Transcranial Low-Intensity Pulsed Ultrasound Stimulation Induces Neuronal Autophagy

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***Abstract*— Autophagy, or cellular self-digestion, is an essential process for eliminating abnormal protein in mammalian cells. Accumulating evidence indicates that increased neuronal autophagy has a protective effect on neurodegenerative disorders. It has been reported that low-intensity pulsed ultrasound (LIPUS) can noninvasively modulate neural activity in the brain. Yet, the effect of LIPUS on neuronal autophagy is still unclear. The objective of this study was to examine whether LIPUS stimulation could induce neuronal autophagy. Primary neurons were treated by LIPUS with a frequency of 0.68 MHz, a pulse repetition frequency (PRF) of 500 Hz, a spatial peak temporal-average intensities (I_{SPTA}) of 70 and 165 mW/cm^2. Then, the immunofluorescent analysis of LC3B was carried out for evaluating neuronal autophagy. Furthermore, 0.5-MHz LIPUS was noninvasively delivered to the cortex and hippocampus of adult mice ($n = 16$) with PRF of 500 Hz and I_{SPTA} of 235 mW/cm^2. The LC3BII/LC3BI ratio and p62 (autophagic markers) were measured by western blot analysis. In the *in vitro* study, the expression of LC3B in primary neurons was statistically improved after LIPUS stimulation was implemented for 4 h ($p < 0.01$). With the increase in the irradiation duration or acoustic intensity of LIPUS stimulation, the expression of LC3B in primary neurons was increased. Furthermore, transcranial LIPUS stimulation increased the LC3BII/LC3BI ratio ($p < 0.05$) and decreased the expression of p62 ($p < 0.05$) in the cortex and hippocampus. We concluded that LIPUS provides a safe and capable tool for activating neuronal autophagy *in vitro* and *in vivo*.**

***Index Terms*— Neurodegenerative disorders, neuronal autophagy, transcranial ultrasound neuromodulation.**

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I. Introduction

AUTOPHAGY, or cellular self-digestion, is an essential process for recycling obsolete cellular constituents and eliminating abnormal protein aggregates and organelles, which is a protective and adaptive mechanism for maintaining cellular health [1], [2]. Accumulating evidence has demonstrated that neuronal autophagy plays an essential role in the development of neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease [3], [4]. Pickford *et al.* [50] have reported that the decreased neuronal autophagy induced by beclin 1 deficiency causes intraneuronal amyloid-β accumulation (a neuropathological hallmark of Alzheimer's disease) and promotes neurodegeneration in mice. Alvarez-Erviti *et al.* [6] have suggested that the chaperone-mediated autophagy activity is decreased in Parkinson's disease brain. Recent studies have demonstrated the potential protective effect of neuronal autophagy against neurodegeneration [4], [7], [8]. Li *et al.* [7] have shown that autophagy is significantly enhanced after long-term carbamazepine treatment, which can alleviate the memory deficits and improve the cerebral amyloid-β pathology in Alzheimer's disease mice. Spencer *et al.* [8] have indicated that increased autophagy ameliorates the synaptic and dendritic pathology and reduces the α-synuclein accumulation in the mouse model of Parkinson's disease. Therefore, a significant step is to develop a technique that can modulate neuronal autophagy, which is promising in the treatment of neurodegenerative disorders.

The low-intensity pulsed ultrasound (LIPUS) has been demonstrated as a promising tool for treating neurodegenerative disorders [9]–[13]. Many studies have proved the feasibility of LIPUS stimulation on cultured neurons [14]–[18], Caenorhabditis elegans [19], [20], rodents [21]–[24], macaques [25], [26], and human brains [27], [28]. Recently, it has been reported that LIPUS stimulation plays a protective role in neurodegenerative disorders [29]–[31]. Burgess *et al.* [29] have found that ultrasound stimulation of the hippocampus improves pathologic abnormalities and behavior in Alzheimer's disease mice. Lin *et al.* [30] have shown that the LIPUS stimulation can enhance the expression of some neuronal protection factors [e.g., brain-derived neurotrophic factor (BDNF)] and significantly increase the memory retention in an Alzheimer's disease rat model. Huang *et al.* [31] have demonstrated that ultrasound

stimulation could enhance the learning and memory abilities in the vascular dementia model of rats. Zhang *et al.* [32] have reported that transcranial ultrasound stimulation promotes the BDNF expression and has an antidepressant-like effect. It has been demonstrated that neuronal autophagy is a protective response to neurodegenerative disorders [3], [33]. Recently, Pandit *et al.* [34] have found that repeated ultrasound treatment with microbubbles can clear neuronal tau by autophagy. However, the effect of LIPUS stimulation on neuronal autophagy is still unclear.

