Original Article

Electroacupuncture accelerates peripheral nerve regeneration via enhancement of BDNF, NGF, and GAP43 in rats

Li Li1, Guoying Li1, Yanyan Dong2, Yilin Ye3

1Department of Pharmacology, Anhui University of Chinese Medicine, China; 2Department of Pharmacy, The Third People’s Hospital of Hefei, China; 3College of Nursing, Anhui University of Chinese Medicine, China

Received September 29, 2018; Accepted February 11, 2019; Epub May 15, 2019; Published May 30, 2019

Abstract: Electroacupuncture (EA) is a modified Traditional Chinese Medicine approach. Acupuncture has been used for treatment of sciatic nerve injuries, showing excellent clinical effects. This study aimed to investigate the effects of electroacupuncture (EA) on peripheral nerve regeneration. A total of 104 Sprague-Dawley rats were randomly divided into four groups, the sham group, sciatic nerve crush model group, EA treatment group, and EA + K252α treatment group. The rats received sham/sciatic nerve crush injury surgery, in respective groups, and adopted different methods of treatment for 8 days. Present results indicate that EA significantly improved axonal regeneration and recovery, both structurally and functionally. Brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and growth associated protein 43 (GAP-43) were significantly increased after EA administration. Interestingly, blockage of BDNF downstream signaling abolished EA regeneration promoting actions. In summary, EA is beneficial for axonal regeneration after sciatic nerve injuries, potentially through elevation of levels of BDNF, NGF, and GAP-43.

Keywords: Electroacupuncture, sciatic nerve crush, BDNF, NGF, sciatic function index

Introduction

Peripheral nerve injuries are devastating to humans, leading to high rates of morbidity and extreme healthcare expenses. Although the peripheral nervous system obtains a certain degree of capacity for axonal regrowth and regeneration, recovery of injured peripheral nerves has been unsatisfactory [1]. As a result, development of reagents or methods that can foster and promote axonal regeneration after peripheral nerve injuries is demanding.

Neurotrophins, including BDNF and NGF, support and nurture growth and regeneration of axons during development and after injuries [2, 3]. BDNF and NGF are members of a small family of secreted proteins. Expression, secretion, and actions of BDNF and NGF have been studied extensively. They regulate neural activity in the mammalian nervous system [2]. BDNF and NGF are secreted by Schwann cells following peripheral nerve injuries, promoting axonal regeneration capacity and enhancing myelination [3, 4].

Growth-associated protein 43 (GAP-43) is a presynaptic protein. Located at the inner surface of axon terminals, it is actively transported at nerve terminals between neurons to regulate neurite outgrowth and branching [5]. GAP-43 is highly upregulated during the regeneration processes when neurons are damaged [6]. Therefore, increased GAP-43 during neural regeneration is beneficial for repair.

EA is a modified Traditional Chinese Medicine approach. EA may improve clinical outcomes in multiple conditions. EA has been recently reported to improve locomotive and sensory functions after spinal cord transaction [7]. EA effects on neuroplasticity have been associated with increased neurotrophins (NGF, BDNF) and GAP-43 [8, 9]. However, underlying mechanisms of EA after sciatic nerve injuries and fine protocols have not been fully elucidated.

Therefore, the current study aimed to investigate the effects of EA on expression of BDNF, NGF, and GAP-43 using the model of sciatic nerve crush lesion. In addition, this study
Electroacupuncture accelerates peripheral nerve regeneration

explored the recovery of sensory and motor function of the sciatic nerve in vivo.

Materials and methods

Experimental animals

Sprague-Dawley rats (180-220 g) were purchased from the Animal Center of Anhui Province. All animal protocols were approved by Anhui Chinese University Animal Care and Use Committee. All procedures were designed to minimize discomfort for the animals.

A total of 104 animals were randomly divided into four groups (n = 26). The sham group received sham sciatic nerve crush injury surgery. The injury group received sciatic nerve crush injury surgery. The injury + EA group received sciatic nerve crush injury surgery and EA treatment on day 1 to day 8 after surgery. The injury + EA + K252α group received sciatic nerve crush injury surgery, injections of BDNF antagonist K252α, and EA treatment on day 1 to day 8 after surgery. As previously reported, stock solutions (10 mM) of K252α were prepared in DMSO and final concentrations of 100 nM were supplemented in the cell culture medium for 1 hour [10].

