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ORIGINAL ARTICLE

Repetitive Transcranial Magnetic Stimulation Alleviates Neurological Deficits After Cerebral Ischemia Through Interaction Between RACK1 and *BDNF exon IV* by the Phosphorylation-Dependent Factor MeCP2

Hongzhan Li[1] · Jianqing Shang[1] · Chengliang Zhang[2] · Rulan Lu[2] · Junpao Chen[1] · Xianju Zhou[1,2]

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Abstract

Repetitive transcranial magnetic stimulation (rTMS) is acknowledged as a form of neurostimulation, especially for functional recovery. The foundational knowledge of molecular mechanism is limited regarding its role in cerebral ischemia, for which the present study was designed. Primary neurons were treated with oxygen-glucose deprivation (OGD) and repetitive magnetic stimulation (rMS), in which brain-derived neurotrophic factor (BDNF) and transcription of *BDNF exons* were examined. Then, adenovirus vectors carrying siRACK1 sequence were delivered to primary neurons, followed by detection of the transcription of *BDNF exons* and the extent of methyl CpG binding protein 2 (MeCP2) phosphorylation. Results showed that BDNF and the transcription of *BDNF exons* were upregulated by rMS and OGD treatment, but decreased by extra treatment of RACK1 siRNA. Then, the mechanism investigations demonstrated that rMS increased the extent of MeCP2 phosphorylation to promote the interaction between RACK1 and *BDNF exon IV*. The aforementioned findings were further confirmed *in vivo* in middle cerebral artery occlusion (MCAO)-induced rat models, as indicated by improved neurological functions and reduced area of cerebral infarction. The study offers potential evidence for improvement of neurological deficits, highlighting the important role of rTMS for treatment of cerebral ischemia.

Key Words Repetitive transcranial magnetic stimulation · cerebral ischemia · BDNF · RACK1 · MeCP2 phosphorylation · neurological function

Introduction

Cerebral ischemia is the most prevalent cause of acute cerebral infarction across the world with high mortality rates [1]. Neurologic deficits relative to cerebral ischemia can be attributed to dysfunction of several neurotransmitter systems, including the cholinergic and glutamatergic systems [2]. In recent years, noninvasive brain stimulation has been widely applied for the regulation of brain functions and neuropsychiatric conditions [3]. For instance, evidence suggests that repetitive transcranial magnetic stimulation (rTMS) can be used to improve cognitive decline in patients with Alzheimer's disease [4], and the recovery of neurological functions after the occurrence of cerebral ischemia [5]. Importantly, the improvement of high-frequency rTMS on neurological functional recovery in ischemic rat models has been linked to brain-derived neurotrophic factor (BDNF) [6]. BDNF that can be produced throughout the brain is capable of regulating neuronal activity and normal neuronal function as well as plays an important role in protection and recovery after stroke [7]. Likewise, BDNF has been highlighted to serve as a contributor in rTMS-produced amelioration of hippocampus-dependent cognitive impairment in rats with ischemic stroke [8]. Therefore, it is reasonable to infer that rTMS may be applied to promote neurogenesis by the neurorehabilitation effects of BDNF.

Hongzhan Li and Jianqing Shang contributed equally to this work.

✉ Xianju Zhou
xianju_zhou@yahoo.com

[1] Department of Neurology, Integrated Hospital of Traditional Chinese Medicine, Southern Medical University, No. 13, Shiliugang Road, Guangzhou 510315, Guangdong Province, China

[2] Department of Neurology, The Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University, No. 29, Xinglong Alley, Changzhou 213003, Jiangsu Province, China

Additionally, BDNF stimulation has been suggested to induce the receptor for activated C kinase 1 (RACK1) to mediate the local translation of synaptic protein, promoting neural development [9]. RACK1, a scaffold protein consisting of 7 tryptophan-aspartate (WD) 40 domains, is capable of regulating interaction between proteins [10]. Targeting the RACK1 signaling pathway has further been highlighted to serve as a therapeutic approach for neurodevelopment disorders [11]. In addition, the tumor necrosis factor-α receptor/RACK1/embryonic ectoderm development signaling pathway also play a role in ischemia-associated neuronal damage [12], suggesting the possible involvement of RACK1 in BDNF-mediated cerebral ischemia. Intriguingly, a previous study revealed that rTMS to cortex promotes the function of BDNF-tropomyosin-related kinase B (TrkB)-*N*-methyl-D-aspartic acid (NMDA) receptor axis in both cortex and lymphocytes [13]. Besides, the neuroprotective effects of NMDA have been found to reduce accompanied by decreased BDNF protein levels in association with neuronal survival [14]. Moreover, BDNF induced methyl CpG binding protein 2 (MeCP2) phosphorylation at S86 and S274, regulating the responses of neuronal chromatin to diverse stimuli [15]. MeCP2, known as an epigenetic factor that is highly abundant in the brain, can bind to both DNA nonmethylated and methylated regions of chromatin, and may be responsible for mediation of responses to immunity and inflammation [16, 17]. As previously described, BDNF promoter IV can be activated by releasing the transcription repressor, MeCP2, from the promoter leading to DNA demethylation [16]. Hereby, we hypothesize whether rTMS could improve neurological functions in cerebral ischemia involving the interaction of BDNF, RACK1, and MeCP2. In the current study, we aim to employ a cellular ischemia model OGD in the primary rat neurons and *in vivo* cerebral ischemia rat model middle cerebral artery occlusion (MCAO) to investigate the role of rTMS in the treatment of cerebral ischemia.

Materials and Methods

Experimental Animals

A total of 75 adult male Sprague-Dawley (SD) rats at specific pathogen free (SPF) level (aged 8–12 weeks, weighing 200–250 g) were obtained from the Hunan SJA Laboratory Animal Co., Ltd. (license key: SCXK (xiang) 2009-0004, Changsha, Hunan, China). All rats were raised under condition of 12-h light/darkness with free access to food and water. Ten rats were selected as normal controls, and the remaining 60 rats were used to establish the cerebral ischemic model. All experimental animals were euthanized by CO_2 asphyxiation.

Adenovirus Vector Construction

The RACK1 sequence was inserted into AAV5-LacZ vector to obtain adenovirus vectors for upregulating RACK1 expression. The AAV5-LacZ cosmid vectors, provided by the adenovirus expression vector kit (TaKaRa Biotechnology Inc., Japan), were co-transfected with relevant reagents into 293 cells in accordance with the manufacturer's instructions. The wild-type strain was screened from the obtained recombinant adenovirus at passage 1 by differential infection method using HeLa cells and 293 cells, followed by amplification using Dulbecco's Modified Eagle Medium (DMEM) to harvest AAV5-LacZ adenovirus at passage 4 with higher titer.

