Effects of Repetitive Transcranial Magnetic Stimulation (rTMS) and Treadmill Training on Recovery of Motor Function in a Rat Model of Partial Spinal Cord Injury

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Statistical Analysis C
Data Interpretation D
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Background: This study aimed to investigate the effects of repetitive transcranial magnetic stimulation (rTMS) and treadmill training (TT) on motor function recovery in rats with partial spinal cord injury (SCI).

Material/Methods: Sixty rats with moderate partial SCI at the 9th thoracic vertebral level induced by a Louisville Injury System Apparatus impactor were randomly allocated to 5 groups: Sham surgery (Intact); Sham rTMS without TT (S-rTMS/Non-TT); Sham rTMS with TT (S-rTMS/TT); rTMS without TT (rTMS/Non-TT); and rTMS with TT (rTMS/TT). Interventions commenced 8 days after SCI and continued for 8 weeks. Outcomes studied were Basso, Beattie, and Bresnahan locomotor scale scores, grid walking test, and biochemical analysis of the brain-derived neurotrophic factor (BDNF), synapsin I (SYN), and postsynaptic density protein-95 (PSD-95) in the motor cortex and spinal cord.

Results: The rTMS/TT contributed to greater Basso, Beattie, and Bresnahan scores compared with the S-rTMS/Non-TT (P<0.01), S-rTMS/TT (P<0.05), and rTMS/Non-TT (P<0.05), and showed obviously reduced numbers of foot drops compared with the S-rTMS/Non-TT (P<0.05). The rTMS/TT significantly increased the expressions of BDNF, SYN, and PSD-95 compared with the S-rTMS/Non-TT, both in the motor cortex (P<0.01, P<0.01, P<0.001, respectively) and spinal cord (P<0.001, P<0.01, P<0.05, respectively).

Conclusions: In a modified rat model of SCI, combined rTMS with TT improved motor function, indicating that this combined approach promoted adaptive neuroplasticity between the motor cortex and the spinal cord. A combined approach to improving motor function following SCI requires further evaluation to determine the possible clinical applications.

Keywords: Neuronal Plasticity • Spinal Cord Injuries • Transcranial Magnetic Stimulation

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Background

Spinal cord injury (SCI) is a neurological disorder caused by interruption of ascending and descending pathways that carry information between the brain and the spinal cord, leading to severe and permanent motor and sensory dysfunction [1-3]. After SCI, plastic changes occur in some neural circuits, not only in the spinal cord but also in cortical structures [3]. Unselective and maladaptive plasticity of the motor and sensory projection may induce further limitation of movement [4]. Promotion of motor function recovery is urgent and challenging for SCI patients [5]. Approaches based on drug administration or cell transplants are aimed at restoring sensorimotor function after SCI [6]. However, clinical conversions often fail due to adverse effects or lack of efficacy [6]. In contrast, noninvasive neurophysical therapy has shown the potential to improve motor function in patients with incomplete SCI [7]. A clinical practice guideline for SCI has suggested that rehabilitation can maximize neural function recovery [8].

Exercise training aimed at promoting motor function has been studied extensively with SCI patients and animal models [9]. The biological effects of exercise training mainly include preventing dendritic atrophy of motor neurons [10], inducing collateral sprouting of axons [11], reducing myelin loss [12], and promoting the expressions of neurotrophic factors [13]. Some studies reported that exercise training is beneficial to improve motor function after SCI [14-16]. However, the effectiveness of exercise training alone on the recovery of motor function is often limited [17-19] and additional intervention may be necessary to activate specific neural networks [1].

Repetitive transcranial magnetic stimulation (rTMS) is a noninvasive brain stimulation technique effected by transmitting bipolar magnetic pulses repeatedly on the cerebral cortex to generate induced current [17], modulating activity of cortical, subcortical, and the corticospinal pathways to improve function [20]. In general, low-frequency rTMS ($\leq$1 Hz) inhibits cortical excitability, whereas high-frequency rTMS ($\geq$5 Hz) increases cortical excitability and induces long-term potentiation, which contributes to the promotion of synaptic plasticity [1,21,22]. rTMS has been considered to be a promising tool for the treatment of somatomotor deficit after SCI [23,24]. However, some studies have shown that the effectiveness of rTMS is limited and variable, which may due to use of different stimulus parameters, number of sessions, targeted region, and the start time of use [20,25,26].

Recently, more and more researchers have realized that combination therapy is more beneficial to the regeneration and reconnection of neural circuits than monotherapy [17,27]. From the spinal to the motor cortex, combination therapy aims to promote plasticity at multiple levels of the neuraxis and may

be the key to improve motor function after SCI [28]. In a clinical study, Benito et al [29] reported the combination of routine rehabilitation training and rTMS is beneficial to functional recovery, but the evaluation indexes are limited to the subjective function assessment scales, such as walking index for SCI, 10-meter walking test, and the lower-extremities motor score. The differences in biological effects between exercise training and rTMS at different levels of motor systems have not been studied, and their synergistic mechanism is still unclear. Thus, further studies in animal models of SCI are needed to explore the biological effects of exercise training and rTMS at different levels of motor systems. We proposed that significant motor function recovery following SCI may require the co-promotion of neuroplasticity both in the motor cortex and spinal cord through a combination therapy using rTMS and exercise training.

Therefore, this study aimed to investigate the effects of rTMS and exercise training on the recovery of motor function in a rat model of partial SCI. The training method was modified treadmill training (TT) to produce a standardized and consistent plantar stepping pattern because the pattern of plantar stepping helps activate motor function [30]. We conducted an animal experiment based on a rat model of partial SCI because rats have been identified to be the primary model to study the underlying mechanisms of function recovery after SCI [31]. We evaluated motor function by using the Basso, Beattie, and Bresnahan locomotor scale scores and the grid walking test. The Basso, Beattie, and Bresnahan scale is a universal, reliable, and valid measure of motor function recovery [32,33]. The grid walking test is valid and sensitive for sensorimotor coordination evaluation [34,35]. For motor cortical and spinal plasticity, we evaluated BDNF, synapsin I (SYN), and postsynaptic density protein-95 (PSD-95). BDNF modulates synaptic efficacy by altering release of presynaptic transmitter or increasing sensitivity of postsynaptic transmitters [36,37], thereby promoting neuroplasticity [38,39]. SYN is a presynaptic marker showing synaptic connectivity changes [40], and PSD-95 is a marker of postsynaptic remodeling [41].

Material and Methods

Animals

All procedures were conducted in accordance with the China Laboratory Animal Guidelines and followed the guidelines of the Institutional Animal Care and Use Committee of Nanjing Medical University. All procedures complied with animal protection, animal welfare, and ethical principles, and were approved by Institutional Animal Care and Use Committee of Nanjing Medical University, China (license no. IACUC-1901039).

Sixty female Sprague-Dawley rats (8-10 weeks, 200-220 g) were provided by the Animal Care Facility, Nanjing Medical University, China. The rats were housed in a temperature-controlled environment (22°C), with free access to food and water and a 12-h light/dark cycle.