Sciatic nerve crush model

Rats were anesthetized with 2% pentobarbital sodium (35 mg/kg, ip). The sciatic nerve of right hind leg was exposed by blunt dissection through the biceps femoris. The sciatic nerve was crushed at 6 mm distal to ischial tuberosity. The crush was achieved by applying consistent pressure with a pair of needles held for 10 seconds. This was repeated three times with intervals of 10 seconds. About 2 mm of the nerve was injured. All crushing was done by one person. Finally, the muscles and skin were sutured separately.

To build a sciatic nerve regeneration chamber model, 3 mm of the sciatic nerve was removed from the middle and bridged by a silicone tube.

Sham surgery (exposure of the sciatic nerves without ligation) was used for the Sham control group.

Electroacupuncture treatment

Two acupuncture needles, 0.25 mm in diameter, were inserted into right hind leg, one in the Zusanli point and the other in the Huanbiao point. Electrical stimulation was applied using a PCE-A acupuncture stimulation machine (Anhui Tianheng Technology, China) for 20 minutes, with 1.5 mA intensity and 2 Hz frequency from day 1 to day 7 after surgery, once a day. Rats in the Sham group and Injury group did not receive electroacupuncture (EA) treatment. After EA treatment, 16 rats in each group were randomly selected for sciatic functional index (SFI) determination, electrophysiological examination, histological examination, immunohistochemical staining, real-time PCR, and Western blotting. For the remaining rats, a sciatic nerve regeneration chamber model was produced. After a week of regular feeding, BDNF concentrations in the regeneration fluid were tested by ELISA.

After SFI determination and electrophysiological testing, the right sciatic nerve was removed and fixed for histological examination and immunohistochemical staining. The ventral horn of L4-L6 spinal cord was also removed and frozen for real-time PCR and Western blotting.

Sciatic functional index (SFI) determination

Functional evaluation of sciatic nerve regeneration was expressed by the sciatic functional index (SFI). The hind paws were dipped in blue ink and the rats walked through a 50 cm × 8 cm tunnel. Thus, the footprints could be recorded on paper loaded onto the bottom of the tunnel, with three or four footprints for each hind paw.

The distance between the third toe ant heel (PL), first and fifth toe (TS), and second and fourth toe (ITS) was measured on the experimental side (EPL, ETS, and EITS, respectively) and the contra lateral normal side (NPL, NTS, and NITS, respectively). SFI was calculated as follows: SFI = -38.3 × (EPL/NPL)/NPL + 109.5 × (ETS - NTS)/NTS + 13.3 × (EITS/NITS)/NITS - 8.8. In general, the SFI oscillates around 0 for normal nerve function. Approximately -100 SFI represents total dysfunction.

Electrophysiological study

Latency of action potential, maximal evoked potential, and nerve conduction velocity of the gastrocnemius motor dominated by the sciatic nerve were determined using a phasis.
Stimulation parameters were set, including square wave 0.2 ms, frequency 2-3 Hz, and supracurrent 2.6 mA. Recording parameters included sensitivity 2 mV/D and scanning speed 2 ms/D. Stimulation and recording were performed twice per time group.

Latency and maximum evoked potential of CMAP, on both sides, were recorded. Nerve conduction velocity (NCV) of the nerve trunk potential was also calculated. During the experiment, the muscles were moistened with warm saline to avoid contact of the stimulating electrode with surrounding tissue. Room temperature was maintained at 25°C. The recovery rate was calculated by dividing the delay rate and maximum evoked potential of sciatic nerve latency on the surgical side by the corresponding measurements on the contralateral side.

**Immunohistochemistry**

Nerve samples were taken at 0.5 cm from the distal and proximal ends of the crushing injury point. Samples were then fixed in 10% formaldehyde (formalin), embedded in paraffin, and cut into 5 μm sections. Hematoxylin-eosin (HE) staining and luxol fast blue staining were performed. After luxol fast blue staining, myelinated fibers (including phospholipids) were blue-green and the background was colorless. Samples were observed using an Olympus biomicroscope.

