rTMS pre-treatment effectively protects against cognitive and synaptic plasticity impairments induced by simulated microgravity in mice

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GRAPHICAL ABSTRACT

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ABSTRACT

During space flight, microgravity has several negative effects on cognitive functions and learning and memory abilities. However, there are few effectively preventive methods that have been developed yet. Previous studies showed that repetitive transcranial magnetic stimulation (rTMS), as a novel non-invasive technique, alleviated cognitive dysfunctions and facilitated synaptic plasticity. In the present study, we used a hindlimb unloading (Hu) mouse model to simulate microgravity conditions. And then, we investigated whether rTMS played a neuroprotective role in a Hu mouse model. Behavioral experiments including Open field test and Novel object recognition test were performed. These results showed that spontaneous activity and recognition memory were reduced by Hu, while rTMS significantly protected against the harmful effect. Furthermore, electrophysiological recordings were performed to examine the level of synaptic plasticity including paired-pulse facilitation (PPF) and long-term potentiation (LTP). In the hippocampus DG and CA1 regions, dendritic spine density was measured using Golgi-Cox staining. Our data showed that rTMS effectively impeded the impairment of PPF and LTP, as well as the decrease of spine density induced by Hu. Subsequently, Western blot assay showed that rTMS inhibited the downregulation of CREB/BDNF signaling network associated proteins in Hu mice. It suggests that rTMS pre-treatment plays a neuroprotective role in protecting against cognitive impairments and synaptic plasticity deficits induced by microgravity stimulation.

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1. Introduction

Microgravity is a part of space environment stress, inducing severe physiological and mental alterations in astronauts. Cognitive deficits may be caused by long-term space flight, which can affect human performance [1]. Laboratory animals, including mice and rats, have been used to perform the investigation of microgravity simulation effects on physiology [2]. Since a variety of limitations of spaceflights, a hindlimb unloading (Hu) model is generally used to simulate microgravity conditions [3]. It was developed in 1980s and used to reproduce a cephalad blood and fluid shift, in which the effects were same as microgravity in various organ systems such as the cardiovascular, immune, and nervous systems [4]. A considerable evidence supports that long-term microgravity simulation in space can also modulate neuronal synaptic plasticity in mice [5]. Moreover several studies showed that exposing to microgravity in rodents could impair learning and memory [6] by altering levels of biomolecules involved in an enhanced ROS level, decreased BDNF expression or changed neurotransmitters level [7–9]. One of our previous studies also showed that the expressions of NR2A and NR2B were significantly decreased in Hu mice [10].

Repetitive transcranial magnetic stimulation (rTMS) is a non-invasive technique of brain stimulation, which can generate a long-term effect in specific region such as hippocampus based on Faraday law of electromagnetic induction. Repeated stimulation with a stripe of magnetic pulses produces a regular magnetic field to generate ion movement and neuronal depolarization, and then modulate brain functions [11]. High-frequency rTMS significantly enhanced the hippocampal neuronal synaptic plasticity and spatial learning via the increase of the levels of NAMD receptors and synapse-associated proteins (SAPs) in the normal rat’s hippocampus [12]. Low-frequency rTMS has been considered as an effective approach to ameliorate cognitive function and synaptic plasticity in AD mouse [13]. Moreover, rTMS is associated with the improvement of memory dysfunction in Human [14]. It suggests that rTMS could be an efficient approach to prevent the harmful effects of Hu on learning and memory.

In the present study, we hypothesized that rTMS pre-treatment played a neuroprotective role in improving cognitive dysfunctions induced by simulated microgravity. Accordingly, we established a hindlimb unloading mouse model and examined if there was a preventively neuroprotective effect of 15 Hz rTMS pre-treatment on cognition and synaptic plasticity in the Hu mouse model. Open field test and novel object recognition (NOR) test were performed to evaluate the cognition and learning and memory abilities. Afterwards, the long-term potentiation (LTP) and paired-pulse facilitation (PPF) from the hippocampal Perforant Path (PP) to Dentate Gyrus (DG) region were recorded. In addition, Golgi-Cox staining and Western blot assay were used to examine spine density and the expression level of NR2B, CAMKII, CREB, BDNF, TrkB, SYP and PSD95.

