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RESEARCH ARTICLE

Low-frequency transcranial magnetic stimulation is beneficial for enhancing synaptic plasticity in the aging brain

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Abstract

In the aging brain, cognitive function gradually declines and causes a progressive reduction in the structural and functional plasticity of the hippocampus. Transcranial magnetic stimulation is an emerging and novel neurological and psychiatric tool used to investigate the neurobiology of cognitive function. Recent studies have demonstrated that low-frequency transcranial magnetic stimulation (≤1 Hz) ameliorates synaptic plasticity and spatial cognitive deficits in learning-impaired mice. However, the mechanisms by which this treatment improves these deficits during normal aging are still unknown. Therefore, the current study investigated the effects of transcranial magnetic stimulation on the brain-derived neurotrophic factor signal pathway, synaptic protein markers, and spatial memory behavior in the hippocampus of normal aged mice. The study also investigated the downstream regulator, Fyn kinase, and the downstream effectors, synaptophysin and growth-associated protein 43 (both synaptic markers), to determine the possible mechanisms by which transcranial magnetic stimulation regulates cognitive capacity. Transcranial magnetic stimulation with low intensity (110% average resting motor threshold intensity, 1 Hz) increased mRNA and protein levels of brain-derived neurotrophic factor, tropomyosin receptor kinase B, and Fyn in the hippocampus of aged mice. The treatment also upregulated the mRNA and protein expression of synaptophysin and growth-associated protein 43 in the hippocampus of these mice. In conclusion, brain-derived neurotrophic factor signaling may play an important role in sustaining and regulating structural synaptic plasticity induced by transcranial magnetic stimulation in the hippocampus of aging mice, and Fyn may be critical during this regulation. These responses may change the structural plasticity of the aging hippocampus, thereby improving cognitive function.

***Key Words:** neural regeneration; non-invasive brain stimulation; transcranial magnetic stimulation; neurotrophic factor; brain-derived neurotrophic factor; neuroplasticity; hippocampus; aging; cognitive function*

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Introduction

Aging is associated with cognitive decline, such that older individuals develop cognitive deficits or age-associated memory impairments. The progressive reduction in structural and functional plasticity, particularly in the hippocampus and the prefrontal cortex, plays a key role in cognitive function (Bisaz et al., 2013). The hippocampus is central to the formation of declarative memory (and other types of memory) and is particularly sensitive to aging (Martin et al., 2014). Modifications to the neural circuit strength have been hypothesized as the foundation for learning and memory. Furthermore, hippocampal neuronal networks, which mediate learning and memory, are particularly vulnerable to aging (Voineskos et al., 2013). However, the precise mechanisms underlying reduced hippocampal plasticity with aging remain unclear.

Transcranial magnetic stimulation (TMS) has been recognized as a novel neurological and psychiatric tool (with both clinical and basic neuroscience applications) because it is non-invasive and painless while stimulating brain regions. TMS modulates cortical plasticity. A brief electrical current is delivered through a coiled wire placed on the scalp resulting in a time-varying magnetic field across the skull, which induces an electric field and subsequently alters neuronal activity (Hallett, 2007; Rossi and Hallett, 2009; Elder et al., 2014) within the cortex (Pleger et al., 2006) and deep brain regions connected to the cortex (Miniussi and Rossini, 2011). Recently, TMS has been shown to affect learning and memory and other brain functions in degenerative brain diseases (Boroojerdi et al., 2001; Wang et al., 2010). Moreover, low-frequency TMS is considered important for regulating neuronal differentiation and neuronal network properties (Saito et al., 2009). Recent studies have demonstrated that low-frequency TMS affects the synaptic plasticity of the hippocampal CA1 area in rats with vascular dementia (VaD) (Wang et al., 2010), ameliorates spatial-cognitive difficulties in mice with amyloid beta $(A\beta)_{1-42}$-mediated memory deficits (Tan et al., 2013), and regulates nerve development and regeneration in cultured hippocampal neurons (Ma et al., 2013). Overall, these studies indicate that low-frequency magnetic stimulation mediates changes in cognition and synaptic plasticity involving the hippocampus. However, the neural mechanisms of TMS on learning and memory during aging remain unclear.

Numerous reports confirm that brain-derived neurotrophic factor (BDNF) depolarizes neurons as rapidly as glutamate by activating tropomyosin receptor kinase B (TrkB), enhancing glutamatergic synaptic transmission, and increasing phosphorylation of the subunits of N-methyl-D-aspartate (NMDA) receptors in the hippocampus. Furthermore, this neurotrophin enhances long-term potentiation (LTP) in the hippocampus (Yamada and Nabeshima, 2003).

