Therapeutic mechanism of transcranial iTBS on nerve regeneration and functional recovery in rats with complete spinal cord transection

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Abstract

Background: After spinal cord transection injury, the inflammatory microenvironment formed in the injury site and the cascade of secondary injury results in limited regeneration of injured axons and the apoptosis of neurons in the sensorimotor cortex (SMC). It is crucial to reverse these adverse processes for the recovery of voluntary movement. In this study, transcranial intermittent theta-burst stimulation (iTBS) was used for the treatment of complete spinal cord transection in rats. The mechanism of transcranial iTBS as a new non-invasive neural regulation paradigm in promoting axonal regeneration and motor function repair was explored.

Methods: Rats from the iTBS group were treated with transcranial iTBS 72h after spinal cord injury (SCI). Each rat was received behavioral testing. Inflammation, neuronal apoptosis, neuroprotective effect, regeneration and synaptic plasticity were measured by immunofluorescence staining, western blotting and mRNA sequencing 2 or 4w after SCI. Each rat was received anterograde tracings in the SMC or the long descending propriospinal neurons and tested for motor evoked potentials. Regeneration of corticospinal tract (CST) and 5-hydroxytryptamine (5-HT) nerve fibers were detected eight weeks after SCI.

Results: Compared with the control group and the sham iTBS group, rats of the iTBS group showed reduced inflammatory responses and neuronal apoptosis in the SMC two weeks after treatment. After four weeks, the neuroimmune microenvironment at the injury site was improved, and neuroprotective effects were seen to promote axonal regeneration and synaptic plasticity. Significantly, eight weeks after treatment, transcranial iTBS also increased the regeneration of CST, 5-HT nerve fibers, and the long descending propriospinal tract (LDPT). Moreover, motor evoked potentials and hindlimb motor function were significantly improved at eight weeks.

Conclusions: Collectively, our results verified that iTBS has the potential to provide neuroprotective effects at early injury stages and pro-regeneration effects related to the 1) CST–5-HT; 2) CST–LDPT; and 3) CST–5-HT–LDPT descending motor pathways and revealed the relationships among neural pathway activation, neuroimmune regulation, neuroprotection, and axonal regeneration, as well as the interaction network of key genes. The proposed non-invasive transcranial iTBS treatment is expected to provide a serviceable practical and theoretical support for spinal cord injury.

Background

Spinal cord injury (SCI) often leads to severe impairment of sensory and voluntary motor function. The acute and chronic stages involve a series of destructive events, such as ischemia, oxidative stress, inflammation, neuronal apoptosis and scar formation [1]. A wide array of studies has confirmed that SCI can cause inflammation in the brain and apoptosis of sensorimotor cortex (SMC) neurons, which greatly affects the regeneration of brain-derived nerve fibers innervating the injury site [2]. It remains undetermined whether brain-derived nerve regeneration and motor function can be promoted by
activating brain motor neural pathways after SCI, with little research on related therapeutic strategies and mechanisms.

As a non-invasive, safe, and easily accepted treatment technique, transcranial magnetic stimulation (TMS) has a wide effect on neural networks in the brain by altering the excitability of neurons \[^{3-7}\]. Despite available clinical data supporting the effectiveness of TMS in the treatment of SCI \[^{8, 9}\], the specific cellular and molecular mechanisms targeted by TMS are still unclear. The stimulation paradigms of TMS used in clinical therapy include traditional repetitive TMS and theta-burst stimulation (TBS). TBS is a novel TMS protocol that rapidly induces synaptic plasticity. During TBS, short bursts of high frequency (50 Hz) stimulation are repeated at 5 Hz (200-ms interval) \[^{10}\]. TBS can be either intermittent (iTBS), with excitatory effects, or continuous, with inhibitory effects, and these are associated with long-term potentiation- and long-term depression-like activity, respectively. In recent years, iTBS has been used increasingly as a treatment to promote recovery of neurological function due to its short treatment time, simple operation procedure, and clinical acceptability \[^{11}\].

At present, research on iTBS is mostly focused on the observation of therapeutic phenomena, and there is a lack of in-depth and systematic research on its mechanisms. Marufa et al. \[^{12}\] explored neuronal plasticity and motor function recovery after iTBS therapy in rats with spinal cord clipping injuries with different compression forces. They observed enhancement in the motor evoked potential (MEP) amplitude and an increase of growth-associated protein 43 (GAP43) expression in the brain and spinal cord, but they did not observe significant improvements of motor function in hindlimbs. This may be related to the fact that they first applied the iTBS therapy four weeks after SCI, and the treatment lasted only two weeks. Delarue et al. \[^{13}\] found that repetitive trans-spinal magnetic stimulation could improve neuroinflammation and regulate neuroglial scar formation by regulating microglia and astrocytes, thus promoting SCI repair. Therefore, we speculated that transcranial iTBS may also play a similar role in the regulation on neuroinflammation and neuroprotection. In fact, neuroprotective effects in the acute stages of SCI are the premise for nerve regeneration. In addition, the protective and activating effects on motor pathways are the key to the recovery of voluntary motor function.

At present, the mechanisms of axonal regeneration based on models of complete spinal cord transection have rarely been studied. We used fully spinally transected SCI rats, in which a 2-mm spinal cord segment was completely removed at the T10 level. The stimulation site was selected on the SMC, which can effectively explain the mechanisms of transcranial iTBS’s effects promoting brain-derived axon regeneration and motor pathway repair. The present study analyzed neuroprotective mechanisms from the level of cells, proteins, and gene interactions in acute and subacute phases of SCI and axonal regeneration and voluntary motor function repair in later phases. Through in-depth mechanistic research, this study offers new insights into the therapeutic value of transcranial iTBS on SCI.

**Methods**
Experimental animals and groups

Eight-week-old Sprague–Dawley rats (180–220 g) were provided by the Guangdong Medical Laboratory Animal Center. All animal protocols and animal handling procedures were approved by the ethics committee of Sun Yat-sen University (Animal Use Protocol no. 2021PS704K).

All rats were divided into the Normal group (n = 10), the Control group (n = 15), the sham iTBS group (n = 15), and the iTBS group (n = 15). Five rats (each) in the Normal group were sacrificed at 2 weeks and 4 weeks after SCI. Five rats (each) from other three groups were sacrificed at 2 weeks, 4 weeks, and 8 weeks after SCI.

Spinal cord transection

The rats were anesthetized by 1% pentobarbital sodium intraperitoneal injection (0.35 ml/100 g). A laminectomy was performed to expose the T9 and T10 spinal cord segments, and the dura was slit vertically with a pair of micro-forceps and micro-scissors under a stereomicroscope. A 2-mm cord segment including the associated spinal roots was completely removed at the T10 level. Using a gel sponge to stop bleeding in lesion area of spinal cord, soft tissues were then sutured layer by layer with 4-0 surgical sutures. After surgery, each rat was intramuscularly injected with penicillin (160,000 U/kg/day) to prevent infection and placed on a heating pad until awakening. Within seven days after SCI, the rats were reared in a single cage and their bladders were massaged three times daily to assist urination. At day seven after SCI, five rats were housed per cage, and urination was assisted twice daily until rats were sacrificed.

Transcranial Magnetic Stimulation

The mean motor evoked potentials of rats (n = 55) were measured using a circular coil for animals (r = 32 mm, Y064; Wuhan Yiruide Medical Equipment New Technology Co., Ltd., Wuhan, China) with a 3-T peak intensity of pulsed magnetic field for further iTBS after SCI. The recording electrode was inserted into the muscle belly of the anterior tibialis in a hindlimb; the grounding electrode was fixed on the rat's back, and the stimulation coil center was placed above skin on the center of the skull. In non-anesthetic state, the intensity started from low-intensity stimulation that evoked ankle dorsiflexion, and the MEP was recorded from the anterior tibialis. The MEP was defined as at least 5 out of 10 stimuli with not less than 100 µV during slight muscle contraction. Meanwhile, the results of the pre-test showed that 25% maximal stimulator output was the rest motor threshold for iTBS.

Rats from the iTBS group were treated 72 h after SCI with TMS (YRD CCY1; Wuhan Yiruide Medical Equipment New Technology Co., Ltd.). Each rat was repeatedly stimulated 20 times (600 pulses in total for 200 s) a day, five days a week until they were sacrificed. iTBS used in this study consisted of three-pulse bursts at 50 Hz repeated at 5 Hz. A 2-s stimulation of TBS was repeated every 10 s for 20 repetitions, for 600 pulses in total. Rats in the sham iTBS group were given sham stimulation with the same parameters 72 h after SCI, in which the coil was placed perpendicular to the skull.
Motor evoked potential detection

In a non-anesthetic state, each rat was tested for MEPs eight weeks after SCI. Each rat was stimulated 20 times (30-s interval), and the amplitudes were recorded.