Therefore, the purpose of this study was to examine whether neuronal autophagy could be induced by noninvasive LIPUS stimulation. The results indicated that ultrasound stimulation is capable of inducing neuronal autophagy, which may suggest that transcranial LIPUS stimulation is a promising therapeutic strategy for neurodegenerative disorders via enhancement of neuronal autophagy.

II. Materials and Methods

A. Neuron Culture

The neuron culture samples were prepared from the fetal rats of 18-day-pregnant rats. The fetal rat brain was immersed into a modified sterile Hank's balanced salt solution (HBSS) (14175095, Invitrogen, Rockford, IL, USA), which was supplemented with 1% Sodium Pyruvate (14175095, Invitrogen, Rockford), 1% HEPES (15630080, Invitrogen, Rockford), and 0.5% of 20% (wt/vol) glucose in double-distilled H_2O. The cortical tissue was separated from the brain, and then, the tissue was fully shredded and digested in the incubator for 35 min. The digested solution contained 99.8% modified sterile HBSS solution and 0.2% papain (3126, Worthington, NJ, USA). The eight-well chamber (154941, Nunc, Roskilde, Denmark) was used for the neuron culture and placed in a humidified incubator at 37 °C and with 5% CO_2. First, neurons were cultured in a modified MEM medium (42360099, Invitrogen, Rockford) for 4 h, which supplemented with 10% fetal bovine serum (Gibco, New York, NY, USA) and 1% penicillin-streptomycin (15140122, Invitrogen, Rockford). After the cells were attached, the medium was replaced with a modified neurobasal medium (21103049, Invitrogen, Rockford), which consisted of 1% GlutaMax (350050061, Invitrogen, Rockford), 2% B27 (17504044, Invitrogen, Rockford), and 1% penicillin-streptomycin. After two weeks, LIPUS stimulation was carried out for the primary cultured neurons.

B. Animal Experiments

All animal experiments in this study were approved by the Use Committee and the Ethics Committee of the Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences. Twenty-eight adult C57Bl/6/C3H mice (seven months) were randomly divided into sham ($n = 14$) and LIPUS ($n = 14$) groups. Four of 28 mice (sham: $n = 2$ and LIPUS: $n = 2$) were assigned to the immunofluorescent analysis of c-Fos. Sixteen mice (sham: $n = 8$ and LIPUS: $n = 8$) were used for the western blot analysis. The remaining eight mice (sham: $n = 4$ and LIPUS: $n = 4$) were applied for histological examination. In the experimental process, mice were anesthetized with isoflurane (2% for induction and 0.6% for maintenance). We removed the hair of mice using a depilatory paste before LIPUS stimulation was implemented. A transducer was coupled with a custom-fabricated collimation cone so that the ultrasound could stimulate the mice cortex and hippocampus.

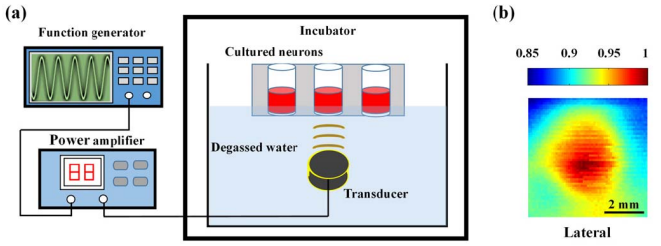

Fig. 1. Experimental apparatus of cultured neurons. (a) Schematic of the LIPUS stimulation setup for cultured neurons. (b) Lateral normalized spatial-peak temporal-peak pressure maps.

