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Treadmill step training promotes spinal cord neural plasticity after incomplete spinal cord injury

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Research Highlights

(1) A rat model of spinal cord contusion at the T_{10} level was used to examine the effect of step training. This study analyzed Basso, Beattie and Bresnahan scores, as well as plasticity at the lesion site and in segments caudal to the lesion site.
(2) We used growth-associated protein-43, which reflects axonal and synaptic plasticity, as an index of plasticity at the lesion site, and we used tyrosine hydroxylase as the main index of interneuron plasticity in segments caudal to the lesion site.
(3) To further explore plastic mechanisms in tissues surrounding the lesion site, brain-derived neurotrophic factor expression was measured at the lesion site.
(4) Treadmill training substantially improved spontaneous motor activity in rats with incomplete spinal cord injury. The improvement in rat behavior was associated with a significant increase in growth-associated protein-43 expression in the injured spinal cord and in tyrosine hydroxylase expression in the second lumbar spinal segment.
(5) Treadmill training significantly improves functional recovery and neural plasticity after incomplete spinal cord injury.

Abstract

A large body of evidence shows that spinal circuits are significantly affected by training, and that intrinsic circuits that drive locomotor tasks are located in lumbosacral spinal segments in rats with complete spinal cord transection. However, after incomplete lesions, the effect of treadmill training has been debated, which is likely because of the difficulty of separating spontaneous stepping from specific training-induced effects. In this study, rats with moderate spinal cord contusion were jected to either step training on a treadmill or used in the model (control) group. The treadmill training began at day 7 post-injury and lasted 20 ± 10 minutes per day, 5 days per week for 10 weeks. The speed of the treadmill was set to 3 m/min and was increased on a daily basis according to the tolerance of each rat. After 3 weeks of step training, the step training group exhibited a significantly greater improvement in the Basso, Beattie and Bresnahan score than the model group. The expression of growth-associated protein-43 in the spinal cord lesion site and the number of tyrosine hydroxylase-positive ventral neurons in the second lumbar spinal segment were greater in the step training group than in the model group at 11 weeks post-injury, while the levels of brain-derived neurotrophic factor protein in the spinal cord lesion site showed no difference between the two groups. These results suggest that treadmill training significantly improves functional recovery and neural plasticity after incomplete spinal cord injury.

Key Words

neural regeneration; spinal cord injury; neurorehabilitation; incomplete spinal cord injury; treadmill training; spinal cord plasticity; growth-associated protein-43; tyrosine hydroxylase; function recovery; grants-supported paper; neuroregeneration

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INTRODUCTION

Step training on a treadmill enhances locomotor function in experimental animals and in humans with spinal cord injury. The improved behavior is associated with a reorganization of spinal circuits that occurs *via* different mechanisms. Training can prevent atrophy of spinal motor neurons and cause changes in their firing threshold and conduction velocity[1], provide sensory feedback to stimulate intrinsic spinal circuits[2], modulate release of neurotransmitters[3-5], and stimulate growth and the expression of neurotrophic factors in the spinal cord[6-7]. A large body of evidence has shown that the spinal circuits that are significantly affected by training and that specifically drive locomotor tasks are located in lumbosacral spinal segments in rats with complete spinal cord transection[8-9]. However, after incomplete lesions, the spontaneous stepping ability largely depends on the amount of remaining descending control[10-11], and the spinal circuit that generates stepping movements also spontaneously reorganizes[12]. Thus, until now, the effect of treadmill training after incomplete injury has been under debate, which is likely due to the difficulty of separating spontaneous stepping from specific training-induced effects[13-14]. In this study, we investigated the effects of step training on a treadmill on the behavioral recovery of rats after a spinal cord contusion. We also examined neuroplasticity at the lesion site and at the lumbar level following spinal cord injury, and we explored the mechanism of action of step training in rats with spinal cord injury.

RESULTS

Quantitative analysis of experimental animals

At the beginning of the study, 20 rats were subjected to an incomplete spinal cord injury in the laboratory, and were equally and randomly assigned to model and step training groups. Rats in the step training group underwent step training on a treadmill commencing at day 7 post-injury, for 15–30 minutes per day, 5 days per week for 10 weeks. The speed of the treadmill was set to 3 m/min and was increased on a daily basis according to the tolerance of each rat. During the study, all animals survived, and hindlimb movement was observed after the injury. At the end of the planned observation period, five rats from each group were used for immunohistochemistry, and the remaining five rats in each group were used for western blot analysis.