Behavioral testing

Double-blinded 5-min Basso–Beattie–Bresnahan (BBB) test scores were used to evaluate hindlimb joint movement, walking ability, coordination, and stability of rats weekly after SCI. A modified inclined-grid climbing test was used to observe and videotape the coordinating movement between forelimbs and hindlimbs to evaluate the overall locomotor ability of animals. Each rat was placed on a grid on a 45° slope and climbed the grid three times. The total number of “pedal steps” on the grid by both hind legs was counted.

Biotinylated dextran amine (BDA) anterograde tracing

BDA tracer in SMC

Three rats were randomly selected from each group for anterograde BDA labeling in the SMC eight weeks after SCI. The head was fixed on the rat brain stereolocator under anesthesia; based on stereotaxic mapping of the brain, two round bone windows (r = 2.5 mm) were made 1 mm anterior to the fontanelle and 1 mm from the sagittal suture bilaterally to expose the SMC in the bilateral cerebral hemispheres. Then, 10% (Molecular weight 10000) BDA solution (Invitrogen D1956; Thermo Fisher Scientific, Waltham, MA, US) was injected slowly under a surgical microscope.

Six injection sites were selected bilaterally located 1 mm, 2 mm, or 3 mm behind the anterior fontanelle and 1 mm or 2 mm from the sagittal suture. At each site, 0.25 µl was injected at a depth of 2 mm and 1 mm from the SMC surface, totaling 0.5 µl at each injection site, and the needle was retained for 2 min before being slowly withdrawn.

BDA tracer in long descending cervical propriospinal neurons

Two rats were randomly selected from each group for anterograde BDA labeling in the long descending cervical propriospinal neurons eight weeks after SCI. C3 and C4 are the segments where neurons in the long descending propriospinal tract (LDPT) are located. After anesthesia, a 2-cm incision was made to fully expose and remove the C3 and C4 spinous processes and vertebral bodies. Five sites on each side were injected with 0.5 µl 10% BDA tracer, with a total of 5 µl bilaterally. Operations were performed at a depth of 1 mm from the surface of the spinal cord, and the needle was retained for 2 min after injection. After surgery, penicillin was injected intramuscularly (160,000 U/kg/day) once a day for five days, and rats were sacrificed after two weeks of single-cage housing.

Perfusion and tissue preparation
For perfusion and preparation of animals, under anesthesia, a needle was inserted through the left apex of the heart into the aorta, and the right atrium was immediately opened after hemostatic forceps fixation for perfusion using pre-cooled phosphate-buffered saline (PBS, 4 °C) until the liver turned white.

Two weeks after SCI, five rats in each group were perfused with PBS as described previously. Whole brains were removed by craniotomy. One side of the brain hemisphere was fixed with 4% paraformaldehyde (PFA) at 4 °C for 3 d, and the SMC of the other side (2 mm off the coronal suture and 3 mm off the sagittal suture) was removed carefully and washed in PBS. Next, tissues were placed directly into an Eppendorf tube and immediately stored at -80 °C.

Four weeks after SCI, five rats in each group were perfused with PBS as described previously. The SMC was removed carefully and washed in diethylpyrocarbonate-treated ddH₂O and immediately put into liquid nitrogen for quick freezing. Additionally, 2 cm of spinal cord with T10 as the center was removed and fixed in 4% PFA at 4 °C for 3 d. Meanwhile, 8-mm spinal cord tissue sections in the rostral injury region were removed on ice and divided into two parts. One part was placed directly into an Eppendorf tube, the other part was washed in diethylpyrocarbonate-treated ddH₂O and immediately put into liquid nitrogen for quick freezing. Frozen tissues were immediately stored at -80 °C.

Eight weeks after SCI, five rats in the Control group, sham iTBS group, and iTBS group were perfused with 4% PFA until shaking their limbs and wagging their tails. The total perfusion amount of each rat was about 100 ml. Then, 2-cm spinal cord tissue sections were removed with T10 as the center and fixed with 4% PFA at 4 °C for 2 d.

**Western Blot analysis**

RIPA Lysis Buffer (P0013; Beyotime, Shanghai, China) mixed with protease inhibitor (05892791001; Roche, Basel, Switzerland) was added to each Eppendorf tube containing spinal cord tissue. The tissue was broken up by ultrasound and was placed on ice for 20 min and centrifuged at 12000 rpm for 20 min; protein supernatant was placed at -80 °C.

Protein samples were diluted with 4-µl 5X loading buffer and ddH₂O to 20 µl volume of protein loading solution and boiled in a water bath for 10 min before centrifugation. The electrophoresis apparatus (1645050; Bio-Rad, Hercules, CA, US) was used to adjust the voltage to 80 V for 30 min to observe that the loading buffer indicator ran out of the concentrated gel. Then, the voltage was adjusted to 100 V for 70 min to observe that the indicator ran to the bottom of the gel. Gels were transfer printed with a "sandwich" clip, the current of the electrophoresis apparatus was adjusted to 0.25 mA, and gels were transferred for 4 h. Membranes were incubated in blocking solution for 20 min and then incubated in specific primary antibodies for one night at 4 °C with nutation. On the next day, the shaker was placed at room temperature for rewarming, and membranes were washed with 1x tris buffered saline with Tween 20 (TBST); the same samples were incubated with horseradish peroxidase for 2 h in 1x TBST. An electrochemiluminescence reagent (BL523A; Biosharp, Anhui, China) was used for the chemiluminescence imaging system (ChemiDoc Touch 1708370; Bio-Rad, Hercules, CA, US). All bands
were analyzed using ImageJ [14] for five rats in each group. A summary of the antibodies used can be found in Table S1.

**Immunofluorescence analysis**

The cerebral hemispheres and spinal cord, after fixation in 4% PFA, were dehydrated in 30% sucrose solution for two weeks. The SMC and the spinal cord from the lesion area were embedded in Tissue-Tek® O.C.T. (Sakura Finetek, Alphen aan den Rijn, Netherlands) for coronal sectioning with a thickness of 25 µm using a cryotome. The tissues were circled with a histochemical pen and sealed with 10% goat serum diluted in PBS with 0.3% Triton X-100 in a thermotank at 37 °C for 30 min. Samples were incubated in specific primary antibodies overnight at 4 °C.

The next day, samples were washed with 0.01 mol/L PBS three times for five minutes and then incubated with secondary antibodies for 2 h at 37 °C. Cell nuclei were counterstained with Hoechst33342 (Hoe) for 15 min. The slides were imaged using a confocal microscope (Dragonfly CR-DFLY-202 2540; Andor Technology, Belfast, UK). Immunofluorescence quantitative analysis was performed on five rats from each group. Three coronal sections selected from each rat from the regions rostral and caudal to/in the injury site of the spinal cord were analyzed using positive cell counting or mean gray values in ImageJ. A summary of the antibodies used can be found in Table S1.

**3,3'-diaminobenzidine staining**

Slices were incubated with 3% H2O2 in deionized water at room temperature to eliminate endogenous peroxidase activity. Slices were incubated in primary antibody against 5-hydroxytryptamine (5-HT) overnight at 4 °C and then rewarmed for 30 min. Tissue slices were incubated with secondary antibodies at 37 °C for 30 min, washed with PBS three times for five minutes each, and streptavidin–biotin complex (SA1021; Boster, Wuhan, China) was added to the tissue for 30 min at 37 °C. A summary of the antibodies used can be found in Table S1.

**mRNA sequencing**

At two and four weeks after SCI, two rats were selected per group for SMC and spinal cord mRNA sequencing. RNA was extracted according to conventional methods. RNA integrity was detected using agarose gel electrophoresis (28S:18S ≥ 1.5), RNA purity was detected using a Nanodrop (OD260/280: 1.8–2.2), and RNA concentration was accurately quantified using Qubit (≥ 500 ng/µl). Qualified samples were sent to Genergy Bio-Technology Co., Ltd (Shanghai, China) for RNA-Seq analysis. The image data measured by a high-throughput sequencer were converted into sequenced reads by CASAVA base calling. Original sequence counts of known genes for all samples were analyzed by StringTie, and expression levels of known genes were calculated by fragments per kilobase of transcript per million fragments mapped. DESeq2 software was used for screening differentially expressed genes between groups, with |log2FC| ≥ 1 and P-values ≤ 0.05.