The effects of BDNF-TrkB signaling are numerous and include increases in cell plasticity, survival, and growth (Green et al., 2013). TrkB signaling uses Ras, phosphatidylinositol-4,5-bisphosphate 3-kinase, and phospholipase C-gamma, and is important for both hippocampal LTP (Minichiello et al., 1999) and memory consolidation (Lee et al., 2004). TrkB increases the arborization and synapse number (Alsina et al., 2001), the survival of neurons (Gorski et al., 2003), neurogenesis (Scharfman et al., 2005), and neuronal morphology (Baquet et al., 2004). Given its widespread distribution throughout the brain and its broad functional role, the BDNF-TrkB pathway interacts with many other signaling systems relevant to psychopathology, including the serotonin, endocannabinoid, and glutamatergic pathways (Andero and Ressler, 2012). Many studies have shown that TMS affects BDNF levels in the brain, cerebrospinal fluid, and blood (Boroojerdi et al., 2001; Wang et al., 2010; Tan et al., 2013). However, the regulation of BDNF-TrkB signaling is multivariate and has not been fully elucidated. Fyn, a Src-family tyrosine kinase, is considered to be involved in the BDNF signal transduction pathway and downregulates TrkB (Mizuno et al., 2003). Fyn is also involved in synaptic plasticity and regulates NMDA receptor and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor activity, trafficking, and degradation (Chen et al., 2013). To our knowledge, there have been no studies reporting on the correlation between TMS and Fyn.

The overall objective of the present study was to better understand the potential of TMS in inducing long-term effects on cognition. Therefore, we investigated the effects of TMS (1 Hz) on spatial memory behavior and on the expression of synaptophysin (SYN) and growth-associated protein 43 (GAP43) (both markers of synaptic plasticity) in hippocampal regions of aging mice.

Materials and Methods

Animals

Fifteen-month-old male Swiss mice, weighing 28–32 g, were obtained from Medical Laboratory Animal Center of Hebei Medical University, China. The animals were housed at room temperature (23 ± 2°C), maintained on a 12-hour light/dark cycle, and had *ad libitum* access to food and water.

All mice were randomly divided into three groups: control, sham, and TMS groups (n = 20 mice/group). The control mice were housed in cages without treatment. The TMS group were treated daily with low-frequency (1 Hz) TMS for 14 consecutive days, and the sham group were handled in a manner similar to that of the TMS group but without TMS stimulation. Mice in the TMS group were awake during the stimulation delivery. Six mice from each group were randomly selected for immunohistochemistry, while eight mice were selected for reverse transcription polymerase chain reaction (RT-PCR) and western immunoblot experiments, and the remaining six mice were used for the Morris water maze (MWM) tests. All of the experimental procedures were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council). The study was approved by the Committee of Ethics on Animal Experiments at Hebei Medical University, China. All efforts were made to minimize animal suffering and the number of animals used.

Application of low-frequency TMS

The procedure was based on previous clinical (Schambra et al., 2003; Piccirillo et al., 2011) and animal (Gersner et al., 2011; Tan et al., 2013) studies. The detailed procedure for TMS stimulation, including stimulation parameters and stimulation patterns, has been described in our previous report (Ma et al., 2013). Briefly, low-frequency TMS was delivered with an MC-B70 butterfly coil (MagVenture, Farum, Denmark; inner diameter 20 mm, outer diameter 100 mm) connected to a MagProX100 magnetic stimulation device (MagVenture; active pulse width 280 μs, 4.2 Tesla maximum output). In our preliminary experiment, the magnetic stimulation intensity was determined according to a previous report (Gersner et al., 2011). Briefly, the coil was placed above the head of an anesthetized mouse, which was aligned with its center on the midline, equidistant between the bregma

Figure 1 Schematic diagram of the transcranial magnetic stimulation (TMS) protocol in aged mice.
Four sessions of low-frequency TMS were performed once daily for 14 consecutive days. The pattern of one session of TMS consisted of 5 burst trains, with each train consisting of 20 pulses and 10-second inter-train intervals, with inter-session intervals of 30 seconds; *i.e.*, 400 pulses in total.

and lambda sutures along the longitudinal body axis (the distance of coil-scalp was 1.0 cm) (Cohen et al., 1990). The resting motor threshold (MT) intensity was then obtained by visual inspection of bilateral forelimb movement elicited by the lowest stimulus intensity (Gersner et al., 2011). The stimulation intensity was found to be 110% of the average MT in these mice (Gersner et al., 2011). Therefore, in the following experiments, the suprathreshold intensities (which corresponded to TMS) were delivered to the mice. After determining the stimulus parameters, the TMS protocol was carried out. First, mice in the TMS group were restrained in lab-made suitable cloth sleeves (Tan et al., 2013) by hand force and without anesthesia (Ogiue-Ikeda et al., 2003; Gersner et al., 2011). They then received four sessions of low-frequency TMS daily (between 9:00 and 12:00 a.m.) for 14 consecutive days. The protocol of TMS is shown in **Figure 1**. The pulse width was 68 μs. Sham mice were handled in a manner similar to that of the TMS group and were exposed to the magnetic apparatus for an identical length of time but were separated from the head using a 3-cm plastic spacer cube (Wang et al., 2011). This ensured that the animal felt the vibrations produced by the click of the TMS coil without brain stimulation. Moreover, according to the manufacturer's manual, the TMS coil penetrates the cortex, as well the sub-cortical structures, such as the hippocampus, because of the small size/volume of the mouse brain. Current rTMS coils for rodents cannot be used to localize stimulation to specific brain regions because of technical limitations of the coil size relative to the mouse brain. Therefore, all brain areas may be affected.