The number of all axons on the proximal and distal cross-sections of the sciatic nerve injury segment was then counted under a magnification of 400 times using an Olympus biomicroscope. The recovery rate was calculated by dividing the distal measurement by the proximal measurement. Using the IMS/color image analysis system, (the magnification of the eye-piece is 40 times and the calibration value is 0.38 μm/pixel), the cross-sectional area of axons in the proximal and distal cross sections of the lesion was determined. A straight line passing through the center was randomly specified and the cross-sectional area of all the nerve axons on the straight line was measured. The average value was taken as the sectional area of the axon of the nerve cross section.

For BDNF staining, tissues were embedded in paraffin, and cut into 5 μm consecutive sections. Antigen retrieval was performed. After exposure to 0.3% hydrogen peroxide and block-

***ELISA***

Sciatic nerve regeneration fluid was extracted and BDNF content was detected using a rat brain BDNF ELISA kit (RA20017, Bioswamp).

**Western blotting**

Extracted tissues were homogenized in ice-cold RIPA lysis buffer (BYL40825, Jrdun biotechnology, Shanghai, China) with 1 mM phenylmethylsulfonyl fluoride, 1 mM phosphatase, and proteinase inhibitors (EDTA-free protease inhibitor cocktail, Roche, USA). Homogenates were centrifuged at 12,000 g for 15 minutes at 4°C and the supernatant was collected. Protein concentrations were measured using a BCA assay kit (Pierce Biotechnology).

A total of 50 μg in each sample was subjected to SDS-PAGE, using 12% running gels, and transferred onto PVDF membranes. Membranes were blocked with 5% BSA in TBST (50 mM tris-HCl at pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 hour at room temperature, then incubated overnight at 4°C with the following primary antibodies: BDNF (1:1000, Ab108319, Abcam), NGF (1:1000, Ab52918, Abcam), GAP-43 (1:200000, Ab75810, Abcam), and GAPDH (1:2000, 5174, CST). HRP-conjugated antibody (1:1000, A0208, A0181, and A0216, Beyotime Biotechnology, China) was used as the secondary antibody. Blots were examined with the lightening chemiluminescence kit (A0216WBKLS0100, Millipore). For analysis, films were scanned using a Tanon-5200 chemiluminescent imaging system (Tanon Science & Technology, Shanghai, China).

**RT-PCR and real-time PCR***

Total RNA was isolated from the L4-L6 spinal ventral horn with TRIzol Reagent (Invitrogen,
Electroacupuncture accelerates peripheral nerve regeneration

Carlsbad, CA, USA). D260 values and D260/280 ratios were determined using an ultraviolet spectrophotometer. Total RNA concentrations and purities were also calculated. Reverse transcription was performed using a RT-PCR kit (Tiangen, Beijing, China).

Primers (Sangon Biotech, Shanghai, China) for real-time PCR were as follows: GAP-43, forward 5’-ACGATCA TGCTGCTGTATGAAG-3’; and reverse 5’-CTGCTGCTTGCCCGGTG-3’; BDNF, forward 5’-TAGGCAGAATGAGCAATGTC-3’, and reverse 5’-CCCAGAGGTAATAAGTGAAG-3’; NGF, forward 5’-GACATCAAGGGCAGGAG-3’, and reverse 5’-GTCGTGGTGCAGTAG-3’; GAPDH (as a loading control), forward 5’-GCTCGTGGTGCAGTAGTGAAG-3’, and reverse 5’-ATCTTCTGTTAGGTCAGTC-3’.

Thermocycling conditions for real-time PCR: Initial denaturation at 95°C for 2 minutes. Denaturation at 94°C for 40 seconds, annealing at 58°C for 40 seconds, and Extension at 72°C for 40 seconds, repeated for 35 cycles in total. Final extension was at 72°C for 5 minutes, held at 4°C.

Statistical analysis

Values are presented as mean ± SEM. GraphPad Prism was used for statistical analysis. One-way ANOVA, followed by Newman-Keuls post hoc tests, was used for immunohistochemistry, real-time PCR, Western blotting, and ELISA data. P < 0.05 indicates statistical significance.

