Final Report to FUSF Research Grant

Project title: "FUS-mediated Neuromodulation from Unanesthetized Freely-moving Animals (FUS461)"

December 15, 2017

Prepared by Wonhye Lee, Phillip Croce, Amanda Cammalleri, and Seung-Schik Yoo

Introduction

The main technical focus of this project was to develop a comprehensive FUS environment that provides (1) wearable miniature FUS headgear setup to implement and apply non-thermal, transcranial FUS to the somatomotor area (SM1) of small animals (rats) under unanesthetized freely-moving awake status or anesthetic conditions, while (2) enabling the motor behavioral monitoring of the FUS-mediated neuromodulatory outcomes, such as the elicited movements of the limb or tail. These capabilities are crucial to investigate the FUS neuromodulatory effects under different anesthetic conditions including awake status.

A. Study Outcome #1: Development of Wearable FUS Headgear Setup for the Stimulation of Rat Brain

A.1. Development of Miniature FUS Transducers

The miniature headgear FUS setup was successfully developed. A FUS transducer, an applicator for holding the transducer, and a base for mechanical support of the head pedestal were all custom-built (Fig. 1a). A 3D printer (Form2; FormLabs Inc.) using photo-crosslinkable resin was utilized for making these parts accurately and reproducibly. The transducer fits into a ball and socket joint of the applicator in order to maneuver the orientation of sonication (Fig. 1a). The applicator is attached to the base using a lock and key mechanism to ensure the reproducible orientation. Two set screws were used to secure the stable locking of the applicator (Fig. 2a). Multiple applicators having different lengths of arm and drop were prepared before FUS session for adjusting the location of transducer in an on-demand fashion (Fig. 1b).

Two different types of miniature FUS transducers were prepared. One type of the miniature transducer was prepared with a lens and operated at 500 kHz frequency (Fig. 1e, on the left side). The other type of miniature FUS transducer was prepared without a lens and operated at 600 kHz frequency (Fig. 1e, on the right side). The acoustic intensity profiles of the FUS transducers were also characterized along the sonication direction as well as on the transversal plane at the focus (Figs. 1f and 1g). The focal size was about 2 mm in diameter and 2.5 mm in length for the 500 kHz transducer and 3.5 mm in diameter and 5 mm in length for the 600 kHz transducer, measured at the full-width at half-maximum (FWHM) of the acoustic intensity profile. The intensity-level measured at the focus has shown to be capable of generating 10 W/cm^2 spatial-peak pulse-average intensity (I_{sppa}) (or higher) and 20 W/cm^2 I_{sppa} (or higher) for the 500 kHz and 600 kHz transducers, respectively. The acoustic profile mapping showed a focal area at a distance of ~10 mm from the exit plane of transducers (Figs. 1f and 1g at 500 kHz and 600 kHz, respectively). Figs. 1c and 1d show the schematics of sonication targeting to the somatomotor area of the rat brain and the sonication parameters used.

