

Basic Science

Improvement of motor function induced by skeletal muscle contraction in spinal cord-injured rats

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Abstract

BACKGROUND: The involvement of neurotrophic factors such as brain-derived neurotrophic factor (BDNF) in functional recovery after spinal cord injury (SCI) by treadmill training has been suggested. The precise mechanism is poorly understood. However, muscle-derived bioactive molecules (myokines) are known to be produced by muscle contraction. Although BDNF is a myokine and is considered to be a potential mediator of neuroplasticity following exercise, its contribution to motor function recovery after SCI has not yet been described in detail.

PURPOSE: To investigate the role of muscle contraction in motor function recovery after SCI, with a focus on BDNF.

STUDY DESIGN: Male Sprague-Dawley rats (aged 8–9 weeks) were used to establish the SCI model. Percutaneous electrical muscle stimulation (10 mA, 2 Hz, 10 minutes) was applied to both hindlimbs of the rats immediately after SCI. The stimulation was performed once per day for 4 weeks. The sham, SCI only (SCI), and SCI with electrical muscle stimulation (SCI+ES) groups were compared.

METHODS: Spinal cord injury was induced by dropping a 20 g rod with an apex diameter of 2 mm from a height of 25 mm onto the spine of an anesthetized rat at the T9 level. Motor function was assessed using the Basso-Beattie-Bresnahan Locomotor Scale, inclined plane test, and rotarod test. One week after injury, terminal deoxynucleotidyl transferase dUTP nick end labeling-positive cells were counted at the injury epicenter, and the level of BDNF was measured in both the spinal cord and the anterior tibial muscle. Four weeks after injury, the cavity volume of the epicenter and the level of phosphorylated growth-associated protein 43 in the spinal cord were measured.

RESULTS: Significantly improved Basso-Beattie-Bresnahan scores and inclined plane test results were observed in the SCI+ES group compared with those in the SCI group at 4 weeks post-SCI. We also observed a decrease in the cavity volume and an increase in phosphorylated growth-associated protein 43 levels in the SCI+ES group. Electrical muscle stimulation decreased the numbers of terminal deoxynucleotidyl transferase dUTP nick end labeling-positive cells in the epicenter and increased the levels of BDNF in the spinal cord and lower limb muscles at 1 week post-SCI.

CONCLUSIONS: Electrical muscle stimulation improved motor function and increased BDNF levels in both the muscles and the spinal cords of rats subjected to SCI. Muscle contraction-induced BDNF expression might be involved in motor recovery during rehabilitation.

CLINICAL RELEVANCE: Our study provides experimental evidence for a possible therapeutic role of peripheral electrical muscle stimulation to enhance motor recovery after SCI. © 2018 Elsevier Inc. All rights reserved.

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Introduction

According to a global survey, the annual incidence of spinal cord injury (SCI) is 10.4–83 per 1 million individuals [1]. The incidence of SCI in Japan has been reported to be 39.4 per 1 million individuals [2]. A sizeable number of patients with SCI have permanent disabilities and experience a reduction in both quality of life and postinjury life expectancy [3]. SCI comprises irreversible central neuronal damage, which oftentimes causes motor and sensory dysfunction related to the spinal cord. Treatments for SCI have been investigated, and the improved understanding of the pathophysiology of SCI has led to the development of novel medical and interventional therapeutic strategies. Physical therapy is one of the major rehabilitation strategies used to mitigate the deleterious subsequent effects caused by the initial injury of SCI. Recent studies have reported that exercise training has beneficial effects on the restoration of function after SCI in animals [4] and humans [5,6]. Exercise seems to be more effective in facilitating motor function recovery when performed with the assistance of robotic walking devices [7,8]. Hinahon et al. have suggested that synaptic connections within the ventral horn of the lumbar spinal cord are increased due to activity-dependent plasticity, which led to enhancement of step height and movement velocity in a rat model of SCI [7]. Moreover, direct stimulation of muscles using functional electrical stimulation during walking can be used to improve speed and other parameters of gait in patients with SCI [9]. Functional electrical stimulation has also been used to augment partial weight-bearing treadmill training with encouraging results [10]. Muscle contraction might be involved in motor function recovery after SCI in addition to peripheral nerve activation.

Brain-derived neurotrophic factor (BDNF) elicits neuroprotective and axonal regeneration effects following both central and peripheral nerve damage [11,12]. BDNF has been suggested to be involved in exercise-induced recovery of motor function after SCI. Jung et al. reported that BDNF levels were increased in the spinal cord after treadmill training in rats subjected to SCI [13]. This increase in BDNF expression in the spinal cord was accompanied by enhanced axonal sprouting and resulted in the promotion of motor recovery. Joseph et al. also reported that treadmill training stimulated BDNF mRNA expression in motor neurons and other ventral horn cells in rats that had undergone spinal transection [14]. However, the mechanism by which exercise leads to motor recovery via BDNF expression is still unknown, particularly as it relates to skeletal muscle.

Recently, various bioactive substances, termed “myokines,” including cytokines and neurotrophins, have been detected in skeletal muscles during exercise. Myokines

have been reported to contribute to the preventive and therapeutic benefits of exercise [15]. Of all known neurotrophins, BDNF is the most affected by exercise [16]. Indeed, exercise-induced BDNF expression is observed in the spinal cord and is suggested to promote synaptic plasticity and the recovery of motor function after SCI [17]. BDNF can also aid repair of motor neurons [18], similar to other muscle-derived neurotrophins such as neurotrophin 4 [19]. Thus, muscle activity itself might play an important role in the restoration of motor function after SCI via the induction of BDNF secretion by exercise training.

Here, we investigated the hypothesis that motor function is restored due to post-SCI neuroprotection and neuroregeneration induced by muscle activity itself, and that muscle-induced BDNF expression is involved in the above mechanism. To test this hypothesis, we established a rat SCI model by dropping a rod onto the spine at the T9 level. We then activated the bilateral hindlimb muscles using percutaneous electrical stimulation to induce muscle contractions in the paralyzed hind limbs. The relevance of BDNF to both muscle and spinal motor function recovery was investigated.