2. Materials and methods

2.1. Animal care

Eighteen male C57BL/6 mice, six-week-old, were purchased from the Laboratory Animal Center, Academy of Military Medical Science of People’s Liberation Army. The animals were housed in cages at a constant temperature (25 ± 2 °C) under a 12 h light/dark cycle (lights on at 7 a.m.) with ad libitum access to food and water. All animals were reared individually in one cage. All experiments were approved by the Animal Research Ethics Committee, School of Medicine, Nankai University (20,160,004), and performed in accordance with the Animal Management Rules of the Ministry of Health of the People’s Republic of China. Every effort was made to minimize animal suffering and the number of animals.

2.2. Repetitive transcranial magnetic stimulation procedure

Mice were randomly divided into three group: Sham group (n = 6), Hu group (n = 6) and rTMS with Hu group (rTMS+Hu, n = 6). After three-day habituation, the mice in the rTMS + Hu group were treated with rTMS for two weeks continuously. A commercially available stimulator, Rapid2 Magstim magnetic stimulation device (UK), was used. The rTMS performance was done as described [15]. Biphasic magnetic pulses were delivered: pulse rise time, 60 μs; pulse duration, 250 μs. The strength of the pulse was 90% of the maximum output of the machine. Animals were gently held by hand with the aid of a plastic cylinder, and a small figure 8-shaped coil for rodents (5 cm ϕ) was then placed over the skull with a direct contact to the head skin. One session (15 Hz, 20 s) of rTMS was applied daily (between 2:00 and 4:00 p.m.). The animals in the both Sham and Hu groups were handled similarly to those treated with rTMS. They heard the same click sound but did not receive the stimulation.

2.3. Hindlimb unloading procedure

Mice in the Hu group were treated with the hindlimb unloading. The hindlimb unloading procedure was conducted for 14 days from the 15th day to the 28th day, as described in a previous study [10]. In a nutshell, the animal was placed in a transparent plastic cage with detachable roof. The tail was cleaned, air dried and bound with medical adhesive tape which attached a small metal ring to the proximal portion of the tail. A small hook was linked to the metal ring through a string and it was attached to a stainless bar across the opening of the cage. At last, the mice were tilted at ~30 head down from the horizontal plane and could get food and water free. At the same time, the mice in the other two groups took the same metal ring as the Hu group but moving on four limbs.

2.4. Behavioral tests

2.4.1. Open field test

Open field tests were carried out with a pervious guideline [16] after two-day adaptation to the lab environment. Mice were placed individually in a corner of a painted wood box (40 × 40 × 40 cm) with an open top and a white floor for 5 min. The floor was divided into 16 equal squares. Rearing was defined as standing upright on hind legs, and crossing was defined as an animal going to central area from the peripheral area or to peripheral area from central area with both front paws. Any rearing, crossing and the speed in the open field were recorded.

2.4.2. Novel object recognition

NOR tests were performed as described in detail [17]. There are two phases in NOR tests: training and testing. Before the test, mice were allowed to acclimate the apparatus of NOR tests for 10 min 1 day. During the training phase, two identical sample objects were put at the backside of left and right corners of the apparatus. Then animals were placed in the apparatus to explore for 10 min. After a training-to-testing interval of 2 h, one of the sample objects was replaced by a novel one. Mice were allowed to explore the apparatus 5 min. The contact durations of both objects and average speed in two phases were recorded by video recording equipment. The total contact duration was the sum of contact duration of familiar object and novel object in testing phase. The discrimination index (DI, ratio of contact duration of the novel object and total contact duration) was qualified subsequently.