Rats were randomly allocated to 5 groups (n=12 per group) with a computer-generated randomization algorithm: Sham surgery (Intact): rats underwent laminectomy without damaging the spinal cord; S-rTMS/Non-TT: Rats with induced SCI that performed sham rTMS without TT; S-rTMS/TT: Rats with SCI that performed TT with sham rTMS; rTMS/Non-TT: Rats with SCI that performed rTMS without TT; and rTMS/TT: Rats with SCI that performed TT immediately after the completion of rTMS.

SCI Model Establishment

A moderate contusive SCI rat model was established with the Louisville Injury System Apparatus (LISA, University of Louisville, USA) impactor. To avoid damaging the respiratory center and urination center, leading to difficulties in postoperative care and high postoperative mortality, we set SCI at the ninth thoracic vertebra level. Surgical procedures followed previously published methods [42]. Briefly, rats were anesthetized via intraperitoneal injection of 10% chloral hydrate (3.5 mg/kg). Once the tail and paw withdrawal reflex disappeared, the back of the rat was shaved and disinfected. The skin and musculature were dissected to expose thoracic vertebrae, and the ninth thoracic vertebra was stabilized with a stabilization device. After that, vertebral laminectomy was performed to expose the spinal cord. Then, rats received a rapid strike via a tip (diameter=2.2 mm) driven with 138 Kpa (**Figure 1A**). The depth of penetration into the spinal cord was 1.2 mm. Finally, muscles and skin were sutured. Rats were placed on a heat-regulated mat until awake. The swing of the tail and paralyzed hind limbs were regarded as a sign of a successful model. The whole procedure was performed under aseptic conditions. Postoperative care was performed to empty bladders of all SCI rats at least twice a day until micturition control re-established.

Interventions

TT

TT commenced 1 week after SCI. The instrument was provided by Indiana University School of Medicine, Indianapolis, USA. The partial body weight of rats was supported with a cloth harness [30], which was connected to a steel bridge. The 2 tracks slide back and forth alternately. Both hind paws wore boots that were immobilized on the pedals of 2 tracks and moved back and forth with the tracks. The pedals moved up and down to make the hind limbs of rats lift and fall in a standardized and consistent plantar stepping pattern. The tail was gently

secured to the steel bridge to prevent it from interfering with the instrument (**Figure 1B**). The training schedule was 5 days per week, 10 min per day, for a total 8-week training period. Based on the situation of locomotion recovery, initial speed was 60 rpm (3.6 m/min) and gradually increased to 100 rpm (6 m/min) within the first training week and then was maintained at that speed until the end of the training period.

rTMS

rTMS commenced 1 week after surgery. Conscious rats were attached to a board with an elastic bandage to keep them stable, and their heads were fixed by pushpins placed around the head. An instrument (CCY-IA, Wuhan Yiruide medical Co., Hubei, China) with a 6-cm-diameter coil was used (**Figure 1C**). The stimulation site was located at the midline between the bregma and lambda, covering bilateral sensorimotor cortices [26]. The orientation of the coil was tangent to the skull. Stimulation parameters were as follows [23,43]: 10 Hz at 90% active motor threshold, 1000 pulses and a 25-s interval between each training. For sham rTMS [44], an inactive coil was placed where the active coil was normally placed, and the active coil was placed vertically above the inactive coil with parameters unchanged, so the rats could feel the same sounds and vibrations without being exposed to the magnetic field. The stimulation schedule was 5 days per week, 10 min per day, for a total 8-week period.

Measures

Behavioral Tests

Basso, Beattie, and Bresnahan Locomotor Scale

Locomotion was evaluated using Basso, Beattie, and Bresnahan locomotor scale scores preoperatively and weekly until the ninth week after surgery, during the days when the rats did not receive interventions. The evaluation process was as previously described [14]. The rats were placed on a 90-cm-diameter open-field platform, and their free movements were recorded for 4 min (**Figure 1D**). The scale ranges from 0 to 21 (from complete flaccid paraplegia to normalization) [32]. The rats were scored independently by 2 observers blinded to the group allocation of the animals according to limb movements, coordination, trunk stability, toe clearance, and tail position.

Grid Walking Test

The grid walking test was performed preoperatively and after completing the final treatment. A metal square grid (1.5×1.5 m, 5×5 cm per cell; height: 50 cm) was used. The rats were placed on the grid and allowed to walk freely (**Figure 1E**). The evaluation process was as previously described [14]. One blind
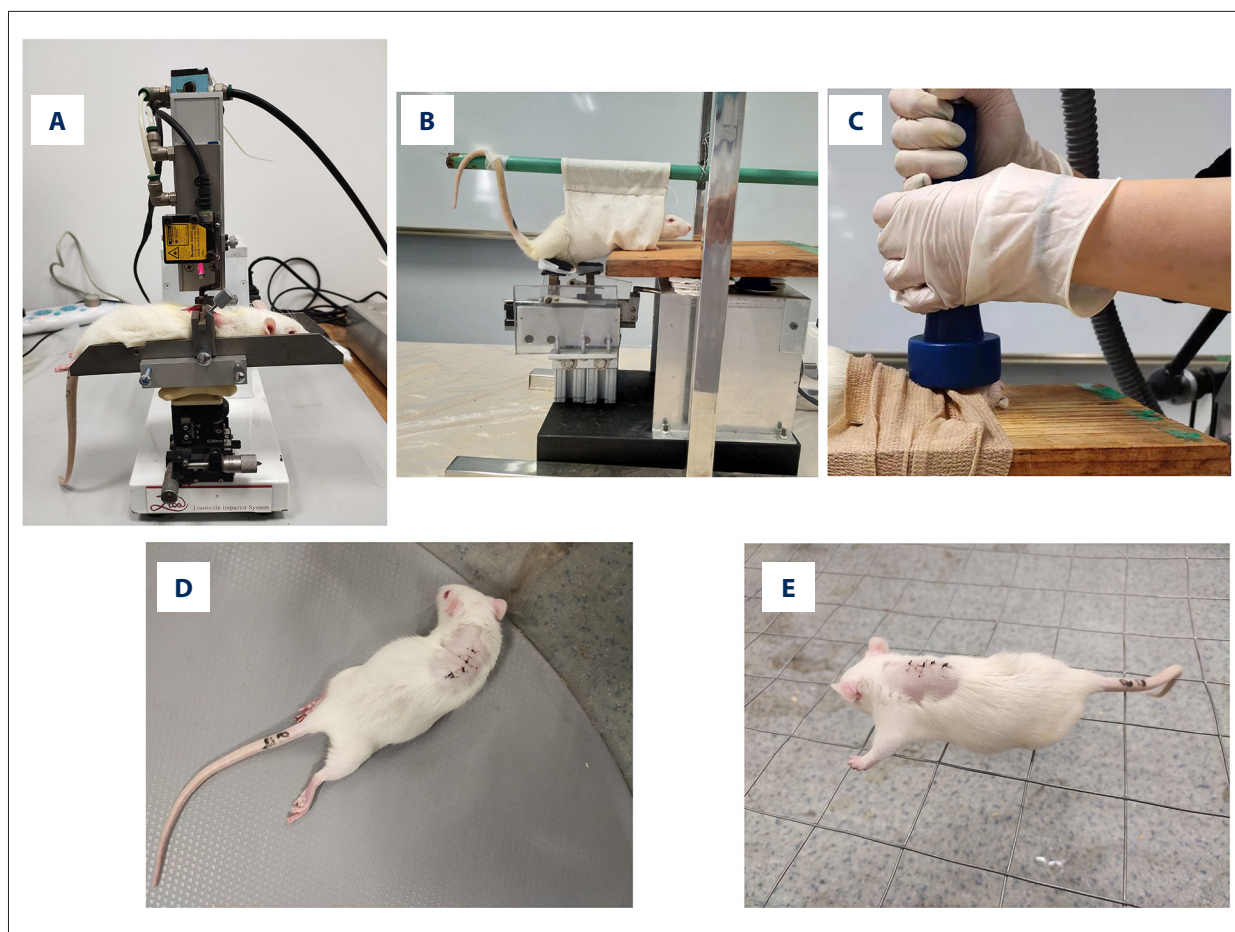
Figure 1. Interventions and measures. (**A**) Spinal cord injury model establishment. (**B**) Treadmill training. (**C**) Repetitive transcranial magnetic stimulation. (**D**) Basso, Beattie, and Bresnahan locomotor scale. (**E**) Grid walking test.

