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Research paper

BDNF promotes the axonal regrowth after sciatic nerve crush through intrinsic neuronal capability upregulation and distal portion protection

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HIGHLIGHTS

- BDNF enhances the outcomes of functional recovery after sciatic nerve crush injury.
- BDNF triggers multiple effects to promote axon regrowth.
- The activation of neuronal intrinsic regeneration is increased.
- The distal portion atrophy is delayed.

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ABSTRACT

Nowadays peripheral nerve injurie occurs more common, the outcome is often poor because of the ineffective treatment. Recent researches revealed the duration of BDNF administration acts a positive role during the nerve regeneration, but its potential mechanisms beneath the behavioral recovery and axonal regrowth after peripheral nerve injury are still controversial. To observe the potential mechanisms we established sciatic nerve injury model and detected the expression of several axonal regeneration and function related genes. The results showed that, BDNF promotes axonal regrowth through increasing the activation of neuronal intrinsic growth capacity and strengthening the deference effects against distal portion atrophy. To further study, we determined the expression of protein associated to neuronal intrinsic growth capacity and investigated the ultrastructure of the distal portion of the injured nerve were analyzed. These data revealed that BDNF triggers multiple effects including neuronal intrinsic growth capacity improvement and distal portion atrophy protection to promote behavioral recovery following sciatic nerve crush injury in mouse.

1. Introduction

Today, injury to the nerve of peripheral nervous system (PNS) occurs commonly and lacks of effective treatment, which results in long-term disabling and reducing the quality of life to clinical patients. According to the previous studies, the prevalence of peripheral nerve injury arises to about 3% among trauma patients [12] which needed surgical repair. Despite decades of significant advances in human nerve repair, the patients would often still facing suboptimal function recovery after clinical surgery [15]. The main issues we need to address are how to promote axonal regeneration while prevent the chronic denervation changes of distal portion [1]. In the recent studies, researchers found that brain-derived neurotrophic factor (BDNF) has play some positive roles during the nerve regeneration in the central nervous system, including promoting the molecules (such as cAMP, Gap-43 and c-jun) expression which contributes to regulating intrinsic axonal regeneration [9,10,5,7]. Additionally, BDNF may also be involved in providing nutritional supports for the distal portion [4,16,8]. In this research we hypothesized that BDNF could increase the behavioral

Abbreviations: BDNF, brain derived neurotrophic factor; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; CREB, cAMP-response element binding protein; Gap-43, growth associated protein 43; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GDNF, glial cell line-derived neurotrophic factor; PNS, peripheral nervous system; qRT-PCR, quantitative real time polymerase chain reaction; TEM, transmission electron microscopy; STAT3, signal transducer and activator of transcription 3.

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function through promoting intrinsic axonal regeneration capability and protecting distal portion from denervation. So we designed and performed the experiments as follow.

2. Experimental procedures

2.1. Animals

Adult male C57BL/6J mice (180 in total) weighing 20–22 g were used in this research. All animals were maintained in a temperature and humidity controlled environment, and allowed for standard mouse chow and water ad lib, with a 12:12 h light: dark cycle.

2.2. Surgery

All experimental procedures were approved by the Administration Committee of Experimental Animals, Nanjing Medical University. All surgical experiments were performed by inhalation anesthetic (2% isofluorane in 98% oxygen) on adult male mice (8–12 weeks old). After that, the sciatic nerve was squeezed with smooth forceps for 60 s. After injury, the wound was sutured and the mice were allowed to recover on heated pad. In the sham-operated controls, the sciatic nerve was only exposed but without crush injury.

2.3. Experimental design

We separated all animals into four groups in this research (n = 10 per group): (1) sham-operated controls; (2) model group which sciatic nerve was performed crash injury; (3) BDNF administration (200 pg/μL in sterile distilled water; Life Technologies, Grand Island, NY, USA) group which mice were treated with BDNF after crash injury; (4) anti-BDNF treatment (1.5 μg/μL in sterile distilled water; Chemicon, Temecula, CA, USA) group which mice received anti-BDNF treatment after crush injury. All drugs were intramuscular injected in plantar muscle on left hindlinb of mouse once daily at a volume of 0.1 mL.

