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Low-frequency (1Hz) repetitive transcranial magnetic stimulation (rTMS) reverses $A\beta_{1-42}$ -mediated memory deficits in rats



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ABSTRACT

Accumulating evidence shows the disruption of hippocampal neurotrophins secretion leads to memory deficits in Alzheimer's disease (AD) animal models. Invasive injection of exogenous neurotrophins into hippocampus reverses spatial memory deficits, but its clinical application is limited by traumatic brain injury during the injection procedure. Notably, recent studies have demonstrated that noninvasive repetitive transcranial magnetic stimulation (rTMS) increases endogenous neurotrophins contents in the brain of normal rats. Whether low-frequency rTMS can reverse $A\beta_{1-42}$ -mediated decrease in hippocampal neurotrophins contents and spatial memory impairment is still unclear. Here, we reported that severe deficit in long-term potentiation (LTP) and spatial memory were observed in an $A\beta_{1-42}$ -induced toxicity rat model. Furthermore, neurotrophins (NGF and BDNF) and NMDA-receptor levels were decreased after $A\beta$ injection. However, low-frequency rTMS markedly reversed the decrease in neurotrophins contents. And the rTMS-induced increment of neurotrophins up-regulated hippocampal NMDA-receptor expression. Moreover, low-frequency rTMS noninvasively and effectively increases hippocampal neurotrophins and NMDA-receptor contents in $A\beta_{1-42}$ -induced toxicity model rats, which helps to enhance hippocampal LTP and reverses $A\beta_{1-42}$ -mediated memory deficits.

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1. Introduction

Irreversible memory decline associated with Alzheimer's disease (AD) is the most common form of dementia in the elderly (Haffen et al., 2011). A host of animal models have been proposed with relevance to model the pathological features including deposits of amyloid-B peptide (AB) and neurofibrillary tangles (NFTs) which characterize AD brain. AB oligomers (main components: $A\beta_{1-40}$ and $A\beta_{1-42}$) are the typical endogenous neurotoxic substances, which not only suppress endogenous neurotrophins contents in vitro (Colaianna et al., 2010) and in vivo (Holsinger et al., 2000), but also cause synaptic dysfunction in the early stage of AD (Shankar and Walsh, 2009). Intra-hippocampal injection with $A\beta_{1-42}$ in mice or rats has been used as an animal model which shows noticeable senile plaques formation associated with persistent memory decline (Nomura et al., 2012). Brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) belong to the family of neurotrophins characterized by the ability to regulate diverse neuronal responses, including augment the type and number of afferent synapses by promoting the survival of discrete neuronal

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subpopulations (Connor and Dragunow, 1998). Strikingly, injection with exogenous neurotrophins (NGF or BDNF) into hippocampus significantly reverses spatial memory deficits in transgenic AD mice (Nilsson et al., 2010; Tian et al., 2012). However, exogenous neurotrophins can't permeate into blood-brain-barrier, its clinical application is limited by lacking of feasible methods for delivering neurotrophins into brain. Developing a simple and practical means of delivering neurotrophins into the brain continuously and safely has presented new significant challenges and opportunities (Covaceuszach et al., 2009).

Notably, recent studies have identified that noninvasive repetitive transcranial magnetic stimulation (rTMS, a method of delivering magnetic stimuli into the brain through the intact scalp) contributes to up-regulate endogenous neurotrophins contents in normal rat brain (Gersner et al., 2011; Wang et al., 2011). Via generating pulses of high-intensity magnetic field by passing brief electric current through an inductive coil, rTMS is able to induce cortical activity and excitability changes that outlast the period of stimulation. The outlasting effect of rTMS on brain are long-term depression (LTD) or long-term potentiation (LTP)-like, because the duration of effects seems to implicate changes in synaptic plasticity (Pilato et al., 2012).

Since the 1980s, as a noninvasive neuromodulation approach, rTMS has been widely used in psychiatry, neurology as well as other clinical specialties (Barker, 1994; Barker et al., 1985). High frequency-rTMS

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has become an important therapeutic tool for part of human neuropsychiatric disorders, such as depression and Parkinson's disease (Gross et al., 2007; Guerra et al., 2011; Wu et al., 2008). rTMS has also been used for pain relief (Wassermann and Zimmermann, 2012) and AD treatment. It is verified that rTMS attenuates $A\beta_{1-42}$ -induced neurotoxicity in hippocampal slices *in vitro* (Kim et al., 2010; Post et al., 1999). Catarina Freitas (Freitas et al., 2011) performed a systematic search of studies using noninvasive stimulation on AD and reviewed all identified articles. Their results show that TMS can induce acute and short-duration beneficial effects on cognitive function. Bentwich (Bentwich et al., 2011) combined high-frequency rTMS (10 Hz, applied on six different brain regions) with cognitive training to treat AD patients, which has proved to be beneficial for improving cognitive ability.