observer counted steps of the rats, while the other blind observer recorded the number of hind-limb foot drops within 30 steps. One step meant the left and right foot respectively touch the grid once. Foot drops were counted if hind paws dropped below the grid when the rats were stepping and paw placement.

Tissue Collection

After completing the final treatment and behavioral assessments, the rats of all groups were deeply anesthetized by 10% chloral hydrate. and then killed by transcardial perfusion with 0.9% saline, then 4% paraformaldehyde. Afterwards, the brain and the lumbar spinal cord (L2-4 level) were carefully separated and fixed in 4% paraformaldehyde overnight at 4°C. After gradient alcohol dehydration, the tissues were paraffin-embedded and sectioned serially to a thickness of 5 μm.

Immunohistochemistry Assay

As deparaffinization finished, slices were repaired with sodium citrate buffer (0.01 mol/L, pH 6.0). After peroxidase inactivation, phosphate-buffered saline washing and 10% goat serum blocking, sections were incubated overnight at 4°C with the following primary antibodies: rabbit anti- BDNF antibody (1: 800, Abcam, UK), anti-SYN antibody (1: 300, Abcam, UK), and anti- PSD-95 antibody (1: 200, Abcam, UK). Then, the tissues were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (1: 300, Zhong Shan Jin Qiao, China) for 1 h at 37°C. 3,3'-diaminobenzidine-4 HCl/H_2O_2 (DAB, Vector Laboratories, Burlingame, CA, USA) was used for visualization. Hematoxylin was counterstained after BDNF and SYN staining. Images of the motor cortex and the ventral horn region of the lumbar spinal cord were obtained using an optical microscope at 200× and 400× view field by Leica Application Suite (Leica Microsystems, Wetzlar, Germany). Image J software (National Institutes of Health, Bethesda, Maryland, USA) was applied to analyze mean optical density values.

Western Blotting

Tissues in the motor cortex and the lumbar spinal cord (L2-4 level) of rats were selected. After homogenization and

centrifugation, the supernatants were diluted with loading buffer. The protein samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel by electrophoresis (SDS-PAGE) and transferred on to polyvinylidene fluoride membranes (Millipore, USA). Then, the membranes were blocked with 5% milk solubilized in Tris-buffered saline with 0.1% Tween 20 at room temperature for 1 h. After that, the membranes were incubated with rabbit anti-BDNF antibody (1: 1000, Abcam, UK), anti-SYN antibody (1: 1000, Abcam, UK), anti- PSD-95 antibody (1: 1000, Abcam, UK), or glyceraldehyde phosphate dehydrogenase (GAPDH) (1: 1000, Proteintech, USA) overnight at 4°C. The next day, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit antibody (1: 2000, Zhong Shan Jin Qiao, China) for 1 h at room temperature. Reacting bands were visualized though enhanced chemiluminescence method. Image J software (National Institutes of Health, Bethesda, Maryland, USA) was applied for semi-quantitative analysis of the density of the immunoreactive bands.

Statistical Analysis

Basso, Beattie, and Bresnahan scores were analyzed with repeated-measures analysis of variance, followed by Bonferroni-corrected post hoc tests. The remaining data were analyzed using one-way analysis of variance, followed by post hoc Tukey t test. All data were presented as mean±standard deviation (SD). Statistical analysis was performed with Statistical Product and Service Solutions (SPSS) 22.0 (IBM Co., Armonk, NY, USA) software. A value of $P<0.05$ indicated a statistically significant difference.

Results

Motor Function Assessment

Basso, Beattie, and Bresnahan Scores

At 1 week after injury, the scores in the S-rTMS/Non-TT, S-rTMS/TT, rTMS/Non-TT, and rTMS/TT groups were 6.79±2.26, 6.67±1.97, 6.38±2.60, and 6.67±2.08, respectively. No statistically significant difference was observed ($P>0.05$). From the seventh week to the ninth week after injury, the rTMS/TT group (7/8/9 weeks: 14.79±1.96, 15.25±1.94, 15.85±1.93) showed noticeably enhanced locomotion compared with the S-rTMS/Non-TT (7/8/9 weeks: 13.32±1.64,

13.77±1.77,13.77±1.77, and $P<0.01$) and rTMS/Non-TT (7/8/9 weeks: 13.71±2.24, 14.17±2.33, 14.29±2.27, and $P<0.05$) groups. At 8 weeks after injury, the rTMS/TT group showed significantly higher scores than the S-rTMS/TT group (8/9 weeks: 14.25±2.03,14.42±1.98, and $P<0.05$). No significant difference was observed among the S-rTMS/Non-TT, S-rTMS/TT, and rTMS/Non-TT rats (**Figure 2A, Table 1**).

Grid Walking Test

At 9 weeks after injury, the number of foot drops in the Intact, S-rTMS/Non-TT, S-rTMS/TT, rTMS/Non-TT, and rTMS/TT groups were 2.75±1.36, 26.82±4.09, 25.75±3.41, 26.17±4.41, and 22.08±4.06, respectively. The number of foot drops in the 4 groups of SCI rats were significantly higher than in the Intact rats ($P<0.001$). The rTMS/TT rats showed obviously reduced number of foot drops compared with the S-rTMS/Non-TT rats ($P<0.05$) (**Figure 2B**). However, there was no statistically significant difference among the S-rTMS/TT, rTMS/Non-TT, and S-rTMS/Non-TT groups.

Immunohistochemistry Staining

Motor Cortex

In the 2 TT-treated groups, the relative densities of BDNF and PSD-95 were significantly higher in the rTMS/TT group (145.81±18.88% and 122.00±8.21%, respectively) compared with the S-rTMS/TT group (110.65±4.95%, 104.60±8.85%, and $P<0.05$, $P<0.05$, respectively, **Figure 3B, 3D**).

