Ultrasonic neuromodulation by brain stimulation with transcranial ultrasound

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Brain stimulation methods are indispensable to the study of brain function. They have also proven effective for treating some neurological disorders. Historically used for medical imaging, ultrasound (US) has recently been shown to be capable of noninvasively stimulating brain activity. Here we provide a general protocol for the stimulation of intact mouse brain circuits using transcranial US, and, using a traditional mouse model of epilepsy, we describe how to use transcranial US to disrupt electrographic seizure activity. The advantages of US for brain stimulation are that it does not necessitate surgery or genetic alteration, but it confers spatial resolutions superior to other noninvasive methods such as transcranial magnetic stimulation. With a basic working knowledge of electrophysiology, and after an initial setup, ultrasonic neuromodulation (UNMOD) can be implemented in less than 1 h. Using the general protocol that we describe, UNMOD can be readily adapted to support a broad range of studies on brain circuit function and dysfunction.

INTRODUCTION

US is an acoustic wave (mechanical pressure wave) occurring at frequencies exceeding the range of human hearing (>20 kHz)[1]. More than 80 years ago, it was first shown to be an effective neuromodulation tool capable of enhancing neural activity in frog and turtle neuromuscular preparations[2]. Nearly 30 years later, William Fry and colleagues[3] showed that US transmitted to the lateral geniculate nucleus of craniotomized cats could reversibly suppress light-evoked potentials recorded in the visual cortex. Since then, US has been shown to be capable of differentially enhancing or suppressing neuronal activity in a variety of experimental preparations across a wide range of acoustic frequencies, intensities and modes of transmission (**Table 1**). Until recently, however, US had only been shown to be effective for modulating the amplitude, duration and/or conduction velocity of electrically evoked or sensory-driven neuronal activity. Using whole-cell electrophysiology and optical recording techniques in hippocampal slice cultures, it was recently shown that US is capable of directly stimulating action potentials, voltage-gated Na^+ and Ca^{2+} transients and synaptic transmission in central neurons[4]. Subsequently, these observations have been translated into whole animal preparations, in which it was discovered that transcranial pulsed ultrasound (TPU) can directly stimulate intact brain circuit activity in mice[5]. The major advantages of TPU for brain stimulation are that it offers a mesoscopic spatial resolution of a few millimeters, while possessing the ability to remain completely non-invasive. Collectively, the above-described observations have opened the rousing possibility of using US for neuromodulation. On the basis of our previous *in vivo* studies designed to investigate the influence of US on mouse brain circuits[5], the protocol provided below describes how to directly stimulate brain activity using transcranial US.

To convey the utility of UNMOD, we provide three basic procedures using pulsed US for the stimulation of intact mouse cortex. One procedure does not require surgical manipulation and details methods for implementing TPU in non-invasive brain stimulation. The second procedure involves a craniotomy such that extracellular recordings can be conducted in response to US stimulus waveforms. The third procedure is based on previous observations demonstrating the effects of US in animal models of epilepsy[6–9], and it provides a protocol using UNMOD to attenuate chemically induced seizure activity in a mouse model of *status epilepticus* (SE). These specific UNMOD protocols have been provided so that others may more easily implement and expand upon the many potential applications of US for brain stimulation.

Brief overview of ultrasound

Besides its global applications in diagnostic medical imaging, US has broad uses in nondestructive testing of materials, ultrasonic cleaning, chemical manufacturing, food processing, physiotherapy, personal hygiene, sonar and communications[1]. For medical imaging, US has a frequency range from about 1 to 15 MHz, whereas therapeutic applications typically use a frequency of ~1 MHz or less[10]. US can be transmitted as pulsed or continuous waves (CWs) through tissues, including bone, and can produce thermal and/or non-thermal (mechanical) bioeffects[10–13]. Therapeutic US can be classified as low power (typically <0.5 W cm^{-2}) or high power (>100 W cm^{-2}) depending on its acoustic intensity[12]. The thermal ablation of tissue is conducted with high-intensity US at power levels usually exceeding 600 W cm^{-2}, whereas therapeutic effects mediated by non-thermal actions of US can occur at power levels <0.5 W cm^{-2} (refs. 10–13). To gain additional insight into the biophysics of US, the reader is referred to recent reviews on the topic[10,12,13].

Comparison of UNMOD with modern brain stimulation methods

Methods and applications of intact brain circuit stimulation have been undergoing a continuous evolution since their first description in the late nineteenth century[14]. At present, neuroscience relies almost exclusively on electrical-, magnetic- and photonic-mediated approaches for modulating neural activity. Recent advances in molecular biology have enabled the use of genetically encoded light-activated sensor and actuator proteins in the study and control of brain circuits ('optogenetics')[15,16]. In terms of spatial

TABLE 1 | Summary of studies demonstrating neural circuit excitation or suppression using ultrasound.

Preparation	US mode	Acoustic frequency	Acoustic intensity	Outcome	Reference(s)
In vitro frog and turtle muscle and heart	CW	0.34 MHz	Unknown	Stimulation of nerve and muscle	2
Craniotomized cat LGN	CW	Unknown	Unknown	Reversible suppression of visually evoked responses in V1	3
Cat spinal cord	PW	2.7 MHz	Unknown	Differential and reversible enhancement and suppression of mono- and polysynaptic spinal reflexes	78
Chemically induced epilepsy in cats	PW	2.7 MHz	840 W cm^{-2}	Attenuation of seizure activity monitored by EEG and decreased morbidity	6
Human somatosensory receptors in hand	PW	0.48–2.67 MHz	8–3,000 W cm^{-2}	Differential touch, pain and thermoreception	79
Human cochlea and auditory nerve	PW; AM (125–8,000 Hz)	2.5 MHz	0.02–1.5 W cm^{-2}	Auditory sensations even in deaf subjects	80
Cat cochlea and auditory nerve	PW	5.0 MHz	30 W cm^{-2}	Stimulation of cochlea and auditory nerve	81
Human brain (TCD of basilar artery)	TCD; PW	2.0 MHz	<0.5 W cm^{-2}	Site-dependent stimulation of auditory sensations	82
Frog sciatic nerve	CW	3.5 MHz	1–3 W cm^{-2}	Increase of evoked CAP amplitude at 1 W cm^{-2} and decrease in CAP amplitude at 2–3 W cm^{-2}	83
Frog sciatic nerve	PW	2.0–7.0 MHz	100–800 W cm^{-2}	Differential increase and decrease of evoked CAP amplitude	84
Rabbit sciatic nerve	CW	3.2 MHz	1940 W cm^{-2}	Suppression of activity; nerve block	85
Acute hippocampal slices	PW	0.5–0.75 MHz	40–110 W cm^{-2}	Suppression of evoked fiber volley and orthodromic population spikes, but enhanced dendritic field potential	86,87
Hippocampal slice cultures	TB	0.44–0.67 MHz	2.9 W cm^{-2}	Stimulation of action potentials and neurotransmitter release	4
Intact mouse cortex and hippocampus	PW	0.25–0.5 MHz	0.75–0.229 W cm^{-2}	Direct stimulation of action potentials in cortex and evoked synchronous oscillations and BDNF expression in the hippocampus	5
Craniotomized rabbit cortex	TB/CW	0.69 MHz	3.3–12.6 W cm^{-2}	Modulation of visually evoked functional MRI BOLD signals; stimulation of motor cortex	40

AM, amplitude modulated; BDNF, brain-derived neurotrophic factor; CAP, compound action potential; CW, continuous wave; EEG, electroencephalogram; PW, pulsed wave; TB, tone burst; TCD, transcranial Doppler sonography. BOLD, blood oxygen level–dependent.

resolution, these genetically mediated neurostimulation methods currently reign superior to all other brain stimulation methods. For optogenetic brain stimulation approaches, the functional resolution limits are determined by the location and density of protein expression rather than the diffraction limits of electromagnetic radiation or light. By transducing the expression of exogenous light-activated ion channels or transporters in neurons, optogenetic methods confer the ability to stimulate and/or inhibit the activity of individual cells in brain circuits[15,16]. These advantages conferred by optogenetic manipulation have led to the prolific expansion of their use for elucidating brain function in cellular models, as well as in whole-animal experimental preparations. However, the major weakness of optogenetics will always be an inherent requirement for genetic modification, which can present its own set of obstacles and complicate implementation.

