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Attenuation of TRPV1 by AMG-517 after Nerve Injury Promotes Peripheral Axonal Regeneration in Rats

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Abstract

Aims: The main objective was to investigate the effects of the transient receptor potential cation channel subfamily V member 1 (TRPV1) on nerve regeneration following sciatic transection injury by functional blockage of TRPV1 using AMG-517, a specific blocker of TRPV1. **Methods:** AMG-517 was injected into the area surrounding ipsilateral lumbar dorsal root ganglia (DRGs) 30 min after unilateral sciatic nerve transection. The number of sciatic axons and the expression of growth associated protein-43 (GAP-43) and glial fibrillary acidic protein (GFAP) were examined using semi-thin sections, western blot, and immunofluorescence analyses. **Results:** Blockage of TRPV1 with AMG-517 markedly promoted axonal regeneration, especially at 2 weeks after sciatic injury; the number of axons was similar to the uninjured control group. After sciatic nerve transection, expression of GFAP was decreased and GAP-43 was increased at the proximal stump. However, the expression of both GFAP and GAP-43 increased significantly in AMG-517-treated groups. **Conclusions:** TRPV1 may be an important therapeutic target to promote peripheral nerve regeneration after injury.

Keywords

Peripheral nerve injury and regeneration; transient receptor potential cation channel subfamily member V1; calcitonin gene-related peptide; glial fibrillary acidic protein; growth associated protein-43

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Introduction

Nerve injury leads to physical and mental damage. Comparatively, peripheral nerves have the capacity to regenerate after injury. Based on that characteristic, it may be possible to cure various types of peripheral nerve damage or diseases. However, the regeneration of peripheral nerves is usually unsatisfactory and delayed. To promote/accelerate regeneration after peripheral nerve injury, many previous efforts have focused on improving surgical procedures, alleviating local inflammatory reactions and supplementing nerve growth factors locally. Severe pain can develop after various peripheral nervous system injuries. TRPV1 is a well-known nociception-mediating ion channel that is abundantly expressed in peripheral sensory system, especially in the terminals and cell bodies of sensory dorsal root ganglion (DRG) neurons, trigeminal ganglion neurons, and vagal neurons[1-3]. It is activated by several physical stimuli, including temperature, voltage, protons, pH, and chemical ligands such as resiniferatoxin and capsaicin[2-5]. TRPV1 is regarded as a nociceptor, meaning that it plays an important role in peripheral neuropathic pain[2,6,7]. In addition, TRPV1 was shown to be involved in neural plasticity, cancer prevention, regulation of obesity, cardiovascular protection, and gastrointestinal mucosa integrity maintenance[6-10]. These different functions suggest that TRPV1 is involved in transmission of a variety of intracellular signals.

Our previous study showed that blockage of TRPV1 before sciatic crush injury prevented the overactivation of TRPV1 and accelerated nerve repair[11]. However, whether the attenuation of TRPV1 after nerve injury can exert the same effects on nerve regeneration, which is a plausible clinical strategy, is not yet clear. In this study, TRPV1 was blocked by AMG-517 applied ipsilaterally 30 min after unilateral sciatic nerve transection injury. We then investigated the functional activity of TRPV1, axonal regeneration in the proximal stump of the injured sciatic nerve, expression of

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growth associated protein-43 (GAP-43) and glial fibrillary acidic protein (GFAP), which is regarded as a marker of Schwann cells[12,13], to determine whether attenuation of TRPV1 after nerve injury promotes nerve regeneration.

Materials and Methods

Animal and the model of rat sciatic nerve transection injury

This is an exploratory research designed to assess the relationship between TRPV1 receptor function and nerve regeneration after sciatic injury.

42 Sprague-Dawley rats, male, 220 -240g, provided by the Experimental Animal Center of Shanxi Medical University, China (license No. SCXK (Jin)2009-0001). This study was approved by the Shanxi Medical University Animal Ethics Committee in China. All experiments were performed in accordance with the use of Laboratory Animals. Animals were kept in a clean environment where temperature and humidity are appropriate.

Animals were divided into 7 groups randomly (n = 6): 1) control group, 2) injury only groups, 3) injury+AMG-517 150 μg/kg groups, 4) injury+AMG-517 300 μg/kg groups. The groups of 1), 2) and 3) were divided into two time periods: 1 week and 2 weeks after injury.

Establish of sciatic nerve transection injury was executed according literatures[1,14]. Briefly, Animals were anesthetized with 1% sodium pentobarbital (4 mL/kg , intraperitoneally), the two hind-limbs were stretched and fixed in position. 1-2 cm incision was made lateral to the left knee, locate and expose the sciatic nerve below muscles. The sciatic nerve was transversely cut off using fine scissors just at 3mm from the bifurcations of sural, fibular and tibial nerve. The distal stump was stitched to the surrounding syndesm, far away from the proximal stump. After surgery,

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animals were housed in temperature (20～24 °C) with 45～50% of humidity and 12-hour light cycles. Normal food and water was ad libitum.

