Nerve root magnetic stimulation improves locomotor function following spinal cord injury with electrophysiological improvements and cortical synaptic reconstruction

From the Contents

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Graphical Abstract *Nerve root magnetic stimulation promotes electrophysiological restoration and cortical synaptic reconstruction following spinal cord injury*
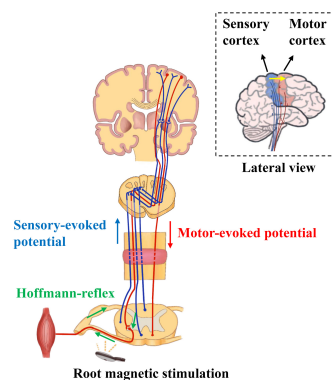
Abstract

Following a spinal cord injury, there are usually a number of neural pathways that remain intact in the spinal cord. These residual nerve fibers are important, as they could be used to reconstruct the neural circuits that enable motor function. Our group previously designed a novel magnetic stimulation protocol, targeting the motor cortex and the spinal nerve roots, that led to significant improvements in locomotor function in patients with a chronic incomplete spinal cord injury. Here, we investigated how nerve root magnetic stimulation contributes to improved locomotor function using a rat model of spinal cord injury. Rats underwent surgery to clamp the spinal cord at T10; three days later, the rats were treated with repetitive magnetic stimulation (5 Hz, 25 pulses/train, 20 pulse trains) targeting the nerve roots at the L5–L6 vertebrae. The treatment was repeated five times a week over a period of three weeks. We found that the nerve root magnetic stimulation improved the locomotor function and enhanced nerve conduction in the injured spinal cord. In addition, the nerve root magnetic stimulation promoted the recovery of synaptic ultrastructure in the sensorimotor cortex. Overall, the results suggest that nerve root magnetic stimulation may be an effective, noninvasive method for mobilizing the residual spinal cord pathways to promote the recovery of locomotor function.

Key Words: evoked potentials; H-reflex; motor activity; nerve conduction; neural plasticity; rehabilitation; sensorimotor cortex; spinal cord injury; synapses; transcranial magnetic stimulation

Introduction

Spinal cord injury (SCI) is a serious disorder of the central nervous system (CNS) (Hu et al., 2020; Sugeno et al., 2020; Zhang et al., 2020) caused by damage to the nerves that run through the spinal canal (Yao et al., 2021). Following SCI, output signals from the upper motor neurons terminate at the proximal end of the nerve injury site, thus interrupting the neural conduction that controls movement (O'Shea et al., 2017). SCI can be classified as either complete or incomplete, depending on the severity of the damage (Marino et al., 2003). Approximately 42% of patients with SCI are clinically diagnosed as having a complete SCI; however, an autopsy study showed that the diagnosis could be confirmed in just 14.3% of cases (Kakulas, 2004), thus indicating that the majority of patients have an incomplete SCI with some intact nerve fibers (Weidner et al., 2001; Kaegi et al., 2002; Rosenzweig et al., 2010). It has been suggested that certain

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interventions could enhance the neural plasticity of these remaining nerve fibers, and that this could rehabilitate neural conduction and function (Iarikov et al., 2007; Serradj et al., 2017; Khorasanizadeh et al., 2019). This is of much clinical importance, because SCI continues to be associated with high rates of disability, despite widespread efforts to treat the disorder (Katoh et al., 2019; Liau et al., 2020).

In recent years, noninvasive magnetic stimulation has become an effective therapeutic intervention in the neuropsychiatric field (Concerto et al., 2015; Lanza et al., 2018; Wessel and Hummel, 2018; Staudt et al., 2019). Recent studies have shown that it can also be used for neural rehabilitation following SCI (Ganzer et al., 2018; Wagner, 2018; Elmgreen et al., 2019). The technique involves selecting stimulation targets; when these are located in the cortex, transcranial magnetic stimulation (TMS) is typically used (Barker et al., 1985). A number of studies have demonstrated that patients with SCI have improved motor function following TMS of the motor cortex (Sato et al., 2018; Guo et al., 2020). However, for a more optimal recovery, the sensorimotor neural circuits in the spinal cord would need to be reconstructed. This would require more than the stimulation of the motor cortex, which can only excite the descending corticospinal tract. Our team therefore developed a neural circuit-magnetic stimulation protocol, which involves stimulating both the motor cortex and the nerve root of the target muscle group, to fully activate the residual intact nerve fibers. In our preliminary study, subjects with chronic incomplete SCI underwent four weeks of treatment using intermittent theta-burst stimulation of the right motor cortex combined with bilateral nerve root stimulation. The patients showed significant improvements in lower limb motor function as well as nerve conduction in the corticospinal tract.

To further evaluate the efficacy of our novel approach for treating SCI, we used a SCI rat model to explore the effects of nerve root magnetic stimulation (NRMS) on motor function, nerve conduction, and the synaptic ultrastructure of the sensorimotor pathway.

Materials and Methods

Animals
The experiments were approved by the Animal Ethics Committee of Tongji Hospital Affiliated to Tongji University School of Medicine on August 31, 2019 (approval No. 2019-DW-(036)). The experimental procedures followed the United States National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) and strictly complied with the guidelines of Animal Research: Reporting of *In Vivo* Experiments (Percie du Sert et al., 2020).

The study was carried out on 45 adult male Sprague-Dawley (SD) rats (specific-pathogen-free, weighing 200–220 g, 2–3 months old), which were obtained from Shanghai Jiesijie Experimental Animal Farm (license No. SCXK (Hu) 2018-0004). The sample size (*n* = 45) was determined using G*Power 3.1 software for a two-way analysis of variance with α = 0.05 and β = 0.95 (Dattalo, 2009; Ko and Lim, 2021).