Materials and methods

SCI model

Male Sprague-Dawley rats (aged 8–9 weeks and weighing 250–290 g) were used for the SCI model. The rats were divided into three groups: sham, SCI only (SCI), and SCI with electrical muscle stimulation (SCI + ES). The SCI operation was performed under intraperitoneal anesthesia (60 mg/kg ketamine and 6 mg/kg xylazine) and sustained intravenous anesthesia delivered via the tail vein (12.5 mg/h ketamine). After laminectomy of the T8–T10 thoracic vertebrae, a 20 g rod with an apex diameter of 2 mm was dropped from a height of 25 mm onto the exposed spinal cord at the T9 level. Body temperature was maintained at 37°C during the operation using a heat pad. After SCI, manual bladder expression was performed once daily until all rats regained autonomic bladder function (within approximately 4 days post-SCI).

A total of 70 rats were used for data assessment. Forty-two rats were used for motor assessment postinjury; of them, 10 and 15 rats were randomly selected for histological (cavity volume and immunohistochemistry for phosphorylated growth-associated protein 43 [pGAP43]) and quantitative (western Blotting for pGAP43) assessment at 4 weeks postinjury, respectively. Twenty-eight rats were euthanized at 1 week postinjury; of them, 15 rats were selected for histological assessment of both BDNF immunohistochemistry and terminal deoxynucleotidyl transferase

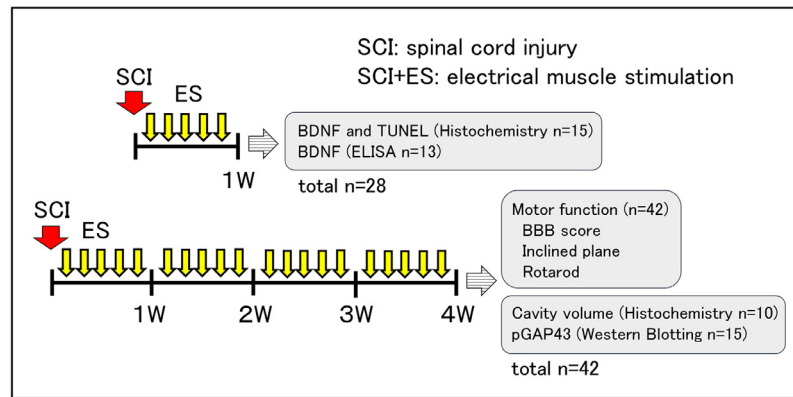


Fig. 1. The experiment schedule. Electrical muscle stimulation was applied immediately after SCI and was continued 5 days per week for 4 weeks. Motor function was assessed every week using the BBB score and 4 weeks postinjury using the inclined plane and rotarod tests. Histological assessments were performed 1 week postinjury for BDNF and TUNEL assays, and 4 weeks postinjury for cavity volume and pGAP43.

dUTP nick end labeling (TUNEL), and 13 rats were assessed for quantity of BDNF (enzyme-linked immunosorbent assay [ELISA]). All experimental procedures were performed in accordance with the National Institutes of Health regulations and approved by the Animal Research Committee of Kawasaki Medical School (approval numbers: 16-037 and 18-006).

Electrical muscle stimulation

Electrical stimulation was applied to both hindlimbs at 10 mA and 2 Hz (double pulse) for 10 minutes 5 days per week for 4 weeks. Flat clip electrodes were placed on the shaved lower hindlimbs, and appropriate muscle stimulation was confirmed based on ankle movement. The overall schedule of the study is provided in Fig. 1.

Motor function assessment

Motor function was assessed for 4 weeks after SCI. The Basso-Beattie-Bresnahan (BBB) Locomotor Rating Scale score was determined every week [20] and was based on assessments of joint movement, weight support, forelimb-hindlimb coordination, and toe clearance (sham: $n=17$, SCI: $n=13$, SCI+ES: $n=12$). The final BBB score was determined 4 weeks postinjury (sham: $n=12$, SCI: $n=16$, SCI+ES: $n=10$). The inclined plane test was performed 4 weeks after injury (sham: $n=11$, SCI: $n=12$, SCI+ES: $n=10$). The rats were placed on a table that was progressively tilted in increments of 5° , with a rubber mat attached to the surface. The score was determined based on the angle at which the rats could no longer remain on the table [21]. The rotarod test was also performed 4 weeks after injury (sham: $n=7$, SCI: $n=7$, SCI+ES: $n=7$). In this test, the rats were forced to run on a rotating rod, the speed of which gradually increased from 2 to 20 rpm [22].

Histological assessment

Animals were anesthetized with pentobarbital sodium (50 mg/kg) and transcardially perfused with 0.01 M phosphate-buffered saline (PBS) (pH 7.4). This was followed by fixation with 4% paraformaldehyde in 0.1 M PBS. The spinal cord was removed, postfixed in the same fixative overnight, and dehydrated in 10%–30% sucrose for 5 days. The tissues were then frozen in optimal cutting temperature compound and sliced into 16 μm thick sections using a microtome. Cavity volumes and cell counts were determined using ImageJ.

Cavity volume

The cavity volume was determined at 4 weeks post-SCI (sham: $n=5$, SCI: $n=5$, SCI+ES: $n=5$). The cavity volume at the epicenter (area within 1 cm of the lesion) was measured in the sagittal plane (11 sections at 200 μm intervals, stained with hematoxylin-eosin). The whole area of the sagittal section at the injury epicenter and the area of the lesion cavities were measured separately. The volumes of the cavity and the whole epicenter of the spinal cord were determined by the integrating the areas of the 11 sections. The final volume of the cavity injury in experimental models of SCI may be significantly influenced not only by factors inherent to the initial injury, but also by the slicing technique itself. To minimize the influence of the slicing technique, the cavity volume was expressed as a percentage rather than raw data in mm^3 by dividing the volume of the cavity by the volume of the whole epicenter.

TUNEL-positive cell counts

Assessment of apoptosis at the lesion site was performed at 1 week post-SCI (SCI: $n=5$, SCI+ES: $n=5$). Horizontal sections including the epicenter were stained using a TUNEL kit (Millipore, Billerica, MA, USA). Sections

(16 μm thick) were prepared and subjected to ethanol dehydration followed by heating in a 0.01 M citrate buffer solution (500 W microwave, 15 minutes). The sections were then permeabilized with 0.5% Triton X-100 in 0.01 M PBS (10 minutes) and incubated with Proteinase K (1:200; Millipore) in 0.01 M PBS (15 minutes). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in 0.01 M PBS (5 minutes). The sections were then incubated with equilibration buffer for 10 minutes. This was followed by a terminal transferase reaction for 1 hour in a warm box at 37°C. The reaction was stopped using termination buffer for 10 minutes at 22±2°C. After washing with 0.01 M PBS, the sections were incubated with antidigoxigenin peroxidase conjugate for 30 minutes in a humid box and washed with 0.01 M PBS. Peroxidase activity was then detected using diaminobenzidine (DAB) (Vectastain DAB Kit, Burlingame, CA, USA) at 22±2°C for 3–5 minutes. TUNEL-positive cells were counted in the field containing the posterior pyramidal tract and the epicenter using a microscope (40× objective lens). The results are expressed as the average number of TUNEL-positive cells per field.

