Repellent transcranial magnetic stimulation promotes neurological functional recovery in rats with traumatic brain injury by upregulating synaptic plasticity-related proteins

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From the Contents

Abstract

Studies have shown that repetitive transcranial magnetic stimulation (rTMS) can enhance synaptic plasticity and improve neurological function. However, the mechanism through which rTMS can improve moderate traumatic brain injury remains poorly understood. In this study, we established rat models of moderate traumatic brain injury using Feeney’s weight-dropping method and treated them using rTMS. To help determine the mechanism of action, we measured levels of several important brain activity-related proteins and their mRNA. On the injured side of the brain, we found that rTMS increased the protein levels and mRNA expression of brain-derived neurotrophic factor (BDNF), trkB, N-methyl-D-aspartic acid (NMDAR), cAMP response element binding protein (CREB), and synaptophysin. rTMS also partially reversed the loss of synaptophysin after injury and promoted the remodeling of synaptic ultrastructure. These findings suggest that upregulation of synaptic plasticity-related protein expression is the mechanism through which rTMS promotes neurological function recovery after moderate traumatic brain injury.

Key Words: brain-derived neurotrophic factor; moderate traumatic brain injury; neurological dysfunction; neurological improvement; N-methyl-D-aspartic acid receptor; repetitive transcranial magnetic stimulation; synaptic plasticity; synaptophysin; traumatic brain injury; TrkB

Introduction

Traumatic brain injury (TBI) refers to neuropathologic damage and dysfunction caused by blunt force to the head (Mckee and Daneshvar, 2015). Patients with TBI typically show motor dysfunction, cognitive impairment, and behavioral abnormalities after regaining consciousness. Although neurological dysfunction usually returns to normal within 1 year in patients with mild TBI, long-term deficits can persist in 10–15% of cases. Further, long-term deficits occur in 50% of moderate injuries and an even greater proportion of severe injuries (Hoskinson et al., 2009). At present, rehabilitation therapy is the best way to treat neurological deficits after TBI, including motor rehabilitative training, hyperbaric oxygen therapy, and electro-acupuncture (Lu et al., 2021). However, as neurological impairment still exists in most survivors of TBI even after rehabilitation therapy, there is an urgent need for new rehabilitation methods.

Repetitive transcranial magnetic stimulation (rTMS) is an efficient and painless brain-stimulation technology that generates a super-threshold current in the brain via electromagnetic induction (Seewoo et al., 2018; Xie et al., 2021). This method was originally used to explore cortical function and physiological and biochemical disorders, all of which result in anatomical and functional changes in synaptic transmission (Algattas and Huang, 2013; Ng and Lee, 2019). Some researchers have suggested that rTMS may promote histologic recovery (Verdugo-Diaz et al., 2017) and enhance brain activity (Yoon et al., 2015) in rat models of TBI. However, in TMS plays a neuroprotective role in many brain diseases, such as depression (Feng et al., 2012), schizophrenia (Eastwood, 2004), Parkinson’s disease (Cacace et al., 2017), vascular dementia (Yang et al., 2015) and stroke (Hong et al., 2021) through changes in synaptic plasticity, particularly long-term depression (LTD) and long-term potentiation (LTP) of synaptic connections.

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rTMS has been reported to significantly reduce neuronal death and glial activation in remote regions, and improve functional recovery in hemi-cerebellar ectomized rats (Sasso et al., 2016). Other research has indicated that high-frequency rTMS promotes cell proliferation and neurogenesis in the dorsolateral subependymal ventricular zone, as well as decreased loss of mature neurons and reduced apoptosis in the perilesional zone of rat models of TBI (Sasso et al., 2016). A recent study (Shin et al., 2018) revealed that cortical excitability and reorganization are possible mechanisms through which rTMS therapy combined with environmental enrichment leads to functional improvement after TBI. Several studies have reported that rTMS can promote histologic recovery (Verdugo-Diaz et al., 2017) and enhance brain activity (Yoon et al., 2015) in rat models of TBI. However, in this study, we established rat models of moderate traumatic brain injury using Feeney’s weight-dropping method and treated them using rTMS. To help determine the mechanism of action, we measured levels of several important brain activity-related proteins and their mRNA. On the injured side of the brain, we found that rTMS increased the protein levels and mRNA expression of brain-derived neurotrophic factor (BDNF), trkB, N-methyl-D-aspartic acid (NMDAR), cAMP response element binding protein (CREB), and synaptophysin. rTMS also partially reversed the loss of synaptophysin after injury and promoted the remodeling of synaptic ultrastructure. These findings suggest that upregulation of synaptic plasticity-related protein expression is the mechanism through which rTMS promotes neurological function recovery after moderate traumatic brain injury.