2.4. Toe spreading test

To assess motor recovery after nerve injury (sciatic nerve crush injury), the movement of the each toe was evaluated. We performed the analysis as described before [2]; Fig. 1A). Full spreading was defined as a complete, wide, and sustained (at least 2 s) spreading of each toe. Mice were scored when a full response was observed on the contralateral side to the injury. Mice were evaluated twice in each experimental session with at least a 45-minute interval. Scoring was done by whom blinded to the group design.

2.5. qRT-PCR

Dissociated L4 and L5 spinal cord were prepared from experimental adult male mice at day 14 after the surgical procedure. For tissue preparation, mice were anesthetized by chloral hydrate (7%), then an incision was made at the back and the spinal cord was carefully exposed. Removed L4 and L5 of the spinal cord then minced with eye scissors on ice. Total mRNA of tissues was isolated by using TRIzol Reagent (Invitrogen, Grand Island, NY, USA) then reverse-transcribed by using the PrimeScript™ RT reagent Kit (TaKaRa, Japan). RT-qPCR was performed with the corresponding primers (Table 1) and the SYBR® Premix Ex Taq™ II (Takara, Japan) by using StepOnePlus™ System (Applied Biosystems). Gene expression was normalized by 18s and data was analyzed with StepOne Software and fold changes determined with GraphPad Prism 5 Software.

2.6. LANCE™ cAMP assay

As mentioned before, L4 and L5 the spinal cord were removed then minced with eye scissors on ice. The samples were then lysed with lysis buffer on ice for 10 min. After that, spun at 14000 rpm (16000 g) for 10 min at 4 °C. Finally collected the supernatant and assessed the cAMP concentration according to LANCE™ cAMP 384 Kit (PerkinElmer Life and Analytical Sciences, Inc. USA) instruction.

2.7. Western blotting assay

As described before, dissociated L4 and L5 spinal cord were prepared from experimental adult male mice at day 14 after the surgical performance. Protein samples from spinal cord were extracted using RIPA buffer (Beyotime, Nanjing, JS, China) supplemented with a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). After protein concentration determination, equal amounts of protein was loaded in each lane, resolved on 10% SDS poly-acrylamide gradient gels, and transferred onto a 0.45-μm NC membrane (Milipore, Billerica, MA, USA). Then membrane was blocked with 5% BSA and incubated with anti-Gap-43 (1:1000; Milipore), anti-*p*-STAT3 (1:1000; Abcam), anti-STAT3 (1:1000; Abcam), anti-*p*-cjun (1:1000; Abcam), anti-cjun (1:1000; Abcam), anti-CREB (1:1000; Abcam) and anti-GAPDH (1:1000; Sigma) overnight at 4° C, washed, and incubated with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies. Immunoreactive bands were visualized by the Immobilon Western Chemiluminescent HRP Substrate (Milipore). Quantification of band intensity was performed by Quantity One software (Bio-Rad, Hercules, CA, USA). The intensity of the Gap-43 band was normalized to GAPDH. Experiments were repeated in triplicate.

2.8. Transmission electron microscopy (TEM)

We dissociated the sciatic nerve from adult male mice at 5 weeks after sciatic nerve crush injury. Samples of the sciatic nerve were prepared as previously described [13]. All specimens were examined with Hitachi H-7000 electron microscope and images were acquired through a SIA-8CCD camera mounted on the microscope for examination with Northern Eclipse Software (Cheektowaga, NY, USA). The reduction in myelin thickness was confirmed by calculating the G-ratio of the individual myelinated fibers (at least 100 per group), which was calculated as axon diameter/fiber diameter.

2.9. Statistical analysis

All data were presented as the mean ± SEM, differences between groups were compared by using a one-way analysis of variance (ANOVA), with post-hoc Student LSD (least significant difference) pairwise comparisons applied as appropriate. For all behavioral test analysis, 2-way repeated measures ANOVA, with post-hoc Bonferroni analysis between groups where appropriate was utilized. Significant differences were considered for $p < 0.05$. Statistical calculations were carried out with GraphPad Prism 5 Software.

