J. Integr. Neurosci. **2025**; 24(3): 26731
https://doi.org/10.31083/JIN26731

Original Research

Transcranial Intermittent Theta-Burst Stimulation Reverses Neurodegeneration in the Somatosensory Motor Cortex after Spinal Cord Transection in Rats

Zhenghong Chen[1,2,†], Yujian Lin[2,3,†], Jing Xu[2,3], Jiawei Sun[2,3], Rui Liu[2,3], Yue Yang[2,3], Zhen Chen[2,3], Mingyu Lv[2,3], Biqin Lai[2,3,4], Ling Zhang[2,5,*]

[1]Rehabilitation Medicine Department, The First Affiliated Hospital of Sun Yat-sen University, 510080 Guangzhou, Guangdong, China
[2]Center for Stem Cell Biology and Tissue Engineering, Department of Histoembryology and Cell Biology, Zhongshan School of Medicine, Sun Yat-sen University, 510080 Guangzhou, Guangdong, China
[3]Guangdong Provincial Key Laboratory of Brain Function and Disease, Zhongshan School of Medicine, Sun Yat-sen University, 510080 Guangzhou, Guangdong, China
[4]Co-innovation Center of Neuroregeneration, Nantong University, 226001 Nantong, Jiangsu, China
[5]Department of Geriatrics, The First Affiliated Hospital, Sun Yat-sen University, 510080 Guangzhou, Guangdong, China
*Correspondence: Zhangling@mail.sysu.edu.cn (Ling Zhang)
†These authors contributed equally.
Academic Editor: Gernot Riedel
Submitted: 25 September 2024 Revised: 6 December 2024 Accepted: 11 December 2024 Published: 7 March 2025

Abstract

Background: Complete spinal cord injury (SCI) leads to a disconnection between the brain and the body below the injury level, resulting in the functional silencing, degeneration, and apoptosis of sensorimotor cortex (SMC) neurons, which is of crucial importance to the pathological process. **Methods**: In this study, a rat model of spinal cord transection was employed to explore the activation of neurons in the SMC and the reversal of neurodegeneration after the rats were treated with transcranial intermittent theta-burst stimulation (T-iTBS). **Results**: The results demonstrated that the expression of the immediate early gene *c-Fos* and the synaptic plasticity-associated activity-regulated cytoskeleton (*Arc*) gene in the neurons of the SMC was increased in the T-iTBS group 4 weeks after SCI. Transcriptome sequencing revealed that neuronal activation-, neuronal metabolism-, synaptic activity-, and neural regeneration-related genes were significantly upregulated in the T-iTBS group compared with those of the sham-iTBS group, but the expression was similar to that in the normal group. Western blot analysis indicated that the expression of Cle-caspase-3 (CC3) in the SMC was significantly reduced in the T-iTBS group, and the number of CD68-positive cells in the SMC was close to that of normal rats but significantly less than that in the sham-iTBS and SCI groups. These results are in line with those of the transcriptome sequencing. Correlation analysis of the expression rate between c-Fos and Arc, CC3, and CD68 further suggested that T-iTBS improved the immune microenvironment and prevented neurodegeneration by regulating the activation and synaptic plasticity of SMC neurons in the early stages of injury. **Conclusions**: Collectively, our findings offer support for the utilization of T-iTBS, a non-invasive neural stimulation treatment, to prevent SMC degeneration following severe SCI.

Keywords: spinal cord transection; transcranial intermittent theta-burst stimulation; sensorimotor cortex; neurodegeneration; neuroplasticity

1. Introduction

Complete spinal cord injury (SCI) gives rise to a loss of neural connections between the sensory motor cortex (SMC) and the spinal cord below the level of injury [1–3]. This brings about neuronal functional silencing, apoptosis, and degeneration of the SMC following the progression of secondary injury and further gray matter atrophy that is observable on imaging [3–5]. Currently, SCI treatment pays more attention to the injury site rather than brain pathology. Nevertheless, it is of crucial significance for nerve regeneration and functional reconstruction to protect neurons in the SMC and prevent the advancement of neurodegeneration [3,6–8].

Several studies have demonstrated that rehabilitation training and physical therapy based on neural stimulation after incomplete SCI have a positive effect on the recovery of sensory and motor function in paralyzed limbs [9–11]. However, transverse SCI induces a complete loss of neural connection between the SMC and the body below the level of injury. Pathological changes occur in SMC neurons in the early stages of injury, including histological, gene expression, and functional changes, and it is urgent to find therapeutic strategies that can reverse these adverse processes in time [3,12]. The aim of this study was to explore the efficacy of a neural stimulation therapy, which is easy to apply clinically, in saving SMC neurons.

Copyright: © 2025 The Author(s). Published by IMR Press.
This is an open access article under the CC BY 4.0 license.

Publisher's Note: IMR Press stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Table 1. Groupings of animals and the treatments administered to the rats.

Group	Surgery	Postoperative care	Treatment
SCI group (n = 15)	T9 spinal cord transection	Penicillin (80,000 units) and analgesics (Meloxicam, 0.1 mg/kg) were administered daily for one week. Urination was artificially induced.	No stimulation treatment
Sham-iTBS group (n = 15)	T9 spinal cord transection	Penicillin (80,000 units) and analgesics (Meloxicam, 0.1 mg/kg) were administered daily for one week. Urination was artificially induced.	sham iTBS stimulation (spurious stimuli, once per day for five days in each week)
T-iTBS group (n = 15)	T9 spinal cord transection	Penicillin (80,000 units) and analgesics (Meloxicam, 0.1 mg/kg) were administered daily for one week. Urination was artificially induced.	T-iTBS treatment (A total of 600 pulses with an intensity of 0.675 Tesla, 3 pulse sequence was emitted at 50 Hz, and the entire sequence was replicated at 5 Hz, once per day for five days in each week)
Normal group (n = 10)	No operation	No postoperative care	No treatment

SCI, spinal cord injury; iTBS, intermittent theta-burst stimulation; T-iTBS, transcranial intermittent theta-burst stimulation.

Repetitive transcranial magnetic stimulation (rTMS) can regulate the plasticity of neural networks by modifying the excitability of neurons at the stimulation site [13–15]. rTMS is extensively utilized as a clinical therapy for numerous central nervous system diseases, such as pain, depression, and multiple-system atrophy [16–19]. In recent years, transcranial intermittent theta-burst stimulation (T-iTBS), which is a novel rTMS paradigm with advantage like non-invasiveness and a short treatment duration [20], has been increasingly used for neural rehabilitation [21–25]. T-iTBS has been certified to promote axonal regeneration and motor function repair at the site of SCI [3,18,26–28]. Nevertheless, the biological effects on the SMC in the early stages of SCI following the application of T-iTBS need to be further investigated.

The SMC initiates a series of nociceptive cascade reactions when it receives damage signals from a SCI [3,29,30]. The timely rescue of SMC degeneration is crucial for preserving the regeneration ability of corticospinal tracts and other brain-spinal tracts and restoring voluntary motor function [31–33]. Therefore, we applied T-iTBS to rats in the early stages of injury after transected SCI to rescue neurodegeneration by regulating the excitability of SMC neurons.

2. Materials and Methods

2.1 Animals

The experimental animals were adult female SD rats (weighing 180–220 g) procured from the Experimental Animal Center of Sun Yat-sen University.