Culture and Treatment of Primary Neurons

The cortex, striatum, and hippocampus were separated from rat brains, washed with D-Hanks for 3 times, respectively, sliced into pieces and detached with 2.5 g/L trypsin at 37 °C for 10 min. The detachment was eliminated by the addition of DMEM containing 10% fetal bovine serum (FBS). The elimination agent was removed through centrifugation at 179×*g* for 10 min, and this step was repeated again. After filtration, the filtrate was centrifuged at 179×*g* for 10 min and washed with D-Hanks for 2 times. A single cell suspension was produced by DMEM containing 10% bovine serum albumin and 10% B-27. With cell concentration adjusted to 2×10^5 cells/mL, the cells were seeded in a 96-well plate coated with 0.05 g/L poly-L-lysine and incubated at 37 °C with 5% CO_2 in air. The following day, the cortex, striatum, and hippocampus neurons were cultured in oxygen-glucose deprivation (OGD)-treated condition (sugar-free DMEM, 5% O_2, $<1\%$ CO_2, and 95% N_2) for 1 h to obtain one-time OGD neurons. In order to simulate a condition of recurrent ischemia, OGD neurons were required for two times. Then, 60 min after the first time of OGD, the sample was cultured in normal medium for 3 h and treated with OGD for 60 min, followed by normal culture. The primary cultured neurons were transfected or infected with sequences of Mock, siRACK1 (20 nt, GACCATCATCATGTGGAAGC), siNC, AV-oe-NC, and AV-oe-RACK1 [18], respectively. Next, the cells were seeded in a 6-well plate 48 h prior to transfection.

When cell confluence reached 80–90%, cell transfection was performed according the instructions of lipofectamine 2000 (11668-019, Invitrogen, NY, CA, USA). Following incubation at 37 °C with 5% CO_2 in air for 6–8 h, the culture was continued for 24–48 h in complete medium for subsequent experimentation.

Focal Cerebral Ischemia Model Induced by Middle Cerebral Artery Occlusion in Rats

Focal cerebral ischemia model was induced by MCAO as previously described [19, 20]. General anesthesia was performed using 3% pentobarbital sodium (P3761, Sigma-Aldrich Chemical Company, St Louis, MO, USA) and sustained by inhalation of 1.5% isofluothane. The rats were placed in an incubator after surgery with their body temperature maintained at 36.5–37 °C. A total of 63 rats were used for modeling, and 3 rats died after the completion of surgery. The remaining 60 rats were subjected to different treatments with 12 rats for each, including MCAO, MCAO +1 Hz rTMS, MCAO +10 Hz rTMS, NVP-AAM007 + MCAO +1 Hz rTMS, and Ro25–6981 + MCAO +1 Hz rTMS. An additional 12 rats were regarded as normal controls without any treatment (mock group).

rTMS Protocols and Neurological Evaluation

The rTMS instrument (Yiruide Medical Instrument Co., Ltd. Wuhan, Hubei, China), was introduced for intervention with the highest frequency of 100 Hz, and external diameter of circular stimulation coil being 6 cm. The primary cultured neurons were placed under rTMS and treated with stimulation of low and high frequency (1 Hz for 900 s, at 10 Hz for 50 s at an interval of 50 s with 900 pulses in total) [21]. No stimulation was given to the neurons for control purpose. Incubation was allowed for 3 h prior to subsequent experiments. Rats treated with rTMS were subjected to rTMS treatment 1 time per day for 2 consecutive weeks at 24 h after surgery. Rats were stimulated using the standard figure-of-eight coil (MagPro Magnetic Stimulator, Farum, Denmark), fixed and oriented to cause synaptic activity in most cortical areas of the left hemisphere. During the stimulation, rats were un-anesthetized, wrapped, and gently fixed on a platform using Velcro straps. Their eyes were partly covered with a thick cloth. Next, the rats were allowed to acclimatize to the condition 1 week prior to the surgery in order to relieve the pressure associated with handling, fixing, and stimulation [22]. All rats were allowed free access to food and drinking water during the experiment.

Neurological evaluations were conducted by 2 blinded observers in the 1st and 2nd week of stimulation. The effect of rTMS in the first week was not obvious, but the effects in the second week were obvious. The grading process included the following: 1) neurological status in accordance with the methods reported by Bederson et al. [23] with 4 categories observed and noted ranging from 0 to 3 points in which 0 = no neurological deficit observed, 1 = contralateral forelimb flexion with wrist flexion and shoulder adduction, 2 = reduced resistance to lateral push, and 3 = circling movements towards the ipsilateral side; 2) wire grasping ability and forelimb strength scoring, ranging from 0 to 3 points that rats were suspended by its forelimbs on a wire stretched between two posts, 45 cm above a foam sheet, and the time was recorded until the rat fell down with the cutoff time set as 2 min; and 3) learning-memory ability in which Y-type electric maze was implemented and the times of correct choice were recorded. Briefly, the elevated plus-maze apparatus (Inco) for rats was composed of a central platform connected to two open arms and two enclosed arms. The maze was elevated to a height of 50 cm above the floor. During training trials, the rats were placed at the end of an open arm with their faces turning away from the central platform of the maze. The time that the rats spent to move from open arm and cross the line marked in enclosed arm with all four paws was recorded as transfer latency (TL) time. If the rat did not enter the enclosed arm within 90 s, it was gently pushed into the enclosed arm and the TL time was set as 90 s. Then, the rats were allowed to stay in the maze for 10 s. The TL measured on the plus maze on the 1st day was considered as the index of acquisition, whereas the TL measured after 24 h of acquisition trial was considered as the index of retrieval [24].

Immunofluorescence

The paraffin-embedded brain tissue slices were placed at 60 °C for 1 h until the paraffin thawed, followed by dewaxing and hydration using xylene and ethanol. After performing antigen retrieval by microwave oven irradiation for 9 min with high heat (3 times), the slices were allowed to cool down to room temperature. After being blocked with 5% goat serum at 37 °C for 30 min, the slices were stained with 5 mg/mL propidium iodide (PI) for 5 min, incubated with 0.5% Triton X-100 at 37 °C for 30 min, and probed with the primary rat antibody to hexaribonucleotide binding protein-3 (NeuN) (dilution ratio of 1:1000, ab104225, Abcam Inc., Cambridge, UK) at 4 °C overnight, followed by rewarming at 37 °C for 1 h. The sample was allowed to bind to the fluorescence

secondary antibody (murine-green) (dilution ratio of 1:1000, ab150117, Abcam Inc., Cambridge, UK) for 2 h, stained with 5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) for 5 min and stored at 4 °C in dark conditions. Observation was performed under a laser scanning confocal microscope (Olympus Corp., Tokyo, Japan). Subsequently, the ratio of NeuN/PI positive cells to NeuN positive cells was calculated and recorded.

2,3,5-Triphenyl Tetrazolium Chloride Staining

2,3,5-Triphenyl tetrazolium chloride (TTC) staining was applied to visualize the ischemic infarction. The fresh brain tissues were stored at − 20 °C for 10 min, sectioned into 1-mm slices, and incubated with 1% TTC PBS (pH 7.4) at 37 °C for 20 min with gentle shaking. The slices were then fixed in 10% formaldehyde overnight, followed by photography using a digital camera. The living tissues were brick red-stained and cerebral infarction area exhibited grayish white-stained. The percentage of infarction area in total area was analyzed using ImagemasterVDS. The cerebral infarction area (%) = cerebral infarction area/total area × 100%.

