Antidepressant-Like Effect of Low-Intensity Transcranial Ultrasound Stimulation

Daqu Zhang, Hangdao Li, Junfeng Sun, Weiwei Hu, Wen Jin, Shengtian Li, and Shanbao Tong

Abstract*—*Objective: **Transcranial ultrasound stimulation (TUS) is a noninvasive neuromodulation technique with good spatial resolution and deep penetration. This study aims to investigate whether TUS has antidepressant-like effect to depressed rats.** ***Methods:*** **Rats were divided into five groups, including two groups (ST-Ctr and ST-Res) for evaluating the short-term impact of restraint stress and three groups (LT-Ctr-ShamTUS, LT-Res-ShamTUS and LT-Res-TUS) for studying the long-term effects of restraint and TUS stimulation. The TUS-treated rats were subjected to 15 min TUS stimulation to the prelimbic cortex every day for 2 weeks after the restraint. Then, depressive symptoms related behavioral outcomes were estimated in ST-Ctr and ST-Res groups (1 week after restraint), as well as in the other three groups (3 weeks after restraint).** ***Results:*** **The 48-h-restraint stress could lead to long lasting reduction of exploratory behavior (1 and 3 weeks after restraint) and protracted anhedonia (only observed 3 weeks after restraint). TUS application successfully reversed the core depressive phenotype, anhedonia, indicated by significantly higher sucrose preference index in LT-Res-TUS group $(88.8\% \pm 2.5\%, \mathrm{n} = 16)$ than LT-Res-ShamTUS group $(72.8\% \pm 7.2\%, \mathrm{n} = 16)$ $(p = 0.046)$. Furthermore, the brain derived neurotrophic factor expression in left hippocampus was significantly promoted in LT-Res-TUS group $(1.53 \pm 0.096, \mathrm{n} = 5)$ compared to LT-Res-ShamTUS group $(0.79 \pm 0.054, \mathrm{n} = 5)$ $(p = 0.009)$. In addition, the histologic results of hematoxylin and eosin staining showed no TUS-induced brain tissue injury.** ***Conclusion:*** **These results demonstrated that low intensity TUS had antidepressant-like effect.** ***Significance:*** **TUS has been speculated to have therapeutic effect in depression. This study provide evidence for the antidepressant-like effects of TUS in rats for the first time.**

***Index Terms*—Low-intensity pulsed ultrasound, transcranial stimulation, antidepressant-like effects, restraint stress, hippocampal BDNF.**

I. Introduction

DEPRESSION is a commonly occurring, recurrent mood disorder linked to quality of life, medical morbidity, and mortality. Currently, there are mainly three types of treatments: drug therapy, psychotherapy and brain stimulation. Despite the remarkable increase of antidepressant medications as the initial main treatments in depression, therapeutics are still plagued by inadequate responses in a significant number of patients [1].

For those patients with medicine-resistant depression, brain stimulation methods including electroconvulsive therapy (ECT), vagus nerve stimulation (VNS), deep brain stimulation (DBS), and transcranial magnetic stimulation (TMS) have been utilized as alternative therapies [2]–[5]. Nevertheless, ECT may cause cognitive side effects, VNS and DBS needs invasive implant surgery. Compared with these invasive treatments, TMS, which can non-invasively stimulate the cerebral cortex, has been widely applied in clinical to depression. However, some patients do not respond to TMS treatment [6]. Furthermore, TMS has relatively low spatial resolution and low penetration, which makes it difficult to modulate the neural activities in subcortical regions [7]. tDCS is a noninvasive neuromodulaion technique with even poor spatial resolution and lower penetration. And recent researches showed that tDCS might be efficacious for treating the major depressive episodes. Nevertheless, the data do not support the use of tDCS in treatment-resistant depression, or as an add-on augmentation treatment [8].

Compared with transcranial direct current stimulation (tDCS) and TMS, transcranial ultrasound stimulation (TUS) is another non-invasive technique with better spatial resolution and deeper penetration depth, and has also been demonstrated to be an effective neuromodulation tool in both basic and clinical neurosciences [9]–[12]. At low intensity, TUS can activate or suppress neuronal activity by delivering mechanical vibration without irreversible damage [13]. TUS could successfully alter behavioral and electrophysiological process by stimulating particular regions of brain [14], [15]. As potential applications, recent studies reported therapeutic effects of TUS in ischemic brain injury

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[16], [17], epilepsy [18], and Alzheimer's disease [19], [20]. There has also been speculation that TUS would be a potential therapeutic strategy for depression [21].

In aspect of neuroplasticity, decreased hippocampal neurogenesis and brain derived neurotrophic factor (BDNF) might be causally related to depression, which is supported by various studies. For examples, hippocampal neurogenesis has been found to be down-regulated during the development of depression and up-regulated by antidepressant treatments [22], [23]. BDNF plays an important role in the pathogenesis of depression, and promoting the BDNF could be therapeutic [24]. On the other side, recent animal studies demonstrated that low intensity ultrasound stimulation could elevate BDNF expression in hippocampus [9] and significantly promote the neural proliferation in the dentate gyrus of the dorsal hippocampus [25]. These results suggest that TUS might be able to relieve the depression symptoms by promoting the BDNF level and neurogenesis. Additionally, TUS could induce temporary and reversible blood-brain barrier (BBB) disruption with hyper-permeability, which might allow delivery of antidepressants or proteins from peripheral blood to the brain [26]. These results rationalize the use of TUS as a potential therapeutics of depression or assistive tool for delivering the antidepressants across the BBB.