3. Results

3.1. BDNF promotes motor functional recovery after sciatic nerve injury in mouse

To investigate the role of BDNF during peripheral nerve repair after injury, we initially established sciatic nerve injury model in C57BL/6J mouse and recorded the recovery process as described before [2]; Fig. 1A). The data showed that the sciatic nerve crush injury induces a severe damage to the motor function of mice

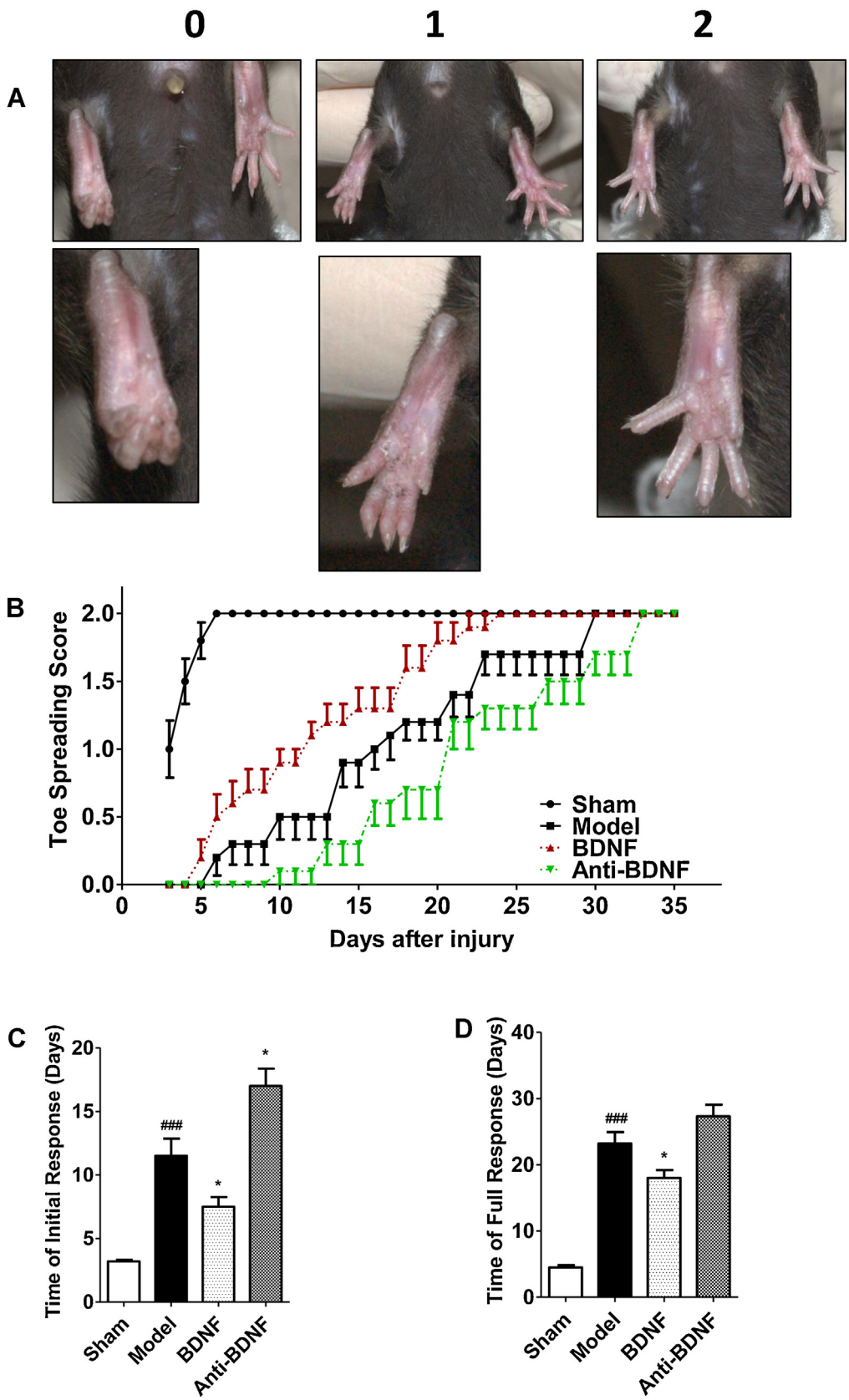


Fig. 1. BDNF promotes the motor functional recovery after sciatic nerve injury in mouse by toe spreading test after sciatic nerve crush injury. (A) Representative pictures of mice at various stages of recovery after sciatic nerve crush. Score 0—no spreading, 1—intermediate spreading with all toes, 2—full spreading. (B) Motor recovery, assessed by toe spreading test. BDNF administration group mice had a faster appearance of the initial (C) and full (D) of the sensory response in comparison to model group after sciatic nerve crush. ($n = 9–10$ per group, data are presented as mean ± SEM; $^{###}p < 0.001$ compared with sham, $^{*}p < 0.05$ compared with model; one-way ANOVA followed by Turkey test).