RNA Isolation and Quantitation

Primary neurons before and after repetitive magnetic stimulation (rMS) stimulation and rat tissues treated with rTMS were collected and treated with 1 mL Trizol (Invitrogen, Carlsbad, CA, USA) in ice bath. Total RNA content was extracted using a RNA extraction kit (QIGEN, Duesseldorf, Germany). The concentration and purity of the obtained RNA were detected using an ultraviolet spectrophotometry (UV1901, Shanghai AuCy Instrument Co., Ltd., Shanghai, China) to ensure that the value of A260/A280 was between 1.8 and 2.0 and the concentration being 50 ng/μL. Then, RNA was reverse transcribed into cDNA (50 ng/μL) according to the instructions of PrimeScriptTM RT reagent Kit (Takara, RR047A, Beijing Think-Far Technology Co., Ltd., Beijing, China). Primer sequences (Table 1) were designed by primer Premier 5.0 and synthesized by Beijing Tsingke Biological Technology Co., Ltd. (Beijing, China). The reaction was performed using the two-step method on an ABI 7900HT real-time quantitative PCR instrument (ABI 7900, Shanghai Pudi Biotechnology Co., Ltd., Shanghai, China) with β-actin serving as the internal reference. The expression of total mRNA and mRNA of *BDNF exons* was calculated using the $2^{-\Delta\Delta Ct}$ method. Three replicates were set for each gene in each sample.

Table 1 Primer sequences for RT-qPCR

Gene	Primer sequences
BDNF	F: 5′-AGACATGTTTGCGGCATCCAG-3′
	R: 5′-CCATAAGGACGGGGACTTGTAC-3′
BDNF exon I mRNA	F: 5′-CTCAAAGGGAAACGTGTCTCT-3′
	R: 5′-TCACGTGCTCAAAAGTGTCAG-3′
BDNF exon II mRNA	F: 5′-CTAGCCACCGGGGTGGTGTAA-3′
	R: 5′-TCACGTGCTCAAAAGTGTCAG-3′
BDNF exon IV mRNA	F: 5′-TGCGAGTATTACCTCCGCCAT-3′
	R: 5′-TCACGTGCTCAAAAGTGTCAG-3′
BDNF exon VI mRNA	F: 5′-TTGGGGCAGACGAGAAAGCGC-3′
	R: 5′-TCACGTGCTCAAAAGTGTCAG-3′
BDNF exon IX mRNA	F: 5′-GAGAAGAGTGATGACCATCCT-3′
	R: 5′-TCACGTGCTCAAAAGTGTCAG-3′
Tubulin, beta 4 mRNA	F: 5′-AGCAACATGAATGACCTGGTG-3′
	R: 5′-GCTTTCCCTAACCTGCTTGG-3′

RT-qPCR = reverse transcription quantitative polymerase chain reaction, BDNF = brain-derived neurotrophic factor, mRNA = messenger RNA, F = forward, R = reverse

Western Blot Analysis

Total protein content was extracted from primary neurons before and after rMS stimulation and rat tissues using Radio Immunoprecipitation Assay lysis (R0010, Solarbio Life Sciences Co., Ltd., Beijing, China) containing phenylmethanesulfonyl fluoride, followed by incubation on ice for 30 min. The protein (supernatant) was collected after centrifugation at 12000×*g* for 10 min at 4 °C for quantification. The collected protein was separated with 10% SDS-polyacrylamide gel electrophoresis, and electroblotted onto a polyvinylidene fluoride membrane. Following blockade with 5% skimmed milk powder at room temperature for 1 h, the membrane was probed at 4 °C overnight with diluted primary rabbit polyclonal antibodies against BDNF (dilution ratio of 1:5000, ab108319, Abcam Inc., Cambridge, UK), RACK1 (dilution ratio of 1:5000, ab62735, Abcam Inc., Cambridge, UK), p-MeCP2 (dilution ratio of 1 mg/mL, IC17598, Shanghai YuBo Biological Technology Co., Ltd., Shanghai, China), and β-actin (dilution ratio of 1:1000, ab8227, Abcam Inc., Cambridge, UK). After being rinsed by Tris-Buffered Saline and Tween 20 (TBST) for 3 times (5 min each time), the membrane was then probed with secondary horseradish peroxidase-labeled goat anti-rabbit Immunoglobulin G antibody (dilution ratio of 1:1000, HA1003, Shanghai Yanhui Biotechnology Co., Ltd.,

Table 2 Neurobehavioral assessment in rats post-modeling in the presence of rTMS

Group	*n*	Neurological deficit score	Wire grasping ability score	The correct choice (n/min)
Mock	5	0.20 ± 0.45	2.80 ± 0.45	13.60 ± 1.14
MCAO	5	2.80 ± 0.45	0.20 ± 0.45	3.20 ± 0.45
MCAO +1 Hz	5	1.20 ± 0.84*	1.80 ± 0.84*	7.40 ± 0.89*
MCAO +10 Hz	5	0.60 ± 0.55*	2.20 ± 0.48*	12.20 ± 1.30*

*$p < 0.05$ *vs* the MCAO group (rats with MCAO alone). MCAO = middle cerebral artery occlusion, rTMS = repetitive transcranial magnetic stimulation

Shanghai, China) for 1 h. Then, the enhanced chemiluminescence method was applied to development for 1 min. The harvested protein was analyzed by Western blot analysis with β-actin serving as the internal reference [25].

Chromatin Immunoprecipitation Assay

The cortical tissues were cut into 2-mm slices, crosslinked by 1% formaldehyde, and stored at −80 °C for further use. The nuclei were obtained by micro-probe, lysed, and treated with ultrasound until DNA fragment length was between 200 and 1000 bp. Protein G beads (40 μL) were incubated with 3 μg antibodies for at least 1 h, followed by overnight incubation for immunoprecipitation. DNA-protein complex was extracted from beads and de-crosslinked at 65 °C overnight. DNA was detached by RNase A and Proteinase K, extracted by phenol/chloroform and precipitated with alcohol. Quantitative real-time PCR was performed using iQ SYBR Green Supermix (Bio-Rad Laboratories Inc., Hercules, CA, USA). The amount of DNA after immunoprecipitation was calculated by comparison to the amount of total input DNA. Each data was obtained from 4 times of independent amplification. Chromatin immunoprecipitation (ChIP) was conducted using RACK1 antibody (dilution ratio of 1:5000, ab62735, Abcam Inc., Cambridge, UK). The quantification of *BDNF exons* after precipitation was carried out by reverse transcription quantitative polymerase chain reaction (RT-qPCR).

Statistical Analysis

Statistical analyses were conducted using the SPSS 21.0 statistical software (IBM Corp. Armonk, NY, USA). If measurement data conformed to normal distribution and homogeneity of variance, measurement data were expressed as mean ± standard deviation and analyzed by one-way analysis of variance among multiple groups, followed by Tukey's post hoc test. As for measurement data with skewed distribution, they were expressed as inter-quartile range and analyzed by Mann–Whitney *U* test. A value of $p < 0.05$ was considered to be statistically significant.

