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Microbubble-enhanced ultrasonic neuromodulation of motor cortex of mouse

Zhiwei Cui[1], Fangyuan Cao[1], ShanShan Xu[1], Dapeng Li[1], Tianqi Xu[1], Shan Wu[1], Ayache Bouakaz[2], Mingxi Wan[1] and Siyuan Zhang[1]

1. The Key Laboratory of Biomedical Information engineering of The Ministry of Education, Department of Biomedical Engineering, School of Life Science and Technology, Xi'an Jiaotong University, Xi'an710049 China

2. UMR1253, iBrain, Université de Tours, Inserm, France

***Abstract*—This study investigated the effect of MB oscillations on neuronal activity in the central nervous system (CNS) of mammals. The motor cortex of mouse was subjected to ultrasound (US) stimulation with and without MBs. Electromyogram (EMG) signals were recorded by tungsten electrodes. An immunofluorescence assay with antibodies against c-fos was performed to examine the evoked neuronal activity. The influence of US stimulation with MBs on blood brain barrier (BBB) integrity was also assessed. The presence of MBs significantly increased the success rate of motor response from 6.5% ± 6% to 28% ± 10% at 0.12 MPa acoustic pressure. At 0.25 MPa, the success rate was 38% ± 9% without MBs and it significantly increased to 77% ± 18% with MBs. Compared to the US stimulation alone group, the density of c-fos$^+$ cells in the region of stimulation was significantly increased for US+MBs group, which indicated that MBs oscillations would enhance neuronal activity. The examination of BBB integrity showed that 0.12 MPa US stimulation with MBs did not result in disruption of BBB integrity while MB oscillations still produced enhanced effect on ultrasonic neuromodulation, suggesting that the neuromodulatory effects induced by MBs is independent on BBB opening. These findings advance the understanding of the effects of MB oscillation on neuronal activity, and break new avenues for the application of MBs in brain.**

Keywords—Ultrasound stimulation, Microbubbles, Motor response, C-fos, Neuronal activity

I. INTRODUCTION

The feasibility of focused ultrasound (FUS) in the treatment of neurological diseases has been extensively investigated [1]. Most of these applications are based on the mechanical effects of ultrasound, which are also considered to be the main potential mechanism of ultrasonic neuromodulation [2]. Furthermore, microbubbles (MBs) always serve as an amplifier of the mechanical effects of ultrasound [3]. Oscillating MBs might be able to produce an active effect on neuronal activity and it is also necessary to investigate their influence on the neuronal activity and neurological function.

Tufail et al. [4] revealed that ultrasound stimulation could excite *in vivo* mouse motor cortex. Lee et al. [5,6] showed that not only can the sonicated brain area be excited by ultrasound stimulation, but the regional network involved in visual and higher-order cognitive processes also be stimulated. Ultrasound has been proved to be an effective tool to treat psychiatric and neurological diseases, with the nature of noninvasive, high spatial resolution and deep penetration.

In addition to ultrasound, the oscillations of MBs also show the ability to alter neuronal activities. The studies on the effects of BBB opening induced by focused ultrasound combined with MBs on the neuronal functions indicated that BBB opening can impact the neuron response [7] and visual-motor decision-making behavior [8,9], which indicate that MB oscillation might affect mammalian neuron activity. However, due to the opening of BBB, ions, nutrients, and hormones in blood may disrupt neuronal activity. Therefore, it is unclear whether diffused blood constituents or the sustained impacts of MB oscillation alter the neuronal activity.

In present study, the effects of MB oscillation on neuronal activity were investigated in mouse. The motor cortex of mice brain was stimulated by ultrasound, and motor responses with and without MBs were compared. Moreover, c-fos antibodies was performed to evaluate the evoked neuronal activity. BBB integrity was also assessed during ultrasound stimulation with MBs.