Key Words: brain-derived neurotrophic factor; moderate traumatic brain injury; neurological dysfunction; neurological improvement; N-methyl-D-aspartic acid receptor; repetitive transcranial magnetic stimulation; synaptic plasticity; synaptophysin; traumatic brain injury; TrkB

Discussion

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promote functional recovery of damaged nerve tissue after TBI by enhancing synaptic plasticity between cortical neurons (Bolognini et al., 2009; Villamar et al., 2012; Nardone et al., 2020). At present, the underlying mechanism is still not completely understood. One possible mechanism through which high-frequency rTMS promotes neural functional recovery after TBI is an increase in the expression of synaptic plasticity-related proteins. Specifically, N-methyl-D-aspartic acid receptor 1 (NMDAR1) (Volianskis et al., 2015) and cAMP response element binding protein (CREB) (Todorovski et al., 2015) have been shown to play a key role in synaptic plasticity. Moreover, brain-derived neurotrophic factor (BDNF) (Miranda et al., 2019) is known as an important upstream regulator of LTP. The BDNF-tropomyosin receptor kinase B (TrkB) pathway plays an important role in neural development, maturation, neurogenesis, and survival (Wurzelmann et al., 2017). Additionally, synaptophysin (SYN) is stored in presynaptic vesicles after neuronal synthesis and reflects the number, density, and distribution of synapses (Yu et al., 2018). We hypothesized that production of these five proteins involved in synaptic plasticity is facilitated with nerve repair and functional recovery following TBI, and tested this hypothesis in a rat model of TBI.

**Methods**

**Animals**

Forty-five male Sprague-Dawley rats (250–300 g, 8 weeks old) were used in this study. Male rats were used because TBI is more common in men than women (Gupte et al., 2019). The rats were purchased from the Experimental Animal Center of Southern Medical University (license No. SCXK [Yue] 2021-0041) and housed in a room with a 12-hour light/dark cycle, maintained at 15–25°C. Food and water were available ad libitum. All animal procedures conformed to the guidelines issued by the Committee on Animal Research of Nanfang Hospital (Guangzhou, China). Methods of care and use of Laboratory Animals from the National Institute of Health (8th, 2011). The study was approved by the Institutional Ethics Committee of Nanfang Hospital (Application No. NFFY-2020-0317) on March 20, 2020.

**TBI model establishment**

The rat model of moderate TBI was established using Feeney’s weight-drop method, as previously described (Feeney et al., 1981). After anesthesia with 1% pentobarbital (40 mg/kg; Experimental Animal Center of Southern Medical University, Guangzhou, China) via intraperitoneal injection, shaving, and disinfection, an incision at the middle of the sagittal plane was made in the scalp. A lesion was generated in the right hemisphere 1.5 mm posterior and 2.5 mm lateral to the bregma (Feeney et al., 1981), and a 5-mm diameter round piece of skull was removed, while the dura mater was kept intact. Then, a footplate (4.5 mm in diameter) was directly attached to the dura, and a weight was dropped onto it with a force of impact to the right hemisphere cortex of 20 × 30 cm. According to a previous study (Gao et al., 2004), this method causes moderate TBI. Next, we closed the bone window with bone wax and sutured the scalp. After recovery from anesthesia, the animals were put back into their cages and provided with water and food. All animal models were made by experienced researchers within 2 days. In the sham group (n = 15), only a 5-mm diameter bone window was opened to ensure integrity of the brain parenchyma. The complete experimental process is shown in Figure 1.