However, high-frequency rTMS may occasionally cause adverse effects, such as stroke and seizure (Wassermann, 1998). And the efficacy and safety of low-frequency rTMS in normal mice/rats and healthy human have been evaluated (Liebetanz et al., 2003; Siebner et al., 2004; Todd et al., 2006). Recently, low-frequency rTMS has been utilized to treat depression and epilepsy in human (Fregni and Pascual-Leone, 2005). Zhang et al. (2007) have reported that low-frequency rTMS augments BDNF secretion in normal rats. Wang et al. (2010) have found that low-frequency rTMS alleviates cognitive impairment in vascular dementia rats model. Since high-frequency rTMS to some extent ameliorates short-term memory of AD (Guerra et al., 2011), it arouses interest in whether low-frequency rTMS has the same effect, which is the main issue to be explored in this research.

NMDA-receptor is a critical molecule that underlies hippocampal LTP and memory formation, for blocking or down-regulation of NMDA-receptor leads to memory impairment (Tang et al., 1999). However, significant reduction in NMDA-receptor subunits expression (including NR1, NR2B and NR2A) has been observed in AD animal model (e.g. APP, APP/PS1 mice), as well as AD patients (Bi and Sze, 2002; Calon et al., 2005; Tsang et al., 2008). Moreover, high-frequency and low-frequency rTMS can up-regulate BDNF and NGF level in normal rats (Gersner et al., 2011; Muller et al., 2000). Other studies have identified that neurotrophins (e.g. BDNF and NGF) can up-regulate hippocampal NMDA-receptor expression of rats *in vitro* and *in vivo* (Bai and Kusiak, 1997; Caldeira et al., 2007; Jarvis et al., 1997). Regulation of neurotrophins and NMDA receptor expression may be the underlying mechanism of low-frequency rTMS affects cognitive function.

In this study, we investigated whether low-frequency rTMS can regulate endogenous neurotrophins contents and rescue spatial memory deficits. At the same time, we also explored the underlying mechanism of low-frequency rTMS affects cognitive function in respect of regulation of NMDA-receptor and synaptic plasticity (LTP).

2. Materials and methods

2.1. Animals

2.5-month-old Male Sprague–Dawley (SD) rats (250–300 g) were obtained from the Experimental Animal Center of Tianjin Medical University, Tianjin, China. Rats were housed two to three per cage with ad libitum access to food and water at room temperature (25 °C), and maintained on a 12/12 h light/dark cycle (lights on at 7:00 a.m.). All experiments were conducted in accordance with ethical procedures and were approved by the Animal Ethics Committee of Tianjin Medical University.

All rats were divided randomly into four study groups (21 rats/group), namely control, control with rTMS, A β injection and A β injection with rTMS group. Rats bilaterally injected with saline (pH 7.4) in dentate gyrus (DG) area of dorsal hippocampus were used as control group. While, rats in A β injection group (treated as A β_{1-42} -induced toxicity rat model) were injected with incubated A β_{1-42} . Rats in control with rTMS group and A β injection with rTMS group were daily treated with one session of low-frequency rTMS between 9:00 and 12:00 a.m. for 14 consecutive days. Three rats of each group were randomly selected for pathological staining experiments, while eight rats for ELISA and Western blot experiments. The other 10 rats were used for Morris water maze test and LTP experiments.

2.2. Preparation of $A\beta_{1-42}$ and $A\beta_{1-42}$ -induced toxicity rat model

 $A\beta_{1-42}$ peptide (Sigma,USA) was prepared as described previously (O'Hare et al., 1999). Briefly, $A\beta_{1-42}$ was freshly prepared from 1 µg/µl soluble $A\beta_{1-42}$ solution, which was dissolved in filtered phosphate buffered saline (PBS: 10 mM NaH₂PO₄–Na₂HPO₄, 100 mM NaCl, dissolved in glass-distilled deionized water, pH = 7.5). $A\beta_{1-42}$ solution was then incubated under vigorous agitation using a Teflon-coated stirbar at 23 °C for 36 h. Following incubation, the incubated $A\beta_{1-42}$ solution was turbid and then for injection.

SD rats were placed in a stereotaxic apparatus under general anesthesia with chloral hydrate (300 mg/kg, i.p.). Incubated $A\beta_{1-42}$ (5 µl, 1 µg/µl) or vehicle (5 µl normal saline, pH = 7.4, used as control) was injected into DG area of dorsal hippocampus bilaterally (anterior posterior, 3.2 mm; lateral, 2.5 mm; horizontal, 3.5 mm from bregma) (Christensen et al., 2008). Rats with spatial memory deficits identified by Morris water maze test were considered as suitable $A\beta_{1-42}$ -induced toxicity model. On the 14th day after $A\beta_{1-42}$ injection, all rats were randomly divided into two groups (n = 21/group): one group as $A\beta$ injection group ($A\beta_{1-42}$ -induced toxicity model) and the other group as $A\beta$ injection with rTMS treatment group.