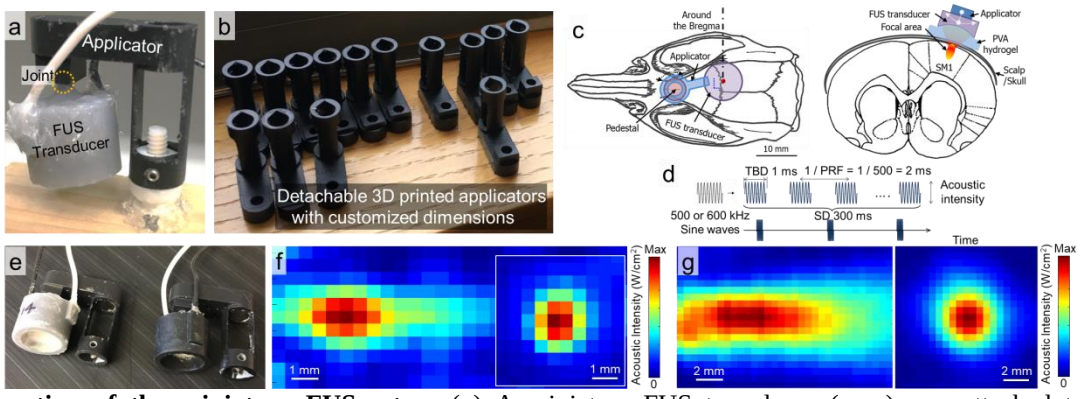

Figure 1. Preparation of the miniature FUS setup. (**a**) A miniature FUS transducer (grey) was attached to a custom-built 'Applicator' (black), which was mounted on a base (white) around the head pedestal. At the 'Joint', the transducer orientation can be adjusted and locked with respect to the applicator. The drop length of the applicator in the photo was 4.5 mm. (**b**) Detachable 3D printed applicators with customized dimensions of drop and arm lengths. The depth of sonication targeting can be adjusted using one of these applicators. (**c**) Schematic drawing of the FUS sonication targeting to the rat somatomotor area (SM1) using the wearable miniature FUS setup. (**d**) The sonication parameters used: fundamental frequencies of 500 kHz or 600 kHz, a tone burst duration (TBD) of 1 ms, a pulse repetition frequency (PRF) of 500 Hz, a sonication/stimulation duration (SD) of 300 ms, an inter-stimulation interval of 3–10 s, and acoustic intensity of maximum ~21 W/cm^2 I_{sppa} or below. (**e**) Two different types of miniature FUS transducers were prepared. The transducer on the left was fabricated with a lens and operated at 500 kHz. The other transducer on the right was made without a lens and actuated at 600 kHz. (**f, g**) The acoustic intensity profile along the sonication direction (from the left to right) generated by the FUS transducer (**f**) with a lens at 500 kHz and (**g**) without a lens at 600 kHz. Inset: transversal intensity profile at ~10 mm away from exit plane of the transducer.

A.2. Rat Animal Surgery for the Wearable FUS Headgear Setup

The surgical procedures for the implantation of the head pedestal (MS333; PlasticsOne) was successfully established with the use of skull-mounted screws and a custom-built base for mechanical support (Figs. 2a-c). Medical device instant adhesive (Loctite 18690) was used as a fixative for the implantation. The pedestals implanted on the rat skull have remained stable by the end of study period (over 8 months). Although this head pedestal was not used to measure EMG data due to signal noise issues from the custom-built transducer, this surgical procedure can also accommodate the inclusion of the subdermal wires (Figs.2d-2f) for the electrophysiological signal recording. The EMG recording capability can be utilized in future research, and we will make sure acknowledging the FUSF grant for the study.

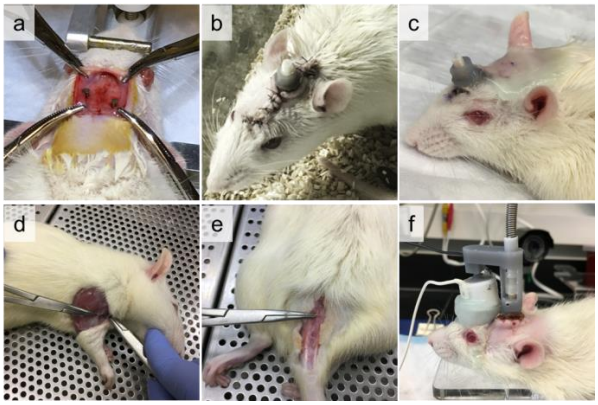

Figure 2. Surgical implantation of the head pedestal. (**a**) Skull surface was exposed by making a midline incision over the scalp. Two screws were mounted on the skull to secure the implanted head pedestal in place. (**b, c**) The head pedestal were implanted on either posterior region (*e.g.*, on lambda, **b**, for rats #1–8) or anterior region (**c**, for rats #9–15) of the skull. (**d, e**) Surgical practice for implanting the subdermal EMG wires to muscles of (**d**) the limb and (**e**) tail base. (**f**) An electrical cable for the EMG recording was connected to the head electrode pedestal implanted on the rat skull. For the EMG measurement setup, the head pedestal can be connected to the subdermal wires during the surgical procedures so that it can be used as the head 'electrode' pedestal. This capability was achieved as a study outcome, but not used due to signal noise issues from the custom-built FUS transducer. When this be used in future study, FUSF will be acknowledged.

A.3. Implementation of the Wearable Headgear FUS Setup and the use of PVA gel acoustic coupling

Upon the completion of the transducer preparation (Fig. 1), the miniature headgear FUS setup was implemented on the pedestal implanted on the rat's head (Figs. 2f and 3). The rat's fur was shaved to expose the scalp. A polyvinyl alcohol (PVA) hydrogel (prepared in degassed water, underwent 2 freeze-thaw cycles) was used for acoustic coupling between the transducer and the scalp. A generic ultrasound hydrogel was applied to the interfaces of the PVA gel (*e.g.*, transducer to PVA gel and PVA gel to the scalp). The preparation of an appropriate PVA hydrogel, in terms of the shape and rigidity, was crucial for acoustic coupling between the transducer and scalp as well as for the sonication targeting. The initial hydrogel was designed to have a flat interface covering the whole scalp area (Figs. 3a, 3b, 3c1). This created difficulties with sonication targeting due to the opacity of the hydrogel. For better sonication targeting, cone shape hydrogels were prepared and utilized (Figs. 3c2 and 3c3). Varying weight percentage of PVA gel, in a range of 6–9% (w/v). has been tested to adjust the hydrogel rigidity for the stable acoustic coupling. 8–9% (w/v) PVA gels were used for the FUS sessions. During the awake rat FUS sessions, the rat's grooming and head shaking behavior kept moving the coupling hydrogels off. In order to secure the hydrogel coupling stably, a long sleeve/wall structure was added to the hydrogel design (Fig. 3c4) and used for the FUS sessions under the awake status.