Determination of magnetic stimulation parameters

After measurement and calculation, the average resting was determined (MT magnetic stimulus intensity of all mice was 1.033 ± 0.056 Tesla). In the following experiments, the stimulation intensities (representing 110% of the average resting MT that corresponded with the TMS) were delivered to the mice at a low frequency (1 Hz). The magnetic stimulus intensity was also documented as approximately 27% of the maximal strength of the magnetic field generated by the coil.

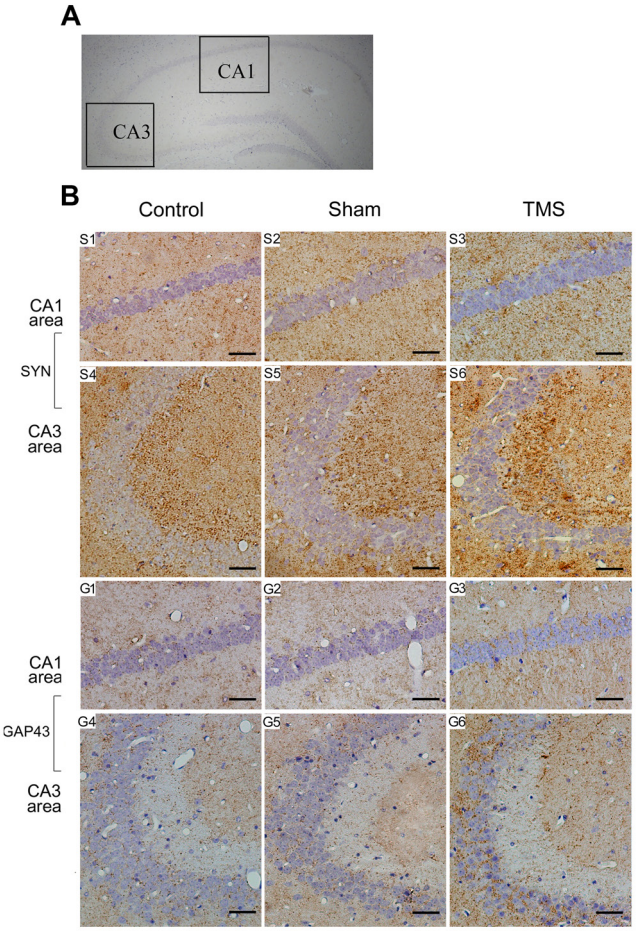

Figure 2 Immunohistochemical staining of SYN and GAP43 in the hippocampus of aged mice.
(A) Hippocampal CA1 and CA3 regions (both boxed) in the control (no primary antibody). (B) Immunoreactivity for SYN (S1–3, CA1 area; S4–6, CA3 area) and GAP43 (G1–3, CA1 area; G4–6, CA3 area) is shown as brownish-yellow granules in pyramidal cells. Scale bars: 100 μm. SYN: Synaptophysin; GAP43: growth associated protein 43.

Immunohistochemical staining for SYN and GAP43

Mice were deeply anesthetized with 6% chloral hydrate (5 mg/kg, intraperitoneal (i.p.) injection) and were transcardially perfused with PBS (0.01 M) and fixative (4% paraformaldehyde, 0.2% picric acid, diluted in 0.1 M phosphate buffer (PB), pH 7.4). Mouse brains were carefully dissected out and post-fixed in 4% paraformaldehyde (diluted in 0.1 M PB buffer) for 24 hours at 4°C. Brain tissue samples were obtained from the superior colliculus to the optic chiasma and postfixed overnight in the same fixative at 4°C. Next, the tissue block was dehydrated in ethanol in a graded series, then cleared in xylene and embedded in paraffin. Based on the mouse brain atlas (Paxinos and Franklin, 2001), paraffin blocks with the CA1 and CA3 hippocampal regions were prepared along the longitudinal axis (anterior-posterior: approximately −2.06 mm to −2.54 mm from bregma). The blocks were then sliced on a sliding microtome (Leica-RM2145, Germany) into consecutive 5-μm-thick coronal sections and placed on individual poly-lysine-coated slides