In the 2 Non-TT-treated groups, the relative densities of BDNF and PSD-95 were significantly higher in the rTMS/Non-TT group (127.49±13.26% and 114.56±7.01%) compared with the S-rTMS/Non-TT group (90.30±18.00%, 88.05±7.40%, and $P<0.05$, $P<0.01$, respectively, **Figure 3B, 3D**).

In 2 rTMS-treated groups, no significant difference was observed between the rTMS/TT and rTMS/Non-TT groups. In the 2 sham rTMS-treated groups, no significant difference was observed between the S-rTMS/TT and S-rTMS/Non-TT groups.

The relative densities of BDNF, SYN, and PSD-95 were far higher in the rTMS/TT group (145.81±18.88%, 115.65±4.93%, 122.00±8.21%) compared with the S-rTMS/Non-TT group (90.30±18.00%, 93.03±4.50%, 88.05±7.40%, and $P<0.01$, $P<0.01$, $P<0.001$, respectively, **Figure 3A-3D, Table 2**). There was no statistically significant difference between the S-rTMS/Non-TT and Intact group.

Lumbar Spinal Cord

Compared with the Intact group, the relative density of BDNF was significantly reduced in the S-rTMS/Non-TT group (82.71±8.18%, $P<0.05$, **Figure 4B**).

In the 2 TT-treated groups, the density of BDNF was significantly higher in the rTMS/TT group (143.75±8.72%) than that in the S-rTMS/TT group (115.18±5.76%, $P<0.01$, **Figure 4B**).
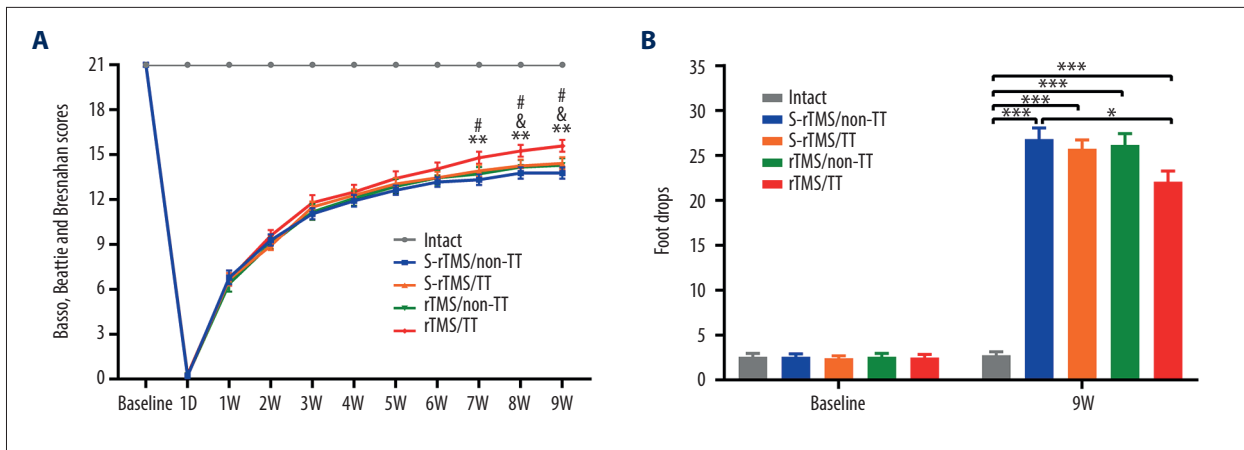
Figure 2. Motor function assessment. (**A**) Basso, Beattie, and Bresnahan scores. ** $P<0.01$, rTMS/TT vs S-rTMS/Non-TT; # $P<0.05$, rTMS/TT vs rTMS/Non-TT; & $P<0.05$, rTMS/TT vs S-rTMS/TT. (**B**) Grid walking test. The number of foot drops in each group. *** $P<0.001$, * $P<0.05$. Twelve rats in each group. At 8 weeks after injury, 1 rat in the S-rTMS/Non-TT group died of abdominal distention. Data presented as mean±standard error of mean. D – day; W ,– week; rTMS – repetitive transcranial magnetic stimulation; TT – treadmill training; S – sham.

Table 1. Motor function assessment.

Behavioral tests	Time	Intact	S-rTMS/Non-TT	S-rTMS/TT	rTMS/Non-TT	rTMS/TT
Basso, Beattie, and Bresnahan scores	Baseline	21	21	21	21	21
	1D	21	0.21±0.41	0.21±0.41	0.25±0.44	0.29±0.46
	1W	21	6.79±2.26	6.67±1.97	6.38±2.60	6.67±2.08
	2W	21	9.29±1.83	8.92±1.44	9.04±1.97	9.58±1.84
	3W	21	11.04±1.81	11.50±1.79	11.17±2.53	11.79±2.43
	4W	21	11.92±1.93	12.29±1.92	12.08±2.45	12.50±2.36
	5W	21	12.63±1.53	13.04±1.90	12.88±2.49	13.42±2.24
	6W	21	13.17±1.55	13.46±1.93	13.46±2.30	14.04±2.10
	7W	21	13.32±1.64**	13.92±2.00	13.71±2.24*	14.79±1.96
	8W	21	13.77±1.77**	14.25±2.03*	14.17±2.33*	15.25±1.94
	9W	21	13.77±1.77**	14.42±1.98*	14.29±2.27*	15.85±1.93
Grid walking test (foot drops)	Baseline	2.58±1.31	2.58±1.08	2.42±0.90	2.58±1.31	2.50±1.17
	9W	2.75±1.36	26.82±4.09###,*	25.75±3.41###	26.17±4.41###	22.08±4.06###

* $P<0.05$, ** $P<0.01$, vs rTMS/TT. ### $P<0.001$, vs Intact. Data presented as mean±standard deviation. rTMS – repetitive transcranial magnetic stimulation; TT – treadmill training; S – sham; D – day; W – week.

In the 2 Non-TT-treated groups, no significant difference was found between rTMS/Non-TT and S-rTMS/Non-TT.

In the 2 rTMS-treated groups, the density of BDNF was significantly higher in the rTMS/TT group (143.75±8.72%) compared with the rTMS/Non-TT (98.58±8.03%, $P<0.001$, **Figure 4B**).

In the 2 sham rTMS-treated groups, the S-rTMS/TT group had significantly higher BDNF density compared with the S-rTMS/Non-TT group ($P<0.001$, **Figure 4B**).