Application of AMG-517 around L_3-L_5 DRGs

The stock solution of AMG-517 (5 mM/L, Selleckchem, USA) was prepared with appropriate amount of DMSO and sterile saline and stored at −80 °C until use. Two doses of AMG-517 (150 μg/ kg or 300 μg/kg) was selected and calculated the required volume of stock solution for each animal depending on body weight.

Before the model was established, the required stock solution for each animal was diluted with sterile saline and brought the volume up to 90 μL.

After 30min of the sciatic nerve section, a dorsal median incision was made between L_2-S_1 vertebra. AMG-517 dilution with a total volume of 90 μL was injected into three sites surrounding lumbar DRGs using a microinjector, 30 μLfor each site and 0.3mm of needle insert depth. The three locations were the interstitial tissue located between two spinal transverse processes of the left L_3-L_4, L_4-L_5 and L_5-L_6. The same volume of sterile saline was applied into the injury only group at the same injection site.

Detection of the TRPV1 function by assessment of CGRP release from the dorsal-horn of spinal cord

The release of CGRP from dorsal-horn of spinal cord was regarded as one of the indicators of periphery TRPV1 activation[15]. The quantification of lumbar dorsal spinal CGRP release was assayed by ELISA. The CGRP ELISA kit was provided by Heng Yuan Biological Technology (Shanghai, China). The operation was executed according to the manufacture's manual. Summarily, lumbar spinal dorsal horns corresponding to L_3, L_4 and L_5 DRGs were harvested after 1 week and 2 weeks of sciaic nerve trasversection. Tissues were grinded and suspended in cold phosphorylate buffer saline (0.01mmol/L). Total protein concentration of each sample was measured using Bradford method. 30 μg of protein for each sample was added into a

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96-well -plate. The absorbance values at 450 nm wavelength was measured and CGRP concentration was calculated using SpectraMax® 190 Microplate Reader (Molecular Devices, USA).

Quantification of the number of sciatic axons and karyocytes

The quantification of the number of sciatic axons and karyocytes was done at semi-thin section with toluidine blue staining[16]. Briefly, the proximal stumps (about 1 cm) of sciatic nerve were harvested at the time points described as above, three rats for each group. The harvested nerve stems were quickly immersed into pre-cold 2.5% glutaraldehyde and fixed for 4 hours, and then the samples were post-fixed in 1% osmium tetroxide for 2 hours. After dehydration with acetone, the samples were embedded with epoxy resin (Araldite Epon-812) and cut into 1μm thickness (semi-thin) of transverse sections by an Ultra-Microtome（LKB，Sweden）, then the sections were stained with 1% of toluidine blue. 8~9 sections were collected for each nerve, which were selected by the appearance of complete epineurium under microscope . Sections were checked under upright light microscopy (Olympus BX43, Japan). 5 high power images (100×, including the up, down, left, right and center areas of the nerve) for each section were captured randomly. Count the number of axons in each image and then calculate the average number of axons in each group. In addition, the counts of axons and karyocytes on each section was finished by the way of double blind trial.

SDS-PAGE/western blot analysis

Sciatic nerves and left L_3-L_5 DRGs were dissected and homogenized rapidly on ice at the time point of 1 week and 2 weeks after sciatic injury. The protein concentration of each sample was determined by Bradford assay (Sangon Biotech, China). 20μg homogenate of DRGs and 30 μg homogenate of sciatic nerves were uploaded onto 10% acrylamide gel for SDS-PAGE. The protein was then transferred onto PVDF membranes. Afterwards, the membranes were blocked with 5% no-fat

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milk solution, and then incubated with mouse anti-TRPV1 (1:4000,Biosensis, USA), or mouse anti-GFAP (1:2500, Novus Biologicals, USA), or rabbit anti-GAP-43 (1:5000, Bioworld, USA) overnight at 4°C. On the second day, the membranes were washed with 0.1% TBST, 3×5min, and then incubated with for 1 hour in horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:5000, Sangon Biotech, China) or HRP-conjugated goat anti-rabbit IgG (1:5000, Sangon Biotech, China). Finally, the protein bands were detected after incubated in Easy See Western Blot ECL reagent (Sangon Biotech, China) and exposed onto X-ray film in dark room. Meanwhile, rabbit anti-GAPDH (1:5000, Bioworld, USA) was used as a loading control.