Antidepressant-like effects of neuromodulation, e.g., TMS, can be achieved by targeting specific brain circuits such as prefrontal cortex (PFC), hippocampus and other limbic structures in reward and affective circuitry [27]. Among these brain regions, PFC is the easiest one to be targeted by TUS, whereas other deep structures are covered with dense white matter tracts which may absorb or scatter low-intensity TUS [9]. In addition, increasing clinical evidences have demonstrated the efficacy and safety of TMS to the left PFC of depression patients [28]. Thus, we propose to apply TUS to the prelimbic cortex (PLC, homologous to human PFC [29]) to study its therapeutic effect on depression.

Based on the above review, this study aimed to investigate the potential antidepressant-like effects of TUS in a rat model. We created a depression model by restraining rats for 48 hours continuously, and then investigated both short-term (1 week after restraint) and long-term (3 weeks after restraint) effects of restraint and the antidepressant-like effects of TUS with a group of behavioral tests. In addition, we measured the hippocampal BDNF by western blotting to test the underlying mechanisms of antidepressant-like effects of TUS on depression, and also assessed the safety of long-term TUS by histologic analysis.

II. Materials and Methods

A. Experimental Paradigm

The experimental design is demonstrated in Fig. 1. Rats were allowed to acclimate for 10 days before experiments. Then sucrose preference test (SPT), or pre-SPT hereafter, was performed in all rats to have the baseline of anhedonia before the restraint. According to the pre-SPT, rats were divided into five groups with similar distribution of sucrose preference index (SPI), including two groups (control/ST-Ctr (N = 14) and restraint/ST-Res (N = 13), Fig. 1(a)) for evaluating the short-term impact of restraint and three groups (control with sham TUS/LT-Ctr-ShamTUS (N = 16), restraint with sham TUS/LT-Res-ShamTUS (N = 16), and restraint with TUS/LT-Res-TUS (N = 17), Fig. 1(b)) for studying the long-term effects of restraint and antidepressant-like effects of TUS. Then the 48-hr restraint was conducted to three restraint groups (ST-Res, LT-Res-ShamTUS and LT-Res-TUS), while the rest two non-restraint groups (ST-Ctr and LT-Ctr-ShamTUS) were remained in the home cages in a separated room. After the restraint, rats were kept in home cages with free access to food and water for one week to recover the weight. Then behavioral tests were carried out to ST-Ctr and ST-Res groups immediately. For the rest rats, after the same period of recovery, TUS was applied to the LT-Res-TUS group for two weeks, while sham TUS operation was applied to LT-Ctr-ShamTUS and LT-Res-ShamTUS groups.

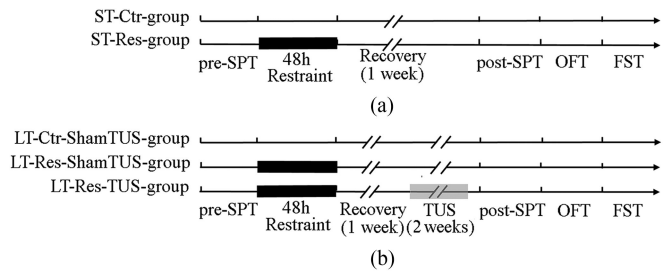

Fig. 1. Experimental paradigm. (a) ST-Ctr and ST-Res groups to investigate the short-term effects of restraint stress. (b) LT-Ctr-ShamTUS, LT-Res-ShamTUS and LT-Res-TUS groups to investigate the long-term effects of restraint stress and antidepressant-like effects of TUS. TUS, transcranial ultrasound stimulation; SPT, sucrose preference test; OFT, open field test; FST, forced swimming test.

In order to distinguish short-term and long-term impacts of the restraint and antidepressant-like effects of TUS, a series of behavioral tests were carried out including sucrose preference test (SPT), open field test (OFT) and forced swimming test (FST). After that, rats were sacrificed for measuring the level of BDNF by western blotting analysis and assessment of safety of long-term TUS by hematoxylin and eosin (H&E) staining.

All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Shanghai Jiao Tong University, and every possible effort was made to minimize the number of animals used and their suffering.

B. Animals

Six weeks old male Sprague-Dawley rats (200–250 g) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). Rats in the same group were housed in pairs in polycarbonate cages (420 mm × 260 mm × 200 mm) with free access to food and water. The rats were kept on a 12 h reversed light/dark cycle (lights on at 9 pm) in a room with temperature at 24–26 °C and relative humidity at 50–66%. The reversed time cycle was aimed to conveniently

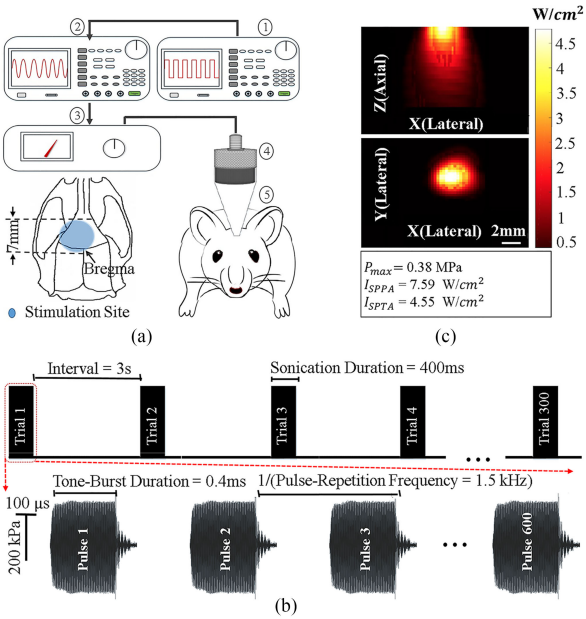

Fig. 2. Construction and parameters of transcranial ultrasound stimulation. (a) Schematic of TUS system: ① function generator #1, ② function generator #2, ③ amplifier, ④ transducer and ⑤ acoustic collimator (d = 7 mm). Anatomy map of rat's head showed the site of stimulated region by TUS. (b) Illustration of the parameters for the pulsed TUS, i.e., acoustic intensities (AI), sonication duration (SD), tone burst duration (TBD), and pulse repetition frequencies (PRF). The tone pulse was composed of several cycles of basic waves. (c) Acoustic intensity profile of 500 kHz transducer in the longitudinal (top) and transversal (bottom) plane to the sonication path.

conduct behavioral testing in rat's active (dark) phase of the light cycle.