Immunohistochemistry and BDNF-positive neuron counts

Histological assessment of BDNF-positive cells was performed at 1 week post-SCI using horizontal sections of the spinal cord. Sections from the epicenter, cephalad, and caudal points (5 mm and 10 mm distant from the epicenter, respectively) were assessed (sham: n=5, SCI: n=5, SCI+ES: n=5). To count the BDNF-positive cells, immunohistochemistry was performed using an ABC staining kit (Vectastain ABC Kit). Tissue sections underwent ethanol dehydration followed by heat-activation of the antigen (500 W microwave, 15 minutes). The sections were permeabilized with 0.3% Triton X-100 in 0.01 M PBS for 30 minutes. Quenching of endogenous peroxidases was performed using 0.3% hydrogen peroxide in methanol for 30 minutes. After washing with 0.01 M PBS, the sections were blocked with 1% bovine serum albumin and goat serum (1:66) in 0.3% Triton X-100 in 0.01 M PBS in a humid box for 30 minutes. The sections were then incubated with rabbit anti-BDNF primary antibody (1:400; Proteintech, Rosemont, IL, USA) in a blocking buffer solution overnight at 4°C. After washing with 0.01 M PBS, the sections were incubated with a biotinylated secondary antibody (1:200) in 0.01 M PBS for 30 minutes. The sections were then incubated with an avidin-biotin-peroxidase complex for 1 hour at 22±2°C. Finally, the sections were incubated in peroxidase substrate (DAB) for 30 seconds to obtain the desired staining intensity. As BDNF-positive cells primarily exist in the ventral horn of the gray matter, BDNF-positive cells were counted in the gray matter. The area of the gray matter was measured using ImageJ, and the total number of BDNF-positive cells was divided by the area to obtain the density of BDNF-positive cells (cells/mm²).

Double-immunostaining of sections containing the lesion was performed to identify the BDNF-producing cells. After ethanol dehydration, antigen activation was performed by heating in a 0.01 M citrate buffer solution (500 W microwave, 15 minutes). The spinal cord specimens were then permeabilized with 0.3% Triton X-100 in 0.01 M PBS and nonspecific reactions were blocked with 5% donkey serum in 0.01 M PBS. The primary antibodies used were rabbit anti-BDNF (1:800; Proteintech), mouse antiglial fibrillary acidic protein (1:300; Dako, Glostrup, Denmark) to identify astrocytes, and mouse anti-NeuN (1:500; Abcam, Cambridge, United Kingdom) to identify neurons. The sections were incubated with the primary antibodies in blocking buffer (5% donkey serum in 0.01 M PBS) overnight at 4°C. After washing with 0.01 M PBS, the sections were incubated with secondary antibody in a dark box for 1 hour (donkey antirabbit immunoglobulin G conjugated to Alexa Fluor 568, or donkey antimouse immunoglobulin G conjugated to Alexa Fluor 488) (Thermo Fisher Scientific K.K., Tokyo, Japan). Analyses were performed using a laser microscope (Confocal Laser Scanning Microscopy, Carl Zeiss MicroImaging GmbH, Jena, Germany).

Immunohistochemistry for pGAP43 was also performed using the sagittal sections from the epicenter of the spinal cord (the sections for cavity volume assessment) in the same manner as that used for BDNF immunohistochemistry (the ABC method). Mouse anti-pGAP43 antibody (1:500; Wako, Osaka, Japan) was used as the primary antibody.

ELISA

Quantitative analysis of BDNF in the spinal cord and the anterior tibial muscle were performed 1 week after SCI using a BDNF ELISA kit (Sigma-Aldrich, St. Louis, MO, USA) (sham: n=4, SCI: n=4, SCI+ES: n=5). Spinal cord specimens were sampled from sites 5 mm cephalad and 10 mm caudal to the epicenter, and from the lumbar spinal cord (L4–L6). For the assessment of BDNF in the hindlimb muscles, the anterior tibial muscles were sampled 2–3 hours after muscle stimulation. Tissue specimens were stored at –80°C prior to analysis. The samples were homogenized in 50 mM Tris-buffered saline (TBS, pH 7.6; Takara Bio, Otsu, Japan) containing 2% Triton X-100, 0.1% protease inhibitor, and 4 mM ethylenediaminetetraacetic acid-2K, and were centrifuged at 14,000×g for 30 minutes at 4°C. The protein concentration of the supernatant was measured using NanoDrop One (NanoDrop One Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA). The plates were incubated overnight at 4°C on a shaker. After washing with washing buffer, the plates were then incubated with anti-BDNF secondary antibody in diluent buffer (1:80) on a shaker for 1 hour. The plates were washed again and incubated with horseradish peroxidase-streptavidin in diluent buffer (1:200) for 45 minutes at 22±2°C. The plates were then washed and the tetramethylbenzidine reaction was performed for 30 minutes in a dark box. A stop solution was added to terminate the tetramethylbenzidine

reaction. Absorbance was read at 450 nm using a Varioskan Flash plate reader (Thermo Scientific, Yokohama, Japan).