2.3. Immunohistochemistry staining

Rats for pathological staining experiments were deeply anesthetized with sodium pentobarbital and were transcardially perfused successively with PBS (0.01 mol/l) and fixative (4% paraformaldehyde, 0.2% picric acid, diluted in 0.1 mol/l phosphate buffer, pH = 7.4). Rat brains were dissected out and post-fixed in 4% PFA (diluted in 0.1 mol/l PB buffer) for 24 h at 4 °C (Nomura et al., 2012). Brains were embedded in paraffin and cut into 5 µm-thick coronal sections, which were then subjected to immunohistochemistry staining. For immunohistochemistry staining, sections were dewaxed in xylene and rehydrated in a series of graded alcohols according to histopathological standards. Sections were treated with 3% H2O2 for 30 min to remove residual peroxidase activity, and rinsed again with PBS. Microwave antigen retrieval was applied with slides immersed in 10 mM citrate buffer (pH = 6.0). Slides were blocked with 10% normal goat serum, and incubated with rabbit polyclonal anti-AB1-42 antibody (1:250, Abcam 10148) at 4 °C for overnight and subsequently treated with appropriate biotinylated secondary antibodies (1:200; Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd. Beijing, China). The immunoreactivity was developed using DAB for 3-10 min.

2.4. Application of low-frequency rTMS

From the 14th day after $A\beta_{1-42}$ injection, rats in $A\beta$ injection with rTMS treatment and Con with rTMS group were treated with one session of low-frequency rTMS daily (between 9:00 and 12:00 a.m.) for 14 consecutive days. Highly focusing magnetic-electric stimulator (High-Speed MES-10, Cadwell, Kennewick, WA, USA) with 9-centimeter diameter round coil was used. The coil was connected to MES-10 magnetic-electric stimulator with monophasic current waveform; and it was held centered tangentially to the center of exposed head of rats (which were fixed in suitable cloth sleeves). The pattern of one session rTMS consisted of 20 burst trains, each train contained 20 pulses at 1 Hz with 10-second inter-train intervals, in total 400 stimuli and pulse width was 70 μ s. Stimulation intensity was presented 100% of average resting motor threshold as determined by visual inspection of bilateral forelimb movement in a preliminary experiment (Gersner et al., 2011) in anesthetized rats (20% of the maximum output, 0.4 Tesla).



Fig. 1. $A\beta_{1-42}$ immunohistochemistry in $A\beta_{1-42}$ -induced toxicity model rats and sham operated rats. A: control group (only injected with saline, pH 7.4). B: negative control of $A\beta_{1-42}$ immunohistochemistry in $A\beta$ injection group without incubated with rabbit polyclonal anti- $A\beta_{1-42}$ antibody. C: $A\beta_{1-42}$ immunohistochemistry in $A\beta_{1-42-}$ induced toxicity model rats. Arrows showed $A\beta_{1-42-}$ positive immunostaining area of hippocampus. Amyloid plaques depositions in hippocampus of $A\beta$ injection group were observed. Bar = 100 µm in A1-C1, 50 µm in A2-C2 and 20 µm in A3-C3.

2.5. Morris water maze test

Morris water maze test was carried out on the 28th day by using Morris Water Maze Tracking System (New universe, Beijing, China). Memory-acquisition trials (training) were carried out 3 trainings (started from different quadrants) daily for consecutively 5 days and escape latencies (time to find the submerged platform) were observed. To assess spatial memory retrieval, a 60-second probe trial with the platform removed from the pool was given on day 6. Swimming time spent in the target quadrant (where the platform was located during hidden platform training) was recorded (Filali et al., 2011).

2.6. LTP recording in vivo

LTP-inducing procedures were described as our previous report after 14 day's rTMS treatment (Tan et al., 2011). Briefly, rats were placed in a stereotaxic frame under 20% urethane anesthesia (1.5 g/kg, i.p.). Population spike (PS) was evoked by a tungsten bipolar stimulating electrode which positioned in the perforant path (anterior posterior, 7.5 mm; lateral, 4.4 mm; horizontal, 3.4–4.0 mm from the bregma), and were recorded with a 2 mol/l sodium chloride-filled glass pipette that positioned in the granular cell body region of DG (anterior posterior, 3.7 mm; lateral, 2.5 mm; horizontal, 3.4–3.6 from the bregma) (Abe et al., 2009) by using Axoclamp-2B amplifier (Axon Instruments, USA). Baseline responses were set to 50% of maximal response and recorded for 30 min. LTP was induced by tetanic stimulation (TS, 10 trains, each train contained 20 pulses of 150 µs at 200 Hz, 2-second interval) using Master-8 stimulator (A.M.P.I, Israel). Evoked responses

recorded before (0.5 h) and after (1 h) LTP induction were stored by Clampex 9 (Molecular Devices, Foster City, CA, USA) for analysis of PS amplitude. Responses were normalized to baseline, and data were analyzed using ANOVA (Bruel-Jungerman et al., 2006; Shin et al., 1997).