II. MATERIAL AND METHODS

A. Animal preparation

All procedures described in this study were approved by the Institutional Animal Care and Use Committee of School of Life Science and Technology of Xi'an Jiaotong University. Adult BALB/c mice were anesthetized with isoflurane (2%, 0.5 L/min O_2) and placed in a mouse stereotaxic apparatus. The focus of the FUS was targeted to the mouse motor cortex. In general, the anesthetics concentration was decreased to 0.5% five minutes before ultrasound stimulation, keeping the animal in a light anesthesia level.

B. Ultrasound stimulation

The experimental setup is shown in Fig. 1. Pulsed ultrasound sequence was used in this work, which were generated by a function generator, and then amplified by an RF power amplifier, and finally sent to a custom 620 kHz frequency FUS transducer (100 mm in aperture, 80 mm in focal depth). The reported acoustic pressures in this work of 0.12 MPa and 0.25 MPa, were peak rarefactional pressures in the focus of FUS after mouse skull attenuation. Besides, the influence of BBB opening can also be excluded by using a lower acoustic pressure (0.12 MPa).

C. Acquisition and electromyogram signals processing

Tungsten electrodes were implanted into the triceps muscles of mouse forelimbs to record ultrasound stimulation evoked EMG signals. An amplifier with a gain of 1000 was used to amplify the EMG signal and bandpass filtered between 300 Hz and 5 kHz. Then EMG signals were acquired at a 10 kHz sampling frequency using a data acquisition card (1550A, Axon

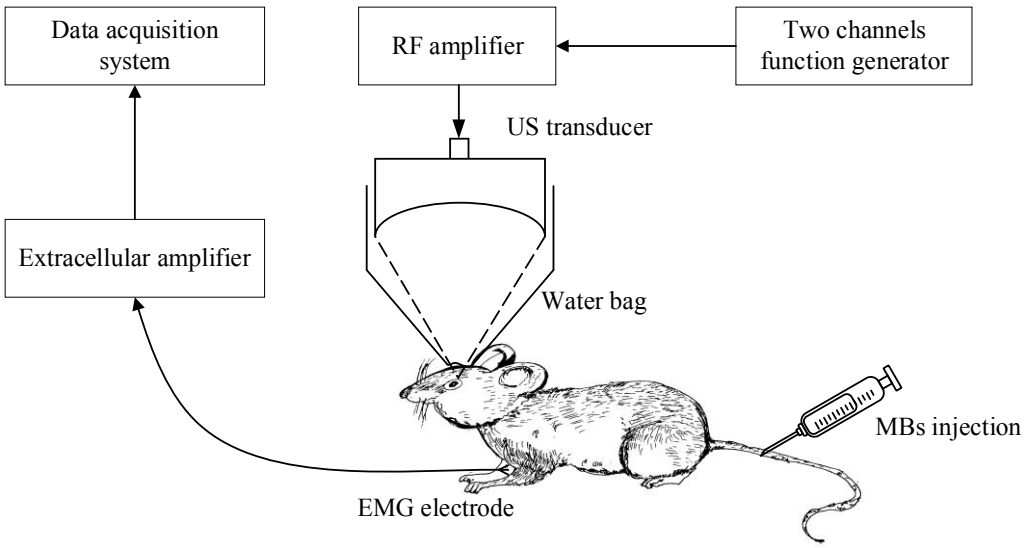

Fig. 1 Block diagram of ultrasound stimulation and EMG acquisition.

Instruments, San Jose, CA, USA) and processed with MATLAB. Twenty ultrasound stimulations mentioned above in 8s intervals were performed for each condition. For the group of ultrasound stimulation with MBs, 60 μL SonoVue MBs were injected intravenously before ultrasound stimulation. When the peak amplitude of EMG was greater than three standard deviations of background noise, it is considered as a successful stimulation. In this study, the success rate was recorded, which is defined as the percentage of the stimulus that succeeds.