**Transmission electron microscopy**

To examine the synaptic ultrastructure, the dissected brain tissue was trimmed to approximately 1 mm thick, fixed with 1% osmic acid (Google Biotechnology, Wuhan, China) for 2 hours, dehydrated in acetone, and embedded in epoxy resin Epon-812 (SPI, Guangzhou, China). Sections with a thickness of approximately 80 nm were cut using an ultramicrotome (Leica EM UC7, Wetzlar, Germany). The sections were double-stained with 2% uranium acetate and lead citrate. Images were observed and recorded using a Hitachi 7700 transmission electron microscope (Electron microscope Experimental Center, Southern Medical University, Guangzhou, China). At least 30 sections per animal were randomly selected from each group, and the average thickness of the postsynaptic density (PSD), the width of the synaptic cleft, and the length of the synaptic active zone (AZ) were quantified and analyzed with NIH Elements BR software (version 4.2.0, Nikon, Tokyo, Japan).

**Immunohistochemistry**

To evaluate immunobiofivity for BDNF, TrkB, NMDAR1, phosphorylated (p)-CREB and SYN around the injured area, the paraffin sections for immunohistochemistry were fixed in 4% paraformaldehyde for 24 hours, then dehydrated with alcohol and embedded in paraffin wax. Five sections (3 μm thick) were cut and guidelines for the Care and Use of Laboratory Animals from the National Institute of Health (8th, 2011). The sections were incubated overnight at 4°C with BDNF (rabbit, 1:2000, Abcam, Cat# ab187043, RRID: AB_285113), NMDAR1 (rabbit, 1:200, Abcam, Cat# ab108319, RRID:AB_10862052), TrkB (rabbit, 1:250, Abcam, Cat# ab187043, RRID:AB_285113), SYN (rabbit, 1:2000, Abcam, Cat# ab268144, RRID: AB_114847), P-CREB (rabbit, 1:100, Abcam, Cat# ab32096, RRID: AB_731734), and SYN (rabbit, 1:200, Abcam, Cat# ab21272, RRID: AB_228694). Next, the sections were rinsed in Tris buffered saline-Tween three times, each time lasting 5 minutes. The sections were then incubated with secondary antibody (goat anti-rabbit, 1:2000, Abcam, Cat# ab205718, RRID: AB_2819160) at 37°C for 45 minutes and 50 μL diaminobenzidine was added. After re-drying with hematoxylin, the sections were dehydrated and sealed. Immunobiofivity was observed under an optical microscope (CX31, Olympus, Tokyo, Japan) at 400× magnification. Image Pro Plus 6.0 (Media Cybernetics, MD, USA) was used to calculate the positive cell number/total cell number for each of the five proteins of interest (BDNF, TrkB, NMDAR1, SYN, and p-CREB).

**Western blot assay**

To assess the protein levels for BDNF, TrkB, NMDAR1, P-CREB and SYN, some of the tissue from the injured cortex was selected for western blot assay, and the remaining tissues were used for RT-PCR detection. The tissue was homogenized in radio-immunoprecipitation assay lysis buffer then sonicated, and concentrations were measured using the bicinchoninic acid method. Samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The blots were blocked with 5% skimmed milk for 1 hour at 37°C, followed by incubation with the following primary antibodies: anti-BDNF (rabbit, 1:1000, Abcam, Cat# ab108319, RRID: AB_10862052), TrkB (rabbit, 1:1000, Abcam, Cat# ab187043, RRID: AB_28392613), NMDAR1 (rabbit, 1:1000, Abcam, Cat# ab68144, RRID: AB_241474), p-CREB (rabbit, 1:1000, Abcam, Cat# ab108319, RRID: AB_731734), and SYN (rabbit, 1:8000, Abcam, Cat# ab32127, RRID: AB_228694) at 4°C overnight. After washing with Tris buffered saline-Tween, the blots were placed in secondary antibody (goat anti-rabbit, 1:10,000, Sungenbiotech, Tianjin, China, Cat# LLK001) for 1 hour at room temperature.
temperature. Next, they were put into the photo-chemiluminescence gel imaging system to determine the corresponding protein band strength. The bands were quantitated using ImageJ software. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal reference to calculate the relative expression of each antibody.