2.2 SCI Surgery

A total of 55 SD rats were divided into four groups: SCI group (n = 15), sham-iTBS group (n = 15), T-iTBS group (n = 15), and normal group (n = 10). The animals were anesthetized with 1% sodium pentobarbital (40 mg/kg, intraperitoneally, No.P3761, Sigma-Aldrich Millipore, Burlington, MA, USA) and the T9-T10 vertebral level of laminae were exposed by cutting through the skin and muscles. The dura matter was cut in a longitudinal incision with microscopic scissors and the segment of spinal cord (2.0 mm) at T9 level was completely removed, including the associated spinal roots. The rats received extensive post-operation care including intramuscular injection of penicillin (80,000 units, No.1224, Shandong Shengwang Pharmaceutical Co., Ltd., Shandong, China) and analgesics (Meloxicam, 0.1 mg/kg, No.T0826, TargetMol Chemicals Inc., Boston, MA, USA) daily for one week, and they were artificially urinated until their autonomous urination function was restored. The SCI group, the sham-iTBS group and the T-iTBS group underwent complete transection of the spinal cord at the T9 level. Rats in the T-iTBS group were treated for four weeks after surgery, the sham-iTBS group received spurious stimulation after SCI for four weeks. The normal group received neither surgery nor stimulation. (Table 1 and Fig. 1E).

2.3 T-iTBS Therapy

The rats in the T-iTBS group received T-iTBS treatment 72 hours subsequent to surgery using a YRD CCY-I Magnetic Stimulator (Wuhan Yiruide Medical Equipment New Technology, Wuhan, Hubei, China), which was equipped with a special circular coil (r = 32 mm) and a

pulsed magnetic field with a peak intensity of 3 Tesla [3]. During the procedure, the focal point of the magnetic field generated by the coil was positioned adjacent to the mid-section of the skull. T-iTBS therapy was administered repeatedly from 3:00 to 5:00 PM five days a week, the output intensity was 0.675 Tesla, with a total of 600 pulses. It encompassed three-pulse bursts delivered at a frequency of 50 Hz, and the entire burst was reiterated at a rate of 5 Hz. In light of relevant literature, a 90% movement threshold (0.75 Tesla) was chosen as the output intensity of the stimulus [3,15]. During the concurrent time period, the rats within the sham-iTBS group were exposed to the spurious stimuli. Since T-iTBS does not engender pain or any other discomfort, rats can undergo T-iTBS treatment while remaining awake, and there is no requisite for restraint.

2.4 Cortical Motor-Evoked Potential Detection

Cortical motor-evoked potentials (CMEPs) were assessed in five rats from each group using the TMS instrument (YRD CCY-I, YIRUIDE Medical Equipment, Wuhan, Hubei, China), four weeks post-injury. For the procedure, the rats were anesthetized with 1% sodium pentobarbital (30 mg/kg, intraperitoneally, No.P3761, Sigma-Aldrich Millipore, Burlington, MA, USA) and then positioned on a wooden board for stability. The electrophysiological setup involved placing the recording electrode in the anterior tibialis muscle of the rat's paralyzed hind limb. Concurrently, the reference electrode was placed adjacent to the calcaneal process, while the ground electrode was inserted into the dorsal skin for effective signal grounding. The instrument stimulation output was set to 100% intensity. The TMS coil was aligned at the center of the rat's skull to ensure precise stimulation. CMEPs were recorded once the rat had regained consciousness. For each rat, 10 separate CMEP records were acquired and expressed as average amplitude.

2.5 RNA Extraction and Sequencing

Three rats from each group were chosen for sampling and detection through RNA sequencing (RNA-seq) 4 weeks after SCI, and the targeted tissue was the SMC. For each rat, 200 mg of SMC tissue was employed for RNA extraction. Total RNA of different samples was lysed and extracted with TRIzol reagent (15596026, Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The qualities of the RNA samples were assessed through a NanoDrop spectrophotometer (ND-1000, Bio-Rad, Hercules, CA, USA), and the concentrations of RNA samples were examined with a Qubit fluorometer (QX200, Bio-Rad, Hercules, CA, USA) before RNA-seq analysis. RNA-seq was run at the laboratory of Genergy Bio-Technology Co., Ltd. (Shanghai, China). The sequencing data were assessed through Consensus Assessment of Sequence And Variation (CASAVA) base calling after data were generated using a high-throughput sequencer. The gene expression levels were calculated with the help of StringTie (StringTie 2.2.1, Johns Hopkins University, Baltimore, MD, USA), and DESeq2 (DESeq2 1.40.0, The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA) was applied to analyze differential genes in pair-wise comparisons. A p value of $<$0.05 and fold change of $>$1.5 or $<$0.667 were set as the criteria for differentially expressed genes (DEGs).

2.6 Western Blotting

Three rats from each group were chosen for sampling and detection through Western blotting 4 weeks after SCI, and the targeted tissue was the SMC. For each rat, 500 mg of SMC tissue was utilized for protein extraction using RIPA lysis buffer (P0013, Beyotime, Shanghai, China). Equal amounts of proteins from each group were electrophoresed through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto 0.2-µm Polyvinylidene fluoride (PVDF) membranes. The membranes were blocked and incubated with primary antibodies at 4 °C overnight. After that, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 2 h. The bands were detected by a chemiluminescence imaging system (Bio-Rad, Hercules, CA, USA) with ECL solution (NO. BIO9934, Bioman Technology Co., Ltd, Beijing, China) and quantified by ImageJ (ImageJ For Mac 1.52, LOCI, University of Wisconsin, Madison, WI, USA). A summary of the antibodies used is provided in **Supplementary Table 1**.

2.7 Immunofluorescence Analyses

All rats intended for immunofluorescence analyses were deeply anaesthetized using 1% sodium pentobarbital (50 mg/kg, intraperitoneally, No.P3761, Sigma-Aldrich Millipore, Burlington, MA, USA), and then they were perfused systemically with 0.9% NaCl containing 0.002% $NaNO_2$ and 0.002% heparin (PHR8927, Merck, Rahway, NJ, USA), followed with 4% paraformaldehyde (PFA, C10814814; Macklin, Shanghai, China). After perfusion, samples of tissues were placed in 30% sucrose solution to dehydrate. Brain slices were cut at a 25-µm thickness with a cryotome, blocked in 10% goat serum (Biosharp, BL210A, Wuhan, Hubei, China) with 0.3% Triton X-100 (EZ7890C254, BioFroxx, Beijing, China) at room temperature for 2 h, and incubated in primary antibody at 4 °C overnight. Then, the samples were washed three times with PBS and incubated with secondary antibodies at 37 °C for 1 h in the dark. The images were captured by fluorescence microscope, and the number of positive cells was calculated by Image J (ImageJ 1.53t, National Institutes of Health, Bethesda, MD, USA). A summary of the primary and secondary antibodies used is provided in **Supplementary Table 1**. Each group consists of five rats, with one rat having four slices chosen (with one taken from every five consecutive slices as a sample) for immunofluorescence analyses.