**Statistical analysis**
All experiments were randomized. Animals were chosen from multiple cages and assigned randomly to each experiment. Data are expressed as the mean ± Standard error and mean (SEM). All experiments were carried out in a double-blinded manner. The data were analyzed by one-way analysis of variance (ANOVA) in GraphPad Prism software (v 8.0; GraphPad Software, San Diego, CA, USA); the least squares difference-t test was used when pairwise comparisons were normally distributed, and Dunnett’s post hoc test was used when the variances were not uniform. For all experiments, values of $P < 0.05$ were considered to be statistically significant.

**Results**

**Transcranial iTBS inhibits inflammation and neuronal apoptosis in the brain**

Transcranial iTBS treatment started 72 h after SCI significantly reduced the expression of the microglia/macrophage marker ionized Ca^{2+}-binding adapter protein 1 (IBA-1) in the SMC measured two weeks after SCI (Fig. 1A and C). To further evaluate the effects of transcranial iTBS on inflammation, we analyzed tissue obtained from the SMC via western blot to discover alterations in microglia/macrophage-associated proteins. The results showed that macrosialin (CD68) and C-C chemokine receptor 7 (CCR7, an M1 macrophage marker) showed a decreasing trend after two weeks of transcranial iTBS (Fig. 1D and F). At the same time, transcranial iTBS increased the expression of mannose receptor (CD206, an M2 macrophage marker) after SCI (Fig. 1D and F). Cleaved caspase 3 (Cle-caspase3) can be used as an indicator of cell apoptosis. After two weeks of transcranial iTBS treatment, we found that the number of apoptotic neurons co-labeled by Cle-caspase3 and NeuN was significantly lower than that in the Control and sham iTBS groups. There were no significant differences between the Control and sham iTBS groups, including in western blot analysis (Fig. 1B, H, E, and I). Differential gene expression related to inflammation and apoptosis further supported this result (Fig. 1G and J). The expression levels of 56 pro-inflammatory genes in the SMC after transcranial iTBS treatment over 4 weeks were significantly lower than in the sham iTBS group, such as *cluster of differentiation 37*[^15] and *soluble calcium-binding protein A9 (S100a9)*[^16] (Table S2). Moreover, compared with the sham iTBS group, the expression levels of 83 anti-inflammatory genes were more similar to those in the Normal group, such as *C-C chemokine receptor 5* (Table S2), which could suppress enhancement of microglia/macrophage through the *chemokine (C-C motif) ligand 3–C-C chemokine receptor 5 axis*[^17]. Meanwhile, heatmap data of 16 differential genes related to supporting apoptosis including *B-cell lymphoma 2-associated X protein* were decreased after transcranial iTBS treatment, and 38 differential genes that promote cell survival and inhibit apoptosis, including *B-cell lymphoma 2*, were increased after treatment compared with the sham iTBS group[^18]. The expression of the immediate early gene c-Fos reflects the activation of neurons. We found that transcranial iTBS could up-regulate the expression of c-Fos in neurons in the SMC (Fig. S1A). Therefore, we speculate that the regulation of transcranial iTBS on SMC inflammation was related to neuron activation. As shown in Fig. 1G, genes in the functional sets between neuronal activation and
inflammation were displayed in another interaction network. Similarly, based on the genes related to neuronal activation and apoptosis, we generated a network to represent the relationship among genes in these two functional sets (Fig. 1J). Noticeably, there was a significantly differently expressed gene named 3-phosphoinositide-dependent protein kinase 1 (Pdpk1) at the center of the network in the two figures. The results suggested that transcranial iTBS can effectively regulate the activation and polarization of inflammatory cells in the SMC triggered by SCI and significantly inhibit neuronal apoptosis to prevent the harmful signal cascades after SCI.

Transcranial iTBS inhibits inflammation and neuronal apoptosis in the SCI site

In the injured spinal cord, pro-inflammatory microglia/macrophage enhance the prolonged inflammatory period, resulting in impaired axon regeneration and neurodegeneration. However, we found that, after four weeks of transcranial iTBS treatment, the expression level of CD68 in the regions rostral and caudal to/in the injury site of the spinal cord in the iTBS group was significantly lower than that in the Control and sham iTBS groups (Fig. 2A, B, C, and D). We also observed a significant decrease in the number of co-expressing Cle-caspase3+ and NeuN+ neurons in the rostral and caudal injury area (arrows in Fig. 3A, B, C, and G); this was also confirmed by western blot of tissues from the rostral injury area (Fig. 3D and F). Then, we investigated the changes in gene expression in the rostral injury region after four weeks of transcranial iTBS treatment (Fig. 2E and 3E). We found that 76 differentially expressed pro-inflammatory genes including complement component 3 (C3) were decreased after four weeks of transcranial iTBS treatment (Table S3). As an inflammatory complement, C3 is widely present in glia and neurons after SCI. The complement cascade system is activated after traumatic brain injury and SCI. Moreover, its lytic products, complement C3a and C3, can mediate inflammatory responses, inhibit the outward growth of axons, reduce neuronal activity, and inhibit myelin regeneration.[19] In addition, the expression levels of 17 genes related to pro-apoptosis including Caspase4 were also significantly decreased in the iTBS group [20], which was closer to the expression level of the Normal group (Table S3). Transcranial iTBS also stimulated c-Fos expression in neurons in the cervical and thoracic spinal cord (Fig. S1B, C and D). Based on the genes related to neuronal activation and inflammation, we generated a network to represent the relationship among genes in these two functional sets (Fig. 2F). In the network, the expression of both S100a9 and S100a8 in the iTBS group was significantly lower than that in the sham iTBS group. The gene ontology interaction network also suggested that transcranial iTBS activating spinal cord neurons not only produced regulatory effects on the inflammation and neuronal apoptosis, but also played an important regulatory role in the activation of astrocytes and microglia/macrophage (Fig. 3H). In conclusion, our results showed that transcranial iTBS can effectively improve inflammation in the spinal cord injury site and significantly inhibit neuronal apoptosis so as to promote axonal regeneration and remodeling.

(A-C) Low magnification of coronal spinal cord sections showing the expression of CD68. (A1, B1, C1) The CD68+ cells in the rostral injury region. (A2, B2, C2) The CD68+ cells in the injury site of spinal cord.
(A3, B3, C3) The CD68+ cells in the caudal injury region. Arrows indicate CD68+ cells. (D) Bar chart showing the number of CD68+ cells in the regions rostral and caudal to/in the injury site (***, **P < 0.001, **P < 0.01, *P < 0.05). (E) Heatmap constructed from 76 significantly differentially expressed genes related to pro-inflammatory in the rostral injury region. In clustering analysis, up-regulated and down-regulated genes are colored in red and green, respectively. (F) Based on the results of the enrichment analysis of the functional sets between the neuronal activation and inflammation in the iTBS group vs. the sham iTBS group, the network diagram among genes inside these two functional sets were constructed. The network was extracted using the strings database of rats (confidence coefficient ≥ 700), and each node was displayed in color according to the Log2FC value. Cell nuclei were counterstained with Hoechst33342 (Hoe). Data are presented as mean ± SEM (n = 5). One-way ANOVA with a least significant difference (LSD)-t (equal variance assumed) or Dunnett’s T3 (equal variance not assumed) was performed. Scale bars = 1000µm in (A) - (C); 50µm in (A1) - (C3).

The neuroprotective effect of transcranial iTBS on the injury site of spinal cord

