Transcranial Magneto-Acoustic Stimulation Improves Neuroplasticity in Hippocampus of Parkinson's Disease Model Mice

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Abstract

In this study, we have, for the first time, demonstrated the beneficial effects of transcranial magneto-acoustic stimulation (TMAS), a technique based on focused ultrasound stimulation within static magnetic field, on the learning and memory abilities and neuroplasticity of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of Parkinson's disease (PD). Our results showed that chronic TMAS treatment (2 weeks) improved the outcome of Morris water maze, long-term potentiation (LTP), and dendritic spine densities in the dentate gyrus (DG) region of the hippocampus of PD model mice. To further investigate into the underlying mechanisms of these beneficial effects by TMAS, we quantified the proteins in the hippocampus that regulated neuroplasticity. Results showed that the level of postsynaptic density protein 95 was elevated in the brain of TMAS-treated PD model mice while the level of synaptophysin (SYP) did not show any change. We further quantified proteins that mediated neuroplasticity mechanisms, such as brain-derived neurotrophic factor (BDNF) and other important proteins that mediated neuroplasticity. Results showed that TMAS treatment elevated the levels of BDNF, cAMP response element-binding protein (CREB), and protein kinase B (p-Akt) in the PD model mouse hippocampus, but not in the non-PD mouse hippocampus. These results suggest that the beneficial effects on the neuroplasticity of PD model mice treated with TMAS could possibly be conducted through postsynaptic regulations and mediated by BDNF.

Keywords Transcranial magneto-acoustic stimulation (TMAS) · long-term potentiation (LTP) · dendritic spine · brain-derived neurotrophic factor (BDNF) · hippocampus

Introduction

Parkinson's disease (PD) is a progressive neuronal degenerative disorder of the central nervous system that mainly affects the motor system, resulting primarily from dopaminergic neuron degeneration in the substantia nigra [1, 2]. With the hope to stimulate or modulate the activities of degenerating

neurons, several brain stimulation techniques have been developed. Deep brain stimulation (DBS) is an advantageous technique based on implanting electrodes into the brain to stimulate deep brain regions electrically [3, 4]. Although DBS could produce effective and immediate beneficial impacts on PD patients, noninvasive techniques are still desirable. Transcranial magnetic stimulation (TMS) is a noninvasive brain stimulation technique that has been widely applied in neurological and psychiatric disease treatment, which utilizes pulsed magnetic stimulation to modulate neuronal activities [5–7]. But due to the intrinsic properties of magnetic stimulation, the spatial resolution of TMS is only several centimeters and is not capable of stimulating deep brain regions.

In recent years, focused ultrasound stimulation (FUS) has evolved to be a noninvasive technique to modulate neuronal functions with high spatial resolution, and being able to target at small and deep brain regions due to the utilization of focused ultrasound [8–11]. Though proved to be beneficial in treating various neuronal diseases in preclinical researches, the unclear mechanisms of neuronal modulation induced by

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FUS made the manipulation of stimulation effects difficult. In 2003, Norton proposed the possibility of utilizing FUS within a static magnetic field, or called transcranial magneto-acoustic stimulation (TMAS), could serve as a novel and potential technique for noninvasive brain stimulation in treating neurological and psychiatric diseases [12, 13]. TMAS treatment is based on applying focused ultrasound to the target brain regions within a static magnetic field. Ionic particles in the conductive brain motivated by ultrasound would induce a transient current generated by Lorentz forces in magnetic field. The proportional relationship between the generated electric field and velocity of ionic particles, according to Faraday's law, made the manipulation of stimulation effect possible. Further, TMAS can provide spatial resolution at low millimeters even in deep brain regions, 10 times more focused than TMS due to the utilization of focused ultrasound [8, 12]. This granted TMAS the advantage on stimulating specific deep brain regions with small size, such as the substantia nigra, an important brain region highly involved in PD. These advantages rendered TMAS possibilities to be applied in brain stimulation in PD treatment in the future as a novel and noninvasive technique with superior advantages.

In this study, we utilized the TMAS technique to treat PD model mice and investigated into its effect on the neuroplasticity, the fundamental neuronal function underlying learning and memory of PD mice. It has long been known that the substantia nigra, an important brain region within dopaminergic pathway, and other components of the basal ganglia play important roles in learning and memory in PD patients suffering memory impairments. This is because the basal ganglia interacted with multiple brain regions that are related with memory, and many types of memory are subjected to regulations from these brain regions [14]. Hence, we hypothesized that TMAS treatment on the substantia nigra could provide beneficial effects on learning and memory abilities of PD mice. To test this hypothesis, we investigated the effect of chronic TMAS treatment (2 weeks) on the outcome of Morris water maze (MWM), a behavior test for spatial learning and memory abilities. To investigate the underlying mechanism, we monitored the electrophysiological signals of long-term potentiation (LTP) and depotentiation (DP), and dendritic spine densities, important features of neuroplasticity in the hippocampus, an important brain region deeply involved in learning and memory [15]. We found that TMAS treatment substantially improved the LTP results and elevated dendritic spine densities in the hippocampus of the PD mouse brain. These improvements could possibly be due to the recovery of postsynaptic density protein 95 (PSD-95) according to our Western blot results. To further clarify the mechanism of the improvements on neuroplasticity, we quantified the proteins related to brain-derived neurotrophic factor (BDNF) pathway. It was found that the levels of BDNF, cAMP response element-binding protein (CREB), and protein kinase B (p-

Akt) were improved by TMAS treatment in the hippocampus of PD mice. These results suggest that the improvement of neuroplasticity in the PD mouse hippocampus treated with TMAS could be mediated through BDNF signaling pathway.