2.7. Western blotting

After the probe trial of Morris water maze test, rats in each group were sacrificed by decapitation immediately. The whole hippocampus of each rat was dissected out rapidly (frozen on dry ice) and then kept at -80 °C until ready for use. The hippocampi were homogenized (1:10, w/v) in ice-cold tissue lysis buffer (137 mM NaCl, 20 mM Tris-HCl pH = 8.0, 1% NP40, 10% glycerol, 1 mM PMSF, 10 μ g/ml aprotinin and 1 µg/ml leupetin). Homogenates were then centrifuged at 12,000 g for 15 min at 4 °C, and supernatants were collected. Protein concentration was determined using BCA assay kit (Pierce Biotechnology, USA). Samples containing equal protein amounts (50 µg/lane, sample volumes usually in the range of 2-10 µl) and prestained molecular weight standards were separated on Tris-SDS-PAGE and blotted onto PVDF membranes (Millipore, USA). Membrane was blocked with 5% non-fat milk in Tris-buffered saline (0.1% Tween-20) for 2 h at room temperature, incubated over night at 4 °C with the following antibodies: polyclonal anti-NR1 (1:2000), polyclonal anti-NR2A (1:1000) and polyclonal anti-NR2B (1:1500). After that, membranes were washed and incubated for 1 hour at 37 °C with appropriate HRP-conjugated secondary antibody, and then developed with enhanced chemiluminescence (ECL kit; Millipore) and visualized using Kodak films. Following



Fig. 2. Low frequency-rTMS rescues spatial memory deficits in $A\beta_{1-42}$ -induced toxicity rats. (A) Mean swimming distance (cm) of rats which were trained in the hidden platform version of Morris water maze test from day 1 to 5 in control, control with rTMS, $A\beta$ injection and $A\beta$ injection with rTMS group. (B) Escape latency (sec) from day 1 to 5 in control, control with rTMS, $A\beta$ injection and $A\beta$ injection, and $A\beta$ injection with rTMS group. (C) Swimming distance in target quadrant of rats which were tested on day 6. (D) Percentage time in target quadrant of rat tested in probe trial. Rats in control, $A\beta$ injection, and $A\beta$ injection with rTMS group n = 7, rats in control with rTMS n = 10. ** p < 0.05 vs. control. ## p < 0.05 vs. $A\beta$ injection.

autoradiography, films were scanned and densitometric analyses were performed using public domain NIH Image Program (Tong et al., 2013).

2.8. Measurement of hippocampal neurotrophins levels determined by ELISA Kit

BDNF and NGF protein levels in hippocampus were quantified by using ELISA according to the manufacturer's protocol (Boster Biological Technology, Wuhan, China). Supernatants from homogenates (as described above) were used. Monoclonal anti-rat BNDF antibody and anti-rat NGF antibody were used in ELISA. Absorbance was determined at 450-nm wavelength. Standard curve was used to demonstrate the linear relationship between optical density (OD) and BDNF/NGF contents. Protein levels were expressed as pg/mg tissue weight as described before (Weskamp and Otten, 1987; Zhou et al., 2008).

2.9. Statistical analysis

Data from each experiment were analyzed using one-way ANOVA, followed by Fisher's least significant difference (LSD) post hoc test comparing both treatment groups to control group. All statistical analysis was performed with IBM SPSS Statistics 19 software. A value of p < 0.05 was considered statistically significant.

In Morris water maze experiment, measures of performance during acquisition trials (i.e., swimming distance, escape latency) were averaged within each day for each animal. To determine the difference between each day, data were analyzed using repeated measures ANOVA's with day as within-subjects factor and different treatment as between-subjects factor. Difference among the four treatment groups within each day were analyzed with one-way ANOVA's test, and Fisher's LSD was used for post-hoc comparisons (Harrison et al., 2009; Spritzer et al., 2011).