Evoking neuronal activity using conventional electrodes represents, by far, the most widely implemented brain stimulation approach[17,18]. Several notable studies have addressed the spatial resolution characteristics of brain circuit stimulation with microelectrodes. The general consensus is that a sparse population of nonspecific cells and cellular processes are activated within a current density volume generated by an electrode. The diameter of a current density volume generated (the effective spatial resolution) varies from several micrometers to several millimeters depending on the electrode size, electrode placement, stimulus amplitude, duration and frequency[19–22]. Similarly, there is a high degree of variability with regard to the numbers and types of cells stimulated within any given current volume at any given time. However, in the study and treatment of brain circuits, basic scientists and clinicians have, for more than a century, successfully accommodated any lack of spatial specificity conferred by stimulating electrodes. The primary disadvantage of using electrodes for intact brain circuit stimulation is that they require direct contact with neural tissue and necessitate surgical procedures, which triggers deleterious processes such as inflammation, bleeding, cell death and gliosis[23]. Thus, less-invasive brain stimulation procedures are often desirable.

Although declining in popularity for various reasons, electroconvulsive shock therapy is a classic brain stimulation method, which does not require surgery and has a long history of use in treating psychiatric disorders[24]. At present, the most recognizable and broadly accepted noninvasive brain stimulation methods are transcranial direct current stimulation (tDCS) and transcranial magnetic stimulation (TMS)[25–28]. These noninvasive brain stimulation methods can be used to nonspecifically activate cells in tissue volumes having diameters of 1 cm or more by transmitting electrical currents (tDCS) or magnetic energy (TMS) through the skull into the brain[25,26]. Although the spatial resolutions for TMS and tDCS are considerably worse than those for microelectrodes, TMS and tDCS do possess a major advantage—they do not require surgery. In rodents, both TMS and tDCS have been found to be effective for stimulating intact mouse and rat brain circuits, including by non-invasive modulation of cortical and subcortical activity[29–31]. Despite their noninvasiveness, it is often preferred to implement brain stimulation methods that offer better spatial resolutions and targeting accuracies than those conferred by tDCS and TMS.

By using acoustic pressure waves rather than light, electrical currents or magnetic radiation, we recently showed that TPU can functionally stimulate mouse brain circuits without requiring surgery or genetic modification, while conferring a spatial resolution of ~3 mm (ref. 5). Our data indicate that low-intensity, low-frequency pulsed US activates a nonspecific population of cells within acoustic pressure field[4,5]. Such nonspecific activation is a property shared by brain stimulation approaches using electrodes, TMS or tDCS in rodents and other animal species including humans, as referenced above. The diffraction-limited resolution of US for brain stimulation presently resides somewhere between those achievable with microelectrodes and TMS. However, implementation of hyperlenses and acoustic metamaterials for focusing US enables subdiffraction-limited spatial resolutions to be achieved[32,33], and it may be useful for improving the spatial resolution of UNMOD in the future.

With respect to the spatiotemporal patterns of brain activity evoked by US, we have shown that UNMOD can be used to stimulate action potentials and synaptic transmission in a manner similar to that of conventional electrodes, without generating macroscopic heating[4,5]. Further, we showed that TPU can be used to stimulate tetrodotoxin-sensitive brain circuit activity in the motor cortex and hippocampus of intact mice[5]. The response latencies of US-evoked brain circuit activity in mice (approximately 20–30 ms) tend to be slightly slower than those achieved using channelrhodopsin-2 (ChR2), electrical stimulation or TMS[5,34–36]. We presume that these kinetic differences in reaching activation thresholds are most likely to stem from the different energy modalities and mechanism(s) of action underlying each method. In fact, the time course for neuronal activation by US (tens of milliseconds) may provide clues to potential mechanisms of action. For example, they are similar to the kinetics described for pore formation triggered by lipid-phase transitions, which are thought to underlie excitatory sound wave propagation in cellular membranes including neuronal ones[37].

Thermodynamic investigations of lipid-phase transitions have shown that mechanical waves can be adiabatically propagated through lipid monolayers and bilayers, as well as neuronal membranes to influence fluidity and excitability[37–39]. Notably, such sound wave propagation in pure lipid membranes has been estimated to produce depolarizing potentials ranging from 1 to 50 mV with negligible heat generation (~0.01 K) due to differences in the viscous and thermal penetration depth-length scales of monolayers and their surrounding aqueous environments[39]. Without producing significant heat, as described above and elsewhere[5], US may initiate mechanical (sound) waves in neuronal membranes thereby depolarizing them sufficiently to activate voltage-gated ion channels and trigger action potentials. This idea represents only one of many testable hypotheses describing how US may mechanically (non-thermally) stimulate neuronal activity. Further studies are required to explore the many potential mechanisms underlying the ability of US to stimulate neuronal activity in the intact brain. Even without knowing the exact mechanisms of action, however, US for brain stimulation represents a powerful new tool for neuroscience.

Limitations of UNMOD

By no means is UNMOD without limitations. One of the major concerns regarding the use of US for neuromodulation is its safety. As US is capable of destroying biological tissues, the potential for biohazardous effects must be taken into careful consideration. Many of the biohazards associated with US stem from its ability to induce cavitational damage in tissues. In soft tissues, including brain, inertial cavitation rarely induces damage at pressures <40 MPa (except in lung, intestinal and cardiac tissues in which damage from

inertial cavitation can occur at pressures of ~2 MPa because of the presence of large gas bodies)[13]. At peak rarefactional pressures <1 MPa, US has been found effective for acutely (tens of hours up to spaced trials repeated across weeks) stimulating brain circuits without producing damage in mice as assessed by cellular, histological, ultrastructural and behavioral methods[4,5]. Similar safety observations have recently been reported for using US to modulate the excitability of intact rabbit and rat brain circuits[9,40]. However, additional investigations across animal species and US dosage levels are required before the safety of UNMOD can be fully ascertained.

It is important for laboratories working on US for brain stimulation applications to share their observations in an open and responsible manner with the scientific community while working to establish a set of standards and safety margins for acoustic intensity, frequency and exposure times. Of particular interest, repeated US exposure has indeed been shown to disrupt neuronal migration in developing mouse embryos[41]. Thus, the potential for damage arising from repeated, long-term US exposure across various stages of brain development (embryonic to mature adult) commands caution and requires rigorous evaluation. Compiling data regarding the safety of UNMOD across animal species in various stages of development and for different applications should be a primary focus of future research in this emerging field.

Road map for the further development and clinical application of UNMOD. Some therapeutic applications using US in intact brain tissues, such as ablation therapies, have been developed and are currently being applied in clinical settings[42,43]. This ablative technology, however, has been engineered primarily by groups that lack an intimate understanding of molecular and cellular neurophysiology. In contrast, novel applications of US showing high promise for applications in translational neuroscience (for example, UNMOD) have been developed by groups that understand neurophysiological processes, but that do not have expertise in engineering sophisticated medical devices. Thus, there is a major need for increasing open communication between engineers designing US-based medical devices, neuroscientists studying the core bioeffects of US on brain physiology and clinicians implementing US for therapeutic interventions. Professional societies and foundations, such as the International Society for Therapeutic Ultrasound (http://www.istu.org/) and the Focused Ultrasound Surgery Foundation (http://www.fusfoundation.org/), have recognized this need and are now serving as forums for overcoming communication barriers between engineers, scientists and clinicians working on US for applications in neuroscience. Improving communication across disciplines will enable global research groups to gain traction in addressing some of the most pressing questions regarding the applications of US for achieving desirable bioeffects in neuroscience.

The biological effects of US on nervous tissues remain poorly understood in comparison with those produced using electromagnetic energy modalities. This lack of knowledge presents a substantial hurdle to making translational progress toward further developing UNMOD approaches and applications. Attention should be paid to how US influences the excitability of nervous tissues, as well as to how it influences more salient cellular molecular signaling cascades in neurons and glia. US has been shown to have clear effects on cellular signaling cascades by regulating intermediate early gene activity, as well as by promoting growth factor signaling in bone and peripheral vascular tissues[44–50]. Whether or not US can impart similar effects on brain circuits and peripheral nervous tissues remains largely unknown, but it will be important to determine through future research.