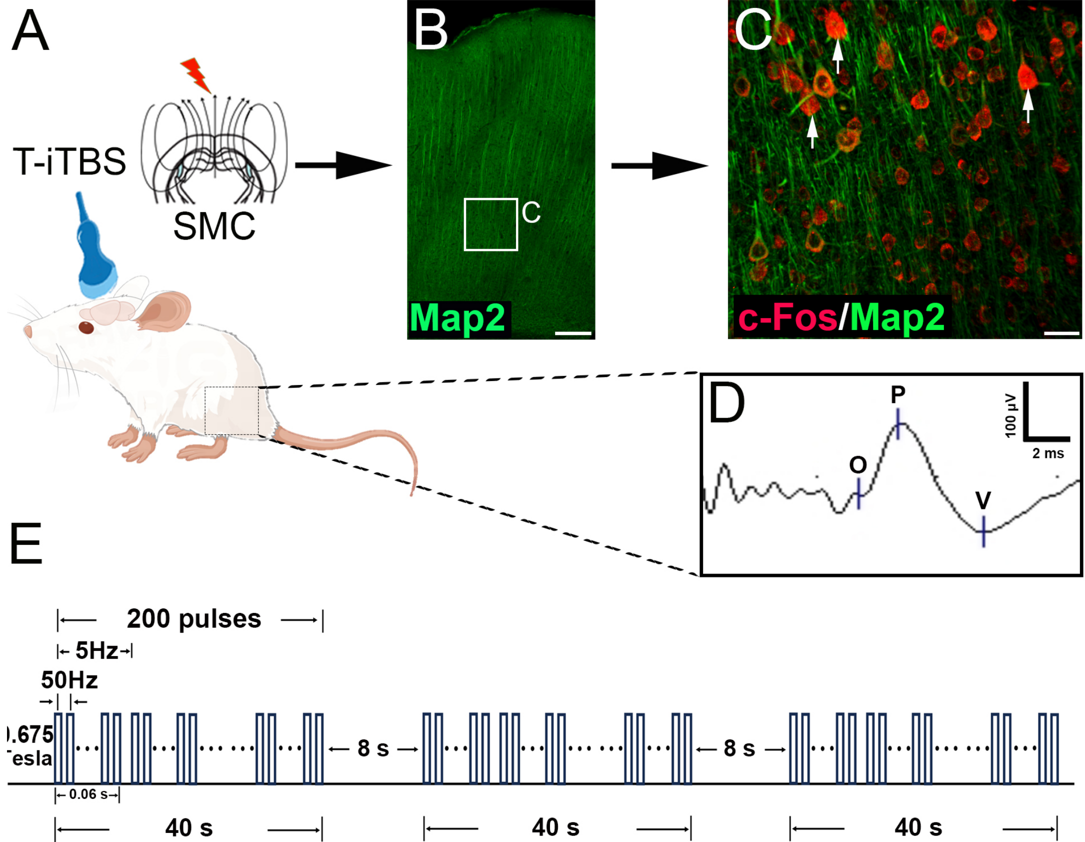

Fig. 1. Experimental strategy diagram. (A) Normal rat was treated with transcranial intermittent theta-burst stimulation (T-iTBS). (B–D) Expression of Arc on the layer V pyramidal neurons (arrow) of the sensorimotor cortex (SMC) was measured, and the cortical motor-evoked potentials (CMEPs) of hind limb skeletal muscles were recorded in normal rats. Scale bars = 200 μm in (B); 30 μm in (C); O, onset; P, peak; V, valley in (D). (E) A graphical delineation of the T-iTBS stimulation pattern.

2.8 Statistical Analyses

All experimental data are presented as the mean ± standard error of the mean from at least three samples. Pearson or Spearman rank correlation test was utilized to determine the association between two variables according to the distribution. Data were analyzed by GraphPad Prism 9.0 software (Dotmatics, Boston, MA, USA) and differences determined by one-way analysis of variance. If $p < 0.05$, the value was considered statistically significant.

3. Results

3.1 Neurons Were Activated in the SMC after T-iTBS Treatment

The results of normal rat showed that c-Fos was activated in the layer V pyramidal neurons of SMC and the action potentials of the hind limb skeletal muscles were recorded (Fig. 1A–D). After T-iTBS treatment for 4 weeks post-SCI, we assessed the expression levels of the immediate early gene *c-Fos* and NeuN by immunofluorescence. The results showed that the numbers of c-Fos and NeuN double-positive neurons were significantly higher in the T-iTBS group than the SCI and sham-iTBS groups (Fig. 2A–F). The above evidence indicates that neurons of SMC are functionally silenced after SCI but can be re-activated by T-iTBS treatment.

3.2 T-iTBS Regulated Neural Regeneration and Synaptic Plasticity in the SMC

To further explore the biological effects of 4 weeks of excitatory stimulation with T-iTBS on SMC neurons, we observed the expression of *Arc*, an activity-regulated cytoskeleton-associated gene, which reflects the occurrence of neuronal regeneration and synaptic plasticity after excitation. The immunofluorescence showed that the T-iTBS treatment group had significantly increased expression levels of Arc on microtubule-associated protein 2-positive ($Map2^+$) neurons in SMC after SCI compared with the control group (Fig. 3A–F).

3.3 SCI-Induced Abnormal Gene Expression was Reversed in the T-iTBS Treatment Group

To detect the biological changes attributed to T-iTBS, we utilized RNA-seq to analyze DEGs. The results showed that the expression levels of mRNA related to neurogenesis, neuronal metabolism, synaptic plasticity, and nerve

Fig. 2. Neurons were activated in the SMC after T-iTBS treatment. (A–D) c-Fos- and NeuN-positive (c-Fos$^+$, NeuN$^+$) cells (arrow) were detected in the SMC of each group; c-Fos$^+$ cells in green and NeuN$^+$ cells in red; scale bars = 150 µm in (A–D); 30 µm in (A1–D3). (E) A quantitative analysis of the number of NeuN$^+$ cells in SMC (n = 5, *$p < 0.05$). (F) Bar chart showing the expression of c-Fos$^+$/NeuN$^+$ (n = 5, ****$p < 0.0001$).

regeneration were upregulated in T-iTBS treatment group compared with the sham-iTBS group (Fig. 4A and **Supplementary Table 2**). We further investigated the DEGs relating to inflammation and apoptosis. In a heatmap showing considerably upregulated (red) and downregulated (green) genes, the expression levels of proinflammatory and pro-apoptosis genes of the T-iTBS group in the SMC were significantly lower than those in the sham-iTBS group (Fig. 4B and **Supplementary Table 3**). In addition, we found anti-inflammatory and anti-apoptosis genes were upregulated in the T-iTBS group (Fig. 4B). Gene ontology (GO)-interaction analysis further showed that, compared with the T-iTBS group, the sham-iTBS group showed the significant upregulation of genes related to inflammation and microglia, whereas genes related to nerve regeneration, neuronal metabolism, and synaptic plasticity were significantly downregulated (Fig. 4C).

Fig. 3. T-iTBS regulated neural regeneration and synaptic plasticity in the SMC. (A–D) Arc- and Map2-positive (Arc$^+$, Map2$^+$) cells (arrow) were detected in the SMC. Arc was highly expressed in the nucleus and perinucleus of neurons; (A1–D1) Arc$^+$ cells in red (arrow), (A2–D2) Map2$^+$ cells in green (arrow), and (A3–D3) Hoechst33342(Hoe) in blue; scale bars = 50 µm. (E) Bar chart showing the number of Map2$^+$ cells in SMC (n = 5, ***$p < 0.001$, **$p < 0.01$). (F) A quantitative analysis of the expression of Arc$^+$/Map2$^+$ (n = 5, ****$p < 0.0001$).