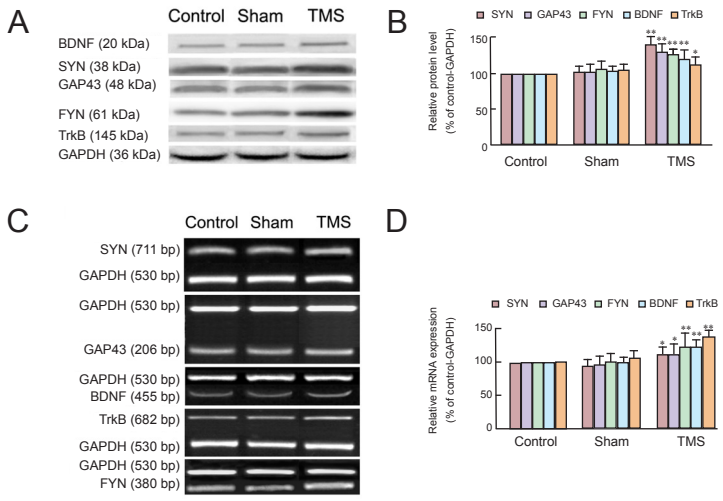

Figure 3 Protein and mRNA expression of SYN, GAP43, BDNF, TrkB and Fyn in the hippocampus of aging mice.
Western immunoblots (A) and their respective quantitative analysis (B) of SYN, GAP43, BDNF, TrkB, and Fyn. mRNA bands *via* reverse transcription polymerase chain reaction (RT-PCR) (C) and their respective quantitative analysis *via* semi-RT-PCR of SYN, GAP43, BDNF, TrkB and Fyn. (B, D) The data are expressed as the mean ± SD (8 mice per group). The expression of protein and mRNA was normalized to GAPDH levels, which was then normalized to the control (defined as 100%.). $*P < 0.05$, $**P < 0.01$, *vs.* control group. One-way analysis of variance followed by the Fisher's Least Significant Difference was used. Each experiment was repeated three times. SYN: Synaptophysin; GAP43: growth associated protein 43; BDNF: brain-derived neurotrophic factor; TrkB: tropomyosin receptor kinase B.

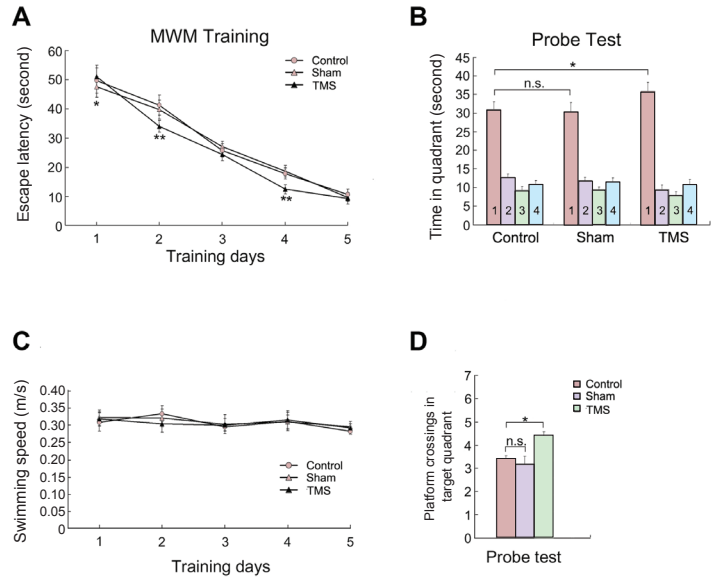

Figure 4 Changes in spatial cognition in aged mice using the Morris water maze test.
(A) Escape latency on each training day. (B) The time spent in the target quadrant during probe trial. The column 1 represents the target quadrant. (C) Mean swimming speed on each training day. (D) Number of platform crossings in the target quadrant during probe test (over 60 seconds). The data are expressed as the mean ± SD (6 mice per group). $*P < 0.05$, $**P < 0.01$, *vs.* the control group (one-way analysis of variance followed by the Fisher's Least Significant Difference test).

according to their coordinates. After the sections were deparaffinized and hydrated, they were treated with 3% H_2O_2 for 30 minutes, to remove residual peroxidase activity, followed by a rinse with PBS. The sections were then incubated with 5% normal goat serum to block nonspecific binding, followed by an overnight incubation with the following primary antibodies (at 4°C): rabbit anti-SYN (1:200; Epitomics, Burlingame, CA, USA) or rabbit anti-GAP43 (1:100; Epitomics). After washing, the sections were incubated with biotinylated goat anti-rabbit IgG (1:200; Sigma-Aldrich, Louis, MO, USA) for 2 hours at room temperature, followed by incubation with avidin conjugated to horse radish peroxidase for 1 hour at room temperature. Finally, immunoreactivity was developed (between 3 and 10 minutes) using a diaminobenzidine (DAB) kit (Sigma-Aldrich). Sections were dehydrated and covered, then stored at 4°C until they were to be used for imaging. To ensure that each primary antibody did not react with the non-corresponding secondary antibody-conjugate, control experiments were performed, as described previously (Diaz et al., 2000; Harris et al., 2010). In these controls, no cross-reactive immunostaining was observed (data not shown).

Western immunoblotting for SYN, GAP43, BDNF, TrkB, and Fyn

Mice were decapitated, their brains removed and then snap-frozen. Hippocampal tissue samples were prepared from these brains. The samples were homogenized with a glass homogenizer in radio immunoprecipitation assay lysis buffer with 1% phenylmethanesulfonyl fluoride on ice. The homogenate was centrifuged (30 minutes at 12,000 × *g*, 4°C) and supernatants were collected and stored at −80°C. Protein concentration was determined using the bicinchoninic acid assay kit (Pierce Biotechnology, Rockford, IL, USA). Samples containing equal amounts of protein (50 μg/20 μL) were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and then transferred onto polyvinylidene fluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked for 1 hour at room temperature and then incubated overnight at 4°C with the following primary antibodies (all from Sigma-Aldrich): rabbit anti-SYN (1:5,000), GAP43 (1:5,000), BDNF (1:2,000), TrkB (1:2,000), or Fyn (1:2,000), and mouse anti-GAPDH (1:1,000). After several washes, membranes were incubated

