil chemotaxis; LIX (CXCL5) is reportedly upregulated soon after SCI, facilitating the neutrophil influx [33]. The reduced astrogliosis presently observed through GFAP immunostaining also indicates reduced neuroinflammation on NTP treatment. In the context of recent findings, our results thus demonstrate that NTP's enhancement of repair after SCI may be attributable to its alleviation of inflammation. NF- κ B is responsible for apoptosis and the inflammatory cytokine. The JNK pathway influenced the inflammatory cytokines.

Although the application of NTP to the treatment of SCI remains nascent, it has been shown to feature multiple potential therapeutic positive effects. Neuropathic pain occurs in 40% of patients with SCI with a mean onset of 1.2 years after injury, thus severely diminishing the patients' quality of life [4]. As NTP is prescribed to alleviate chronic pain—it activates the descending pain suppression system by enhancing noradrenergic receptors and serotonergic systems [6]—whether the drug could be used to alleviate neuropathic pain after injury warrants research. One report documented the case of a 7-year-old girl with SCI whose condition was complicated by epidural anaesthesia [34]. Moreover, NTP also reportedly features potential as an antidepressant [35]. As the psychological well-being of patients with SCI is gaining increasing attention, whether NTP could ameliorate depression after SCI is an additional promising direction for research.

Conclusion

This study has found that NTP promotes SCI functional repair in a rat contusion model. Our results indicate that NTP treatment after SCI spares spinal cord tissue, reduces gliosis and apoptosis, upregulates neurotrophic factors, and downregulates proinflammatory cytokines. Although the molecular mechanisms of this multicomponent drug are not yet fully understood, the neuroprotection profile of this safe drug in the animal model evinces its promise for its translation as an effective therapy for SCI.

Conflict of Interest

The authors have no conflicts of interest to disclose in relation to this article.

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