Materials and Methods

Materials and Chemicals

Chemicals for Golgi-Cox staining were purchased from Tianjin Yanqiao Chemical Company (Tianjin, China). Chemicals for Western blot assay were purchased from Beyotime Biotechnology (Haimen, China).

1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was purchased from Sigma. All the solutions were prepared in distilled water.

Animals and TMAS Treatment

Adult, specific-pathogen-free (SPF) male C57BL mice of 8 weeks of age (52 mice in total) were purchased from the Experimental Animal Center of Chinese Academy of Medical Sciences. Mice were housed at a 12-h:12-h light/dark cycle with food and water *ad libitum*. All experiments were conducted according to protocols approved by the Committee for Animal Care of Nankai University and in accordance with the practices outlined in the NIH Guide for the Care and Use of Laboratory Animals.

At first, half of the mice were randomly chosen to receive a dosage of 25 mg/kg MPTP intraperitoneally each day for 5 days. This dosage was approved to be sufficient to generate PD-like behavioral phenotypes on mice [16]. At the same time, the other half of mice received intraperitoneal injection of blank saline solution. After 5 days of injection, all the mice were subjected to pole test (Fig. 1) (pole, 50 cm long; 1 cm O.D. with a 1.5-cm O.D. ball on the top) for PD model confirmation [17, 18]. Then, the mice that received MPTP administrations were equally divided into 2 groups: PD and PD-TMAS groups. At the same time, the mice that received blank saline injections were divided into 2 groups: control (con) and TMAS groups. TMAS and PD-TMAS groups received TMAS treatment conducted by the transcranial magneto-acoustic stimulation experimental system for animals (TMAS-ESA-BME01) designed by the Institute of Biomedical Engineering, Chinese Academy of Medical Sciences & Peking Union Medical College [19]. The ultrasound stimulation (1 MHz, 8 pulses, 100 Hz PRF, 60 s) was focused at the substantia nigra of the mouse brain (centered at anterior-posterior (AP), -3.4 mm; L, 1.5 mm; dorsal-ventral (DV), 4.5 mm) within a static magnetic field of approximately 0.17 T. The focused ultrasound region was 2 mm in diameter. The ultrasound intensity was $I_{\text{spta}} = 63 \text{ mW/cm}^2$ and generated

Fig. 1 Body weights and pole test results for PD model confirmation. **(a)** Mouse body weights during 5-day MPTP or saline injections ($n = 29$). **(b)** Turning over time spent by mice that received blank saline or MPTP injections. **(c)** Climbing down time spent by mice that received blank saline or MPTP injections. Data were plotted as mean $\pm$ SEM. * $p < 0.00005$; # $p < 0.05$

a transient pressure of 2.35 MPa within the mouse skull as measured by a standard reference hydrophone. During stimulation, mouse was anesthetized by isoflurane, and mouse head was fixed on a stereotaxic frame with its head hair shaved. The position of the substantia nigra for ultrasound probe targeting was measured on the surface of mouse head according to its interaural line (interaural, 0.40 mm which is equal to AP, -3.4 mm). The direction of magnetic field was perpendicular to the ultrasound direction, and this setup could produce an ionic current pulse along the sagittal direction in the mouse brain. Mice of Con and PD groups received sham stimulation by keeping the turned-off ultrasound probe on the mouse head located within the same static magnetic field for the same amount of time as TMAS and PD-TMAS groups. All the mice received TMAS or sham stimulation once each day for 2 weeks. After the 2-week treatment, mice were subjected to *in vivo* electrophysiological experiments or Morris water maze test.

Morris Water Maze Test

After 14 days of TMAS or sham stimulation, 24 mice (6 in each group) were subjected to the MWM test (RB-100A type; Beijing, China) to assess the spatial learning and memory abilities. The MWM system contained a 90-cm-diameter swimming arena filled with water that was stained white with nontoxic TiO_2 powder, and water temperature was kept at $25^\circ\text{C} \pm 1^\circ\text{C}$. The swimming activities of mice were recorded by a video camera and analyzed by a personal computer. On the computer software, the swimming arena was equally divided into 4 quadrants (I–IV) and a 9-cm-diameter platform was placed in the center of quadrant I and submerged 0.5 to 1 cm below water surface during the initial training stage.