The 45 rats were divided into three equal-sized groups using a random number table (*n* = 15 in each group): (1) sham operation + sham stimulation (sham + SS), (2) SCI + sham stimulation (SCI + SS), and (3) SCI + nerve root magnetic stimulation (SCI + NRMS). All of the rats were kept at a constant temperature of 25°C with a 12-hour light-dark cycle. Food and water were well supplied ad libitum. The rats were preadapted for one week prior to SCI surgery.

Rat spinal cord injury model
The 30 rats in the SCI + SS and SCI + NRMS groups underwent clip compression using a method developed by Rivlin and Tator (Rivlin and Tator, 1978) for the rat SCI model. The rats underwent preoperative fasting and water deprivation for at least 6 hours. They were then anesthetized by an intraperitoneal injection with 1% pentobarbital sodium (4 mL/kg; Sigma-Aldrich, St. Louis, MO, USA), placed on a bench in the prone position, and shaved. An incision was made, and the muscles were separated; a laminectomy was performed at the T9–T11 level and the spinal cord was exposed at the T10 level. An aneurysm clip (50 g, Fine Science Tools, Heidelberg, Germany) was placed so that the lower blade passed extradurally and completely around the spinal cord and nerve roots at the T10 vertebra. This was then rapidly released from the applicator, producing a bilateral impact force and sustained dorsal-ventral compression. This was maintained for 15 seconds before the clip was removed, resulting

in a sudden, violent convulsion of the hindlimbs and tail swing, which indicated the success of the SCI model. The rats from the sham + SS group underwent the same laminectomy but without the aneurysm clip compression. For all of the rats, the muscle and skin incisions were closed, they were injected with normal saline solution according to the intraoperative blood loss, and they were placed on a heating pad until fully awake. Early postoperative care included keeping the rats singly in cages with enough food and water, a daily intraperitoneal injection of penicillin (200,000 IU/d) for one week to prevent infection, and an abdominal massage to induce micturition twice a day until the recovery of autonomous urination.
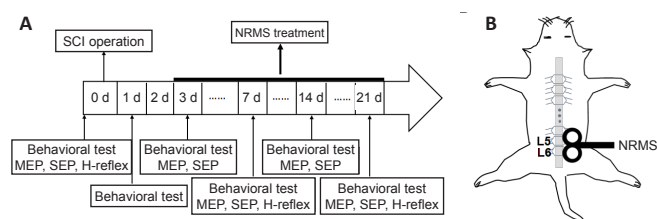
Nerve root magnetic stimulation treatment
We started NRMS treatment on day three after the SCI surgery, when the blood-borne monocytes had started to infiltrate the spinal cord to decrease the apoptosis of neurons (Kjell and Olson, 2016). We used the MagPro R30 magnetic stimulator (MagVenture Co., Farum, Denmark) with a 25 mm, figure-of-eight, custom rodent coil. The rats were treated in the prone position, in a holder made of plastic resin. The stimulation sites were the nerve roots at the L5–L6 lumbar segment, which target the gastrocnemius (GAS) muscles, on both paravertebral sides. The correct location for the NRMS was identified using palpation along with the anatomical landmarks (e.g., the anterior superior iliac spine).

To determine the NRMS stimulation intensity, it was first necessary to ensure that motor-evoked potentials (MEPs) were elicited. For this, the coil was held over the motor cortex, and the stimulation intensity was gradually increased from zero; recordings from the right GAS muscle were observed on a real-time digital oscilloscope so that MEPs could be detected. If none were observed at low intensities, the position of the magnetic coil was adjusted by a few millimeters, and the procedure was repeated. When the optimal position had been found, the resting motor threshold (rMT) was determined, which is the lowest stimulation intensity that induces at least three MEPs of similar amplitude (~100 μV) for five consecutive, single TMS pulses (Rossini et al., 1994). For the treatment, the NRMS was delivered in a series of 20 pulse trains, each containing 25 pulses at a rate of 5 Hz and a stimulus intensity of 100% rMT (500 pulses in total). For the SCI + NRMS group, the rats were treated five times a week for 3 weeks; the other two groups received sham stimulation, which involved treatment with the coil placed perpendicular to the spine, thus giving the same level of sound stimulation. The NRMS treatment was always run between 6 p.m. and 8 p.m.

Evaluation of locomotor function
The rats' locomotor function was assessed at different time points using the Basso-Beattie-Bresnahan (BBB) scale (Basso et al., 1996), the inclined plane test (Duan et al., 2018), the rotarod test (Sauer et al., 2017), and the modified Tarlov score (Jiang et al., 2016). These were run the day before surgery and on days 1, 3, 7, 14, and 21 after surgery (**Figure 1**). To enable the rats to adapt to the tests, they were run five times before the official tests took place. Each measure was blindly and independently assessed by two observers, and the average scores were recorded for each rat. All of the tests were run on all 15 rats in each group.

Figure 1 | Study timeline and model of the root magnetic stimulation (NRMS) treatment.

(A) The timeline shows the overall study schedule. NRMS treatment began on the third day after the SCI operation. Behavioral tests were run to evaluate the recovery of motor function on days 1, 3, 7, 14, and 21 post-surgery. Electrophysiological measures (MEP, SEP, and H-reflex) were obtained to assess nerve conduction on days 3, 7, 14, and 21 following SCI. (B) The diagram displays the stimulation site and the coil used for the NRMS treatment. The rat was placed in the prone position, and the nerve roots were stimulated at the L5–L6 level on both sides of the intervertebral foramen with a figure-of-eight rodent coil to activate the gastrocnemius muscle. H-reflex: Hoffmann reflex; MEP: motor-evoked potential; NRMS: nerve root magnetic stimulation; SCI: spinal cord injury; SEP: sensory-evoked potential.