Western blotting

Quantitative analysis of pGAP43 in the lesion areas containing the epicenter (length of spinal cord: 1 cm) was performed 4 weeks after injury (sham: $n=5$, SCI: $n=5$, SCI+ES: $n=5$). The samples were homogenized and centrifuged, as described in the ELISA protocol. The protein concentrations of the samples were adjusted to $4 \mu\text{g}/\mu\text{l}$ using NuPAGE LDS Sample Buffer and NuPAGE Sample Reducing Agent (Thermo Fisher Scientific, Tokyo, Japan). The samples were incubated for 10 minutes at 70°C to denature the proteins. Western blotting was performed using the semi-dry method (XCell SureLock Mini-Cell, Thermo Fisher Scientific, Tokyo, Japan). The denatured samples ($7 \mu\text{l}/\text{lane}$) were loaded and separated on NuPAGE 4%–12% Bis-Tris Gels using NuPAGE MOPS SDS Running Buffer (Thermo Fisher Scientific, Tokyo, Japan). The proteins were then electrophoretically transferred to nitrocellulose membranes using NuPAGE Transfer Buffer (Thermo Fisher Scientific, Tokyo, Japan). The membranes were blocked in 0.5% skim milk dissolved in 50 mM TBS containing 0.1% Triton X-100 (TBST) for 30 minutes. They were then incubated overnight at 4°C with mouse anti-pGAP43 primary antibody (1:500; Wako, Osaka, Japan) to detect regenerating axons, and with rabbit anti-glyceraldehyde 3-phosphate dehydrogenase antibody (1:10,000; Cell Signaling Technology, MA, USA) as a loading control. The membranes were washed with TBST and incubated for 2 hours at $22\pm 2^\circ\text{C}$ with horseradish peroxidase-conjugated secondary antibodies (1:5,000) in blocking buffer. The membranes were washed again with TBST and the reactive bands were visualized using Western Lightning ECL Pro (PerkinElmer, Inc., Waltham, MA, USA). Chemiluminescence was detected and quantified using the Amersham Imager 600 (Thermo Fisher Scientific, Tokyo, Japan). The expression of pGAP43 was normalized to that of glyceraldehyde 3-phosphate dehydrogenase.

Statistical analysis

Data are expressed as the mean \pm standard error of the mean. The Mann-Whitney U test was used for analysis of the cavity volume and the number of TUNEL-positive cells. The Kruskal-Wallis test (Steel-Dwass multiple comparisons) was used to determine all other p values. p values $< .05$ were considered to be statistically significant in this study.

Results

Transition and recovery of motor function

The BBB score improved in the SCI+ES group from 2 weeks after SCI. This improvement persisted until the end

of the observation period (Fig. 2a). Four weeks after SCI, a significant improvement in the BBB score was observed in the SCI+ES group compared with that in the SCI group (sham: 20.83 ± 0.11 , SCI: 6.37 ± 1.35 , SCI+ES: 17.40 ± 1.35 , $p<.01$) (Fig. 2b). The angle (degree) at which the rats fell during the inclined plane test was also improved in the SCI+ES group compared with that in the SCI group (sham: 88.63 ± 2.24 , SCI: 60.83 ± 2.44 , SCI+ES: 79.00 ± 3.85 , $p<.01$) (Fig. 2c). In addition, the running time (s) in the rotarod test tended to increase in the SCI+ES group compared with that in the SCI group, although the difference in running time between the SCI and SCI+ES groups was not significant (sham: 155.14 ± 10.06 , SCI: 17.00 ± 6.10 , SCI+ES: 35.00 ± 12.09 , $p=.52$) (Fig. 2d). Evaluations using more complicated motor ability tests, such as the rotarod test, might be needed for longer recovery periods, as motor function improvement had not reached a plateau by 4 weeks postinjury.

Differences in cavity volume at the site of the spinal cord lesion

Four weeks post-SCI, the cavity volume (%) was significantly decreased in the SCI+ES group compared with that in the SCI group (SCI: 17.55 ± 0.97 , SCI+ES: 9.52 ± 2.36 , $p<.05$). We observed partial scar formation around the cavity, and a few cells had infiltrated the cavity (Fig. 3a–c).

Apoptotic cell counts at the site of the spinal cord lesion

One week post-SCI, the density of apoptotic cells was determined in horizontal sections containing the injury epicenter (Fig. 4a and b). Apoptotic cells in the pyramidal tract of the posterior funiculus were less dense (cells/field) in the SCI+ES group than in the SCI group (SCI: 12.40 ± 2.37 , SCI+ES: 3.40 ± 0.74 , $p<.05$) (Fig. 4c).

Histological assessment of BDNF in the spinal cord

One week after the injury, histological assessment of BDNF-positive cells was performed using horizontal sections of the spinal cord (Fig. 5a–c). BDNF-positive cells were primarily present in the gray matter, and almost all BDNF-positive cells in the anterior area were also NeuN-positive and glial fibrillary acidic protein-negative, indicating that they were motor neurons (Fig. 5d–i). The density of BDNF-positive cells in the gray matter (cells/ mm^2) was significantly higher in the SCI+ES group than in the SCI group (sham: 12.69 ± 1.05 , SCI: 15.84 ± 1.60 , SCI+ES: 24.56 ± 2.60 , $p<.05$; Fig. 5j).

Quantitative assessment of BDNF in the spinal cord and lower hindlimb muscle

A significant increase in the BDNF level was observed around the epicenter and in the lumbar spinal cord (L4–6) in the SCI+ES group compared with that in the SCI group (5 mm cephalad, SCI: 0.67 ± 0.15 , SCI+ES: 1.58 ± 0.21 ,