with the appropriate HRP-conjugated secondary antibodies at room temperature for 2 hours, and then detected by enhanced chemiluminescence (ECL, Sigma-Aldrich). Band signals were acquired using the LAS-4000 gel imaging system (LAS-4000; Fuji Film, Tokyo, Japan) and quantified using Image Quant software (Image Gauge 4.0; Fuji Film, Japan). The average background intensity was subtracted from each pixel within each band of interest. The labeling densities for SYN, GAP43, BDNF, TrkB, and Fyn were compared with those of GAPDH, which was the internal control. The protein levels were normalized to GAPDH and then expressed as a percentage of the control samples (which were defined as 100%).

RT-PCR for SYN, GAP43, BDNF, TrkB and Fyn

RNA isolation and cDNA synthesis

Mice were deeply anesthetized with 6% chloral hydrate (5 mg/kg, i.p.), and total RNA samples were extracted from the hippocampus using Trizol reagent (Invitrogen, Madison, WI, USA) according to the manufacturer's instructions and our previous experiments (Cui et al., 2012). Frozen tissue was weighed and immediately homogenized (on ice) in Trizol Reagent using a pellet pestle cordless motor (Kimble®/Kontes LLC). Then, 1 mL of Trizol Reagent per 50–100 mg of tissue was added. After incubation at room temperature for 5 minutes, 0.2 mL of chloroform was added per 1 mL of Trizol Reagent. The samples were shaken (by hand) for 15 seconds and then further incubated at room temperature for 3 minutes followed by centrifugation (12,000 × *g* for 15 minutes at 4°C). After the upper aqueous phase was transferred to a fresh tube, 0.5 mL of isopropyl alcohol per 1 mL of Trizol Reagent was added, and the samples were incubated at room temperature for 10 minutes followed by centrifugation (12,000 × *g* for 10 minutes at 4°C). The supernatant was removed and 1 mL of 75% ethanol per 1 mL Trizol Reagent was added to wash the RNA pellet using a vortex. After centrifugation (7,500 × *g* for 5 minutes at 4°C), the RNA pellet was dried (for 5 minutes), then dissolved in 20 μL of RNase-free water. The concentration and purity of RNA preparations were measured under the optical densities of 260 and 280 nm using an ultraviolet spectrophotometer. The ratio of optical density exceeded 1.9 for all preparations. Total RNA (1 μg) was reversely transcribed in 20 μL of reaction system using the M-MLV First Strand Kit (Invitrogen) with oligo dT primers, according to the manufacturer's instructions. The synthesized cDNA was stored at −80°C before use.

Semi-quantitative reverse transcription-polymerase chain reaction (semi-RT-PCR)

The resulting cDNA template was then amplified by PCR using PCR Supermix (Invitrogen). The PCR primers for SYN, GAP43, BDNF, and TrkB were designed by using Primer premier 5.0 software, and were specifically tested by Primer-BLAST software and synthesized by TaqMan Gene Expression Assays (Applied Biosystems, Foster, CA, USA). Amplification was performed in Thermal Cycler 2720 (Applied Biosystems). Initial denaturation occurred at 94°C for 3 minutes. This was followed by 30 cycles for BDNF, TrkB and Fyn, 28 cycles for SYN and GAP43, and 25 cycles for GAPDH at 94°C for 45 seconds. Annealing at 55°C lasted for 45 seconds. Extension occurred at 72°C for 1 minute followed by a final extension step at 72°C for 5 minutes. Gene expression for SYN, GAP43, BDNF, TrkB, and Fyn was determined by normalizing to the average levels of the internal control, GAPDH. Every amplification procedure was repeated twice from different reverse transcription preparations, and the data were averaged. RT-PCR products were subjected to electrophoresis through an agarose gel (15 g/L), and relative mRNA levels of SYN, GAP43, BDNF, TrkB, Fyn, and GAPDH were quantified by band densitometry of gel images using Gel-Pro Analyzer Analysis software (Media Cybernetics). RT-PCR signals were acquired and analyzed identically to western immunoblotting signals. The expression of mRNA was normalized to GAPDH and then expressed as a percentage to the control samples (defined as 100%).