Some of the specific questions that need to be addressed by forthcoming studies and then cross-validated by independent research groups across animal species are the following: How are neurons and glia outside of the focal volumes affected by standing waves and unfocused US? Do different focusing strategies influence those outcomes? If so, how? Which UNMOD applications require precise focusing and which do not? What are the differences in terms of safety between chronic and acute brain exposure to US stimulus waveforms? What are the exposure time cutoffs when using pulsed US waveforms compared with CW US waveforms? Do differences exist in these exposure time safety margins for different pulsed US waveforms? Do differences exist in the safety margins when US is used for ablation compared with diagnostic imaging or neuromodulation? Are these safety margins the same or different for use in organisms across different lifespan stages (embryonic, juvenile, adult and aged) or different disease states? The basic protocols described below are intended to further enable neuroscientists, engineers and clinical research groups to begin elucidating some of these unresolved issues.

Experimental design

Choice of UNMOD stimulus waveform parameters. The acoustic frequency and intensity characteristics of an UNMOD stimulus waveform underlie its core effect on brain activity. As highlighted in **Table 1,** the acoustic frequencies used to manipulate neuronal activity range from 0.25 (ref. 5) to 7.0 MHz[51]. Although lower frequencies of US have longer wavelengths and thus lower spatial resolutions compared with higher US frequencies, we recommend the use of acoustic frequencies <1 MHz for stimulating intact brain circuits with US. This is primarily because US frequencies <0.7 MHz represent a range in which optimal gains between transcranial transmission and brain absorption of US have been observed[52–54]. In mice, we have found that the optimal waveforms for evoking intact brain circuit activity are composed of acoustic frequencies ranging between 0.25 and 0.65 MHz[5]. Although we anticipate that other acoustic frequencies will be useful for UNMOD, we recommend initially implementing transducers having a center frequency between 0.2 and 0.7 MHz for intact brain stimulation with US. It is also important to use immersion-type (water-matched) transducers coupled to the skin with US gel in order to minimize acoustic impedance mismatches when transmitting acoustic pressure waveforms from a transducer into the brain.

In addition to acoustic frequency and transducer variables, several waveform characteristics such as mode of transmission (CW versus pulsed wave) and pulse profile (cycles per pulse, c.p.p.; pulse-repetition frequency, PRF; and number of pulses, np) affect the intensity characteristics and outcome of any particular UNMOD stimulus on brain activity. We have previously discovered that stimulus waveforms constructed of US pulses having a low PII (<0.1 mJ cm^{-2}), which are repeated at high PRFs (1.0–3.0 kHz) for short durations (<0.4 s), are effective for stimulating normal brain circuit activity in intact mice[5]. Our previous observations indicate that the optimal US waveforms for stimulating brain activity in mice have temporal average intensity values between 30 and 300 mW cm^{-1} (ref. 14). Using functional MRI and electroencephalography, others have recently reported that similar acoustic

intensities and pulse profiles are effective for modulating the excitability of intact brain circuits in rabbits and rats[9,40].

As we have shown[5], short bursts of pulsed US can stimulate brief (tens of milliseconds) periods of physiologically normal neuronal activity. In addition, US transmitted in short-lasting CWs or through rapidly repeated tone bursts are also capable of modulating the excitability of intact brain activity[9,40]. For example, using the protocol described below, you can observe how US stimuli delivered in CW-mode for 5 s to normal mice can induce seizure activity lasting >20 s and can disrupt chemically induced electrographic seizure activity in epileptic animals as previously reported[6–9]. Collectively, these observations for UNMOD are similar to those made using electrical-, magnetic- and optogenetic-mediated brain stimulation approaches where the influence of stimuli on brain activity patterns depends on stimulus amplitude, duration, frequency, and the baseline state of brain activity when stimulation ensues. The implementation of any particular UNMOD stimulus waveform or transmission mode will largely depend on the outcome sought by the operator. Below, we describe protocols based on our previous work[5] for implementing US in the modulation of brain activity, so that individual investigators may make more informed decisions regarding the use of UNMOD in their specific applications.

US focusing strategies and brain circuit targeting. The skull reflects, diffracts and absorbs acoustic energy fields during transcranial US transmission. The acoustic impedance mismatches between the skin-skull and skull-brain interfaces present additional challenges for transmitting and focusing US through the skull into the intact brain. One of the most important variables for delivering transcranial US to the intact brain is the acoustic frequency. As mentioned above, the optimal gains for transcranial transmission and brain absorption of US occurs at acoustic frequencies <0.70 MHz[52–54]. Although we have primarily implemented mouse models in our studies, we initially developed the basic UNMOD method using the above acoustic frequency ranges, so that frequency-dependent effects may not be such a concern when scaling to larger organisms having thicker craniums than mice[5].

There are several methods for delivering US across the skin and skull in order to conduct UNMOD manipulations. The most easily implemented method described in the protocols below is to use unfocused US for stimulating broad, nonspecific brain regions. When using water-matched transducers, the transmission of US from the transducer into the brain only occurs at points at which acoustic gel physically couples the transducer to the head. On the basis of this acoustic transmission property, coupling the transducer to the head through small gel contact points represents one physical method for transmitting US into restricted brain regions as we have reported[5]. Be cautioned that the entire face of the transducer should always be covered with acoustic gel to prevent heating and damage of the transducer face. The area of gel coupling the transducer to an animal's head, however, can be sculpted to restrict the lateral extent through which US is transmitted into the brain. Although this method does provide an effective approach for stimulating coarsely targeted brain regions as shown[5], calculating acoustic intensities transmitted into the brain with this method can be difficult because of nonlinear variations in the acoustic pressure fields generated. Therefore, we most routinely restrict the lateral extent of the spatial envelope of US transmitted into the brain by using acoustic collimators. The use of acoustic collimators can easily facilitate the targeted stimulation of brain regions with US as previously described[5] and as outlined in the protocol below. Single-element transducers having concave focusing lenses (**Fig. 1c**, fourth transducer from left) can also be used for delivering focused acoustic pressure fields to brains. Such single-element focused transducers can be manufactured having various focal lengths depending on the lens curvature, as well as the physical size and center frequency of the transducer.

The most accurate yet complicated US focusing method involves the use of multiple transducers operating in a phased array. US can be focused through the skulls of mice[55], rats[9,56], rabbits[40,57], monkeys[58] and humans[42] to targeted brain regions using phased arrays. Focusing with phased arrays can be further combined with magnetic resonance (MR) imaging to enhance the spatial precision of US localization in a technique known as MR-guided focused ultrasound (MRgFUS)[58–61]. In MRgFUS procedures, wave equations are applied to predict the scattering, reflection and diffraction of US on the basis of skull bone density and other acoustic impedance mismatch layers. The timing and phase of US emitted from multiple transducers is then modulated to control the location of intersection for US beams in intact brain tissue. With millimeter accuracy, MRgFUS has been shown to be useful for performing noninvasive thalamotomies (d~4 mm) in people suffering from chronic neuropathic pain[42]. Recent evidence indicates that MRgFUS can also be used for targeting and modulating the excitability of intact brain circuits in both rats and rabbits[9,40]. We suspect that globally increased research into the use of US for neuromodulation will begin to reveal even more practical brain circuit–targeting approaches in the near future.

Choice of experimental models for implementing UNMOD protocols. Observed initially in dogs[14] and then in a human[62], the first demonstrations of electrical brain stimulation showed that stimuli delivered to the motor cortex evokes body movements. TMS was also first shown to stimulate intact brain circuit activity by triggering body movements when applied over human motor cortex[63]. Similarly, one of the most common optogenetic probes was first shown to be capable of stimulating intact mammalian brain circuits by evoking locomotive behaviors in rodents, while light was delivered to pyramidal neurons in the motor cortex expressing ChR2 (ref. 64). Following tradition, we first showed that TPU can stimulate intact brain circuit activity and movement behaviors using mouse motor cortex as an experimental platform[5]. We extended these primary observations by showing that TPU can also drive spiking and synchronous oscillations in the intact mouse hippocampus[5]. On the basis of those observations, we believe that the UNMOD procedures provided below will be particularly useful in the study of brain circuit function and plasticity, in which non-invasive brain stimulation methods are desired in order to minimize the influence of confounding variables such as gliosis and inflammation.