During the initial training stage, all the mice were trained for 4 days with 4 trials each day. In each trial, the mouse was gently put onto water surface at a random point of each

quadrant. The time spent to find the hidden platform (escape latency) and the swimming speed of each mouse were monitored. If the mouse failed to find the platform within 60 s, the experimenter would guide the mouse and keep the mouse stay on the platform for 10 s, and the escape latency of this mouse would be recorded as 60 s. The time interval between trials was no less than 10 min to make sure all the mice got sufficient rest. After 4 days of training, all the mice were subjected to spatial probe test 24 h after the last training trial. During the spatial probe test, the platform was removed and the mouse was gently put into water at the opposite quadrant (IV). The mouse was allowed to swim freely for 60 s, and the target quadrant dwell time (percentage of time spent in quadrant I) and platform crossings were recorded on the computer.

In the following re-acquisition stage, the platform was placed in the center of quadrant IV to train the learning flexibility of mice. During this stage, all the mice were allowed 3 days with 4 trials each day for training and a following spatial probe test was conducted 24 h after the last trial. All the procedures were similar to those of the initial training stage.

In Vivo Electrophysiological Study

After 14 days of TMAS or sham stimulation, 28 mice (7 in each group) were subjected to electrophysiological experiments. LTP and DP protocols were similar to those described in our previous study [20–22]. Briefly, after being anesthetized with 30% (w/w) urethane (0.4 mL/kg, i.p.), mice were positioned on a stereotaxic frame (SR-6N; Narishige, Japan). After incision of the mouse scalp to allow for the exposure of the skull, a hole was drilled in the skull for the location of both the recording electrode and the stimulating electrode. A bipolar stimulating electrode was implanted in perforant pathway (PP) (-3.8 mm AP, 3.0 mm medial-lateral (ML), 1.5 mm

DV). The recording electrode was positioned in the dentate gyrus (DG) region of the hippocampus (−2.0 mm AP, 1.4 mm ML, 1.5 mm DV). The positions of both the recording electrode and the bipolar stimulating electrode were optimized by slowly dialing down the electrodes while recording the response evoked by single stimulation pulse with 0.2 ms duration and 0.4 mA stimulation intensity. Then, an I–O curve was obtained by tuning the stimulation intensity from 0.1 to 1 mA with a 0.1-mA increment each time. The stimulation intensity that could evoke a field excitatory postsynaptic potential (fEPSP) of 50 to 60% of the maximal fEPSP was used for the following experiments. After the stimulation intensity optimization, basal fEPSP evoked by single stimulation pulse was recorded every 60 s for 30 min. Then, a theta burst stimulation (TBS) (30 trains of 6 pulses at 100 Hz) was delivered to induce LTP. Then, the single stimulating pulse-evoked fEPSP was recorded every 60 s for 90 min after TBS. Afterwards, low-frequency stimulation (LFS) (900 pulses of 1 Hz for 15 min) was delivered to induce DP. Then, the single stimulating pulse-evoked fEPSP was recorded every 60 s for 90 min. All the raw data were analyzed in Clampfit 10.0 (Molecular Devices, Sunnyvale, CA). After the electrophysiological experiment, mice were sacrificed and brains were removed for Golgi-Cox staining or Western blot assay.

Golgi-Cox Staining

The protocol of Golgi-Cox staining was similar to those described in previous literatures [23] with modifications. Briefly, the bulk solution for Golgi-Cox staining was prepared with the following solutions: A, 200 mL of 5% (w/w) $K_2Cr_2O_7$; B, 200 mL of 5% (w/w) $HgCl_2$; and C, 560 mL of 5% (w/w) K_2CrO_4 . First, solution A and solution B were mixed slowly and thoroughly. Then, the mixture of solution A and solution B was added into solution C and mixed thoroughly. After staying in the dark for 3 h, the mixture solution was filtered and stored in the dark for later use.

After mouse sacrifice, the whole mouse brain was dissected out carefully. Then, the cerebellum was removed with a sharp blade. The hemispheres with the hippocampus inside were impregnated in the mixture solution for Golgi-Cox staining for 2 weeks. Each stained hemisphere was cut into coronal sections with the thickness of 150 μ m on a vibrating blade microtome (Leica VT1000S; Leica Biosystems, Nussloch, Germany). The brain slices with the hippocampus inside were immersed into 6% Na_2CO_3 solution for 20 min, then transferred into a series of EtOH solutions for dehydration: 70% EtOH for 10 min, 90% EtOH for 15 min, and 100% EtOH for 20 min. After dehydration, the brain slices were immersed into xylene for 20 min. Then, the brain slices were mounted onto glass slides and sealed with neutral balsam (Solarbio, Beijing, China) and covered with a coverslip. The slides were left air-dried for imaging and dendritic spine analysis later.

Imaging and Dendritic Spine Analysis

An upright fluorescence microscope system integrated with a digital camera (Leica DFC420 (CCD), Leica DM3000 (lens); Leica Biosystems, Nussloch, Germany) was used for imaging the Golgi-Cox-stained brain slices. The dendritic spines of neurons in the DG region were analyzed with ImageJ and NeuronStudio software programs [23–25]. Twenty dendrites with a length of no less than 10 μ m were analyzed in each group of mice.