C. Procedure of 48-Hour Restraint

Referring to the work that 24-hour restraint stress could induce long-term depressive phenotypes in mice [30], we adapted this acute restraint method to build depression model in Rats. Each rat was individually placed in a ventilated transparent plastic cylindrical container (60 mm to 65 mm in diameter and 240 mm in length) for 48 hours (Supplementary Fig. S1(a)). According to the weight of rats, deformable plastic sheets of 1 mm to 3 mm in thickness were put inside the container to restrain the animals as tight as possible but not hurting them. During the restraint, rat could move its head and anterior limb, but was not able to move or turn around. The container was designed with holes for air flow (diameter: 1 cm, interval: 2 cm). There were not food and water supply until the end of the restraint.

D. Transcranial Ultrasound Stimulation

The TUS system (Fig. 2(a)) consists of four major parts: (i) two serially connected function generators (AFG3022B, Tektronix, USA), (ii) a custom-designed radio frequency amplifier (HGX100, Nanjing, China), (iii) a single element immersion type planar transducer (25.4 mm in diameter, V301, Olympus, USA), and (iv) a custom-designed acoustic collimator (7 mm diameter output aperture). The first function generator controlled the pulse repetition frequency (PRF), sonication duration (SD) and inter-trial-interval, triggering the second function generator, which controlled the fundamental frequency and tone-burst duration (TBD). The pulse sequence generated by function generator module was then amplified to 180 Vpp to drive the transducer. After collimation, the pulsed ultrasound waves were transmitted into the left PLC (3.5 mm anterior and 0.75 mm lateral to the bregma) of rats [31], using the stereotaxic apparatus (brain stereotaxic, RWD, Shenzhen, China).

Pulsed ultrasound waveforms were constructed as illustrated in Fig. 2(b). For each trial, ultrasound pulses were repeated at PRF of 1.5 KHz for a SD of 400 ms. Each ultrasound pulse contained 200 sinusoidal waves corresponding to the TBD of 0.4 ms at the fundamental frequency of 0.5 MHz. The ultrasound pressure field generated by the planar transducer (mounted on collimator) using above acoustic parameters was measured in degassed water tank, by a needle-type hydrophone (0.5 mm, 1622, Precision Acoustics, UK). The change of ultrasound pressure measured by hydrophone at one point during one pulse is shown in Fig. 2(b). The acoustic pressure was measured in a space spanning 12 mm × 12 mm in transversal plane (XY) and 12 mm × 30 mm in longitudinal plane (ZX) relative to the sonication path with 0.3 mm steps. The spatial-peak temporal-peak pressure is 0.38 MPa. Based on the acoustic pressure field, the spatial-peak pulse-average intensity (I_{SPPA}) was estimated to be 7.59 W/cm^2 and the spatial-peak temporal-average intensity (I_{SPTA}) was 4.55 W/cm^2 at duty cycle of 60%. The measured ultrasound intensity profile of I_{SPTA} is shown in Fig. 2(c), with full-width-at-half-maximum of 4.3 mm in XY plane. According to the absorption and refraction properties of ultrasound in rat's skull and brain tissue [32], [33], the calculated acoustic attenuation was about 33%. Details of the acoustic intensity calculation are described in Appendix A.

TUS at above acoustic parameters was reported to activate neural activity [16], so that we used it to enhance the function of PLC in depressed rats. Interval of 3s was adopted between two stimulation trails to avoid thermal effect accumulating. Rats were treated by TUS 15 min every day for 2 weeks. This dose of TUS treatment was designed according to clinical TMS [28]. During TUS stimulation, rats were anesthetized by inhalation isoflurane (5% isoflurane initial, and 2% isoflurane for maintenance) and fixed on the stereotaxic apparatus and their hair was trimmed. An acoustic collimator (7 mm diameter output aperture) was used to refine the ultrasound waves within the area of PLC. The LT-Ctr-ShamTUS and LT-Res-ShamTUS groups received sham TUS, that is, LT-Ctr-ShamTUS and LT-Res-ShamTUS groups were anesthetized, fixed, trimmed and applied with ultrasound gel over the scalp following the same protocols as the TUS group. The only difference was that there was no ultrasound stimulation in the ShamTUS group. Moreover, the shamTUS was used as the reference for studying the effect of TUS.

E. Sucrose Preference Test

Before pre-SPT test, rats were trained to drink water with sucrose for two-day adaptation. During this period, rats were individually caged with two side-by-side bottles containing pure

water (200 ml) and 1% sucrose solution (200 ml) respectively. The position of sucrose solution and water was exchanged to avoid the side preference after 24 hours. After the sucrose adaptation, rats were deprived of water and food for 12 hours and then resume the sucrose solution and pure water supply. The fluid consumption was recorded 12 hours later, and then the sucrose and water bottles was exchanged and the fluid consumption was recorded after another 12 hours. SPI was defined as the ratio of the sucrose consumption to the total fluid consumption. Decline in SPI represents the typical anhedonia symptom in depression.

F. Open-Field Test

The OFT is commonly used to measure both the quantity and quality of general activity of rodents. It is not only simply a measure of motor activity, but also involves factors like exploratory drive (curiosity) and anxiety [34]. The exploratory activity is emphasized by the novelty of environment in the initial period of the test. Because repeating this test would reduce the novelty (stress), we only conducted the OFT once in all groups. Each rat was individually and gently placed into the center of a square plexiglass box (90 cm × 90 cm × 50 cm) to freely explore the chamber for 10 min. Its activity was recorded by ANY-maze (Stoelting, USA) and the number of its rearing was counted by an experienced technician who was blind to the rat's groups. The box was cleaned after the OFT of each rat. OFT videos were analyzed by a technician in aspects of moving distance, time in center zone, and number of rearing.