After SCI via transection, astrocytes labeled by neuroglial fibrillary acidic protein (GFAP) surrounding the lesion developed hypertrophy and protrusions (Fig. 4A-2, B-2, and C-2). These reactive astrocytes migrate toward the central injury site in the spinal cord (Fig. 4A2-2, B2-2, and C2-2) and eventually form glial scars that secrete axon growth inhibitors and prevent axon regeneration [21]. Noticeably, after four weeks of transcranial iTBS treatment, the GFAP+ area in the injury site of the spinal cord was significantly reduced (Fig. 4E), thus alleviating the adverse effects on axonal regeneration. Moreover, this result was further supported by differential gene expression in astrocytes (Fig. 4F). The expression levels of 37 differential genes related to astrocytes in the iTBS group were significantly down-regulated compared with the sham iTBS group, including c-x-c motif chemokine ligand 1 released by astrocytes [22] (Table S3). In addition, activating transcription factor 3 (ATF3) is one of the earliest genes responding to axon injury [23]. After SCI, ATF3 was not expressed, due to the death of neurons in the central injury site (Fig. 4A2-1, B2-1, and C2-1), while the number of ATF3+ cells in the regions rostral and caudal to the injury site was significantly increased (Fig. 4A1-1, A3-1, B1-1, B3-1, C1-1, C3-1, and 4D). However, after effective treatment with transcranial iTBS, we found that the number of ATF3+ cells in the regions rostral and caudal to the injury site in the iTBS group was significantly reduced compared with the Control and sham iTBS groups (Fig. 4D). At the same time, we made a heat map of the differential gene expression of 19 different types of neurons. After four weeks of transcranial iTBS treatment, the gene expression in different types of neurons in the iTBS group was most similar to the Normal group. However, the expression trend of the sham iTBS group was clearly opposite to that of the Normal group; this was seen, for example, in neurogranin, which mediates postsynaptic membrane excitability [24] (Fig. 4F). Figure 4G shows that four-week treatment with transcranial iTBS significantly up-regulated neurotrophic factors and receptor-related genes including Sodium voltage-gated channel alpha
subunit 5 (Scn5a), cytochrome P450 family 26 subfamily C member 1 (Cyp26c1), wingless and integration-1 (Wnt1), and C-C chemokine ligand 21 (Ccl21), and down-regulated the genes that inhibit nerve regeneration including collagen type III alpha 1 (Col3a1), lethal with sec-thirteen (Lst1), and organic anion transport 1 (Slc22a6) (Table S3). Compared with the Control group and the sham iTBS group, more brain-derived neurotrophic factor (BDNF) expression was detected in the rostral region of the injury site in the iTBS group (Fig. S5). Finally, our results showed that transcranial iTBS reduced the activation of astrocytes and neuroglial scar formation and played a neuroprotective role, contributing to the survival of neurons by downregulating the neuron injury factor genes and upregulating excitatory neuron genes, neurotrophic factors, and receptor genes.

Transcranial iTBS activates nerve fiber regeneration and synaptic plasticity

To further quantitatively evaluate nerve fiber regeneration, we measured the fluorescence intensity of neurofilament-positive (NF+) nerve fibers in the regions rostral and caudal to/in the injury site. As displayed in Fig. 5A–E, the iTBS group showed an increased area of NF+ nerve fibers in comparison to the Control and sham iTBS groups. The result of western blot followed this (Fig. 6B, D). Overall, with the help of transcranial iTBS, more regenerated nerve fibers were found in the regions rostral and caudal to/in the injury site of the spinal cord.

GAP43 is closely related to nerve fiber growth and plays an important role in synaptic plasticity. It is highly abundant in the dynamic structure of the presynaptic membrane and growth cone. Changes in synaptic morphology are caused by neuronal differentiation during nervous system development, and nerve fiber regeneration and neural repair after injury can lead to the increased expression of GAP43. Noticeably, compared with the Control and sham iTBS groups, the iTBS group showed that the area of GAP43+ nerve fibers was increased after four weeks of SCI (Fig. 5A1-1–B3-2, D). In the iTBS group, the area of GAP43+ nerve fibers was further enhanced in the regions rostral and central to the injury site (Fig. 5). Western blot further showed that GAP43 expression was enhanced in the rostral region of the injury site in the iTBS group compared with the Control and sham iTBS groups (Fig. 6C, D).

To assess whether transcranial iTBS could promote the synaptic plasticity, Western blot was performed. Our results showed that synaptic formation-related proteins including synapsin (SYN) and postsynaptic density 95 (PSD95) were significantly decreased in the Control and sham iTBS groups compared with the Normal group, whereas the iTBS group manifested significantly higher expression of SYN and PSD95 levels than the other groups (Fig. 6A, D). These findings indicated that transcranial iTBS may promote the formation of synapses between neurons in the rostral region of the injury site.

In the gene ontology enrichment bubble diagram (Fig. 6E), compared with the sham iTBS group, the iTBS group had a higher degree of enrichment of mRNAs involved in neuronal activation, neurotransmitters, neuronal metabolism, axonal regeneration, and synaptic plasticity. Among these, the mRNAs related to axon regeneration showed the most significant differences. Based on the mRNA in these five aspects, we
generated a gene ontology net to represent the connection of genes in different functional sets (Fig. 6F). We found that there were several node genes with significantly different expressions in relating to neuronal activation, neurotransmitters, neuronal metabolism, and axonal regeneration. Transcranial iTBS down-regulated genes that negatively regulate neurotransmitters and neuronal metabolism, such as *PYD and CARD domain-containing* (*Pycard*), *cysteine-rich 61* (*Cyr61*), *Cd74*, *Cytochrome P450 1B1* (*Cyp1b1*), *triggering receptor expressed on myeloid cells 2* (*Trem2*), *C-X-C motif chemokine ligand 17* (*Cxcl17*) and *F2R-like trypsin receptor 1* (*F2rl1*). Transcranial iTBS in turn down-regulated genes including *Twist-related protein 1* (*Twist1*), *prostaglandin E receptor 3* (*Ptger3*), *S100a8*, and *S100a9*, which inhibit nerve regeneration through a gene interaction regulatory network (Table S3). On the contrary, we did not observe a relationship between neuronal activation, neuroinflammation, neuronal apoptosis, axonal regeneration, and neuroplasticity in the gene interaction network formed by comparison between the sham iTBS and iTBS groups (Fig. S6).

**Transcranial iTBS improves motor function of paralyzed hindlimbs**

We performed behavioral observations in all SCI rats, including the modified grid climbing test, BBB scoring, and electrophysiological examinations, to assess the recovery of motor function after transcranial iTBS treatment. After eight weeks of transcranial iTBS treatment, the 45° sloping grid climbing method was used to evaluate the spontaneous placement reflex triggered by direct touch (Fig. 6G). There was no placement reflex in the Control and sham iTBS groups. When the forelimbs climbed the diagonal grid, the hindlimbs were dragged behind and often fell. In contrast, rats in the iTBS group generally showed pronounced placement reflexes on their hindlimbs, and their hind feet could occasionally tread on the grid (Fig. 6H, Video S1, S2 showing the grid climbing test). Finally, BBB assessments showed that all rats had complete hindlimb paralysis after T10 spinal cord transection. Compared with the Control group, the iTBS group showed significant improvement in hindlimb motor function beginning six weeks after treatment (Fig. 6I, Video S3, S4 showing open-field locomotor test). At eight weeks, the motor function of the hindlimbs in each group improved; the mean BBB score was 4.3, indicating that the hind hips, knees, and ankles could move slightly, and it was observed that even the hind ankles could move substantially. However, the average BBB score between the Control group and the sham iTBS group was 3.0; the rats only had slight two-joint movements, but there was no movement of all three joints (Fig. 6G). In the Normal group, stimulation of the SMC with magnetic stimulation coil could induce MEPs with large response amplitudes, while rats in the Control and sham iTBS groups only showed small response amplitudes. Compared with the Control and sham iTBS groups, transcranial iTBS treatment significantly increased the response amplitude of MEPs (Fig. 6J and K). These results suggested that transcranial iTBS treatment of eight weeks had a significant effect on improving the motor function of paralyzed hindlimbs in SCI rats.

**Transcranial iTBS promotes regeneration of corticospinal tract (CST) and 5-HT positive axons**
BDA is mainly used as an anterograde tracer for the long conduction tracts of nerve fibers because it can realize long-distance axon transport after uptake by neurons [26]. We injected BDA into the SMC. After 14 days, BDA+ CST nerve fibers appeared more in the rostral region to the injury site of spinal cord in the iTBS group compared with the Control and sham iTBS groups. Moreover, BDA+ nerve fibers were mainly distributed in the dorsal deep white matter area near the midline and arranged in a relatively orderly manner and parallel to the longitudinal axis of the spinal cord in the iTBS group (Fig. 7C1). Some disordered and enlarged ends of BDA+ nerve fibers were observed in the injury site (Fig. 7C2), while no BDA+ nerve fibers were found in the caudal injury area (Fig. 7C3). However, there were fewer and more disordered BDA+ nerve fibers in the rostral region of the injury site in the Control and sham iTBS groups (Fig. 7A1, B1, G), and no BDA+ nerve fibers were found in the regions central and caudal to/in the injury site (Fig. 7A2, A3, B2, B3).