3. Results

3.1. rTMS rescues spatial memory in $A\beta_{1-42}$ -induced toxicity rat model

First, we investigated whether $A\beta_{1-42}$ can be detected in hippocampus of rats with or without $A\beta_{1-42}$ intrahippocampal injection. Using immunohistochemistry, we found that there were no $A\beta_{1-42}$ -positive immunostaining in hippocampus in sham operated rats (only injected with saline, pH = 7.4) (Fig. 1A1–A3). However, a number of $A\beta_{1-42}$ deposits were obviously observed in DG area and CA1 region of hippocampus in rats with $A\beta$ injection (Fig. 1C1–C3). There's no $A\beta_{1-42}$ -positive immunostaining can be detected in negative control of $A\beta$ injection group without incubated with rabbit polyclonal anti- $A\beta_{1-42}$ antibody (Fig. 1B1–B3). These results indicate that the incubated $A\beta_{1-42}$ had been successfully injected into hippocampus of rats in $A\beta$ injection group.

Then, we examined whether low-frequency rTMS can reverse spatial memory impairment in A β_{1-42} -induced toxicity model rats. To investigate the potential effects of rTMS (1Hz) on the ability of hippocampal dependent spatial learning and memory retrieval in A β_{1-42} -induced toxicity rat model, Morris water maze test was carried out. Learning ability of animals was measured by escape latency and mean swimming distance from day 1 to day 5 in memory-acquisition trials (training). To





Fig. 3. Low frequency-rTMS facilitates hippocampal LTP which is suppressed in A β_{1-42} -induced toxicity rats after 14 days rTMS treatment. (A) Hippocampal LTP was obviously impaired in rats with A β injection than control rats *in vivo*. Amplitude of hippocampal LTP was increased in A β injected rats after low frequency-rTMS treatment. (B) PS percentage change from baseline on 5, 30 and 60 min after TS (** p < 0.01 vs. control; ## p < 0.01 vs. A β injection; ANOVA; n = 8). Values are representative of mean \pm SEM.

evaluate the accuracy of encoding platform coordinates, probe trial was carried out 24 h after the last day of training (on day 6). Percentage time and swimming distance in target quadrant (without the platform) were used to evaluate retrieval ability of spatial memory.

In the present study, all rats showed a significant decrease in escape latency and swimming distance over 5-day's acquisition trials of Morris water maze test [Fig. 2A, B; swimming distance: F(4, 108) = 120.290, P < 0.001; escape latency: F(4, 108) = 79.897, P < 0.001]. It indicates that rats have learned the location of platform in acquisition trial. Meanwhile, day × treatment interaction was not significant (both P > 0.1, swimming distance: F (12, 108) = 1.809, p = 0.094; escape latency: F (12, 108) = 1.327, p = 0.247). But the main effect of treatment (different groups) was significant (both p < 0.05; swimming distance: F (3, 27) = 9.102, p < 0.001; escape latency: F (3, 27) = 10.555, P < 0.001), indicating that different treatments (A β_{1-42} injection, rTMS treatment) influenced spatial learning ability of rats.

Analyses of swimming distance within each day revealed a significant effect of treatment on day 2 [F (3, 27) = 5.506, P = 0.004], day 3 [F (3, 27) = 10.463, P < 0.001] and day 5 [F (3, 27) = 3.860, p = 0.020]. Post hoc analyses for day 2 and 3 showed A β injection group

had significantly longer swimming distance than control group (p = 0.035 on day 2, p = 0.001 on day 3) (Fig. 2A). While analyses of escape latency within each day revealed a significant effect of treatment on day 2 [F (3, 27) = 4.75, p = 0.009], day 3 [F (3, 27) = 2.97, p = 0.050], day 4 [F (3, 27) = 3.297, p = 0.035] and day 5 [F (3, 27) = 4.272, p = 0.014]. Post hoc analyses for day 2, 3 and 4 showed rats in A β injection group had significantly longer escape latency than control group (p < 0.05) (Fig. 2B). In the probe trial test, rats with A β injection stayed less time in the target quadrant compared with control group (p < 0.01) (Fig. 2C, D). These results identified that A β_{1-42} obviously impaired spatial learning ability of A β_{1-42} -induced toxicity model rats.

However, mean swimming distance and escape latency in each day were not significantly changed between control group and $A\beta$ injection with rTMS group (p > 0.05). And on day 3, both swimming distance and escape latency were decreased significantly (p < 0.05) in AB injection with rTMS group compare to $A\beta$ injection group (Fig. 2A, B). After low-frequency rTMS treatment, rats in AB injection with rTMS treatment group obviously remembered the hided platform, as mean swimming distance and percentage time in target quadrant were much longer than that of AB injection group (p < 0.05) (Fig. 2C, D). This implies that low-frequency rTMS rescues spatial learning ability in $A\beta_{1-42}$ -induced toxicity model rats. Similarly, mean swimming distance and escape latency in each day were not significantly changed between control group and control with rTMS group (p > 0.05) (Fig. 2A, B). But notably, swimming distance and percentage time in target quadrant in control with rTMS treatment group were longer than that of control group (p < 0.05, one-way ANOVAs). This suggests that low-frequency rTMS also enhances memory retrieval ability in normal rats to a certain degree (Fig. 2C, D).