C. Pulsed Ultrasound Apparatus

In cell experiments, a transducer with a fundamental frequency of 0.68 MHz was used, which was fabricated in our lab. As shown in Fig. 1(a), the LIPUS was transmitted across the bottom of culture chambers. The experimental setup is composed of an arbitrary waveform generator (DG4000, RIGOL, Suzhou, China) and a power amplifier (325LA, Electronics & Innovation Ltd, New York). The following parameters were used: sonication duration of 1 s, the interstimulus interval of 1 s, the duty cycle of 5%, and pulse repetition frequency (PRF) of 500 Hz. A calibrated hydrophone (Precision Acoustics Ltd, Dorchester, U.K.) was used to evaluate the acoustic intensity. After about 10% attenuation of the chamber bottom, the spatial peak temporal-average intensities (I_{SPTA}) were 70 and 165 mW/cm^2. The experimental process of LIPUS stimulation was performed in a 37 °C incubator with 5% CO_2 for 4 or 6 h.

In animal experiments, a 19-mm diameter transducer (IBP0.506, NdtXducer, Northborough, MA, USA) with 0.5 MHz of center frequency, sonication duration of 1 s, the interstimulus interval of 1 s, the duty cycle of 5%, and PRF of 500 Hz was used (see Fig. 2). After through the mice skull, the acoustic intensity was attenuated by about 35%, and I_{SPTA} was 235 mW/cm^2 (corresponding with peak negative acoustic pressure of 0.34 MPa). As shown in Fig. 2(c), the transducer generated a 3 × 5.5 mm focal spot (full-width at half-maximum) that can target the cortex and hippocampus. Mice were subjected to transcranial LIPUS stimulations for 4 h. In the sham group, mice underwent the same treatment as mice in the LIPUS group, while the ultrasound was switched OFF. To evaluate the heat produced by 4-h LIPUS stimulation *in vitro*, an infrared thermal imaging camera (R300, NEC Avio, Tokyo, Japan) was used.

D. Immunofluorescence

In a cell experiment, the expression of LC3B was detected to evaluate the level of neuronal autophagy according to

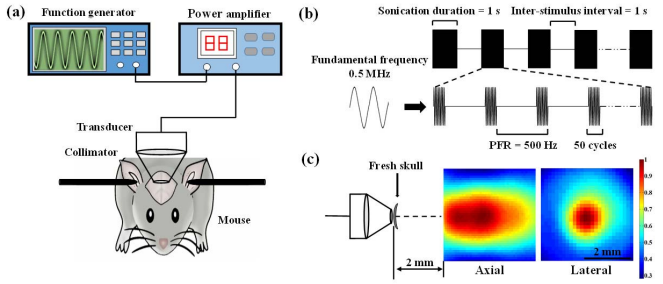

Fig. 2. Overview of experimental design and parameters of transcranial LIPUS stimulation. (a) Diagram of the LIPUS stimulation setup. (b) Illustration of transcranial LIPUS sonication, PRF. (c) Lateral and axial normalized spatial-peak temporal-peak pressure maps.

previous studies [35]–[37]. After ultrasound stimulation, the cultured neurons were fixed in 4% paraformaldehyde solution for 20 min. Then, the culture dish was washed triple with [phosphate buffer saline (PBS)] for 5 min. The neurons were blocked and permeabilized with PBS containing 3% bovine serum albumin (Genview, FA016) and 0.3% Triton X-100 (Sigma, T8787) for 1.5 h. The rabbit antibody to LC3B (1:500, Cell Signaling Technology) and the chicken antibody to MAP2 (1:500, Abcam) were coincubated for 12 h at 4 °C, and then, the donkey antirabbit Alexa Fluor 488 secondary antibody (1:500, A21206, Invitrogen, Rockford) and goat antichicken Alexa Fluor 633 secondary antibody (1:500, Invitrogen, A-21103) were coincubated for 2.5 h at room temperature. For each group, at least five images were obtained by a Leica TCS SP5 laser confocal microscope. The fluorescence intensity was measured using ImageJ software.