3.4 T-iTBS Treatment Suppressed Inflammation and Apoptosis in the SMC after SCI

To further investigate the anti-apoptosis effect of T-iTBS in the SMC after SCI, we examined the protein levels of pro-caspase-3, Cleaved-caspase-3 (CC3), and β-actin. Western blotting showed that the expression levels of CC3 and the ratio of activated caspase-3 were downregulated in the T-iTBS group compared to the SCI and sham-iTBS groups (Fig. 5A,B). For all original western blot figures of Fig. 5A see **Supplementary Figures**. To further evaluate the level of inflammation after T-iTBS in the SMC, a immunofluorescence assay was performed, which showed that the number of CD68-positive (CD68$^+$) cells was significantly reduced after T-iTBS treatment (Fig. 6A–E). These

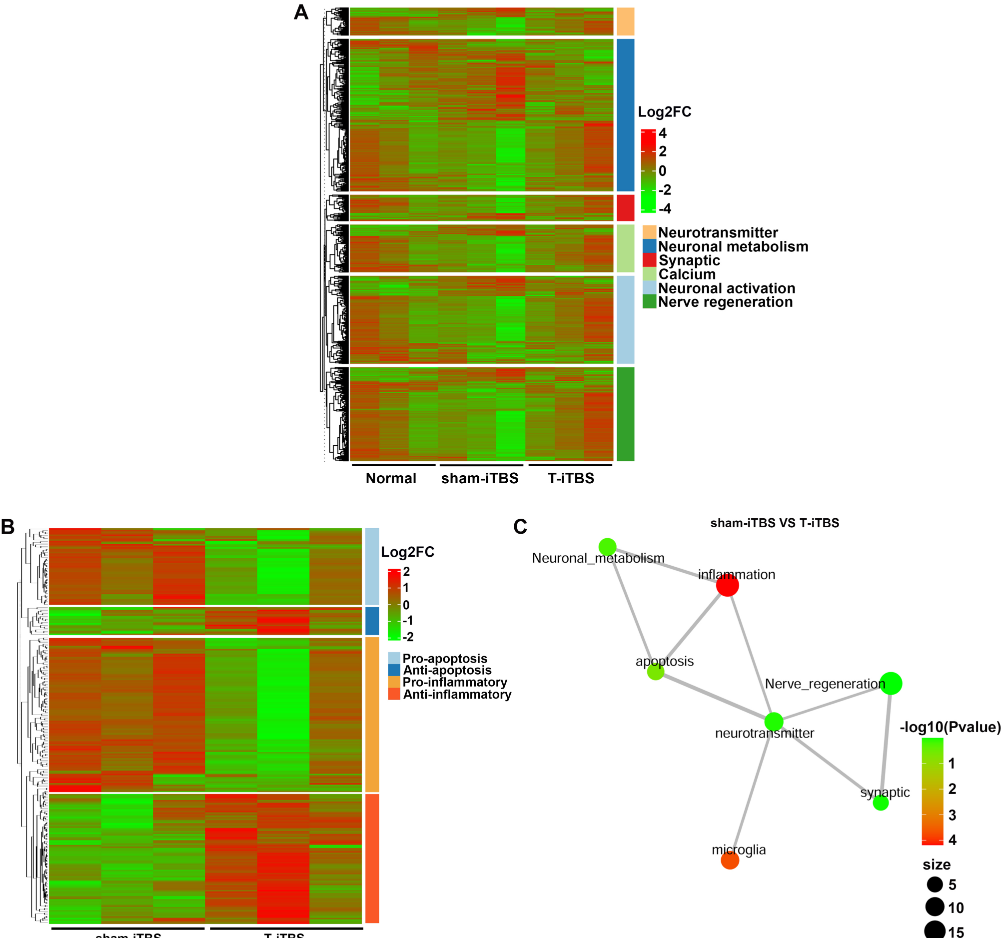

Fig. 4. Differentially expression expressed genes in T-iTBS treatment group. (A) Heatmap constructed from differentially expressed genes related to neurotransmitter, neuronal metabolism, synaptic, calcium, neuronal activation, and nerve regeneration in the SMC. The results of the clustering analysis indicate upregulated and downregulated genes with green and red, respectively, based on the Log2 fold change (FC) value. (B) In the SMC, a heatmap constructed from differentially genes related to apoptosis and inflammation. Upregulated and downregulated genes are respectively presented in green and red based on the Log2FC value. (C) Based on the enrichment analysis of the functional sets in the T-iTBS group versus the sham-iTBS group, the network diagram shows the genes inside the functional sets, with each node shown in color according to the -log10 value.

results demonstrated that T-iTBS treatment alleviates the inflammation and apoptosis in the SMC caused by SCI.

3.5 Correlation Analysis of Neuron Activation, Synaptic Plasticity, and Inflammation in the SMC

To determine the relationship between neuron activation, synaptic plasticity, and inflammation in the SMC, we analyzed the correlation between the expression levels of c-Fos and Arc, CD68, and activated caspase-3 from each group. The number of c-Fos$^+$/NeuN$^+$ cells was positively correlated with the number of Arc$^+$/Map2$^+$ cells (Fig. 7A). When we analyzed the correlation between neuron activation and inflammation, we found that the number of c-Fos$^+$/NeuN$^+$ cells was negatively correlated with the number of CD68$^+$ cells (Fig. 7B). Moreover, in the correlation between neuron activation and apoptosis, the number of c-Fos$^+$/NeuN$^+$ cells was negatively correlated with the ratio of activated caspase-3 (Fig. 7C). Four weeks after SCI, the CMEPs of each group of rats indicated that the latency of CMEPs in the T-iTBS group was significantly shorter than that of the control group (Fig. 7D,E). There was a tendency for a higher amplitude, but it was not statistically significant (Fig. 7D,F).

4. Discussion

It is of considerable significance that the SMC neurodegeneration resulting from complete SCI is mitigated promptly to facilitate the reconstruction of voluntary motor function [3]. In this study, we employed T-iTBS to treat rats transected at the T9 level. Our results suggested that treatment with T-iTBS for 4 weeks effectively mitigated the damage cascade caused by SCI by stimulating SMC neu-

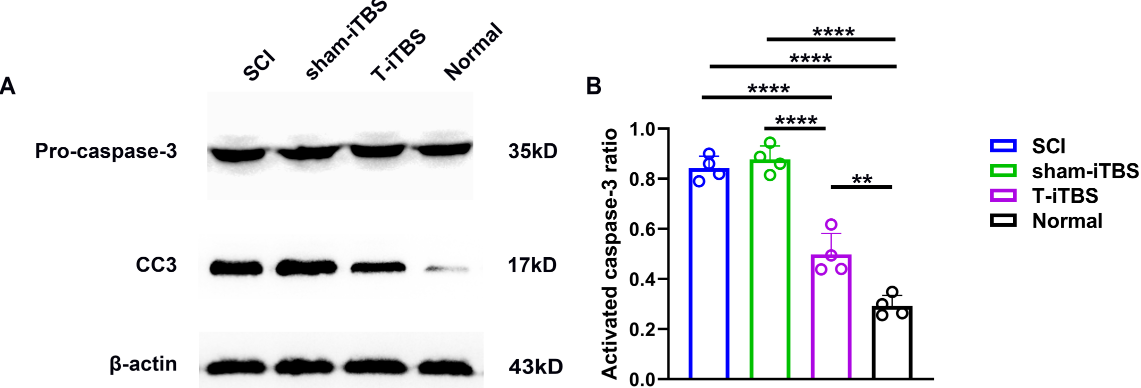

Fig. 5. T-iTBS treatment suppressed apoptosis in the SMC after spinal cord injury (SCI). (A) Pro-caspase-3, CC3, and β-actin expression levels in each group, as assessed by western blot. (B) Bar chart showing protein expression level of activated caspase-3 (CC3/Pro-caspase3) (n = 4, ****$p < 0.0001$, **$p < 0.01$). CC3, Cleaved-caspase-3.