The rTMS/TT group (143.75±8.72%, 118.34±12.33%, 117.68±10.12%) had higher relative densities of BDNF, SYN, and PSD-95 compared with the S-rTMS/Non-TT group (82.71±8.18%,
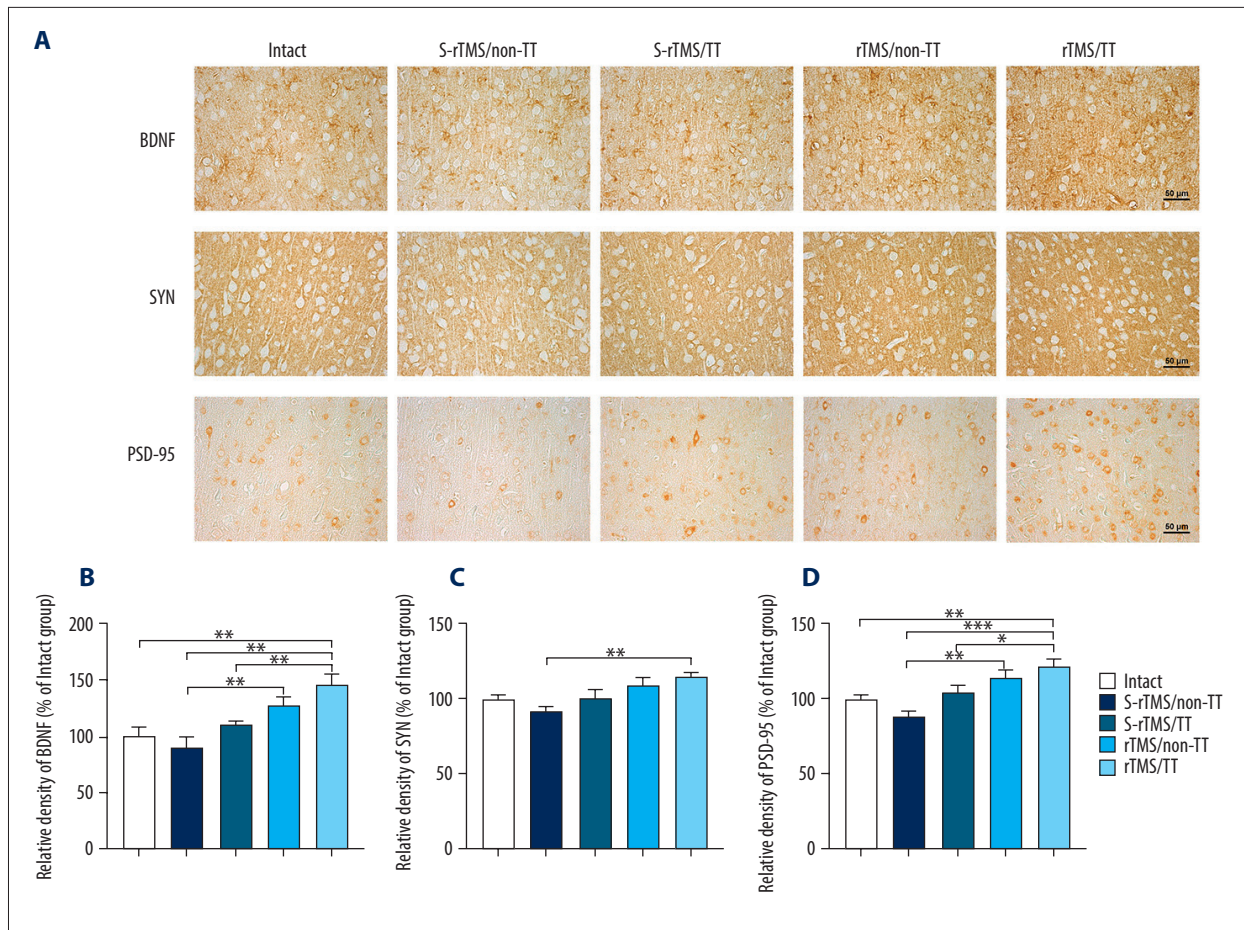
Figure 3. Immunohistochemistry analysis of motor cortex. (**A**) Immunohistochemistry staining of BDNF, SYN and PSD-95. (**B**) Relative density of BDNF. (**C**) Relative density of SYN. (**D**) Relative density of PSD-95. Data presented as mean±standard error of mean. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Four rats in each group. Scale bar: 50 μm. BDNF – brain-derived neurotrophic factor; SYN – synapsin I; PSD-95 – postsynaptic density protein-95; rTMS – repetitive transcranial magnetic stimulation; TT – treadmill training; S – sham.

84.06±9.75%, 93.11±8.57%, and $P<0.001$, $P<0.01$, $P<0.05$, respectively, **Figure 4A-4D**).

Western Blotting

Motor Cortex

In the 2 TT-treated groups, the expressions of BDNF and PSD-95 were significantly higher in the rTMS/TT group (300.35±87.44%, 194.33±37.98%) compared with the S-rTMS/TT group (170.77±31.81%, 131.23±31.74%, and $P<0.05$, $P<0.05$, respectively, **Figure 5B, 5D**). In the 2 Non-TT-treated groups, the rTMS/Non-TT group (195.51±53.88%, 137.00±29.49%) also had significantly higher expressions of BDNF and PSD-95 compared with the S-rTMS/Non-TT group (82.67±22.59%, 77.03±16.43%, and $P<0.05$, $P<0.05$, respectively, **Figure 5B, 5D**).

The expressions of BDNF, SYN, and PSD-95 were higher in the rTMS/TT group (300.35±87.44%, 178.91±48.27%, 194.33±37.98%) compared with the S-rTMS/Non-TT group (82.67±22.59%, 86.95±27.29%, 77.03±16.43%, and $P<0.001$, $P<0.05$, $P<0.001$, respectively, **Figure 5A-5D**). Taken together, these results further confirmed the results of immunohisto-chemistry analysis of the motor cortex.

Lumbar Spinal Cord

Compared with the Intact group, the BDNF expression was significantly reduced in the S-rTMS/Non-TT group (34.52±3.88%, $P<0.05$, **Figure 6B**). In the 2 TT-treated groups, the BDNF expression was significantly higher in the rTMS/TT group (225.52±47.22%) than that in the S-rTMS/TT group (155.47±21.91%, P<0.05, **Figure 6B**). In the 2 Non-TT-treated groups, no significant difference was found between rTMS/Non-TT and S-rTMS/Non-TT. In the 2 rTMS-treated groups,

Table 2. IHC and WB.

Tissues	Methods	Measures	Intact	S-rTMS/Non-TT	S-rTMS/TT	rTMS/Non-TT	rTMS/TT
Motor cortex	IHC	BDNF	100.00± 15.08%**	90.30± 18.00%#,**	110.65± 4.95%*	127.49± 13.26%	145.81± 18.88%
		SYN	100.00± 6.82%	93.03± 4.50%**	101.36± 11.07%	109.62± 9.62%	115.65± 4.93%
		PSD-95	100.00± 7.32%**	88.05± 7.40%##,***	104.60± 8.85%*	114.56± 7.01%	122.00± 8.21%
	WB	BDNF	100.00± 27.19%***	82.67± 22.59%#,***	170.77± 31.81%*	195.51± 53.88%	300.35± 87.44%
		SYN	100.00± 36.73%	86.95± 27.29%*	136.13± 47.51%	146.29± 44.11%	178.91± 48.27%
		PSD-95	100.00± 11.66%**	77.03± 16.43%#,***	131.23± 31.74%*	137.00± 29.49%	194.33± 37.98%
Spinal cord	IHC	BDNF	100.00± 7.14%***	82.71± 8.18%&,&&&,***	115.18± 5.76%**	98.58± 8.03%***	143.75± 8.72%
		SYN	100.00± 8.53%	84.06± 9.75%**	102.06± 6.46%	98.49± 8.49%	118.34± 12.33%
		PSD-95	100.00± 8.86%	93.11± 8.57%*	110.16± 8.05%	106.95± 9.36%	117.68± 10.12%
	WB	BDNF	100.00± 23.17%***	34.52± 3.88%&,&&&,***	155.47± 21.91%*	95.24± 24.99%***	225.52± 47.22%
		SYN	100.00± 23.45%	66.98± 29.71%*	109.21± 36.51%	87.96± 29.06%	137.78± 38.33%
		PSD-95	100.00± 23.52%	72.73± 35.31%*	131.24± 42.86%	101.39± 23.98%	163.87± 45.26%