MWM task

On the day following the 14-day magnetic stimulation, all mice were subjected to the MWM procedure (between 9:00 and 12:00 a.m.) to assess hippocampus-dependent learning and memory performance *via* a spatial orientation task. This task was performed as previously described but with slight modifications (Pan et al., 2012). The task consisted of a tank (120 cm in diameter and 60 cm in height), which was filled with water to the depth of 45 cm and maintained at 25 ± 1 °C. The tank was divided into four equal quadrants (labeled 1, 2, 3, and 4) by two imaginary perpendicular lines crossing the center of the tank. A round platform (8 cm in diameter) was hidden 2 cm below the surface of opaque water in one of the four quadrants (*i.e.*, the target quadrant) of the basin. Mice were randomly placed in drop zones, facing the wall, in any of the three quadrants without the platform at the beginning of the trial or test. A total of 20 trials (4 trials per day for 5 consecutive days) were performed during the training session. Mice were allowed to swim for a maximum of 60 seconds to find the platform, or guided to the platform if it was not located by this time. After each trial, the mice were allowed to stay on the escape platform for 20 seconds. Twenty-four hours after the last trial (*i.e.*, on day 6), a probe test was performed in which the escape platform was removed and mice were allowed to swim for 60 seconds in search of the escape platform. A camera located above the center of the maze and a computerized animal tracking system (Ethovision2.0, Noldus, Wagenigen, the Netherlands) were used to monitor and relay images. The time that each mouse spent in finding the platform in each trial was recorded. Performance parameters included latency to the platform, quadrant dwell time, number of crossings, and swim speed. The MWM task was conducted from day 1 to day 6, *i.e.*, from day 7 (day 1r) to day 12 (day 6r).

Statistical analysis

All data are expressed as the mean ± SD. We performed tests of normality (using the Kolmogorov-Smirnov test) and homogeneity variance (using the Levene's test) on all data. If the distribution was normal and the variance homogenous,

one-way analysis of variance was then performed, followed by the Fisher's Least Significant Difference test to determine significance between the groups. Significance was reached at values of $P < 0.05$. The data were analyzed by the Statistical Analysis System V8 software (Raleigh, NC, USA).

Results

TMS regulated the mRNA and protein expression of SYN and GAP43 in the hippocampus of aging mice

Strong punctate expression of SYN was observed in the neuropil but was not present in the nucleus and perikaryon (**Figure 2S1–S6**). GAP43 was mainly located in the membrane, lamellae, and dendrites around spines (**Figure 2G1–G6**).

We next investigated the protein and mRNA levels of SYN, GAP43, BDNF, TrkB, and Fyn. Consistent with the immunohistochemical results, the immunoblots (**Figure 3A**) and their respective quantitative analysis (**Figure 3B**) revealed that SYN and GAP43 protein in the TMS group were significantly ($P < 0.01$ or $P < 0.05$ respectively) increased compared with controls (SYN, $P < 0.01$, GAP43, $P < 0.01$) and shams (SYN: $P = 0.003$, GAP43: $P = 0.002$). There were no significant differences between the sham and control groups. The results from semi-RT-PCR corroborated with those of the western immunoblotting analysis. As the semi-RT-PCR results shown in **Figure 3C**, **D**, compared with control mice, mRNA expression of SYN and GAP43 was significantly increased in TMS-treated mice ($P < 0.05$; SYN: $P = 0.026$, GAP43: $P = 0.031$). No significant differences were found between sham mice and controls (**Figure 3C**, **D**). The present findings demonstrated that TMS increased the mRNA and protein expression of markers for synaptic plasticity.

TMS modulated the mRNA and protein expression of BDNF, TrkB, and Fyn in the hippocampus of aging mice

Results from the western immunoblots (**Figure 3A**) and semi-RT-PCR (**Figure 3C**) showed that TMS-treated mice significantly upregulated the mRNA and protein expression of BDNF, TrkB, and Fyn (**Figure 3B**, **D**) compared with control ($P < 0.01$ or $P < 0.05$; western blot analysis: TMS *vs.* control, BDNF: $P = 0.001$, TrkB: $P = 0.002$, Fyn: $P = 0.032$; PCR: TMS *vs.* control, BDNF: $P = 0.003$, TrkB: $P = 0.005$, Fyn: $P = 0.004$). No significant differences were found between control and sham mice. These results suggested that the BDNF-TrkB signal may be involved in the regulation of brain aging and TMS.

TMS influenced spatial cognition in aged mice

To determine if the administration of TMS improved learning and memory in mice, we investigated the results of hippocampus-dependent spatial cognition test, the MWM task. The escape latency progressively ($P < 0.01$) decreased in mice from all groups during the 5 training days. No difference was found on day 5 relative to day 4 in the TMS-treated mice. All of the mice found the submerged platform within 20 seconds at the end of the trial on day 5, suggesting that they learnt the location of platform through the acquisition trials. Overall, the latency on days 2 and 4 was markedly reduced in TMS-treated mice compared with the control and sham groups (**Figure 4A**, $P < 0.01$; TMS *vs.* control, $P = 0.001$, TMS *vs.* sham, $P = 0.001$). TMS-treated animals performed significantly better than control and sham mice during this trial ($P < 0.05$; TMS *vs.* control, $P = 0.016$, TMS *vs.* sham, $P = 0.021$). There was no significant difference in the escape latency between control and sham groups. For the probe test, TMS-treated mice spent significantly ($P < 0.05$; TMS *vs.* control, $P = 0.034$, TMS *vs.* sham, $P = 0.028$) more time in the target quadrant compared with the control and sham groups (**Figure 4B**). Platform crossings were significantly ($P < 0.05$; TMS *vs.* control, $P = 0.037$, TMS *vs.* sham, $P = 0.035$) higher in TMS-treated mice (**Figure 4D**). There were no significance differences between control and sham groups. These results suggested that TMS treatment improved cognitive deficit in the aged mice. In our preliminary experiment using a maze with a visible platform, the mean swimming speed was found to not be affected by TMS (**Figure 4C**), indicating that motor ability and vision do not influence learning and memory events. Furthermore, the improvement in learning and memory in TMS-treated mice implied that TMS enhanced cognition in the aged animals. Overall, the results demonstrated that learning and memory deficits in normally aged mice were reduced with TMS treatment. Sham magnetic stimulation had no effect on the overall spatial cognition capacity.