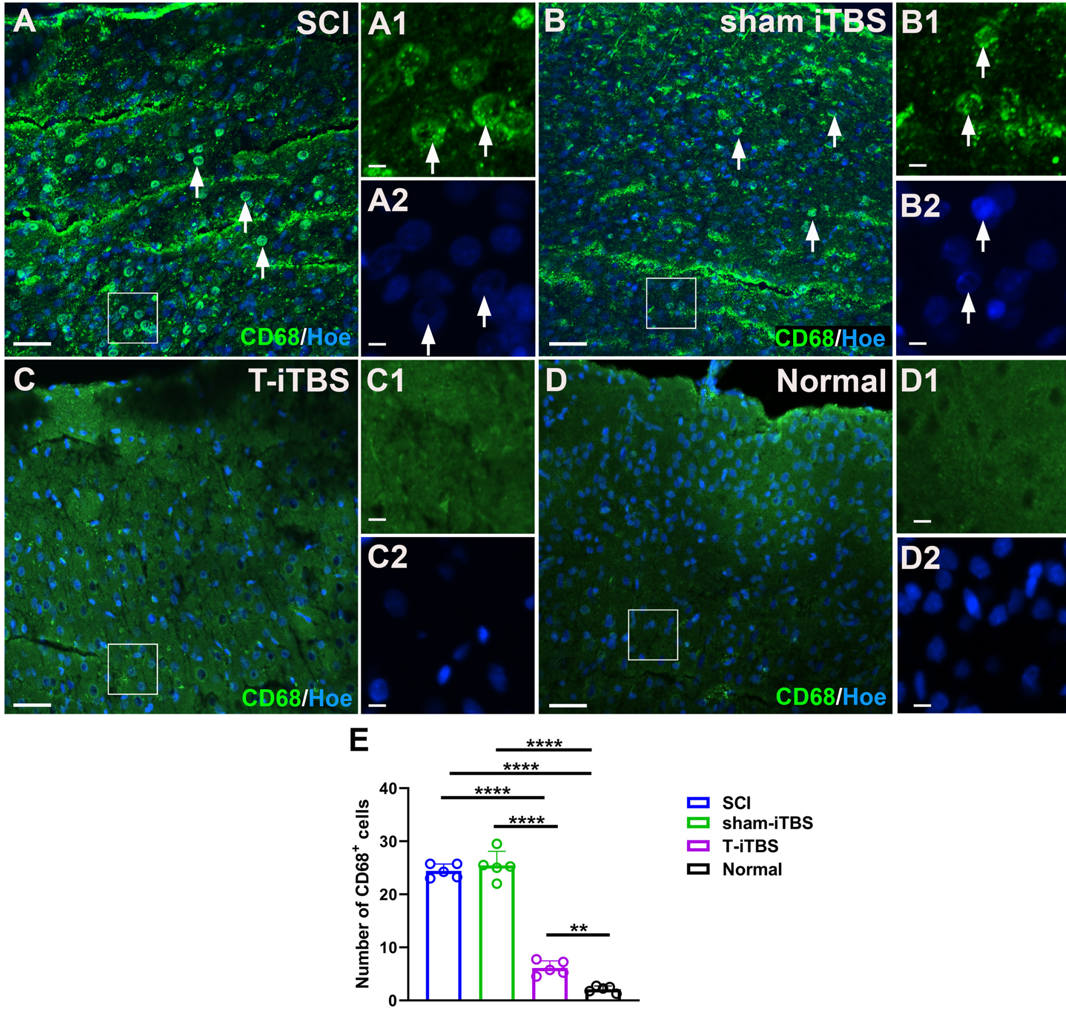

Fig. 6. T-iTBS treatment suppressed inflammation in the SMC after SCI. (A–D) $CD68^+$ cells detected in the SMC of each group; $CD68^+$cells in green (arrow); 20x microscope, scale bars = 50 µm. (A1–D2) show magnifications of (A–D), 40x microscope, scale bars = 10 µm. (E) Bar chart showing the number of $CD68^+$ cells (n = 5, ****$p < 0.0001$, **$p < 0.01$).

rons and regulating neuroplasticity. The expression levels of mRNA related to SMC neuronal activation, neuronal metabolism, synaptic activity, and neural regeneration were significantly downregulated in the sham-iTBS group, but

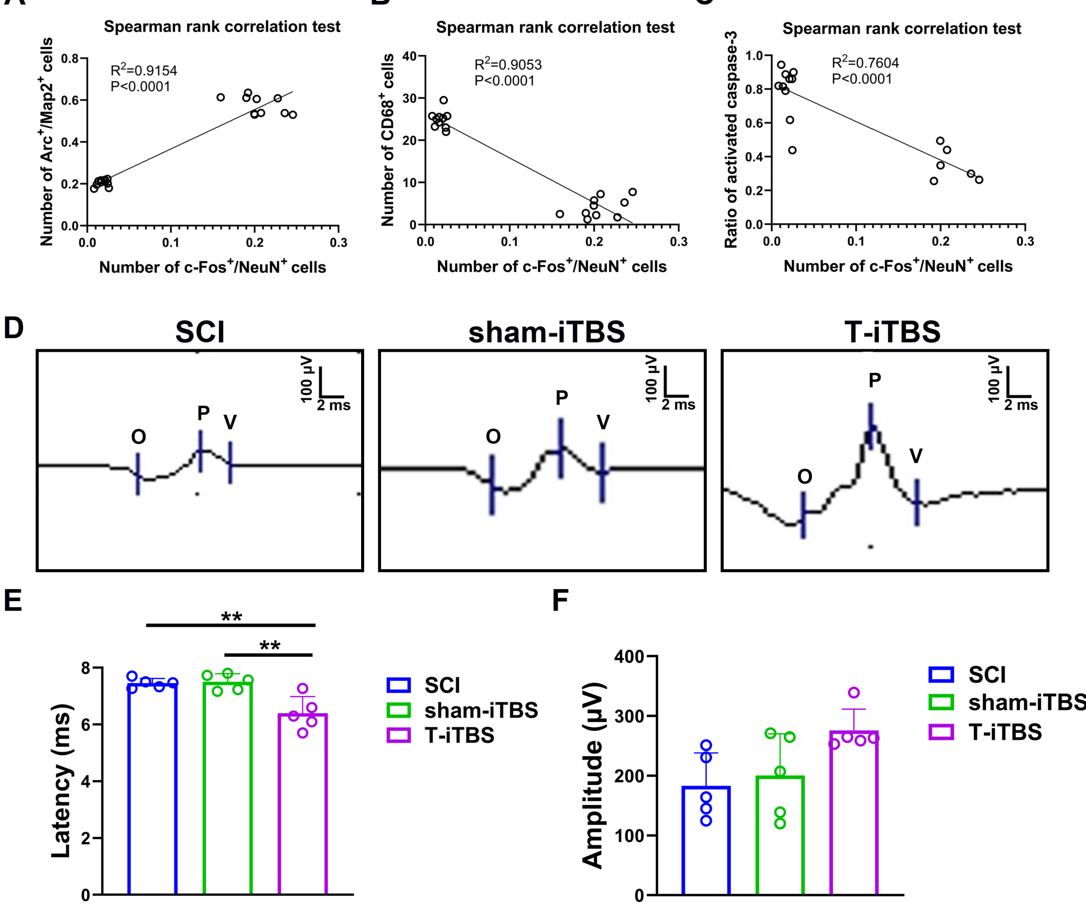

Fig. 7. Correlation analysis of neuronal activation, synaptic plasticity, and immune microenvironment in the SMC. (A) Correlation analysis of the number of c-Fos$^+$/NeuN$^+$ cells and Arc$^+$/Map2$^+$ cells. (B) Correlation analysis of the number of c-Fos$^+$/NeuN$^+$ cells and CD68$^+$ cells. (C) Correlation analysis of the number of c-Fos$^+$/NeuN$^+$ cells and ratio of activated caspase-3. (D) The CMEPs were recorded in SCI, sham-iTBS and T-iTBS groups four weeks after SCI. (E) Bar chart showing the latency of each group (n = 5, $^{**}p <$ 0.01). (F) A quantitative analysis of the amplitude of each group (n = 5). O, onset; P, peak; V, valley in (D).

upregulated in the T-iTBS group to nearly normal levels. The expression level of CC3, which is related to apoptosis, and the number of CD68$^+$ cells, which are related to inflammation, were significantly lower in the T-iTBS group. These findings imply that T-iTBS could serve as a treatment that is facile to apply clinically for the early prevention of cerebral neurodegeneration after severe SCI.