Basso-Beattie-Bresnahan locomotor rating scale

Two rats were placed in an open field (2 m in diameter) and were free to move around for 5 minutes. The experimenters observed each rat's hindlimb locomotor function, including the joint movements, coordination, paw placement, and toe clearance. Each hindlimb was given a score ranging from 0 to 21, and the average for both limbs was calculated for each rat. A score of zero indicated complete paralysis without any hindlimb movement, whereas a score of 21 indicated unimpaired locomotion, as observed in normal, uninjured rats.

Inclined plane test

The rats were placed on a smooth slanting board with freely adjustable angles, with their heads facing the upper end of the board. The angle was gradually increased in 5° steps until the rat could no longer remain stable for five seconds. The test was repeated three times and the average inclined angle was recorded.

Rotarod test

The rats were placed on a rotating rod in a rotarod apparatus (Shanghai Xinruan, Shanghai, China), with a rotation speed of 20 r/min. The experimenters recorded the length of time that the rats were able to remain on the rod. This was repeated four times for each rat, with a 10-minute interval between the tests, and the average on-rod time was calculated.

Modified Tarlov scoring system

The rats were given a modified Tarlov score based on a six-point scale (0–5): 0) complete paralysis of both hindlimbs without any function; 1) the hindlimbs can move slightly without bearing weight; 2) the hindlimbs can move freely without bearing weight; 3) the hindlimbs can support enough weight to walk a few steps; 4) the rat can walk with a slight impairment; 5) the rat walks normally.

Neuroelectrophysiological measurements

Neuroelectrophysiological tests were carried out to assess nerve conduction in the injured spinal cord following NRMS treatment. These were carried out before the SCI surgery, and at 1, 2, and 3 weeks after the surgery. For these tests, the rats underwent inhalation anesthesia with 5% isoflurane followed by a steady level of 2% isoflurane in 97–98% O_2, administered via a nose cone. The rats were placed horizontally in the prone position, and needle electrodes in the Keypoint 4-evoked Potential System (Beijing Weidi Kangtai Medical Instrument Co., Ltd., Beijing, China) were used to measure the MEPs, somatosensory-evoked potentials (SEP), and the Hoffmann reflex (H-reflex). These disposable subdermal needle electrodes were inserted into the hindlimbs, cortex, and tail, and acted as the stimulating, recording, reference, and ground electrodes. The interelectrode impedances were kept ≤ 3 kΩ. Each test was performed on both sides of the rat's body and the average values were calculated.

Motor-evoked potential

The stimulating electrode was inserted under the skull into the motor cortex, 2 mm anterior to the coronal suture and 2 mm lateral to the sagittal suture. Direct square-wave electrical pulses from the electrode stimulated the motor cortex to elicit slight hindlimb tics. The pulse intensity was 32 mA, the width was 0.1 ms, the frequency was 1 Hz, the sensitivity was 2 mV/D, and the scanning speed was 2 ms/D. Muscle compound action potentials were recorded in the middle of the GAS muscle in each hindlimb. The reference electrode was inserted into the Achilles tendon, and the grounding electrode was placed under the skin of the tail. The time delay between the start of the electrical pulse and the onset of the MEP response was referred to as the onset latency. The amplitude of the MEP was taken as the height of the wave from the peak to the trough and was measured for five rats in each group.

Sensory-evoked potential

A stimulating electrode was used to stimulate the hindlimb tibial nerve, and a recording electrode was placed under the skull at the somatosensory cortical area for the hindlimbs, at the intersection of the coronal and sagittal sutures. A reference electrode was placed 0.5 cm posterior to the recording electrode. Slight tics of the hindlimb indicated that the stimulating electrode had been correctly inserted. The current intensity was 1.5 mA, the pulse width was 0.1 ms, the frequency was 1.5 Hz, the sensitivity was 2 mV/D, the scanning speed was 2 ms/D, the filter was 10–3000 Hz, and the waveform was superimposed 50 times. Four rats were tested from each group, and the SEP latency and amplitude were recorded.

H-reflex

For the H-reflex, the hindlimb tibial nerve was stimulated and recordings were obtained in the second dorsal interosseous muscle of the hind paw, with the reference electrode placed in the muscle tendon, and the ground electrode placed subcutaneously near the base of the tail (Zhang et al., 2007). The electrical stimulus intensity was set so that the toes alone were stimulated; the current intensity was 0–0.5 mA, the pulse width was 0.5 ms, the frequency was 0.5 Hz, the sensitivity was 1 mV/D, the scanning speed was 2 ms/D, the filter was 10–10000 Hz, and the waveform was superimposed 50 times. The test was run on five rats from each group, and there were 10–15 recordings for each side. For the data analyses, the latency and amplitude of the H-reflex H-wave and M-wave were determined, and the H/M amplitude ratio was calculated.