2.5. In vivo electrophysiological recordings

After behavioral test, electrophysiological recordings were performed from the hippocampal PP to DG region on the basis of previous studies [18]. Mice in the three groups (Sham: n = 6, Hu: n = 6, and...
rTMS + Hu: n = 6) were fixed by a stereotaxic frame (SN-3, Narishige, Japan) after anesthetized with urethane (1.2 g/kg body weight). An incision at the electrode inputting region was made and a small hole on the skull was drilled by a dental drill on the brain. After that, a bipolar stimulating electrode was slowly implanted into PP (3.8 mm posterior to the bregma, 3.0 mm lateral to midline, 1.5 mm ventral below the dura) and the recording electrode was positioned into DG (2.0 mm anterior to the bregma, 1.4 mm lateral to midline, 1.5 mm ventral below the dura). Initially, a stable normalized baseline was recorded for 20 min and then the test stimuli were made every 30 s at an intensity (range 0.3–0.5 mA) which can evoke a response of 50% of its maximum amplitude. Pairs of stimuli with inter-stimulus intervals (ISI) of 50, 100, 150 and 200 ms were applied to evoke paired pulse facilitation (PPF) and 4 trials were recorded at each interval. The ratio of the second pulse-evoked EPSP slope to the first evoked was used to determine the percentage PPF (%). Afterwards, a theta burst stimulation (TBS) consisting of 30 trains of 12 pulses (200 Hz) at 5 Hz was used to evoke LTP. Following that, the single-pulse stimulation was resumed to collect the evoked response every 60 s for 1 h at the baseline intensity (Scope software, PowerLab; AD Instruments, New South Wales, Australia). Initial data were measured using Clampfit 10.2 (Molecular Devices, Sunnyvale, CA, USA).

After in vivo recordings, the contralateral hippocampi that were not electrophysiologically recorded were randomly chosen for Golgi–Cox staining (Sham group: n = 3, Hu group: n = 3, and rTMS + Hu group: n = 3) or Western blotting assay (Sham group: n = 3, Hu group: n = 3, and rTMS + Hu group: n = 3).

2.6. Golgi-Cox staining

According to the previous description [19] Golgi–Cox staining was performed. Firstly, the brain of mouse was removed from skull quickly and carefully from the anesthetized animals with sodium pentobarbital. Following that, the brain was sectioned into 120 μm thick coronal slices with a vibratome (Campden Instrument Ltd., MA752, England). After that, the slices were transferred into 6% sodium carbonate solution (pH = 10.06) for 20 min and then were treated with varying concentrations of alcohol: 70% alcohol (10 min), 90% (15 min), and absolute alcohol (20 min). Then the slices were cleaned by xylene for 20 min. Finally, the slices were mounted on slides with DPX and cover glassed. Then the dendrite spines in DG and CA1 region were evaluated morphologically using a light microscope (Magnus MLX) after dried naturally at room temperature.

2.7. Western blot assay

The Western blot assay was performed [12]. Briefly, the mouse hippocampus was mashed by a grinder and homogenized in lysis buffer (Beyotime Biotechnology, Haimen, China) containing Phenylmethylsulfonyl fluoride (PMSF, 1:100 dilutions). Then the homogenates were centrifuged at 12,000 rpm for 20 min at 4 °C and the supernatant was collected. The concentration of protein was determined by using enhanced BCA protein assay kit (Beyotime Biotechnology, Haimen, China). Next, the same amount of hippocampal protein lysates (40 μg) was electrophoresed by SDS-PAGE 10–13% gels and then transferred onto 0.45 μm polyvinylidene difluoride (PVDF) membranes (Millipore Corporation) at 4 °C. Subsequently, the PVDF membranes were blocked in Tris-buffered saline with Tween-20 (TBST) including 5% skimmed milk for 1.5 h at room temperature. They were incubated at 4 °C overnight with primary Rabbit anti-mouse polyclonal antibodies (β-actin, 1:2000 dilution; Anti-NR2B, 1:1000 dilution; CAMKII, 1:2000 dilution; p-CAMKII, 1:2000 dilution; CREB, 1:2000 dilution; p-CREB, 1:2000 dilution; BDNF, 1:1000 dilution; SYP, 1:2000 dilution; TrkB, 1:2000 dilution; PSD95, 1:1000 dilution), which were purchased from Abcam (Cambridge, UK). After washed by TBST for 4 × 10 min, the membranes were incubated with secondary HRP-conjugated antibodies of anti-rabbit (1:2000 dilution; Cell Signaling Technology, Beverly, MA, USA) for 1 h at room temperature. They were washed by TBST for 4 × 10 min again and then the protein band intensities were detected with HRP substrate (Millipore, USA) by computerized chemiluminescent imaging system (Tanon Science & Technology, China) and quantified by ImageJ program.

The timeline of all above experimental procedures was shown in Fig. 1.