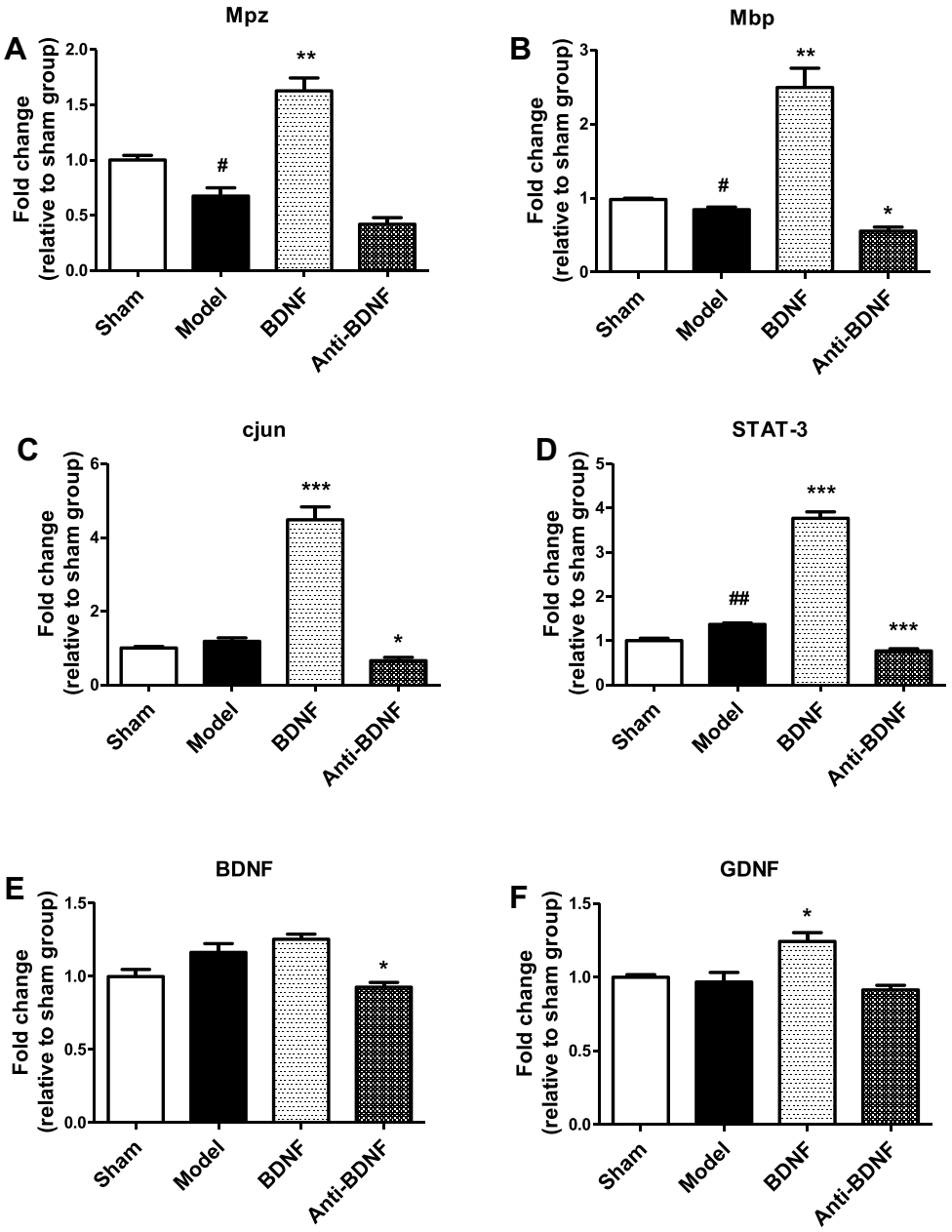


Fig. 2. The mRNA expression tested by qPCR after sciatic nerve crush injury in mouse. BDNF increases the mRNA expression of Mpz (A), Mbp (B), cjun (C) and STAT3 (D), but without changing the mRNA expression of BDNF (E) and GDNF (F). (n = 3 per group, data are presented as mean ± SEM; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with sham; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with model; one-way ANOVA).

