Neuroprotection of netrin-1 on neurological recovery via Wnt/β-catenin signaling pathway after spinal cord injury

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The neuroprotective effects of netrin-1 after spinal cord injury and its specific molecular mechanisms have not been elucidated. In our study, Western blot, transferase UTP nick end labeling staining and immunofluorescence staining first showed that netrin-1 significantly decreased the expression levels of caspase-3, caspase-9, transferase UTP nick end labeling-positive neurons, nuclear factor kappa-B, and tumor necrosis factor-α after spinal cord injury, which inhibited neuronal apoptosis and inflammatory response. Using Nissl and HE staining, we also found that netrin-1 significantly increased the number of Nissl bodies in the anterior horn of spinal cord and promoted the recovery of injured tissue after spinal cord injury, consequently providing a good microenvironment for recovery of motor function. Finally, the results of Basso, Beattie, and Bresnahan score further confirmed that netrin-1 promoted the recovery of neurological function after spinal cord injury. Furthermore, netrin-1 significantly promoted the expression of β-catenin and inhibited the expression of glycogen synthase kinase-3β, which activated Wnt/β-catenin signaling pathway after spinal cord injury. However, XAV939 inhibited Wnt/β-catenin signaling pathway, which significantly inhibited the regulatory effect of netrin-1 on apoptosis, inflammation, Nissl bodies, damaged tissues, and neuroprotection. These results demonstrate for the first time the correlation between netrin-1 and Wnt/β-catenin signaling pathway after spinal cord injury and show that netrin-1 exerts its neuroprotective effect by activating this signaling pathway after spinal cord injury. *NeuroReport* 31: 537–543 Copyright © 2020 The Author(s). Published by Wolters Kluwer Health, Inc.

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Introduction

Netrin family is a highly conserved family of small-molecule secreted proteins associated with laminin, including netrin-1, netrin-2, netrin-3, and netrin-4, which play important roles in cell migration and axon-directing activities [1,2]. Studies have found that the expression level of netrin-1 is increased after spinal cord injury (SCI), and netrin-1 protein has an effective protective effect on axon development between spinal cord and axon development of motor neurons [3,4]. Netrin-1 regulates autophagy activity of neurons after SCI and promotes neurological recovery [5,6]. However, the regulation of netrin-1 protein on the recovery of neurological function after SCI and its specific molecular mechanism needs further clarification.

Wnt family is a secreted glycoprotein family, mainly including Wnt1, Wnt3, Wnt-3a, and other proteins, which play a key role in embryonic development, tissue regeneration, bone growth, stem cell differentiation, nervous system diseases, and human cancer [7]. In central nervous system diseases, it affects the proliferation, composition, and survival of nerve cells [8]. Wnt signaling pathway is mainly divided into three signaling pathways: classical signaling pathway (Wnt/β-catenin), non-canonical signaling pathway (Wnt/JNK), and Wnt–Ca^{2+} signaling pathway [7]. Our previous studies have also shown that after SCI, simvastatin, methylprednisolone, and atorvastatin inhibit apoptosis by activating Wnt/β-catenin signaling pathway of neurons and promote the recovery of motor function [9–11].

However, the correlation between netrin-1 protein and Wnt/β-catenin signaling pathway after SCI has not been reported. Our study first reported that after SCI, netrin-1 protein inhibits neuronal apoptosis and inflammatory response, reduces the loss of anterior horn motoneurons, promotes the repair of spinal cord tissue, and ultimately promotes the recovery of motor function. This neuroprotective effect of netrin-1 is achieved by activating Wnt/β-catenin signaling pathway after SCI. And, the study provides a theoretical and experimental basis for treatment of SCI and promotes the clinical transformation of netrin-1.

Materials and methods
Animals and groups
Male SD rats (age: 8–10 weeks old, weight: 250–280 g) were purchased from Beijing Weitong Lihua Company and were raised in the Animal Experimental Center of Jining Medical College. All animal experiments complied with the National Health Research Program. All experimental procedures were approved by the Animal Ethics Committee of Jining Medical College.