In addition, we used 3,3'-diaminobenzidine immunohistochemistry to display the descending 5-HT+ nerve fibers eight weeks after SCI. Although 5-HT+ nerve fibers reached the rostral region of the injury site in spinal cord (Fig. 7D1, E1, S4A1, B1), fewer 5-HT+ nerve fibers crossed the injury site in the Control and sham iTBS groups (Fig. 7D2, E2, D3, E3, S4A2, B2, A3, B3) than the iTBS group. Remarkably, a large number of 5-HT+ nerve fibers were observed in the rostral region to/in the injury site in the iTBS group (Fig. 7F1, F2, S4C1, S4C2), and significantly more 5-HT+ nerve fibers regenerated into the caudal region to the injury site (Fig. 7F3, S4C3, H). Transcranial iTBS could stimulate the expression of c-Fos in the raphe nucleus of the brain stem (Fig. S2). More convincingly, BDA injected into the SMC may induce synapse formation with neurons in the raphe nucleus in rats treated with transcranial iTBS for eight weeks (Fig. S2, S3). These results suggested that transcranial iTBS could activate SMC neurons and affect 5-HT neurons in the raphe nucleus to promote 5-HT+ nerve fiber regeneration.

In conclusion, in order to explore the associations between CST and 5-HT nerve fiber regeneration and motor function recovery, we performed linear calculations of the number of BDA+ nerve fibers and the number of 5-HT+ nerve fibers with BBB scores. We found positive relationships between the number of BDA+ nerve fibers, the number of 5-HT+ nerve fibers, and BBB scores (Fig. 7I, J), suggesting that, by activating neurons in the SMC and raphe nucleus, transcranial iTBS promotes regeneration of CST nerve fibers and 5-HT+ nerve fibers which can lead to the recovery of motor function.

**Transcranial iTBS promotes regeneration of long descending nerve fibers of cervical propriospinal neurons**

To further explore the specific mechanism of transcranial iTBS improvement of motor function, rats in each group were injected with BDA, eight weeks after SCI, into the cervical propriospinal neurons that project long descending nerve fibers (propriospinal tract). Interestingly, in the iTBS group, some nerve fibers labeled with BDA were found in the rostral injury region, and BDA+ nerve fibers were arranged parallel to the longitudinal axis of the spinal cord (Fig. BC1). Moreover, there were also BDA+ nerve fibers...
in the center of the injury site (Fig. 8C2). Most significantly, a small number of BDA\textsuperscript{+} nerve fibers were observed in the caudal injury regions (Fig. 8C3). However, compared with the iTBS group, few BDA\textsuperscript{+} nerve fibers in the rostral injury region were observed in the Control group (Fig. 8A1) and sham iTBS group (Fig. 8B1). We only found a few BDA\textsuperscript{+} nerve fibers in the caudal region (Fig. 8A2, A3, B2, B3). These findings suggest that transcranial iTBS may promote regeneration of the LDPT (Fig. 8E).

Discussion

SCI leads to the rupture of brain-derived nerve fibers, causing neuronal apoptosis and further loss of motor function. The regeneration of brain-derived nerve fibers and reconstruction of motor pathways is the most difficult problem in SCI repair. Previous treatments for SCI paid more attention to neuroprotection and axonal regeneration in the lesion area. However, in this study, we focused on exploring the therapeutic effects and mechanisms of transcranial iTBS on the SMC on neuroprotection, axonal regeneration, and voluntary motor function reconstruction in acute, subacute, and chronic stages of completely transected spinal cords in rats. Our results indicated that the timely implementation of transcranial iTBS in the acute stage of transected SCI played an important role in regulating the microenvironmental homeostasis of the brain and spinal cord, preventing neuronal apoptosis and initiating the regeneration of brain-derived nerve fibers. The results also revealed the mechanisms by which transcranial iTBS-activated neurons in the brain and spinal cord initiate axonal regeneration and regulate neural plasticity and further unraveled the basis of the neural pathways of voluntary motor function repair in rats.

Neuroprotective effects of transcranial iTBS on SMC

Many studies suggested that SCI could trigger a series of traumatic cascade reactions in the brain, which are manifested as inflammation, and lead to the progression of neurological dysfunction\textsuperscript{[27,28]}. Our results suggested that microglia/macrophage were the dominant immune cells in the SMC two weeks after transection SCI. SCI induced an increase in the number and cell size of microglia/macrophage, which had increased expressed of CD68 and CCR7, suggesting that they differentiated into the M1 phenotype and participated in secreting pro-inflammatory factors to trigger inflammation. However, transcranial iTBS treatment on the SMC significantly reduced the number of microglia/macrophage. CD206, as a marker of the M2 phenotype, was detected, suggesting that transcranial iTBS had a positive role in regulating the immune balance of the brain. Previous studies suggested that SCI could cause atrophy and apoptosis of SMC neurons, but the time point of observation was mostly in the chronic stage (i.e., two months after SCI). Our results demonstrated that SMC neurons initiated apoptosis two weeks after SCI, and the number of apoptotic neurons was consistent with the number of microglia/macrophage, suggesting that the progression of brain neuroinflammation was closely related to the apoptosis of brain neurons. It has been reported that neurons in the brain send out nociceptive stimulus signal after SCI to induce the aggregation and activation of microglia/macrophage before
neuronal apoptosis. Our results suggested that timely control of microglia/macrophage activation by transcranial iTBS was important to prevent programmed apoptosis of neurons.

There are few preclinical and clinical studies on the application of transcranial iTBS in SCI, and most SCI models involve incomplete transection. The therapeutic effects have mainly been directed at the rehabilitation of motor function and the neuroplasticity of the spinal cord, and few studies involved the mechanisms of regulation of neuroinflammation and neuroprotection in the brain and spinal cord. We found that extensive expression of the immediate early gene c-Fos was detected in SMC neurons after transcranial iTBS treatment, suggesting that the direct effect of transcranial iTBS was neuronal activation, which is consistent with the reported effects of TMS on neuronal activation. We speculated that the activation of neurons by transcranial iTBS reduced the number of nociceptive signals sent out by neurons, avoiding further recruitment and stimulation of microglia/macrophage activation. In addition, decreased activation of microglia/macrophage further reduced damage to the neurons caused by the secretion of inflammatory factors. The mechanism probably lies in the therapeutic effect of transcranial iTBS on the regulation of internal genes and the microenvironment of neurons. Therefore, the synergistic regulation of internal and external factors reduces neuronal apoptosis.

After four weeks of transcranial iTBS, transcriptome sequencing of SMC tissue suggested that the results from analysis of inflammation- and apoptosis-related mRNA were similar to the immunofluorescence results from after two weeks of iTBS. The mRNA levels of these pro-inflammatory genes were up-regulated after SCI but were significantly down-regulated to near normal levels after transcranial iTBS treatment. The mRNA of anti-inflammatory genes were significantly down-regulated in the sham iTBS group and up-regulated in the iTBS group to nearly normal level. Thus, transcranial iTBS played an important role in regulating the balance of the inflammatory microenvironment in the SMC. In the sham iTBS group, the mRNA levels of pro-apoptotic genes were still high, while the mRNA levels of anti-apoptotic genes were significantly lower. In the iTBS group, the pro-apoptotic genes were significantly down-regulated, while the anti-apoptotic genes were up-regulated to levels similar to normal rats. A possible reason for this is that continuous iTBS activation of SMC neurons reduced the emission of nociceptive signals and, thus, decreased microglia/macrophage aggregation and inflammatory factor expression, decreasing neuronal apoptosis.

To further confirm the mechanism, we focused on interaction network analysis of genes related to neuron activation, regulating inflammation and apoptosis in the SMC. Notably, Pdpk1 at the core regulatory position was significantly expressed after transcranial iTBS treatment. Pdpk1 is regulated by c-Fos and regulates the protein kinase families A, G, and C by activating phosphorylation of ribosomal protein S6, affecting the survival and metabolism of neurons and participating in the regulation of immune microenvironment homeostasis. In addition, Pdpk1 also plays an important role in neuronal survival, axonal regeneration, and synaptic plasticity by regulating the protein kinase B–mechanistic target of rapamycin pathway. Regulation of individual genes, such as phosphatase and tensin homolog (PTEN), mechanistic target of rapamycin, or Pdpk1, can promote the regeneration of brain-derived nerve fibers to a certain extent after SCI. However, our study suggested that maintaining microenvironmental
homeostasis was essential to promote the regeneration of brain-derived nerve fibers in the injury area while regulating the endogenous regeneration signal of SMC neurons.

**Neuroprotective effect of transcranial iTBS on spinal cord**

Inflammation, oxidative stress and tissue necrosis at the site of SCI are much more serious than in the SMC. If the pathological processes at the site of injury are not controlled in time, the spinal cord neurons in the regions rostral and caudal to the injury site will continue to be lost. Understanding whether transcranial iTBS on the SMC can regulate the microenvironment in the site of injury and reduce the inflammation and neuronal apoptosis as much as possible is crucial for promoting nerve regeneration and functional reconstruction, but there have been few reports on this.