Ultrastructure of the sensorimotor cerebral cortex

After three weeks of NRMS treatment, the rats were sacrificed under anesthesia. The brains were collected on an ice plate, the sensorimotor cortex was dissected, and the tissue was cut into 1 mm × 1 mm × 1 mm pieces. The samples were then fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 3–4 hours at 4°C, followed by post-fixation in 1% OsO4 in 0.1 M phosphate buffer in the dark for two hours at room temperature. After dehydration in graded ethanol and embedding in EMBed 812 resin, the samples were moved into a 65°C oven for polymerization for more than 48 hours, and then sliced into 60–80 nm thick slices using an ultramicrotome (Leica, Solms, Germany). The slices were double-stained using uranium acetate for eight minutes and then lead citrate for eight minutes; they were then photographed using a HT7800 transmission electron microscope (Hitachi Electronic Instruments, Tokyo, Japan). Ten non-overlapping tissue samples were photographed for each rat, and the synaptic ultrastructure was quantified using Image Pro Plus 6.0 software (Media Cybernetics, MD, USA). The synaptic curvature was measured using a method described by Jones (1993); the thickness of the post-synaptic density (PSD) and the length of the synaptic active zones were measured using a method described by Güldner and Ingham (1980); and the width of the synaptic cleft was determined using the multi-point averaging method. Each of these measures was obtained for four rats from each group, with a total of 40 tissue sample images per group.

Statistical analysis

Statistical analyses were conducted, and figures were generated using GraphPad Prism 7 software (GraphPad Software, San Diego, CA, USA). All of the data were expressed as the mean ± standard error of the mean (SEM) and analyzed using unpaired t-tests, one-way analysis of variance, or two-way analysis of variance, followed by Tukey's *post hoc* tests. A level of $P < 0.05$ was considered to be statistically significant.

Results

NRMS improves locomotor function in SCI rats

All 45 SD rats completed the whole battery of behavioral tests assessing motor function. The scores on the tests prior to surgery did not differ significantly between the three groups ($P > 0.05$; **Figure 2**). On the first day post-surgery, all of the measures (the BBB score, the inclined plane angle, the on-rod time, and the modified Tarlov score) were significantly lower in the SCI + SS group and the SCI + NRMS group compared with the sham + SS group ($P < 0.001$; **Figure 2**). On the third day following SCI, there were no significant differences between the SCI + SS group and the SCI + NRMS group ($P > 0.05$; **Figure 2**); however, on the seventh day following SCI, the BBB score, the inclined plane angle, and the on-rod time were all significantly higher in the SCI + NRMS treatment group compared with the SCI + SS group ($P = 0.0036$, $P = 0.0019$, and $P = 0.0257$, respectively; **Figure 2**). By the end of the second and third weeks, remarkable group differences could be observed for all four tests, with the rats in the SCI + NRMS group having higher scores than the SCI + SS group ($P < 0.001$ for all tests; **Figure 2**). These results imply that NRMS treatment leads to improved recovery of locomotor function following SCI.

NRMS improves nerve conduction in SCI rats

To investigate whether NRMS can improve nerve conduction, we recorded MEPs, SEPs, and the H-reflex. These can be used to assess neuronal excitability and conduction within the spinal cord nerve tracts.

NRMS enhances nerve conduction in the sensory neural pathway

Prior to surgery, there were no significant differences between the three groups in terms of both the latency and amplitude of the SEP. On the third day following surgery, the two groups of SCI rats were found to have prolonged SEP latencies compared with the sham + SS group ($P < 0.001$; **Figure 3A**, **B**), whereas no significant differences were observed between the two SCI groups; there were no significant differences in the SEP amplitudes between the three groups ($P > 0.05$; **Figure 3A**, **C**). After the first week, the SEP latencies in the SCI rats gradually decreased, with a greater reduction seen in the NRMS-treated SCI rats (two-way analysis of variance with Tukey's *post-hoc* test: $P_{7\,d} = 0.004$, $P_{14\,d} = 0.0137$, $P_{21\,d} < 0.001$). However, there were no significant differences in the SEP amplitude between the two SCI groups during the study period ($P > 0.05$).

NRMS increases the excitability of the corticospinal tract

Prior to SCI surgery, there were no significant MEP latency differences between the three groups of rats. On the third day after surgery, the SCI rats had decreased MEP amplitudes and significantly prolonged MEP latencies compared with the rats in the sham + SS group (**Figure 4A–C**); there was no significant MEP latency difference between the SCI + NRMS and SCI + SS groups ($P > 0.05$). For the later time points, the NRMS treatment was found to attenuate the prolonged MEP latency (two-way analysis of variance with Tukey's *post hoc* tests: $P_{7\,d} = 0.0023$, $P_{14\,d} < 0.001$, $P_{21\,d} < 0.001$), thus indicating that it induces elevated excitability in the corticospinal tract.

NRMS improves spinal presynaptic inhibition

The H-reflex recordings (**Figure 5A**) revealed that there was a significantly longer H-waveform latency in the SCI + NRMS group compared with the SCI + SS group 1 week after surgery ($P < 0.01$; **Figure 5D**). For the H-waveform amplitude, although there were noticeable differences between the two groups, this was only significantly different at the end of the first week ($P < 0.01$; **Figure 5C**). For the H/M ratio, the percentage of excited alpha motor neurons responding to the electrical stimulation decreased noticeably in the SCI + NRMS group compared with the SCI + SS group on the seventh day after surgery ($P < 0.001$; **Figure 5B**), as well as on day 21 after surgery ($P < 0.001$; **Figure 5B**).