Taken together, all these data indicate that low-frequency rTMS treatment not only improves spatial memory retrieval ability in normal rats, but also ameliorates $A\beta_{1-42}$ -mediated memory deficits.

3.2. rTMS rescues $A\beta_{1-42}$ -suppressed hippocampal LTP in vivo

Accumulating evidence shows that LTP represents synaptic plasticity. And maintaining hippocampal LTP sustains spatial memory (Pastalkova et al., 2006; Whitlock et al., 2006). Hippocampus is a brain region involved in spatial learning (Andersen et al., 2007; Bliss and Collingridge, 1993). To elucidate whether rTMS can modulate putative cellular processes related to learning and memory, we further investigated the effect of rTMS on hippocampal synaptic plasticity. There was a strong potentiation of PS amplitude in PP to DG pathway evoked by TS maintained over 60 min compare with baseline recordings (Fig. 3A). PS amplitude values at 5 min, 30 min and 60 min after TS were 324.88 \pm 7.40%, 280.49 \pm 5.49% and 274.04 \pm 9.39% in rats of Con with rTMS group. While in control group, values were 282.12 \pm 11.92% at 5 min, 271.01 \pm 22.35% at 30 min and 250.31 \pm 29.31% at 60 min, respectively (Fig. 3B). Compared with normal rats, there was a statistical difference in LTP amplitude of control with rTMS treatment only within 5 min after TS, but declined to normal level within 60 min (Fig. 3A, B).

Previous studies have showed that acute $A\beta_{1-42}$ injection inhibited hippocampal LTP *in vitro* and *in vivo* (Cullen et al., 1997; Lanctot et al., 2008; Nomura et al., 2012). In this study, we found that hippocampal LTP was impaired in $A\beta_{1-42}$ -induced toxicity rat model after 30-day $A\beta_{1-42}$ injection *in vivo*. PS amplitude values in $A\beta$ injection group were 184.88 \pm 8.55%, 160.49 \pm 7.43% and 149.04 \pm 9.39% at 5 min, 30 min and 60 min after TS; PS potentiation of $A\beta$ injected rats was markedly suppressed (p < 0.05) compared with control group (Fig. 3B). However, PS amplitude of $A\beta$ injection with rTMS group rats was significantly higher than that of $A\beta$ injected rats, yet still lower than control group at 5 min, 30 min and 60 min after TS (Fig. 3B). These results imply that $A\beta_{1-42}$ injection impairs hippocampal synaptic plasticity of rats; however, low-frequency rTMS treatment partially rescues $A\beta_{1-42}$ -mediated hippocampal LTP impairment, but can't recover to the normal level.



Fig. 4. Low-frequency rTMS up-regulates NMDA receptor (NR1, NR2A and NR2B) which is suppressed by $A_{\beta_{1-42}}$ injection. (A) Representative immunoblots showed hippocampal NR1, NR2A and NR2B expression levels in different groups (Sham: home cage; Con: control; $A\beta$ injection; and $A\beta$ injection with rTMS treatment). (B, C, D) The density of auto-radiographic bands. β -actin was probed as internal control for NR1, NR2A and NR2B. The relative density of sham group was set as 100%. Results were mean \pm SEM of at least six hippocampal extracts from each group. n = 6; * P < 0.05; ** P < 0.01. vs. sham group.

3.3. rTMS reverses $A\beta_{1-42}$ -suppressed NMDA-receptor expression

NMDA-receptor is a critical molecule for LTP and memory formation (Tang et al., 1999). To investigate the possible mechanism of low-frequency rTMS reverses deficits in LTP and spatial memory of $A\beta_{1-42}$ -induced toxicity rats, we first measured hippocampal NMDA-receptor expression by Western blot. NMDA-receptor expression is obviously decreased in AD patients and AD animal models such as APP, APP/PS1 mice (Bi and Sze, 2002; Calon et al., 2005; Tsang et al., 2008). Therefore, low-frequency rTMS facilitates hippocampal LTP and spatial memory may due to increases NMDA-receptor expression. In this study, Western blot results revealed that protein levels of NR1 and NR2B in A_β injection group were significantly lower than that of control group (Fig. 4B, D). Moreover, there was obvious increased expression of NMDA-receptor subunits including NR1, NR2A, and NR2B in hippocampus of A β injection with rTMS group than those of A β injection group (Fig. 4B–D). At the same time, we found that there was distinct higher expression in NMDA receptor (NR1 and NR2B) in control group rats after Morris water maze than sham rats without behavioral testing. These results indicate that behavioral testing increases NMDA receptor content in normal rats. And $A\beta_{1-42}$ injection suppresses NMDA-receptor expression. Importantly, low-frequency rTMS obviously reversed $A\beta_{1-42}$ -suppressed NMDA-receptor expression in hippocampus.