Our results suggested that transcranial iTBS could activate neurons in the spinal cord that receive neural information from the brain. After four weeks of transcranial iTBS treatment, the results of inflammation and apoptosis analysis showed that the number of CD68+ microglia/macrophage and Cle-caspase3+ apoptotic neurons in the regions rostral and caudal to the injury site, as well as CD68+ microglia/macrophage in the injury site, were significantly lower in the iTBS group than in the Control and the sham iTBS group. It is suggested that the activation of spinal cord neurons may be involved in regulating neuroinflammation to reduce apoptosis after receiving iTBS stimulation transmitted by brain-derived nerve fibers.

The transcriptome sequencing of injured spinal cord tissue suggested that a large number of pro-inflammatory genes and pro-apoptotic genes were up-regulated after injury, while the pro-inflammatory genes and pro-apoptotic genes were significantly decreased after transcranial iTBS treatment compared with the sham iTBS group, although they were still higher than those in the Normal group. Further analysis of the interactions between genes related to neuronal activation and inflammation suggested that the downregulation of **TYRO protein tyrosine kinase-binding protein** after activation of spinal cord neurons played a key role in regulating complement genes C1qa, C1qb, and C1qc to maintain the stability of the immune microenvironment [34, 35]. On the other hand, the down-regulated stimulus-response related genes **interferon regulatory factor 7**, **myxovirus resistance 1**, MHC class II member **RT1-Ba**, **S100a8**, and **S100a9** help avoid hyperactivation of aberrant immune cells by transcranial iTBS. Therefore, this plays an important role in regulating the balance of the immune microenvironment and avoiding the over-activation of inflammatory cells [36–38]. In the sham iTBS group, we detected significant downregulation of genes related to neuronal activation, while microglia/macrophage activation and pro-inflammatory genes were significantly up-regulated. The gene ontology network diagram also suggested that transcranial iTBS activating spinal cord neurons not only produced multidirectional regulatory effects on the immune microenvironment, but also played an important regulatory role in neuronal survival and astrocyte activation.

Four weeks after SCI, the expression of **ATF3**, a marker of injured neurons, was also significantly higher in the rostral and caudal injury area, suggesting that the progression of inflammation in the subacute phase was still damaging neurons at both ends of the injury area. The expression of ATF3 was significantly
decreased in the iTBS group, which is consistent with the results of iTBS inhibiting neuroinflammation and reducing neuronal apoptosis, suggesting that transcranial iTBS played an important role in regulating microenvironmental homeostasis, reducing neuronal injury and apoptosis. In addition, we also found that iTBS significantly reduced the expression of GFAP in the regions rostral and caudal to/in the injury site of spinal cord, and transcriptome sequencing also suggested that genes related to astrocyte activation were significantly up-regulated in the sham iTBS group compared with the iTBS group. This is consistent with the effects of inflammatory factors on astrocyte activation and proliferation mentioned in previous studies [39]. The inhibitory effect of transcranial iTBS on the excessive proliferation of astrocytes is beneficial to alleviate the obstacles of nerve fiber regeneration across the injured area, which is of great significance for the recovery of voluntary motor function. Meanwhile, the scatter plot of identified mRNA showed that genes related to nerve regeneration were significantly up-regulated in the iTBS group, while genes inhibiting nerve regeneration were significantly up-regulated in the sham iTBS group. This is consistent with the effects of inflammatory factors on astrocyte activation and proliferation mentioned in previous studies [39].

**Mechanism of transcranial iTBS on neuronal activation to regulate axonal regeneration and neuroplasticity**

After confirming the neuroprotective effects of transcranial iTBS in the acute and subacute phases of SCI, we detected axonal regeneration at the injury site of the spinal cord. The results showed that the number of GAP43+ and NF+ nerve fibers in the regions rostral and caudal to/in the injury site in the iTBS group was significantly higher than in the Control group and sham iTBS group four weeks after SCI, confirming the hypothesis that transcranial iTBS could activate neurons in the brain and spinal cord and regulate the immune microenvironment to achieve neuroprotective effects and initiate axon regeneration. In addition to significantly higher expression of GAP43 and NF, we also detected significantly higher expression of the synapse-associated proteins PSD95 and SYN, suggesting that transcranial iTBS not only promoted axonal regeneration but also affected synaptic plasticity. Many studies have confirmed that regulating axon regeneration and neural pathway remodeling after nerve injury is an important prerequisite for the recovery of function [40]. We analyzed the interactions of genes related to neuronal activation, neurotransmitters, neuronal metabolism, nerve regeneration, and synaptic plasticity to explore the key genes that regulate axonal regeneration and neural pathway remodeling after activation of spinal neurons by transcranial iTBS.

The results showed that the down-regulating of genes *Pycard*, *Cd74*, and *Cyr61* regulated the expression of genes related to neurotransmitters and neural metabolism after the activation of spinal cord neurons by transcranial iTBS. Subsequently, transcranial iTBS regulated neuron ion channels by upregulating *Scn5a* and regulated the neuronal cytoskeleton and axon regeneration by up-regulating *Avil*. It also regulated neuronal dendritic remodeling and synaptic plasticity through downregulation of *Twist1*. Finally, we found that, after transcranial iTBS treatment, the joint motor ability of paralyzed hindlimbs was enhanced, and the injured spinal cord could transmit motor evoked potentials with the highest peak amplitude. The results of behavioral tests fully confirmed the therapeutic action and regulatory effect of
transcranial iTBS on immune regulation, neuroprotection, axon regeneration, and neuroplasticity in the injured spinal cord.

The neural pathway of transcranial iTBS promoting the recovery of voluntary motor function

In order to further reveal the neural pathways through which transcranial iTBS promotes the restoration of voluntary motor function after spinal cord transection in rats, we performed BDA tracing on the SMC and the LDPT of the cervical spinal cord after eight weeks of transcranial iTBS treatment. The results showed that BDA⁺ CST could regenerate more to the rostral injury region after treatment, but it was difficult to cross the injured area and regenerate the caudal injury region. This is inconsistent with our previous observation that GAP43⁺ and NF⁺ nerve fibers were significantly increased in the regions central and caudal to the injury site in the iTBS group.

BDA injected into the SMC was detected in the raphe nucleus of the brain stem in the iTBS group, which suggested that, after transcranial iTBS treatment, the motor neural circuit produced structural and functional remodeling, and the CST might regenerate to the red and raphe nuclei in the midbrain (Fig. 1D, E), in which the neurons could project to the spinal tracts or to the neurons of the LDPT in the rostral injury area, forming synapses with these neurons (Fig S1H, I). After receiving neuronal information via the CST, these neurons might regenerate and cross the injury area and eventually established synapses with the neurons that control hindlimb movement, ultimately mediating the restoration of voluntary motor function and electrophysiological function of the spinal cord.

The cell bodies of 5-HT⁺ nerve fibers originate from the raphe nucleus of the midbrain. A large number of studies have confirmed that 5-HT⁺ nerve fibers have a stronger regenerative ability through injury sites than CST nerve fibers, and this regeneration plays an important role in the repair of voluntary motor function. In particular, it plays an important compensatory role in the limited regeneration of CST. We found that, after transcranial iTBS, neurons in the raphe nucleus, which projects 5-HT nerve fibers, were heavily activated and expressed c-Fos. Moreover, compared to the other groups, more 5-HT⁺ nerve fibers were observed to regenerate into the regions rostral and caudal to/in the injury site eight weeks after SCI. The regeneration of CST and 5-HT nerve fibers was positively correlated with the recovery of hindlimb motor function.

Noticeably, c-Fos expression was detected in neurons activated by transcranial iTBS in both the cervical spinal cord and the rostral region to the injury site of the thoracic spinal cord. The results of BDA tracing in the LDPT of the cervical spinal cord suggested that there were more BDA⁺ nerve fibers in the regions rostral and caudal to/in the injury site in the iTBS group than the other groups. It is suggested that the neurons in the LDPT of the cervical spinal cord are most likely to regenerate across the injury site after receiving the signals from CST or 5-HT nerve fibers and relay these to the motor neurons in the caudal injury regions, thus further mediating the repair of motor function.
In conclusion, the results showing neuronal activation, axonal regeneration, and nerve tracing suggested that the neural pathways of transcranial iTBS promoting the restoration of voluntary motor function after transected SCI in rats might include the 1) CST–5-HT pathway; the 2) CST–LDPT pathway; and the 3) CST–5-HT–LDPT pathway. The remodeling of these neural pathways by transcranial iTBS ultimately promoted the restoration of voluntary motor function.