2.8. Data and statistical analysis

The statistical analyses were performed by using SPSS (version 13.0) software. All the data were presented as mean ± SEM. The normality was verified by Kolmogorov-Smirnov test and the homogeneity of variance was assessed with Levene’s test for the data of each group. Data from behavioral tests, Western blot assay, Golgi–Cox staining and LTP recordings were further analyzed by using a one-way ANOVA followed by Bonferroni post hoc test. The body weight and PPF data were analyzed by using a two-way repeated-measures ANOVA followed by Bonferroni post hoc test for multiple comparisons. Differences with p < 0.05 were considered statistically significant. The correlation analysis was carried out by Pearson correlation analysis. Significant differences were defined at p < 0.05.

3. Results

3.1. rTMS pre-treatment protected against cognitive and memory impairments induced by Hu

There was no statistical difference of body weight among all three groups during the treatment (Fig. 2A, day * group interaction F (14,210) = 1.423, group F (2,15) = 0.043, p > 0.05). In order to detect the effect on emotion and cognition ability, the mice were examined by Open Filed tests (Fig. 2B). The data showed that the three groups had no significant difference in average speed (Fig. 2C, F (2,15) = 0.167, p > 0.05). There were statistical differences of the duration in the center square among three groups (Fig. 2D, F (2,15) = 4.017, p < 0.05). The mice in the Hu group spent much less time in the center square than that in the Sham group (Fig. 2D, F (2,15) = 4.017, p < 0.05). It was found that the numbers of both rearing and crossing had statistical differences among three groups (Fig. 2E, F (2,15) = 24.929, p < 0.001; Fig. 2E, F (2,15) = 7.583, p < 0.01). Which were significantly decreased in the Hu group.
compared to that in the Sham group (Fig. 2E, p < 0.001; Fig. 2F, p < 0.05). Interestingly, rTMS significantly increased them in the rTMS+Hu group compared to that in the Hu group (Fig. 2E, p < 0.001; Fig. 2F, p < 0.01).

In order to detect the effect of rTMS on recognition memory abilities, the mice were examined by Novel Object Recognition tests (Fig. 2G). The data showed that there was no statistical difference of speed among all three groups (Fig. 2H, F(2,15) = 0.928, p > 0.05). However, the percentage of discrimination index (Fig. 2I, F(2,15) = 7.445, p < 0.01) was significantly reduced in the Hu group compared to that in the Sham group (Fig. 2I, p < 0.01), while it was increased in the rTMS+Hu group compared to that in the Hu group (Fig. 2I, p < 0.05).

Pearson correlation analysis showed that there was an evidently positive correlation between the total number of rearing and the total number of crossing in Open Field Tests (Fig. 2J, r^2 = 0.6303, p < 0.001). And there were obviously positive correlations between the discrimination ratio and the total number of rearing (Fig. 2K, r^2 = 0.7450, p < 0.001), as well as between the discrimination ratio and the total number of crossing (Fig. 2L, r^2 = 0.7130, p < 0.001).

3.2. rTMS pre-treatment protected against synaptic plasticity deficits induced by Hu

After the behavior tests, the electrophysiological recordings were performed. To examine postsynaptic function, LTP was measured. Fig. 3A shows the time course of in vivo electrophysiological recordings for these three groups. As shown in the inset of Fig. 3A, there are examples of fEPSP under baseline and stimulation conditions from three mice, one for Sham, one for Hu, and another for rTMS+Hu, respectively. It can be seen that the slopes are stable and congruous among these three groups, suggesting that recording and stimulating electrodes are placed properly. All fEPSP slopes were immediately enhanced after TBS. The mean slopes of fEPSP in last 15 min were evaluated as LTP level for each mouse. The data showed that the LTP level (Fig. 3B, F(2,15) = 524.129, p < 0.001) was significantly decreased in the Hu group (Fig. 3B, p < 0.001), which was considerably increased in the rTMS+Hu group compared to that in the Hu group (Fig. 3B, p < 0.001). In order to clarify if the presynaptic mechanism was involved in the effects of both Hu and rTMS on the hippocampus synaptic plasticity, the PPF was measured. A two-way repeated measures ANOVA showed that there was no significant interaction between ISI*group (F(3,45) = 0.682, P > 0.05). Bonferroni post hoc test showed that there were significant differences of the ISIs between all pair of groups (F(2,15) = 7.335, P < 0.05). And the PPF level was same as the LTP level, decreasing in the Hu group compared to that in the Sham group (Fig. 3C, p < 0.01 (ISI = 50 ms); p < 0.05 (ISI = 100 ms); p < 0.001 (ISI = 150 ms); p < 0.01 (ISI = 200 ms)), however, increasing in the rTMS+Hu group compared to that in the Hu group (Fig. 3C, p < 0.01 (ISI = 50 ms); p < 0.05 (ISI = 100 ms); p < 0.01 (ISI = 150 ms); p < 0.05 (ISI = 200 ms)).