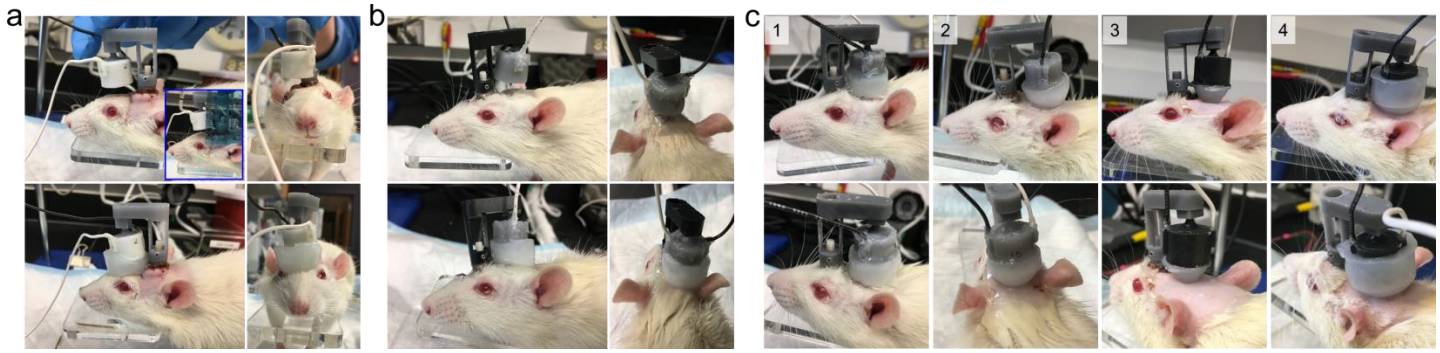

Figure 3. Implementation of the wearable headgear FUS setup. A miniature FUS transducer with a custom-built applicator was attached to the base/pedestal mounted on the (**a**) posterior or (**b**) anterior region of the rat's skull. In each panel of **a** and **b**, side and front/back views of the rat wearing the FUS setup were shown. The gap between the transducer exit plane and the scalp was shown in the upper pictures. A PVA hydrogel was used for acoustic coupling between the transducer and the scalp. A generic ultrasound hydrogel was applied to all the interfaces on the sonication path. **(c)** Various designs of acoustic coupling hydrogel for the wearable headgear FUS setup. Several shapes of the hydrogel were prepared using 6–9% (w/v) polyvinyl alcohol hydrogel and used for acoustic coupling between the transducer and the scalp. The hydrogels were prepared with (**c1**) a flat-bottom shape, (**c2**) a cone shape for the transducer with lens, (**c3**) a cone shape for the transducer without lens, and (**c4**) a cone shape and a long sleeve/wall structure for awake rat sessions. A generic ultrasound hydrogel was applied to all the interfaces on the sonication path.

B. <u>Study Outcome #2:</u> Examine the FUS stimulatory efficiency under different anesthetic conditions

B.1. Validation of the wearable FUS system to elicit movements:

We have established an experimental setup that can accommodate both anesthetized and awake rats during the sonication sessions. The use of a swivel cable connector (slip ring with flange; Adafruit) located above middle of the cage grants the unrestricted motion of the rats, while providing power to the FUS transducer (as well as the electrical connections to EMG electrodes). A data acquisition system (PowerLab and LabChart; ADInstruments) is prepared for recording time-series data of sonication (onset timing and duration) with syncronized video with/without EMG recording. A surveillance system having 4 channel cameras (QTH44-4CH-1; Q-See) is instrumented for recording high-resolution videos in order to analyze the behavioral responses elicited by the sonication under the three different experimental conditions (Figs. 4, upper panels and 5). With this setup, we were able to record and analyze the rat's motor responses elicited by the sonication (Figs. 4 and 5, lower panels).