**Conclusion**

In this study, we used a model of transected spinal cords in rats to reveal that transcranial iTBS treatment performed starting three days after injury could activate the brain and spinal cord neurons to play a regulatory role in the homeostasis of the neuroimmune microenvironment of the brain and spinal cord. This could prevent neuronal injury and apoptosis and initiate axon regeneration, ultimately restoring voluntary motor function by reconstructing motor pathways. At the same time, our study revealed the key genes that regulate the neuroimmune microenvironment and initiate axonal regeneration and neuroplasticity after activating the neural pathway by transcranial iTBS, and these genes are expected to be used as regulatory targets to enhance therapeutic effects in subsequent studies. Transcranial iTBS is commonly used in clinical practice as a non-invasive and safe treatment with few side effects. In the past, it has been mostly used for the treatment of neurological abnormalities and mental disorders \cite{45, 46}. There is still a lack of systematic and in-depth research on the therapeutic mechanisms of repairing tissue after SCI. Our study elucidated new mechanisms of transcranial iTBS on the repair of severe SCI at the level of cells, proteins, and gene interactions. However, it should also be considered that it is difficult to achieve truly ideal motor function restoration with a single treatment for severe SCI. In addition to affirming the therapeutic value of transcranial iTBS, the value of transcranial iTBS combined with cutting-edge therapeutic strategies such as stem cell transplantation, bioactive scaffolding, and tissue engineering therapy should be considered to jointly solve the problems associated with recovering voluntary motor function and sensation after SCI.

**Abbreviations**

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SCI</td>
<td>Spinal cord injury</td>
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<tr>
<td>SMC</td>
<td>Sensorimotor cortex</td>
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<tr>
<td>iTBS</td>
<td>Intermittent Theta-Burst Stimulation</td>
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<td>CST</td>
<td>Corticospinal tract</td>
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<td>5-HT</td>
<td>5-hydroxytryptamine</td>
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<td>LDPT</td>
<td>Long descending propriospinal tract</td>
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<tr>
<td>TMS</td>
<td>Transcranial magnetic stimulation</td>
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<tr>
<td>MEP</td>
<td>Motor evoked potential</td>
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<tr>
<td>GAP43</td>
<td>Growth-associated protein 43</td>
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<tr>
<td>BBB</td>
<td>Basso–Beattie–Bresnahan</td>
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<tr>
<td>BDA</td>
<td>Biotinylated dextran amine</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
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<tr>
<td>CC3</td>
<td>Cleaved caspase 3</td>
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<tr>
<td>GFAP</td>
<td>Glial fibrillary acidic protein</td>
</tr>
<tr>
<td>ATF3</td>
<td>Activating transcription factor 3</td>
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<tr>
<td>NF+</td>
<td>Neurofilament-positive nerve fibers</td>
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<tr>
<td>SYN</td>
<td>Synapsin</td>
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<tr>
<td>PSD95</td>
<td>Postsynaptic density 95</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>SEM</td>
<td>Standard error and mean</td>
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**Declarations**

**Ethics approval and consent to participate**
The author(s) declare that they have no conflict of interest. Animals were used with the approval of the ethics committee of Sun Yat-sen University (Animal Use Protocol no. 2021PS704K).

**Consent for publication**

Not applicable.

**Availability of data and materials**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

Biqin Lai, Yuanshan Zeng and Lixin Zhang designed and supervised the study. Jialin Liu and Shuai Wang performed the experiments and collected the data. Jialin Liu, Shuai Wang, Biqin Lai, Yuanshan Zeng and Lixin Zhang summarized, analyzed, and plotted the data and drafted the manuscript. Zhenghong Chen, Rongjie Wu, Haiyang Yu, Shangbin Yang, Jing Xu, Yinan Guo, Ying Ding, Ge Li, Xiang Zeng, Yuanhuan Ma, Yulai Gong and Chuangran Wu helped with study planning and critically reviewed the manuscript. Jialin Liu, Biqin Lai and Shuai Wang wrote and finalized the article.

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**References**


Figures
Transcranial iTBS inhibits inflammation and neuronal apoptosis in the SMC. (A) IBA-1+ microglia/macrophage (arrows) in the Normal, Control, sham iTBS, and iTBS groups two weeks after SCI. The expression of IBA-1+ microglia/macrophage in the iTBS group was lower than that in the Control and sham iTBS groups. (B) The co-expression of Cle-caspase3 and NeuN (arrows in B) in the four groups. The expression of Cle-caspase3+ in NeuN+ neurons in the iTBS group was lower than that in the Control.
group and sham iTBS group. (C) Bar chart showing the number of IBA-1+ cells in the four groups (**P < 0.0001). (D) Western blot of CD206, CD68, CCR7, and GAPDH in the SMC. (E) Western blot of Pro-caspase3, Cle-caspase3, and β-actin in the SMC. (F) Bar chart showing quantification of the protein expression based on western blot. Compared to the Control and sham iTBS groups, the levels of CD68 and CCR7 were decreased in the iTBS group; however, the levels of CD206 were increased in the iTBS group (**P < 0.0001, *P < 0.01, *P < 0.05). (G) Heatmap constructed from 139 significantly differentially expressed genes related to inflammation in the SMC. In clustering analysis, up-regulated and down-regulated genes are colored in red and green, respectively. Based on the results of enrichment analysis of the functional sets between the neuronal activation and inflammation in the iTBS group vs. the sham iTBS group, the network diagram among genes inside these two functional sets were constructed. The network was extracted using a strings database of rats (confidence coefficient ≥ 700), and each node was displayed in color according to the Log2FC value. (H) Bar chart showing the relative expression of Cle-caspase3 (CC3+) in NeuN+ cells in the four groups (**P < 0.01, *P < 0.05). (I) Bar chart showing quantification of the activated caspase3 (Cle-caspase3/Pro-caspase3) protein expression based on western blot; the activated caspase3 ratio in the iTBS group was lower than that in the Control and sham iTBS groups (**P < 0.0001). (J) Heatmap constructed from 54 significantly differentially expressed genes related to apoptosis in the SMC; in clustering analysis, up-regulated and down-regulated genes are colored in red and green, respectively. Based on the results of enrichment analysis of the functional sets between the neuronal activation and apoptosis in the iTBS group vs. the sham iTBS group, the network diagram among genes inside these two functional sets were constructed. The network was extracted using the strings database of rats (confidence coefficient ≥ 700), and each node was displayed in color according to the Log2FC value. Cell nuclei were counterstained with Hoechst33342 (Hoe). Data are presented as mean ± SEM (n = 5). One-way ANOVA with a least significant difference (LSD)-t (equal variance assumed) or Dunnett's T3 (equal variance not assumed) was performed. Scale bars = 50 μm in (A1) - (B4).
Figure 2

Transcranial iTBS inhibits inflammation in the injury site of spinal cord.

(A-C) Low magnification of coronal spinal cord sections showing the expression of CD68. (A1, B1, C1) The CD68+ cells in the rostral injury region. (A2, B2, C2) The CD68+ cells in the injury site of spinal cord. (A3, B3, C3) The CD68+ cells in the caudal injury region. Arrows indicate CD68+ cells. (D) Bar chart
showing the number of CD68+ cells in the regions rostral and caudal to/in the injury site (****P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05). (E) Heatmap constructed from 76 significantly differentially expressed genes related to pro-inflammation in the rostral injury region. In clustering analysis, up-regulated and down-regulated genes are colored in red and green, respectively. (F) Based on the results of the enrichment analysis of the functional sets between the neuronal activation and inflammation in the iTBS group vs. the sham iTBS group, the network diagram among genes inside these two functional sets were constructed. The network was extracted using the strings database of rats (confidence coefficient ≥ 700), and each node was displayed in color according to the Log2FC value. Cell nuclei were counterstained with Hoechst33342 (Hoe). Data are presented as mean ± SEM (n = 5). One-way ANOVA with a least significant difference (LSD)-t (equal variance assumed) or Dunnett’s T3 (equal variance not assumed) was performed. Scale bars =1000μm in (A) - (C); 50μm in (A1) - (C3).
Figure 3