The upregulation of c-Fos expression indicates that neurons were activated by stimulation, which can be used as an indirect marker of neuronal activity [38], [39]. In previous studies, c-Fos has been used as a strategy to confirm the stimulation capability of the ultrasonic system [21], electrical system [39], and optogenetic system [40]. Immunofluorescence staining of c-Fos was performed to verify the effectiveness of our LIPUS system in stimulating the mouse cortex and hippocampus. After 40-min transcranial LIPUS stimulation, the mice were perfused immediately with PBS and 4% paraformaldehyde. The mice brain was fixed in 4% paraformaldehyde overnight and then was cryoprotected in 10%, 20%, and 30% sucrose solutions. The brain tissue was frozen in the Tissue-Tek OCT medium and then was sliced into 20-μm coronal sections using a cryostat. The section was blocked and permeabilized with the same solution in the cell experiment for 2 h. The rabbit primary antibody to LC3B c-Fos (1:500, OSR00004W, Invitrogen) was incubated for 12 h at 4 °C, and then, the donkey antirabbit Alexa Fluor 488 secondary antibody (1:500, A21206, Invitrogen) was incubated for 2.5 h at room temperature. Then, images of the cortex and CA1 hippocampus were obtained by the confocal microscope of dendritic length.

E. Western Blotting

To evaluate the levels of neuronal autophagy in the cortex and hippocampus, the western blotting analysis was carried out. Mice were sacrificed after a 4-h LIPUS stimulation. The fresh cortical and hippocampal tissue was separated and frozen at −80 °C. After the tissues were fully shredded, the lysate was added for homogenizing. Then, the sample liquid medium was centrifuged at 12 000 rpm for 5 min, and the supernate of mouse brain lysate was obtained. The protein concentration was measured by BCA protein assay and then was adjusted to 2 mg/ml. We separated the proteins by using the SDS-PAGE gels, and then, the separated proteins were transferred on the PVDF membrane. Then, the membrane was blocked with 5% skim milk for 1 h. The rabbit primary antibody to LC3B (1:1000, 2775s, Cell Signaling Technology, Danvers, MA, USA) or rabbit primary antibody to p62 (1:1000, ab109012, Abcan, Cambridge, MA, USA) was incubated for 12 h at 4 °C, and then, the goat antirabbit secondary antibody HRP conjugated (1:1000, GB23303, Servicebio, Wuhan, China) was incubated for 1 h at room temperature. By using Image J software, the densities of the western blot bands were quantified and normalized to GAPDH (1:25000, GB12002, Servicebio, Wuhan). The ratio of LC3BII/LC3BI was calculated and expressed as a percentage.

F. Histological Assessment

A total of eight mice (sham: $n = 4$ and LIPUS: $n = 4$) were prepared for histological evaluation. After LIPUS stimulation, the animals were perfused transcardially with PBS and then changed into PBS with 4% paraformaldehyde. The mice brains were removed from the skull and fixed in PBS with 4% paraformaldehyde overnight. The 8-μm coronal sections were prepared by using the paraffin section method. Then, histological staining (Hematoxylin and eosin (H&E) and Nissl staining) was performed for at least five sections. H&E staining is a common technique that can assess hemorrhaging and damage in tissue. In short, the hematoxylin solution was used for staining the coronal sections 5 min, and then, stained sections were washed with running water, differentiated with 1% glacial acetic acid, terminated by running water. Eosin solution was used for staining the sections for 2 min. After dehydrated in graded alcohol, the sections were cleared in xylene for 5 min, and then, the resinous medium sealed the pieces. Nissl staining analysis is usually used for observing the distribution of neurons [41]. Briefly, after coronal sections were washed with running water, a 0.5% thionine blue solution (G1032, Servicebio, Wuhan) was used for staining for 5 min. Then, sections were differentiated with 1% glacial acetic acid (G10000218, Servicebio, Wuhan), and the reaction was terminated by running water. After cleared in xylene for 5 min, the resinous medium sealed the pieces. A digital microscope slide scanner was used for imaging the stained sections.

G. Statistical Analysis

Data collected are expressed as mean ± SEM. An independent sample t-test was performed for comparison between two groups, and one-way analysis of variance (ANOVA) followed by LSD was implemented for comparison between multiple groups (SPSS, version 13.0.). The significance level was set at a p-value < 0.05.