Immunofluorescence of sciatic nerve

Sciatic nerves were dissected rapidly at the time point of 1 week and 2 weeks after sciatic nerve transection, and then were freshly embedded with OCT and fixed in the liquid nitrogen. Samples were stored at −80°C till use. Nerves were longitudinally cut into sections of 12μm thickness. Put the slices onto glass slides which were pre-coated with 0.1% polylysine. The sections were dried in the air completely and immersed into 4% paraformaldehyde for 12 hours of fixation. Sections were first washed three times with 0.01M PBS, and incubated in 0.1% Triton for 10 minutes. After 1 hour of incubation with 10% normal serum, two primary antibodies [mouse anti-GFAP (1:1000, Novus Biologicals, USA) and rabbit anti-GAP-43 (1:500, Bioworld, USA)] were applied onto slices, incubated overnight at 4°C. On the following day, the secondary antibodies of Alexa Fluor® conjugated with 488 or 594 (1:500, Lifetechnology, USA) were loaded onto slices after substantially washing with 0.01M PBS. Finally, the sections were mounted with an antifade-mount medium (Lifetechnology, USA). Immunofluorescence imags of GFAP and GAP-43 were observed under uprighted immnofluorescence microscope (Olympus BX43, Japan).

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Statistical Analyses

All of the data were expressed as mean ± standard deviation (SD). The date was analyzed using Graph-pad prism 5.0 software, two-way ANOVA. $p < 0.05$ was regarded as statistically significance.

Results

AMG-517 blockage of TRPV1 decreases calcitonin gene-related peptide (CGRP) release from spinal dorsal horn

Enzyme-linked immunosorbent assay (ELISA) showed that there was no significant difference in lumbar dorsal CGRP release between 1 week after sciatic transection injury and the control group (Fig. 1, the concentration of CGRP (C_{CGRP}) in these two groups was approximately 0.15 ng/g, $p > 0.05$). However, the release of CGRP was increased 2 weeks after sciatic injury (Fig. 1, $C_{CGRP} = 0.17$ ng/g, $p < 0.05$), which indicated that TRPV1 was overactivated 2 weeks after peripheral nerve injury.

The administration of AMG-517 to both 1 week and 2 week groups after injury induced a decrease of CGRP release (Fig.1, 1 week: $C_{CGRP\text{-}150\mu g/kg} = 0.11$ ng/g, $C_{CGRP\text{-}300\mu g/kg} = 0.11$ ng/g, 2 weeks: $C_{CGRP\text{-}150\mu g/kg} = 0.095$ ng/g, $C_{CGRP\text{-}300\mu g/kg} = 0.11$ ng/g, $p < 0.001$). This suggested that the injection of AMG-517 around DRGs was efficient to inhibit the function of TRPV1. Further, both doses of AMG-517 (150 μg and 300 μg/kg) were sufficient to block TRPV1, and there was no linear dose response for inhibition of CGRP release (Fig.1, $p > 0.05$).

Expression of TRPV1 in DRGs

Western blot for TRPV1 expression in DRGs showed that the expression of TRPV1 increased significantly after sciatic injury, which was semi-quantitatively determined to be almost twice of the expression in the control group (Fig. 2, $p < 0.001$), with much highly increased expression at 2 weeks. However, a strong decrease in expression was seen in tissue samples treated with AMG-517 (Fig. 2, $p < 0.001$). This result indicated that TRPV1 was upregulated both functionally and structurally after

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sciatic injury. The activation of TRPV1 after nerve injury may be regarded as overexpression, which was inhibited by injecting AMG-517 in the area surrounding DRGs.

Attenuation of TRPV1 function facilitated axonal regeneration

Semi-thin transverse sections (1-μm thickness) from the proximal stump of transected sciatic nerves were stained by toluidine blue. Sections from the different experimental groups exhibited some interesting morphological patterns, providing evidence that TRPV1 is involved in nerve regeneration. Normal sciatic nerves mainly consist of bundles of myelinated axons, with few cells that can be seen under the light microscope (Fig. 3A). One week after sciatic nerve injury, the normal structures disappeared, a lot of debris was observed at the terminus of the proximal stump and few axons could be found (Fig. 3A). At a longer time after injury (2 weeks), some myelinated axons were found amongst many cells and tissue debris (Fig. 3A). There was no obvious difference in the number of axons in the 1 week injury-only group compared with the group treated with AMG-517. However, 2 weeks of AMG-517 treatment induced strong regeneration of axons in the proximal stump compared with the untreated control group (Fig. 3, $p < 0.001$). Moreover, the sciatic nerve axons with 2 weeks of AMG-517 treatment were well-organized and included both myelinated and non-myelinated regenerating axons (Fig. 3A).