qRT-PCR
To assess the mRNA expression levels for BDNF, TrkB, NMDAR1, P-CREB and SYN, total RNA was extracted from the injured cerebral hemisphere using Trizol reagent (Aidlab Biotechnology, Beijing, China). An equal amount of RNA was converted into complementary DNA using ReverTraAce reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA). Using the glyceraldehyde-3-phosphate dehydrogenase gene as the internal reference, mRNA expression of the BDNF, TrkB, NMDAR1, P-CREB, and SYN genes was detected by PCR. For each gene, two specific PCR primers were used: BDNF (forward: 5′-AGA ATG ACA AGG CAT AGG AAG G1 T-3′; reverse: 5′-CCA AGA GGT AAG TAG TAG AAG GGA-3′), Ntr2 (forward: 5′-GGA TTT GGT GTA CCG AGC CT-3′; reverse: 5′-TGG ATG TCG CCG AGG TTG-3′), Gln1 (forward: 5′-CGT GGA CCA CAA CAT C-3′; reverse: 5′-AGT CAC CAC AGC CAG GTG GGA-3′), CREB (forward: 5′-GAC AAC CAG CAG AGT GGA GAT G-3′; reverse: 5′-TCA TGG TGC CAG GAT AGC AGC-3′, Syp1 (forward: 5′-TGG GTG GTA CCG AGC ATC TAA-3′; reverse: 5′-GAG TAC GCC CTC CTG CTT TTA-3′), and glyceraldehyde-3-phosphate dehydrogenase (forward: 5′-GGC TAA CAT CAA ATG GGG TG-3′; reverse: 5′-TTG CGT ACA ATC TTG AGG GAG-3′). RT-PCR reactions were performed in a 20-μL total volume with 5 μL cDNA diluted 10 times, 0.4 μL each for forward and reverse primers, 10 μL AceQ Universal SYBR qPCR Master Mix (2× mix), and 4.2 μL ddH2O. The amplification protocol consisted of one cycle at 95°C for 5 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 10 seconds. The formula \(2^{-\Delta\Delta C_T}\) was used to indicate the relative expression level of each gene (Tabatabaeian and Hojati, 2013).

Statistical analysis
No statistical methods were used to predetermine sample sizes; however, our sample sizes are similar to those reported in a previous publication (Zhou et al., 2022). No animals or data points were excluded from the analysis. The evaluators were blinded to the assignments. All data are reported as mean ± standard deviation (SD). Group differences were determined by Student’s t-test using SPSS 25.0 (IBM, Armonk, NY, USA). The level of statistical significance was set at \(P < 0.05\).

Results
TBI model establishment
Magnetic resonance imaging results of rat brain tissue 3 days after TBI are shown in Figure 2. In the sham group, only the scalp was damaged and the brain tissue was intact. There was no significant difference in the ratio between the area of the injury and the area of the uninjured hemisphere between the TBI and rTMS groups (Figure 2B). Moreover, there were no differences in body weight (Figure 3A) or mNSS values among the three groups. These data support the conclusion that the TBI model was stable.

**Figure 2 | Stability of the TBI model.**
(A) Magnetic resonance imaging indicates no significant difference in the size of parenchymal injury between the TBI and rTMS groups. (B) Quantitative result for the ratio of injured area to ipsilateral area 3 days after TBI. Data are expressed as mean ± SD (n = 15/group) and were analyzed by Student’s t-test. rTMS: Repetitive transcranial magnetic stimulation; TBI: traumatic brain injury.

**Figure 3 | Body weight (A) and mNSS (B) for rats in each of the three groups 3 days after TBI.**
Data are expressed as mean ± SD (n = 15/group), and were analyzed by Student’s t-test. mNSS: Modified neurological severity score; rTMS: Repetitive transcranial magnetic stimulation; TBI: traumatic brain injury.

**Figure 4 | TMS improve neurological functional recovery after TBI.**
(A) Experimental schedule. (B) Quantitative result for mNSS score. Data are expressed as mean ± SD (n = 15/group). *P < 0.05, rTMS vs. TBI (Student’s t-test). mNSS: Modified neurological severity score; rTMS: Repetitive transcranial magnetic stimulation; TBI: traumatic brain injury.