Step training improved functional recovery after incomplete spinal cord injury

Twelve hours after spinal cord injury, the Basso, Beattie and Bresnahan scores for both groups were 0. The animals in the model group exhibited a characteristic improvement in open-field locomotor activity that plateaued 6 weeks after injury. However, the step training group exhibited a significantly greater improvement in the Basso, Beattie and Bresnahan score beginning 3 weeks post-step training ($P < 0.01$; Table 1).

Step training increased expression of growth-associated protein-43 at the site of spinal cord injury and the number of neurons expressing tyrosine hydroxylase in the second lumbar spinal cord (L_2) segment.

Growth-associated protein-43 was expressed around the lesion in the model and step training groups. Although growth-associated protein-43-positive axons were observed surrounding the lesion site in both groups, they occupied the lesion site only in the step training group (Figure 1). Growth- associated protein-43 expression around the lesion occupied (16.71 ± 4.27)% of the total lesion area in the step training group, which was higher than that in the model group [(11.00 ± 3.69)% of the total lesion area] ($P = 0.02$).

Tyrosine hydroxylase was expressed predominantly in ventral horn neurons and in some fibers surrounding neurons in the second lumbar spinal cord (L_2) segment in the model and step training groups. We also observed a few bouton-like tyrosine hydroxylase accumulations around neurons in

both groups. A quantitative increase in the number of neurons expressing tyrosine hydroxylase was observed in the step training group (33.86 ± 4.88/per section) compared with the model group (19.86 ± 8.55/per section) (P = 0.004; Figure 1).

Table 1 Effect of step training on Basso, Beattie and Bresnahan scores for rats with incomplete spinal cord injury

Time after injury (week)	Model group	Step training group	P
1	2.21±0.86	2.07±0.98	0.776
2	4.21±1.52	4.07±0.53	0.819
3	6.93±0.84	7.50±0.41	0.131
4	9.21±0.39	10.00±0.91	0.006
5	9.57±0.35	11.64±0.94	0.000
6	10.36±0.38	12.57±0.83	0.000
7	10.50±0.41	12.79±0.86	0.000
8	10.64±0.63	12.64±0.99	0.001
9	10.64±0.63	12.71±1.04	0.001
10	10.71±0.95	12.86±0.94	0.001
11	11.00±0.76	13.00±1.12	0.003

Rats in the step training group underwent treadmill step training commencing at day 7 post-injury. Data are expressed as mean ± SD of 10 rats in each group. A mixed factorial (repeated measures) analysis of variance followed by the Tukey-Kramer test was used for the comparison of functional recovery after injury.

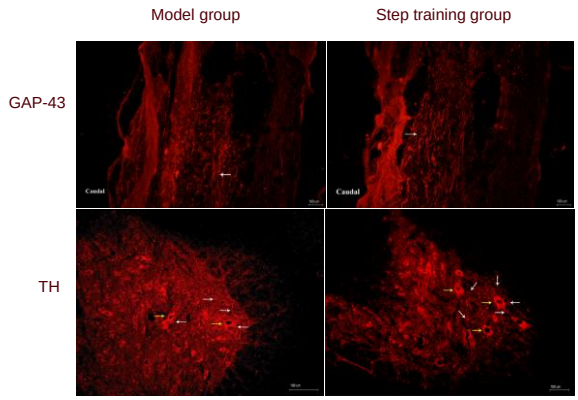

Figure 1 Effect of step training on immunostaining for growth-associated protein-43 (GAP-43) at the site of the spinal cord lesion and for tyrosine hydroxylase (TH) in the L_2 segment in rats with spinal cord injury (fluorescence microscopy).

At 11 weeks after injury, an increase in the expression of GAP-43 at the lesion site and in the number of TH positive neurons in the L_2 segment was observed. In the model group, a few GAP-43 positive fibers around the lesion site and a few TH positive neurons in the L_2 segment were found. In the step training group, there was a dramatic increase in the percentage of GAP-43 positive processes that invaded the cystic cavity and in the number of TH positive neurons in the ventral horn in the L_2 segment. The white arrows indicate GAP-43 positive axons in the upper figure, while they indicate TH positive labeling in the lower figure. The golden arrows show TH positive neurons in the ventral horn. Scale bars: 100 μm.