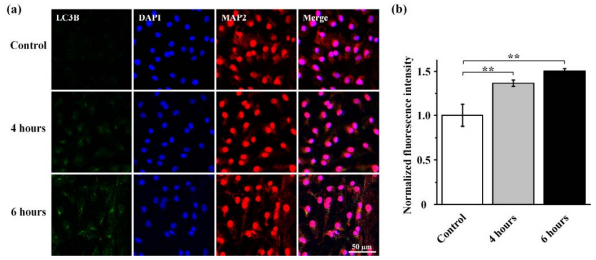

Fig. 3. Level of autophagy in primary cultured neurons was increased following prolonged ultrasound stimulation. (a) Representative images of ultrasound-induced neuronal autophagy. Multiple labeled primary cultured neurons with DAPI (blue) for nuclei, MAP2 (red) for neuron, and LC3B (green) (autophagy marker). (b) LIPUS stimulation (spatial peak temporal-average intensity of 70 mW/cm^2) significantly increased the expression of LC3B in primary cultured neurons. LIPUS = Low-intensity pulsed ultrasound. ($^{**}p < 0.01$).

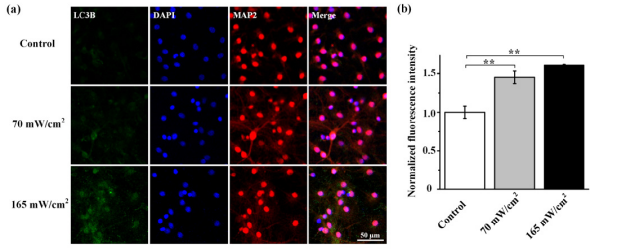

Fig. 4. Level of autophagy in primary cultured neurons was increased by ultrasound stimulation with increased intensity. (a) Representative images of ultrasound-induced neuronal autophagy. Multiple labeled primary cultured neurons with DAPI (blue) for nuclei, MAP2 (red) for neuron, and LC3B (green) (autophagy marker). (b) 4-h LIPUS stimulation with spatial peak temporal-average intensity of 70 mW/cm^2 or 165 mW/cm^2 improved the expression of LC3B primary cultured neurons. LIPUS = Low-intensity pulsed ultrasound. ($^{**}p < 0.01$).

III. Results

A. LIPUS Stimulation Increased Expression of LC3B in Primary Cultured Neurons

After cultured for 14 days, the primary cultured neurons were subjected to LIPUS stimulation. Immunofluorescence analysis was implemented for LC3B, a marker for autophagy [35], to evaluate the neuronal autophagy *in vitro*. As shown in Fig. 3, when LIPUS stimulation (I_{SPTA} = 70 mW/cm^2) was performed, the expression of LC3B was increased statistically after 4 h ($p < 0.01$) and was increased to about 1.5-fold after 6 h ($p < 0.01$). Moreover, we stimulated the primary cultured neurons by LIPUS for 4 h with different acoustic intensities and found that the expression of LC3B increased with I_{SPTA} (see Fig. 4). Overall, the neuronal autophagy increased with acoustic intensity and irradiation duration of LIPUS stimulation, demonstrating that ultrasound neuromodulation was a feasible method for regulating autophagy of primary cultured neurons.

B. Transcranial LIPUS Stimulation Increased c-Fos Expression in the Cortex and Hippocampus

Before carrying out the animal study, we examined the effectiveness of LIPUS on stimulating neurons in the mouse cortex and hippocampus. Fig. 5(a) and (b) shows that, compared with the sham group, c-Fos expression in the cortex was significantly increased after a 40-min LIPUS stimulation ($p < 0.01$). Similarly, the expression of c-Fos was also statistically enhanced in the CA1 hippocampus after LIPUS stimulation ($p < 0.01$) [see Fig. 5(c) and (d)]. Therefore, the LPIUS system in this study was feasible to investigate the ultrasound biological effects of cortical and hippocampal neurons.