G. Forced Swimming Test

The FST is to measure the despair behavior (immobility) of the rats under the stress of being forced to swim. As the extreme stress might have effect on rats' mental state, we only conducted the FST once at the end of behavioral test. This test consisted of two sessions. In the first session, rats were trained to discover the test without being scored in the first day. In the second session on the next day, the performance of FST was recorded with ANY-maze (Stoelting, USA) for further analysis. In each session, rats were placed individually into a transparent plexiglass cylinder (60 cm in height, 30 cm in diameter) for 5 min. The cylinder was filled with fresh water (25 ± 1 °C, 35 cm in depth) and the swimming animal could not touch the bottom of the container with their posterior limbs or tails. Immobility was defined as the status in which animals floated in water with only occasional slight movements to keep their balance. The immobility time of each rat was measured from the average of two separate measures by a technician who was blind to the groups.

H. Western Blotting Analysis

Rats of LT-Ctr-ShamTUS (n = 4), LT-Res-ShamTUS (n = 5) and LT-Res-TUS (n = 5) were sacrificed on the next day after FST. Left hippocampus were harvested and frozen at −80 °C. After homogenized in ice-cold radio immunoprecipitation assay buffer containing protein inhibitors (Roche Diagnostics, USA), the protein concentration of each sample was measured using BCA Protein Assay Kit (Sangon Biotech Co., Ltd, Shanghai, China). Equivalent amounts of total protein (30 μg protein for each sample) were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to immunoblot polyvinyldifluoride (PVDF) membranes (0.22 μm). After blotting, the membranes were blocked for 1 hour at room temperature using 5% skimmed milk, and then the membranes were incubated with antibody raised in rabbit against BDNF (1:1500, ab108319, Abcam, Cambridge, UK) over night at 4 °C. After that, the membranes were incubated with the secondary antibody for 1 hour at room temperature. To examine expression of BDNF, we selected a signal band around 14 kDa (mature BDNF) referring to [35]. The signal intensity of the protein was quantified with ChemiDoc Touch imaging system (Bio-Red, USA).

I. Histology

For LT-Ctr-ShamTUS, LT-Res-ShamTUS and LT-Res-TUS groups, three rats were chosen from each group for histological assessment after all behavioral tests. After being perfused with saline and 10% formalin sequentially, the brains were then harvested and immersed in 10% formalin for 24 hours before being embedded in tissue freezing medium (Leica, Germany). Each brain was coronally sectioned into at least 15 slices (20-μm-thick) from 1 to 5 mm anterior to the bregma. All slices were stained with H&E. Photomicrographs were obtained using a digital microscope camera (DS-Ri2, Nikon, Japan) and then were observed by a certified pathologist who was blind to the groups.

J. Statistical analysis

The analysis of variance was adopted when data met following assumptions: independence of observations, normality and equality of variances. And one-way ANOVA was performed among at least three groups while t-test was used in two-group case. Otherwise, nonparametric tests were applied. All statistical analyses were performed by SPSS. All data are expressed as mean ± SEM. The level of statistical significance was set at p-value ≤ 0.05.

III. Results

A. Sucrose Preference Test

No significant difference of sucrose preference index (SPI) or sucrose consumption was observed between ST-Ctr and ST-Res rats one week after the restraint (Fig. 3(a) and (b)).

Nonparametric test was performed on the SPI of the rest three groups due to the failure of the test of homogeneity of variances. The statistical analysis showed marginally significant difference in SPI among groups (p = 0.064, Kruskal Wallis Test) at the 24th hour (Fig. 3(c)). Post hoc analysis showed a significant decrease of the SPI in LT-Res-ShamTUS group (72.8% ± 7.2%, n = 16) compared with LT-Ctr-ShamTUS group (90% ± 1.7%, n = 16) ($p = 0.042$, Mann − Whitney), and a significant increase of the SPI in LT-Res-TUS rats (88.8% ± 2.5%, n = 16) compared with LT-Res-ShamTUS rats (72.8% ± 7.2%, n = 16) ($p = 0.046$, Mann − Whitney).

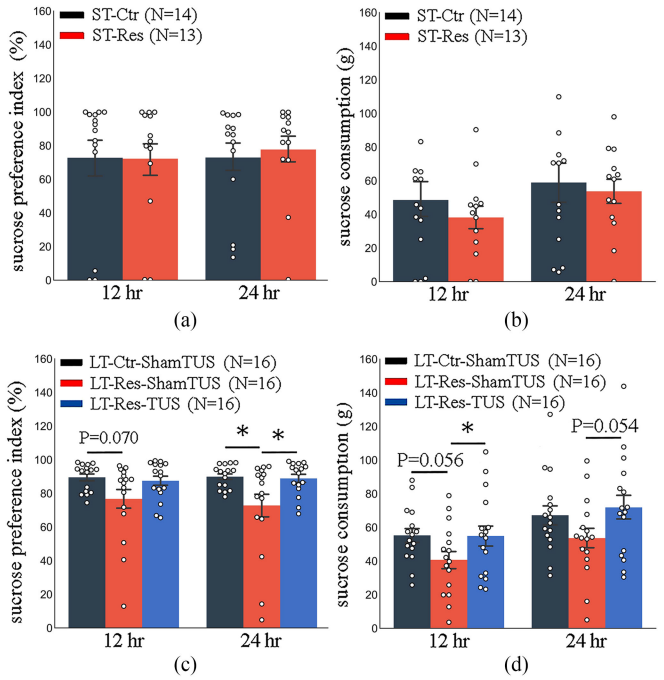

Fig. 3. Sucrose preference test after restraint stress. Means ± SEMs of (a) sucrose (1%) preference index and (b) sucrose consumption in ST-Ctr and ST-Res groups at two time points (12th hour & 24th hour). Means ± SEMs of (c) sucrose preference index and (d) sucrose consumption in LT-Ctr-ShamTUS, LT-Res-ShamTUS and LT-Res-TUS groups at two time points (12th hour & 24th hour). Symbol "*" denotes p-value<.05, as revealed by Mann-Whitney test compared with LT-Res-ShamTUS subjects in (c) and by LSD post hoc comparisons with LT-Res-ShamTUS subjects in (d).