Rats were divided into four different treatment groups: sham, SCI, SCI + netrin-1, and SCI + netrin-1 + XAV939. The sham operation group only underwent laminectomy, and the spinal cord tissue was not damaged. Rats were intraperitoneally injected with saline at 5 minutes after SCI. The rats in the SCI + netrin-1 and SCI + netrin-1 + XAV939 groups were intraperitoneally injected with netrin-1 recombinant protein (800 ng/ml, 1 ml, Creative Biomart, Shirley, New York, USA) and XAV939 (Wnt antagonist, 1 µM, 1 ml, Selleckchem, Houston, Texas, USA)[5,6,12] at 5 minutes after SCI and then once per day at the same time point for three days.

Spinal cord injury model
Rats were anesthetized by intraperitoneal injection of chloral hydrate (0.33 ml/kg). The T10 spinal cord was exposed, and the modified Allen's device was used. The device first placed a circular gasket with a diameter of approximately 3 mm on the surface of the T10 spinal cord. The device then dropped it from a height of 25 mm with a machine with a diameter of approximately 2 mm and a weight of approximately 10 g and hit the circular gasket, causing rapid congestion and edema of spinal cord in the T10 region. The lower limbs quickly retracted, tail swayed left and right, and incontinence was noted. Finally, the wound was washed with sterile warm saline and sutured in turn. After 3 days of intramuscular injection of penicillin for infection, rats were massaged twice a day to promote the recovery of spontaneous urination.

Motor function evaluation
Basso, Beattie, and Bresnahan (BBB) open sport scoring criteria was used to assess motor neurological recovery in rats after SCI [13]. This experiment was carried out using three scorers who were blinded to treatment condition. Three independent investigators scored three treatment groups before SCI and on days 1, 3, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, and 7 weeks after SCI, with scores ranging from 0 to 21 points. A score of 0 indicates complete paralysis of the hind limbs of the rats, and 21 represents a normal state. The recovery of motor function in each group was reflected according to the average score of each group at each time point.

Tissue preparation
Seven days after SCI, each group of rats (n = 6/group) was anesthetized. Rats were perfused with 4% paraformaldehyde and 0.9% normal saline. The 2 cm-long spinal cord tissue containing the lesion center segment was taken out. The spinal cord tissue was immersed in 4% paraformaldehyde for 3 days, and tissue was removed and immersed in a 30% sucrose solution for 3 days. Finally, tissue sections were performed using a cryostat, 5 µm-thick lateral sections (3 mm from the epicenter) for transferase UTP nick end labeling (TUNEL), and immunofluorescence staining and 20 µm-thick lateral sections (3 mm from the epicenter) for Nissl and HE staining.

Immunofluorescence double staining
The sections were placed in boiling 0.01 M citric acid (pH = 6) for antigen retrieval for 15 minutes. The repaired sections were blocked with goat serum blocking solution at 4°C for 2 hours, and the following primary antibodies were added to incubate overnight at 4°C: anti-nuclear factor kappa-B (NF-κB) (1:1000, Abcam, Cambridge, UK) and anti-tumor necrosis factor-α (TNF-α) (1:1000, Abcam, Cambridge, UK). On the next day, a fluorescent secondary antibody Alexa Fluor 568 (1:400; Life Technology, USA) was added to the spinal cord tissue, and the tissues were combined for 2 hours at room temperature. Subsequently, the sections were incubated with a second primary antibody (anti-neuronal nuclei [NeuN], 1:400; Novus Biologicals) for 2 hours at room temperature and then the tissues were incubated at room temperature for 2 hours with fluorescent secondary antibody Alexa Fluor 488 (1:400; Life Technology, USA). Next, the nuclei were counterstained by staining with 4′,6-diamidino-2-phenylindole (1:1000) and sealed with a neutral gum (Sigma-Aldrich, St. Louis, Missouri, USA). Tissue staining was observed under a fluorescence microscope (Leica, Heidelberger, Germany). Neurons in the anterior horn of the spinal cord were observed to evaluate changes in the inflammatory response of neurons.