Western Blot Assay

After mouse sacrifice, its hippocampus was dissected out carefully and kept frozen at −80 °C until use. For Western blot analysis, brain tissue (hippocampus) was lysed in 200 μ L buffer containing phenylmethanesulfonyl fluoride (PMSF) (1:100 dilutions; Beyotime Biotechnology, Haimen, China). The lysate was then centrifuged at 12,000g for 20 min at 4 °C. Then, the supernatant was sucked out and mixed thoroughly with loading buffer (4:1). The mixture was later boiled for 20 min and then electrophoresed in a 10 to 13% SDS-PAGE gel to produce separate protein bands. Then, the protein bands were transferred onto polyvinylidene fluoride (PVDF) membranes (0.45 μ m), on which the protein bands were incubated with 5% skim milk followed with primary antibody overnight at 4 °C. The next day, after being washed for 3 times with TBST, PVDF membranes with protein bands were incubated with secondary antibody. Protein bands were visualized on a chemiluminescence detection kit (Pierce) and exposed to X-ray film (Eastman Kodak, Rochester, NY). Equal protein loading was ensured by using β -actin expression using a mouse monoclonal antibody (1:1000; Santa Cruz, CA).

Hematoxylin/Eosin Staining

After sacrifice, mice were perfused with 0.1 mol/L phosphate buffer (pH 7.4) immediately. Then, the brains were removed and embedded in OCT compound (Tissue-Tek, Miles) and kept frozen at −20 °C. After tissue sectioning, the brain slices (10 μ m) stained with hematoxylin/eosin (HE) were photographed on a Leica microscope (Wetzlar, Germany).

Statistics

OriginPro 8 was used to plot and analyze all the data of this study. Two-way ANOVA with repeated measurements was conducted on the data in Figs. 2 and 3. One-way ANOVA was conducted on the data in Figs. 4, 5, 6, 7, and 8. *Post hoc* comparison was performed with Tukey's test, and a *p* value < 0.05 was considered as significantly different.

Fig. 2 MWM training and test results. (a) Escape latencies during training days. (b) Swimming speeds during training days. (c) Platform crossings on probing test day. (d) Percentage of duration spent in the target quadrant. * $p < 0.05$; # $p < 1 \times 10^{-10}$

Results

PD Model Confirmation

In the experiment, 25 mg/kg MPTP caused slight body weight loss at the 2nd day of injection but recovered in the following days to the level similar to saline-injected mice (Fig. 1a). These results suggested that MPTP administration did not cause substantial damage to the whole healthiness of mice. The pole test was performed on mice to test if the PD model was successfully established. In the mice that received MPTP injections, the turning over time was significantly longer than that in mice that received blank saline injections ($n = 29$, $F(1,56) = 26.6$, $p = 3.4 \times 10^{-6}$). And, the time spent for climbing down the pole by the mice that received MPTP injections was significantly longer than the climbing down time of mice that received saline injections ($n = 29$, $F(1,56) = 4.8$, $p = 0.033$). These results suggested that MPTP administration damaged the motor function of mice as approached by the pole test, and PD models were established in these mice.

TMAS Treatment Improved MWM Outcomes in PD Model Mice

After TMAS or sham stimulation, mice were subjected to MWM for spatial memory test. Data in Fig. 2a were subjected to 2-way ANOVA with repeated measurements. The training day factor (repeated) was significant ($n = 6$, $F(3,60) = 317.6$, $p = 9.1 \times 10^{-37}$), and the escape latencies in all groups decreased significantly in the following days as compared to the 1st training day ($p < 1 \times 10^{-10}$), suggesting the efficient learning during training days. Though the group factor was significant ($n = 6$, $F(3,20) = 4.64$, $p = 0.013$), neither MPTP treatment (con vs PD, or TMAS vs PD-TMAS) nor TMAS stimulation (con vs TMAS, or PD vs PD-TMAS) played a significant role in the escape latencies of mice as analyzed by *post hoc* comparison. Further, the swimming speeds (Fig. 2b) were not significantly different between either groups ($n = 6$, $F(3,20) = 0.03$, $p = 0.99$) or days ($n = 6$, $F(3,60) = 1.34$, $p = 0.27$), suggesting that the swimming abilities of mice in 4 groups were similar.

Fig. 3 Reverse MWM training and test results. **(a)** Escape latencies during training days. **(b)** Swimming speeds during training days. **(c)** Platform crossings on probing test day. **(d)** Percentage of duration spent in target quadrant. * $p < 0.05$; # $p < 1 \times 10^{-10}$

After 4 days of training, mice were subjected to probing test, and the platform crossings and the percentage of duration spent in the target quadrant were analyzed. As demonstrated in Fig. 2c, the group factor of platform crossings was significant ($n = 6$, $F(3,20) = 3.63$, $p = 0.031$) and the difference between PD and PD-TMAS was significant ($p < 0.05$), while the difference between PD-TMAS and con groups was not significant ($p > 0.05$) as analyzed by *post hoc* comparison. For Fig. 2d, the group factor of the percentage of duration spent in the target quadrant was significant ($n = 6$, $F(3,20) = 3.83$, $p = 0.026$), and the difference between PD and PD-TMAS groups was significant ($p < 0.05$), while the difference between PD-TMAS and con groups was not significant ($p > 0.05$). These results suggested that TMAS could improve the spatial learning and memory abilities in PD model mice while its impact on non-PD mice was minimal (con vs TMAS, $p > 0.05$).