Blockage of TRPV1 function modified the cellular response after sciatic nerve injury

Interstitial cellular reactions were another feature of injured sciatic nerves. There were a few karyocytes in normal sciatic nerve transverse sections (Fig. 4, the number of karyocytes ($N_{karyocyte}$) was approximately 16). Quantitation of karyocytes in injured sciatic nerve transverse sections showed an obvious interstitial cellular response in each group at 1 week, which was higher in the AMG-517 intervention groups (Fig.4, injury-only group: $N_{karyocyte} = 61$, AMG-517 150 μg/kg: $N_{karyocyte} = 85$, AMG-517 300 μg/kg: $N_{karyocyte} = 112$, $p < 0.001$). Based on the presence of intracellular engulfed

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components, most of the cells appeared to be phagocytes (Fig. 4A). Two weeks after sciatic nerve injury, there was a decrease in the cellular response in nerves from the AMG-517 treatment groups (Fig. 4, injury-only group: $N_{karyocyte} = 101$, AMG-517 150 μg/kg: $N_{karyocyte} = 33$, AMG-517 300 μg/kg: $N_{karyocyte} = 24$). Further, tissue debris was scattered and the number of phagocytes decreased. Instead, Schwann cells enclosed regenerating axons in well-organized axon bundles, although myelination was not as good as in the non-injured controls (Fig. 4). Overall, the sciatic nerve transverse structure 2 weeks after injury with AMG-517 treatment appeared more normal. The results suggested that AMG-517 accelerated nerve regeneration by promoting elimination of disintegrated axons and myelin, benefitting remyelination by Schwann cells and increasing axon regeneration.

Increased Schwann cell responses by blockage of TRPV1

The cellular response described above indicated that Schwann cells were modulated by the inhibitory effect of AMG-517 on TRPV1. Morphologically, the cells that were found around regenerating axons looked like Schwann cells. To verify the cell type, immunofluorescence analyses and western blot for GFAP were performed. The results revealed that most of the cells 2 weeks after injury with AMG-517 treatment were Schwann cells. Western blot for GFAP showed that the expression of GFAP was low 1 week after injury ($p < 0.001$) but was almost recovered 2 weeks after injury (Fig. 5B). GFAP was upregulated by TRPV1 blockage using AMG-517 (both 1 week and 2 weeks,Fig. 5). Apart from this, the GFAP immunofluorescence formed a trimmed cord-like pattern, similar to the uninjured control (Fig. 5A). There is no doubt that the proliferation and reorganization of Schwann cells facilitated the axonal regeneration.

Blockage of TRPV1 upregulates the expression of GAP-43

To verify the role of TRPV1 blockage in nerve regeneration, immunofluorescence and western blot of GAP-43 was also applied after sciatic transection injury with/without AMG-517 application. Semiquantitative analysis showed that the GAP-43 intensity at

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the proximal stump of the sciatic nerve was eventually increased after injury. However, the administration of AMG-517 made the increased intensity more marked (Fig. 6, $p < 0.001$). GAP-43 positive fibers eventually became well-ordered with AMG-517 treatment, which coincided with the regenerated axons shown in Fig. 3. The two dosages selected in this study showed no significant differences. These results indicate that inhibition of TRPV1 may activate regeneration-associated signaling pathways to overcome insufficient nerve repair resulting from microenvironmental alteration caused by the overactivation of TRPV1.

Discussion

The peripheral nervous system is susceptible to a variety of injuries but has the ability to regenerate after injury. Successful regeneration or increased speed of injury repair would positively impact on patients' quality of life. Prompt and well-established nerve regeneration following peripheral injury promotes nervous function recovery. Peripheral nerve regeneration involves the regrowth of axons and the proliferation and myelination of Schwann cells. Alterations in the local environment after injury can interrupt the regenerative procedure and delay repair. The local microenvironment changes include accumulation of tissue debris, an excessive inflammatory reaction and activation of TRPV1[1,17]. Therefore, a variety of factors can impact nerve regeneration after peripheral nerve injury. TRPV1, a receptor that mainly plays an important role in the transmission of pain signals, has long been regarded as one of the targets for pain therapeutics. The mechanism by which TRPV1 induces hyperalgesia or allodynia is by contributing to an increase in the release of pain neuropeptides (substance-P, CGRP) from the dorsal horn of the spinal cord. Apart from this, TRPV1 is involved in other intracellular signaling pathways through increasing calcium influx and interacting with other membranous receptors involved in neural plasticity, cancer prevention, cardiovascular protection[6-10], inflammation, regeneration and infarcts[18]. In our previous study[11], TRPV1 was first blocked by the