**Figure 5 | rTMS promotes the remodeling of synaptic structures after TBI.**
(A) After 20 Hz rTMS, synaptic ultrastructure was significantly improved compared with the TBI group (20,000× magnification; scale bars: 0.5 μm). The red arrow indicates the presynaptic membrane; the blue arrow indicates the postsynaptic membrane; the yellow arrow indicates the postsynaptic density, width of the synaptic cleft, and length of the synaptic active zone. Data are expressed as mean ± SD (n = 6/group). **P < 0.01, vs. TBI group (Student’s t-test). AZ: Active zone; PSD: postsynaptic density; rTMS: repetitive transcranial magnetic stimulation; TBI: traumatic brain injury.

SYN protein and mRNA levels were examined at 16 days after TBI using immunocytochemistry and western blot assay. Weak immunopositive SYN staining was observed in the TBI group but not in the sham group (F = 1.198, t = 8.204, dt = 10, P < 0.01). After rTMS treatment, strong immunopositive SYN staining was observed in the TBI group (20,000× magnification; scale bars: 0.5 μm). The red arrow indicates the presynaptic membrane; the blue arrow indicates the postsynaptic membrane; the yellow arrow indicates the postsynaptic density. Data are expressed as mean ± SD (n = 6/group). **P < 0.01, vs. TBI group (Student’s t-test).

**Figure 6 | rTMS increases the expression of synaptic functional plasticity-related proteins in injured brain of TBI rats**
Protein levels for BDNF, TrkB, NMDAR1, and P-CREB in the injured cortex were assessed at 16 days after TBI using immunocytochemistry. The cytoplasm of cells around the injury site showed stronger immunohistochemical staining for SYN was observed in the cytoplasm of cells around the injury site (Figure 6A). SYN protein levels in the TBI group were significantly lower than those in the sham group, indicating loss of synapses. (Figure 6B) Immunopositive SYN staining was observed in the cytoplasm of cells around the injury site (F = 1.031, t = 2.70, dt = 10, P < 0.01). After rTMS treatment, strong immunopositive SYN staining was observed in the TBI group (20,000× magnification; scale bars: 0.5 μm). The red arrow indicates the presynaptic membrane; the blue arrow indicates the postsynaptic membrane; the yellow arrow indicates the postsynaptic density, width of the synaptic cleft, and length of the synaptic active zone. Data are expressed as mean ± SD (n = 6/group). **P < 0.01, vs. TBI group (Student’s t-test). AZ: Active zone; PSD: postsynaptic density; rTMS: repetitive transcranial magnetic stimulation; TBI: traumatic brain injury.
Figure 6 | rTMS reduces the loss of SYN after TBI. (A) After 20 Hz rTMS, the number of cells around the injury site that were positive for SYN were higher than they were for the TBI group (*p < 0.05). Scale bars: 50 μm. (B) Quantification of SYN-positive cells. (C) Quantification of SYN mRNA expression in the injured hemisphere. (D) Western blot images of SYN protein level in the injured hemisphere 16 days after TBI. (E) Relative protein expression of SYN, which was normalized by GAPDH. Data are expressed as mean ± SD (n = 6/group). *p < 0.05, vs. TBI group (Student’s t-test). SYN: synaptophysin; TBI: traumatic brain injury.

Figure 7 | rTMS increases the number of BDNF, TrkB, NMDAR1, and P-CREB positive cells around the injured area after TBI. (A) After 20 Hz rTMS, the number of BDNF, TrkB, NMDAR1, and P-CREB positive cells around the injury site were higher than they were in the TBI group. Scale bars: 50 μm. (B) Quantification BDNF, TrkB, NMDAR1, and P-CREB-positive cells. Data are expressed as mean ± SD (n = 6/group). *p < 0.05, vs. TBI group (Student’s t-test). BDNF: Brain-derived neurotrophic factor; NMDAR1: N-methyl-D-aspartic acid receptor 1; P-CREB: phosphorylated cAMP response element binding protein; rTMS: repetitive transcranial magnetic stimulation; TBI: traumatic brain injury; TrkB: tropomyosin receptor kinase B.