Results

rMS Promotes BDNF Expression in Primary Cultured Neurons Treated with OGD

Initially, primary cultured neurons obtained from the cortex, hippocampus, and striatum were subjected to OGD conditions to investigate the effects of rMS on BDNF expression. After OGD treatment, the BDNF expression was found to decrease significantly (Fig. 1A, B). Then, OGD-treated primary cultured neurons were subjected to rMS of low and high frequency. The primary cultured neurons for controls were also placed under rMS without stimulation. Subsequently, RT-qPCR and Western blot analysis were used to determine the BDNF expression pattern. As shown in Fig. 1A, B, compared with neurons following OGD treatment alone, 1 Hz rMS and 10 Hz rMS both upregulated the BDNF expression in OGD-treated neurons ($p < 0.05$), with especially significant upregulation in the cortex. The above findings suggested that rMS promoted the BDNF expression in OGD-treated primary cultured neurons.

rMS Regulates the Transcription of *BDNF exons* in Primary Cultured Neurons

Next, the expression patterns of *BDNF exons* were examined by means of RT-qPCR. Primary cultured neurons of cortex, hippocampus, and striatum were subjected to OGD conditions, followed by rMS of low and high frequency. As illustrated in Fig. 2, both the 1 Hz rMS and 10 Hz rMS induced different expression patterns of different *BDNF exons* in neurons of cortex, hippocampus, and striatum, with the most significant change of *BDNF exon IV* noted in the primary neurons isolated from

cortex. These results suggested that rMS regulated the transcription of *BDNF exons* in different neurons.

siRNA-Mediated Depletion of RACK1 Downregulates the Transcription of *BDNF exons* in OGD-Treated Primary Cultured Neurons Stimulated by rMS

After rMS treatment, Bdnf IV expression was the highest and most obvious in the primary neurons isolated from cerebral cortex. Thus, the primary neurons isolated from cerebral cortex were selected for subsequent experiments. After discovering that rMS regulated the transcription of *BDNF exons* in different neurons, we elucidated whether RACK1 mediates the regulatory role of rMS in the transcription of *BDNF exons* in OGD-treated neurons. We attained knockdown of RACK1 by employing the siRNA approach. Firstly, we confirmed that the interference efficacy of siRACK1 by RT-qPCR and Western blot analysis. As compared with siNC-transfected neurons, the addition of siRACK1 was found to significantly reduce the expression of RACK1 ($p < 0.05$, Fig. 3A, B). Next, we explored whether siRACK1 affected the expression of *BDNF exons* (Fig. 3C). RT-qPCR demonstrated that expression of *BDNF exons* (I, II, IV, and VI) was significantly decreased in neurons after OGD treatment ($p < 0.01$). Additionally, after OGD treatment, no differences in expression of *BDNF exons* were noted between siRACK1-transfected neurons and siNC-transfected neurons ($p > 0.05$). Then, the effect of rMS on BDNF expression after knockdown of RACK1 was examined (Fig. 3D, E). After OGD treatment followed by either 1 Hz rMS or 10 Hz rMS, the BDNF expression was observed to be obviously decreased in siRACK1-transfected neurons as compared to siNC-transfected neurons ($p < 0.05$). Further, we examined by what means RACK1 affected the BDNF expression and the expression of exons (I, II, IV, VI) of BDNF in rMS-stimulated OGD neurons (Fig. 3F, G). After 1 Hz or 10 Hz rMS treatment, BDNF expression in the OGD-treated neurons was found to be downregulated by siRACK1 treatment, especially the *BDNF exon IV* ($p < 0.05$). Knockdown of RACK1 significant decreased the expression of exons (I, II, IV, VI) of BDNF in the OGD-treated neurons after 1 Hz or 10 Hz rMS, and over-expression of RACK1 resulted in

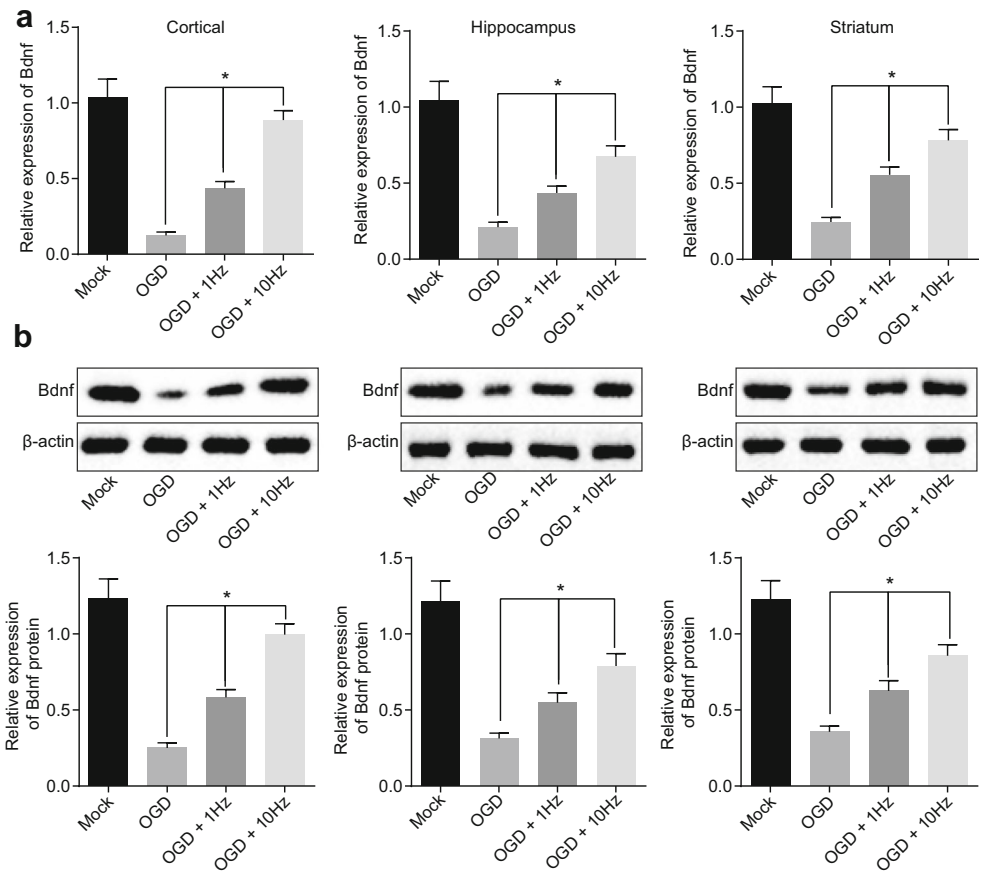

Fig. 1 rMS upregulates the expression of BDNF in primary cultured neurons exposed to OGD. (A) mRNA expression patterns of BDNF in cortex, hippocampus, and stratum determined by RT-qPCR. (B) Relative expression patterns of BDNF protein in cortical, hippocampus, and stratum determined by Western blot analysis relative to β-actin. *$p < 0.05$ *vs* the OGD group (neurons treated with OGD alone). **$p < 0.01$ *vs* the OGD group (neurons treated with OGD alone). The measurement data were expressed as mean ± standard deviation. Comparison among multiple groups was analyzed by one-way analysis of variance. The experiment was repeated 3 times independently