Pulsed sonication events were administered to the somatomotor areas in the left or right (randomized) hemisphere of the rat brain (Figs.1c and 1d). Sprague-Dawley rats (N = 7, all male, 361.7 ± 23.4 g in 500 kHz Ketamine/Xylazine session, 388.6 ± 55.5 g in 500 kHz Isoflurane session, 395.3 ± 55.0 g in 600 kHz Ketamine/Xylazine session, 388.3 ± 39.6 g in 600 kHz Isoflurane session, 412.7 ± 33.8 g in 600 kHz Awake session) were used under local IACUC approval. In the course of adjusting the orientation of the FUS transducer, the motor responses were elicited by the sonication from various anatomical locations. Table 1 shows the locations of movement elicited by the sonication using the wearable miniature headgear FUS setup under five different experimental conditions, including (1) anesthetic conditions of Ketamine/Xylazine, Isoflurane, and Unanesthetized freely-moving Awake status and (2) fundamental frequencies of 500 kHz and 600 kHz. An acoustic intensity of up to 11.4 W/cm^2 I_{sppa} with 500 kHz ultrasound and that of 21.7 W/cm^2 I_{sppa} with 600 kHz ultrasound were applied. The elicited movements were examined across a total of seven rats (ID #9–#15), and detected from whisker, forelimb, hindlimb, and tail under every different experimental condition from at least one or more animals, which validated the use of the wearable FUS system to elicit the movements from rats.

Although the number of animals responsive to the FUS sonication seems similar among the different experimental conditions (Table 1), it was difficult to elicit reproducible movements under Isoflurane anesthesia except whisker movements, compare to Ketamine/Xylazine condition. To our knowledge, this was the first trial to test the use of Isoflurane on rat for the ultrasonic neuromodulation, while there were mice studies under Isoflurane anesthesia reported from Stanford group (King/Pauly et al. 2013, 2014, Ye/Pauly et al. 2016 UMB). Stimulatory efficiencies (or response rates) were further examined and compared in section B.2. with Fig. 5.

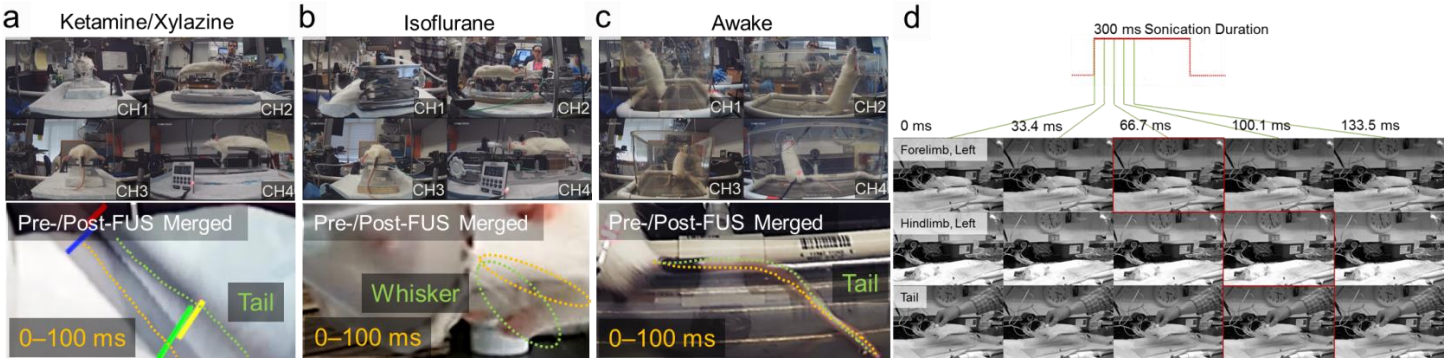

Figure 4. Validation of the wearable FUS system to elicit movement from rats under different anesthetic conditions. The ultrasonic neuromodulation-mediated elicitation of motor response has been investigated by using the wearable headgear FUS setup, under three different anesthetic conditions of (**a**) ketamine/xylazine, (**b**) isoflurane, and (**c**) unanesthetized awake status. The upper panel shows the video data from the left, front, back, and right sides of the rat/cage that were recorded for behavioral analysis during the sonication sessions. The lower panel of each column shows an exemplar movement elicited during each different experimental condition. (**d**) Exemplar temporal sequences of video frames recorded during a pulsed sonication event of 300 ms. Using the miniature FUS setup, pulsed sonication events were administered to around the somatomotor areas on the unilateral hemisphere of the rat brain.

Table 1. Locations of movement elicited by the sonication using the wearable miniature headgear FUS setup under five different experimental conditions, including anesthetic conditions of Ketamine/Xylazine, Isoflurane, and Unanesthetized freely-moving Awake status and 500 kHz and 600 kHz ultrasound. The elicited movements from whisker, forelimb, hindlimb, and tail were examined across seven rats.