Transcranial iTBS inhibits neuronal apoptosis in the injury site of spinal cord. (A-C) Low magnification of coronal spinal cord sections showing the expression of Cle-caspase3 and NeuN. (A1, B1, C1) The co-expression of Cle-caspase3+ and NeuN+ neurons in the rostral region to the injury site of spinal cord. (A2, B2, C2) The expression of Cle-caspase3+ in the injury site of spinal cord. (A3, B3, C3) The co-expression of Cle-caspase3+ and NeuN+ neurons in the caudal region to the injury site of spinal cord. Arrows indicate...
the co-expression of Cle-caspase3+ and NeuN+ neurons. (D) Western blot for Pro-caspase3, Cle-caspase3, and β-actin in the rostral injury region. (E) Heatmap constructed from 17 significantly differentially expressed genes related to pro-apoptosis in the rostral injury region. In clustering analysis, up-regulated and down-regulated genes are colored in red and green, respectively. (F) Bar chart showing quantification of the activated caspase3 (Cle-caspase3/Pro-caspase3) protein expression based on western blot. The activated caspase3 ratio in the iTBS group was lower than that in the Control and sham iTBS groups (*P < 0.05). (G) Bar chart showing the relative expression of Cle-caspase3 (CC3+) in NeuN+ neurons in the regions rostral and caudal to the injury site (****P < 0.0001). (H) The network diagram showing the association of enriched genes in iTBS group compared with sham iTBS group in six different functional sets, including neuronal activation, apoptosis, inflammation, microglia/macrophage, astrocyte and neuron type. The node size of the function set represents the total number of candidate genes belonging to gene ontology. Cell nuclei were counterstained with Hoechst33342 (Hoe). Data are presented as mean ± SEM (n = 5). One-way ANOVA with a least significant difference (LSD)-t (equal variance assumed) or Dunnett’s T3 (equal variance not assumed) was performed. Scale bars =1000μm in (A) - (C); 50μm in (A1) - (C3).
Figure 4

C3-2) The GFAP+ cells in the caudal injury region. Arrows indicate GFAP+ cells. (D) Bar chart showing the number of ATF3+ cells in the regions rostral and caudal to the injury site (**P < 0.0001, *P < 0.01). (E) Bar chart showing the relative density of GFAP+ cells in the regions rostral and caudal to/in the injury site (**P < 0.01, *P < 0.05). (F) Heatmap constructed from 56 significantly differentially expressed genes related to types of neurons and astrocytes in the rostral region to the injury area of spinal cord. In clustering analysis, up-regulated and down-regulated genes are colored in red and green, respectively. (G) Each point in the scatter plot represents a gene. The abscissa represents the gene expression levels in the sham iTBS group, and the ordinate represents the gene expression levels in the iTBS group. Therefore, the genes significantly up-regulated in the iTBS group compared with the sham iTBS group are shown in red, including Ccl21, Wnt1, Cyp26c1, and Scn5a; the genes significantly down-regulated in the iTBS group compared with the sham iTBS group are shown in green, including Slc22a6, Lst1, S100a8, S100a9 and Col3a1, and the gray dots represent genes with no significant difference. Cell nuclei were counterstained with Hoechst33342 (Hoe). Data are presented as mean ± SEM (n = 5). One-way ANOVA with a least significant difference (LSD)-t (equal variance assumed) or Dunnett’s T3 (equal variance not assumed) was performed. Scale bars =1000μm in (A) - (C); 50μm in (A1) - (C3).
**Figure 5**

(A2-2, B2-2, C2-2) The NF+ nerve fibers in the injury site. (A3-2, B3-2, C3-2) The NF+ nerve fibers in the caudal injury region. Arrows indicate NF+ nerve fibers. (D) Bar chart showing the area of GAP43+ nerve fibers in the regions rostral and caudal to/in the injury site (****P < 0.0001, **P < 0.01). (E) Bar chart showing the area of NF+ nerve fibers in the regions rostral and caudal to/in the injury site. Cell nuclei were counterstained with Hoechst33342 (Hoe). Data are presented as mean ± SEM (n = 5). One-way ANOVA with a least significant difference (LSD)-t (equal variance assumed) or Dunnett’s T3 (equal variance not assumed) was performed. Scale bars =1000μm in (A) - (C); 50μm in (A1) - (C3).
Transcranial iTBS promotes nerve fiber regeneration and synaptic plasticity in the injury site of spinal cord and improves motor function of paralyzed hindlimbs. (A) Western blot for PSD95, SYN, and β-actin in the rostral injury area. (B) Western blot for NF and β-actin in the rostral injury region. (C) Western blot for GAP43 and GAPDH in the rostral injury region. (D) Bar chart showing quantification of the protein expression based on western blot. Compared to the Control and sham iTBS groups, the levels of GAP43, NF, SYN, and PSD95 were increased in the iTBS group (****P < 0.0001, **P < 0.01). (E) The gene ontology enrichment bubble diagram represents the expression levels of different gene sets in the iTBS group compared with the sham iTBS group. The abscissa represents the Rich factor (the ratio of the total number of candidate genes to the total number of all genes belonging to this gene ontology). The larger is the Rich factor, and the greater is the degree of enrichment. The ordinate represents different function sets and the color represents -Log10 (P-value). (F) The node size of the function set represents the total number of candidate genes belonging to gene ontology; individual nodes between function sets represents individual genes; and the color represents Log2FC value. (G) Grid climbing test and BBB assessment were performed in the Control, sham iTBS and iTBS groups after eight weeks. (H) Bar charts of the number of climb steps, showing more climb steps in the iTBS group compared with the Control (***P < 0.001) and sham iTBS (**P < 0.01) groups. (I) Comparison of BBB score for hindlimb locomotor function in the Control, sham iTBS and iTBS groups. (&, ✶ indicate statistical significance when compared with the Control and sham iTBS groups, respectively, P < 0.05; && indicates P < 0.01; *** indicates P < 0.001). (J) MEPs were obtained by electrophysiological analysis in the Control, sham iTBS and iTBS groups eight weeks after SCI. (K) Bar charts of the MEP amplitude, showing higher amplitudes in the iTBS group compared with the Control and sham iTBS groups (****P < 0.0001). Data are presented as mean ± SEM (n = 5). One-way ANOVA with a least significant difference (LSD)-t (equal variance assumed) or Dunnett’s T3 (equal variance not assumed) was performed.
Figure 7

Transcranial iTBS promotes regeneration of CST nerve fibers and 5-HT positive nerve fibers. (A-C) Lower magnification displaying sagittal spinal cord sections labeled by BDA. (A1, B1, C1) Representative images showing the anterograde nerve fibers labeled with BDA (red, arrowheads) in the rostral injury region. (A2, B2, C2) Representative images showing anterograde nerve fibers labeled with BDA in the injury site. (A3, B3, C3) Representative images showing anterograde nerve fibers labeled with BDA in the caudal injury region.
region. (D-F) Low magnification of sagittal spinal cord sections showing the expression of 5-HT. (D1, E1, F1) The 5-HT+ nerve fibers in the rostral injury region. (D2, E2, F2) 5-HT+ nerve fibers in the injury site. (D3, E3, F3) 5-HT+ nerve fibers in the caudal injury region. Arrows indicate 5-HT+ nerve fibers. Inserts in the lower left corner of the images are the magnification of the area indicated by the arrow. (G) Bar chart showing the number of BDA+ nerve fibers in the regions rostral and caudal to/in the injury site (** **P < 0.0001). Data are presented as mean ± SEM (n = 3). (H) Bar chart showing the number of 5-HT+ nerve fibers in the regions rostral and caudal to/in the injury site (** **P < 0.0001, ***P < 0.001, **P < 0.01, *P < 0.05). Data are presented as mean ± SEM (n= 5). (I) Line chart showing the linear relationship between the number of total BDA+ nerve fibers and BBB score (r = 0.9604, **P < 0.01). (J) Line chart showing the linear relationship between the number of total 5-HT+ nerve fibers and BBB score (r = 0.9567, *P < 0.05). Cell nuclei were counterstained with Hoechst33342 (Hoe). One-way ANOVA with a least significant difference (LSD)-t (equal variance assumed) or Dunnett’s T3 (equal variance not assumed) was performed. Scale bars =2000μm in (A) - (F); 50μm in (A1) - (C3); 25μm in (D1) - (F3).
Figure 8

Transcranial iTBS promotes regeneration of LDPT. (A-C) Lower magnification displaying sagittal spinal cord sections labeled by BDA. (A1, B1, C1) Representative images showing the LDPT labeled with BDA (red, white arrows) in the rostral injury region. (A2, B2, C2) Representative images showing the LDPT labeled with BDA in the injury site. (A3, B3, C3) Representative images showing the LDPT labeled with BDA in the caudal injury region. (D) Experimental paradigms illustrating the timelines of the major
experimental manipulations. (E) A schematic diagram of reconstructing descending motor neural pathways. The numbers represent neural pathways including: CST–LDPT pathway; CST–5-HT–LDPT pathway; CST–5-HT pathway. Cell nuclei were counterstained with Hoechst33342 (Hoe). Scale bars = 2000 μm in (A) – (C); 50 μm in (A1) – (C3).

**Supplementary Files**

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