D. BBB opening evaluation

The effect of ultrasound stimulation with MBs on the integrity of BBB was evaluated. After intravenous injection of EB dye and MBs, twenty ultrasound stimulations in 8s intervals were applied with the same parameters as described above. After one hour, the animals were sacrificed and the mouse brains were removed to assess the BBB integrity.

E. Histological evaluation

In order to further illustrate the effect of MBs' oscillations on neuronal activity, an immunofluorescence assay (IFA) with antibodies against c-fos was performed to assess the neuronal activity induced by ultrasound stimulation with and without MBs. The average density of c-fos$^+$ cells under ultrasonic stimulation of 0.12 MPa and 0.25 MPa was detected to analyze the effect of MB oscillation on neuronal activity.

III. Results

EMG signals were acquired respectively when the motor cortex was subjected to ultrasonic stimulation with and without MBs. Fig. 2(a) shows typical EMG signals from the triceps muscles of mouse forelimbs evoked by 0.25 MPa ultrasound stimulation with and without MBs. Fig. 2(b) shows the mean success rates with and without MBs as a function of acoustic pressure. The results revealed that MBs and acoustic pressure have significant effects on the success rate of motor response. It was found that MB oscillation produced higher success rate, and the success rate was generally proportional to the acoustic pressure ($p < 0.001$; Fig. 2(b)). The injection of MBs significantly increased the success rate of motor response from 0.065 ± 0.06 to 0.28 ± 0.10 when stimulation was applied at 0.12 MPa and from 0.38 ± 0.09 to 0.77 ± 0.18 at 0.25 MPa ($p < 0.001$).

C-fos expression in US stimulated brain sections with and without MBs was compared and Fig.3 shows the representative fluorescent images of the c-fos immunofluorescence assay. The

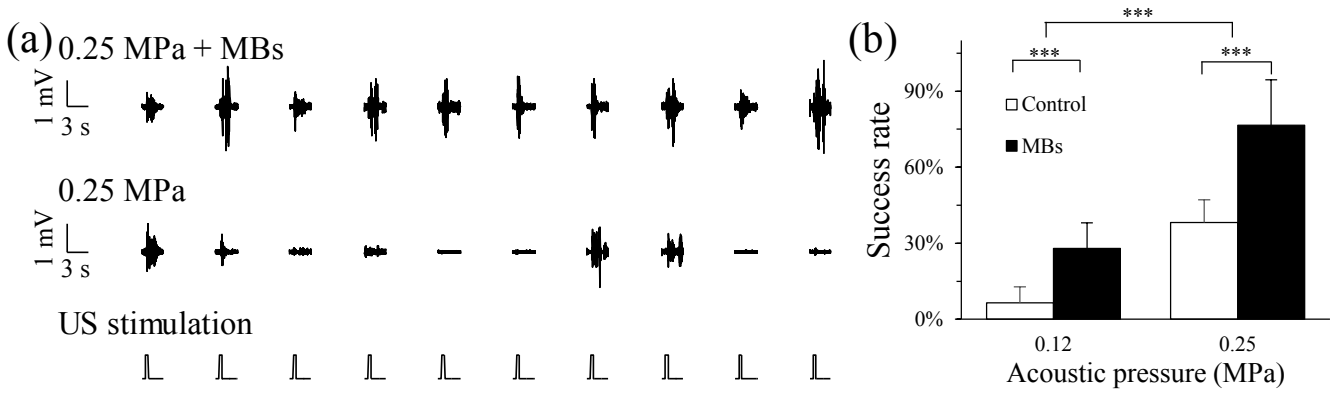

Fig. 2 Typical EMG signals with and without MBs. (b) Mean success rates with and without MBs as a function of acoustic pressure (n = 10 mice per group). Group differences were analyzed by two-way repeated ANOVA, with * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and N.S. for not significant.