A similar trend was also found at the 12th hour ($p = 0.070$, LT − Ctr − ShamTUS vs LT − Res − ShamTUS; $p = 0.132$, LT − Res − ShamTUS vs LT − Res − TUS). One-way ANOVA on the sucrose consumption indicated marginal group effect at the 12th hour ($p = 0.084$) (Fig. 3(d)). Post hoc analysis revealed significant increase of sucrose intake in LT-Res-TUS rats ($p = 0.05$) compared with LT-Res-ShamTUS at the 12th hour, but LT-Res-ShamTUS rats showed marginal decrease of sucrose computation compared with LT-Ctr-ShamTUS rats ($p = 0.056$) at the 12th hour.

Although both SPI and sucrose consumption did not differ between the restraints and controls in short-term condition (Fig. 3(a) and (b)), both sucrose indicators decreased in the restraint subjects after two more weeks' accumulation [36]. However, such a sucrose intake decrease was recovered by TUS treatment (Fig. 3(c) and (d)). Additionally, both the SPI and sucrose consumption elevated in LT-Ctr-ShamTUS rats compared to ST-Ctr groups. It might relate to the increase of weight in long term rats.

B. Open Field Test

The exploratory behavior and locomotor activity of rats in a novel environment were measured for a period of 10 minutes. We not only measured total moving distance, rearing, and time in center zone, but also assessed the variability of these parameters within this period [37].

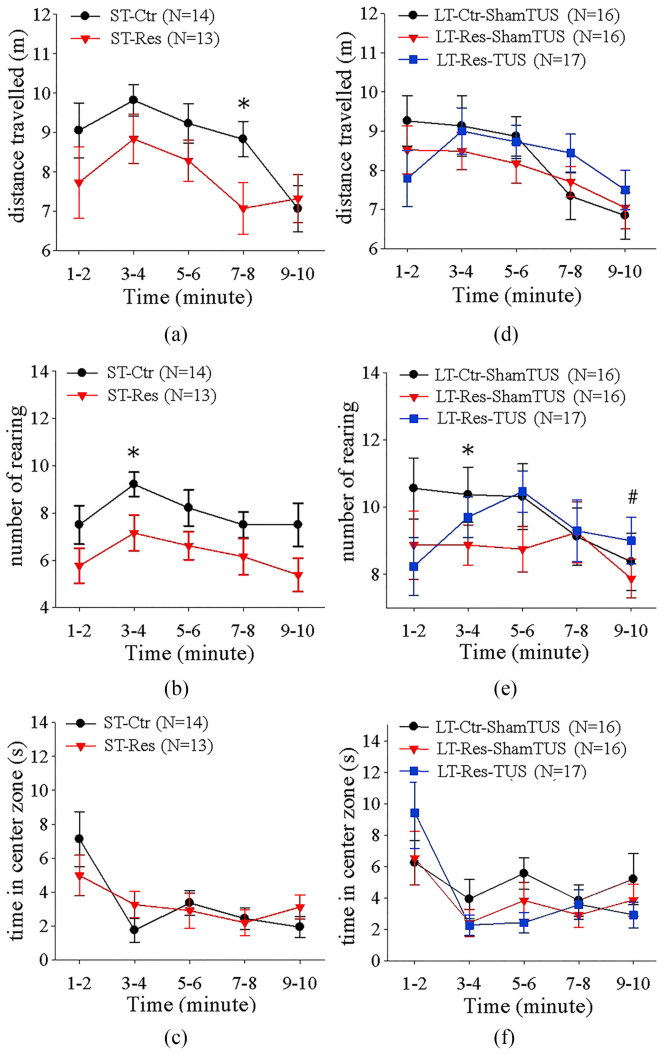

Fig. 4. Open field test after restraint stress. (a) Moving distance. (b) Number of rearing. (c) Time in center zone in ST-Ctr and ST-Res groups. (d) Moving distance. (e) Number of rearing. (f) Time in center zone in LT-Ctr-ShamTUS, LT-Res-ShamTUS and LT-Res-TUS groups. All these data were presented as Means ± SEMs. Symbol "*" denotes p-value<.05, as revealed by LSD post hoc comparisons between restraint and non-restraint subjects. Symbol "#" denotes p-value<.05, as revealed by LSD post hoc comparisons between LT-Res-ShamTUS and LT-Res-TUS subjects.

The number of rearing in ST-Res subjects decreased compared with ST-Ctr throughout 10 minutes, though there was not differences in either moving distance or time in center zone (Supplementary Fig. S4). More specifically, there was significant decreases of moving distance and number of rearing during 7–8 minutes ($p = 0.034$, independent samples t-test) and 3–4 minutes ($p = 0.032$, independent samples t-test) respectively (Fig. 4(a) and (b)).