Results

Elevated BDNF, NGF, and GAP-43 mRNA expression in right L4-L6 spinal ventral horn

The current study examined changes in BDNF, NGF, and GAP-43 mRNA expression levels after EA in the right L4-L6 spinal ventral horn. Effects of K252α, a pharmacological blocker of Trk signaling, were evaluated by real-time PCR. BDNF, NGF, and GAP-43 mRNA expression levels were significantly increased in the Injury group, compared to the Sham group (Figure 1). An increase in BDNF, NGF, and GAP-43 mRNA expression levels was observed in the Injury + EA group (compared with the Injury group), suggesting that EA treatment upregulated mRNA expression. Moreover, a reduction in BDNF, NGF, and GAP-43 mRNA expression levels was observed in the Injury + EA + K252α group (compared with the Injury + EA group), suggesting that K252α reversed the effects of EA on expression of BDNF, NGF, and GAP-43 mRNA.

Elevated BDNF, NGF, and GAP-43 protein expression in the right L4-L6 spinal ventral horn

BDNF, NGF, and GAP-43 protein expression levels in the ipsilateral (right side) L4-L6 spinal ventral horn were analyzed by Western blotting. BDNF, NGF, and GAP-43 were significantly upregulated in the Injury group, compared with the Sham group, suggesting that peripheral nerve injuries induced BDNF, NGF, and GAP-43 expression in the spinal ventral horn. The Injury + EA group showed significant increases in

![Figure 1](attachment:image.png)

Figure 1. Changes in GAP-43, NGF, and BDNF mRNA expression in the L4-L6 spinal ventral horn after different treatments on day 8 after surgery. Real-time PCR analysis of (A) GAP-43, (B) NGF, and (C) BDNF mRNA expression in L4-L6 spinal ventral horn after different treatments. Columns represent means ± SEM of relative mRNA expression levels. GAPDH was used as a loading control. (**** P < 0.001, compared with the Sham group; #### P < 0.001, compared with the Injury group; $$$ P < 0.001, compared with the Injury + EA group).
Electroacupuncture accelerates peripheral nerve regeneration

K252α reversed the effects of EA on BDNF release.

Effects of EA and K252 on structures of injured sciatic nerves

H&E staining was performed to observe the structures of ipsilateral sciatic nerves. No inflammatory cell infiltration was observed in the Sham control sample (Figure 4). After nerve injury, inflammatory cells, including neutrophils, T lymphocytes, and macrophages, were noted in sciatic nerves from animals in the Injury group. Notable Schwann cell proliferation was found in the Injury group. There was less inflammatory cell infiltration in the Injury + EA group, compared with the Injury group, suggesting that EA relieved local inflammatory response. There was more inflammatory cell infiltration in the Injury + EA + K252α group, compared with the Injury + EA group, suggesting that K252α reversed the effects of EA on local inflammatory response (Figure 4).

Elevated BDNF concentrations in sciatic nerve regeneration fluid

BDNF concentrations in sciatic nerve regeneration fluid were examined by ELISA. BDNF concentrations were significantly upregulated in the Injury group, compared to the Sham group. The Injury + EA group showed a significant increase in BDNF concentrations, compared to the Injury group, suggesting that EA stimulated BDNF release after sciatic nerve injury. BDNF concentrations were significantly decreased in the Injury + EA + K252α group, compared to the Injury + EA group (Figure 3), indicating that K252α reversed the effects of EA on BDNF release.

Figure 2. Changes in GAP-43, NGF, and BDNF protein expression in the L4-L6 spinal ventral horn after different treatments on day 8 after surgery. (A) Representative Western blots of GAP-43, NGF, and BDNF in L4-L6 spinal ventral horn on day 8 after different treatments. GAPDH was used as a loading control. Quantification analysis of the optical density of these bands, (B) GAP-43, (C) NGF, and (D) BDNF. Columns represent means ± SEM from two separate experiments. (** P < 0.001, compared with the Sham group; ### P < 0.001, compared with the Injury group; $$$ P < 0.001, compared with the Injury + EA group).

Figure 3. Changes in BDNF concentrations in sciatic nerve regeneration chambers after different treatments on day 14 after surgery. BDNF was quantified by standard ELISA (*** P < 0.001, compared with the Sham group; ### P < 0.001, compared with the Injury group; $$$ P < 0.001, compared with the Injury + EA group).