* $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs rTMS/TT; # $P<0.05$, ## $P<0.01$, vs rTMS/Non-TT; & $P<0.05$, vs Intact; &&& $P<0.001$, vs S-rTMS/TT. Data presented as mean ± standard deviation. IHC – immunohistochemistry; WB – western blotting; BDNF, brain-derived neurotrophic factor; SYN – synapsin I; PSD-95 – postsynaptic density protein-95; rTMS – repetitive transcranial magnetic stimulation; TT – treadmill training; S – sham.

the BDNF expression was significantly higher in the rTMS/TT group compared with the rTMS/Non-TT ($95.24\pm24.99\%$, $P<0.001$, **Figure 6B**). In 2 sham rTMS-treated groups, the S-rTMS/TT group had significantly higher BDNF expression compared with the S-rTMS/Non-TT group ($P<0.001$, **Figure 6B**). The rTMS/TT group ($225.52\pm47.22\%$, $137.78\pm38.33\%$, $163.87\pm45.26\%$) had higher expressions of BDNF, SYN, and PSD-95 compared with the S-rTMS/Non-TT group ($34.52\pm3.88\%$, $66.98\pm29.71\%$, $72.73\pm35.31\%$, and $P<0.001$, $P<0.05$, $P<0.05$, respectively, **Figure 6A-6D**). Taken together, these results further confirmed the results of immunohistochemistry analysis of the spinal cord.

Discussion

In the present study, we evaluated the effects of rTMS and TT on motor function recovery and motor cortical and spinal plasticity in a rat model of partial SCI. Our data indicated that application of rTMS or TT alone had limited effect on

motor function recovery. The therapeutic influences were better in the combination therapy group than in the single-therapy groups. In the spinal cord, TT effectively induced BDNF expression, but had the limitation of inducing the expression of synaptic plasticity markers. In the motor cortex, rTMS effectively promoted cortical plasticity. Moreover, rTMS combined with TT remarkably promoted the plasticity in the motor cortex and spinal cord. These results greatly support the feasibility and efficacy of the combined therapeutic strategy for SCI.

Effects of TT

We found the effect of TT with sham rTMS on motor function recovery was limited. Nicola et al [18] also found no significant differences in Basso, Beattie, and Bresnahan scores between TT alone and non-trained SCI rats. Research on TT applied following SCI has often shown controversial results in motor function recovery [9]. Some studies found that TT alone improved motor function, but it often took considerable time
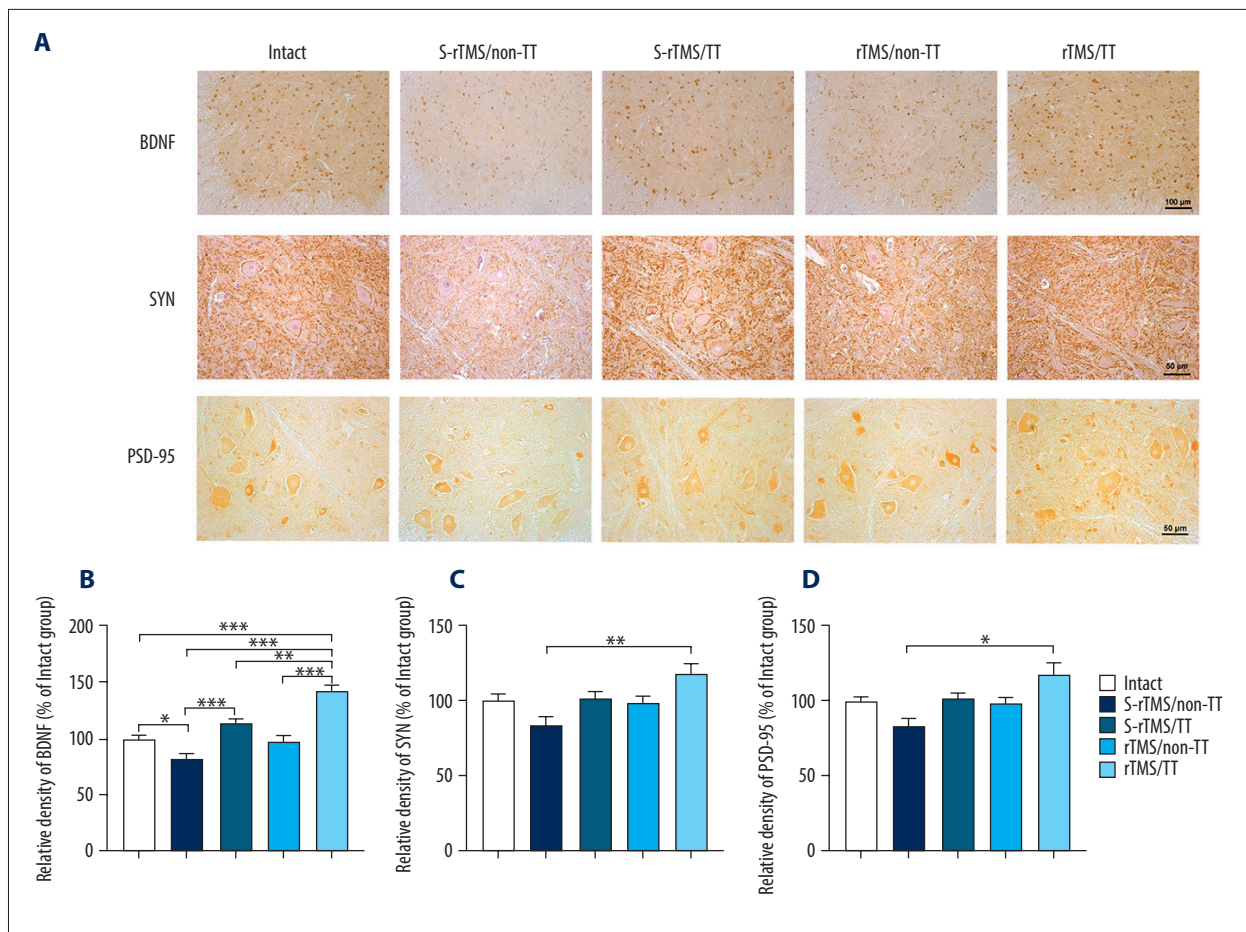
Figure 4. Immunohistochemistry analysis of spinal cord. (**A**) Immunohistochemistry staining of BDNF, SYN and PSD-95. (**B**) Relative density of BDNF. (**C**) Relative density of SYN. (**D**) Relative density of PSD-95. Data presented as mean±standard error of mean. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Four rats in each group. Scale bar: 100 μm in BDNF. 50 μm in SYN and PSD-95. BDNF – brain-derived neurotrophic factor; SYN – synapsin I; PSD-95 – postsynaptic density protein-95; rTMS – repetitive transcranial magnetic stimulation; TT – treadmill training; S – sham.

and effort, such as multiple sessions per day [14] and longer duration (30 min) [15] and periods (more than 9 weeks) [19].