C. LIPUS Stimulation Improved the LC3BII/LC3BI Ratio and Decreased the Expression of p62

Furthermore, an animal study was performed to define the effectiveness of LIPUS stimulation in inducing autophagy. Mice ($n = 16$) were subject to transcranial LIPUS stimulation, and then, the western blot analysis was performed for the detection of LC3B and p62 in the cortical and hippocampal tissue. During the process of autophagosome formation, LC3B convert from its cleaved cytosolic (LC3BI) form to its lapidated autophagosomal membrane-bound (LC3BII) form is a marker for autophagy via the ratio of LC3BII/LC3BI [36], [37]. Fig. 6(b) indicates that a 4-h LIPUS stimulation significantly increased the ratio of LC3BII/LC3BI in the cortex of adult mice ($p < 0.05$). Note that p62, also known as sequestosome 1, is associated with LC3B, and it can be selectively degraded through the autophagic pathway [42], [43]. Increased autophagic degradation causes a decrease in p62, and inhibited autophagic degradation contributes to an increase in p62. Fig. 6(c) shows that a 4-h LIPUS stimulation statistically decreased the expression of p62 in the cortical tissue ($p < 0.01$). Similarly, LIPUS stimulation statistically increased the ratio of LC3BII/LC3BI ($p < 0.05$) and decreased the p62 expression ($p < 0.05$) in hippocampus [see Fig. 6(d)–(f)]. Thus, transcranial LPIUS was an effective tool for modulating autophagy *in vivo*.

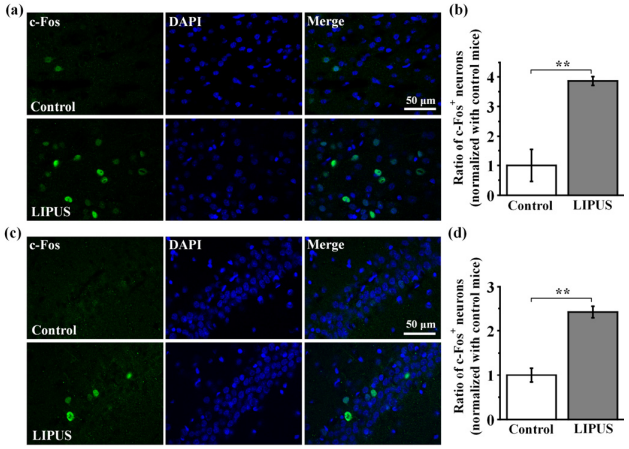

Fig. 5. Transcranial LIPUS stimulation increased the expression of c-Fos in the cortex and CA1 hippocampus of adult mice. Representative images of c-Fos protein in (a) cortex and (c) CA1 hippocampus after LIPUS stimulation for 40 min. Double-labeled brain sections with DAPI (blue) for nuclei and c-Fos (green) (a marker of neural activation). The ratio of c-Fos positive neurons in (b) cortex and (d) CA1 hippocampus was normalized with the sham group. LIPUS = Low-intensity pulsed ultrasound. ($^{**}p < 0.01$).

D. Histological Evaluations

Histological staining was carried out to verify the safety after 4-h ultrasound stimulation (LIPUS $n = 4$ and sham $n = 4$). Fig. 7(a) illustrates the representative images of the

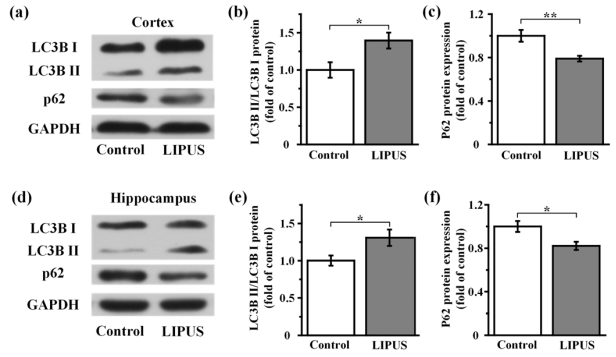

Fig. 6. Transcranial LIPUS stimulation-induced autophagy in the cortex and hippocampus of adult mice. Representative western blots, in which each lane contained a sample from (a) cortex and (d) hippocampus of an individual mouse. The expression levels of LC3BI, LC3BII, and p62 were determined by western blotting after treatment with LIPUS stimulation for 4 h. LIPUS stimulation increased the LC3BII/LCB3I intensity of (b) cortex and (e) hippocampus. LIPUS stimulation decreased the p62 intensity of (c) cortex and (f) hippocampus. LIPUS = Low-intensity pulsed ultrasound. ($^{*}p < 0.05$ and $^{**}p < 0.01$).