In diseased circuits, brain stimulation has been used to study, map and treat epileptic seizure activity since the late nineteenth century[18]. Penfield and Jasper[65] provided the earliest accounts of electrically stimulating the cortex in response to spontaneously occurring epileptiform activity in humans. Since then, a large number of research studies and clinical trials have convincingly shown that various brain stimulation methods are effective for

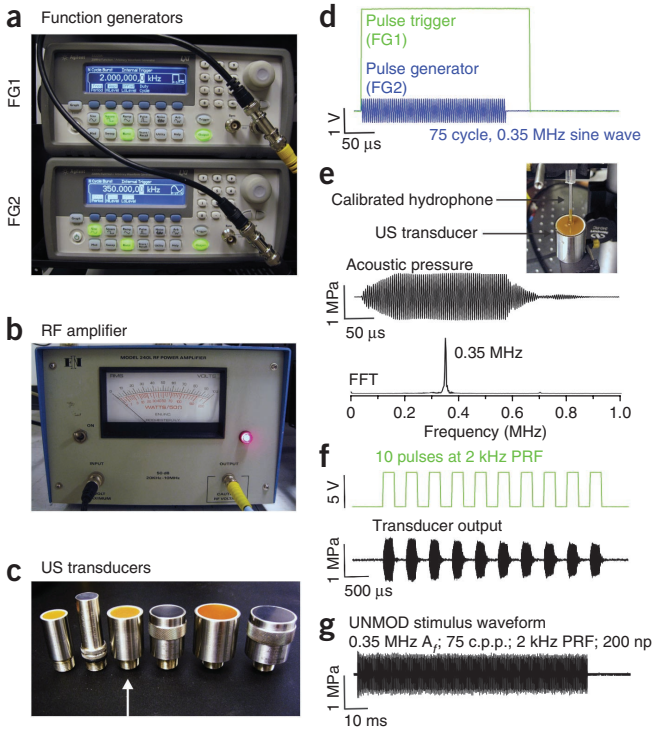

Figure 1 | Basic ultrasonic brain stimulation rig and UNMOD waveform generation. (**a**) Top, function generator 1 (FG1) used to trigger US pulses, establish the pulse-repetition frequency (PRF) and define the number of pulses (np) in an UNMOD stimulus waveform is shown. Bottom, function generator 2 (FG2) used to establish the acoustic frequency (A_f) and the number of cycles per pulse (c.p.p.) in an UNMOD stimulus waveform is shown. (**b**) Shown is an RF amplifier, which receives an input voltage waveform from FG2 to provide the output power to an US transducer for producing the acoustic pressure profile of an UNMOD stimulus waveform. (**c**) Various immersion-type US transducers used for UNMOD are shown. Transducer models shown from left to right are as follows: Ultran GS500-D13, NDT Systems IBMF0.53, Ultran GS350-D19, Olympus Panametrics V318 focused transducer 0.5 MHz/0.75" F = 0.85", Ultran GS200-D25 and Olympus Panametrics V301S 0.5 MHz/1.0". An arrow distinguishes an Ultran GS350-D19 transducer used in the present protocol. (**d**) Illustrated are example voltage traces generated to drive the emission of a single US stimulus pulse. The pulse trigger (green) is a 5-V_{pp} square wave generated by FG1, which triggers FG2. In response to the trigger, FG2 produced a 1-V_{pp} sine wave pulse (blue) consisting of 75 c.p.p. at a frequency of 0.35 MHz (used to establish A_f) as shown. The voltage waveform from FG2 is used to drive the RF amplifier, which provides the final plate voltage delivered to the US transducer. (**e**) Driven by the pulse generator waveform shown in (**d**), a hydrophone voltage trace, which has been converted into acoustic pressure (MPa), is shown. An FFT of the acoustic pressure profile illustrates that the major frequency component of the acoustic pressure waveform is 0.35 MHz. (**f**) Similar to (**d**), except FG1 was set to deliver 10 square wave pulses (np) at a PRF of 2 kHz as shown (green). In response to each one of the square waves produced by FG1, a 75-cycle 0.35 MHz sine wave was produced by FG2 to generate the pressure profile emitted from the US transducer and recorded by the hydrophone (black). (**g**) Example of a typical UNMOD stimulus waveform generated as described above is shown as an acoustic pressure wave with the following properties: A_f = 0.35 MHz, c.p.p. = 75, PRF = 2 kHz, np = 200.

treating medically refractory epilepsy in human patients, as well as in animal models of epilepsy[27,66–71]. More specifically, TMS and tDCS have been shown to be effective for attenuating seizure activity in rodent models of epilepsy[72,73]. Similarly, US has also been shown to be capable of attenuating chemically induced epileptic activity in cats[6] and rats[9]. Thus, we chose to implement a common model of SE in order to highlight a translational application of UNMOD. Below we describe how to implement UNMOD for attenuating kainic acid (KA)-induced seizure activity, a widely implemented model of epilepsy in rodents[74]. The advantage of UNMOD in neurocritical emergencies such as SE is that it can be rapidly applied with little preparation. The protocols described below should further encourage studies exploring the use of UNMOD in neurological disease models in which various brain stimulation strategies have demonstrated therapeutic promise. The non-invasive nature of transcranial US for brain stimulation indeed makes it amenable to a variety of experimental demands in systems and translational neuroscience, and thus there are many options to explore.

MATERIALS

REAGENTS

- C57BL/6 mice (male or female; postnatal day > 21; The Jackson Laboratory) ▲ **CRITICAL** The use of animals for these experiments requires the approval of your Institutional Animal Care and Use Committee or an equivalent regulatory organization. ▲ **CRITICAL** Ensure that juvenile mice (postnatal day 35–50) are used for experiments implementing KA models of SE as mice of different age have different sensitivities to KA.
- Kainic acid monohydrate (Sigma-Aldrich, cat. no. K0250-10MG)
- Aquasonic clear ultrasound gel Parker Labs
- Sodium chloride (Sigma-Aldrich, cat. no. S3014-1kg)
- D(+) Glucose monohydrate (EMD Chemicals, cat. no. 1.08342.1000)
- Diazepam (5 mg ml^{-1}, Hospira, NDC 0409-3213-12) ! **CAUTION** Diazepam is a controlled substance and should be handled properly according to institutional guidelines.
- Ketamine HCl (100 mg ml^{-1}, Bioniche Pharma USA LLC, NDC 67457-034-10) ! **CAUTION** Ketamine is a controlled substance and should be handled properly according to institutional guidelines.
- Xylazine sterile solution (20 mg ml^{-1}, Akorn, NADA no. 139-236)
- PBS (pH 7.4, Sigma-Aldrich, cat. no. P3813-10PAK)
- Sterile sodium chloride solution (10 ml, 0.9%, wt/vol, Sigma-Aldrich, cat. no. S8776)
- Ophthalmic ointment (Pharmaderm, cat. no. NDC 0462-0211-38)

EQUIPMENT

Transcranial UNMOD

- Cunningham mouse stereotax (myNeuroLab, product no. 39462950)
- Depilator lotion (Nair) or small scissors
- Heating pad (Mastek Industries, Model 500/6000)
- Immersion-type ultrasonic transducer (0.35 MHz; Ultran, model no. GS 350-D19)
- Two arbitrary function generators (Agilent Technologies, model no. 33220A)
- RF amplifier (ENI 240L /or/ Electronics & Innovation, model no. 240L)
- Two-channel high-speed oscilloscope (Agilent Technologies, model no. DSO6012A)
- Eight BNC cables (50 Ω)

- Two BNC T-type connectors
- BNC-to-UHF adaptor for transducer
- Positioning arm with magnetic base (Flexbar, model no. 18059)

Electromyography acquisition
- Analog-to-digital converter (ADC) board (DataWave Technologies)
- Differential AC amplifier (A-M Systems, model no. 1700)
- Teflon-coated stainless steel wire (California Fine Wire, cat. no. 316LVK, size 0.0018)

Extracellular physiology acquisition
- Microelectrode AC amplifier (A-M Systems, model no. 1800)
- Tungsten electrodes 0.5 to 1 MΩ (A-M Systems, cat. no. 573220)
- Stereomicroscope (Olympus, model no. SZ61)
- Foredom electric dental drill (Foredom Electric Company, model no. MH-170)
- Drill bits (105 1/32″ engraving cutter, Dremel, model no. 5000105)

Ultrasound waveform intensity measures
- Calibrated hydrophone (Onda, Model HNR 500) ▲ **CRITICAL** When ordering the calibrated hydrophone, ensure that its calibration curve extends into the low MHz range (≥ 0.2 MHz). Calibration of the hydrophone is important as it has different response characteristics across a range of US frequencies. Thus, you will need to know the voltage response for US frequencies used in constructing UNMOD waveforms. The calibrated hydrophone will be provided with a look-up table of voltage responses at different frequencies. On the basis of the data in this table, you will be able to convert hydrophone voltage traces to pressure.