NRMS promotes recovery of the synaptic ultrastructure in the sensorimotor cortex

The synaptic ultrastructure in the sensorimotor cortex was examined to determine the effect of NRMS on structural plasticity in the sensorimotor neural pathways. In the SCI + SS group, we observed marked damage to the ultrastructure of the synapses, with a flat synaptic morphology (see triangle symbol in **Figure 6A**), fewer synaptic vesicles, and more vacuoles than in the sham + SS group. For the SCI + NRMS group, the synaptic structure was closer to normal compared with the SCI + SS group, thus suggesting that there had been a certain amount of recovery to restore the synaptic damage. The significant changes in synaptic ultrastructure that followed SCI included the thickness of the PSD, the length of the synaptic active zone, and the curvature of the synaptic interface ($P < 0.001$; **Figure 6C–E**), but not the width of the synaptic cleft ($P > 0.05$; **Figure 6B**). Importantly, the length of the synaptic active zone increased substantially with NRMS treatment ($P < 0.001$; **Figure 6D**).

Discussion

Damage to the spinal cord neural pathways following SCI leads to varying degrees of motor paralysis and sensory disturbance (Zijdewind and Thomas, 2003). In patients with incomplete SCI, the intact nerve fibers can enable partial spontaneous recovery of sensorimotor function through neural plasticity (Weidner et al., 2001; Kaegi et al., 2002; Rosenzweig et al., 2010), although this remains limited (Cafferty et al., 2008; Boulenguez and Vinay, 2009; Lovett-Barr et al., 2012). Studies have shown that TMS, a technique that was first introduced to activate the cerebral cortex (Barker et al., 1985), has the potential to increase the excitability of certain electrically conductive tissues and improve neural plasticity following CNS injury (Wagner and Valero-Cabre, 2007).
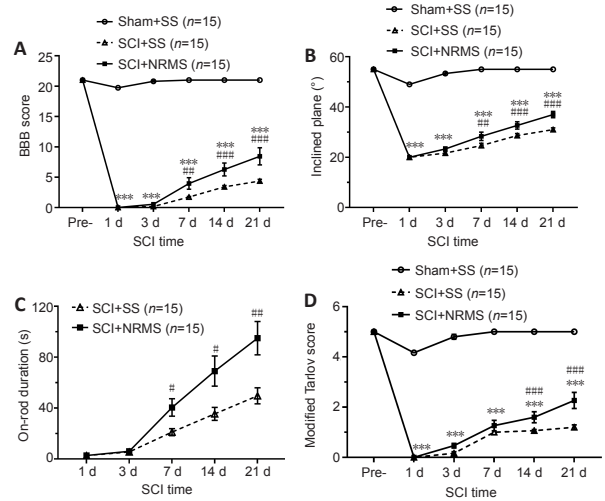
Figure 2 │ **Repetitive magnetic stimulation of the nerve root improves motor function in rats with spinal cord injury (SCI).**
(A–D) The rats' motor function was assessed using four behavioral tests: the Basso-Beattie-Bresnahan (BBB) locomotor rating score (A), the inclined plane test (B), the rotarod test (C), and the modified Tarlov scoring system (D). The line charts show impaired motor function in the SCI rats. With nerve root magnetic stimulation (NRMS) treatment following SCI, there were greater performance improvements over time compared with the sham stimulation. The data are presented as the mean ± SEM for each group ($n = 15$ rats in each group). For comparisons between the three groups, the continuous variables were analyzed using a two-way analysis of variance followed by Tukey's *post hoc* tests (A, B and D); for comparisons between two groups, the continuous variables were analyzed using an unpaired *t*-test at each time point (C). ***$P < 0.001$, *vs.