Tissue from the injured hemisphere was used for western blot assay and RT-PCR at 16 days after TBI. Protein levels (BDNF: F = 0.022, t = −5.964, dt = 10, p < 0.01, TrkB: F = 0.505, t = −2.416, dt = 10, p = 0.036, NMDAR1: F = 0.610, t = −2.576, dt = 10, p = 0.038, P-CREB: F = 4.076, t = −3.915, dt = 10, p < 0.01, Figure 8) and mRNA expression (BDNF: F = 14.233, t = −4.634, dt = 10, p < 0.01, TrkB: F = 28.250, t = −4.861, dt = 10, p < 0.01, NMDAR1: F = 16.088, t = −6.487, dt = 10, p < 0.01, CREB: F = 43.436, t = −7.350, dt = 10, p < 0.01, Figure 9) for BDNF, TrkB, NMDAR1, and P-CREB were higher in the TBI group than in the sham group. Notably, levels were further upregulated after rTMS treatment.

Figure 8 | rTMS increases BDNF, TrkB, NMDAR1, and P-CREB protein levels in the injured hemisphere after TBI. (A) Western blot images representing protein expression levels of BDNF, TrkB, NMDAR1, and P-CREB in the injured hemisphere of rats 16 days after TBI. (B) Relative protein expression of BDNF, TrkB, NMDAR1 and P-CREB, normalized by GAPDH. Data are expressed as mean ± SD (n = 6/group). *p < 0.05, vs. TBI group (Student’s t-test). BDNF: brain-derived neurotrophic factor; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; NMDAR1: N-methyl-D-aspartic acid receptor 1; P-CREB: phosphorylated cAMP response element binding protein; rTMS: repetitive transcranial magnetic stimulation; TBI: traumatic brain injury; TrkB: tropomyosin receptor kinase B.

Figure 9 | Quantification of BDNF, TrkB, NMDAR1, and CREB mRNA expression in the injured hemisphere. Data are expressed as mean ± SD (n = 6/group). *p < 0.05, vs. TBI group (Student’s t-test). BDNF: Brain-derived neurotrophic factor; CREB: cAMP response element binding protein; NMDAR1: N-methyl-D-aspartic acid receptor 1; rTMS: repetitive transcranial magnetic stimulation; TBI: traumatic brain injury; TrkB: tropomyosin receptor kinase B.

Discussion
The current results showed that the expression of synaptic plasticity related proteins (BDNF, TrkB, NMDAR1, P-CREB, and SYN) in injured cerebral cortex was significantly higher after high-frequency rTMS. In addition to these neuroprotective effects, we demonstrated that high-frequency rTMS improved functional recovery in TBI rats, as evidenced by a reduction in mNSS value, suggesting that this method is a promising strategy for TBI rehabilitation.

Modulation of synaptic transmission following TBI likely includes axonal sprouting, synaptogenesis, synaptic remodeling, and synaptic strengthening, which are all processes that promote synaptic plasticity (Alvarez and Lago, 2013; Ng and Lee, 2019). Thus, we directly examined synaptic ultrastructure via transmission electron microscopy. We found greater PSD thickness, lower synaptic cleft width, and longer synaptic active zones after rTMS treatment. Furthermore, rTMS reduced the loss of SYN. SYN exists widely in nerve endings in the central and peripheral nervous system and is specifically distributed in presynaptic vesicle membranes. It is considered a biomarker of presynaptic terminals and has been extensively used to quantify the number, density, and distribution of synapses (Yu et al., 2018; Ji et al., 2021). It has also been demonstrated that BDNF knockout mice showed significant synaptic fatigue at the CA1 synapse under high-frequency stimulation, and that the number of docked vesicles in the presynaptic active region was significantly reduced (Pozzo-Miller et al., 1999). Surprisingly, BDNF may suppress autophagy via the TrkB and the phosphatidylinositol 3 kinase (PI3K)/Akt pathway, reducing the degradation of synaptic proteins and promoting the reconstitution of synaptic structures (Hernandez et al., 2012; Nikoletopoulou et al., 2017). Here, we also report that BDNF and TrkB expression were also higher after rTMS treatment in TBI rats.

rTMS can cause long-lasting changes in cortical excitability. A stimulation frequency ≤ 1 Hz suppresses cortical excitability and produces LTD, while a stimulation frequency ≥ 5 Hz facilitates cortical excitability and generates LTP (Page et al., 2015; Iglesias, 2020). LTP and LTD are the main types of functional plasticity (Levy et al., 2018; Koller and Chakrabarty, 2020) that can be assessed by electrophysiologically. Therefore, it is not surprising that we detected proteins closely related to the occurrence of LTP.