Step training has no effect on levels of brain-derived neurotrophic factor protein at the spinal cord lesion site

Based on western blot analyses, the levels of brain-derived neurotrophic factor protein at the spinal cord lesion site in the step training group showed no difference from those in the model group at 11 weeks post-injury (P = 0.054; Figure 2).

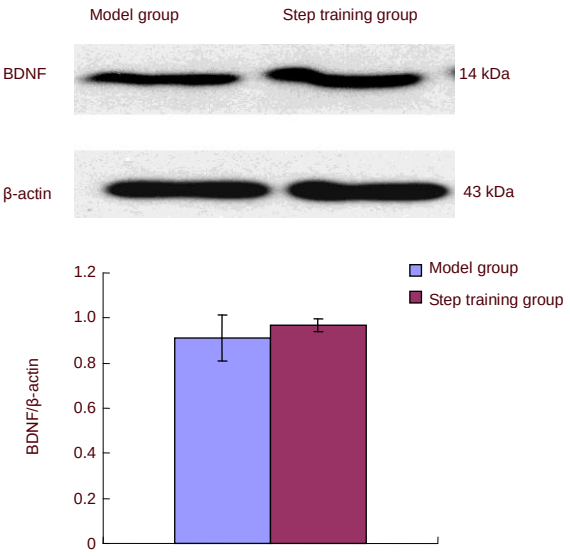

Figure 2 Effect of step training on levels of brain-derived neurotrophic factor (BDNF) protein analyzed by western blot assay in rats with spinal cord injury.

There is no difference in the levels of BDNF protein at the spinal cord lesion site between the step training and model groups at 11 weeks after injury (Mann-Whitney U Test). Data are expressed as mean ± SD of five rats in either group. The expression of BDNF was expressed as an absorbance ratio of BDNF and β-actin.

DISCUSSION

In this study, we found a significant improvement in hindlimb locomotor function and neuroplasticity in spinal cord neural circuits after regular treadmill training in rats with spinal cord contusion injury. In accordance with other reports that rats with severe spinal cord contusion can spontaneously generate partial weight bearing stepping and that treadmill training can help to restore normal patterns of hindlimb movements[15], rats with moderate spinal cord contusion in the treadmill training group demonstrated improved hindlimb Basso, Beattie and Bresnahan scores and more coordinated step cycles than the model group. Neuroplasticity in spinal neural circuits increased after treadmill training. We observed a

better preservation of growth-associated protein-43-positive axons at the spinal cord lesion site and an increase in tyrosine hydroxylase expression in the L_2 segment.

Effects of treadmill training on locomotor recovery

Although treadmill training is known to improve stepping in animals with complete spinal cord injury, few studies have examined the effect of treadmill training on locomotor recovery in animals following incomplete spinal cord injury. In this study, rats with moderate spinal cord contusions were subjected to treadmill training. The experimental protocol was driven by the idea that spontaneous stepping ability largely depends on the amount of remaining descending control[10-11]. Thus, if treadmill training is introduced, it may further promote locomotor recovery. As shown in our results, the spontaneous improvement in hindlimb locomotor activity occurred primarily from 1 to 4 weeks post-injury, and the Basso, Beattie and Bresnahan scores of rats in the model group plateaued between 4 to 6 weeks after injury. In comparison, rats subjected to regular treadmill training exhibited a significantly greater improvement in Basso, Beattie and Bresnahan score from 4 weeks post-injury to the endpoint. Andrade and colleagues[16] reported that treadmill training may favor better functional recovery in the acute period after spinal cord contusion. These results demonstrate that the improved locomotor activity in rats with a moderate spinal cord contusion depends on treadmill training rather than the spontaneous reorganization of spinal circuits. In accordance with the report of Ung *et al* [17], our results also suggest that training-dep- endent spinal plasticity does not simply depend on stepping activity because incomplete lesions allowed the rats to spontaneously recover a certain degree of hindlimb use by self-training in their cages. One explanation for the difference in our results on locomotor recovery in the chronic period and the transient recovery in the acute period is the onset of treadmill training[16]. Spinal learning and recovery critically depend on the training modality, such as the type, quality, quantity, and onset of the training protocol[17-19].