Fig. 3D shows the representative pictures of tertiary dendritic profile of granule cells in the hippocampal DG and CA1 regions, obtained from Golgi–Cox staining (bar = 20 μm). And there were statistical differences of spine density in both DG (Fig. 3E, F(2,6) = 14.641, p < 0.01) and CA1 (Fig. 3E, F(2,6) = 34.049, p < 0.001) area. The
spine density was lower in the Hu group than that in the Sham group in the DG area (Fig. 3E, p < 0.05), and in the CA1 area (Fig. 3F, p < 0.001). However, it was higher in the rTMS+Hu group than that in the Hu group in the DG area (Fig. 3E, p < 0.01), and in the CA1 area (Fig. 3F, p < 0.001).

3.3. rTMS pre-treatment protected against CREB/BDNF signaling network downregulation induced by Hu

We measured the proteins expressions, which were associated with NR2B-CAMKII-CREB signaling pathway, the downstream proteins BDNF and SYP and the synaptic function-associated proteins TrkB and PSD95 by Western Blot assay. The results showed that the expression level of all proteins and/or the relative levels of protein phosphorylation had statistical differences among three groups (Fig. 4A-G, F(2,6) = 18.573, p < 0.01, NR2B; F(2,6) = 10.844, p < 0.01, p-CAMKII/CAMKII; F(2,6) = 15.066, p < 0.01, p-CREB/CREB; F(2,6) = 187.906, p < 0.001, BDNF; F(2,6) = 13.567, p < 0.01, SYP; F(2,6) = 206.503, p < 0.001, TrkB; F(2,6) = 15.904, p < 0.01, PSD95).

Specifically, they were significantly decreased in the Hu group compared to that in the Sham group (Fig. 4A-G, p < 0.01, NR2B; p < 0.05, p-CAMKII/CAMKII; p < 0.01, p-CREB/CREB; p < 0.001, BDNF; p < 0.01, SYP; p < 0.001, TrkB; p < 0.01, PSD95). However, all of them were significantly upregulated by rTMS in the rTMS+Hu group compared to that in the Hu group (Fig. 4A-G, p < 0.01, NR2B; p < 0.05, p-CAMKII/CAMKII; p < 0.05, p-CREB/CREB; p < 0.001, BDNF; p < 0.05, SYP; p < 0.001, TrkB; p < 0.05, PSD95).

Moreover, Pearson correlation analysis showed that there were prominently positive correlations between the relative level of CREB phosphorylation and the expression level of NR2B (Fig. 4H, r² = 0.8678, p < 0.001); between the expression level of BDNF and the relative level of CREB phosphorylation (Fig. 4I, r² = 0.8221, p < 0.01); between the expression level of SYP and the relative level of CREB phosphorylation (Fig. 4J, r² = 0.8037, p < 0.001), and between the expression level of PSD95 and the expression level of TrkB (Fig. 4K, r² = 0.8092, p < 0.01).

3.4. Measurements of correlation between proteins and synaptic plasticity

We focused on NR2B, CREB and BDNF, which are vital nodes in the signaling network. Pearson correlation analysis showed that there were clearly positive correlations between the relative level of CREB phosphorylation and the LTP level (Fig. 5A, r² = 0.8624, p < 0.001, ISI = 100 ms), the PPF level (Fig. 5B, r² = 0.6514, p < 0.01, ISI = 100 ms), the discrimination ratio (Fig. 5C, r² = 0.7617, p < 0.01), and the rearing (Fig. 5D, r² = 0.9818, p < 0.001). The data further showed that there was a significantly positive correlation between the LTP level and the expression of NR2B (Fig. 5E, r² = 0.8181, p < 0.01, NR2B), and BDNF (Fig. 5F, r² = 0.9507, p < 0.001, BDNF). The correlation between the synaptic function-associated proteins and synaptic plasticity was also measured. The data showed that there was a positive correlation between the LTP level and the PSD95 expression (Fig. 5G, r² = 0.8391, p < 0.01), as well as between the PPF level and the SYP expression (Fig. 5H, r² = 0.7339, p < 0.01).
4. Discussion