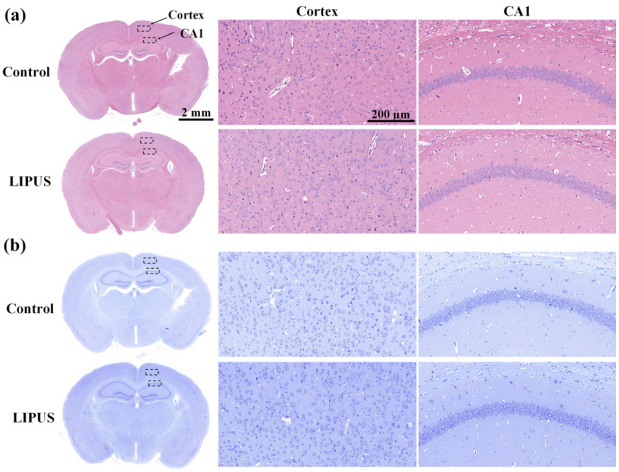

Fig. 7. Histological analysis of mice in sham and ultrasound stimulation groups. Representative images of (a) H&E staining and (b) Nissl staining (b). LIPUS = Low-intensity pulsed ultrasound.

H&E staining in two groups. In the LIPUS group, hemorrhaging and tissue damage were not seen in the cortex and CA1 hippocampus. Nissl-stained brain sections indicated that there are no apparent neuron losses in the LIPUS group [see Fig. 7(b)]. Thus, our results suggest that ultrasound neuromodulation is a safe technique for improving neuronal autophagy *in vivo*.

IV. Discussion

Many studies have demonstrated that neuronal autophagy involves the progress of neurodegenerative disorders, and it is a potential therapeutic strategy for neurodegenerative disorders by targeting the autophagy pathway [3], [4], [33]. Hippocampus is a key region for memory and plays an essential role in the development of neurodegenerative diseases [44]. Previous studies have reported that the cortex and hippocampus are the two targets of brain stimulation in Alzheimer's disease [45], [46]. In this study, we designed a LIPUS system to investigate the effect of LIPUS on cortical and hippocampal neurons. Our results demonstrated that ultrasound stimulation is a feasible technique for inducing neuronal autophagy. Further studies are needed to investigate the therapeutic effect of LIPUS-induced autophagy in the antiaging and neurodegenerative disorders.

Recent studies have reported that mechanical loading (e.g., fluid shear stress) is capable of activating autophagy in different types of cells [47]–[49]. Guo *et al.* [47] have indicated that the autophagy of vascular endothelial cells is promoted under exposure to steady laminar shear stress for 1 h. Zhang *et al.* [48] have demonstrated that osteocytes undergo increased autophagy upon fluid shear stress loading for 2 h. Orhon *et al.* [49] have reported that autophagy increased significantly when the Madin–Darby canine kidney cells were subjected to flow for 4 h. Ultrasound as a mechanical wave has been applied for exploring its effect on neuronal autophagy in this study. The neuronal autophagy was increased significantly after subjecting to LIPUS stimulation for 4 h (see Figs. 3, 4, and 6). Compared with the method based on fluid shear stress, the noninvasive ultrasound can be used for remote stimulation, which shows an advantage in investigating the mechanism of neuronal autophagy responding to mechanical loading *in vivo*. LIPUS stimulation was performed with a sonication duration of 1 s, interstimulus interval of 1 s, and a low duty cycle of 5% and did not cause temperature rise. Thus, our findings may uncover the mechanical function of ultrasound on the neuronal autophagy. Recently, Pandit *et al.* [34] have reported that the repeated 6-s ultrasound treatment with microbubbles can clear neuronal tau by autophagy, which may suggest that autophagy can be increased by a relatively short LIPUS stimulation.

Ultrasound stimulation is a method with adjustable multiple parameters (such as acoustic intensities, PRFs, and duty cycle). Previous studies have illustrated that ultrasound stimulation (PRF = 500 Hz) can impact the morphology of cultured neurons [14], modulate the visual evoked potentials [50], and inhibit the recurrent seizures [51]. In this study, it has indicated that neuronal autophagy could be induced by LIPUS stimulation with a PRF of 500 Hz. Moreover, previous studies have indicated that the ultrasound stimulation with different parameters has either an excitatory effect [27], [52] or inhibitory effect [52]–[54]. Tsui *et al.* [55] have shown that ultrasound radiation at 1 W increased the compound action potential amplitude in neural tissue, whereas progressively decreased for at 2 and 3 W. In a recent study with New Zealand white rabbits, it has reported that LIPUS administered at lower PRF of 10 Hz (corresponding with a tone burst duration of 50 ms) elicited the contralateral motor responses, whereas a higher PRF of 100 Hz (corresponding with a tone burst duration of 0.5 ms) suppressed the visual activity *in vivo* [52]. These findings may suggest that transcranial ultrasound stimulation is a potential tool for increasing or decreasing autophagy in the brain, which needs to be further studied. In addition, in the study of Mott *et al.* [56], ultrasound stimulation was performed with peak negative pressures of 0.2, 0.3, 0.4, or 0.5 MPa for the cardiac endothelial cells *in vitro*, whereas with peak negative pressures of 1 MPa for myocardium *in vivo*. Yang *et al.* [15] treated cultured astrocyte by ultrasound with I_{SPTA} of 110 mW/cm^2 and further stimulated the brain