REAGENT SETUP

Preparation of anesthetic cocktail Add 1 ml ketamine HCl stock (100 mg ml^{-1}) and 0.5 ml xylazine stock (20 mg ml^{-1}) to 2.5 ml sterile NaCl solution (0.9%, wt/vol). To anesthetize mice with this ketamine/xylazine cocktail, i.p. inject 3.5 µl per g body weight and wait for 10–15 min before assessing the level of anesthesia. If subsequent injections are needed to induce deeper planes of anesthesia, or to maintain mice under anesthesia for longer periods of time, then supplemental injections can be given at a dose of 2.0 µl per g body weight.

Preparation of KA solution Dissolve 10 mg KA in 5 ml of sterile 0.9% (wt/vol) NaCl solution to prepare a 2 mg ml^{-1} stock solution. Prepare 0.5-ml aliquots in microcentrifuge tubes. Store unused aliquots at −20 °C for up to 1 month. The concentration of KA used to induce seizure activity in mice is between 15 and 20 mg kg^{-1} (7–10 µl per g body weight of the 2 mg ml^{-1} stock KA solution).

PROCEDURE

Setup of the UNMOD rig ● TIMING 1 h

1| Establish which function generator will be used as the pulse trigger (FG1) to establish the US PRF and number of US pulses (np) for a given UNMOD stimulus waveform. Set the other as the pulse generator (FG2) to generate the acoustic frequency (A_f) and the number of acoustic c.p.p. for the individual US pulses that make up an UNMOD stimulus waveform (**Fig. 1a**).

2| By using a BNC cable, connect the output from the front of FG1 to a BNC T-type connector. Using another BNC cable, connect one output from the 'T' to the input of channel (CH) 1 on the oscilloscope. With another BNC cable, connect the 'T' output from FG1 to the external trigger input located on the back of FG2.
▲ **CRITICAL STEP** Electrical impedance matching should be maintained by connecting equipment using 50-Ω BNC cables and connectors. The digital oscilloscope used in this protocol enables voltage traces to be downloaded to a PC for later offline analysis. This is important for measuring US waveform intensities and for capturing FG outputs if desired.

3| By using another BNC cable, connect the output from the front of FG2 to a BNC T-type connector. Use another BNC cable to connect one output from the 'T' to the input of CH2 on the oscilloscope. With another BNC cable, connect the other 'T' output to the input of the RF amplifier (**Fig. 1b**).

4| Use another BNC cable to connect the output of the RF amplifier to the transducer. A BNC to UHF adaptor may be required depending on the connector type.
▲ **CRITICAL STEP** As discussed above in EXPERIMENTAL DESIGN, there are many transducer options that can be used here (**Fig. 1c**). As we provide a general UNMOD protocol below using 0.35-MHz US stimulus waveforms, we describe the use of a 0.35-MHz center frequency Ultran GS-350 D-19 transducer. We have achieved success to varying degrees using several different immersion-type (water-matched) transducers; therefore, it should be recognized that there is flexibility in terms of the transducers used for UNMOD.
▲ **CRITICAL STEP** Extreme care should be taken to avoid overloading transducers with high-amplitude drive voltages (>1 V_{pp}) delivered to the RF amplifier; otherwise, the transducers can be permanently damaged.

Configure function generators for UNMOD waveform construction ● TIMING 15 min

5| Turn on the power for FG1 and FG2.
▲ **CRITICAL STEP** Do not turn on the RF amplifier at this point. If the RF amplifier is on and the FGs were accidently triggered with the wrong settings, then this may cause permanent damage to the connected transducer and/or amplifier. We have blown several costly US transducers and an RF amplifier by accidently tripping the FGs when not intended. Thus, we advise the experimenter to keep the RF amplifier off when not actively transmitting US waveforms.
▲ **CRITICAL STEP** The following steps explain how pulsed waveforms can be constructed with reference to A_f, c.p.p.,PRF and np. The waveform we describe below is illustrated in **Figure 1d–g**. The corresponding parameters for this waveform are A_f = 0.35 MHz, c.p.p. = 75, np = 200 and PRF = 2.0 kHz. These parameters can be varied to develop different pulsed US stimulus waveforms as described in **Box 1**.

BOX 1 | DESIGN OF DIFFERENT UNMOD STIMULUS WAVEFORMS

Many different US waveforms are capable of achieving UNMOD. Typical parameter ranges used for generating pulsed US stimuli are as follows: A_f from 0.25 to 0.50 MHz, c.p.p. from 50 to 490, PRF from 1 to 3 kHz and np from 250 to 1,000. We refer the reader to Tufail *et al.*[5] for specific waveforms serving as starting points in UNMOD stimulus waveform generation. In addition to pulsed US, one may choose to use CW US for stimulation, which tends to produce different outcomes on brain activity compared with pulsed waveforms, as discussed in ANTICIPATED RESULTS. Methods for generating CW US waveforms are described in option A below. Pulsed US is discussed in option B.

(A) Delivering UNMOD to epileptic brain circuits using CW US

The easiest method for generating CW US waveforms is to use the 'Burst' key on FG2 as an on/off switch. Alternatively, FG2 can be set to deliver CW waveforms by changing its trigger function and mode of operation such that it generates continuous voltage waveforms in response to sustained TTL inputs lasting 1 to 10 s. The acoustic frequency range of CW UNMOD waveforms useful for modulating seizure activity is the same as described above for evoking brain activity with pulsed US. After the CW waveforms have been set and confirmed, you may choose to apply UNMOD to normal mice or a KA-injected mouse at any time before or after seizures emerge; this will allow you to examine how US affects epileptic activity/severity. If seizure elimination is desired, we recommend visually observing motor seizures or monitoring EMG activity in order to time the delivery of CW UNMOD waveforms to the intact brain at a point when prominent seizures are present and have lasted for more than a few seconds. In this particular convention, CW US should be applied to the brain in a responsive manner to sustained seizure activity. If applied correctly, you should observe a brief increase in motor and/or EMG activity responding to CW UNMOD, followed by a lasting decrease in seizure activity (**Fig. 4c** and **Supplementary Video 2**). You may repeat as necessary or modify the general approach of using CW US to study differential effects on normal brain activity or KA-induced seizure activity.

▲ **CRITICAL STEP** If you do use CW stimulus waveforms, then do so with great care. Operating transducers in CW for long periods can cause permanent damage to them. We do not recommend using a sine wave with an amplitude >0.5 V_{pp} for more than 10 s to drive transducers. Many transducers are not designed to tolerate CW excitation for long periods of time. We advise you to check with manufacturers regarding further specifications of transducer load capacities.

(B) Delivering UNMOD to epileptic brain circuits using pulsed US

Pulsed US can be used in a manner similar to CW US described in option A above for attenuating seizure activity. The parameter ranges for constructing pulsed UNMOD waveforms designed to disrupt seizures are similar to those described above for conducting normal brain stimulation with pulsed US. The major difference, however, is that to disrupt seizure activity with pulsed UNMOD, we recommend delivering TPU stimuli to the brain once every 0.5 to 2 s. This can most easily be achieved by changing the trigger mode of FG1 such that it can be controlled using an external TTL trigger as described in the option at Step 8 of the PROCEDURE. As with CW US for attenuating seizure activity, pulsed UNMOD stimuli should be applied to the brain in response to sustained periods of seizure activity. You may repeat as necessary or modify the general pulsing strategy/parameters to study differential effects of TPU on KA-induced seizure activity.

6| Set FG1 to generate square wave pulses serving as the PRF (trigger) driving FG2. To do this on the Agilent 33220A, select and press the 'Square' wave panel key. The button will illuminate green when the 'Square' option is active.

7| For pulsed waveforms, set FG1 to deliver a burst of square waves. To do this on the Agilent 33220A, press the 'burst' option. When the burst menu is displayed, enter '200' and then press 'Cyc' on the subparameter menu to accept this value. If the value is accepted, the 'burst' menu with the value '200' should be displayed (this value represents np).