Transferase UTP nick end labeling/NeuN double staining
Sections were permeabilized at 4°C for 2 minutes using a permeabilizing solution (0.1% Triton X-100), then the tissues were incubated at 37°C for 1 hour in the dark with the TUNEL staining mixture (In Situ Cell Death Detection Kit, TMR red; Roche, Mannheim, Germany). Then, the tissues were washed twice with PBS and incubated at 4°C overnight in the dark with the primary antibody (anti-NeuN, 1:400; Novus Biologicals). The second day, tissues were incubated with fluorescent secondary antibody Alexa Fluor 568 (1:400; Life Technology, USA) for 2 hours at room temperature. Subsequently, 4′,6-diamidino-2-phenylindole (1:1000) was added and incubated for 15 minutes in the dark, and tissue was sealed with glycerol, and the apoptosis of nerve cells was observed under a fluorescence microscope. Neurons in the anterior horn of the spinal cord were observed to evaluate changes in neuronal apoptosis.

Nissl staining

Twenty-micrometer sections were placed in a mixed liquid (anhydrous ethanol:chloroform = 1:1) and allowed to infiltrate at room temperature overnight. Next, the tissues were passed through 100% absolute ethanol, 95% alcohol, and three steamed water. The differentiated sections were stained in pre-warmed tar purple (0.1%) at 37°C for 10 minutes, washed once with triple distilled water, differentiated with 95% alcohol for 5 minutes, and placed in 100% absolute ethanol and xylene. Finally, the tissue sections were mounted and placed under a microscope for observation. The number of mean motor neurons in the five stained sites of the anterior horn of each group of spinal cords was randomly selected to evaluate the effect of each treatment factor on motor function.

HE staining

Twenty-micrometer sections were immersed in hematoxylin solution for 3 minutes. The sections were washed with distilled water and differentiated in a mixed solution (0.5 ml of hydrochloric acid and 100 mL of 75% ethanol) for 6 seconds, and they were then washed with distilled water. The sections were sequentially dehydrated with 95% ethanol for 2 minutes, 100% ethanol for 2 minutes, and xylene for 2 minutes. The tissues were mounted with a neutral gum, and the sections were observed under a light microscope.

Western blot

Rats in the four treatment groups were anesthetized on the seventh day after SCI, and a spinal cord tissue (2 mm cephalad and caudally around the epicenter), including the central part of the SCI, was taken. The protein concentration in each group was 2 μg/μl. The separation gel and the concentrated gel were dispensed. Next, 30 μg of the protein was added to the corresponding well. The gel was separated after electrophoresis, and the protein on the separation gel was transferred to the polyvinylidene fluoride (PVDF) membrane in the transfer liquid. The blocking solution was allowed to stand for 2 hours at room temperature. The following primary antibodies were added to incubate overnight at 4°C: anti-β-catenin (1:1000, Abcam, Cambridge, UK); anti-glycogen synthase kinase glycogen synthase kinase-3β (GSK-3β) (1:1000, Abcam, Cambridge, UK); anti-cleaved-caspase-9 (1:1000, Abcam, Cambridge, UK); anti-cleaved-caspase-3 (1:1000, Abcam, Cambridge, UK); and anti-β-actin (1:2000, Abcam, Cambridge, UK). On the next day, the PVDF membrane was added to the secondary antibody (1:2000; Abcam, Cambridge, UK) and incubated for 2 hours at room temperature. Finally, ECL chromogenic solution was added to display in the gel imaging system. The results were analyzed in ImageJ2x software.

Statistical analysis

All experimental data were expressed as mean ± SD and were analyzed using GraphPad Prism version 5.0 software. Unpaired *t*-test was used to compare the two groups. One-way analysis of variance was used for comparison between groups. The Mann–Whitney *U* test was used for BBB score data analysis. All experiments were carried out using three scorers who were blinded to treatment condition. Statistical significance was considered at $P < 0.05$.

Results

Netrin-1 promoted the recovery of motor function after spinal cord injury

BBB motor scoring criteria were used to observe the recovery of motor function in rats before SCI and on the 1 day, 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, and 7 weeks after SCI (Fig. 1). The average score over time was plotted as a line graph. The results showed that the score of the four groups before SCI was 21 points. On the first day of SCI, the SCI, SCI + netrin-1, and SCI + netrin-1 + XAV939 groups scored 0 points (complete paralysis). No significant difference in BBB scores was observed among the three groups before 2 weeks after SCI ($P > 0.05$). At 2 weeks after SCI and between 2 and 7 weeks, the BBB score of rats treated with netrin-1 was significantly higher than that of rats in SCI group. After co-treatment with netrin-1 + XAV939, BBB score was significantly reduced. This difference meant that netrin-1 significantly promoted the recovery of motor function in rats with SCI, and this beneficial effect was inhibited by XAV939.