Table 1
The primers used in RT-qPCR.

Gene	Forward PCR primer	Reverse PCR primer	Product length
STAT3	CAATACCATTGACCTGCCGAT	GAGCGACTCAAACTGCCCT	109
Jun	CCTTCTACGACGATGCCCTC	GGTTCAAGGTCATGCTCTGTTT	102
BDNF	TCATACTTCGGTTGCATGAAGG	AGACCTCTCGAACCTGCCC	137
GDNF	TATGGGATGTCGTGGCTGTC	CAGGCATATTGGCGGCGG	78
Mbp	AATCGGCTCACAAGGGATTCA	TCCTCCCAGCTTAAAGATTTTGG	74
Mpz	CGGACAGGGAAATCTATGGTGC	TGGTAGCGCCAGGTAAAAGAG	106

(Fig. 1B). At the meanwhile, BDNF administration accelerated the recovery process while anti-BDNF treatment delayed. For further confirmation of the functional recovery promotion, we analyzed the time needed for initial response and the time of full response (Fig. 1C and D). After the sciatic nerve crush injury, the mice showed a time of initial and full response at about 12 and 24 days after the injury. These two parameters in mice with BDNF treatment were remarkably reduced to about 7 and 18 days. In contrast, anti-BDNF administration significantly delayed these processes to nearly 17 and 30 days.