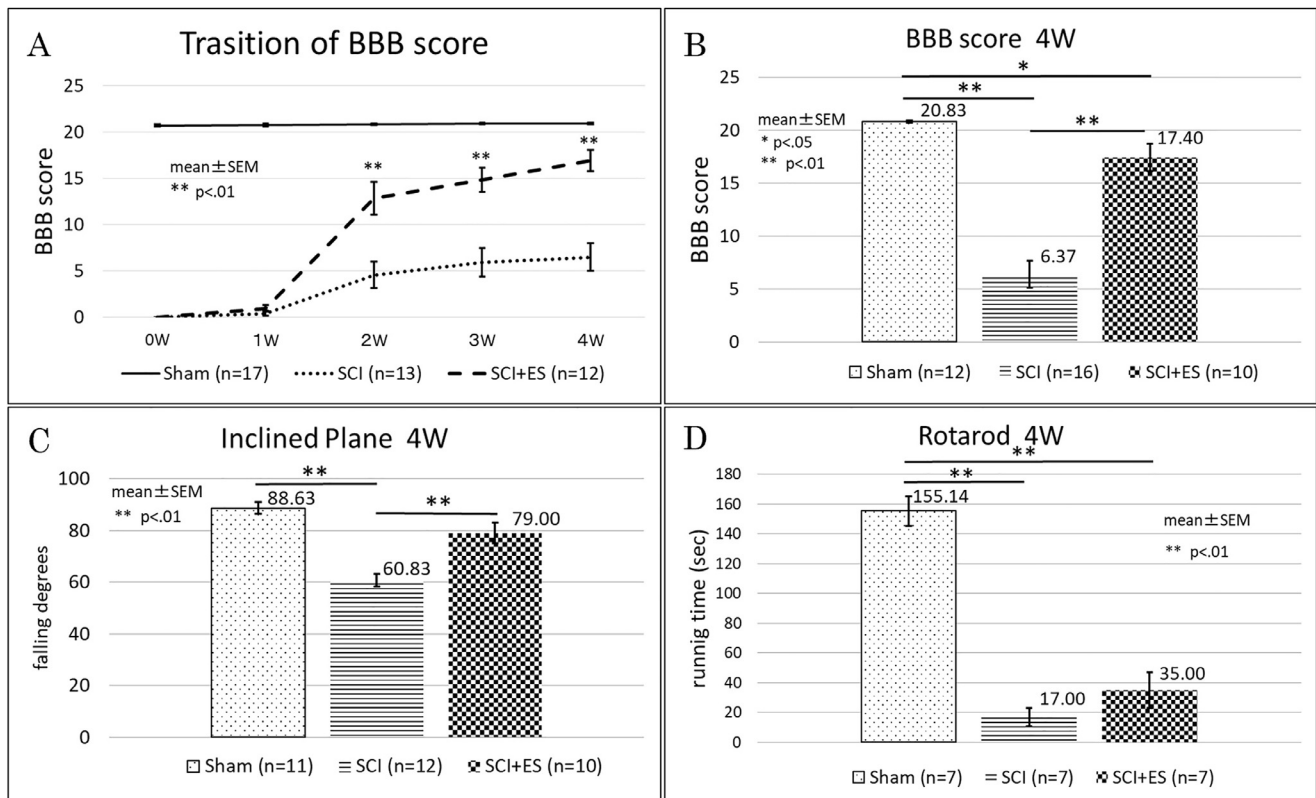


Fig. 2. Motor function recovery. (A) Significant recovery of BBB score was observed 2 weeks postspinal cord injury in the SCI+ES group ($p < .01$ vs. SCI). (B) BBB scores 4 weeks postinjury. An improvement in the BBB scores was evident in the SCI+ES group ($p < .01$). (C) The inclined plane test was performed 4 weeks postinjury. The angle at which the rat fell from the inclined plane was significantly larger in the SCI+ES group than in the SCI group ($p < .01$). (D) The Rotarod test was performed 4 weeks postinjury. No difference was observed between the SCI and SCI+ES groups in the rotarod test.

$p < .05$ vs. sham; 10 mm caudal, SCI: 0.94 ± 0.03 , SCI+ES: 4.75 ± 0.75 , $p < .05$ vs. sham; L4–6, SCI: 0.53 ± 0.12 , SCI+ES: 1.93 ± 0.53 , $p < .05$ vs. sham; Fig. 6a–c). The level of BDNF in the lower skeletal muscle was assessed after 2–3 hours of electrical muscle stimulation. BDNF levels were significantly higher in the SCI+ES group than

in the sham group (SCI: 0.31 ± 0.09 , SCI+ES: 1.41 ± 0.36 , $p < .05$ vs. sham; Fig. 6d).

pGAP-43 at the spinal cord lesion site

Histological and quantitative assessments of pGAP-43 at the lesion site were performed 4 weeks post-SCI. pGAP-43-

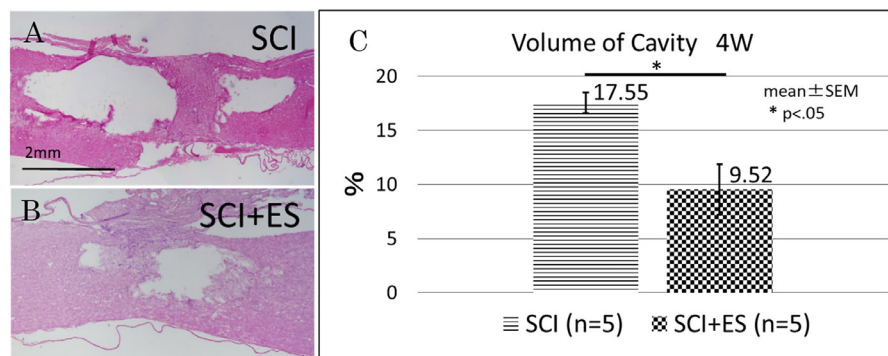


Fig. 3. Comparison of cavity volume 4 weeks postinjury. (A and B) Cavity formation at the lesion site at 4 weeks postinjury (A: spinal cord injury [SCI], B: SCI+electrical muscle stimulation [ES]). (C) Comparison of the cavity volume at the lesion site (1 cm in length). The whole area of the sagittal section at the injury epicenter and the area of the lesion cavities were measured separately. The volumes of the cavity and the whole epicenter of spinal cord were determined by integrating the areas of 11 sections. The cavity volume is expressed as a percentage rather than raw data in mm^3 by dividing the volume of the cavity by the volume of whole epicenter. A significant decrease in cavity volume was observed in the SCI+ES group compared with that in the SCI group ($p < .05$).

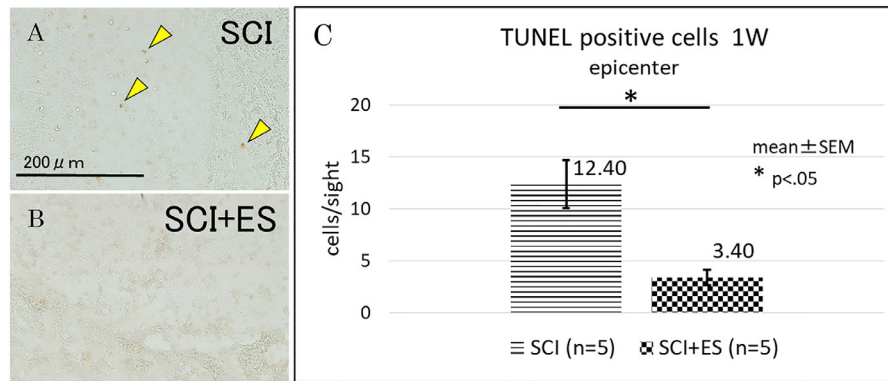


Fig. 4. Numbers of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells (cells/site) in the pyramidal tract of the posterior funiculus at 1 week postinjury (A: spinal cord injury [SCI], B: SCI+electrical muscle stimulation [ES], C: Numbers of TUNEL-positive cells in the pyramidal tract of the posterior funiculus). There were significantly fewer TUNEL-positive cells in the SCI+ES group than in the SCI group ($p < .05$). A decreased number of TUNEL-positive apoptotic cells were observed in the pyramidal tract in the SCI+ES group.

positive neurons with meandering axons were observed at the lesion epicenter (Fig. 7a and b). A significant increase in the pGAP-43 level was detected in the SCI+ES group compared with that in the SCI group (SCI: 0.93 ± 0.08 , SCI+ES: 1.63 ± 0.21 , $p < .05$ vs. sham; Fig. 7c).