Fig. 1
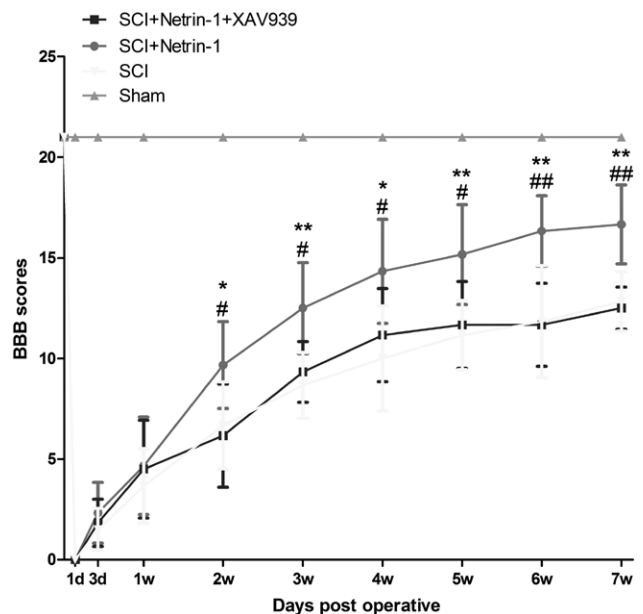
BBB scores before SCI and on days 1, 3, 7, 14, 21, 28, 35, 42 after injury (ordinate: 0–21 points) was used for evaluating the motor function of the four groups (n = 6/group, *$P < 0.05$ and **$P < 0.01$ compared with the SCI group, #$P < 0.05$ and ##$P < 0.01$ compared with the SCI + netrin-1 + XAV939 group). BBB, Basso, Beattie, and Bresnahan; SCI, spinal cord injury.

Netrin-1 promoted the repair of damaged tissue after spinal cord injury and reduced the loss of the Nissl bodies

After SCI, Nissl (Fig. 2a) and HE (Fig. 2b) staining were used to observe the survival rate of neurons and damage tissue repair of the anterior horn of each group. HE staining results showed that the proportion of lesions in the spinal cord tissue in SCI group was significantly higher than that in the sham group, whereas netrin-1 treatment significantly reduced the proportion of lesions after SCI (Fig. 2c). This effect of netrin-1 was evidently inhibited by XAV939. In addition, more Nissl bodies were detected in the anterior horn of the spinal cord in SCI + netrin-1 group compared with the SCI group (Fig. 2d). Similarly, the number of Nissl bodies in the spinal anterior horn was significantly reduced after treatment with netrin-1 and XAV939. The results showed that after SCI, netrin-1 promoted the survival of spinal cord anterior horn motor neurons and inhibited the tissue damage of the spinal cord. This beneficial effect was significantly inhibited by XAV939.

Netrin-1 inhibited the expression of inflammatory factors after spinal cord injury

After SCI, the expression of inflammatory factors NF-κB (Fig. 3a) and TNF-α (Fig. 3c) was observed by immunofluorescence staining. The results showed that compared with the sham group, the expression levels of inflammatory factors NF-κB and TNF-α were significantly increased after SCI, which means inflammation occurred. Netrin-1 could significantly inhibit the expression levels of NF-κB and TNF-\α, but their expression levels were significantly increased after the combination of netrin-1 and XAV939 (Fig. 3b and c). The results indicated that after SCI, netrin-1 inhibited the inflammatory response of neuron, and the beneficial effects were inhibited by XAV939.