After the probing test, mice were subjected to reverse MWM test for monitoring the flexibility of learning and

memory re-formation abilities. The reverse training day factor (repeated) was significant ($n = 6$, $F(2,40) = 74.9$, $p = 3.0 \times 10^{-14}$), and the escape latencies in all groups decreased significantly in the following days as compared to the 1st training day ($p < 1 \times 10^{-10}$), suggesting the efficient learning during training days. During the 3 days of reverse training, the group factor of escape latencies was not significant ($n = 6$, $F(3,20) = 0.90$, $p = 0.46$). Further, there were no significant differences in swimming speeds between either groups ($n = 6$, $F(3,20) = 0.21$, $p = 0.89$) or days ($n = 6$, $F(2,40) = 1.87$, $p = 0.17$).

After 3 days of reverse training, mice were subjected to the probing test. As demonstrated in Fig. 3, the group factor of neither the platform crossings ($n = 6$, $F(3,20) = 0.67$, $p = 0.58$) nor the percentage of duration spent in the target quadrant was significant ($n = 6$, $F(3,20) = 1.03$, $p = 0.40$). These results suggested that neither TMAS nor MPTP treatment produced a significant impact on the learning flexibility and memory re-formation abilities of mice.

Fig. 4 Normalized slopes of fEPSP in the DG of the mouse hippocampus. (a) Normalized slopes of fEPSP of con (black) and TMAS (red) groups. (b) Normalized slopes of fEPSP of PD (black) and

PD-TMAS (red) groups. (c) Averages of the last 10 min of LTP. (d) Averages of the last 10 min of DP. Data were plotted as mean $\pm$ SEM. * $p < 0.05$

TMAS Treatment Improved LTP in the PD Model Mouse Hippocampus

After MPTP administration for 5 days, the LTP effect in the DG region of the mouse hippocampus was substantially suppressed as compared to that of blank saline-treated mice (black lines in Fig. 4a vs b). At the end of LTP recordings, the normalized slope of fEPSP was only $97.3\% \pm 22.9\%$ of the baseline level in the PD group, while the normalized slope of fEPSP reached $181.7\% \pm 32.3\%$ of the baseline level in the con group. This implied a suppression of neuroplasticity caused by MPTP treatment in PD model mice.

In MPTP-treated mice, TMAS treatment improved LTP substantially. At the end of LTP recording, the slope of fEPSP reached $262.8\% \pm 46.7\%$ of baseline in the PD-TMAS group, which was much higher than that of the PD group ($97.3\% \pm 22.9\%$). These results suggested that TMAS

treatment could potentially alleviate the memory impairments caused by MPTP treatment by improving the LTP effect in the hippocampus. On the other hand, TMAS treatment did not induce a notable change of LTP in saline-treated mice, and the normalized slope of fEPSP in the TMAS group ($142.3\% \pm 35.5\%$) was even slightly lower than that of the con group ($181.7\% \pm 32.3\%$). This implied that the neuroplasticity of non-PD mice may not benefit from TMAS treatment.

We quantified the extent of LTP and DP by calculating the averages of the normalized fEPSP slopes of the last 10 min of LTP or DP recording of each group of mice (Fig. 4c, d). The group factor of the normalized fEPSP slopes of the last 10 min of LTP reached a significant level ($n = 7$, $F(3,24) = 4.48$, $p = 0.012$), and the difference between PD and PD-TMAS groups was significant ($p < 0.05$) (Fig. 4c), while the difference between con and PD-TMAS groups was not significant. These results suggested that TMAS treatment could improve the

Fig. 5 Normalized slopes of fEPSP of DP in the DG of the mouse hippocampus (normalized against slopes at the end of LTP recordings). (a) Normalized slopes of fEPSP of con (black) and TMAS (red) groups.

(b) Normalized slopes of fEPSP of PD (black) and PD-TMAS (red) groups. (c) Averages of the last 10 min of DP. Data were plotted as mean $\pm$ SEM

LTP effect in the DG of the hippocampus in PD model mice and could potentially improve the learning and memory abilities of PD mice. This agreed with our MWM results.

On the other hand, the DP results (Fig. 4d) did not show any significant difference between groups ($n = 7$, $F(3,24) = 1.17$, $p = 0.34$). Since the baseline level of DP (the end of LTP) was different in each mouse, we further quantified the DP extent by re-normalizing the fEPSP slopes of DP against those at the end of LTP of each mouse as shown in Fig. 5a, b. The normalized fEPSP slope decreased in the TMAS group, which was similar to that of the con group right after LFS, but increased gradually in the following recording time. As for the averages of the normalized fEPSP of the last 10 min of DP, the group factor did not reach a significant level ($n = 7$, $F(3,24) = 2.27$, $p = 0.11$). These results suggested that TMAS treatment preferentially improved neuroplasticity related to memory formation but had a minimal impact on the neuroplasticity related to memory re-formation. This agreed with our MWM results.