Discussion

Over the last several decades, normal aging has been characterized by a gradual decline in cognitive function associated with the progressive reduction in structural and functional plasticity of brain regions (such as the hippocampus and the prefrontal cortex) that play key roles in cognition (Bisaz et al., 2013). Furthermore, cognitive deficits result from aging-mediated neurodegeneration; however, the precise mechanisms underlying this relationship are not well understood (Ramírez et al., 2012). During aging, cognitive decline can be measured *via* short- and long-term memory, psychomotor speed, attention, and executive function (Lyketsos et al., 2002).

Numerous reports have suggested that cognitive capacity can be affected by low-frequency TMS, although the underlying causes remain unknown (Boroojerdi et al., 2001; Wang et al., 2010; Tan et al., 2013). We have previously shown that low-frequency TMS (1 Hz, 110% of threshold) improves spatial cognition in aged mice (Ma et al., 2014) and enhances the synaptic strength of primary hippocampal neurons (Ma et al., 2013). Therefore, in the present study, we used this stimulation treatment to explore the mechanisms by which TMS improves cognition and synaptic plasticity.

Synaptic plasticity is the neurobiological basis for learning and memory, and the structural and functional modifications of hippocampal synapses result in cognitive changes (Hasan et al., 2011). The synaptic structure and convective functions have been recognized to correlate with changes in

proteins, receptors, neurotransmitters, and messenger molecules in neurons and at synapses (Sheng et al., 2002). Long-term memory formation requires the transcription and synthesis of new proteins (Hernandez and Abel, 2008). We therefore hypothesized that in rodents, differential changes in gene expression between the animals may be responsible for the variability in memory-related behaviors in this aged population. Moreover, the mechanisms by which TMS modulates synaptic plasticity in the aging hippocampus are unclear. Therefore, we focused on synaptic plasticity markers, namely SYN and GAP43, to explore the regulatory mechanisms and neurological basis of the effects mediated by TMS. These markers are involved in synaptic transmission and LTP in hippocampal pyramidal cells (Donovan et al., 2002; Kwon and Chapman, 2011). SYN is a presynaptic vesicle protein and a marker of synaptic density. This protein affects the release of neurotransmitters. GAP43 is synthesized during outgrowth and regeneration at an increased rate, and is enriched in nerve growth cones (Donovan et al., 2002), thereby prompting additional nerve regeneration. Compelling evidence supports the role of SYN and GAP43 in stimulating synapse formation and reconstruction (Donovan et al., 2002; Kwon and Chapman, 2011). Synaptic markers are considered to be reflective of changes in synaptic structure (Donovan et al., 2002; Kwon and Chapman, 2011). The present immunohistochemical and western immunoblotting results demonstrated that TMS upregulated the expression of SYN and GAP43 in aged mice. Therefore, this upregulation may be reflective of the effect of TMS on synaptic plasticity.

Furthermore, numerous reports have indicated that neuronal plasticity markers are dependent on both the BDNF and subsequent TrkB signaling pathways (Li and Keifer, 2012; Huang et al., 2013). BDNF plays an important role in the the survival, development, differentiation, and regeneration of neurons (Huang and Reichardt, 2001). It also mediates synaptic transmission and remodeling (Waterhouse and Xu, 2009). Therefore, in the current study, we measured the expression of BDNF and TrkB to illustrate the possible mechanisms underlying the effects of TMS. Our present results showed that in aged mice, the mRNA and protein expression of BDNF and TrkB was reduced, while TMS increased these levels in addition to the synaptic markers. Our present results showed that in aged mice, the mRNA and protein expression of BDNF and TrkB was reduced, while TMS increased these levels in addition to the synaptic markers. These results suggest that aging inhibits BDNF-mediated TrkB signaling and that TMS reverses this effect. Hippocampal BDNF levels have been shown to be significantly decreased in a rodent Alzheimer's disease (AD) model (Calon et al., 2005). Brain infusion of BDNF has been shown to reverse synaptic loss, and learning and memory deficits in AD rodents (Tian et al., 2012). Therefore, these findings and our current results may point to a relationship between cognitive enhancement and improved synaptic plasticity with TMS-mediated up-regulation of the BDNF-TrkB signaling pathway.