### Netrin-1 inhibited the apoptosis of nerve cells after spinal cord injury

To further verify the neuroprotective effects of netrin-1 on SCI, we observed neuronal apoptosis by Western blot and TUNEL double staining. Western blot results (Fig. 4a) showed that the expression levels of cleaved-caspase-9

Fig. 2
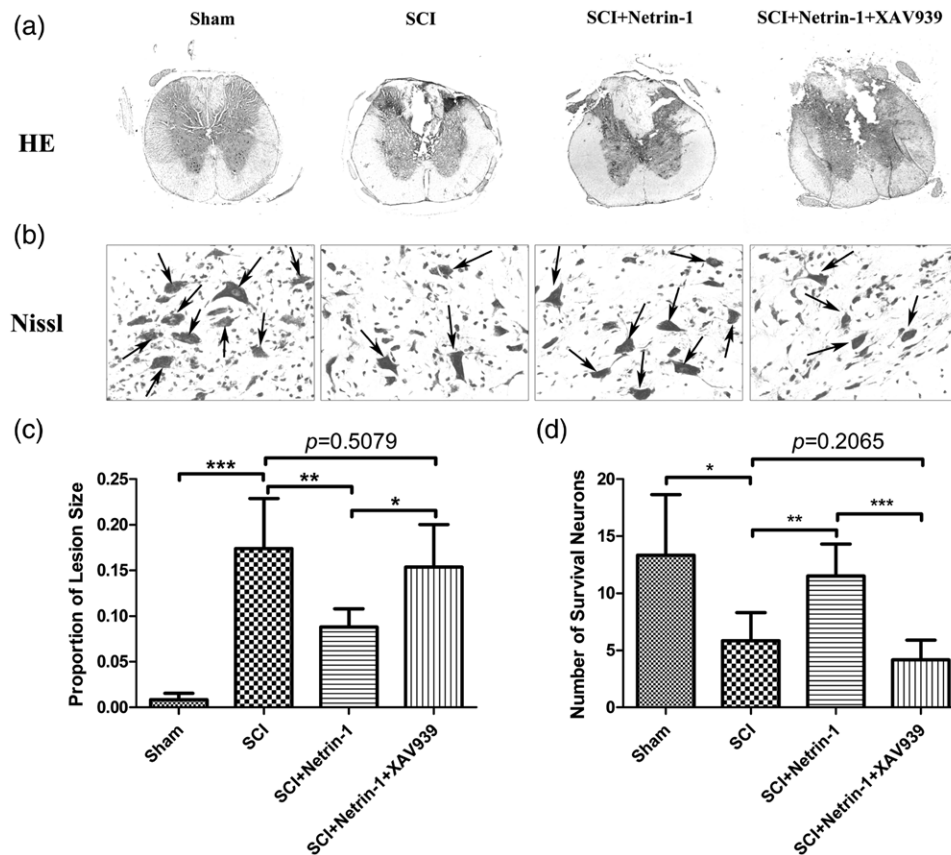
HE (a) and Nissl (b) staining was used to detect area of damaged tissue (c) and Nissl bodies in the anterior horn of spinal cord (d) in four groups on the 7th day after SCI. Scale bar = 500 μm (HE) and 100 μm (Nissl), n = 6/group, *P < 0.05, **P < 0.01, **P < 0.001. SCI, spinal cord injury.