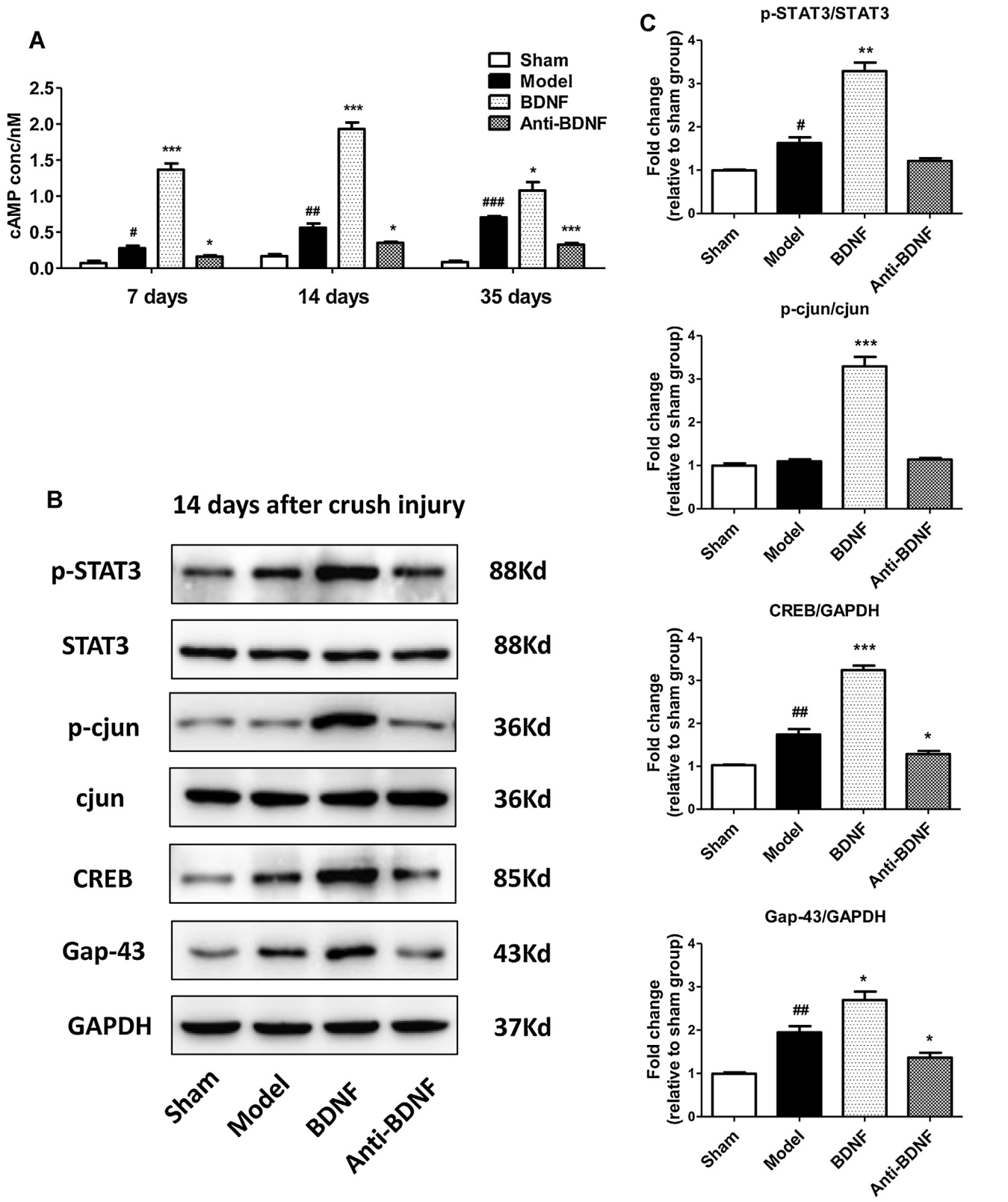
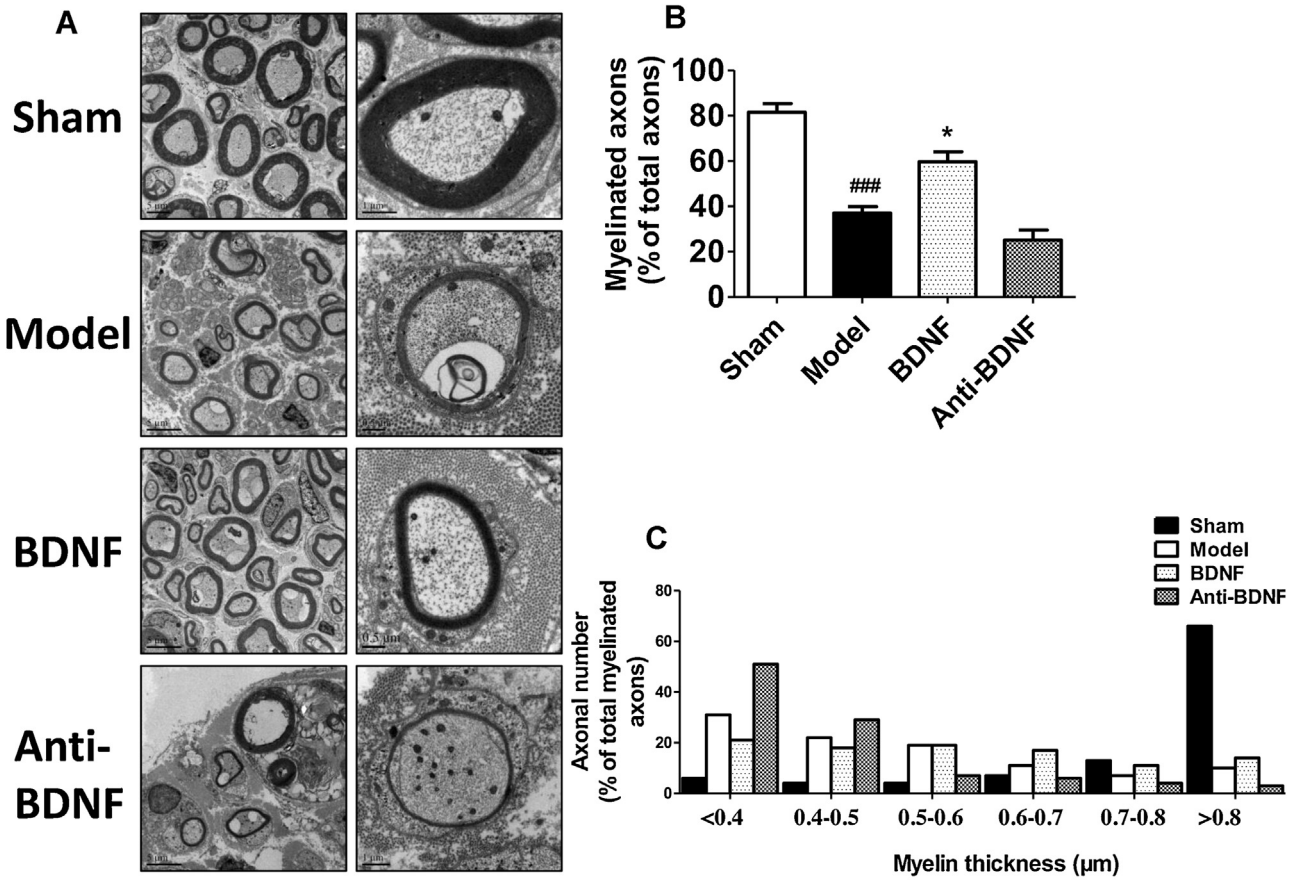
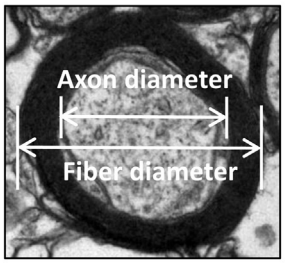