Effects of treadmill training on plasticity of neural circuits in the spinal cord

In accordance with previous reports that treadmill training helps to induce plasticity and reorganization of sublesional neuronal networks[20-22] and restore normal patterns of hindlimb movements in rats with severe spinal cord contusion[23], our results show that 30 minutes of daily treadmill training over 10 weeks produces a significant increase in tyrosine hydroxylase-positive neurons in laminae VII and X in the L_2 segment of rats with moderate spinal cord contusion. These results demonstrate the important roles of interneuron plasticity and tyrosine hydroxylase in the L_2 segment on functional recovery induced by treadmill training. Furthermore, our results show that growth-associated protein-43 expression at the spinal cord lesion site is increased in the treadmill training group. Growth-associated protein-43 is present in growing axon terminals and is an indicator of axonal regeneration and synaptogenesis, suggesting that exercise-related increases in growth-associated protein-43 are associated with axonal sprouting, regeneration and the maintenance of synaptic function. This is consistent with the finding that voluntary exercise accelerates axonal sprouting and enhances locomotor performance in mice after spinal cord injury[24], and that treadmill running helps to protect the spinal cord contusion site from secondary degeneration and favors better functional recovery in rats[16]. Sprouting of injured or spared axons may compensate for the disrupted connections after spinal cord injury, which is consistent with the results of a previous published study[25].

A number of reports have shown that exercise elevates brain-derived neurotrophic factor levels in lumbosacral spinal segments[26-29] and brain-derived neurotrophic factor-wmediated mechanisms promote neuroplasticity[27, 30-33] and the recovery of locomotor performance in spinal cord injury animal models[34]. However, data on the effect of treadmill training on brain-derived neurotrophic factor protein expression at the spinal cord lesion site following spinal contusion are scarce. In this study, we did not observe any difference in brain-derived neurotrophic factor protein expression at the spinal cord lesion site between the treadmill training and model groups. A previous study showed that moderate voluntary physical exercise does not have an effect on brain-derived neurotrophic factor mRNA levels in segments caudal to the lesion site in the contused spinal cord[35], and that treadmill training changes the distribution of brain-derived neurotrophic factor in the processes and fibers rather than the overall levels of brain-derived neurotrophic factor immunoreactivity in lumbar segments in rats with complete spinal cord transection[34]. Another study demonstrated that weight-supported treadmill training increases brain-derived neurotrophic factor immunoreactivity in the ventral horn of the lumbar spinal cord; however, the recovery of stepping function was not correlated with brain-derived neurotrophic factor expression[36]. Evidently, the effects of the exercise-induced changes in brain-derived neurotrophic factor expression on neu-

ronal plasticity are equivocal and need further study.

It is now recognized that enhanced neural activity above the lesion site[24], intrinsic changes below the lesion in the central pattern generator, and afferent inputs[37] are correlated with the reorganization of spinal circuitry and locomotor recovery after incomplete spinal cord injury. Here, we found that neuroplasticity both at the lesion site and lumbar spinal cord plays an important role in locomotor recovery. Based on the observations described above, we infer that locomotor recovery after incomplete spinal cord injury relies on the reorganization of spinal circuitry and that training-dependent reorganization may occur at different levels of the spinal cord, not only at the lumbar level. This is in agreement with observations of incomplete human spinal cord injury[38] and with other reports of incomplete spinal cord injury in rats; the degree of locomotor recovery may be attributable to the formation of new compensatory connections and activity-dependent reorganization of spared neuronal pathways[39-40]. Although we do not know how the enhanced injury-induced plasticity at both the lesion and sublesion sites promotes the formation of new compensatory connections and activity-dependent reorganization of spared neuronal pathways, it is evident that the locomotor recovery in rats with spinal cord contusion after step training results from an increase in spinal cord plasticity. This plasticity is a result of the retraining process, and is not associated with spontaneous recovery that is dependent on the remaining descending control.

In summary, step training following a moderate spinal cord contusion in rats promotes injury-induced plasticity at both the lesion and sublesion sites and substantially promotes locomotor recovery. Our results demonstrate that step training promotes plasticity that is not limited to the lumbar spinal cord, but rather occurs at various levels.

MATERIALS AND METHODS

Design

A randomized controlled animal study.