The moving distance and time in center zone did not significantly change across groups (Fig. 4(d) and (f)), while the number of rearing was affected by both restraint and TUS. LT-Res-ShamTUS rats showed a significant decrease of number of rearing compared with LT-Ctr-ShamTUS ($p = 0.029$, One-way ANOVA, post hoc) during 3–4 minutes, whereas LT-Res-TUS rats had significantly greater number of rearing than the

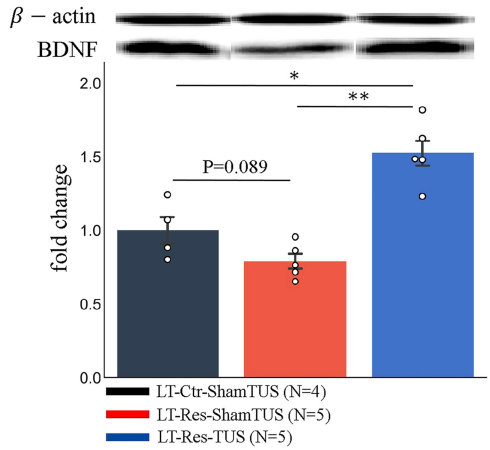

Fig. 5. Effects of the 48-hour-restraint stress and 2 weeks' TUS on the expression of hippocampal BDNF. The hippocampus was subjected western blotting to examine the level of BDNF. β-actin served as a loading control. Symbol "*" denotes p-value$<$.05 and symbol "**" denotes p-value$<$.01 in the BDNF fold change.

LT-Res-ShamTUS rats ($p = 0.022$, One-way ANOVA, post hoc) during 9–10 minutes (Fig. 4(e)).

The locomotor activity of rats decreased one week after the restraint (Fig. 4(a)). However, it recovered with time and no difference of moving distance was observed two weeks later (Fig. 4(d)). In addition, the number of rearing decreased in both ST-Res and LT-Res-ShamTUS rats (Fig. 4(b) and (e)). The results showed that TUS could relieve the reduction of exploratory behavior due to restraint stress.

C. Forced Swimming Test

Neither restraint process nor ultrasound stimulation treatment significantly altered the forced swimming performance (Supplementary Fig. S5).

D. Brain-Derived Neurotrophic Factor in Left Hippocampus

We tested whether 48-hour-restraint or 2 weeks' TUS could change the expression of BDNF in hippocampus of rats. Western blotting was applied to measure the protein expression of BNDF in rats (Fig. 5). Results showed that two weeks' TUS significantly increased the expression of BDNF in LT-Res-TUS rats (1.53 ± 0.096, $n = 5$) compared with LT-Ctr-shamTUS rats (1.00 ± 0.098, $n = 4$) ($p = 0.027$, Mann − Whitney) and LT-Res-shamTUS rats (0.79 ± 0.054, $n = 5$) ($p = 0.009$, Mann-Whitney). However, restraint stress only induced marginally significant decrease of BNDF in LT-Res-shamTUS subjects compared with LT-Ctr-shamTUS 3 weeks after the restraint ($p = 0.089$, Mann-Whitney).

E. Safety of Long-Term TUS

To evaluate the safety of long-term TUS, we analyzed the possible hemorrhage in brain tissue of rats using H&E staining by referring to [14]. Nine brains from LT-Ctr-shamTUS, LT-Res-shamTUS and LT-Res-TUS groups were sectioned into slices (3 rats from each group, and at least 15 slices for each brain) and mounted on glass slides. All slices were stained and carefully inspected by microscope. The H&E staining did not show the presence of any tissue damage or hemorrhage after the sonication (Fig. 6).

IV. Discussion

Current therapeutic strategies for depression suffer from several drawbacks, such as inadequate responses, side effects, high risks associated with implanting surgeries, etc. Therefore, novel physical therapies are highly demanded. TUS has been hypothesized to be an effective treatment for depression for its advantages in spatial resolution and non-invasiveness [21]. To study whether TUS has antidepressant-like effects, we firstly created a depressed rat model induced by 48-hour-restraint stress, and then applied daily TUS for 2 weeks to the prefrontal cortex. Results showed that the 48-hour-restraint stress led to long lasting reduction of exploratory behavior (one week and three weeks after restraint) and protracted anhedonia (three weeks after restraint) in rats. Furthermore, we found the recovery of depression-like phenotypes, i.e., anhedonia and reduced exploratory behavior, and increase of left hippocampal BDNF after TUS. H&E staining indicated no tissue damage or hemorrhage in the brain.

A. Antidepressant Effects of TUS

There have been few researches on the antidepressant effects of TUS on either human subjects or animal depression model. In this study, based on the restraint stress model of rats, we discovered the depression-like phenotypes induced by 48-hour-restraint including anhedonia and reduced exploratory behavior, which, however, could be reversed by 2-week-TUS treatment.

In addition, significant increase of hippocampal BDNF expression was observed in ultrasound treated rats (Fig. 5). It has been extensively proved that BDNF plays an important role in the survival, differentiation, and proliferation of neurons and the formation of new synapses [38]. TUS could elevate hippocampal BDNF expression in healthy mice [9]. We therefore suppose that TUS might promote the expression of BDNF and protect the neurons from the damage induced by restraint stress. In this study, TUS stimulation on the left PLC could interestingly increase the expression of endogenous BDNF in hippocampus. Noted that the left PLC has remarkable projections to subcortical limbic regions with excitatory pathways to hippocampus [39]. These prefrontal-hippocampal pathways had also been used in other brain stimulations, e.g., DBS and ECT for preclinical research [40].

Apart from the BDNF, decrease in hippocampal neurogenesis is also related to depression. It has been demonstrated that TUS could increase the hippocampal neurogenesis [25]. Moreover, BDNF has been shown to be related to the regulation of neurogenesis in hippocampus [41]. Thus, the effect of TUS on neurogenesis is worthy of further investigation to elucidate the mechanism of TUS treatment on depression.