TMAS Treatment Increased Dendritic Spine Densities in the DG Region of the PD Mouse Hippocampus

After electrophysiology experiments, the mice were sacrificed and the brain tissues were treated by Golgi-Cox staining. Figure 6 shows example pictures of dendritic spines and dendritic spine densities of neurons in the DG region of the mouse hippocampus. The group factor was significant ($n = 20$, $F(3,76) = 7.9$, $p = 1.1 \times 10^{-4}$). The total dendritic spine densities were significantly lower in the PD group as compared to those of the con group ($p < 0.05$). Dendritic spine density was significantly higher in PD-TMAS mice than that of PD mice ($p < 0.01$), and the difference was not significant between con and PD-TMAS groups. These results suggested that TMAS treatment could alleviate the suppression of postsynaptic sites in the PD mouse hippocampus and hence contribute to an enhanced neuroplasticity, which agreed with our LTP results.

Fig. 6 Dendritic spine densities in the DG of the mouse hippocampus. Up left, the mouse hippocampus and the position of the DG (black arrow). Up right, neurons in the DG. Down left, example pictures of dendrites in

the DG (dendrite length, 20 μm). Down right, dendritic spine densities of DG neurons. Data were plotted as mean ± SEM. * $p < 0.05$; # $p < 0.01$

TMAS Treatment Improved Neuroplasticity-Related Proteins in the PD Model Mouse Hippocampus

To further explore the mechanisms underlying the enhanced LTP effect in the mouse hippocampus, we quantified the proteins related to neuroplasticity in this brain region. According to our Western blot results, PSD-95, an important protein that almost exclusively located on the postsynaptic density of neurons, played important roles in neuroplasticity. The group factor of PSD-95 contents reached a significant level ($n = 6$, $F(3,20) = 5.72$, $p = 0.005$). PSD-95 was extensively suppressed in PD model mice as compared to control mice (con vs PD, $p < 0.05$) and was elevated by TMAS treatment (PD-TMAS vs PD, $p < 0.05$), to the level that was not significantly different from the con group (PD-TMAS vs con, $p > 0.05$). While at the same time, TMAS treatment did not bring a significant change in the PSD-95 contents in non-PD mice (con vs TMAS). These results suggested that TMAS treatment rescued the LTP effect of PD model mice probably through a postsynaptic regulation.

While for another important protein related to neuroplasticity that is located on the presynaptic terminals, synaptophysin (SYP), the group factor was not significant ($n = 7$, $F(3,24) = 1.20$, $p = 0.33$). These results suggested that the presynaptic protein SYP was not affected by either MPTP or TMAS treatment.

BDNF and Other Neuroplasticity-Related Proteins in the Hippocampus

To clarify the mechanism underlying the improved neuroplasticity of PD model mice by TMAS treatment, we further quantified several proteins tightly related to neuroplasticity. Our Western blot results indicated that the level of BDNF, an important protein deeply involved in the differentiation and survival of neurons and regulated neuroplasticity mechanisms underlying the function of learning and memory [26–29], played a significant role in our experiments. The group factor of BDNF contents was significant ($n = 8$, $F(3,28) = 14.3$, $p = 7.6 \times 10^{-6}$). BDNF was

Fig. 7 PSD-95 and SYP levels in the mouse hippocampus. Left, examples of protein bands in each group. Right, TMAS effect on PSD-95 (up) and SYP (down) contents. Data were presented as mean $\pm$ SEM. * $p < 0.05$

substantially declined in the PD mouse hippocampus as compared to that of the con group (con vs PD, $p < 0.05$). On the other hand, BDNF was significantly higher in the PD-TMAS group as compared to that of the PD group (PD vs PD-TMAS, $p < 0.05$), to the level that was not significantly different from that of the con group (PD-TMAS vs con, $p > 0.05$). These results suggested that the elevated BDNF could possibly contribute to the recovery of neuroplasticity in the PD-TMAS group. On the other hand, TMAS treatment increased the BDNF level in non-PD mice to a significant level as well (con vs TMAS, $p < 0.05$). These results suggested that TMAS treatment had beneficial effects on both non-PD and PD mice, and the improvement of neuroplasticity in PD mice could be possibly mediated by the BDNF pathway.

BDNF modulated membrane excitability through tropomyosin receptor kinase B (TrkB), a receptor of BDNF, which mediated multiple effects initiated by BDNF, such as neuronal differentiation and survival [26]. In our results, though the TrkB level was suppressed in PD model mice and partially recovered by TMAS treatment, the difference did not reach a significant level ($n = 5$, $F(3,16) = 0.64$, $p = 0.6$). Hence, the neuroplasticity may be predominantly contributed by the

elevation of BDNF rather than the change of its membrane receptors.

p-Akt is the phosphorylated form of Akt, also known as protein kinase B, which plays important roles in the BDNF-TrkB signaling pathway. The group factor of p-Akt contents was significant ($n = 5$, $F(3,16) = 5.37$, $p = 0.01$). The elevation of p-Akt level induced by TMAS was significant in PD mice (PD vs PD-TMAS, $p < 0.05$) while the difference in non-PD mice did not reach a significant level (con vs TMAS, $p > 0.05$), and the difference was not significant between PD-TMAS and con groups ($p > 0.05$). These results agreed with our LTP results, suggesting that the level of p-Akt was affected by BDNF-TrkB upregulation and was an important mediator of LTP in our experiment.