Fig. 3
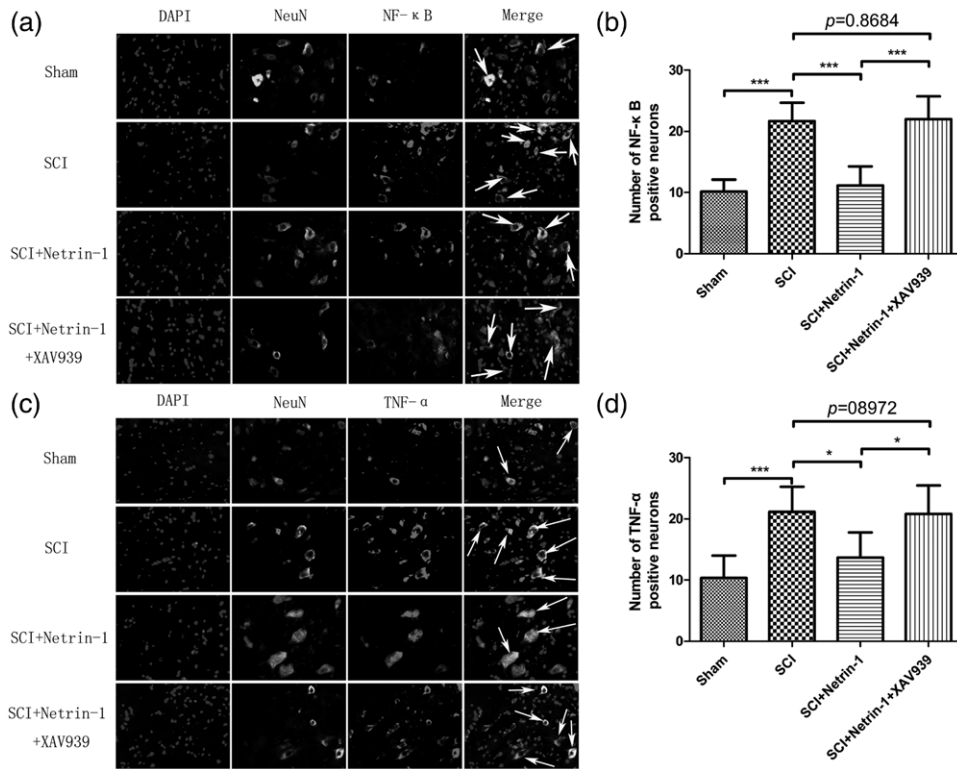
Immunofluorescence double staining (a and c) was used to detect the regulation of netrin-1 on inflammation proteins (NF-κB and TNF-α) (b and d) on the seventh day after SCI. Scale bar = 100 μm, n = 6/group, *$P < 0.05$, ***$P < 0.001$. SCI, spinal cord injury. NF-κB, nuclear factor kappa-B, TNF-α, tumor necrosis factor-α.

Fig. 4
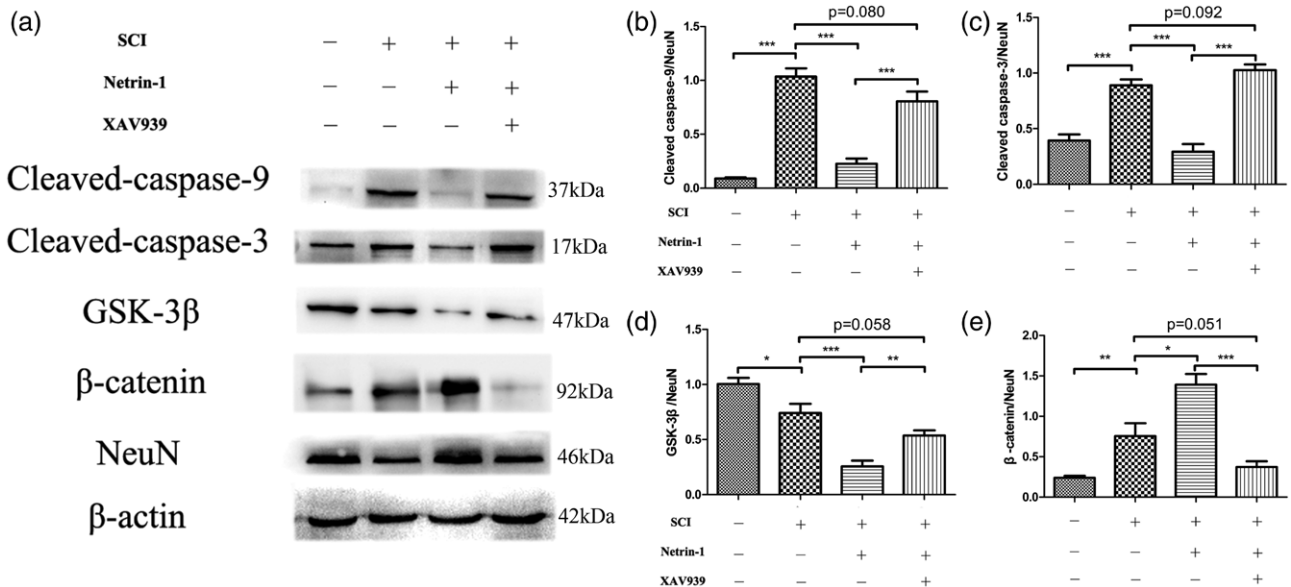
Western blot (a) was used to detect the expression of apoptosis-related proteins: cleaved-caspase-9 (b) and cleaved-caspase-3 (c) and Wnt/β-catenin signaling pathway proteins: GSK-3β (d) and β-catenin (e) in the spinal cord tissue of four groups of rats on the seventh day after SCI (n = 6/group, *$P < 0.05$, **$P < 0.01$, **$P < 0.001$). SCI, spinal cord injury, GSK-3β, glycogen synthase kinase-3β.