Fig. 3. BDNF up-regulates intrinsic growth capacity in mouse after sciatic nerve crush injury. (A) The LANCE cAMP assay in L4-L5 of the spinal cord after sciatic nerve crush injury. (B) The regulation of pathways related to intrinsic growth capacity in spinal cord L4-L5 at 2 weeks after sciatic nerve crush injury. (C) The quantification of band intensity (n = 3 per group, data are presented as mean ± SEM; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared with sham; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with model; one-way ANOVA).



D

G-Ratio=
Axon diameter / Fiber diameter



G-ratio	Sham	Model	BDNF	Anti-BDNF	Optimized
Mean	0.61	0.78	0.71	0.83	0.55-0.68
SD	0.064	0.097	0.086	0.110	

Fig. 4. BDNF protects the distal portion of the nerve and muscles. (A)Transmission electron microscope (TEM) analysis was performed on the proximal nerve lesions at 35 days after sciatic nerve crush injury. (B) Detection of the myelinated axons (% of total axons). (C) The distribution of myelin thickness. (D) The G-ratio analysis which was performed as axon diameter/fiber diameter. (n = 3 per group, data are presented as mean ± SEM; $^{###}p < 0.001$ compared with sham; $^{*}p < 0.05$ compared with model; one-way ANOVA).

3.2. BDNF regulates the mRNA expression associated with axonal regeneration

As previously research revealed [1], there has two important features of axonal regeneration. One is the activation of neuronal intrinsic growth capacity and the other is the degeneration in the distal portion. To reveal the potential mechanisms, we quantified the mRNA expression which is associated to the intrinsic growth capacity, trophic support and distal portion hypotrophy (Fig. 2). Our results showed that BDNF increases the levels of Mpz, Mbp, cjun and STAT3 (Fig. 2A-D) directly indicating the degree of distal portion hypotrophy and the activation of neuronal intrinsic growth

capacity in peripheral axonal regeneration, but without changing the BDNF and GDNF mRNA expression (Fig. 2E and F) which implicated in trophic support. So we next focused on evaluating the intrinsic growth capacity and distal portion degeneration.

3.3. BDNF up-regulates intrinsic growth capacity in mouse after sciatic nerve crush injury

How do peripheral axon lesions increase the activation of intrinsic growth capacity? Researchers had revealed that intracellular cAMP level is a critical molecule that upregulated after peripheral nerve injury, and cAMP analog injection is sufficient to initiate and promote neuronal regrowth [11,9]. And the action of cAMP proceeded through blocking PKA, upregulating cjun pathway and increasing Gap-43 expression to promote axonal elongation. Furthermore, these transcriptional effects seemed to be regulated by CREB and STAT3 activation [3,6]. To assess the regulation of BDNF in intrinsic growth capacity, we chose these critical factors for evaluation. From the data, it indicated that BDNF significantly increases the cAMP expression level (Fig. 3A) and upregulates the cjun pathway through elevating the CREB and STAT3 activation (Fig. 3B).