by ultrasound with I_{SPTA} of 528 mW/cm^2 [15]. Our study is consistent with the previous studies, and I_{SPTA} used *in vivo* (235 mW/cm^2) is higher than that *in vitro* (70 or 165 mW/cm^2). The abovementioned analysis may suggest that cultured cells can be easily affected by ultrasound as cultured cells have fewer neural connections.

Previous studies have reported that the concurrent elevation of BDNF and autophagy contribute to neuroprotection after spinal cord injury [57] and treatment of posttraumatic stress disorder [58]. It has demonstrated that BDNF increases autophagosome formation but prevents excessive autophagic degradation [59]. Many recent findings have shown that LIPUS stimulation plays a role in the neuroprotective effects by increasing the expression of BDNF in neurodegenerative disorders [15], [30]–[32]. In this study, a 4-h LIPUS stimulation significantly enhanced the autophagy in mice cortex (see Fig. 6), which may imply that the neuronal autophagy is mediated by LIPUS-induced BNDF. Further research is needed to examine the relationship between neuronal autophagy and BNDF increased by LIPUS.

This study has several limitations. First, we found that LIPUS can improve neuronal autophagy *in vitro* and hippocampal and cortical autophagy *in vivo*. Neuron and glia are two major types of cells in the brain; thus, the role of glial autophagy *in vivo* is not been excluded. It has been reported that the autophagy of astrocytes and microglia plays an essential role in neurodegeneration, neuroinflammation, and aging [60], [61]. In studies using aged and Alzheimer's mice, Leinenga and Gotz [62], [63] have found a role of microglia in the phagocytosis of amyloid-β plaques after ultrasound treatment. Further studies will be needed to elucidate the effect of LIPUS on the autophagy of neuron and glia. Second, in order to provide the optimal environment for cell culture during LIPUS stimulation, we designed a small-sized transducer that can be conveniently located in the incubator. However, the transducer fabricated in our lab (frequency: 0.68 MHz) has a slight difference in the fundamental frequency with the transducer using *in vivo* (frequency: 0.5 MHz). Tufail *et al.* [21] have reported that transducer with a frequency of 0.25 and 0.5 MHz can reduce electromyography amplitude. Kim *et al.* [64] have indicated that transducers with a frequency of 0.35 and 0.65 MHz were feasible for eliciting motor responses. The findings of Ye *et al.* [65] have demonstrated that the frequency band of 0.3–2.9 MHz was effective for generating motor responses, and a smaller variation in threshold intensities was observed in the submegahertz range. The effect of ultrasonic parameters (frequency, PRF, duty cycle, and so on) on autophagy needs to further study. Third, we used the normal wild type mice in this study, and the behavioral test was not conducted. It is significant and necessary to address how prolonged LIPUS impact the cognitive impairment in the degenerative disease by upregulating autophagy in the brain.

V. Conclusion

Overall, this study has demonstrated that transcranial LIPUS stimulation is capable of modulating neuronal autophagy in the cortex and hippocampus of adult mice, which offers new insight for the neurobiological effects of LIPUS stimulation. This study already suggests the possibility of LIPUS-induced autophagy, and further studies are needed to investigate the mechanisms and the effect of LIPUS-induced neuronal autophagy in aging and neurodegenerative disorders.

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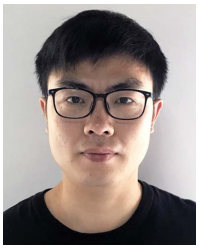

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