* sham + SS group; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, *vs.* SCI + SS group. d: Day; pre-: pre-SCI; SCI: spinal cord injury.
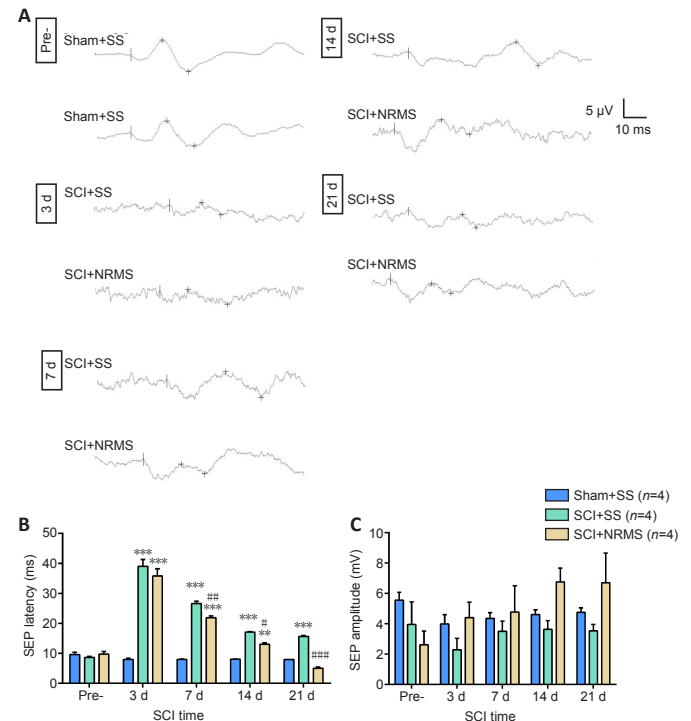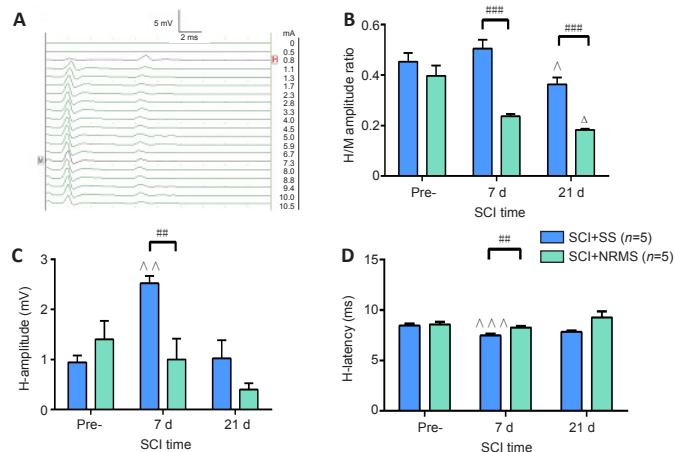
Figure 3 │ **Effects of nerve root magnetic stimulation (NRMS) on spinothalamic nerve conduction in the spinal cord injury (SCI) rats.**
(A) Typical SEP traces that show the differences between sham-operated rats, SCI sham-treated rats, and SCI NRMS-treated rats. The X-axis and Y-axis represent recording time and wave amplitude, respectively. (B) The bar graph shows a prolonged SEP latency in the SCI rats that showed a greater reduction over time with NRMS treatment. (C) No significant differences were found among the three groups at each time point. The mean ± SEM are shown; these are taken from recordings for four rats from each group at each time point. The comparisons used two-way analysis of variance followed by Tukey's *post hoc* tests. **$P < 0.01$, ***$P < 0.001$, *vs.* sham + SS group; #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$, *vs.* SCI groups. SEM: Standard error of the mean; SEP: somatosensory-evoked potential; pre-: pre-SCI; d: day.
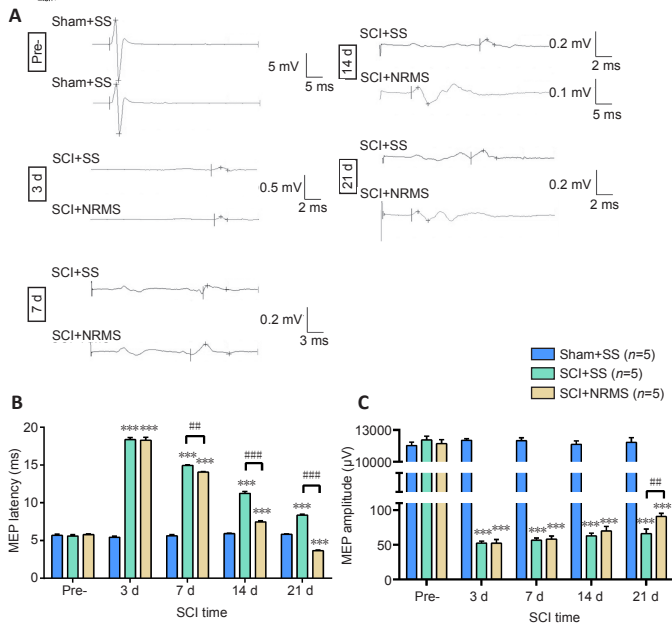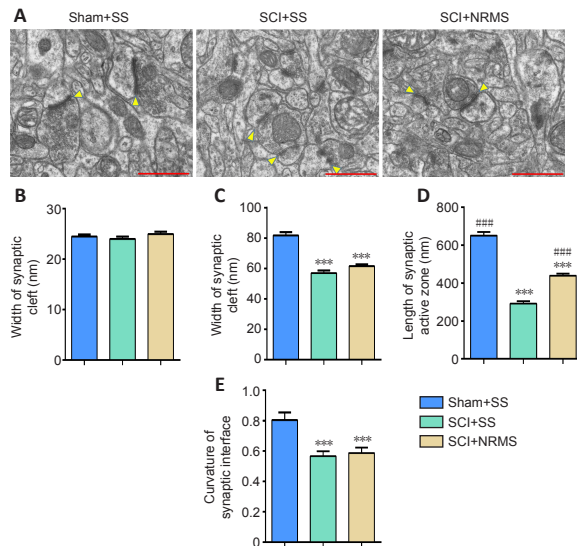
Figure 4 | Effects of nerve root magnetic stimulation (NRMS) on corticospinal nerve conduction in the injured spinal cord of rats.
(A) Representative MEP traces using electrical stimulation with the same intensity (32 mA) that show changes following SCI and NRMS treatment. The X-axis and Y-axis represent the recording time and wave amplitude, respectively. (B, C) The bar graph shows significantly longer onset latencies and decreased amplitudes in the SCI rats compared with the sham-operated rats following SCI. Over time, the SCI rats treated with NRMS had considerably shorter MEP latencies and increased amplitudes compared with the sham-stimulation group. The data are presented as the mean ± SEM, based on recordings for five rats from each group. The groups were compared using two-way analysis of variance followed by Tukey's *post hoc* tests. ***$P < 0.001$, *vs.* Sham + SS group; ##$P < 0.01$, ###$P < 0.001$, *vs.* two SCI groups. MEP: Motor-evoked potential; pre-: pre-SCI; SCI: spinal cord injury; SEM: standard error of the mean.