Different Conditions	Ketamine/Xylazine @ 500kHz	Isoflurane @ 500kHz	Ketamine/Xylazine @ 600kHz	Isoflurane @ 600kHz	Awake @ 600kHz
Locations	Count of Responsive Rats (Out of Total 7 Rats: ID #9–#15)				
Whisker	6 / 7	5 / 7	4 / 7	3 / 7	4 / 7
Forelimb	6 / 7	4 / 7	3 / 7	6 / 7	5 / 7
Hindlimb	5 / 7	4 / 7	3 / 7	6 / 7	1 / 7
Tail	5 / 7	1 / 7	2 / 7	5 / 7	7 / 7

B.2. Response Rates of Movement Elicitations under Different Anesthetic Conditions

Response rates of movement elicitation by sonication under different anesthetic conditions were examined and compared (Fig. 5). An acoustic intensity of 11.4 W/cm^2 I_{sppa} with 500 kHz ultrasound and that of 21.7 W/cm^2 I_{sppa} with 600 kHz ultrasound were used. Compared to the 500 kHz sessions, a higher acoustic intensity (i.e., 21.7 W/cm^2) was used for the 600 kHz session because it was difficult to elicit reliable movements using lower intensity (e.g., 11.4 W/cm^2 I_{sppa}). Similar tendency was previously reported (Kim/Yoo et al. 2014 Brain Stimul). Compared to our (the Yoo group) previous studies of the ultrasonic neuromodulation in rodent, higher acoustic intensities were needed to elicit movement in this project, which likely due to the use of different sonication setup (the wearable miniature FUS headgear). Figures 5a–5c show individual rat (ID #9–#15)'s averaged response rate from multiple sessions with (1) 500 kHz ultrasound under Ketamine/Xylazine anesthesia, (2) 600 kHz ultrasound under Isoflurane anesthesia, and (3) 600 kHz ultrasound under Unanesthetized freely-moving Awake status. Each session consisted of 10 FUS sonication events targeting the limb or tail. These data show the variabilities of the response rates (1) across all the rats and (2) even within a rat through multiple sessions under the same experimental condition (see error bar on each bar plot). It is also noteworthy that there were one or more non-responsive animal(s) (Fig. 5a, rat #9 or Fig. 5b, rat #10)) to the sonication under anesthesia, but all the animals from the same group were responsive under awake status (Fig. 5c). In Fig. 5d, grand mean response rates across responsive rats under the different experimental conditions were compared. At 500 kHz ultrasound, Ketamine/Xylazine condition showed a significantly higher response rate compared to the that under Isoflurane (*t*-test, one-tailed, $P = 0.02$). At 600 kHz ultrasound, there was marginal difference in response rate between Ketamine/Xylazine and Isoflurane sessions (*t*-test, one-tailed, $P = 0.09$). There was no significant differences between different frequency conditions under the same anesthesia (*t*-test, one-tailed, $P > 0.2$) while a higher acoustic intensity was used for 600 kHz sessions. The response rate of awake session did not show significant differences compared to those from other sessions.

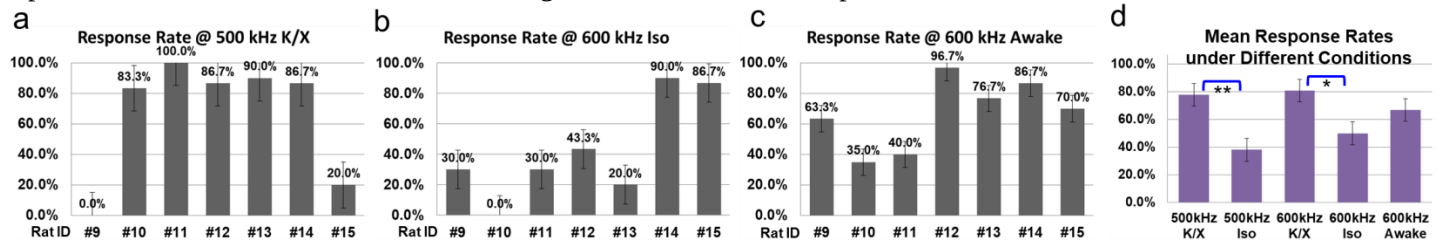

Figure 5. Response rates of movement elicitation by sonication from the wearable FUS headgear. Individual rat (ID #9–#15)'s averaged response rate from multiple sessions with (**a**) 500 kHz ultrasound under Ketamine/Xylazine anesthesia, (**b**) 600 kHz ultrasound under Isoflurane anesthesia, and (**c**) 600 kHz ultrasound under Unanesthetized freely-moving Awake status. Each session consisted of 10 FUS sonication events targeting the somatomotor area. (**d**) Grand mean response rates across the rats responsive to the sonication under different experimental conditions, including the anesthetic conditions of Ketamine/Xylazine, Isoflurane, and Unanesthetized freely-moving Awake status and the ultrasound frequencies of 500 and 600 kHz. An acoustic intensity of 11.4 W/cm^2 I_{sppa} with 500 kHz ultrasound and that of 21.7 W/cm^2 I_{sppa} with 600 kHz ultrasound were used. *t*-test, one-tailed, $^{**}P = 0.02$, $^{*}P = 0.09$.