3.4. rTMS rescues $A\beta_{1-42}$ -mediated disruption of neurotrophins contents

We further investigated the possible mechanism of increased NMDA-receptor expression in rats of A β injection with rTMS treatment. Previous studies have identified that neurotrophins (such as BDNF and NGF) are critical factors for up-regulating hippocampal NMDA-receptor

expression in rats (Bai and Kusiak, 1997; Caldeira et al., 2007; Jarvis et al., 1997). Therefore, we measured hippocampal neurotrophins contents by using BDNF and NGF ELISA kit.

Absorbance was determined at 450 nm. Linear relationship between OD and BDNF/NGF contents was showed as standard curve of BDNF and NGF (Fig. 5A, B). We found that hippocampal BDNF and NGF levels were not only increased in control with rTMS group compared with control group, but also in A β injection with rTMS group compared with A β injection group (Fig. 5C, D). Especially, BDNF and NGF levels were decreased in A β injection group, but markedly increased in A β injection with rTMS group (Fig. 5C, D). Therefore, above results indicate low-frequency rTMS treatment rescues A β_{1-42} -suppressed neurotrophins contents in hippocampus. This is also consistent with previous reports that low-frequency rTMS augmented neurotrophins contents in normal rats or vascular dementia rats (Wang et al., 2010; Zhang et al., 2007).

4. Discussion

rTMS is a technique for noninvasive stimulation of brain via generation of high-intensity magnetic field pulses by passing brief electric currents through an inductive coil. The induced current can be sufficient to cause depolarization of corticospinal tract neurons either directly at the axon hillock or indirectly via depolarization of interneurons (Pell et al., 2011). rTMS may affect Ca²⁺ metabolism, cell hydration and GABA content. The crucial role of Ca²⁺ metabolism in realization of the biological effect of electromagnetic fields have been clearly demonstrated (Adey, 1981; Blackman et al., 1982). Meanwhile, Danielyan and his colleagues have found that static magnetic fields induced dehydration effect on brain tissues of rats (Danielyan et al., 1999). Furthermore, levels of fluorodeoxyglucose



Fig. 5. Low-frequency rTMS up-regulates BDNF and NGF levels which were suppressed by $A_{\beta_{1-42}}$ injection. (A, B) Standard curve of BDNF and NGF, respectively. (C, D) Hippocampal BDNF and NGF levels of control, control with rTMS, A_{β} injection and A_{β} injection with rTMS group, respectively. n = 8, * p < 0.05; ** p < 0.01, vs. control.

(FDG) and GABA synthesis proteins have been increased for up to 8 and 7 days following 5 Hz and 1 Hz rTMS respectively (Hayashi et al., 2004; Trippe et al., 2009).

Beneficial effects of rTMS on cognitive performance (Boroojerdi et al., 2001; Foltys et al., 2001; Wassermann et al., 1996), NMDA receptors (Wang et al., 2010, 2011) and neurotrophin levels (Yukimasa et al., 2006; Zanardini et al., 2006; Zhang et al., 2007) in normal subjects have been reported. The current findings indicate that low-frequency rTMS up-regulates hippocampal endogenous neurotrophins and NMDA receptors both in normal and AB injected rats, which helps to rescue A_{β1-42}-induced spatial memory deficits. Indeed, effects of rTMS in normal rats essentially mirror molecular changes in AB injected rats, except for the LTP results. rTMS-induced LTP amplification was significantly increased only in AB injected rats, but without effecting LTP in normal rats (Fig. 3). Initial studies in vitro (hippocampal slices) (Tokay et al., 2009) and in vivo (subsequent LTP induction in rat models) (Levkovitz et al., 2001; Ogiue-Ikeda et al., 2003) have showed an enhancive effect of high frequency rTMS on LTP formation in normal animals. It also have indicated that low-frequency rTMS (1 or 8 Hz) affects memory retention in novel recognition test, but has no influence on LTP in normal mice (Ahmed et al., 2006). This is accordance with our results and might relates to a "ceiling" effect (Pennisi et al., 2011): there is lack of effects of rTMS on LTP formation in normal rats; while LTP is significantly impaired in AB injected rats, rTMS facilitates LTP but not to normal level.