4.1 Effect of T-iTBS on Regulating the Excitability and Neuroplasticity of SMC

The reconstruction of voluntary motor function after complete SCI is still a global medical challenge [34,35]. SMC is the core cortex of the brain that integrates somatosensory information and emits motor control information. Recent studies suggest that protecting SMC neurons can play a key role in repairing voluntary motor function [3,25,36,37]. Our results showed that the expression of c-Fos in SMC neurons in T9 transverse SCI rats was significantly reduced after 4 weeks compared to that of normal rats. This demonstrated that the functional silencing of SMC neurons begins in the early stages of SCI; it also predicted that further apoptosis and atrophy would occur. It is necessary to perform early stimulation to regulate the excitability of the neurons.

We established a site for T-iTBS stimulation that completely activated layer V pyramidal neurons. Action potentials of hindlimb muscles could be detected during T-iTBS treatment [3,38]. T-iTBS treatment increased the expression of c-Fos in SMC neurons after SCI, achieving their excitatory regulation. To assess the mitigation of neuronal degeneration, we observed the expression of the activity-regulated cytoskeleton-associated protein *Arc*, an immediate early gene related to neural regeneration and synaptic plasticity regulation [39]. The results showed SCI caused neuronal silencing in the SMC and decreased the expression of Arc. The expression of Arc is often used as a sensitive indicator to evaluate the progression of neurodegeneration [39]. The number of Arc$^+$ neurons of the SMC was upregulated in the T-iTBS group to almost normal levels, which indicated that this excitatory neural regulation may prevent

the progression from neuronal functional silencing to neurodegeneration in the early stage of injury. Transcriptome sequencing also demonstrated that mRNA related to neuronal activation, synaptic activity, neuronal metabolism, and regeneration were significantly upregulated in the SMC after T-iTBS treatment and were almost at the levels seen in normal rats. According to above results, we speculated that T-iTBS reversed the progression toward neuronal apoptosis and atrophy by promoting the metabolism and regeneration abilities of neurons. It is necessary to treat in the acute or subacute phase after injury in order to prevent neuronal functional silencing progressing into irreversible apoptosis and atrophy.

4.2 Effect and Mechanism of T-iTBS in Reversing SMC Neurodegeneration

After 4 weeks of T-iTBS treatment, RNA-seq analysis revealed that mRNA related to neuronal apoptosis were significantly downregulated compared with those in the SCI and sham-iTBS group, but mRNA related to anti-apoptosis were significantly upregulated to almost normal levels. The detection of apoptosis protein CC3 also confirmed the amelioration of apoptosis by T-iTBS treatment. According to these results, it can be inferred that T-iTBS promotes neuronal metabolism and regeneration abilities by regulating the excitability of SMC neurons while reversing the apoptosis of these neurons.

Many studies have indicated that neuronal functional silencing is an important factor in neuronal apoptosis, with another important factor being the activation of neuroinflammation [20,40–42]. Due to the damage signals caused by SCI, SMC neurons are in a stressed state and release inflammatory factors to induce a cascade of inflammation [43,44]. After 4 weeks of SCI, the results of transcriptome sequencing indicated that mRNA related to inflammation were significantly upregulated in the SCI group, but anti-inflammatory genes were significantly downregulated, compared to the T-iTBS group. Moreover, the expression levels of mRNA related to anti-inflammatory activity were upregulated to almost normal levels after T-iTBS treatment. These results suggested that T-iTBS treatment maintained the homeostasis of immune microenvironment, avoided neuronal stress, and synthetized inflammatory factors by regulating the excitability of SMC neurons, in which mRNA for anti-inflammation, synaptic activation, and nerve regeneration were upregulated. Our immunohistochemical analysis confirmed that the number of $CD68^+$ cells was reduced and was similar to that of the normal rats in the T-iTBS group, whereas the SCI and sham-iTBS group showed the significant activation and aggregation of $CD68^+$ cells. $CD68^+$ cells may release inflammatory factors, further inducing neuronal apoptosis and finally leading to the gray matter atrophy that is visible on imaging [3,45]. We speculated that the functional silencing of neurons and the inflammatory microenvironment induced the downregulation of regenerative genes and increased the expression of apoptotic genes. Hence, after SCI, it is necessary to improve the immune microenvironment of neurons, reverse apoptosis, and enhance the intrinsic regeneration ability of brain-derived axons through excitatory regulation with T-iTBS [3,46,47]. After four weeks of T-iTBS treatment, the results of CMEPs imply that the capacity of the spinal cord to convey nerve impulses has begun to show an improvement tendency. We hypothesize that this improvement will become more prominent with the prolongation of T-iTBS treatment.

5. Conclusions

In this study, we analyzed the regulation of neuronal excitability and neuroplasticity by T-iTBS in the SMC by establishing a spinal cord-transected model in rats. Based on the findings, we propose that T-iTBS can regulate the expression of genes and the external microenvironment of SMC and has potential application value for the reconstruction of voluntary motor function. However, this study did not explore the cumulative therapeutic effects of T-iTBS over a longer period, particularly regarding immune regulation and brain-derived nerve regeneration at the injury area. Although T-iTBS has demonstrated positive effects in protecting SMC neurons, it still cannot solely reconstruct motor voluntary function. It is necessary to combine T-iTBS with advanced regenerative medicine technologies such as stem cell tissue engineering to further achieve neural pathway reconstruction at the site of SCI, which will be the direction of our research in the future.

Availability of Data and Materials

Data and materials could be obtained upon reasonable request to the corresponding author.

Author Contributions

BQL and LZ—conception and design of the study; ZHC, YJL, JX, JWS, RL, YY, ZC and MYL—experimental implementation and data collection; BQL, ZHC, YJL and LZ—analysis and interpretation of the data; BQL, ZHC, YJL and LZ—manuscript drafting. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental procedures involving animals were conducted in accordance with the guidelines of the Guide for the Care and Use of Laboratory Animals and were approved by the the Animal Care and Use Committee of Sun Yat-sen University (SYSU-IACUC-2024-B0100, Guangzhou, China).

Acknowledgment

We thank all the peer reviewers for their opinions and suggestions.

Funding

This research was supported by grants from the National Key R&D Program of China (No. 2022YFA1105900); the Chinese National Natural Science Foundation (No. 82371400); the Natural Science Foundation of Guangdong Province, China (No. 2021B1515020045); and the Science and Technology Planning Project of Guangdong Province (No. 2023B1212060018).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/JIN26731.

References

[1] Martin JH. Neuroplasticity of spinal cord injury and repair. Handbook of Clinical Neurology. 2022; 184: 317–330. https://doi.org/10.1016/B978-0-12-819410-2.00017-5.