Summary of the effects of muscle stimulation

The summary of the obtained results are shown in Table. Percutaneous electrical muscle stimulation after SCI was effective in improving the BBB score and performance on the inclined plane test. Histological assessment revealed decreased cavity volumes, increased pGAP43 levels, decreased numbers of TUNEL-positive cells, and elevated BDNF levels in the spinal cord and in the stimulated muscle, which may have contributed to motor function recovery.

Discussion

Motor recovery by muscle stimulation and the role of BDNF

Four weeks of electrical muscle stimulation resulted in motor function recovery, as assessed using the BBB Locomotor Scale and the inclined plane test (Fig. 2). The improvement became prominent 2 weeks after injury. The number of fibers that were positive for pGAP-43, which is a marker of neuronal sprouting postinjury, also increased at the epicenter of the injury site in the SCI+ES group (Fig. 7). In addition to decreasing the cavity volume (Fig. 3), axonal regeneration was thought to lead to motor function recovery in the SCI+ES group. Furthermore, immunohistochemistry revealed an increase in the number of BDNF-positive neurons in the ventral horn in rats in the SCI+ES group at 1 week postinjury (Fig 5). ELISAs also revealed that the BDNF level had increased in both the lower hindlimb muscles and the spinal cord (Fig. 6). In addition, increased BDNF expression was observed in both the lumbar and thoracic spinal cord areas. These results are

in concordance with those of previous reports suggesting that treadmill training upregulates BDNF mRNA expression in motor neurons of the lumbar spinal cord of rats subjected to SCI induced by midthoracic transection [14].

BDNF, which acts via tropomyosin-related kinase B receptor (TrkB), and BDNF/TrkB signaling are essential for exercise-induced recovery of motor function after SCI [18,23]. In a previous study, when 1 Hz of electrical stimulation was applied to the phrenic nerve, BDNF levels increased in the diaphragm. This observation led to the hypothesis that retrograde communication occurs between myocytes and axon terminals via BDNF/TrkB signaling [24,25]. Wu et al. detected the expression of BDNF and TrkB in the spinal cord after treadmill training. The expression levels of these two proteins decreased following administration of botulinum toxin A to block the neuromuscular activity of the rat gastrocnemius muscle [26]. The toxin treatment also inhibited the effects of treadmill training on motor function recovery. This observation led the authors to suggest that neuromuscular interactions are required for neurotrophin-mediated locomotor recovery following treadmill training in rats subjected to SCI. These reports, together with our results, suggest that BDNF is produced in muscle fibers in an activity-dependent manner and may promote BDNF-TrkB signaling, which in turn would lead to the enhancement of BDNF production in motor neurons in the spinal cord by facilitating presynaptic activity. The present findings also support the notion that postsynaptic release of BDNF from motor neurons contributes to synaptic plasticity in the injured spinal cord [14].

Although the precise mechanism of motor recovery induced by peripheral stimulation is still unclear in the present study, we can assume two possible therapeutic pathways. First, electrical stimulation of the lower hindlimb muscle enhances proprioceptive afferent activity, the signal of which is transferred to both motor neurons in the ventral horn of the lumbar and thoracic spinal cord via the local neural circuit and the ascending sensory pathway [27].

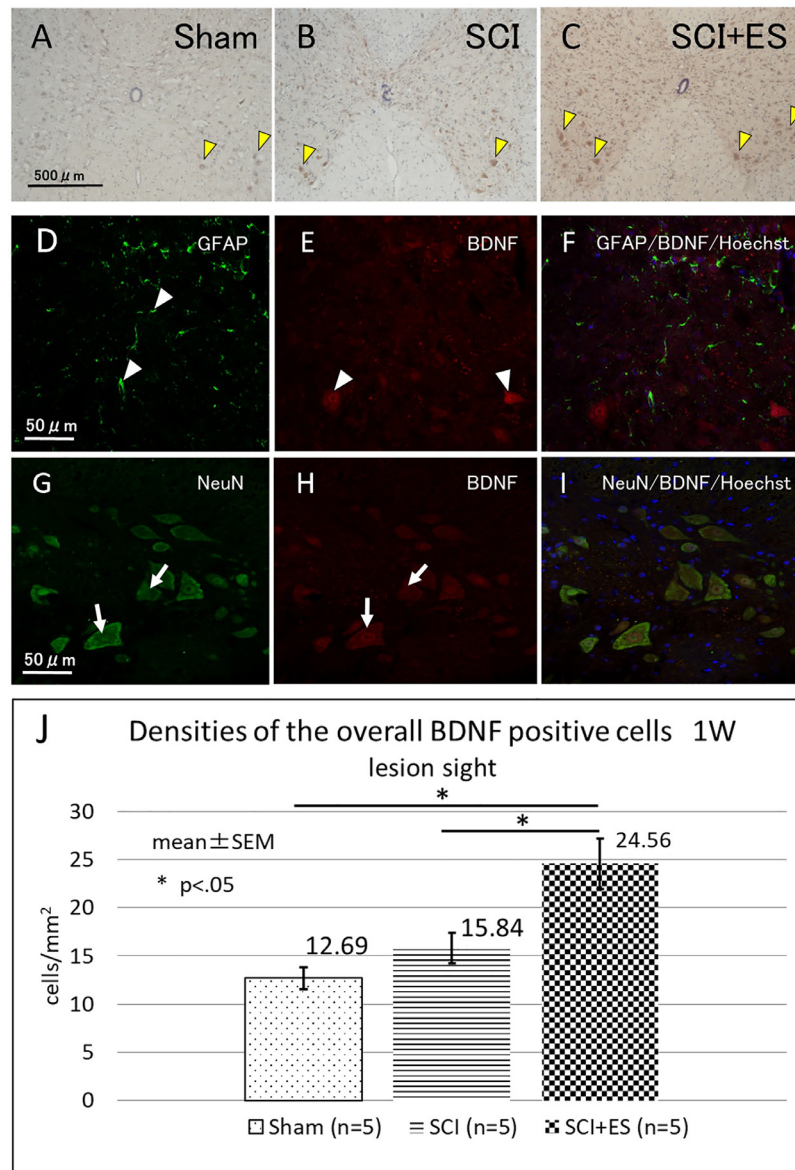


Fig. 5. Histological assessment of brain-derived neurotrophic factor at 1 week postinjury. (A–C) Horizontal sections containing the lesion (10 mm caudal from the epicenter) were stained using an antibody against brain-derived neurotrophic factor (BDNF). BDNF-positive cells were observed in the anterior horn of the gray matter (arrowheads). (D–I) Double-immunostained horizontal sections containing the lesion from rats in the spinal cord injury (SCI)+electrical muscle stimulation (ES) group. Many NeuN/BDNF-positive neurons (arrows) were observed, in contrast to the number of glial fibrillary acidic protein (GFAP)/BDNF-negative glial cells (arrowheads) observed in the anterior horn at the lesion site. (J) The overall densities of BDNF-positive cells (cell/mm²) were significantly increased in the SCI+ES group compared with those in the SCI group ($p < .05$). The results indicate that BDNF production in the spinal cord was enhanced by electrical muscle stimulation and primarily originated from neuron cells.