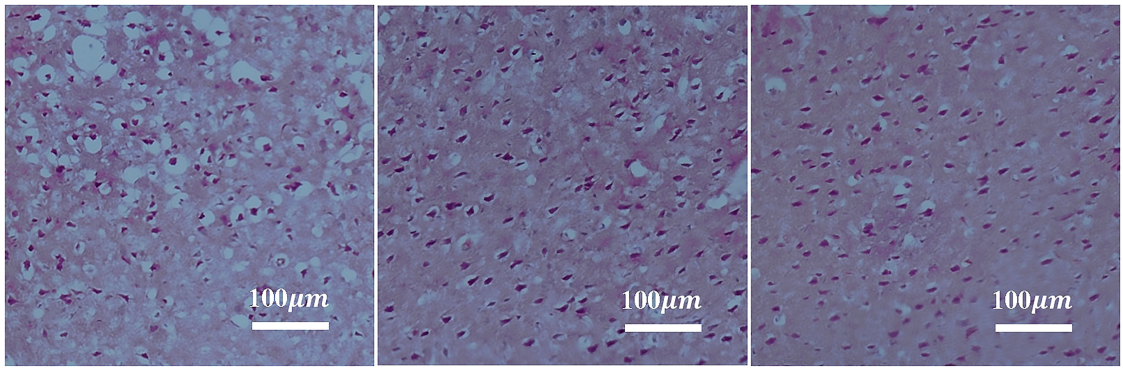

Fig. 6. Examples of the histological analysis of rats of the LT-Ctr-ShamTUS, LT-Res-ShamTUS and LT-Res-TUS groups respectively.

B. Safety Issues

In this study, the I_{SPPA} in water was estimated to be 7.59 W/cm^2, with corresponding I_{SPTA} of 4.55W/cm^2. The acoustic intensity attenuation of ultrasound through skull and brain of rat was calculated to be about 33% (see Appendix A). Thus the I_{SPTA} transmitted into rat's brain could be about 3 W/cm^2. The biological effect of TUS involves thermal effect and non-thermal effect (mainly referring to cavitation). For thermal effect, our theoretical calculations showed that the maximum temperature increase in this study was only 0.02 °C (see Appendix B), far away from harming tissue. Other studies also reported that the thermal effect induced by low intensity TUS was rather small (generally less than 0.1 °C in temperature increase) in both real experiments and theoretical calculations [9], [16]. As for the cavitation, it depends on the acoustic pressure amplitude and the gas bodies of tissue. In brain tissue which is lack of gas, literature reported that cavitation might occur only when the ultrasound pressure was over 40 Mpa [42]. However, in this study, the spatial-peak temporal-peak pressure was 0.38 Mpa, which was not likely to cause cavitation.

Low intensity TUS was generally safe according to literature. For example, TUS of I_{SPTA} at 6.3 W/cm^2 for sonication duration of 2 s [15] or even continuous stimulation with I_{SPPA} of 2.9 W/cm^2 for 48 hours [43] did not show any tissue damage. While at much higher intensity, TUS may result in hemorrhaging or BBB disruption, which needs to be carefully examined and controlled. It was reported that exposure to relatively high acoustic intensity (e.g., $I_{SPPA} = 22.4$ W/cm^2, $I_{SPTA} = 11.2$ W/cm^2) may result in focal bleeding in rat's brain [12]. In this study, low intensity of TUS ($I_{SPPA} = 7.59$ W/cm^2) was implemented for a short sonication duration of 0.4 s with intervals of 3s in each trial to prevent potential tissue damage.

Apart from the intensity, accumulation of TUS effects also need to be carefully examined when it comes to long duration repeated TUS stimulation. Kobus *et al.* investigated the multiple sessions of BBB disruption using focused ultrasound (FUS) in combination with micro-bubbles over a range of acoustic exposure levels (6 weekly sessions of FUS, using ultrasound pressure between 0.66 and 0.80 MPa). They found that the process of repeatedly disrupting the BBB itself did not produce additional significant side effects compared with a single session [44]. Po-Chun Chu *et al.* also found that daily repetitive FUS at mechanical index (MI) of 0.55 with micro-bubbles elicited no accumulative effects on somatosensory evoked potentials or tissue integrity [45]. However, studies on the safety of long duration repeated TUS without micro-bubbles are rare. More studies on the accumulation effects of long-term TUS are needed before the clinical translation.

Presently, several studies of TUS on human brain generally reported no harmful effects. A pilot study reported that application of TUS (duration of 15 s, MI at 0.7) to frontal-temporal cortex could safely improve the mood and slightly reduce chronic pain [46]. Lee *et al.* targeted FUS (duration of 3 s, I_{SPPA} at 3 W/cm^2) to the human somatosensory cortex, and results showed that FUS could elicit transient tactile sensations on the hand. The neurological and neuroimaging assessments showed no changes in mental or physical status or discomforts associated with TUS procedure [47].

In our study, histological results did not show any tissue damage or hemorrhage in the sonication region receiving daily TUS (15 min) for two weeks (Fig. 6). It should also be noted that, in our experiment, the motor cortex was inevitably stimulated as it is on the pathway of TUS to the PLC. Thus the safety of long-term TUS could also be partially assessed by any impairment in motor behavior. We observed no difference in the locomotor activity (OFT experiment) or the performance in swimming test between TUS treated and the sham-control animals. These observations suggest that the low-intensity TUS in this study is basically safe. Nevertheless, more histochemical examination on the ultrastructure would be necessary to confirm the safety of TUS.