[2] Zeng H, Cheng L, Lu DZ, Fan S, Wang KX, Xu LL, *et al*. Unbiased multitissue transcriptomic analysis reveals complex neuroendocrine regulatory networks mediated by spinal cord injury-induced immunodeficiency. Journal of Neuroinflammation. 2023; 20: 219. https://doi.org/10.1186/s12974-023-02906-7.

[3] Liu JL, Wang S, Chen ZH, Wu RJ, Yu HY, Yang SB, *et al*. The therapeutic mechanism of transcranial iTBS on nerve regeneration and functional recovery in rats with complete spinal cord transection. Frontiers in Immunology. 2023; 14: 1153516. https://doi.org/10.3389/fimmu.2023.1153516.

[4] Jiang F, Xia M, Zhang Y, Chang J, Cao J, Zhang Z, *et al*. Cannabinoid receptor-2 attenuates neuroinflammation by promoting autophagy-mediated degradation of the NLRP3 inflammasome post spinal cord injury. Frontiers in Immunology. 2022; 13: 993168. https://doi.org/10.3389/fimmu.2022.993168.

[5] Hou Y, Luan J, Huang T, Deng T, Li X, Xiao Z, *et al*. Tauroursodeoxycholic acid alleviates secondary injury in spinal cord injury mice by reducing oxidative stress, apoptosis, and inflammatory response. Journal of Neuroinflammation. 2021; 18: 216. https://doi.org/10.1186/s12974-021-02248-2.

[6] Hu X, Xu W, Ren Y, Wang Z, He X, Huang R, *et al*. Spinal cord injury: molecular mechanisms and therapeutic interventions. Signal Transduction and Targeted Therapy. 2023; 8: 245. https://doi.org/10.1038/s41392-023-01477-6.

[7] Nicolas N, Kobaiter-Maarrawi S, Georges S, Abadjian G, Maarrawi J. Motor Cortex Stimulation Regenerative Effects in Peripheral Nerve Injury: An Experimental Rat Model. World Neurosurgery. 2018; 114: e800–e808. https://doi.org/10.1016/j.wneu.2018.03.090.

[8] Zhang Y, Yin YL, Jin ZY, Hu QP, Wu XG. Electroacupuncture Activates Neuroplasticity in the Motor Cortex and Corticospinal Tract via the mTOR Pathway in a Rat P-MCAO Model. BioMed Research International. 2022; 2022: 3470685. https://doi.org/10.1155/2022/3470685.

[9] Sutor TW, Kura J, Mattingly AJ, Otzel DM, Yarrow JF. The Effects of Exercise and Activity-Based Physical Therapy on Bone after Spinal Cord Injury. International Journal of Molecular Sciences. 2022; 23: 608. https://doi.org/10.3390/ijms23020608.

[10] Zeng YS, Ding Y, Xu HY, Zeng X, Lai BQ, Li G, *et al*. Electro-acupuncture and its combination with adult stem cell transplantation for spinal cord injury treatment: A summary of current laboratory findings and a review of literature. CNS Neuroscience & Therapeutics. 2022; 28: 635–647. https://doi.org/10.1111/cns.13813.

[11] Liu JL, Chen ZH, Wu RJ, Yu HY, Yang SB, Xu J, *et al*. Effects of tail nerve electrical stimulation on the activation and plasticity of the lumbar locomotor circuits and the prevention of skeletal muscle atrophy after spinal cord transection in rats. CNS Neuroscience & Therapeutics. 2024; 30: e14445. https://doi.org/10.1111/cns.14445.

[12] Hu R, He K, Chen B, Chen Y, Zhang J, Wu X, *et al*. Electroacupuncture promotes the repair of the damaged spinal cord in mice by mediating neurocan-perineuronal net. CNS Neuroscience & Therapeutics. 2024; 30: e14468. https://doi.org/10.1111/cns.14468.

[13] Jo HJ, Richardson MSA, Oudega M, Perez MA. The Potential of Corticospinal-Motoneuronal Plasticity for Recovery after Spinal Cord Injury. Current Physical Medicine and Rehabilitation Reports. 2020; 8: 293–298. https://doi.org/10.1007/s40141-020-00272-6.

[14] Caipa A, Alomar M, Bashir S. TMS as tool to investigate the effect of pharmacological medications on cortical plasticity. European Review for Medical and Pharmacological Sciences. 2018; 22: 844–852. https://doi.org/10.26355/eurrev_201802_14321.

[15] Wang Y, Dong T, Li X, Zhao H, Yang L, Xu R, *et al*. Research progress on the application of transcranial magnetic stimulation in spinal cord injury rehabilitation: a narrative review. Frontiers in Neurology. 2023; 14: 1219590. https://doi.org/10.3389/fneur.2023.1219590.

[16] Evancho A, Tyler WJ, McGregor K. A review of combined neuromodulation and physical therapy interventions for enhanced neurorehabilitation. Frontiers in Human Neuroscience. 2023; 17: 1151218. https://doi.org/10.3389/fnhum.2023.1151218.

[17] Mosilhy EA, Alshial EE, Eltaras MM, Rahman MMA, Helmy HI, Elazoul AH, *et al*. Non-invasive transcranial brain modulation for neurological disorders treatment: A narrative review. Life Sciences. 2022; 307: 120869. https://doi.org/10.1016/j.lfs.2022.120869.

[18] Chen M, Chen Z, Xiao X, Zhou L, Fu R, Jiang X, *et al*. Corticospinal circuit neuroplasticity may involve silent synapses: Implications for functional recovery facilitated by neuromodulation after spinal cord injury. IBRO Neuroscience Reports. 2022; 14: 185–194. https://doi.org/10.1016/j.ibneur.2022.08.005.

[19] Rosson S, de Filippis R, Croatto G, Collantoni E, Pallottino S, Guinart D, *et al*. Brain stimulation and other biological non-pharmacological interventions in mental disorders: An umbrella review. Neuroscience and Biobehavioral Reviews. 2022; 139: 104743. https://doi.org/10.1016/j.neubiorev.2022.104743.

[20] Lin BS, Zhang Z, Peng CW, Chen SH, Chan WP, Lai CH. Effectiveness of Repetitive Transcranial Magnetic Stimulation Combined With Transspinal Electrical Stimulation on Corticospinal Excitability for Individuals With Incomplete Spinal Cord Injury: A Pilot Study. IEEE Transactions on Neural Systems and Rehabilitation Engineering: a Publication of the IEEE Engineering in Medicine and Biology Society. 2023; 31: 4790–4800. https://doi.org/10.1109/TNSRE.2023.3338226.

[21] Poologaindran A, Profyris C, Young IM, Dadario NB, Ahsan SA, Chendeb K, *et al*. Interventional neurorehabilitation for promoting functional recovery post-craniotomy: a proof-of-concept. Scientific Reports. 2022; 12: 3039. https://doi.org/10.1038/s41598-022-06766-8.

[22] Dhami P, Moreno S, Croarkin PE, Blumberger DM, Daskalakis ZJ, Farzan F. Baseline markers of cortical excitation and inhibition predict response to theta burst stimulation treatment for youth depression. Scientific Reports. 2023; 13: 19115. https://doi.org/10.1038/s41598-023-45107-1.

[23] Jannati A, Oberman LM, Rotenberg A, Pascual-Leone A. Assessing the mechanisms of brain plasticity by transcranial magnetic stimulation. Neuropsychopharmacology: Official Publication of the American College of Neuropsychopharmacology. 2023; 48: 191–208. https://doi.org/10.1038/s41386-022-01453-8.