BDNF, NGF, and GAP-43 expression, compared to the Injury group, suggesting that EA further stimulated BDNF expression in the spinal ventral horn. Expression levels of these proteins were significantly decreased in the Injury + EA + K252α group, compared to the Injury + EA group (Figure 2), indicating that K252α reversed the effects of EA on BDNF, NGF, and GAP-43 protein expression.
Cross-sections of sciatic nerves were stained with luxol fast blue, a stain commonly used to observe myelin, revealing the structure of the myelin sheath. The sciatic nerve myelin sheath was found to be regular and uniform in the Sham control group (Figure 5). In contrast, the sciatic nerve myelin sheath was irregular, suggesting that myelins were degenerated and demyelination occurred after injury. The structure of sciatic nerve myelin sheath was partially recovered after EA treatment, suggesting that EA promoted sciatic nerve myelin sheath regeneration and that K252α blocked this effect of EA (Figure 5).

**Elevated BDNF expression in injured sciatic nerves**

Expression of BDNF in injured sciatic nerves was detected by immunohistochemical staining. Results showed that, in the Sham control group, BDNF was mildly expressed in the cytoplasm (Figure 6). In the Injury group, sciatic nerves showed degeneration and BDNF expression was slightly increased. BDNF staining was stronger in the Injury + EA group, suggesting that EA treatment increased BDNF expression. A reduction in BDNF expression was observed in the Injury + EA + K252α group (compared with Injury + EA group), suggesting that K252α reversed the effects of EA on expression of BDNF (Figure 6).

**Discussion**

Current study observations provide compelling evidence that BDNF, TrkB, and GAP-43 contribute to sciatic nerve injuries and that EA exaggerates the upregulation of BDNF, TrkB, and GAP-43. In addition, EA improves the functional and electrical recovery of nerves after damage. The current study demonstrated that EA may serve as an efficient approach to promote recovery from sciatic nerve injuries in future clinical settings.

Currently, methods used for spinal cord injuries are limited due to severe limb movement dysfunction and locomotor impairment found in patients. In recent years, the recovery of motor function after spinal cord injuries has been studied extensively [11]. Electrical stimulation...
Electroacupuncture accelerates peripheral nerve regeneration

Figure 6. Changes in BDNF immunoreactivity in ipsilateral sciatic nerves after different treatments on day 8 after surgery. Representative immunohistochemistry image showing expression of BDNF in ipsilateral sciatic nerves for (A) Sham, (B) Injury, (C) Injury + EA, (D) Injury + EA + K252α group; (E) Average BDNF positive area in ipsilateral sciatic nerves after different treatment. Data are presented as mean ± SEM. (### P < 0.001, compared with the Injury group; $$$ P < 0.001, compared with the Injury + EA group). Scale bar: 100 μm.

NGF and BDNF promote axonal regrowth after sciatic nerve injuries. The use of induced pluripotent stem cells to differentiate into Schwann cells to boost the elevation of NGF and BDNF is an ongoing novel approach towards the treatment of spinal cord regeneration [17]. EA has been demonstrated to enhance Wnt/β-catenin signaling, which promotes the proliferation and differentiation of neural stem cells [18]. These results partially explain the elevation of NGF and BDNF after EA administration. However, further studies are warranted to elucidate the detailed signaling transduction.

GAP-43 is a rapidly transported axonal protein, significantly upregulated after sciatic nerve injuries. GAP-43 is localized to growth cones and mediates axonal outgrowth and synaptogenesis. GAP-43 expression levels decrease after regeneration. Therefore, it has been considered the internal mediator for axonal growth and branching. Recent research has suggested that BDNF affects GAP-43 and promotes neuronal regeneration after injuries. Present results demonstrate that GAP-43 is highly regulated after sciatic nerve injuries. In addition, EA (14 days after treatment) promotes GAP-43 expression, suggesting that EA mediated GAP-43 is BDNF-related.

Acknowledgements

This study was supported by the Natural Science Foundation of Ministry of Education of Anhui Province (KJ2015A116).
Disclosure of conflict of interest

None.

Address correspondence to: Yilin Ye, College of Nursing, Anhui University of Chinese Medicine, B7-459, No. 1 Qianjiang Road, Xinzhan District, Hefei 230012, Anhui Province, China. Tel: +86 0551-68129263; Fax: +86 0551-68129165; E-mail: yliny2000@126.com

References