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subcutaneous injection of AMG-517 into the ipsilateral plantar 60 minutes prior to sciatic nerve crush. Two weeks after injury, the number of axons after TRPV1 blockage was increased compared with no TRPV1 inhibition. The results indicated that inhibition of TRPV1 prior to injury aided nerve rehabilitation following injury. However, clinically, therapeutics for nerve injury is generally administered after the event. Thus, more clinically relevant confirmation that the functional attenuation of TRPV1 facilitates nerve repair after injury was required. As we have already known, TRPV1 is also abundantly expressed in the neuronal membrane of DRGs as what its expression in peripheral nerve terminals. Thus, the blockage of TRPV1 in L_3~L_5 DRGs by injection of AMG-517 surrounding these ganglias will affect both the intracellular signal pathways in both afferent and efferent processes from DRGs. The decrease of CGRP release can be regarded as the result of the intracellular signal pathway alteration by TRPV1 blockage to the afferent process of DGR. On the other hand, the difference of sciatic nerve regeneration between the groups of sciatic injury only and injury plus AMG-517 can be contributed to the peripheral effect of TRPV1 blockage in DRGS. In this study, the blockage of TRPV1 commenced 30 min after sciatic transection injury, which increased the number of sciatic axons and the expression of GAP-43 and GFAP over the same time periods after injury. This investigation provides further evidence that TRPV1 is actively involved in nerve regeneration after injury, beyond its role in nociception signaling pathways. The results also indicate that the severe pain induced by the overactivation of TRPV1 after peripheral nerve injury can delay nerve repair and rehabilitation. Therefore, specific strategies for blocking TRPV1 after nerve injury are suggested and should be examined clinically in the future.

AMG-517 is a potent and selective TRPV1 antagonist that has been extensively applied in many investigations for interruption of TRPV1 function[19,20]. Based on dosages mentioned in the literature, two doses (150 μg/kg and 300 μg/kg) were selected in this study and injection of either dose around DRGs was very effective at

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inhibiting spinal CGRP release, showing that local AMG-517 injection around DRG can efficiently block overactivation of TRPV1. No dose dependence was found in the parameters explored, therefore, 150 μg/kg of AMG-517 is suggested as the better dosage for research. It is interesting that the application of AMG-517 in this study not only inhibited TRPV1 function but also reduced its expression in DRGs. But this decrease in TRPV1 expression needs to be verified in future studies.

Peripheral nerve regeneration after injury involves the regeneration of axons and the proliferation of Schwann cells. Moreover, the proliferation and rearrangement of Schwann cells is essential for the accomplishment of complete structural and functional nerve repair. This study showed that functional block of TRPV1 increased the elimination of degraded debris by infiltration of macrophages and pinocytosis, providing a good environment for nerve branching and Schwann cell proliferation.

With lasting TRPV1 blockage, the number of Schwann cells was increased and they appeared to be wrapped around newly regenerated axons. It is well known that Schwann cells can produce various neurotrophic factors[12,13]. Generally, Wallerian degeneration and nerve structure collapses occur after nerve transection injury, leading to lose of neurotrophic factors from Schwann cells. Any therapeutic that can promote Schwann cell recovery after Wallerian degeneration will therefore likely promote nerve repair after injury. From the results of this study, we speculated that prevention of TRPV1 overactivation will benefit not only pain relief but also promote nerve regeneration after peripheral injury.

Peripheral nerve injury leads to upregulation of many growth factors and regeneration-associated proteins, including actin, tubulin and GAP-43[21,22]. GAP-43, a phosphoprotein in the axoplasm, is mainly expressing in the immature brain and in regenerating neurites as part of the neuronal growth cone[22-24]. GAP-43 is regarded as a marker for regeneration of axons[22,25,26] and increased expression of axonal GAP-43 is important evidence of nerve regeneration. This study showed an increase in axonal expression of GAP-43 in the sciatic nerve after AMG-517 intervention. We propose

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that inhibition of the excessive activation/overexpression of TRPV1 after injury by AMG-517 injection around DRGs accelerates nerve regeneration by promoting both axonal GAP-43 expression and Schwann cell proliferation.

In summary, our study revealed a direct effect of TRPV1 on peripheral nerve regeneration after injury. Therapeutic studies on various peripheral nerve injuries should be aimed at the functional modulation of TRPV1 to relieve severe pain and promote faster nerve repair. However, the specific mechanisms by which TRPV1 promotes nerve regeneration need more detailed investigation to be fully elucidated.

Author contributions

Xia-Qing Li was responsible for the study design. Juan Bai drafted manuscript, collected data and performed analysis. Fu Liu, Li-fei Wu and Ya-Fang Wang reviewed and inspected the manuscript. All authors read and approved the final manuscript.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical Approval

Research involving animals were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996. This study was approved by the Shanxi Medical University Animal Ethics Committee in China. In this study all efforts were made to minimize the number of animals used and their suffering. All experiments were performed in accordance with the use of Laboratory Animals.