B.3. Onset Latency of the Elicited Movements under Different Anesthetic Conditions

Histograms of the elicited movement onset latency after the onset timing of the sonication were shown for each different anesthetic condition at 600 kHz (Figs. 6a–c) as a group-level analysis, regardless of the locations of movement elicited. The latencies over ~100 ms are congruent with previous studies of FUS-mediated response onset latency (mice data from King et al. 2013 UMB, rat data from Kim/Yoo et al. 2014 UMB, sheep data from Lee/Yoo et al. 2016 UMB), and the latencies shorter than ~33 ms were also shown in the present study (mice data from Tufail et al. 2010 Neuron, rat data from Gulick et al. 2017 Ultrasound Med Biol). It is noteworthy that the responses with a short latency (< ~33 ms) were observed more frequently in the awake sessions (Fig. 6c) than in the anesthetic sessions (Figs. 6a and b).

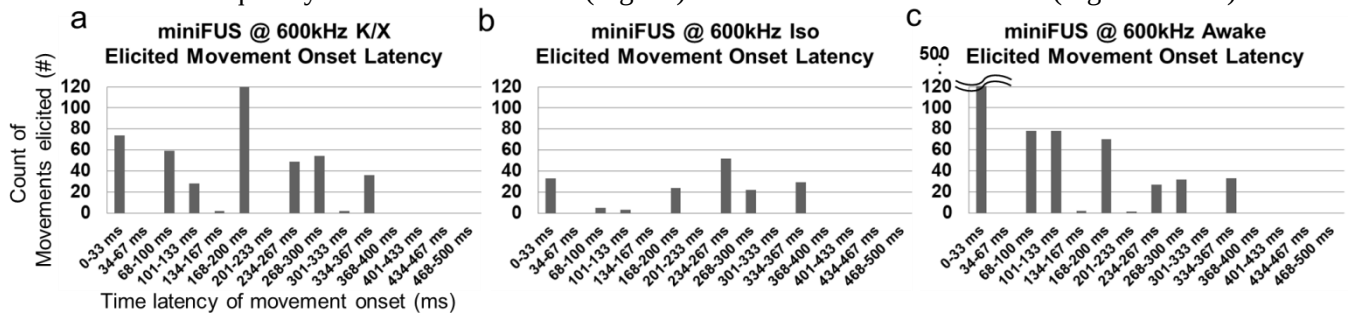

Figure 6. Histograms of the elicited movement onset latency after onset timing of sonication at 600 kHz. (**a**) Ketamine/Xylazine anesthesia ($N = 4$ responsive rats, from 424 sonication events), (**b**) Isoflurane anesthesia ($N = 6$ responsive rats, from 168 sonication events), and (**c**) Unanesthetized freely-moving Awake status ($N = 7$ responsive rats, from 891 sonication events).

C. <u>Study Outcome #3:</u> Unanesthetized Freely-moving Awake Rat's Behavioral Responses to FUS Brain Stimulation

For unanesthetized freely moving awake rats, the somatomotor area of the rat brain was also targeted by the sonication using the wearable FUS headgear. Varying acoustic intensities up to a maximum of 21.7 W/cm^2 I_{sppa} were applied at 600 kHz ultrasound. From awake rats, we were also able to see the elicited movements from the whisker, forelimb, hindlimb, and tail (Table 2), where the movements were elicited under the anesthetic sessions (Table 1). Additionally, elicited movements were also shown from back/lower back and genital parts in the awake sessions. Compared to the elicited movements under anesthetic conditions, the degree of movement were smaller so that it was not easily shown from captured image series, but only seen from the recorded video while playing. The hindlimb movement was detected only from one awake rat (ID #10) likely due to rat's standing postures. We believe that the elicited responses from the hindlimb will be detected more reliably from most of rats if EMG recording is used in future study. Similar undetected elicited responses from the video analysis (due to the absence of EMG recording) may have reduced the mean response rate of the awake session in Fig. 5. Regarding the onset latency of movement, as shown in Fig. 6, a larger portion (~60%) of the elicited movements in awake status were shown with a short onset latency (~33 ms), while in anesthetic conditions, lower than ~25% of the movements was shown with that short latency.