8| Set the desired trigger mode option on FG1. To set the Agilent 33220A to a manual trigger mode, press the 'Trigger Setup' button on the submenu, and then press the 'Source' option, then press 'Manual' and finally press 'DONE'. This configuration allows the user to manually trigger US waveforms by depressing the 'Trigger'.

▲ **CRITICAL STEP** It is often desirable to trigger FG1 such that stimulus waveforms can be delivered at some predetermined rate using an external transistor-transistor logic (TTL). To perform such external triggering using the Agilent 33220A, select 'Ext' as the trigger mode rather than 'Manual' mode as described above. Here, we often use a TTL signal connected to the external trigger input of FG1 in order to deliver constant spaced US stimulus waveforms at some given frequency (0.5 or 0.1 Hz, for example, to deliver a US stimulus waveform every 2 or 10 s, respectively).

9| Set the PRF on FG1. With the Agilent 33220A, press 'Square' on the FG1 control panel. By using the number keypad on FG1, enter '2', and then select 'kHz' for the frequency unit.

10| Set the amplitude of square waves to be generated by FG1. To do this using the Agilent 33220A, press the 'Ampl/HiLevel' option once on the submenu. Enter '5' and select 'V_{pp}' as the unit. This value is the amplitude of the square wave generated, which is used to trigger FG2. At this point, FG1 is now set to drive a 5-V_{pp} pulsed waveform having 200 pulses delivered at a PRF of 2.0 kHz.

11| Set FG2 to generate sine waves at the acoustic frequency of UNMOD waveforms to be used. If using the Agilent 33220A, first push the 'Sine' wave button on FG2 and then press 'Freq' under the 'Freq/Period' submenu. Finally, enter '0.35' and choose 'MHz' as the unit to set the A_f of the US pulses to 0.35 MHz.

12| Set the desired number of acoustic c.p.p. on FG2. Using an Agilent 33220A for FG2, enter '75' as the number of cycles under the 'Cyc' submenu as described in Step 7 for FG1 in establishing np. On FG2, the value will represent the c.p.p. of an individual US pulse.

13| Set the FG2 trigger mode to external. Use an Agilent 33220A for FG2 to choose the 'trigger setup' menu and set the trigger 'source' as 'Ext'. FG2 is now set to produce individual US pulses having 75 acoustic c.p.p. with A_f = 0.35 MHz every time it is triggered by a square wave generated by FG1.

14| Set the amplitude of sine waves to be generated by FG2. With an Agilent 33220A set as FG2, choose the 'Sine' submenu and change the voltage to 1.0 'V_{pp}' in a similar manner for setting the voltage amplitude as explained for FG1 in Step 10 above.
▲ **CRITICAL STEP** It is advisable not to exceed 1.0 V_{pp} on FG2 to generate US pulses, as this is the maximum input voltage rating for the RF amplifier. There is a great risk of damaging the amplifier or transducers if too much power is delivered to them. Depending on the acoustic power desired, we most typically use voltage sine waves between 0.2 and 1.0 V_{pp} produced by FG2 for driving the RF amplifier, which is in turn amplified to provide final plate voltages to the transducer. At this point, ensure that the RF amplifier is powered 'off' before proceeding, as accidently triggering an amplified voltage waveform could produce damage to the transducer before it has been set up properly.

15| Turn 'on' the outputs for FG1 and FG2. On Agilent 33220A function generators, a green button backlight will illuminate when the outputs are active.

16| Turn on the oscilloscope to observe the voltage waveforms generated by FG1 and FG2. Set up the oscilloscope mode using standard practices such that it captures voltage traces for both CH1 and CH2. The oscilloscope should be set to threshold trigger in response to the input from CH1, which corresponds to the output of FG1. When the oscilloscope is ready to acquire on appropriate amplitude and timescales, depressing the 'trigger' on FG1 will enable you to observe two voltage traces on the scope. Variably adjust the amplitude and timescales while scrolling through the voltage traces captured.
As illustrated in **Figure 1d**, you should be able to observe how each 5-V_{pp} square wave generated by FG1 (oscilloscope CH1) triggers a 1-V_{pp} sine wave containing 75 c.p.p. at 0.35 MHz (A_f). The square waves from FG1 triggering sine wave pulses produced by FG2 occur at a frequency of 2 kHz (PRF) until 200 square waves have been produced (np).
▲ **CRITICAL STEP** You may wish to spend some time familiarizing yourself with the function generators and their role in producing UNMOD waveforms before proceeding. We recommend starting from Step 7, replacing individual parameter values and observing differences in the voltage traces produced on the oscilloscope. For example, see if you can construct voltage waveforms to drive an UNMOD waveform having the following characteristics: A_f = 0.5 MHz, c.p.p. = 10, PRF = 1 kHz and 10 np. Although, on the basis of our observations, this particular example is not representative of an UNMOD waveform capable of stimulating neuronal activity, it serves to further familiarize the user with waveform parameters and the construction of UNMOD stimuli. If you have set up the aforementioned waveform correctly, then you should be able to observe voltage traces on the oscilloscope showing 10 square waves (np) on CH1 occurring at 1 kHz (PRF), which will each trigger a 0.5 MHz (A_f) pulse having 10 sine waves (c.p.p.). You may need to make appropriate amplitude and timescale adjustments on the oscilloscope to observe these voltage traces appropriately.
▲ **CRITICAL STEP** Be certain to reconfigure FG1 and FG2 such that you are generating the original waveform described: A_f = 0.35 MHz, c.p.p. = 75, PRF = 2.0 kHz and np = 200. Refer back to Steps 7–14 for additional guidance if needed.
? TROUBLESHOOTING

Monitoring the acoustic pressure variation of US waveforms ● TIMING 30 min
17| To measure US intensity using a scanning hydrophone approach, begin by positioning the transducer in an upright position using a Flexbar and place a liberal amount of US gel over the active surface of the transducer.

18| By using a micromanipulator, carefully affix a calibrated hydrophone such that its face and aperture are positioned parallel to and vertically over the center of the transducer. Slowly lower the hydrophone into the US coupling gel so that its face tip resides approximately 1–2 cm from the face of the transducer (**Fig. 1e**).
▲ **CRITICAL STEP** The face of the hydrophone is a very sensitive surface with a small aperture, which can be easily damaged. Extreme care should be taken to avoid touching or bumping the hydrophone face, as this could affect its response sensitivity and characteristics.

19| Disconnect the input from FG2 going to CH2 on the oscilloscope and plug the BNC terminal of the hydrophone cable into the CH2 input on the oscilloscope.

20| Switch on the RF amplifier and ensure that FG1 and FG2 are configured as described in Steps 7–14. Ensure that the oscilloscope is set to threshold trigger in response to input from CH1 (the output signal from FG1).

21| Push the 'Trigger' button on FG1 to evoke a stimulus waveform. On CH1 of the oscilloscope, you should be able to observe a voltage trace from FG1 corresponding to the PRF (US pulse trigger). On CH2 of the oscilloscope, you will need to increase the amplitude gain to resolve the voltage trace produced by the hydrophone. Once the gain has been appropriately adjusted, you should observe a voltage trace generated by the hydrophone in response to the acoustic pressure wave emitted by the US transducer. Using the appropriate conversion factor listed under the 'Pa/V' (Pascals per volt) column on the look-up table, which accompanied the calibrated hydrophone, you will be able to convert the hydrophone voltage trace waveform into an acoustic pressure waveform measured in Pa (**Fig. 1e**,**f** and **g**).

22| Once you record US pressure profiles in one location over the transducer, use the micromanipulator to begin scanning the hydrophone across different *xyz* locations of the transducer surface while monitoring the variable pressure profiles emitted as a function of space. At this point, you can simply monitor this variation by measuring the peak-to-peak amplitude of the voltage trace on CH2 of the oscilloscope. The major point of this exercise is to recognize that there is variation in the pressure amplitude across the emitted acoustic field. Not only is there natural variation of pressure amplitude within an acoustic field, other influences stemming from the presence of standing waves or reflections spawned by gel-air or water-air interfaces can also lead to constructive and/or deconstructive interference patterns.
▲ **CRITICAL STEP** Because of the nature of pressure variation within an acoustic field, measuring US intensities can be a difficult and complex process. On the basis of the extensive use of US in medicine, there are many established technical standards and guidelines for measuring US intensity[75]. In addition, some consulting information provided by the United States Food and Drug Administration may be useful for understanding some of these procedures, as well as the terms associated with them, such as Information for Manufacturers Seeking Marketing Clearance of Diagnostic Ultrasound Systems and Transducers (http://www.fda.gov/downloads/MedicalDevices/DeviceRegulationandGuidance/GuidanceDocuments/UCM070911.pdf) and 21CFR1050 Performance Standards for Sonic, Infrasonic and Ultrasonic Radiation-emitting Products (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=1050&showFR=1).