In the present work, we explored if rTMS pre-treatment could play a neuroprotective role in a simulated microgravity mouse model. The data showed that rTMS pre-treatment effectively protected against the impairments of cognitive functions and synaptic plasticity, which were induced by hindlimb unloading. Moreover, it was found that the underlying mechanism of the preventively neuroprotective effects was possibly associated with CREB/BDNF signaling network.

First of all, we established a hindlimb unloading mouse model, by which microgravity-induced cognitive deficits were simulated. And then, we chose 90% of the maximum output of the machine as a stimulation intensity, since the effect of high stimulation intensity was more obvious and stable [20,21]. The rTMS effect could be affected by not only the stimulation intensity, but also the stimulation frequency. A previous study showed that the rTMS treatment tended to be an inhibitory effect at low stimulation frequencies ( < 1 Hz) [22]. Whereas, it is thought to have an excitatory effect at high-frequency ( > 1 Hz), [23]. Accordingly, the stimulation at 15 Hz frequency has been applied in establishing rTMS+Hu model in this study.

Behavioral tests are designed to assess the emotion, cognition functions and learning and memory abilities [16,17]. It has been reported that rat’s cognitive functions and spatial learning and memory are impaired by simulated weightlessness [8]. Our data showed that the spontaneous activity was evidently reduced and the recognition ability was significantly impaired by Hu, while the impairments were efficiently protected against by rTMS pre-treatment. It was in accord with the effect of rTMS on Parkinson’s disease and Alzheimer’s disease.

Fig. 4. The measurements of proteins level. A. Mean expressions of NR2B. B. Mean relative levels of phosphorylation of CAMKII. C. Mean relative levels of phosphorylation of CREB. D. Mean expressions of BDNF. E. Mean expressions of SYP. F. Mean expressions of TrkB. G. Mean value of PSD95 expressions. H. A relative expression between NR2B and CREB (r² = 0.8678 p < 0.001). I. A relative expression between BDNF and CREB (r² = 0.8221 p < 0.01). J. A relative expression between SYP and CREB (r² = 0.8037 p < 0.01). K. A relative expression between PSD95 and TrkB (r² = 0.8092 p < 0.01).

Data are expressed as mean ± SEM and the representative immunoreactive bands of associated proteins and β-actin are inset in each diagram. *p < 0.05, **p < 0.01, ***p < 0.001 comparison between the Sham group and the Hu group; #p < 0.05, ##p < 0.01, ###p < 0.001 comparison between the Hu group and the rTMS+Hu group; n = 3 in each group.
The duration in center square in Open Field test could be an implicit of anxiety and depression in Hu mice. It was found that rTMS pre-treatment did not play a neuroprotective role in preventing the occurrence of anxiety and depression induced by Hu. Moreover, the data, obtained from the correlation analysis, suggested that the change of spontaneous activity was primarily caused by altered cognition function rather than emotion.

Synaptic plasticity plays a key role in learning and memory [26,27]. LTP is a form of long-term synaptic plasticity. The expressions of them are associated with pre- and/or post-synaptic function changes [28]. Moreover, as we know, PPF is associated with enhanced presynaptic transmitter release during the second paired-pulse [29], representing short-term plasticity and the memory function. PPF and LTP from PP to DG region were recorded to investigate the underlying mechanism of cognition and memory variation. Our data showed that rTMS pre-treatment significantly protected against the impairment of both pre- and post-synaptic plasticity induced by Hu. Furthermore, it is well known that spine is a basic neuronal structure of synapse. The formation and elimination of synapses represent synaptic structural plasticity and are also closely related with learning and memory [26]. Our data showed that there was a harmful effect of DG spine density on the Hu mice, which was in line with chronic ultra-mild stress model [31] and 3×Tg AD model [32]. Nevertheless, the damage was significantly improved in the rTMS + Hu group.