In the awake sessions, not only the elicited movements, but behavioral responses to the FUS brain stimulation were also shown such as the movements of ear/head or chewing. Startle-like responses were detected from some (but not all) of the sonication events. The grimace response was also observed, mostly during the wearing procedure of the miniature FUS headgear setup including the PVA gel acoustic coupling. Once the grimace response was shown from a rat during this wearing procedure, the rat kept showing the grimace from some of the sonication events. On the other hand, when animals were lightly sedated using Isoflurane for the necessary fur shaving to expose the scalp (so that the wearing procedure was also performed under the sedation) before some of the awake sessions, these rats did not show grimace response during the sonication session.

For conducting more stable and reliable awake sessions in future, more elaboration will be necessary (1) to prepare more stable wearing mechanism of the FUS headgear setup and acoustic coupling mechanism for the PVA gel (Fig. 3c), and (2) to remove signal noise from the FUS transducer/sonication for measuring EMG data.

Table 2. Locations of elicited movement and behavioral responses from awake rats during FUS stimulation. The somatomotor area of the rat brain was targeted by the sonication using the wearable headgear FUS setup. Varying acoustic intensities up to 21.7 W/cm^2 I_{sppa} were applied at 600 kHz ultrasound. The elicited/behavioral responses across a total of seven rats (ID #9–#15) were examined. Compared to the elicited movements under anesthetic conditions, the degree of movement were smaller. It is also notable that chewing, startle, or grimace responses were detected from some but not all of the sonication events.

Rat ID	#9	#10	#11	#12	#13	#14	#15	Counts of Responsive Rats
Location of movement								
Whisker	Yes	Yes	Yes	Yes	No	No	No	4 / 7
Forelimb	Yes	Yes	Yes	Yes	No	Yes	No	5 / 7
Hindlimb	No	Yes	No	No	No	No	No	1 / 7
Tail	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7 / 7
Back/Lower Back	Yes	Yes	Yes	Yes	Yes	Yes	Yes	6 / 7
Genital	Yes	Yes	No	Yes	Yes	Yes	Yes	5 / 7
Behavioral responses								
Head	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7 / 7
Ear	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7 / 7
Chewing	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7 / 7
Startle	Yes	Yes	Yes	Yes	Yes	Yes	Yes	7 / 7
Grimace	Yes	Yes	Yes	Yes	Yes	No	No	5 / 7

D. <u>Study Outcome #4:</u> FUS brain stimulation to anesthetized rats induces long-term changes in SEP

Previous studies have identified transient effects of FUS on the brain excitability and accompanying physiological responses. Yet the presence of long-lasting effects of FUS, which extend on the order of half an hour or more, has not been probed. We were also motivated to test long-lasting effects of FUS. We administered FUS to the somatosensory areas of the anesthetized rats for 10 min at a low duty cycle (5%) and acoustic intensity of 4.2 W/cm^2 I_{sppa}. Concurrently, we measured electroencephalographic (EEG) somatosensory evoked potentials (SEP) induced by the electrical stimulation of the unilateral hind limb before and after the sonication (Fig. 7). Compared to the control condition (no sonication), differential SEP features were evident and persisted beyond 35 min after the administration of FUS. The presence of this non-transient neuromodulatory effect may provide early evidence that FUS-mediated brain stimulation has the potential to induce neuroplasticity. The detailed methods and data were described in the manuscript (NeuroReport, Appendix 2).