Measurement of US waveform intensity characteristics ● **TIMING ~20 min**

23| Return the hydrophone to a location over the *xy* center of the transducer.

24| Trigger a waveform. Download the recorded hydrophone voltage traces data onto a PC or USB drive for later offline analysis while recording the position of the hydrophone in relation to the transducer for each trace.

25| Record several more positions across and away from the transducer face by scanning the hydrophone through several *z* axis points across its *xy* face. Useg this approach to record voltage traces from several locations in the acoustic field where you observe the maximum hydrophone voltage (pressure) amplitudes.

26| To calculate acoustic intensity characteristics, such as pulse intensity integral, spatial-peak pulse-average intensity (I_{SPPA}), the spatial-peak temporal-average intensity (I_{SPTA}) and mechanical index, use the equations in **Box 2** as outlined by technical standards established by the American Institute for Ultrasound in Medicine and the National Electronics Manufacturers Administration[75].

27| To stimulate fully intact brain circuits using UNMOD, follow option A. To carry out *in vivo* extracellular recordings of US-induced cortical activity, follow option B. To apply UNMOD to mice experiencing KA-induced seizures, follow option C.

(A) Ultrasonic stimulation of intact brain circuits ● **TIMING minutes to hours**

(i) Anesthetize a mouse with an i.p. injection (3.5 µl per g of body weight) of ketamine/xylazine cocktail (see REAGENT SETUP). If needed, i.p. administer supplemental doses of the ketamine/xylazine anesthetic cocktail at a dose of 2.0 µl per g of body weight.
▲ **CRITICAL STEP** Use a heating pad to keep mouse body temperature at 35–37 °C.
Ensure that the mouse is at a stable plane of anesthesia by monitoring whisker movement and digit/tail pinch reflex. Lighter planes of anesthesia tend to work best for obtaining motor responses evoked by motor cortex stimulation with US. Thus, it is desirable to obtain a plane of anesthesia at which mild responsiveness to tail/digit pinch is observed.

BOX 2 | ACOUSTIC INTENSITY CALCULATIONS ● TIMING 30 MIN

The equations given below are intended for pressure measurements made at locations in the acoustic field where the peak acoustic pressures were recorded. This is most often in the far field of a transducer emission profile, which is a typically a few centimeters away from the transducer face for the types of transducers that we have recommended using for UNMOD. Where you report the intensity from, however, will be dependent on where you are stimulating in the brain. To estimate the acoustic intensity in the brain, we routinely place hydrophones inside *ex vivo* mouse heads with the brain excised and the cranial cavity filled with acoustic gel, which is a reasonable approximation of brain tissue because of its similar acoustic properties. If measures of UNMOD waveform intensities are not made in the acoustic field where the spatial-peak amplitude occurs, then the calculations described below can still be used for estimating intensity, but they will then represent the pulse-average intensity (I_{PA}) as opposed to spatial-peak, pulse-average intensity (I_{SPPA}) and temporal-average intensity (I_{TA}) as opposed to spatial-peak temporal-average intensity (I_{SPTA}).

The pulse intensity integral (PII) is defined as: $\mathrm{PII} = \int \frac{p^2(t)}{Z_0} dt$ where p is the instantaneous peak pressure, Z_0 is the characteristic acoustic impedance in Pa·s m^{-1} defined as ρc, where ρ is the density of the medium and c is the speed of sound in the medium. We estimate ρ to be 1028 kg m^{-3} and c to be 1515 m s^{-1} for brain tissue on the basis of previous reports[88].

The I_{SPPA} is defined as: $I_{SPPA} = \frac{\mathrm{PII}}{\mathrm{PD}}$, where PD is the pulse duration defined as (t)(0.9PII–0.1PII) 1.25 as outlined by technical standards established by the American Institute for Ultrasound in Medicine and National Electronics Manufacturers Administration[75].

The I_{SPTA} is defined as: $I_{SPTA} = \mathrm{PII(PRF)}$ where PRF is represented in Hertz.
The mechanical index was defined as: $\mathrm{MI} = \frac{p_r}{\sqrt{f}}$ where p_r is the peak rarefactional pressure and f is the acoustic frequency.

(ii) Once the animal reaches the appropriate level of anesthesia, use a pair of small scissors or Nair to remove hair from the scalp. If you are performing electromyography (EMG), carry out the procedure described in **Box 3** before proceeding with Step 27A(iii).

(iii) With the mouse in the stereotax, gently apply a liberal amount of US gel over its scalp, as well as over the face of the transducer (**Fig. 2**).
▲ **CRITICAL STEP** The US gel functions as a coupling medium between the transducer and the mouse head. If air bubbles are present in the gel, it could interfere with the transmission of US. Thus, carefully examine the applied gel to ensure that no air bubbles are present in the gel on the head of the mouse or on the transducer.

(iv) Fix the transducer over the head of the mouse so that there is good coupling between the gel on the transducer face and the gel on the head of the mouse. Start with a working distance of approximately 2–8 mm between the head and the face of the transducer (**Fig. 2q**,**r**).
▲ **CRITICAL STEP** In addition to mounting the entire transducer over the head of the mouse, simple acoustic collimators (**Fig. 2s**,**t** and **Fig. 3**) can be used to direct the US beams into specific brain regions. Acoustic collimators can be easily constructed by filling tubes with US gel and affixing them to the gel on the face of the transducer. One of the simplest embodiments of an acoustic collimator is a 1-ml Luer lock syringe, which has had the Luer lock portion of the syringe cut off and filled with US gel. A collimator can provide lateral restriction of the US beam in order to restrict the area of brain activation based on the requirements or geometrical constraints of an experiment. In addition, collimators can be used for stimulation by delivering US to brain regions in the far field of the transducer output where relatively homogenous spatial-peak intensities are observed.

(v) Once the transducer or acoustic collimator has been coupled to the mouse head, trigger the function generator by pushing the 'Trigger' button on FG1 (see **Box 1** for waveform options and parameters) and monitor for behavioral motor responses, as well as for EMG responses if recording electrophysiological motor activity (see **Box 3**).
▲ **CRITICAL STEP** The level of anesthesia will greatly affect the responses to stimulus waveforms. Lightly anesthetized animals will tend to respond immediately, whereas heavily sedated animals will require more time to reach a lighter plane of anesthesia before responding. Our notes arise from observations during experiments only under conditions using a ketamine-xylazine anesthetic cocktail. If other anesthetics are used, then it is highly suggested that a thorough exploration of stimulus parameters and anesthetic planes be carried out to achieve greater success and reliability. You will need to define the appropriate levels of anesthesia for your particular application. For example, in some applications, we use restraining tubes to facilitate UNMOD in fully conscious animals.
▲ **CRITICAL STEP** We recommend beginning UNMOD protocols with the pulse sequence described in Steps 7–14 above. These can be expanded to explore US waveform parameter space, as many different waveforms are capable of stimulating activity with various degrees of effectiveness (**Box 1**).
? TROUBLESHOOTING

BOX 3 | EMG MONITORING OF US BRAIN STIMULATION OF MOTOR CORTEX ● TIMING 1 H

Although EMG monitoring is not necessary for stimulating brain circuits with transcranial US, we describe these procedures to enable an indirect yet quantifiable measure of activity evoked in the motor cortex. A basic working knowledge of electrophysiology is necessary to carry out these steps.

Recording wire preparation ● TIMING 10 min

1. Cut Teflon-coated stainless steel wire into a length of approximately four inches. Each EMG recording channel requires three leads. Thus, if two EMG recording channels are to be used, then you would need to cut six lengths of wire (**Fig. 2a,b**).
2. Using fine-grit sandpaper (or a razor blade), gently scrape the wire to remove an ~6-mm length of Teflon coating from one end of each wire (**Fig. 2c**).