3.4. BDNF postpones the de-generation in the distal portion

The function of myelin in CNS is to promote the speed of conduction, increase conduction fidelity and reduce energy consumption, however, the biological structure of myelin is complex and vulnerable to injury. It is widely recognized that the g-ratio calculated as axon diameter/fiber diameter is highly reliable for assessing axonal myelination. To evaluate the degree of denervation at the distal portion from injury site, we used transmission electron microscopy (TEM) to detect the cross sections of sciatic nerve segments (~3 mm distal to lesion) at day 35 after sciatic nerve crush injury. Those results showed deficits in myelinated fiber population in model group compared to sham group (Fig. 4A and B). An increase of myelinated fibers was noted in BDNF administration mice, but anti-BDNF administration displayed a significant deterioration situation (Fig. 4A and B). From the composite histogram, it suggested that the myelin thickness statistics was also correlated to the result of the number of myelinated fibers (Fig. 4C). In order to present the data more impressive, we performed the G-ratio analysis. According to the previously study, the optimized value of G-ratio ranged 0.55–0.68 [14]. And in this study, the mean G-ratio of BDNF group was 0.7 ± 0.086 which was much greater than that obtained from model and anti-BDNF group (Fig. 4D).

4. Discussion

Compared to central nervous system (CNS) which usually cannot regenerate, injury occurred in peripheral nerves system (PNS) often initiates spontaneously regeneration, owing to the activation of intrinsic regrowth capacity of neurons and permissive environment [5]. However, despite the significant advances achieved over the past century, functional recovery after peripheral nerve injury is often poor and results in long-term disabling among patients. Our results suggested that BDNF remarkably promotes the outcomes of functional recovery in mice after sciatic nerve crush injury. Moreover, BDNF not only notably accelerated the presence of the initial response, and also accelerated the full recovery in different test facility. These collectively evidence indicated that BDNF administration is a potential approach to treat peripheral nerve injury.

For a successful nerve regeneration, it should include processes as follow: first, the cell body of an injured neuron must receive accurate and timely information about the site and extent of axonal damage in order to increase its intrinsic growth capacity and successfully regenerate. Second, the balance between inhibitory cues present in the environment and the intrinsic growth capacity of the injured neurons determines the proper extent of axonal regeneration following injury. Finally with the assistant of clues in the microenvironment, axons regrow through the lesion and reconnect with the target organ to repair the function. Here, to reveal the potential mechanisms, we chose several typical proteins related to the intrinsic axon growth ability (STAT3, cjun), trophic support (BDNF, GDNF) and myelin function (Mpz, Mbp) for analysis. The results displayed that BDNF could significantly upregulate the mRNA of STAT3, cjun, Mpz and Mbp expression, but not mRNA of BDNF and GDNF. These clues suggested that BDNF may accelerate the functional recovery through improving the intrinsic axon growth ability and myelin function.

So we next evaluated the intrinsic growth capacity and the degree of denervation at the distal portion. The data showed BDNF significantly increase the cAMP level during the regeneration process. And the CREB-cjun-STAT3-Gap-43 pathway which associated with the intrinsic axonal growth capacity was also upregulated. Thus, BDNF could increase and maintain the intrinsic growth capacity to a high level. At the meanwhile, BDNF also delayed the atrophy of distal portion, and these effects eventually contribute to accelerating functional recovery.

There is compelling evidence indicating that combination use of multiple neurotrophic factors have been shown to promote peripheral regeneration better than monotherapy [18,17], but the exact role of each neurotrophic factor acts during the repair process is not clear. For the further application, we need to investigate the role of each neurotrophic factor and the optimal proportion. In this study, we reported that BDNF promotes neuronal intrinsic growth capacity and delays portion atrophy, but its role in reducing portion atrophy is not so excited compared to its role on promoting neuronal intrinsic growth capacity, suggesting that BDNF combined with a medicine which could inhibit the distal atrophy more significantly will come into play.

5. Conclusion

Collectively, our data showed that BDNF administration result in significantly promoting functional recovery in mice after sciatic nerve injury, and the potential mechanisms are mainly via the neuronal intrinsic growth capacity improvement and distal portion atrophy protection.

Conflict of interest

The authors declare no financial conflict of interest.

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