Time and setting

The study was performed at Beijing Army General Hospital, China from May 2008 to April 2009.

Materials

Twenty adult female specific pathogen free Sprague-Dawley rats were obtained from the Weitonglihua (Beijing, China, license No. SCXK (Army) 2007-004). They were 75 ± 1 days of age and weighed 220–250 g at the time of spinal cord injury surgery. All rats were individually housed in a light- and temperature-controlled room and were given free access to food and water. All experiments were performed according to the National Institutes of Health (publication No. 80-23) standards and the guidelines of Beijing Army General Hospital in China for management of laboratory animals.

Methods

Preparation of incomplete spinal cord injury model

Contusion injuries were induced with the MASCIS impactor developed at the W.M. Keck Center for Collaborative Neuroscience of Rutgers University. According to Young's method[41], all rats were anesthetized with an intraperitoneal injection of 4% pentobarbital (35 mg/kg), and the spinal cord was exposed by laminectomy at thoracic vertebra T_{10} while maintaining the dura mater intact. The exposed spinal cord at the T_{10} level was moderately injured by dropping a 10 g rod from a height of 25 mm. This procedure was designed to only produce an incomplete injury. After injury, the muscles and skin were sutured in layers. The rats were allowed to recover in a warmed cage with free access to water and food. Penicillin (20 mg/kg, intramuscular; Beijing Shuanglu Medicine Company, Beijing, China) was administered immediately after surgery and then daily for 7 days. Spinal cord injury was confirmed by the flaccid paralysis of the hindlimbs following transient spasm of the tail and hindlimbs just after the spinal cord was impacted[41].

Step training

Animals in the step training group performed daily quadrupedal locomotion on a treadmill (Simplex II, Hangzhou Medical Instruments Company, Hangzhou, Zhejiang Province, China). Training began on day 7 post-injury for the step training group and lasted for 20 ± 10 minutes per day, 5 days per week for 10 weeks. The speed of the treadmill was set to 3 m/min and was increased on a daily basis according to the tolerance of each rat, up to 11 ± 13 m/min. For the first 3 weeks, the tail was suspended during step training to ensure that the toes were extended and that the footpads contacted the treadmill in the stance phase. Inter-limb coordination was maintained throughout the training to maximize the response. The angle between the horizon of the treadmill and the back of the rat trunk was initially set to 45° and then decreased by 15° on a weekly basis, according to the tolerance of each rat, until 0° was reached (without tail suspended) by the 4th week. The animals in the model group did not receive training.

Assessment of functional recovery after spinal cord contusion injury

Functional recovery was evaluated weekly from 1 to 11 weeks post-injury using the Basso, Beattie and Bresnahan score with assessments made on a scale of 0 (complete paralysis) to 21 (normal mobility)[42]. The locomotor activities of the hindlimbs, the trunk and the tail were observed weekly in an open field for 4 minutes. Two observers scored independently, and the scores were averaged between the observers. The observers were blinded to each rat in the experimental group.

Immunohistochemical staining for growth-associated protein-43 at the site of spinal cord injury and for tyrosine hydroxylase at the L_2 level