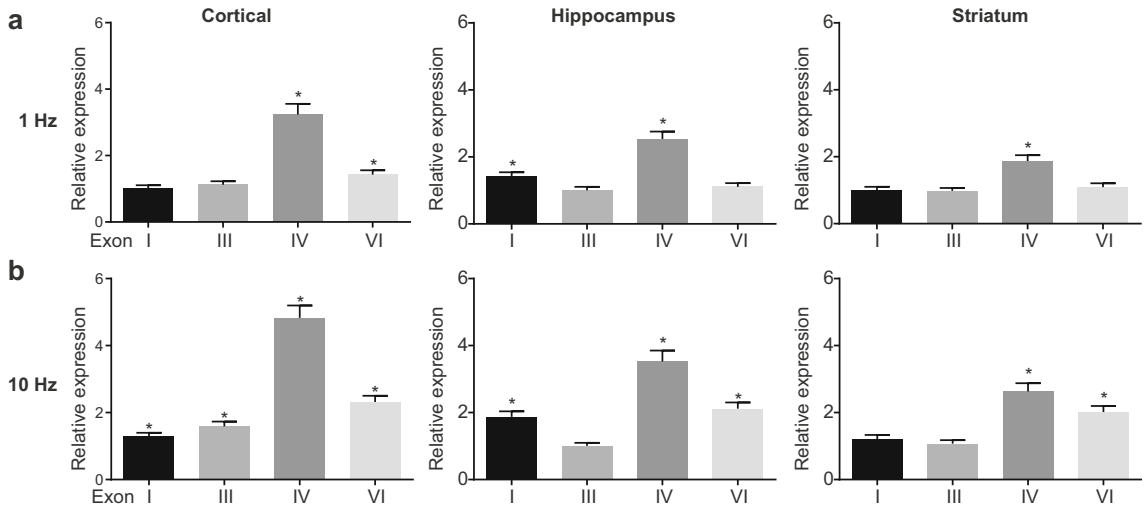

Fig. 2 rMS upregulates the transcription of different *BDNF exons* in primary cultured neurons. RT-qPCR was used to determine the relative expression patterns of different *BDNF exons* (I, II, IV, VI) in cortex, hippocampus, and stratum following 1 Hz or 10 Hz rMS. *$p < 0.05$ *vs* the OGD group (neurons treated with OGD alone). **$p < 0.01$ *vs* the OGD group (neurons treated with OGD alone). Measurement data were expressed as mean ± standard deviation. Comparison among multiple groups was analyzed by one-way analysis of variance. The experiment was repeated 3 times independently

obviously increased expression, most strikingly in the *BDNF exon IV* ($p < 0.01$). Therefore, *BDNF exon IV* was selected for subsequent experimentation. These findings suggested that in the absence of rMS stimulation, downregulation of RACK1 exerted little effect on the transcription of *BDNF exons* in OGD-treated neurons, whereas RACK1 downregulation inhibited the transcription of *BDNF exons* in OGD-treated primary cultured neurons in the presence of rMS stimulation.

rMS Increases MeCP2 Phosphorylation to Promote the Interaction Between RACK1 and *BDNF exon IV* in Primary Cultured Neurons

Then, we tested the involvement of MeCP2 in the interaction between RACK1 and *BDNF exon IV*. ChIP assay revealed that in comparison to OGD-treated neurons, *BDNF exon IV* was significantly enriched in OGD-treated neurons stimulated with 1 Hz or 10 Hz rMS ($p < 0.05$, Fig. 4A). Western blot analysis demonstrated significantly higher levels of MeCP2 phosphorylation after 1 Hz or 10 Hz rMS compared to no rMS stimulation in OGD-treated neurons ($p < 0.05$, Fig. 4B). NR2A and NR2B are two subunits of NMDA receptor whose overexpression is associated with neurodegeneration [26, 27]. Then, in order to study how rMS regulates *BDNF exon IV*, NR2A specific inhibitor (NVP-AAM077) and NR2B specific inhibitor (Ro25-6981) were added prior to OGD and rMS treatments, followed by Western blot analysis to determine the levels of MeCP2 phosphorylation. We observed that NVP-AAM077 or Ro25-6981 led to a significant decrease in MeCP2 phosphorylation (Fig. 4C). Then, ChIP assay was performed again, which revealed that the addition of NVP-AAM007 or Ro25-6981 decreased the enrichment of *BDNF exon IV* in the presence of both OGD and 1 Hz or 10 Hz rMS (Fig. 4D). NVP-AAM007 and Ro25-6981 inhibited the phosphorylation of MeCP2 induced by rMS stimulation, and the effect of 10 Hz rMS stimulation was more apparent. Furthermore, the effect of Ro25-6981 was more obvious than that of NVP-AAM077. Taken together, rMS increased MeCP2 phosphorylation, in turn improved the interaction between RACK1 and *BDNF exon IV*, eventually enhancing the expression of *BDNF exon IV*.

rTMS of Different Frequencies Improves the Neurological Functions of Rats After MCAO

Subsequently, in order to verify the improvement of neurologic functions caused by rTMS, we evaluated various neurobehavioral changes. As shown in Table 2, 1 Hz or 10 Hz rTMS remarkably improved neurologic functions and abilities of movement balance, learning, and memory in MCAO rats ($p < 0.05$). Cell apoptosis analysis in the cortex of MCAO rats showed that 1 Hz or 10 Hz rTMS resulted in significantly lower apoptosis rates ($p < 0.05$, Fig. 5A). TTC staining revealed that the percentage of cerebral infarction area was much smaller in the brain