Fyn kinase is found in the post-synaptic density (PSD), which is the primary post-synaptic site for signaling transduction and processing in neurons. It is a receptor for the major excitatory transmitter glutamate (Kennedy et al., 2000; Nakazawa et al., 2001; Prybylowski et al., 2005; Yang et al., 2011), which plays a critical role in synaptic function. These subunits are critical for both LTP and long-term depression, which are considered to be central for learning and memory (Massey et al., 2004). In a previous study, Fyn is shown to be involved in the BDNF signal transduction pathways downstream of TrkB (Mizuno et al., 2003). The activation of receptor tyrosine kinases (RTKs), specifically TrkB, has also been implicated in Fyn regulation (Rajagopal and Chao, 2006; Xu et al., 2006). The present findings showed that the protein and mRNA expression level of Fyn increased concomitantly with increased synaptic markers and cognitive behavior in the normally aged hippocampus. These results are further corroborated by the finding that the overexpression of a constitutively active Fyn transgene in mice reduces the threshold for LTP induction (Lu et al., 1999), and the lack of Fyn expression promotes a blunted LTP response and impaired function of contextual fear memory (Mizuno et al., 2003; Isosaka et al., 2008; Babus et al., 2011).

Therefore, these findings suggest a crucial interaction between BDNF-TrkB signaling, the downstream effector proteins SYN and GAP43, and spatial memory. The collective effect of TMS on the synaptic markers and cognitive behavior suggest a regulatory influence of this treatment on synaptic plasticity and memory. However, the mechanisms by which TMS regulates cognition and plasticity requires further investigation. Fyn regulates key aspects of synaptic physiology. When activated, Fyn enhances synaptic function, and therefore, it can enhance the vulnerability of neurons to synaptotoxicity. A reduction in Fyn activation reverses this effect, and while this may be neuroprotective, excessive inhibition may lead to impaired LTP and hence, cognitive dysfunction. Therefore, maintaining a delicate balance between the activation and inhibition of Fyn may optimize the function of individual synapses, and this aspect requires further exploration.

The vast majority of animal studies addressing the consequences of aging on brain function have focused on topics related to memory, particularly spatial memory (Rosenzweig and Barnes, 2003). This form of memory is well known to be hippocampus-dependent (Voineskos et al., 2013). The MWM task is a standard test for hippocampus-dependent spatial memory (Kesner and Churchwell, 2011). Our results from the MWM task showed that although all aged mice learned and remembered the location of the platform, TMS treatment improved their learning performance compared with control and sham mice. The probe test showed that exploratory behavior in TMS mice was higher than that of control and sham animals. These results indicate that TMS reduces memory impairment in normally aged mice. In contrast, aged-matched control and sham mice showed deficits in the learning and memory (*i.e.*, a continuous long escape latency, unbiased navigating time in new and original quadrants, and

very few platform crossings).

This pattern of behavior resembles previous reports that suggest that hippocampal and frontal cortex damage can disrupt spatial reversal learning (Kesner and Churchwell, 2011). Cognitive decline during normal aging has been correlated with a decrease in hippocampal activity (Konishi and Bohbot, 2013). Therefore, we hypothesize that the aging-induced deficit in spatial learning and memory in the current study was mediated by the inhibition of hippocampal function which may have been reflective of hippocampal damage. TMS has been shown to improve cognitive performance (Boroojerdi et al., 2001). Our present findings suggest that low-frequency TMS improves spatial memory retrieval in aged mice. Other studies have reported that low-frequency TMS improves $A\beta_{1-42}$-mediated memory in addition to enhanced spatial cognition in normal rodents (Tan et al., 2013). Furthermore, this treatment has been shown to affect synaptic plasticity of the hippocampal CA1 area in VaD rats (Wang et al., 2010). Our results show a similar positive effect, and suggest that cognitive improvement may reflect the maintenance and increase of synaptic plasticity in the hippocampus. We have thus demonstrated cognitive impairment in the aged brain and suggest that TMS improves hippocampal-dependent spatial cognitive ability in these mice. Moreover these functions may reflect the changes in hippocampal plasticity.

In addition, spatial cognition and expression of the neurochemicals remained unchanged between the control and sham groups in the present study. We also found that placing normal aged mice into a magnetic stimulator environment and restraining them by hand without TMS application had little impact on their cognition and plasticity.

Brain aging triggers cognitive decline and reduces neuroplasticity. Decreased hippocampal synaptic plasticity may be a possible neurobiological mechanism underlying spatial memory deficits during aging. Taken together, this study suggests that TMS plays an important role in sustaining and regulating structural synaptic plasticity in the hippocampus of aged mice. Our results showed that TMS enhanced synaptic markers and activated the BDNF-TrkB pathway as well as downstream Fyn, suggesting that these events lead to the changes in structural plasticity in the aged hippocampus and improve cognitive function. Therefore, these neurochemical signaling pathways may be the underlying mechanisms of TMS. Overall, TMS-mediated BDNF-TrkB downstream signaling effector (particularly Fyn) and LTP improvement are important topics for ongoing research, which should help in developing and selecting feasible and effective treatments for the normal aging population.

Author contributions: *JM, ZCZ, and YYW conceived and designed the study. ZCZ, FL, DDG, and CYX analyzed the data. ZCZ, FL, and DDG provided the reagents, materials, and analysis tools. JM, ZCZ, FL, and DDG wrote the paper. All authors performed experiments and approved the final version of the paper.*

Conflicts of interest: *None declared.*

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