Figure 5 | Effects of repetitive magnetic stimulation of the nerve root on peripheral nerve conduction in the injured spinal cord of rats.
(A) Examples of the H-reflex waveforms. The X-axis and Y-axis represent the recording time and wave amplitude, respectively. (B) The bar graph of the H/M amplitude ratio shows a slight increase after surgery due to the increase in the H-amplitude (C). There was significant difference in the H/M amplitude ratio for the nerve root magnetic stimulation (NRMS)-treated rats and the untreated rats (SCI + SS group) on days 7 and 21; there were also significant differences in the H-latency (D) on day 7. The data are presented as the mean ± standard error of the mean (SEM), with $n = 5$ for each group. Statistical analyses were performed using a two-way analysis of variance followed by Tukey's *post hoc* tests. ^$P < 0.05$, ^^$P < 0.01$, ^^^$P < 0.001$, *vs.* for comparisons with pre-SCI in the SCI + SS group; Δ$P < 0.05$, *vs.* pre-SCI in the SCI + NRMS group; ##$P < 0.01$, ###$P < 0.001$, *vs.* SCI + SS group and the SCI + NRMS group on the same day. H: H-wave; pre-: pre-SCI; NRMS: nerve root magnetic stimulation; SCI: spinal cord injury.

Figure 6 | Effects of nerve root magnetic stimulation (NRMS) on the ultrastructure of synapses in the sensorimotor cortex of rats with spinal cord injury (SCI).
(A) Typical images of the ultrastructure of the sensorimotor cortex for each group of rats (transmission electron microscope, × 15 000). The yellow arrows show representative synapses for each group. Scale bar: 1 μm. (B–E) Bar graphs for four synaptic structure measurements show that SCI leads to considerable synaptic ultrastructure damage in the sensorimotor cortex, affecting the thickness of the PSD, the length of the synaptic active zone, and the curvature of the synaptic interface, but not the width of the synaptic cleft. The NRMS treatment improved the synaptic ultrastructure in terms of the length of the synaptic active zone. The mean ± SEM are shown for each group ($n = 40$ images for each group). Statistical analyses were performed using a one-way analysis of variance followed by Tukey's *post hoc* tests. ***$P < 0.001$, *vs.* sham + SS group; ###$P < 0.001$, *vs.* SCI + SS group. PSD: Post-synaptic density; SEM: standard error of the mean.

The present study used NRMS, a novel approach that aims to stimulate the sensory tract to improve motor function. We were able to demonstrate the following: (1) treatment using repetitive magnetic stimulation of the spinal cord nerve root induces functional recovery following SCI; (2) NRMS leads to changes in the excitability of the sensorimotor pathway and improves inhibition in spinal pathways; (3) NRMS promotes the recovery of synaptic ultrastructure in the sensorimotor cortex; and (4) NRMS can activate the ascending sensory pathways leading to an increase in the corticospinal output and motor function improvement, using the lowest level of the high-frequency magnetic stimulation settings (5 Hz). It can therefore be seen that repetitive high-frequency NRMS has considerable potential for the treatment of SCI and could be used in conjunction with TMS and skilled motor training.

It is widely accepted that the primary motor cortex plays a critical role in the flexible control of spinal circuits during sensorimotor learning (Lemon, 2008). However, while cortical activation can potentially excite the descending corticospinal tract, this is not the case for the ascending sensory tract; thus, for functional recovery from SCI, TMS alone cannot activate the sensory tract that can contribute toward improved motor function. Our team therefore designed a novel neural circuit-magnetic stimulation (NC-MS) protocol that includes two stimulation targets: the motor cortex and the spinal nerve roots (**Additional file 1**). Our NC-MS protocol was inspired by work on paired associative stimulation (PAS), which involves spike-timing-dependent plasticity (Song et al., 2000; Urbin et al., 2017; Bunday et al., 2018) that modifies the synaptic efficiency in accordance with Hebbian theory (Hebb, 1949). Our NC-MS protocol may involve similar neural mechanisms to PAS (Stefan et al., 2000; Stefan et al., 2002), and initial results have shown that it effectively improves the recovery of motor function in the lower extremities of both SCI patients and rats (Mao et al., 2019; Zhao et al., 2020). The present study demonstrated the efficacy of NRMS alone on SCI functional recovery using the lowest level of high frequency magnetic stimulation (5 Hz).

The behavioral tests showed that the locomotor function of SCI rats improved following NRMS treatment. The motor recovery was first

assessed using the BBB scale (Basso et al., 1996), which has been extensively used to evaluate motor function in SCI rats. Each score (0–21) represents a combination of movements, which accurately reflects the degree of functional motor recovery during rehabilitation, with higher scores reflecting better motor function. The results showed a significant increase in BBB scores in the NRMS-treated rats compared with the sham-stimulated rats, with improvements seen in the hindlimb joint movements, coordination, paw placement, and toe clearance. Other tests also showed NRMS-related improvements in the hindlimb grasping ability, specifically the inclined plane test and the rotarod test. The latter test also reflects the rats' balance, which also improved with the NRMS treatment. However, the modified Tarlov score did not differ significantly between the NRMS treatment and sham treatment groups on the seventh day post-surgery; this may relate to the lower sensitivity of the modified Tarlov score rating system compared with the BBB scoring system, because each score represents a wide range of movements. Despite this disadvantage, the modified Tarlov score nevertheless provides an overview of hindlimb movement restoration. Taken together, the four behavioral tests provide a comprehensive evaluation of hindlimb motor function, and show improvements following NRMS in SCI rats.

To test our hypothesis that there are changes due to neuroplasticity with NRMS treatment, a series of electrophysiology experiments were conducted. The first measure was SEP, which was used to examine changes in the excitability of the ascending sensory pathway by determining the time it took for a signal to pass from the stimulating electrode to the sensory cortex. We found that NRMS treatment shortened the SEP latency in the SCI rats. As this measure reflects the nerve conduction velocity, the integrity of peripheral nerve fibers, and the functioning of the sensory system pathway, this result supports the notion that NRMS enhances nerve conduction and elevates the excitability of the sensory pathway. We also measured the SEP amplitude, a sensitive measure that can be used to indicate the severity of spinal cord neurological damage (Petersen and Crochet, 2013; Toledo et al., 2016; Sakmann, 2017). However, no significant differences were found between the three groups. This may be because the SEP is subject to a variety of factors, including mechanical, local ischemic, and physiological (age, height, limb length, etc.) factors. In addition, hypotension, decreased erythrocyte volume, hypothermia, and anesthetic drugs can all weaken the SEP.