NMDARs are thought to play a bidirectional role in the occurrence and development of TBI. In the acute phase, excitotoxicity caused by glutamate accumulation is mainly mediated by NMDARs, which leads to secondary neuronal injury and various behavioral dysfunctions. However, in the...
subacute and chronic phases, NMDAR activation can resist synaptic transmission disturbances caused by the continuous increase in inhibitory neurotransmitters. To avoid the aggravation of nerve tissue damage caused by acute excitatory toxicity, it is recommended that rTMS treatment be initiated 24–72 hours in rat models of TBI. However, the application of rTMS at this stage is likely to be impractical clinically (Levy et al., 2018; Koller and Chakraborty, 2020). Therefore, we gave 20 Hz rTMS to injured rats on the 4th day after TBI. After 1 week of rTMS treatment, we found that mNSS values were lower in the TMS group than in the TBI group, but the difference was not statistically significant. However, when tested after 2 weeks of rTMS treatment, we found that a statistical difference had emerged. These results suggest that rTMS might improve neurophysiological function in rats with brain injury, but that the effect is cumulative, and the course of treatment should be at least 2 weeks.

NMDARs are an important mediator of brain plasticity and can transform specific neuronal activity patterns into changes in synaptic structure and function (Malenka and Nicoll, 1989), which is a key role in the back-up of learning and memory behavior. NMDAR1 is present in all endogenous NMDARs and is widely expressed throughout development (Ju and Cui, 2016; Zhang et al., 2018). High frequency rTMS promotes the release of presynaptic glutamate after TBI, which binds to the corresponding NMDAR in the postsynaptic membrane. When the postsynaptic membrane is sufficiently depolarized, magnesium ions are expelled from the cation channel, allowing sodium and calcium ions to flow into the cell. This influx of calcium is thought to be the cause of LTP induction (Malenk et al., 1988; Volianski et al., 2015). Evidence from human studies also suggests that the effect of rTMS depends critically on the activation of the glutamatergic NMDAR since these effects are blocked by the NMDAR antagonist dextromethorphan (Stefan et al., 2002). In the present study, we found that the NMDAR1 levels in the cerebral cortex were higher after rTMS treatment in TBI rats. This result is similar to that of a previous study (Lisany and Belmaker, 2000), which found an increase in the expression of NMDAR1 in the ventromedial thalamus and parietal cortex after rTMS treatment. Therefore, we speculate that rTMS enhances NMDAR1 protein expression in rats with TBI and eventually leads to the induction of LTP.

LTP maintenance is split into early and late phases. Early LTP involves changes in synaptic strength following the redistribution of medium and ion activity, lasting for 20–60 min (Malenka and Nicoll, 1989). In contrast, late LTP (L- LTP) is associated with variations in gene expression and protein synthesis that last for hours, days, or even weeks (Cheryvayok et al., 2015). CREB is an important transcription factor that is thought to contribute to the formation of L- and long-term LTP (LTP). It is localized at the sites by initiating transcription of proteins (Glazewski et al., 1999). It has been reported that LIM1-knockout mice, drastically impaired in long-term memory and selectively defective in L- LTP, were rescued by increasing the activity of CREB (Todorovski et al., 2017). Furthermore, LTP does not last >90 minutes in the hippocampus of animals lacking most of the major isoforms of CREB (Barth et al., 2000). These results suggest that CREB-dependent signaling cascades play an important role in L- LTP. CREB transcriptional and transactivation activity in cultured postnatal dorsal root ganglion neurons from rats (Yan et al., 2016). Furthermore, P-CREB can be reduced by blocking CaMKII with inhibitors KN93 or KN62, and suppressed by specific knockdown of the CaMKIIα or CaMKIIβ subunit (Glazewski et al., 1999).