[24] Antal A, Luber B, Brem AK, Bikson M, Brunoni AR, Cohen Kadosh R, *et al*. Non-invasive brain stimulation and neuroenhancement. Clinical Neurophysiology Practice. 2022; 7: 146–165. https://doi.org/10.1016/j.cnp.2022.05.002.

[25] Lee KZ, Vinit S. Modulatory effect of trans-spinal magnetic intermittent theta burst stimulation on diaphragmatic activity following cervical spinal cord contusion in the rat. The Spine Journal: Official Journal of the North American Spine Society. 2024; 24: 352–372. https://doi.org/10.1016/j.spinee.2023.09.013.

[26] Ginanneschi F, Messa LV, Battisti C, Rossi A. Changes in corticomotor pathway excitability after exercise training in Parkinson's disease. Neurological Sciences: Official Journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology. 2021; 42: 3375–3381. https://doi.org/10.1007/s10072-020-04960-y.

[27] Patel D, Banerjee R, Farooque K, Gupta D, Garg B, Kumar N, *et al*. Restoring initial steps by intermittent theta burst stimulation in complete spinal cord injury patient: a case report. Spinal Cord Series and Cases. 2024; 10: 56. https://doi.org/10.1038/s41394-024-00669-8.

[28] Deng L, Song N, Wang J, Wang X, Chen Y, Wu S. Effect of Intermittent Theta Burst Stimulation Dual-Target Stimulation on Lower Limb Function in Patients with Incomplete Spinal Cord Injury: A Randomized, Single-Blind, Sham-Controlled Study. World Neurosurgery. 2024; 190: e46–e59. https://doi.org/10.1016/j.wneu.2024.06.141.

[29] Chen SY, Yang RL, Wu XC, Zhao DZ, Fu SP, Lin FQ, *et al*. Mesenchymal Stem Cell Transplantation: Neuroprotection and Nerve Regeneration After Spinal Cord Injury. Journal of Inflammation Research. 2023; 16: 4763–4776. https://doi.org/10.2147/JIR.S428425.

[30] Bao SS, Zhao C, Chen HW, Feng T, Guo XJ, Xu M, *et al*. NT3 treatment alters spinal cord injury-induced changes in the gray matter volume of rhesus monkey cortex. Scientific Reports. 2022; 12: 5919. https://doi.org/10.1038/s41598-022-09981-5.

[31] González P, González-Fernández C, Javier Rodríguez F. Effects of Wnt5a overexpression in spinal cord injury. Journal of Cellular and Molecular Medicine. 2021; 25: 5150–5163. https://doi.org/10.1111/jcmm.16507.

[32] Noristani HN. Intrinsic regulation of axon regeneration after spinal cord injury: Recent advances and remaining challenges. Experimental Neurology. 2022; 357: 114198. https://doi.org/10.1016/j.expneurol.2022.114198.

[33] Xu AK, Gong Z, He YZ, Xia KS, Tao HM. Comprehensive therapeutics targeting the corticospinal tract following spinal cord injury. Journal of Zhejiang University. Science. B. 2019; 20: 205–218. https://doi.org/10.1631/jzus.B1800280.

[34] Duguay M, Bonizzato M, Delivet-Mongrain H, Fortier-Lebel N, Martinez M. Uncovering and leveraging the return of voluntary motor programs after paralysis using a bi-cortical neuroprosthesis. Progress in Neurobiology. 2023; 228: 102492. https://doi.org/10.1016/j.pneurobio.2023.102492.

[35] Arora M, Craig AR. Special Issue-Spinal Cord Injuries: Advances in Rehabilitation. Journal of Clinical Medicine. 2024; 13: 1782. https://doi.org/10.3390/jcm13061782.

[36] Sanganahalli BG, Chitturi J, Herman P, Elkabes S, Heary R, Hyder F, *et al*. Supraspinal Sensorimotor and Pain-Related Reorganization after a Hemicontusion Rat Cervical Spinal Cord Injury. Journal of Neurotrauma. 2021; 38: 3393–3405. https://doi.org/10.1089/neu.2021.0190.

[37] Benedetti B, Dannehl D, Janssen JM, Corcelli C, Couillard-Després S, Engelhardt M. Structural and Functional Maturation of Rat Primary Motor Cortex Layer V Neurons. International Journal of Molecular Sciences. 2020; 21: 6101. https://doi.org/10.3390/ijms21176101.

[38] Schlag MG, Hopf R, Redl H. Serial recording of sensory, corticomotor, and brainstem-derived motor evoked potentials in the rat. Somatosensory & Motor Research. 2001; 18: 106–116. https://doi.org/10.1080/135578501012006219.

[39] Steward O, Coulibaly AP, Metcalfe M, Yonan JM, Yee KM. AAVshRNA-mediated PTEN knockdown in adult neurons attenuates activity-dependent immediate early gene induction. Experimental Neurology. 2020; 326: 113098. https://doi.org/10.1016/j.expneurol.2019.113098.

[40] Shao Z, Lv G, Wen P, Cao Y, Yu D, Lu Y, *et al*. Silencing of PHLPP1 promotes neuronal apoptosis and inhibits functional recovery after spinal cord injury in mice. Life Sciences. 2018; 209: 291–299. https://doi.org/10.1016/j.lfs.2018.08.030.

[41] Lu X, Xue P, Fu L, Zhang J, Jiang J, Guo X, *et al*. HAX1 is associated with neuronal apoptosis and astrocyte proliferation after spinal cord injury. Tissue & Cell. 2018; 54: 1–9. https://doi.org/10.1016/j.tice.2018.07.001.

[42] Xu A, Yang Y, Shao Y, Jiang M, Sun Y, Feng B. FHL2 regulates microglia M1/M2 polarization after spinal cord injury via PARP14-depended STAT1/6 pathway. International Immunopharmacology. 2023; 124: 110853. https://doi.org/10.1016/j.intimp.2023.110853.

[43] Wu Z, Zhu R, Yu Y, Wang J, Hu X, Xu W, *et al*. Spinal cord injury-activated C/EBPβ-AEP axis mediates cognitive impairment through APP C586/Tau N368 fragments spreading. Progress in Neurobiology. 2023; 227: 102467. https://doi.org/10.1016/j.pneurobio.2023.102467.

[44] Lei Z, Krishnamachary B, Ritzel RM, Khan NZ, Li Y, Li H, *et al*. Age-related changes in plasma extracellular vesicles influence neuroinflammation in the brain and neurological outcome after traumatic spinal cord injury. Research Square. 2023. (preprint) https://doi.org/10.21203/rs.3.rs-2821858/v1.

[45] Li D, Zhao S, Zhu B, Zhao W, Ding Y, Li X, *et al*. Cinepazide maleate promotes recovery from spinal cord injury by inhibiting inflammation and prolonging neuronal survival. Drug Development Research. 2023; 84: 736–746. https://doi.org/10.1002/ddr.22052.

[46] Li Z, Zhao T, Ding J, Gu H, Wang Q, Wang Y, *et al*. A reactive oxygen species-responsive hydrogel encapsulated with bone marrow derived stem cells promotes repair and regeneration of spinal cord injury. Bioactive Materials. 2022; 19: 550–568. https://doi.org/10.1016/j.bioactmat.2022.04.029.

[47] Liu R, Li Y, Wang Z, Chen P, Xie Y, Qu W, *et al*. Regulatory T cells promote functional recovery after spinal cord injury by alleviating microglia inflammation via STAT3 inhibition. CNS Neuroscience & Therapeutics. 2023; 29: 2129–2144. https://doi.org/10.1111/cns.14161.