To determine the effect of NRMS on corticospinal plasticity, we recorded the MEP. This reflects the transmission of signals along the motor nerves, from the cerebral cortex to the muscles, thus providing an evaluation of the overall synchronization and integrity of the conduction pathway (Ng et al., 2018). Unlike the SEP, the MEP is not affected by body temperature and blood volume changes, and in intraoperative electrophysiological monitoring, the response time for changes is 5 minutes shorter than for the SEP (Harel, 2017). We found that NRMS treatment reduced the MEP latency in the SCI rats. This suggests that NRMS increases the excitability of motor neural circuits and improves neural conduction in the corticospinal tract, thereby enhancing corticospinal plasticity. Interestingly, after three weeks of NRMS treatment, the MEP latency became shorter than in rats with the sham operation. This may relate to the fact that the electrophysiological tests were conducted after the NRMS treatment, with the MEP latency reflecting the excitability of the motor neural pathway at that moment. The reduced MEP latency following NRMS treatment indicated enhanced synaptic transmission and synaptic strength (long-term potentiation, LTP) due to the continuous high-frequency synaptic activity (Zhang et al., 2015; Shang et al., 2016). As LTP can last for hours or even days, it may underlie long-lasting functional remodeling and has potential implications for neurorehabilitation (Cirillo et al., 2017; Di Lorenzo et al., 2020; Jo and Perez, 2020).

The H-reflex, a single synapse reflex of the spinal cord, was also examined to evaluate the strength and distribution of the stimulus input from group Ia sensory fibers in the muscle spindles to the motor neuron pool in the anterior horn of the spinal cord. The maximum amplitude of the H-wave reflects the number of alpha motor neurons that are mobilized. After CNS injury in adults, more muscles are found to elicit the H-reflex, and the reflex can be larger than usual, thus implying that more motor neurons are activated in the spinal cord. The results of our study showed that in the SCI rats with sham stimulation the H-wave amplitudes increased while the latencies decreased a week following SCI; this was not found for the NRMS-treated rats, where smaller amplitudes and longer latencies were seen. This reveals that NRMS treatment leads to a more normal H-reflex earlier on, with fewer activated alpha motor neurons. We found that with time, the amplitude and latency of the H-reflex gradually returned to normal for both SCI groups, and that this related to the extent of injury to the spinal cord. Another measure that was considered was the H/M ratio, which indicates the excitability of alpha motor neurons in the anterior horn of the spinal cord. This was found to be smaller in the SCI rats with NRMS treatment, thus suggesting that NRMS may improve movement at least partially through the resynaptic inhibition of afferent transmission.

Synaptic plasticity affects both structure and function and is the basis for neurological recovery. It is influenced by a variety of factors. In the case of SCI, there is substantial reorganization in both the sensory and motor cortices, especially the somatosensory cortex (Chand and Jain, 2015) and the primary motor cortex (Oudega and Perez, 2012). There is mounting evidence that enhancing the afferent input can strengthen sensory-motor connections; this is of great importance for functional recovery following SCI (Edgerton et al., 2008; Sonksen and Hillier, 2010; Harkema et al., 2011). In our study, we investigated whether the sensory input induced by NRMS can affect the structural plasticity of synapses in the sensorimotor cortex. Using a transmission electron microscope, we observed marked damage to the cortical synaptic ultrastructure in SCI rats, which was at least partially restored following NRMS treatment. This finding is important, as synaptic ultrastructure changes reflect synaptic plasticity and can indicate neurotransmitter release and synaptic transmission efficiency (Weeks et al., 2000). An in-depth analysis of the synaptic ultrastructure revealed that the thickness of the PSD, the length of the synaptic active zone, and the curvature of the synaptic interface all decreased following SCI, but not the width of the synaptic cleft. Notably, the NRMS treatment significantly increased the length of the synaptic active zone, which represents the area enabling synaptic transmission. We hypothesize that this enlarged synaptic area enhances synaptic plasticity in the sensory-motor cortex, which may underlie a compensatory mechanism induced by NRMS.

The limitations of the present study include the lack of data concerning the long-term effectiveness of NRMS and the molecular mechanisms that underlie the NRMS-induced neuroplasticity. Despite this, the findings provide preliminary support for the rationality and validity of our NC-MS protocol.

In conclusion, this study demonstrates that NRMS can increase the neural activity in the ascending sensory pathway and the descending motor pathway; it can also improve the presynaptic inhibition of the spinal pathways in SCI rats. The changes in nerve conduction and synaptic ultrastructure are thought to relate to the improved motor performance, and so may contribute to improved quality of life following SCI. The effectiveness of the NRMS treatment may result from the cortical integration of the ascending sensory inputs and the strengthening of corticospinal connections.

As a final note, we have been able to confirm the long-term therapeutic effects of NRMS in a preliminary study on patients: the improvements in motor function were found to persist for half a year in all of the patients. However, most patients (more than 90%) were still unable to walk backwards a year after the treatment. These results will be published separately and will improve our understanding of how targeted neuromodulation techniques can increase neuroplasticity in neurological disorders.

Additional file:
Additional file 1: Novel neural circuit-magnetic stimulation (NC-MS) mode.

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