Ca++ influx in the postsynaptic membrane activates calcium-sensitive signaling, such as calcium/calmodulin-dependent kinase II (CaMKII), which triggers CREB phosphorylation in the nucleus (P-CREB) (Wang and Peng, 2016; Gandolfi et al., 2014). In the present study, we found that endogenous BDNF plays a response element in the BDNF gene (Yan et al., 2016; Esvald et al., 2020). In a previous study, blocking Ca++-sensitive CREB phosphorylation, and P-CREB downregulation reduces BDNF transcription triggered by electrical stimulation in cultured postnatal dorsal root ganglion neurons from rats (Yan et al., 2016). Furthermore, P-CREB can be reduced by blocking CaMKII with inhibitors KN93 or KN62, and suppressed by specific knockdown of the CaMKIIα or CaMKIIβ subunit (Glazewski et al., 1999).

BDNF belongs to the neurotrophic factor family, which is mainly synthesized by neuronal and glial cells, and highly expressed in the cerebral cortex and hippocampus of rodents and humans. Not only does BDNF regulate neuronal development, growth, and survival, it also plays a role in synaptic transmission and LTP (Namakawa et al., 2018; Miranda et al., 2019). The BDNF receptor TrkB is highly expressed in the postsynaptic membrane, causing depolarization of synaptosomes in the hippocampus and cortex (Leal et al., 2014). At the postsynaptic level, TrkB receptor activation phosphorylates plasma membrane-associated NMDAR by activating the PI3K/Akt pathway (Zhang et al., 2011; Lin et al., 2014). A recent study also showed that 5 Hz rTMS for 5 days improves BDNF-TRkB signaling and NMDAR interaction in lymphocytes (Wang et al., 2011). Moreover, when TrkB binds to the TrkB receptor, it activates the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK) pathway to enhance phosphorylation at Ser123 of CREB (Liu, 2010; Liu et al., 2010). Therefore, the BDNF-TRkB pathway and NMDAR/CREB form a positive feedback circuit that promotes LTP. The long-lasting effects of BDNF-TRkB and LTP and alterations in the synaptic proteome, magnified from the regulation of multiple mRNA expression (Smalheiser et al., 2010; Jaeter et al., 2016). BDNF might activate synaptic consolidation through transcription and rapid dendritic trafficking of mRNA encoded by the immediate early gene, Arc (Bramham and Messaudo, 2005; Wibrand et al., 2006), thus making LTP exist in a more stable state. The formation of LTP increases the excitability of the injured brain hemispheres, thus maintaining the balance between hemispheres and ultimately promoting the recovery of neurological function (Talelli and Rothwell, 2006; Di Filippo et al., 2008). Consistent with previous studies, mRNA expression and protein levels of BDNF and TrkB were significantly higher in the cerebral cortex of rats with TBI after treatment with high-frequency rTMS. Similar to previous studies (Felderhoff-Mueser et al., 2002; Feng et al., 2017), the enhanced expression of BDNF, TRkB, NMDAR1 and P-CREB is thought to be the result of increased self-compensatory synthesis of neurons.

This study has several limitations. The first is the experimental equipment and technology. We did not directly measure LTP, but instead measured the levels of proteins related to LTP. Second, the effects of rTMS on neurological function were not observed when LTP was blocked. Third, although we used the smallest round coils available, the size of the coil was still larger than the rat brain. Thus, we could not ensure that stimulation was applied to a specific brain region. Fourth, as rats were only observed for 2 weeks after TBI, the subsequent therapeutic effects of rTMS remain unknown. Fifth, we only used mNSS as an index of neural function, but these values can be greatly influenced by subjective factors.

In conclusion, our findings suggest that rTMS may promote the recovery of neurological function in TBI rats through the following mechanisms: (1) high-frequency rTMS could enhance SYN protein levels to promote synaptic reconstruction after TBI; (2) high-frequency rTMS could affect the expression of certain synaptic proteins, such as NMDAR1, P-CREB, BDNF, and TrkB, which have been shown to be closely related to the occurrence of LTP.

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