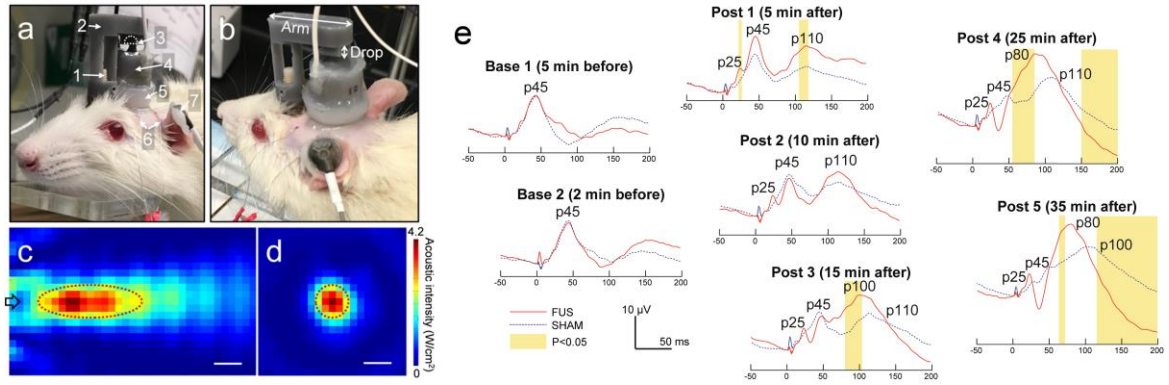

Figure 7. The experimental setup and the acoustic intensity profile of the wearable FUS transducer. (**a**) The transducer worn by a rat during sonication. 1: skull-mounted pedestal, 2: detachable applicator, 3: ball-and-socket joint, 4: FUS transducer, 5: coupling gel, 6: subdermal EEG electrodes, 7: ground EEG electrode. (**b**) the detachable applicator with customizable dimension. The acoustic intensity profile across the (**c**) longitudinal plane and (**d**) transversal plane at 10.5 mm from the exit plane of the transducer. Dotted lines in panels (**c**) and (d) depict the FWHM of the intensity profile from the miniature FUS transducer operated at 650 kHz. The arrow in panel (**c**) indicates the direction of acoustic beam. Scale bar = 2 mm. (**e**) Time course of the grand-average (N = 11 rats) SEP comparing the FUS and sham conditions. The time segments that showed the differential signal features (two-tailed t-test, $P < 0.05$) are highlighted in yellow. (Yoo *et al.*, 2018 NeuroReport, in press).

E. <u>Study Outcome #5:</u> Post-sonication Behavioral Monitoring and Histological Analysis after the repeated FUS

All animals have shown normal behavior during and after the sonication experiments, which suggests safe administaration of the FUS. Acoustic intensities up to 11.4 W/cm^2 I_{sppa} with 500 kHz ultrasound and up to 21.7 W/cm^2 I_{sppa} with 600 kHz ultrasound were applied with an inter-stimulation interval of 5–10 s. There was no loss of life during the study period and the rat's health status was normal throughout the whole duration of the study period before being sacrificed. Post-sonication animal behaviors of all rats were normal across survival durations. Post-sonication brain extractions from the animals were performed considering a short-term (1.0 ± 0.9 days) or long-term duration (41.5 ± 0.6 days days) after the last FUS session. Before being sacrificed, two rats were also underwent tail-vein trypan blue perfusion procedure to detect any blood-brain barrier disrupstion (BBBd) induced by FUS sonication. There were no signs of BBBd detected. After the transcardial formalin perfusion procedure on the rats, the extracted brains were preserved in formalin and further processed for histological analysis of hematoxyline & eosin (H&E) staining to examine cell necrosis or local recruitment of inflammatory cells, Vanadium Acid Fuchsin (VAF)-Toluidine Blue staining to visualize ischemic neurons, glial fibrillary acidic protein (GFAP) staining to examine glia infiltration or signs of neurodegeneration, and Caspase-3 to detect apoptotic activity. The histology revealed that all the brain tissues were normal. Fig. 8 shows example slides from rat #14.

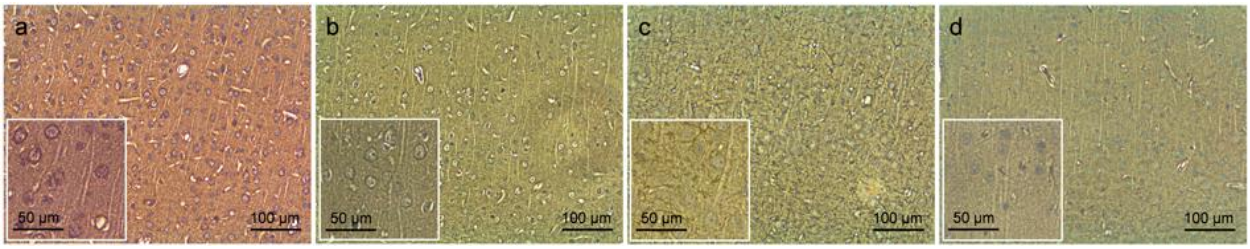

Figure 8. Example of the histology results from the motor cortex of rat #14 after the repeated sonication sessions with 100× magnification (Insets with 200× magnification). (**a**) Hematoxyline & Eosin (H&E) (**b**) Vanadium Acid Fuchsin (VAF)-Toluidine Blue staining, (**c**) Glial fibrillary acidic protein (GFAP) staining, and (**d**) Caspase-3 staining. The histology revealed that all the brain tissues were normal.

Summary of Performance Indices

1. Planned Presentations: (Appendix 1) Development of Wearable Focused Ultrasound Transducer for the Stimulation of Rat Brain, BMES 2017 Conference (presented in oral session), SBMT 2018 Symposium (invited), ISTU 2018 Symposium (to be applied), FUSF 2018 Symposium (to be applied).

2. Planned Publications: (Appendix 2) Focused ultrasound brain stimulation to anesthetized rats induces long-term changes in somatosensory evoked potentials, in press, NeuroReport, and Development of wearable focused ultrasound transducer for the stimulation of rat brain, in preparation, to be submitted to BMC Neuroscience or similar.

3. Planned Research Activity: NIH R21 to be applied in 2018.