CREB is a transcription factor that regulates the transcription of genes such as c-fos, BDNF, tyrosine hydroxylase, and numerous neuropeptides and is well known for its involvement in learning and memory [30]. CREB could be activated by multiple pathways mediated by BDNF-TrkB activation [26]. Our results demonstrated that the group factor of CREB contents was significant ($n = 5$, $F(3,16) = 4.31$, $p = 0.02$). TMAS treatment significantly elevated the level of

Fig. 8 BDNF and other related protein levels in the mouse hippocampus. Left, examples of protein bands in each group. Right, contents of BDNF, NR2A, NR2B, TrkB, CREB, and p-Akt. Data were presented as mean $\pm$ SEM. * $p < 0.05$

CREB in the PD model mouse hippocampus (PD-TMAS vs PD, $p < 0.05$), while the difference in non-PD mice was not significant (con vs TMAS) and the difference was not significant between PD-TMAS and con groups. These results suggested CREB was involved in regulating neuroplasticity of PD model mice.

N-Methyl-D-aspartate receptors (NMDARs) are a family of glutamate receptors and ion channels that are deeply involved in synaptic neuroplasticity and synapse formation in the brain [31–35]. NR2A and NR2B are 2 important variants of NMDAR subunits underlying neuroplasticity mechanisms of learning and memory [31–35]. NR2B is predominant in early postnatal brain and present in immature neurons and extrasynaptic locations. In the later stage of life, the number of NR2A grows and eventually outnumbers that of NR2B [36]. Our Western blot results demonstrated that the group factor of NR2A contents was significant ($n = 5$, $F(3,16) = 3.88$, $p = 0.03$). The NR2A content of the PD group was significantly lower as compared to the con group ($p < 0.05$). Though TMAS treatment did not produce a significant elevation of NR2A in PD model mice (PD-TMAS vs PD, $p > 0.05$), it did elevate NR2A content to the nonsignificant level as compared to the con group (PD-TMAS vs con, $p > 0.05$). These results suggested an impairment of NR2A in PD model

mice that TMAS treatment could partially rescue. While on the other hand, though the content of NR2B showed a similar trend as NR2A, the group factor did not reach a significant level ($n = 5$, $F(3,16) = 2.83$, $p = 0.07$). This may be due to the less effect of TMAS treatment on NR2B.

Histological Observation

As demonstrated in Fig. 9, HE-stained brain tissues treated and untreated with TMAS in both the stimulation site (substantia nigra (SN)) and the recording site (DG) were shown. Under the current experimental setup, neurons from the TMAS-treated mice were full and compactly arranged, suggesting minimal damage caused by TMAS.

Discussion

FUS was a technique firstly predicted to be able to impact neuronal functions for treating chronic pain and PD more than 60 years ago [37]. Then, the following studies in early years used high-intensity ultrasound as a noninvasive neurosurgery approach to irreversibly ablate target brain regions [10, 38–40] with high power usually exceeding 1000 W/cm². Later

Fig. 9 HE-stained brain tissues of the SN and DG regions of mice. Scale bars, 500 μm

researches utilized low-intensity ultrasound (no higher than 500 mW/cm^2) whose effect was reversible on modulating neuronal excitation and inhibition and avoided the thermal injury caused by high-intensity ultrasound [8, 41, 42]. Though still in preclinical stage, low-intensity FUS has been demonstrated to be beneficial in treating traumatic brain injury in rodent models [11, 43]. Other studies showed that FUS could improve hippocampal neurogenesis in mice, suggesting its potential application in treating Alzheimer's disease and other dementia in the future [44]. And a recent study demonstrated that FUS could improve motor function and antioxidative capacity of PD mice [45].

In spite of the advantages and beneficial effects above, the mechanism of the neuromodulation effects of low-intensity FUS was not clear [8]. Gas bubble formation and explosion was proposed to be a possible mechanism [46] while other studies suggested gas bubbles may not be a major cause [47]. A recent study by Gulick et al. [48] demonstrated that the effect of ultrasound stimulation on the rat motor cortex was completely different from that by electrical stimulation, and they suggested this could be a microbubble-related effect.

With the unclear mechanism due to the intrinsic properties of ultrasound stimulation, the outcomes of FUS became complicated to understand, and manipulation of the stimulation effect was difficult. On the other hand, the possibility of an alternative method of generating electrical field with ultrasound within a magnetic field was proposed by Norton in 2003 [13], mentioned as TMAS in this article, and its mechanism can be explained by Faraday's law. Basically, the ionic particles in the conductive tissue (brain) moved by ultrasound wave generate an electrical current induced by Lorentz forces within a magnetic field. The Hall electric field generated by ion movement within static magnetic field motivated by ultrasound is proportional to the speed of ionic particles, implying the possibility of manipulating the stimulation effect of TMAS by adjusting ultrasound. Yuan et al. [12] simulated the effect of TMAS on Hodgkin–Huxley neuron model, suggesting that the stimulation effect could be possibly monitored via tuning ultrasound intensity due to the mechanism of electrical field generation by ultrasound within magnetic field.