In the spinal cord, the BDNF expression was significantly reduced after SCI. We found TT alone reversed this change, enhancing BDNF expression, as reported previously [14,16].

BDNF acts as a mediator or instructor in inducing activity-dependent plasticity [45] by stimulating dendritic spines and regulating synapses [39,42]. As a crucial downstream effector for BDNF [46], SYN is phosphorylated by BDNF, which mediates synaptic vesicle circulation and neurotransmitter release [47]. PSD-95 facilitates downstream intracellular signaling and modulates receptor stability, thus contributing to synaptic plasticity [41,48-50]. In our study, the higher expressions of presynaptic and postsynaptic plasticity markers SYN and PSD-95 were not observed in the TT alone group. These results indicated that the activation of BDNF in the spinal cord induced by TT was

still insufficient to extend to downstream effectors and failed to produce an important effect on synaptic plasticity, which may partially explain the limited motor function recovery.

In the motor cortex, we found that TT alone was not effective in increasing expressions of BDNF and synaptic plasticity markers. However, Ilha et al [51] showed that overground gait training was able to promote motor cortical plasticity. Similar repetitive motor training in our study seemed to train paralyzed limbs without the need for cortical relearning, such as step initiation and forward progression, thus improving the motor learning and control ability of the motor cortex and subsequently enhancing supraspinal drive to the spinal motor circuit, which may be an important way to promote motor function recovery after SCI [51].
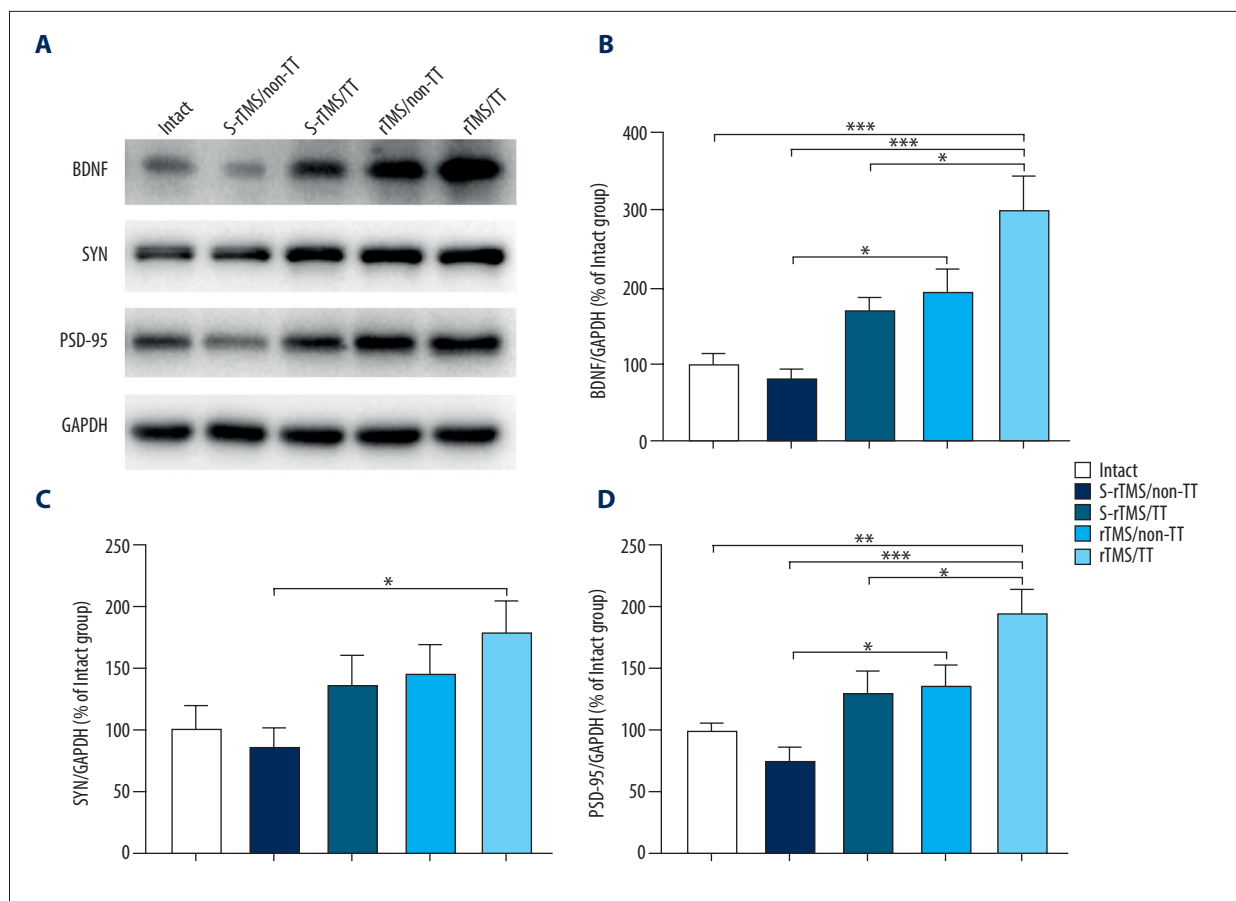
Figure 5. Western blotting analysis of motor cortex. (**A**) Western blotting representative bands of BDNF, SYN and PSD-95. (**B**) Quantification of BDNF expression. (**C**) Quantification of SYN expression. (**D**) Quantification of PSD-95 expression. Data presented as mean±standard error of mean. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Four rats in each group. BDNF – brain-derived neurotrophic factor; SYN – synapsin I; PSD-95 – postsynaptic density protein-95; GAPDH – glyceraldehyde phosphate dehydrogenase; rTMS – repetitive transcranial magnetic stimulation; TT – treadmill training; S – sham.

Effects of rTMS

In the motor cortex, we corroborated that the expressions of BDNF and PSD-95 were increased by rTMS. Recently, Fujiki et al [22] also found that high-frequency rTMS strongly activated molecular pathways critical for synaptic plasticity in the motor cortex of lightly anesthetized rats. It has been reported that synaptic remodeling in the motor cortex is a target to improve function recovery following SCI [28]. Hence, rTMS is an effective combined treatment to promote cortical plasticity, remedying some shortcomings of TT.

However, applying rTMS without TT was insufficient to promote spinal plasticity. It is known that the increase of BDNF has an activity-dependent property [52], and exercise is able to upregulate BDNF genes in the spinal cord [53]. It is reasonable to think that rTMS has limitations in the absence of TT.