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List of abbreviations

TRPV1: transient receptor potential cation channel subfamily V member 1

DRG: dorsal root ganglia

CGRP: calcitonin gene-related peptide

GAP-43: growth associated protein-43

GFAP: glial fibrillary acidic protein

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electropheresis

ELISA: enzyme-linked immunosorbent assay

PVDF: polyvinylidene fluoride

TBST: Tris-Buffered Saline and Tween 20

PBS: phosphate buffered solution

HRP: horseradish peroxidase

OCT: optimal cutting temperature compound

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Figure 1. Quantization for the release of CGRP from dorsal-horn of lumbar spinal cord by ELISA method ($^{\#}p < 0.05$ *vs.* control group, $^{\#\#\#}p<0.001$ *vs.* control group, *** $p< 0.001$ *vs.* injury only groups).

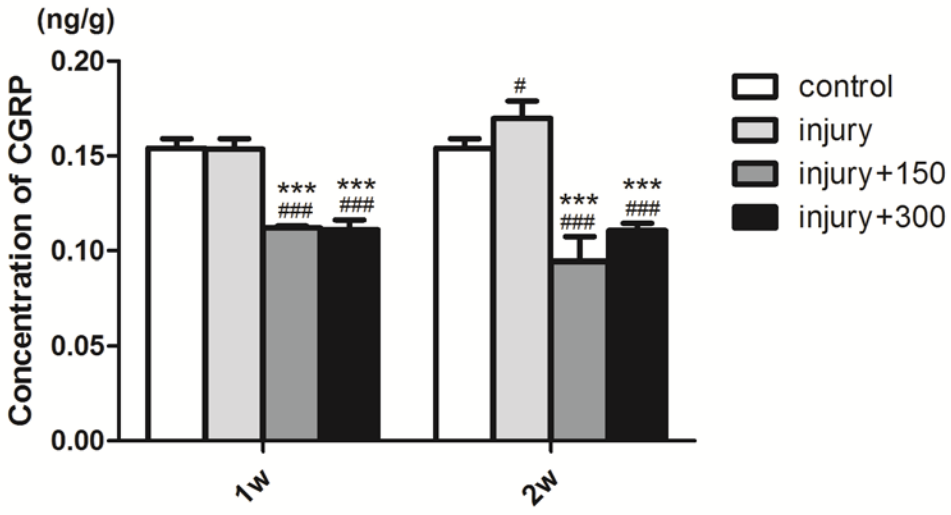

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Figure 2. The expression of TRPV1 on DRG in each group

(A) Bands of western blot for TRPV1. (B) Statistic graph for integrated optical density of TRPV1 in DRG ($^{\#\#}P<0.01$ *vs.* control group, $^{\#\#\#}P<0.001$ *vs.* control group, *** $P< 0.001$ *vs.* injury only groups).