Since exogenous neurotrophins can't permeate through the blood–brain-barrier, direct intravenous injection of exogenous neurotrophins is not used clinically. Intrahippocampal injection with exogenous neurotrophins (BDNF or NGF) reverses hippocampal LTP and spatial memory impairment in AD model rat (Arancibia et al., 2008; Nagahara

et al., 2009; Tian et al., 2012), but this invasive injection causes traumatic brain injury and limits clinical application of exogenous neurotrophins (Covaceuszach et al., 2009). Previous reports have showed that lowfrequency rTMS augmented neurotrophins contents in normal or vascular dementia rats (Wang et al., 2010; Zhang et al., 2007). In this study, we found that low-frequency rTMS obviously up-regulated hippocampal BDNF and NGF, as hippocampal BDNF and NGF contents of AB injection with rTMS group were higher than that of A β injection group (Fig. 5C-D). Other studies also have reported that multiple rTMS treatment can elevate plasmatic BDNF level both in rat and human (Yukimasa et al., 2006; Zanardini et al., 2006; Zhang et al., 2007). Much evidence have verified that neural glia or astrocytes can secret neurotrophins including BDNF and NGF (Lessmann et al., 2003; Zafra et al., 1992). The up-regulated hippocampal BDNF and NGF induced by rTMS may mainly derive from neural cell secretion (glia, astrocytes or neurons). This study along with previous reports all support that noninvasive low-frequency rTMS contributes to augment endogenous neurotrophins contents in normal rats, vascular dementia rats and A β_{1-42} -induced toxicity rats (Wang et al., 2010; Zhang et al., 2007). This suggests that low-frequency rTMS may be a simple and practical means of noninvasively increasing endogenous neurotrophins in brain to rescue memory deficits.

A presumed explanation for therapeutic effects of low-frequency rTMS is to scavenge $A\beta$ neurotoxicity by enhancing neurotrophins contents. Previous studies have identified that $A\beta$ induces NGF dysmetabolism in AD (Bruno et al., 2009). Hippocampal BDNF and NGF levels are obviously decreased in AD rat model and AD patients (Bi and Sze, 2002; Calon et al., 2005; Tsang et al., 2008). However, low-frequency or high-frequency rTMS up-regulates BDNF and NGF

contents in blood, hippocampus and cortex (Angelucci et al., 2004; Gersner et al., 2011; Muller et al., 2000). Other studies have indicated that up-regulated BDNF and/or NGF can inhibited A β neurotoxicity by enhancing secreted amyloid precursor protein (sAPP) expression (Kim et al., 2010; Post et al., 1999). Therefore, BDNF or NGF injection can improve learning and memory ability of rats (Arancibia et al., 2008; Cosgaya et al., 1996; Tian et al., 2012) and AD animal model (Covaceuszach et al., 2009; Nilsson et al., 2010).

The enhancing effect of neurotrophins contents by rTMS treatment may due to up-regulation of NMDA-receptor. NMDA-receptor is a critical molecule for synaptic plasticity (hippocampal LTP) and memory formation (Cooke and Bliss, 2006). Down-regulation of NMDA-receptor obviously causes memory impairment (Tang et al., 1999). Previous study has identified that low-frequency rTMS can increase mRNA and protein level of BDNF and NMDA-receptor (Wang et al., 2010, 2011). Moreover, BDNF and/or NGF can up-regulate NMDA-receptor expression in vitro and in vivo (Bai and Kusiak, 1997; Caldeira et al., 2007; Jarvis et al., 1997). Up-regulation of NMDA-receptor by neurotrophins is also observed to enhance hippocampal LTP and memory formation in rats (Bai and Kusiak, 1997; Bekinschtein et al., 2008; Caldeira et al., 2007; Lu et al., 2008). In the present study, low-frequency rTMS improved hippocampal BDNF and NGF levels associated with enhancing NMDA-receptor expression in AB injection group (Figs. 4, 5). These results indicate that low-frequency rTMS improves BDNF and NGF levels; furthermore, neurotrophins up-regulates NMDA-receptor expression for maintaining LTP and memory in $A\beta_{1-42}$ -induced toxicity rat model.

It has been manifested that standard TMS with figure-of-eight coil (8-coil) is able to modulate cortical excitability up to a maximum depth of 1.5–2.5 cm from scalp (Bersani et al., 2012). In this study, rTMS was performed by using a standard round coil (9 cm in diameter), which penetrated not only cortex but also sub cortical structures such as hippocampus due to the small size/volume of rat brain. Deep rTMS is able to modulate cortical excitability up to a maximum depth of 6 cm (Bersani et al., 2012). It is reasonable to speculate that deep rTMS (with H-coil or other special designed coils considerate for deep TMS such as C-core coil and circular crown coil), might be more effective in treating AD patients, for electromagnetic field generated by deep rTMS can affect deeper brain region including hippocampus. However, the initial characterization of cognitive effects and safety of deep rTMS for treating AD patients needs further investigation.

In all, all the data indicate that low-frequency rTMS may be a potential novel therapy for memory-deteriorating disease such as Alzheimer's disease. But the molecular biological mechanism of observed beneficial effects of rTMS in $A\beta_{1-42}$ -induced toxicity rats awaits more detailed investigation by means of adequate methods in the future. And there is still a long way to go for treating AD patients by using rTMS.

Conflict of Interest

All the authors do not have any conflict of interest.

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