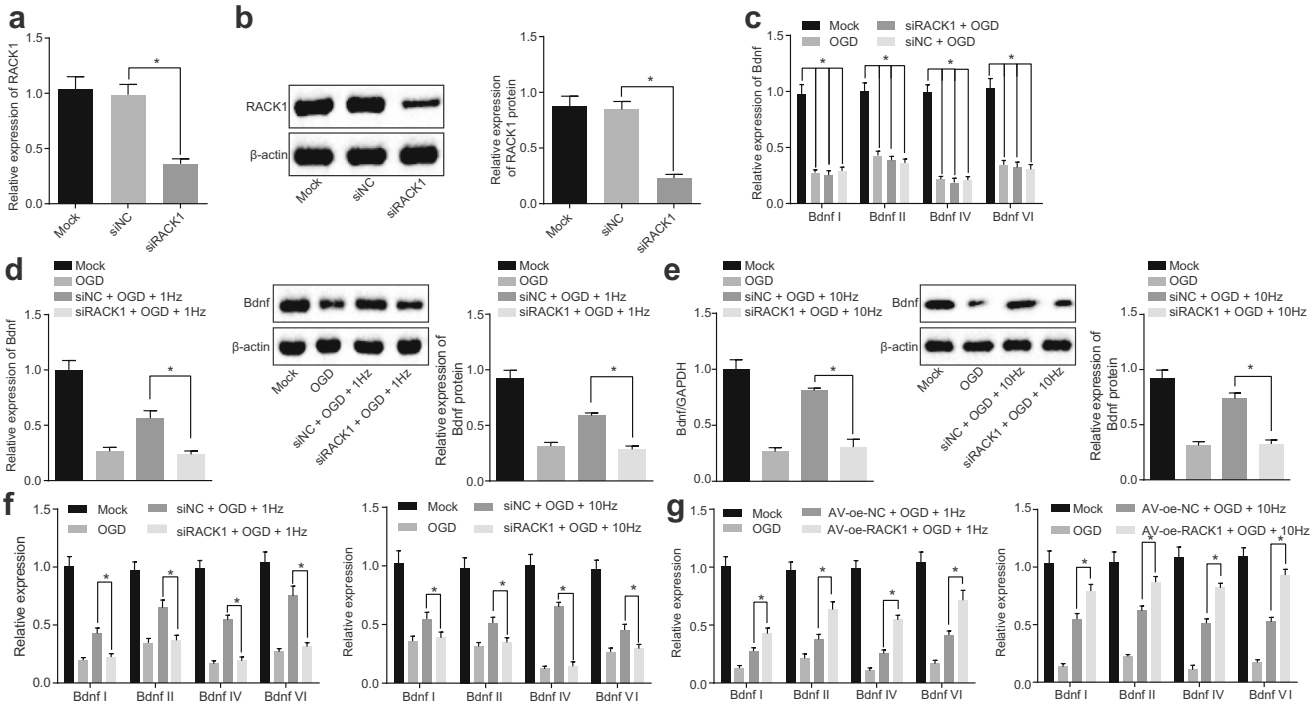

Fig. 3 Silencing of RACK1 inhibits the promoting effects of rMS on transcription of different *BDNF exons* in OGD-treated primary cultured neurons. (A) The interference efficacy of siRACK1 determined by RT-qPCR. (B) The interference efficacy of siRACK1 determined by Western blot analysis relative to β-actin. $*p < 0.05$ *vs* the siNC group (siNC-transfected neurons). (C) Relative expression patterns of different *BDNF exons* (I, II, IV, VI) in OGD-treated neurons in the presence of silenced RACK1 determined by RT-qPCR. $*p < 0.05$ *vs* the mock group (normal primary cultured neurons). (D) Relative expression patterns of BDNF following treatment of 1 Hz rMS, OGD, and siRACK1 determined by RT-qPCR and Western blot analysis, $*p < 0.05$ *vs* the siNC + OGD + 1 Hz group (neurons treated with all siNC, OGD, and 1 Hz rMS). (E) Relative expression patterns of BDNF following treatment of rMS of 10 Hz, OGD, and siRACK1 determined by RT-qPCR and Western blot analysis. $*p < 0.05$ *vs* the siNC + OGD + 10 Hz group (neurons treated with all siNC, OGD, and 10 Hz rMS). (F) Relative expression patterns of different *BDNF exons* (I, II, IV, VI) following 1 Hz or 10 Hz rMS in the presence of silenced RACK1 determined by RT-qPCR. $**p < 0.05$ *vs* the siNC + OGD + 1 Hz or siNC + OGD + 10 Hz group (neurons treated with all siNC, OGD, and 1 Hz or 10 Hz rMS). (G) Relative expression patterns of different *BDNF exons* (I, II, IV, VI) following 1 Hz or 10 Hz rMS in the presence of over-expressed RACK1 determined by RT-qPCR. $**p < 0.01$ *vs* the AV-oe-NC + OGD + 1 Hz or AV-oe-NC + OGD + 10 Hz group (neurons treated with all AV-oe-NC, OGD, and 1 Hz or 10 Hz rMS). Measurement data were expressed as mean ± standard deviation. Comparison among multiple groups was analyzed by one-way analysis of variance. The experiment was repeated 3 times independently

tissue from 1 Hz or 10 Hz rTMS-stimulated MCAO rats in comparison to MCAO rats in the absence of rTMS ($p < 0.05$, Fig. 5B). In conclusion, low-frequency or high-frequency rTMS improved the neurologic functions in MCAO rat models.

rTMS Promotes the Interaction Between RACK1 and *BDNF exon IV* by Enhancing MeCP2 Phosphorylation in a Rat Model of MCAO

Finally, we speculated whether the action mechanisms of rTMS occurred in MCAO rat models as we observed in a cellular stroke model, that is, OGD cultured neurons. We selected the cortex in which rTMS directly act on. The extent of MeCP2 phosphorylation and *BDNF exon IV* enriched by RACK1 was determined. We found higher levels of MeCP2 phosphorylation and *BDNF exon IV* enriched by RACK1 in MCAO rats stimulated by rTMS (1 Hz or 10 Hz) relative to MCAO rats without rTMS ($p < 0.05$, Fig. 6A, B). Application of NR2A-specific inhibitor (NVP-AAM077) or NR2B-specific inhibitor (Ro25-6981) blocked the increase in MeCP2 phosphorylation levels and *BDNF exon IV* enrichment caused by rTMS ($p < 0.05$, Fig. 6C, D). Thus, rTMS promoted the interaction between RACK1 and *BDNF exon IV* by increasing MeCP2 phosphorylation in MCAO rat models.

Discussion

In recent years, mounting evidence has revealed rTMS as a promising therapeutic tool in neuromodulation, such as dysphagia after stroke, psychiatric disorders, and cerebral functional reorganization in ischemic stroke [28–30]. Considering