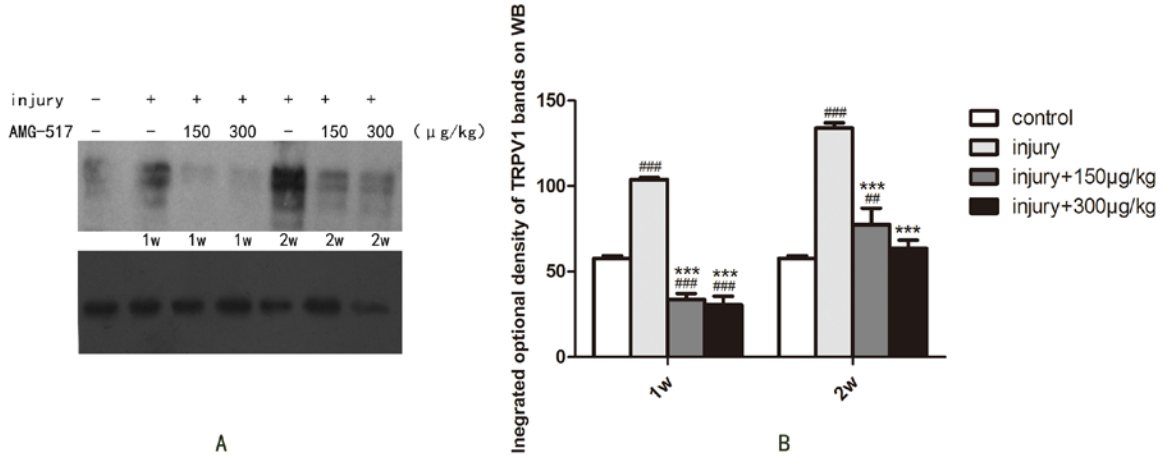

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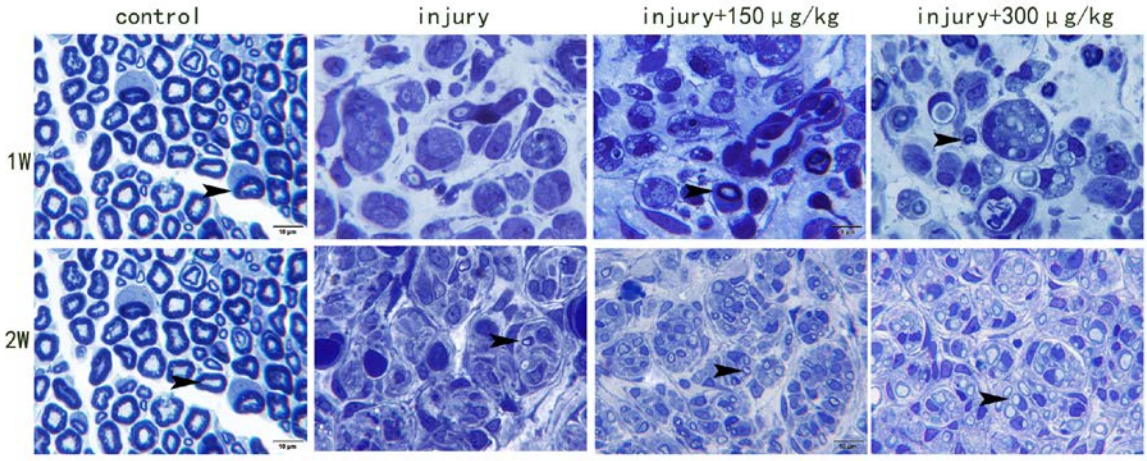
Figure 3. Semi-thin transverse sections of sciatic nerves and axon quantification analysis

(A) The transverse sections of sciatic nerve in each group. (B) Statistic graph for the axonal number in each group. $^{\#}P<0.05$ compared to the control group, $^{\#\#\#}P<0.001$ compared to the control group, *** $P< 0.001$, compared to the injury only groups. ➤ indicating axons, Scale bar =10 μm.



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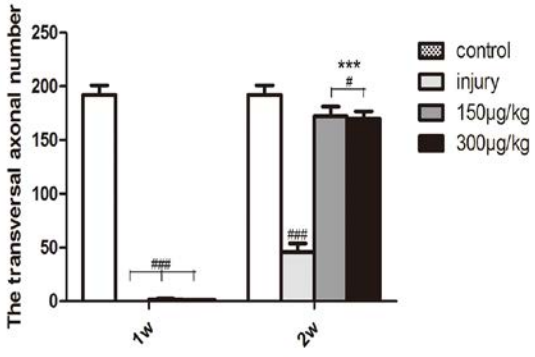

B

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Figure 4. Semi-thin transverse sections of sciatic nerves and karyocyte quantification analysis

(A) Observation of the sciatic transverse sections in each group. (B) Statistic graph for the number of karyota in each group. $^{\#}P<0.05$ compared to the control group, $^{\#\#\#}P<0.001$ compared to the control group, *** $P< 0.001$ compared to the injury only groups. ➡ the macrophages, ➤ the Schwann cells, ➙ the interstitial tissue, Scale bar=10 μm.