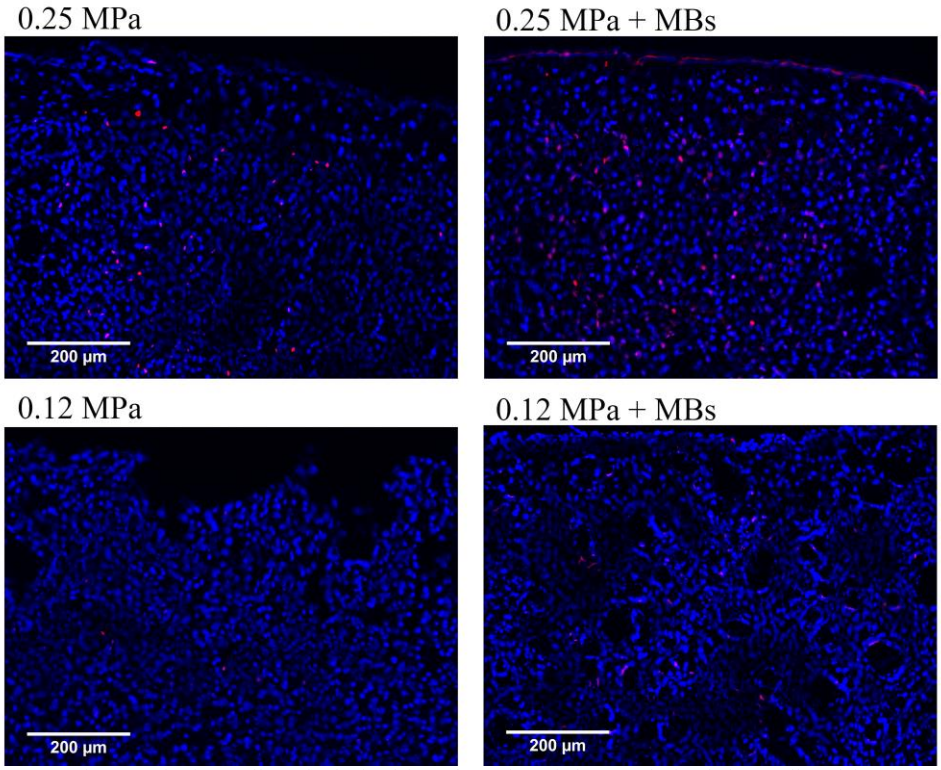

Fig. 3 Representative fluorescent images of c-fos+ cells (red) obtained from the region of ultrasound stimulation. Cell nuclei were stained by DAPI (blue).

mean densities of c-fos$^+$ cells stimulated with 0.12 MPa and 0.25 MPa ultrasound were shown in Fig. 4. The presence of MBs significantly increased the density of c-fos$^+$ cells from 53.01 ± 9.54 cells/0.58 mm^2 ($p<0.05$) to 15.67 ± 3.51 cells/0.58 mm^2 at 0.12 MPa acoustic pressure. At 0.25 MPa, the mean density of c-fos$^+$ cells was 81 ±10.97 cells/0.58 mm^2 without MBs and it significantly increased to 124.12 ± 25.71 cells/0.58 mm^2 with MBs.

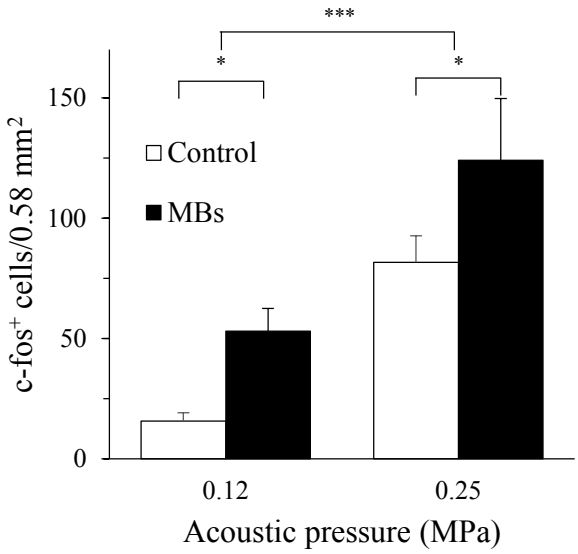

Fig. 4 The mean density of c-fos$^+$ cells in response to ultrasound stimulation with and without MBs (n = 3 mice per group). Group difference was analyzed by two-way repeated ANOVA, with * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and N.S. for not significant.