At 11 weeks post-injury, the rats were deeply anesthetized (45 mg/kg pentobarbital) and transcardially perfused with 100 mL of cold (4°C) 0.9% NaCl and then with 200 mL of 4% paraformaldehyde (0.1 mol/L, pH 7.4). The spinal cords were removed and post-fixed overnight in 4% paraformaldehyde at 4°C and then transferred to phosphate-buffered 30% sucrose for 48 hours at 4°C for tissue cryoprotection. The 15-mm-long T_{8-11} segments of the thoracic spinal cord, containing the entire lesion site, and the 5-mm-long L_2 segments of the spinal cord of five rats from each group were dissected, removed, and embedded in Tissue-Tek optimal cutting temperature compound (Leica, Wetzlar, Germany). The embedded 15-mm-long and 5-mm-long spinal cord segments were sagittally sectioned and cross-sectioned, respectively, in 15-μm-wide slices using a freezing microtome and were mounted on a series of 8 slides such that each slide contained every 8th section. Sections were washed three times with PBS and incubated with 0.1% Triton X-100 (Sigma, Ronkonkoma, New York, NY, USA) containing 1% normal goat serum in 0.1 mol/L phosphate buffer (pH 7.4) for 30 minutes at room temperature. Incubations with the mouse anti-growth-associated protein-43 (1:200; Beijing Bofeikang Biotechnology Company, Beijing, China) and rabbit anti-tyrosine hydroxylase polyclonal antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were performed overnight at 4°C. After repeatedly washing with PBS, sections were incubated with Alexa Fluor 546 goat anti-mouse antibody (1:400; Molecular Probes Inc, Eugene, OR, USA) and Alexa Fluor 546 goat anti-rabbit antibody (1:400; Molecular Probes) for 60 minutes at room temperature, washed three times with PBS, coverslipped, and observed using a fluorescence microscope (Olympus BX 51, Tokyo, Japan). Image-Pro Plus V 6.0 (Media Cybernetics, Silver Spring, MD, USA) was used for quantitative analysis. During image acquisition, the illumination level of each imaging session was maintained by stabilizing the light source, and the settings of the camera and the lamp were constant. The data were expressed as the percentage of the total stained area (pixels) per total lesion site (pixels) in each section, and six sections per spinal cord were measured and summed for each rat. The total sum divided by 6 (the number of sections examined) represented the number of fibers per section for each rat. Five rats were inspected from each group.

Western blot analysis for brain-derived neurotrophic factor protein at the spinal cord lesion site

Four rats from each group were deeply anesthetized at day 77 post-injury (10 weeks post-training), and a 6-mm segment of the spinal cord encompassing the injured region was rapidly removed and immediately frozen at –80 °C in liquid nitrogen. Approximately 100 mg of tissue was homogenized on ice in 400 μL of radioimmunoprecipitation buffer (50 mmol/L Tris-HCl pH 7.4, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L ethylenediamine tetraacetic acid, 1% Triton X-100, 1% Na-deoxycholate, and 0.1% sodium dodecyl sulfate) for 30 minutes. After 5 minutes of centrifugation at 12 000 r/min and 4°C, the supernatant was collected. One microliter of the sample was used for the bicinchoninic acid protein assay. An appropriate volume of sample was heated to 95°C with loading buffer for 5 minutes, and 20 μL extract from each fraction was loaded per lane, separated on a 12% sodium dodecyl sulfate-polyacrylamide gel and transferred to a polyvinylidene fluoride membrane. The membrane then was incubated with 5% milk for 1 hour at room temperature or overnight at 4°C and washed three times for 5 minutes each with Tris-buffered saline-Tween. The membrane and the rabbit anti-brain-derived neurotrophic factor antibody (1:200; Santa Cruz Biotechnology) were incubated with gentle agitation for 1 hour at room temperature and washed three times for 5 minutes each with Tris-buffered saline-Tween. The membrane and horseradish peroxidase-conjugated AffiniPure goat anti-rabbit IgG (1:2 000; Beijing Zhongshan Golden Bridge Biotechnology Company, Beijing, China) were incubated with gentle agitation for 1 hour at room temperature and washed three times for 5 minutes each with Tris-buffered saline-Tween. The membrane was then incubated with SuperSignal West Pico chemiluminescent substrate (Pierce Biotechnology Inc., Rockford, IL, USA) with gentle agitation for 1 minute at room temperature away from light. The membrane was drained of excess developing solution, maintained wet, wrapped in preservative film and exposed to X-ray film. An initial 10-second exposure indicated the proper exposure time. Finally, the results were assayed with

Quantity One software (Bio-Rad, Waltham, MA, USA), which performs peak area integration to determine the area of each band in pixel units. Brain-derived neurotrophic factor protein level was expressed as a ratio of absorbance for brain-derived neurotrophic factor to that for β-actin.

Statistical analysis

Data were expressed as mean ± SD. Statistical analyses were carried out using SPSS 13.5 (SPSS, Chicago, IL, USA). A two-tailed Student's *t*-test was used for comparing growth-associated protein-43 expression and tyrosine hydroxylase-positive neuron counts after immunochemical detection. The levels of brain-derived neurotrophic factor protein were analyzed using a Mann-Whitney *U* test. A mixed factorial (repeated measures) analysis of variance followed by the Tukey-Kramer test was used for the comparison of the weekly functional recovery patterns after injury. A value of $P < 0.05$ was considered statistically significant.

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