In our earlier work [19], we have showed that TMAS could generate electrical field in conductive media whose direction

was perpendicular to the direction of magnetic field and acoustic field, and the distribution of electric field was highly consistent with acoustic field. Along with the high spatial resolution (2 mm focus diameter), TMS could be possibly applied in stimulating small brain regions, such as the substantia nigra. In this study, we, for the first time, demonstrated the beneficial effects of TMS treatment on the neuroplasticity of PD model mice *in vivo* and explored the underlying mechanisms. Unlike the currently widely applied TMS that utilized repetitive magnetic stimulation at a certain frequency, our TMS technique used a static magnetic field. Previous literatures demonstrated high intensity (1.5 T) and long exposure (3 h) of static magnetic field did impact biological samples, such as Na^+ and Ca^{2+} levels in the rat brain [49]. To avoid this problem, we used low intensity (0.17 T) and short exposure time (60 s) of magnetic field in our study to minimize possible tissue injury. To further exclude the possible impact of static magnetic field on our results, the mouse groups in our study that were subjected to sham stimulation were also placed in the same magnetic field and received the same amount of static magnetic field exposure as TMS groups.

MWM is a commonly used method to study spatial learning and memory abilities of rodents [50], and various research studies demonstrated that MPTP caused cognition impairments in mice [51–54], including impairments of MWM outcomes [51, 53]. In the work of Deguil et al. [51], subchronic administrations of MPTP (MPTP-HCl (30 mg/kg/day), i.p., 5 days) caused deficits on learning and memory of mice in 3 versions of water maze. Prediger et al. [53] showed that a single intranasal (i.n.) administration of MPTP (1 mg/nosril) resulted in deficits in elevated plus maze, social cognition, and water maze tests. In this study, we observed suppression on MWM outcomes of MPTP (25 mg/kg/day, 5 days)-treated mice that agreed with these literatures.

Hippocampal neuroplasticity is an important underlying mechanism of learning and memory on the cellular level [55]. The impairment of neuroplasticity in the hippocampus caused by MPTP treatment was confirmed by several recent studies [56–58]. The suppressed LTP induction was observed in hippocampal slices of mice pretreated with MPTP [56]. In a study by Zhu et al. [58], the impairment of both LTP and long-term depression (LTD) was observed on hippocampal slices treated with MPTP. In another study by Zhu et al. [57], a suppressed LTP but a prolonged LTD was observed on hippocampal slices of mice pretreated with MPTP. Our results on the suppression of LTP induction in the mouse hippocampus treated with MPTP agreed with these previous literatures.

In both MWM and LTP results, TMS showed substantial improvements on PD model mice, suggesting the facilitation of spatial learning and memory and the recovery of the underlying neuroplasticity mechanisms induced by TMS treatment. Unfortunately, we did not observe any improvement

on the reverse phase of MWM or in the correlating DP results. Since there is no *in vivo* study reported on TMS yet, we speculate that it may be due to the mild stimulation intensity used in this study. In the future, experimental conditions of TMS should be optimized for pursuing beneficial effects of both memory and memory re-formation abilities.

Our Western blot results demonstrated a postsynaptic regulation mechanism (elevated PSD-95) underlying the improved LTP in the PD mouse hippocampus. The correlated findings of the elevated dendritic spine densities in PD mice could serve as the structural support. This postsynaptic regulation could possibly be mediated by BDNF. In previous literatures [58], the decrease of BDNF expression was observed on hippocampal slices treated with MPTP that agreed with our results. TMS treatment could elevate BDNF expression that possibly contributed to the recovery of LTP induction. On the other hand, though BDNF level was also elevated in the non-PD mouse brain that was supposed to increase the excitability in non-PD mice, we did not see an elevation of LTP in the TMS group as compared to the con group. While at the same time, the reduction of the levels of NR2A and NR2B (though not to significant levels) may partially undermine the upregulation of postsynaptic excitability caused by the elevated BDNF, which may contribute to the nonsignificant difference between LTP results in non-PD mice. CREB is essential for memory formation and serves as a positive regulator for LTP [59]. CREB indirectly controls short-term memory by regulating the expression of BDNF and also enhances long-term memory consolidation via a positive feedback loop. In our results, the significant increase of CREB in PD-TMS mice suggested that CREB regulations could contribute to the improvements of LTP and spatial recognition abilities.

In this study, we investigated the effect of TMS treatment under only 1 setup of parameters, and our current TMS setup did not reach the threshold that would cause action potential firing in neurons, which made our treatment a “sub-threshold stimulation.” In the future, experimental parameters should be investigated to manipulate and optimize stimulation effect.

Conclusions

In this study, we, for the first time, demonstrated the beneficial effects of TMS treatment on the neuroplasticity of the hippocampus of PD model mice and explored the underlying mechanism. This study provides a theoretical basis for TMS, a novel and noninvasive technique with high spatial resolution to be applied in neurological disease treatment in the future.

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