Fig. 5
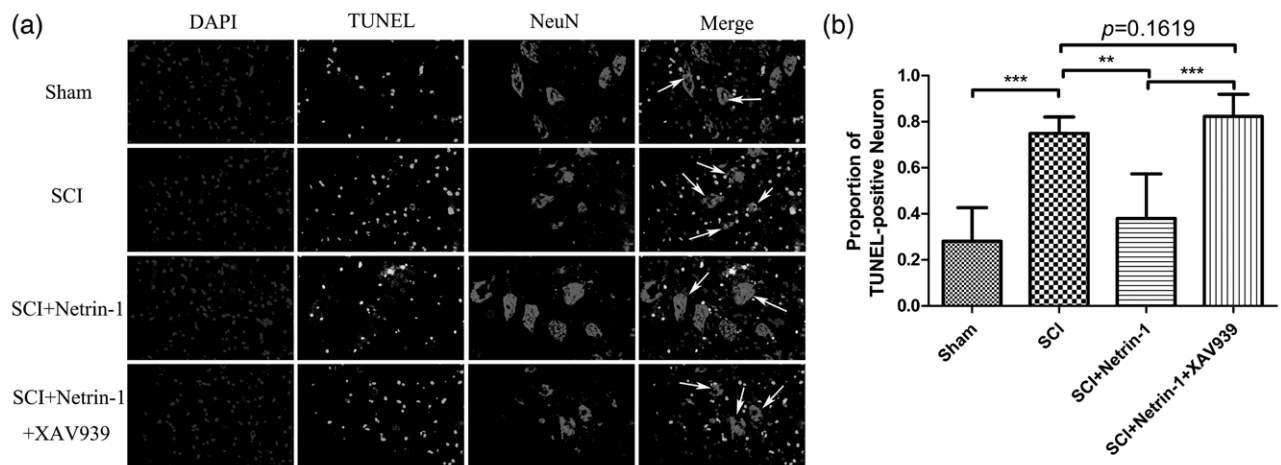
TUNEL staining (a) was used to detect the apoptosis of spinal cord anterior horn cells (b) in the four groups on the seventh day after SCI. DAPI (blue) indicates the nucleus, TUNEL (green) indicates the DNA fragment after apoptosis, and NeuN (red) indicates the nerve cell. Scale bar = 100 μm, n=6/group, **$P < 0.01$, **$P < 0.001$. DAPI, 4′,6-diamidino-2-phenylindole; NeuN, neuronal nuclei; SCI, spinal cord injury; TUNEL, transferase UTP nick end labeling.

(Fig. 4b)and cleaved-caspase-3 (Fig. 4c) in neurons were significantly upregulated after SCI. Compared with the SCI group, netrin-1 significantly decreased the expression levels of cleaved-caspase-9 and cleaved-caspase-3, suggesting that netrin-1 inhibited the apoptosis of nerve cells after SCI. However, the antineuronal apoptosis effect of netrin-1 was significantly inhibited by XAV939.

In addition, we used TUNEL/NeuN double staining (Fig. 5a) to detect changes in neuronal apoptosis in the four groups. The number of TUNEL-positive neurons after SCI was significantly higher than that in the sham group, indicating that neuronal apoptosis activity was activated after SCI (Fig. 5b). Netrin-1 treatment significantly reduced the number of TUNEL-positive neurons compared with the SCI group after SCI. Similarly, the regulation of neuronal apoptosis by netrin-1 was evidently inhibited in XAV939.