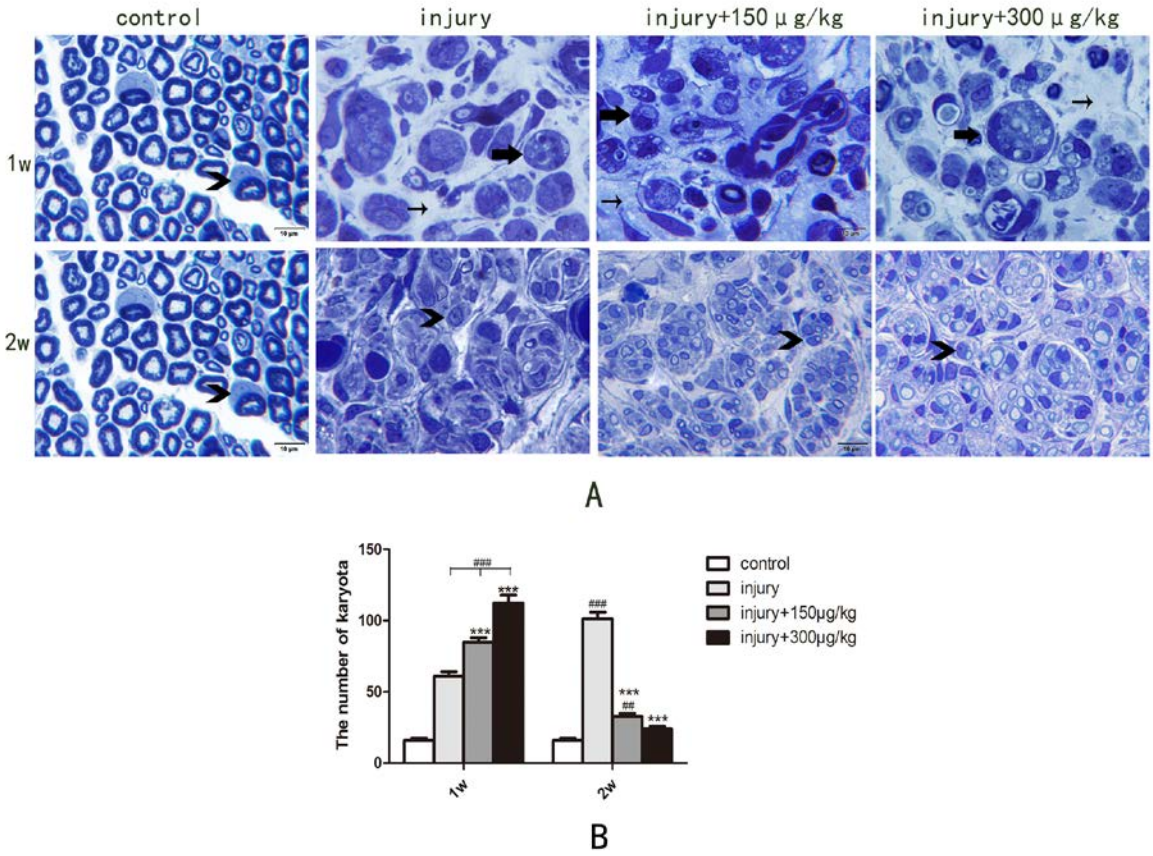

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Figure 5. Immunofluorescence and western blot for GFAP

(A) Immunofluorescence of longitudinal section of sciatic nerve in each group. The green fluorescence represents GFAP positive fibers. (B) Bands of western blot for GFAP. (C) The statistic graph for integrated optical density of western blot bands ($^{###}P<0.01$ compared to the control group, *** $P < 0.001$ compared to the injury only groups. Scale bar=50 μm).

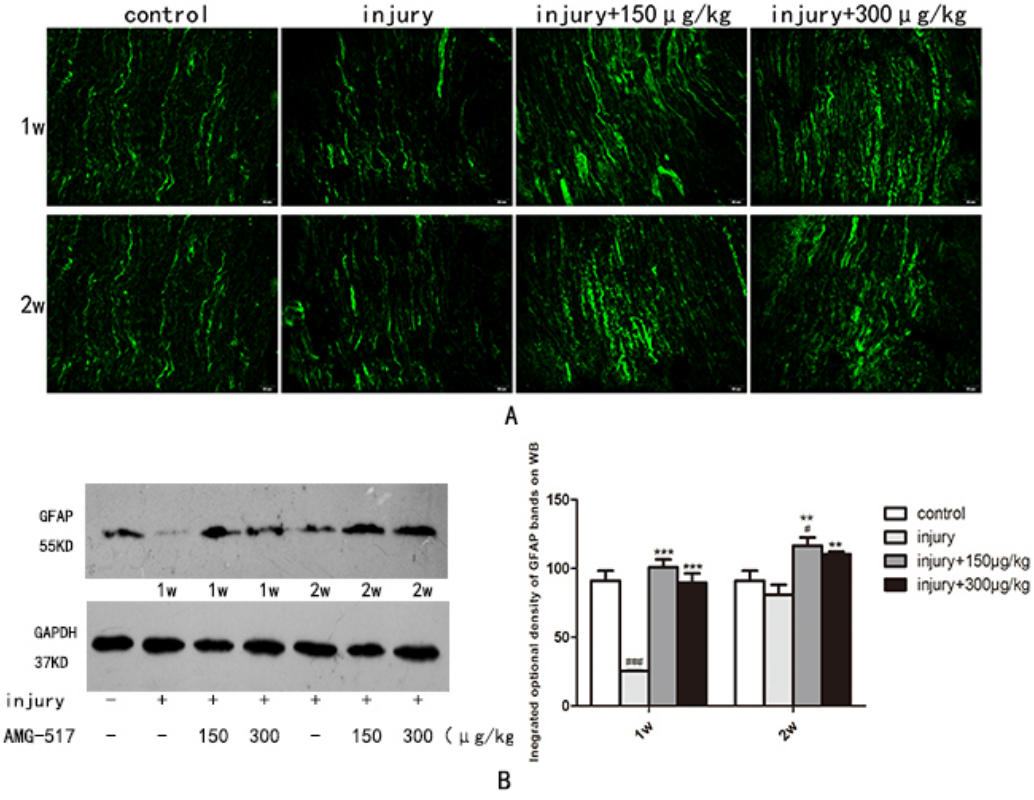

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Figure 6. Immunofluorescence and western blot for GAP-43

(A) Immunofluorescence of longitudinal section of sciatic nerve in each group. The red fluorescence represents GAP-43 positive fibers. (B) Bands of western blot for GAP-43. (C) The statistic graph for integrated optical density of western blot bands ($^{###}P<0.01$ compared to the control group, $^{**}P<0.01$ compared to the injury only groups. $^{***}P<0.001$, compared to the injury only groups; Scale bar=50 μm).