C. Depressed Rat Model

In this study, we found that the impact of 48-hour-restraint stress was long lasting in terms of the reduced exploratory behavior as well as the protracted anhedonia (>3 weeks) in SPT. In short term, the locomotor activity in OFT decreased after restraint stress, but recovered sooner (<3 weeks, Fig. 4(a) and (d)) than the exploratory behavior and anhedonia measurements, because locomotion ability is assessing the status of the

animal more physically than psychologically. However, immobility measured in FST and center exploration in OFT (Supplementary Figs. S4 and S5) showed no despair or anxiety after the 48-hour-restraint. Nevertheless, the behavioral results are mostly consistent with Li's observations [30] that 24-hour restraint mice showed long-term depressive-like phenotypes (anhedonia and despair) but no anxiety-like behaviors either. The difference lies in the absence of increased immobility in FST, which might be related to the distinct responses of mice and SD rats to forced swimming. For the control non-restraint mice in Li's study, the immobility time was more than 100 s on average [30], whereas the control rats in our experiments did not have obvious immobility performance (~10 s immobility time on average).

Previous studies reported that restraint stress, either acute or chronic, may reduce the expression of BDNF in rats or mice [48]. In chronic restraint stress [49], reduction of hippocampal BDNF was observed on the first day after the restraint stress modeling (4-hour-restraint/day for 3 days), which recovered to normal level three weeks later. Interestingly, in another acute experiment (2-hour restraint), the hippocampal BDNF level reduced immediately, but fully recovered 24 h later [50]. In the present study, we checked the hippocampal BDNF four weeks after restraint and only observed marginal decrease (Fig. 5). We speculate that the BDNF was likely to nearly recover from the post-restraint drop by that moment. Unfortunately, we did not have the earlier BDNF data to confirm the initial BDNF response.

Depression features multiple and complex syndrome [51], [52], some of which, e.g., feeling of guilty and sadness, are difficult to be mimicked and measured in animal models. In contrast, despair, hopelessness, anxiety, and anhedonia are commonly examined in animal models. However, it is still impractical to create all symptoms in a rodent model. In practice, a model is thought to be successful if several symptoms are presented [53]. SPT, FST and OFT are the general methods to evaluate the depressive behaviors in rodent. For example, the unpredictable chronic mild stress (UCMS) model could induce decrease of reward sensitivity and anhedonia [54]; and the chronic restraint stress (CRS) model would lead to anhedonia and anxiety [55]. Usually, anhedonia is considered as the fundamental characteristic of depression and major symptom in animal model. In this study, the anhedonia was observed in restraint subjects, indicating 48-hour-restraint as a successful depression model in rats.

V. Conclusion

We conducted daily TUS for 2 weeks on a rat model of depression and found antidepressant-like changes in both behaviors and BDNF expression. In particular, our experiments revealed that 48-hour-restraint stress would induce long lasting reduction of exploration activity and protracted anhedonia in rats. The safety of the long-term TUS on brain tissues was also assessed and no damage was found. To the best of our knowledge, this study is the first attempt to discover the antidepressant-like effects of TUS on animal model, and the results suggest that TUS might be a promising therapeutic strategy for depression. However, stimulation parameters, targets of brain regions, treatment efficacy and the underlying mechanisms are needed to be further studied toward the clinical translation of TUS.

Appendixes

A. Acoustic Intensity Calculation

Using the measured acoustic pressure field, we could calculate several acoustic intensity parameter [9]. The mechanical index (MI) was defined as

$$MI = \frac{p_r}{\sqrt{f}}, \tag{1}$$

where p_r is the peak negative pressure of the ultrasound wave, and f is the frequency of acoustic waves.

The pulse intensity integral (PII) is defined as

$$PII = \int \frac{p^2(t)}{Z_0} dt, \tag{2}$$

where $p(t)$ is the instantaneous pressure, Z_0 is the characteristic acoustic impedance ($Pa \cdot \mathrm{s/m}$) defined as ρc, where ρ is the density of the medium and c is the speed of sound transmitted in the medium. We measured the acoustic pressure in water, so we assessed ρ to be 1000 $\mathrm{kg/m^3}$ and c to be 1540 m/s. The spatial-peak pulse-average intensity (I_{SPPA}) is defined as

$$I_{SPPA} = \frac{PII}{PD}, \tag{3}$$

where PD is the pulse duration. The spatial-peak temporal-average intensity (I_{SPTA}) is defined as $I_{SPPA} \cdot (DC)$, where DC is the duty cycle.

In order to get the acoustic intensity transmitted into the brain, the absorption and refraction properties of ultrasound in rat's skull and brain tissue need to be taken into consideration. The acoustic intensity (I) after passing through skull can be estimated as [32]

$$\mathrm{I} = I_0 e^{-\alpha l}, \tag{4}$$

where I_0 is the initial intensity, α is the attenuation coefficient (3.5 dB/cm in rat's skull at 0.5 MHz [56]), and l is the thickness of skull of rat (about 0.5 mm [57]). Moreover, when ultrasound waves crossed from skull to brain tissue, the transmission intensity coefficient (T) is defined as [58]

$$T = \frac{4Z_1 Z_2}{(Z_1 + Z_2)^2}, \tag{5}$$

where Z_1 and Z_2 are the acoustic impedance in skull and brain tissue respectively.

B. Thermal Effect of TUS

Under the assumption that no heat is lost, the maximum temperature increase ($\Delta T_{\max}$) in brain tissue induced by TUS for short exposure time can be described by [32]

$$\Delta T_{\max} = \frac{Q \Delta t}{C_v}, \tag{6}$$

where Δt is the TUS exposure time, C_v is the heat capacity per unit volume for brain tissue defined as

$$C_v = C\rho, \tag{7}$$

where C is the heat capacity in brain tissue (about 3.6 J/g/°C) and ρ is the density of brain tissue (1028 kg/m^3). The heat generation per volume (Q) is defined as

$$Q = 2\alpha I_{\mathrm{SPTA}}, \tag{8}$$

where α is the absorption coefficient in brain tissue (about 0.03 Np/cm at 0.5 MHz [59]), I_{SPTA} is the temporal-average intensity.

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