Cyclic AMP response element binding protein (CREB) is known to be a critical transcription factor that is activated by posttranslational modifications such like phosphorylation [33]. The impaired hippocampal LTP has been showed in CREB-/- knock-out mice [34]. Long-term memory deficits has been ameliorated by CREB overexpression in dorsal CA1 in aged rats [35]. Otherwise, the inhibition of CREB disrupted spatial memory [36]. The activation of CREB is triggered by diverse signaling processes, including the activation of Ca^{2+} Channels [37]. Moreover, a relative high level of NR2B and NR2A in the hippocampus is closely related to the high level of LTP [38,39]. The activation of NMDA receptors and CAMKII Pathway can trigger the phosphorylation of CREB [37]. Our data showed that the NR2B expression and the phosphorylation relative level of CAMKII and CREB were significantly downregulated by Hu, while rTMS effectively protected against them. It suggests that either the effect of Hu or the neuroprotective role of rTMS is feasibly associated with NR2B-CAMKII-CREB pathway. The correlation analysis provided further evidences, in which there were positive correlations between NR2B and CREB, CREB and LTP, PPF and behavioral changes, as well as between NR2B and LTP.

CREB, a critical transcription factor, can trigger the promotor of many kinds of synaptic function-associated proteins such as BDNF and SYP and then increase the expression level of them [40]. BDNF is one of the most important family members in brain neurotrophic factor and expressed in several brain regions. BDNF was associated with the hippocampal LTP and could increase spine density [41–43]. In vivo studies, the BDNF levels could be significantly enhanced by rTMS in the hippocampus [20,44], cortex [45,46], and superior colliculus [47]. In the present study, our data show that the expression of BDNF was lower in the Hu group than that in either the Sham group or the rTMS + Hu group, suggesting that the underlying mechanism of neuroprotection was possibly associated with the BDNF level. As a presynaptic vesicle protein and a molecular marker of presynaptic density, SYP affects the release of neurotransmitters [48] and plays an important role in synaptic plasticity [49]. Our data were similar with the results of BDNF and CREB. The correlation analysis showed that there was a positive correlation between BDNF and CREB as well as SYP and CREB. It implies that BDNF and SYP are possibly regulated by transcription factor CREB. Moreover, there was a positive correlation between BDNF/PSD95, as well as between SYP and PPF. Accordingly, the mechanism of rTMS-neuroprotection on synaptic plasticity was possibly associated with CREB signaling pathway. Besides, it is reported that BDNF needs to bind to TrkB, a cell surface receptor of BDNF, to perform functions and the activation of TrkB can increase the open probability of NMDA receptor [50]. BDNF/TrkB signaling can promote PSD95 recruitment [51]. The functions of PSD95 include maintaining the PSD size, providing mechanical support to synaptic strength and membrane protein like NMDA receptors and contributing to LTP [41]. In this study, we measured the BDNF/TrkB signaling pathway as the downstream of CREB signaling. The results showed that the expression levels of both TrkB and PSD95 were similar with the expression level of BDNF. In addition, the positive correlation between BDNF and PSD95 suggested that PSD95 was involved in this pathway. Therefore, an underlying mechanism of rTMS-neuroprotective role in the cognition functions is possibly associated with at least two signaling pathways, which are the NR2B-CAMKII-CREB and the BDNF/TrkB.

In one of our previous studies, it was found that high frequency rTMS was able to increase the level of BDNF expression and synapse-associated protein expression in Wistar rats, which was probably
associated with the enhancement of working memory [12]. There are also several other studies related to rTMS applied in healthy human, reporting that rTMS can improve the memory performance [14,52]. Nevertheless, it is not always beneficial to increase hippocampal BDNF and synaptic efficacy as these are in fact the basis of hippocampal kindling in the development of seizure disorders.

5. Conclusions

In summary, rTMS pre-treatment could somehow suppress the downregulation of CREB/BDNF signaling network in Hu mice. Since the presynaptic protein (SYV) and postsynaptic protein (NR2B, PSD95, TrkB) were enhanced, the release of neurotransmitters was increased and the NMDA receptor channel open frequently. And the effectiveness of the synapses was further facilitated. Ultimately, rTMS pre-treatment played a preventively neuroprotective role in protecting against cognitive impairment induced by Hu.

Conflict of interest

The authors declare no conflict of interest.

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