Such communication might induce BDNF production in the both the lumbar and the lesioned spinal cord, leading to regeneration of corticospinal axons. Administration of SCI+ES to posterior rhizotomy rats is one method to demonstrate participation of this pathway in motor recovery. Second, an increase in muscle activity induces BDNF production, and muscle BDNF is transported to the motor neurons of the spinal cord from the skeletal muscles in a retrograde manner [28]. Presynaptically, BDNF promotes regeneration of the descending tract [29]. Presumably, both

of these pathways may engage in motor recovery by peripheral stimulation after SCI.

Apoptosis in the acute phase and axon regeneration in the chronic phase

The natural course of SCI in rats has been described as being comprised of an early phase (hyperacute phase to acute phase, 0–1 week after injury), a subacute phase (1–4 weeks after injury), and a chronic phase (4 weeks after injury) [30].

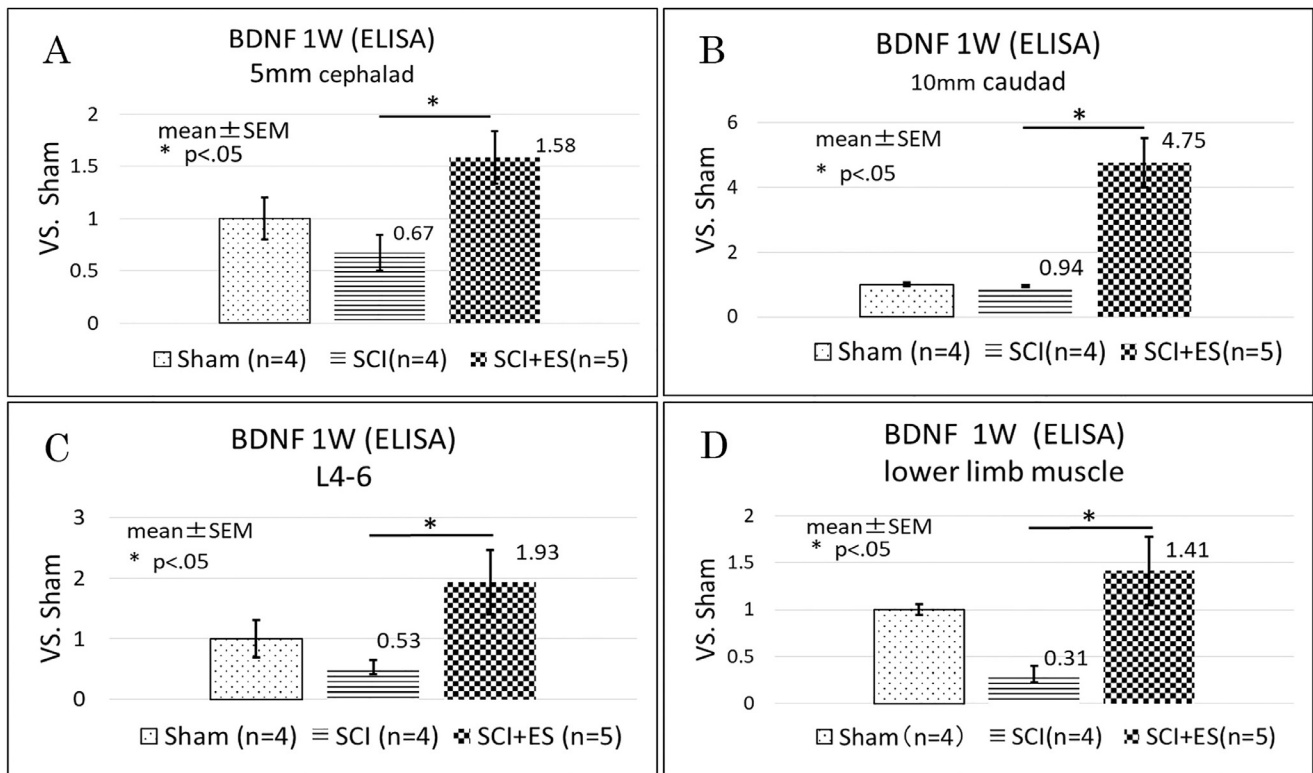


Fig. 6. Quantitative assessment of brain-derived neurotrophic factor at 1 week postinjury. The brain-derived neurotrophic factor (BDNF) level is expressed as a relative value to that in the sham group. Significantly increased BDNF levels were observed at the lesion site (5 mm cephalad and 10 mm caudal to the epicenter) (A–B), in the lumbar spinal cord (L4–6) (C), and in the tibialis anterior muscle (D) in the spinal cord injury (SCI)+electrical muscle stimulation (ES) group using an enzyme-linked immunosorbent assay ($p < .05$). Electrical muscle stimulation resulted in enhancement of the production of BDNF in the stimulated muscle, in the lumbar spinal cord corresponding to the stimulated muscle, and in the spinal cord at the lesion site, which may have contributed to the observed neuroplasticity and neuroprotection after SCI.