Netrin-1 activated the Wnt/β-catenin signaling pathway of neuronal cells after spinal cord injury

To verify the specific molecular mechanism of neuroprotective effect of netrin-1 on SCI, we observed the expression levels of β-catenin and GSK-3β in neuronal cells by Western blot (Fig. 4a). The results showed that the expression levels of β-catenin were increased (Fig. 4e), whereas GSK-3β expression (Fig. 4d) was inhibited after SCI. Netrin-1 further upregulated the expression levels of β-catenin and downregulated the expression levels of GSK-3β, indicating that Wnt/β-catenin signaling pathway was activated by netrin-1. When netrin-1 was co-treated with XAV939, the expression levels of β-catenin and GSK-3β were significantly reversed, which mean that netrin-1 activated Wnt/β-catenin signaling pathway of neuronal cells after SCI.

Discussion

SCI has always been a hot topic in medical research and a global medical problem [14]. SCI mainly includes two pathological processes: primary injury and secondary injury [15]. Secondary injury is caused by primary injury, oxidative stress, immune regulation disorder, inflammatory reaction, autophagy, and activation of apoptotic activity [16–18]. It plays an important role in SCI and is also an important target for the clinical treatment of SCI.

Netrin-1 protein can be combined with its specific membrane receptor—down syndrome cell adhesion molecule —to participate in the regulation of axonal branching and commissural axon transformation [19]. In addition, a study found that the mRNA and protein levels of netrin-1 decreased significantly after SCI, which lasted for several months [20]. By contrast, studies have also shown that the expression level of netrin-1 is increased after SCI, and netrin-1 protein has an effective protective effect on the spinal cord and axonal development of motor neurons [3,21]. In addition, netrin-1 can regulate autophagy activity and promote the recovery of motor function after SCI [5,6]. These two different conclusions suggest that netrin-1 may play a crucial role in SCI. In our study, we found that after SCI, netrin-1 can inhibit the expression levels of apoptosis-related proteins caspase-3 and caspase-9, inhibit the number of TUNEL-positive neurons, and inhibit the apoptosis of spinal cord neurons. Netrin-1 also inhibits the expression of neuronal inflammatory response proteins TNF-α and NF-κB and the inflammatory response following SCI. Our results also suggest that netrin-1 reduces the loss of anterior corpus callosum in SCI, promotes the repair of injured spinal

cord tissue, provides a good microenvironment for motor function recovery, and ultimately exerts neuroprotective effects. However, the specific molecular mechanism of neuroprotective effects of netrin-1 on SCI needs further clarification.

The Wnt signaling pathway is mainly divided into three signaling pathways: classical signaling pathway (Wnt/β-catenin), non-classical signaling pathway (Wnt/JNK), and Wnt–Ca^{2+} signaling pathway [7]. Recent studies have shown that Wnt classical signaling pathway (Wnt/β-catenin) plays an important role in SCI [8,22,23]. After SCI, the Wnt/β-catenin signaling pathway is activated. β-catenin is then separated from GSK-3, receives upstream molecular signals, and is transported to the nucleus to activate target gene, ultimately promoting axonal regeneration after SCI and accelerating SCI. In our previous studies on the process of recovery of posterior nerve function, we observed that methylprednisolone, rapamycin, and simvastatin exert neuroprotective effects by activating Wnt/β-catenin signaling pathway after SCI [9–11]. However, the correlation between netrin-1 and Wnt/β-catenin signaling pathway after SCI has not been elucidated.

In this study, we found that netrin-1 can significantly promote the expression of β-catenin and GSK-3β after SCI, which means that netrin-1 activates Wnt/β-catenin signaling pathway. However, when Wnt/β-catenin signaling pathway was inhibited by XAV939, the effect of netrin-1 on the regulation of apoptosis, inflammation, Nissl bodies, and damaged tissue repair on SCI was significantly inhibited.

We first studied the correlation between netrin-1 and Wnt/β-catenin signaling pathway after SCI. We found that netrin-1 exerts a neuroprotective effect by activating Wnt/β-catenin signaling pathway. Our study further validated the neuroprotective effects of netrin-1 and demonstrated the specific molecular mechanism of its neuroprotective effect on SCI, which may further promote not only the basic research of netrin-1 in the treatment of SCI but also its clinical efficacy on SCI.

Acknowledgements

Conflicts of interest
There are no conflicts of interest.

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