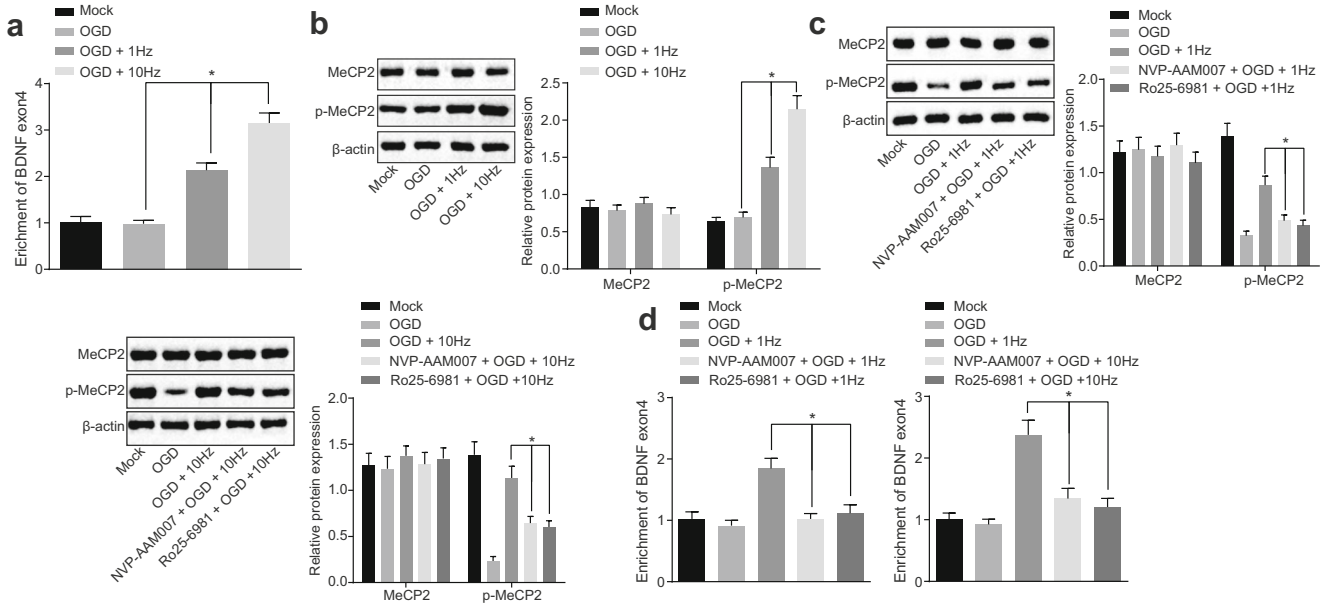

Fig. 4 rMS enhances the interaction between RACK1 and *BDNF exon IV* by promoting the extent of MeCP2 phosphorylation. (A) Enrichment of *BDNF exon IV* by RACK1 detected by ChIP assay. (B) Extent of MeCP2 phosphorylation after rMS treatment detected by Western blot analysis relative to β-actin. $*p < 0.05$ *vs* the OGD group (neurons treated with OGD alone). (C) Extent of MeCP2 phosphorylation in the presence of NR2A specific inhibitor (NVP-AAM077) or NR2B specific inhibitor (Ro25-6981) detected by Western blot analysis relative to β-actin. (D) Enrichment of *BDNF exon IV* by RACK1 in the presence of NR2A specific inhibitor (NVP-AAM077) or NR2B specific inhibitor (Ro25-6981) detected by ChIP assay. $*p < 0.05$ *vs* the OGD + 1 Hz or OGD + 10 Hz group (neurons treated with both OGD and 1 Hz or 10 Hz rMS). Measurement data were expressed as mean ± standard deviation. Comparison among multiple groups was analyzed by one-way analysis of variance. The experiment was repeated 3 times independently

the limitation of in-depth investigation of the molecular mechanisms underlying rMS and rTMS, the current study examined the OGD-treated neurons and rat models of cerebral ischemia and confirmed the neuroprotective effect of rTMS on cerebral ischemia. The mechanism was speculated to involve the interaction between RACK1 and *BDNF exon IV* by MeCP2 phosphorylation.

Initially, the current study demonstrated that rMS and rTMS upregulated the expression of BDNF in primary cultured neurons with OGD and MCAO rats in which the transcription of *BDNF exon IV* was promoted to interact with RACK1 by MeCP2 phosphorylation. *BDNF exon IV* has been regarded as a leading cause of BDNF expression depending on neuronal activity and RACK1 can regulate the exon-specific expression of BDNF expression as a transcription regulator [18]. In addition, RACK1 has been recognized to regulate chromatin remodeling, leading to the exon-specific expression of BDNF, the mechanism of which might influence the mediation of neuronal functions [18]. A previous study has pointed out that BDNF upregulation can be induced by MeCP2 phosphorylation [31]. Another study speculated that the methylation of *BDNF exon IV* CpG-87 may cause the specific MeCP2 binding [32]. In consistency with our results, significantly higher BDNF mRNA expression has been found in the cortex after rTMS than contralateral one [33]. Similarly, the mechanism of BDNF upregulation has been previously elucidated to be related to rTMS in the hippocampus [34], which was additionally confirmed in the current study.

Importantly, the MCAO-induced cerebral ischemia model in rats showed that the neuropsychological assessment results of rats treated with 1 Hz or 10 Hz rTMS were more satisfactory than those without rTMS treatment. Furthermore, our findings indicated that rTMS was effective in the treatment of cerebral ischemia as evidenced by lower neurological deficit scores, inhibited neuronal apoptosis, and smaller cerebral infarction area. Following ischemic insult, if neurons die, it is troublesome to reconstruct the damaged neural circuits, which is also the key to effective treatment [35]. Evidences have highlighted that the repeated magnetic pulses caused by rTMS are capable of modifying the neural activity and behavior and rTMS possesses the ability to reconstruct functions of the injured neural network [36, 37]. Largely in agreement with the observations of our study, a MCAO-induced rat model of transient cerebral ischemia following rTMS was observed with higher neurological scores and reduced infarct volumes of cortex and striatum [38]. Because the MCAO model of stroke is known to result

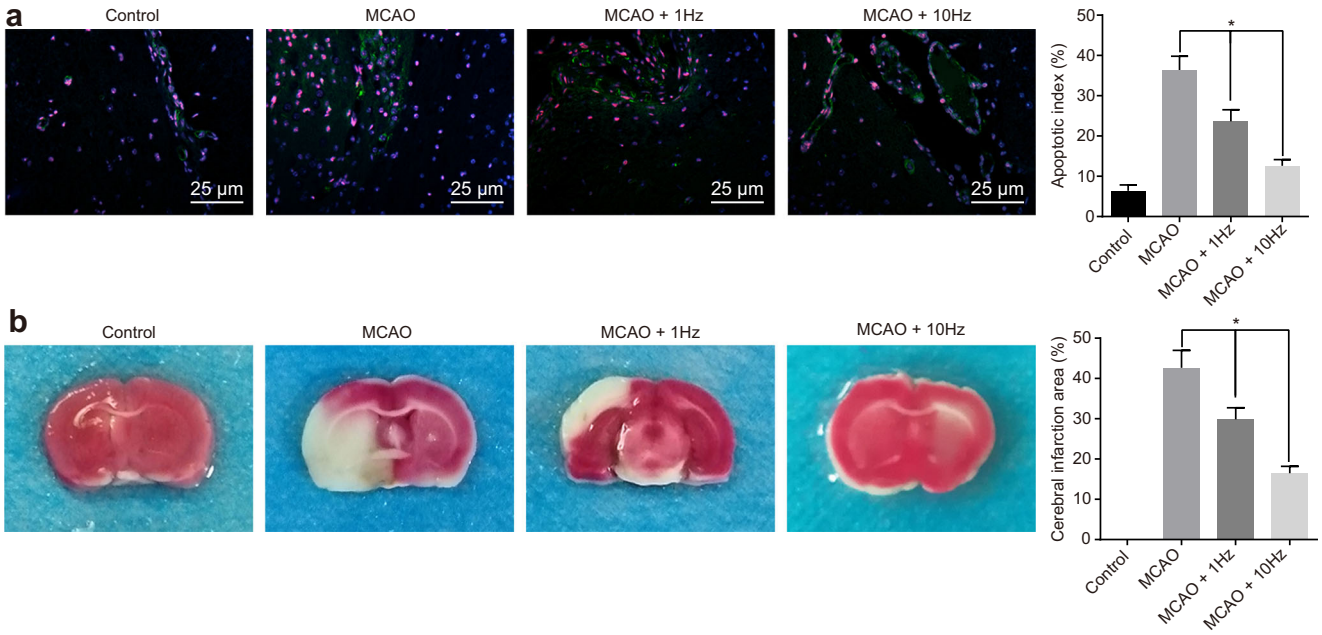

Fig. 5 rTMS of different frequencies leads to improved neurologic function in MCAO rats. (A) Neuron apoptosis in the cortex tissue identified by immunofluorescence (× 400). (B) Cerebral infarction area identified by TTC staining. *$p < 0.05$ *vs* the MCAO group (rats that underwent MCAO). Measurement data were expressed as mean ± standard deviation. Comparison among multiple groups was analyzed by one-way analysis of variance. $n = 5$

in heterogeneous infarcts of various sizes, therefore comparing infarct sizes post intervention in order to demonstrate neuroprotective effects is controversial. The large differences in infarct size between treatment groups could even suggest a problem with randomization prior to treatment. Because neurological function largely depends on