In the early phase of SCI, strong inflammation around the lesion site leads to apoptosis secondary to necrosis due to direct damage [31]. With progression to the chronic phase, stiff scar tissue is formed around the lesion site. Although the glial scar has been viewed as a barrier to CNS regeneration, recent studies have suggested that the glial scar can also support CNS repair depending on the heterogeneous components [32]. Thus, the physiological role of the glial scar remains controversial. Considering the natural course of SCI, avoidance of cell loss in the early phase seems to be the key to preserving motor function. During the initial 3–7 days of the acute phase of SCI, neurons and oligodendrocytes undergo apoptosis due to oxidative stress [33]. Therefore, apoptotic cell death might be prevented by BDNF [34]. In the present study, the decrease in the cavity volume following electrical stimulation may be explained by the decrease in the number of TUNEL-positive cells 1 week postinjury (Fig. 4) due to BDNF action. However, motor function may also be recovered by enhancement of axon sprouting following the early phase [30]. The axon sprouting induced by treadmill exercise has also been reported to result from increased BDNF expression in several studies [13,14]. The elevated levels of pGAP-43 after 4 weeks of electrical muscle stimulation may be explained by the axon sprouting effect of BDNF. Jung et al.

reported that BDNF levels were significantly elevated around the lesion site following treadmill training after SCI. The authors performed double-immunostaining for BDNF and neurofilament-200 and reported the presence of many BDNF-positive myelinating cells [13]. The same group has also reported the ability of myelin-forming cells to produce BDNF and promote axon regeneration [35]. During axon sprouting, which occurs after SCI, the increase in BDNF expression in the spinal cord may be due to increased expression in myelin-forming cells that myelinate the newly sprouted axons in addition to that in neurons, as presented here.

Limitations to the study

In the present study, we demonstrated the involvement of muscle stimulation-induced BDNF in both the muscle and the injured spinal cord as a possible mechanism of motor recovery by exercise post-SCI. Furthermore, the present study also indicates the possibility of regeneration of the injured corticospinal tract by muscle stimulation alone, and the results of the study could be translated to novel rehabilitation strategies. However, there are several limitations to this study. First, although BDNF has been identified as an important neurotrophic factor induced by

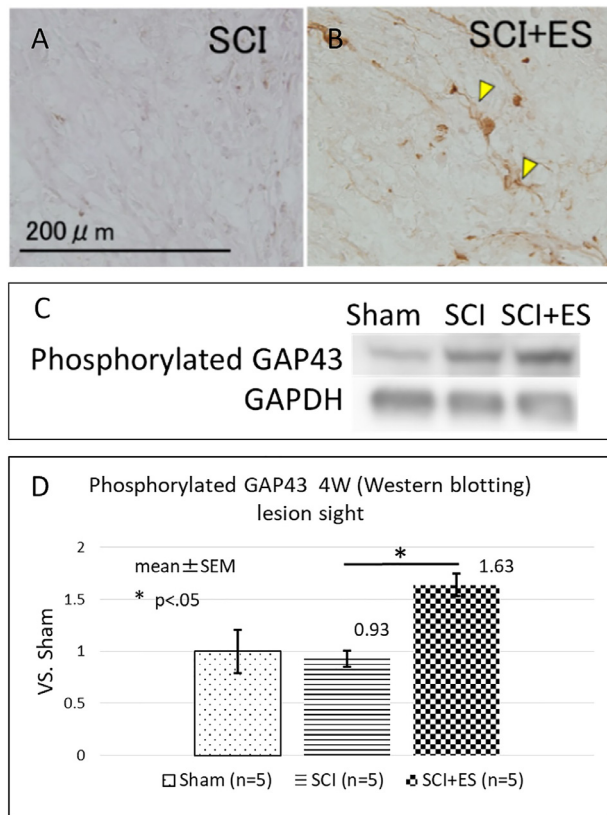


Fig. 7. Assessment of regenerating axons at 4 weeks post-injury

(A and B) Phosphorylated growth-associated protein 43 (pGAP43)-positive meandering axons were observed at the lesion epicenter (arrowheads). (C) Representative western blotting results. An anti-pGAP43 antibody was used to detect regenerated axons, and an anti-glyceraldehyde 3-phosphate dehydrogenase antibody was used as a control. (D) The pGAP-43 level is expressed as a relative value to that in the sham group. A significant increase in the pGAP43 level was observed at the lesion site (1 cm in length) in the spinal cord injury (SCI)+electrical muscle stimulation (ES) group using western blotting ($p < .05$). The enhancement of neuroplasticity was observed by the elevation of pGAP43 in the SCI+ES group.

exercise, other molecules such as glial cell-derived neurotrophic factor have also been suggested to induce neuroplasticity/neuroprotection after nerve injury [36]. To prove

Table
The result summary of the influence of ES

	SCI	SCI+ES
BBB score	NI	I
Inclined plane	NI	I
Rotarod	NI	NI
Cavity volume	NI	I
pGAP43	NI	I
TUNEL	NI	I
BDNF	NI	I

SCI, spinal cord injury; ES, electrical muscle stimulation; I, improved; NI, not improved; BBB, Basso-Beattie-Bresnahan; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; BDNF, brain-derived neurotrophic factor; pGAP43, phosphorylated growth-associated protein 43.

the key role of BDNF in the improvement of motor function in the SCI+ES group, further investigations using, for example, TrkB blockers are needed as per a previous study [23]. Second, the therapeutic mechanisms observed in the study might be directly related to the stimulation itself and not necessarily related to muscle fiber activation. To determine whether muscle contraction is essential for motor recovery after SCI, other control groups that receive electrical stimulation without muscle contraction by using muscle relaxants such as botulinum toxin and dantrolene are needed in further studies. Third, collateral sprouting of pyramidal tract axons into the gray matter after SCI [37] and enhancement of axon regeneration following treadmill exercise has been reported to be correlated with motor function recovery [38]. To determine whether motor function recovery resulted from axon sprouting, tracing of the pyramidal tract and double-staining for myelinated axons and oligodendrocytes should have been performed, as described previously [37]. Last, the presence of the lumbar radicularis magna artery (the Adamkiewicz artery) is well established and it supplies a considerable amount of the blood in the spinal cord [39]. The equivalent of the Adamkiewicz artery is not invariably present in the rat, and the certain prevalence of Adamkiewicz artery is observed at the T10-T11 level, one spinal level below the weight drop in our SCI model [40]. Although we carefully produced an SCI at T9, whether the unexpected additional spinal cord damage caused by injury to the Adamkiewicz artery resulted in influences on motor recovery remains to be investigated.

Conclusions

Percutaneous electrical stimulation of paralyzed lower hindlimb muscles after SCI was useful in improving motor function in a rat model of SCI. Increased BDNF expression was detected in the spinal cord and in the hindlimb muscles after electrical stimulation, and was considered to be associated with motor function recovery. These phenomena are thought to be mediated by the ability of BDNF to exert a neuroprotective effect and facilitate axon sprouting. This study supports the beneficial effects of exercise training after SCI and also provides experimental evidence for a possible therapeutic role of peripheral electrical muscle stimulation to enhance motor recovery after SCI [41].

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