We observed that applying rTMS alone did not lead to behavioral improvement in partial SCI rats. In a clinical study, rTMS also did not induce changes in sensory and motor function in patients with chronic SCI [25]. Krishnan et al [26] determined the effective time range for rTMS intervention in rats with incomplete SCI, and the results showed that rTMS performed 2 weeks after injury was not effective in improving motor function, but it was effective within 10 minutes. A meta-analysis showed that the efficacy of rTMS gradually decreased with the prolongation of disease duration [54]. These results indicate that the application of rTMS alone may have a limited time window, because a decrease in cortical spontaneous neuronal activity was observed within a few minutes after SCI [55], which was associated with poor recovery. Reducing and reversing the reduction of early cortical spontaneous activity as soon as possible plays a key role in the neurological recovery after SCI [26]. It is better to start using rTMS at a very early stage after SCI, at 2 days [23] or even 10 minutes after injury [26], which is difficult to carry out in clinical practice.
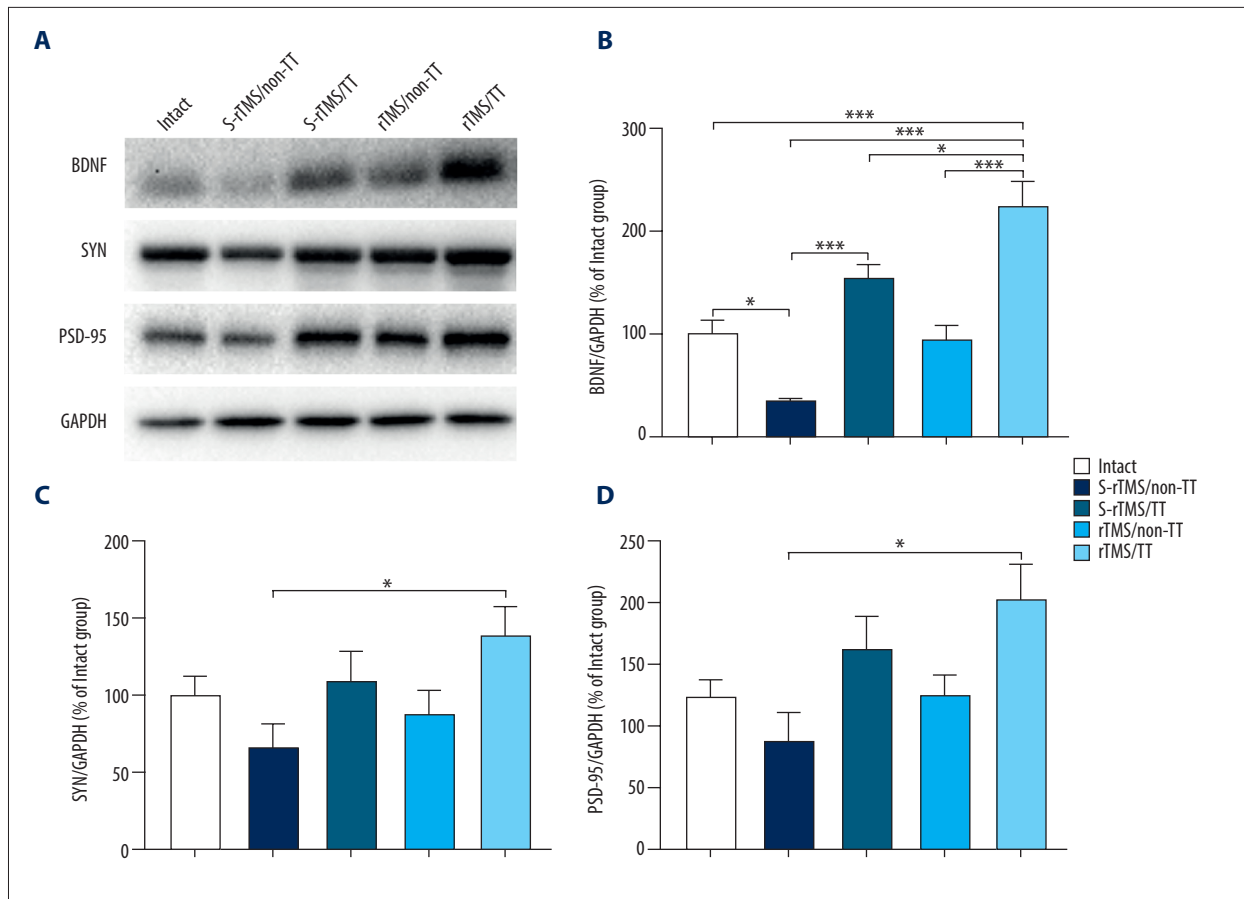
Figure 6. Western blotting analysis of spinal cord. (**A**) Western blotting representative bands of BDNF, SYN, and PSD-95. (**B**) Quantification of BDNF expression. (**C**) Quantification of SYN expression. (**D**) Quantification of PSD-95 expression. Data presented as mean±standard error of mean. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Four rats in each group. BDNF – brain-derived neurotrophic factor; SYN – synapsin I; PSD-95 – postsynaptic density protein-95; GAPDH – glyceraldehyde phosphate dehydrogenase; rTMS – repetitive transcranial magnetic stimulation; TT – treadmill training; S – sham.

Effects of Combination Therapy of rTMS and TT

In our study, combination therapy achieved optimal locomotion recovery, with higher Basso, Beattie, and Bresnahan scores and fewer foot drops. Yang et al [17] reported the possible relationship between rTMS and exercise training in promoting motor function recovery as follows: exercise training can establish motor memory through repeated practice of specific movements, which is a form of use-dependent plasticity, and rTMS enhances the encoding of motor memory related with use-dependent plasticity in the motor cortex. We consider that rTMS excites the motor cortex and related subcortical structures inducing the activation of particular neural networks, but it does not lead to long-term motor behavior changes by itself. Then, TT provides correct motor guidance to make target behaviors correlate with activated networks, thus improving motor performance.

In addition, the combination therapy created a cumulative impact on increased BDNF expression in both the motor cortex and spinal cord. A recent study also showed that combined TT and injury site magnetic stimulation increased the BDNF expression following cervical SCI in rats [4]. These results raise the question as to whether the synergistic activation of BDNF is the key to the improvement of locomotion in combination therapy. Although there is no direct verification in this study, recent studies have identified that both rTMS and TT promote neurological recovery though the BDNF-TrkB signaling pathway [16,40].

We also found that combination therapy further increased the expressions of synaptic plasticity markers in the motor cortex and spinal cord, suggesting that the plastic changes were deeper in the combination therapy group than those in the single-therapy groups. We consider that there were interconnections between cortical and spinal motor systems, because plastic changes occurring at one level may be influenced and/

or result in plasticity at other levels [56]. The adaptive plasticity occurring in a coordinated manner at different levels of the motor system is an important factor for promoting locomotion recovery after incomplete SCI.

Limitations

The three-dimensional analysis of gait kinematics may be beneficial to observe the slight changes of hind-limb motor performance. Additionally, there are anatomical differences between rats and humans. The characteristics of pathophysiological changes and spontaneous function recovery are also different after SCI [31], so further evaluation may be needed to determine its possible clinical effects.

Conclusions

The present study demonstrated that combined treatment using rTMS with TT remarkably promoted motor function recovery in a modified rat model of SCI, which involved promoting neuroplasticity in a coordinated manner at the motor cortex and spinal cord levels. This combined approach to improving motor function following SCI requires further evaluation to determine the possible clinical applications.

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Conflict of Interest

None.

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