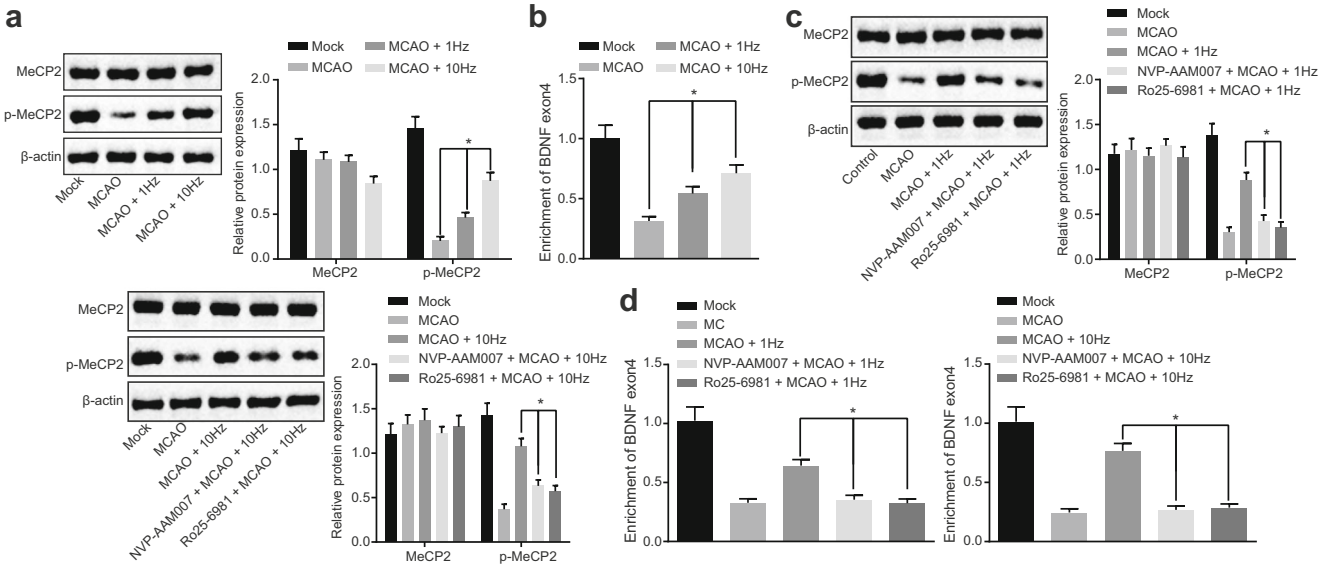

Fig. 6 rTMS promotes the interaction between RACK1 and *BDNF exon IV* by increasing the extent of MeCP2 phosphorylation in MCAO rats. (A) Extent of MeCP2 phosphorylation detected by Western blot analysis relative to β-actin. (B) Enrichment of *BDNF exon IV* by RACK1 detected by ChIP assay. *$p < 0.05$ *vs* the MCAO group (rats that underwent MCAO alone). (C) Extent of MeCP2 phosphorylation in the presence of NR2A specific inhibitor (NVP-AAM077) or NR2B specific inhibitor (Ro25-6981) detected by Western blot analysis relative to β-actin. (D) Enrichment of *BDNF exon IV* by RACK1 in the presence of NR2A specific inhibitor (NVP-AAM077) or NR2B specific inhibitor (Ro25-6981) detected by ChIP assay. *$p < 0.05$ *vs* the MCAO +1 Hz or MCAO +10 Hz group (rats that underwent MCAO and 1 Hz or 10 Hz rTMS). Measurement data were expressed as mean ± standard deviation. Comparison among multiple groups was analyzed by one-way analysis of variance. $n = 5$

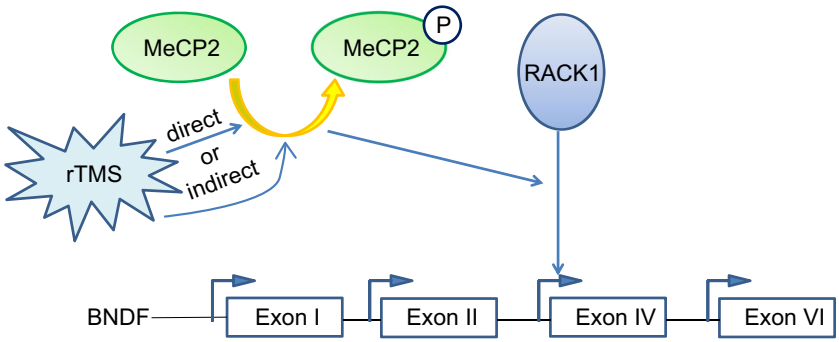

Fig. 7 Mechanistic diagram depicting the effect of rTMS on cerebral ischemia via BDNF. In cerebral ischemia, rTMS increases the extent of MeCP2 phosphorylation, further promoting the interaction between RACK1 and *BDNF exon IV*, whereby neurologic deficits are improved

lesion size, these thoughts apply to the behavioral data as well. However, owing to limited time and cost, our study fails to further demonstrate these issues with a comment on technicality and study design, which will be the focus of our future study. Likewise, the anti-apoptotic property of rTMS has been illustrated in rat models of subacute cerebral ischemia [39], supporting the presence of a regulatory mechanism of rTMS in cerebral ischemia. Additionally, another study indicated that the motor function and disabilities caused by ischemic stroke can be improved sustainably by 1 Hz or 5 Hz rTMS [40]. Notably, low-frequency rTMS (1 Hz) has been considered to be efficient in patients suffering from aphasia after the occurrence of stroke [41]. The frequency-specific influences on the nodes of the motor network induced by rTMS have been identified to exert its optimal effects at a frequency of 5 Hz [42]. However, in an ischemic rat model, better effects were detected considering neurogenesis enhancement with frequency of rTMS being 20 Hz via the BDNF/TrkB signaling pathway [6]. The circumstantial evidence in our study suggested that the application of a stimulation of 10 Hz was better than 1 Hz, indicating the need of further research to determine the optimum frequency. Moreover, a 6-year follow-up research of patients with depression that underwent rTMS shows the great clinical significance of rTMS considering its advantages of safety and efficacy, highlighting the potential role of rTMS as a useful treatment alternative [43].

Taken together, the findings of the current study strongly support the notion that rTMS exerts neuroprotective effects in cerebral ischemia and is capable of improving neurological deficits by promoting the interaction between RACK1 and *BDNF exon IV* through the increase of the extent of MeCP2 phosphorylation (Fig. 7). Ultimately, our study sheds new light on a novel therapeutic target for future treatment of cerebral ischemia from on-bench evidence to clinical situations. However, rTMS protocols deserve more rigorous researches to optimize technical realization and to provide proper care of patients as well as to increase success rate [44], thereby taking the findings of the study to the next level theoretically or practically.

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Authors' Contributions HZL and JQS designed the study. CLZ collated the data and designed and developed the database. RLL and JPC carried out data analyses and produced the initial draft of the manuscript. HZL, JQS, and XJZ contributed to drafting the manuscript. All authors contributed to the revision and approved the final manuscript.

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Compliance with Ethical Standards

Animal use and experimental procedures were carried out with the approval of the Experimental Animal Ethics Committee of Integrated Hospital of Traditional Chinese Medicine, Southern Medical University and the Affiliated Changzhou No. 2 People's Hospital of Nanjing Medical University. All experimental animals operating procedures strictly conformed to the recommendations in the Guide for the Care and Use of Laboratory Animals. Best efforts were made to minimize the suffering of the included animals

Conflict of Interest The authors declare that they have no conflict of interest.

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