The integrity of BBB during ultrasound stimulation with MBs was also assessed. As shown in Fig. 5, the MB-associated ultrasound stimulation at 0.12 MPa acoustic pressure did not cause destruction of the BBB and no EB leakage was observed, while the EB leakage was produced in the group of 0.25 MPa ultrasound stimulation with MBs. The results show that 0.25 MPa ultrasound stimulation with MBs destroyed the integrity of BBB.

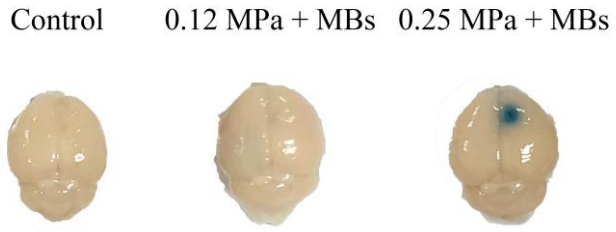

Fig. 5 BBB opening evaluation for the ultrasound stimulation with MBs.

IV. Discussion

This study demonstrated that the introduction of MBs can effectively improve the success rate of motor response to ultrasound stimulation and c-fos activity, which demonstrated that the presence of MBs would induce increased neuronal activity in mammals CNS. Previous studies have suggested that the enhanced neuronal activity induced by MBs might be largely related to the additional mechanical effects induced by the MBs

oscillation. Simulations by Krasovitskia et al. indicated that the scattered pressure from the pulsating bubble may even be several times greater than the amplitude of ultrasonic pressure [10]. Therefore, the scattered pressure generated by pulsating bubbles is supposed to be a potential reason for the enhanced neuronal activity induced by MBs. Additionally, vessel deformations caused by bubble-vessel wall interactions during ultrasound exposure, such as distention and invagination [11], might be another potential mechanical action.

Disruption of the BBB will cause ions, nutrients, and hormones in blood to diffuse into the brain tissue, which may also influence neuronal functions. In this study, we evaluated the integrity of the BBB during ultrasound stimulation with MBs. The examination of BBB integrity showed that 0.12 MPa US stimulation with MBs did not result in disruption of BBB integrity and MB oscillation still produced enhanced effect on ultrasonic neuromodulation, suggesting that the enhanced neuronal activity induced by MBs is likely to be independent of BBB opening.

Ultrasonic neuromodulation provides superior spatial specificity than other noninvasive neuromodulation methods. However, its resolution is still inferior to several invasive neuromodulation approaches. Increasing ultrasound frequency, enlarging geometric size of transducer, and sonogenetics are existing methods to enhance the resolution and specificity of ultrasonic neuromodulation, which will produce some limitations, such as increased acoustic attenuation, larger acoustic window, and genetic alteration. The finding in this study indicated that the introduction of MBs can produce enhanced neuronal activity even at low acoustic pressures, suggesting that not only the acoustic field of ultrasound transducer, but also by the distribution of MBs can adjust the resolution of ultrasonic neuromodulation. Therefore, concentrating MBs within smaller region or targeting MBs to specific regions might realize a new high-specificity method for ultrasonic neuromodulation.

V. CONCLUSIONS

This study demonstrated that the oscillation of MBs have ability to enhance neuronal activity in the CNS of mammals. Furthermore, the evaluation of BBB opening indicates that the enhanced neuronal activity induced by MBs may be irrelevant to BBB opening. These results promote the understanding of the effects of MB oscillation on neuronal activity, and break new avenues for the application of MBs in brain.

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