! CAUTION Handle all sharp objects, including needles and razor blades, with extreme care. You should also practice good laboratory techniques by immediately disposing of used sharp objects in appropriate receptacles.

3. Thread the stripped end of the wire through a 30-gauge hypodermic needle so that the bare wire lead is exposed through the sharp end of the hypodermic needle (**Fig. 2d**). Repeat this step such that one needle will carry two recording wires (the positive and negative recording leads) and a second needle will only carry one wire to be used as the reference lead.
4. Pull the wires through the hypodermic needle while leaving approximately 1–2 mm of bare wire exposed from the bevel end of the needle. Use a razor blade to gently fold the wires over the bevel to create a small barb (**Fig. 2e,f**).
5. Anesthetize and shave the mouse as described in Steps 27A(i) and 27A(ii) of the PROCEDURE.
6. Insert the hypodermic needle containing two wire leads into one of the triceps brachii muscles. After insertion into the muscle, gently retract the needle and slide it off the wires (**Fig. 2g,h**). The small barbs should hold the wires in the muscle. This same procedure can also be performed for tail, hind limb or back muscles if desired (**Fig. 2i**). For the second needle containing a single wire (reference lead), pinch the skin on the dorsal surface of the animal's back or neck and gently pull up while subcutaneously inserting the reference lead so that it positions just under the skin. Remove the hypodermic needle such that the steel wire remains under the skin.
7. Connect the three leads (positive, negative and reference) to a differential amplifier (**Fig. 2j–l**) and place the mouse in the stereotax (**Fig. 2m**).

Electromyography setup ● TIMING 10 min

8. Connect the differential amplifier to an ADC board and computer to amplify, filter and acquire EMG signals. Typical on-board amplifier gain and filter settings used are 1,000× and 10–1,000 Hz, respectively.

▲ CRITICAL STEP Many different ADC data acquisition interfaces and electrophysiology software packages can be used for EMG data acquisition and signal processing[89,90]. A working knowledge of data acquisition techniques used in electrophysiology will be beneficial to your experimental success. Again, you will be able to monitor evoked motor responses in mice being stimulated without using an electrophysiological technique. The purpose for applying such techniques is to provide the experimenter with a simple means of quantitatively assessing neurostimulation produced by US.

(B) *In vivo* monitoring of US-induced cortical activity ● TIMING 2–3 h

(i) Anesthetize the mouse as described in Steps 27A(i) and 27A(ii).

(ii) Turn on the microelectrode amplifier and the computer with software compatible for acquiring extracellular recordings. Sampling rate for these recordings should be 24.4 kHz and notch-filtered at 60 Hz. You may wish to apply additional on- or offline filtering, depending on the signal you are attempting to record. For example, you may wish to apply local field potentials (LFP) or multiunit activity (MUA) as discussed elsewhere[5].

(iii) Affix the mouse head in a stereotactic frame outfitted with mountable *xyz* translators for electrode placement.

▲ CRITICAL STEP Take extra caution when positioning the mouse in the stereotactic frame. Maintaining stability of the mouse head is crucial for these experiments, as the quality of electrophysiological recordings depends on it.

(iv) Apply ophthalmic ointment to the eyes to prevent dehydration.

(v) Use surgical scissors to cut and remove the scalp to expose the skull.

▲ CRITICAL STEP These procedures are not designed for survival or chronic experiments. However, we recommend practicing good aseptic techniques as it will greatly enhance the quality of recordings, especially in longer-term recordings lasting 2 h or more.

(vi) Clean and remove any blood or membranes with PBS and gentle suction.

(vii) Using a mouse brain atlas[76], locate and mark the cortical area of interest.

(viii) Using a dental drill outfitted with a 1/32-inch engraving cutter, gently perform a craniotomy over the area of interest.

▲ CRITICAL STEP Intermittently cool the skull using cold PBS and clean the area of debris. Moreover, do not drill into the dura as this can cause physical damage and bleeding, which may lead to dead tissue and compromised recordings.

(ix) Perform another small craniotomy at another region on the opposite hemisphere. This location will be used for insertion of a reference electrode.

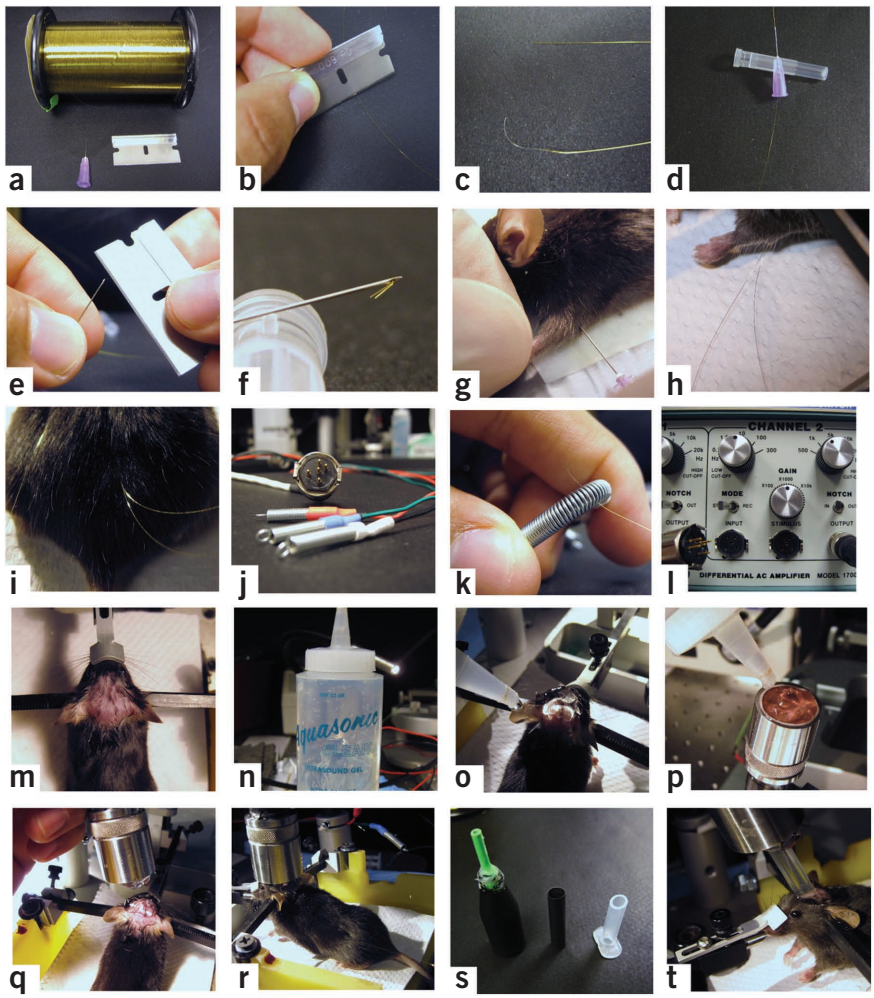

Figure 2 | Preparation of electromyographic recordings to monitor US-evoked stimulation of intact motor cortex. (**a**) Materials used for making fine-wire EMG electrodes; shown are a spool of Teflon-coated stainless steel wire, a 30-gauge hypodermic needle and a razor blade. (**b**,**c**) The razor is used to cut the length of a steel wire and strip the teflon coating off of one end of the wire. (**d**) Hypodermic needle threaded with stainless steel wire by inserting the teflon-stripped side through the beveled end of the hypodermic needle. (**e**,**f**) With the wire minimally exposed from the beveled end, the razor is used to gently fold the bare wire over to create a small barb. (**g**–**i**) Placement of EMG leads into desired arm and/or tail muscles. (**j**–**l**) Positive, negative and reference steel wires are shown being connected to the EMG amplifier leads using small steel springs. (**m**) A mouse placed into a stereotactic device with its hair removed from the scalp is shown. (**n**–**p**) Ultrasound gel is shown being placed on top of the scalp and on the face of the transducer while minimizing the introduction of air bubbles. (**q**,**r**) The transducer is shown being positioned and affixed over the head of the mouse using an adjustable magnetic base Flexbar. (**s**,**t**) Custom-fabricated acoustic collimators are shown (**s**), which are useful for laterally restricting the size of acoustic pressure fields transmitted into the brain (**t**).

(x) Mount the head-stage on the stereotactic manipulator arm. Connect a 0.5- to 1-MΩ tungsten electrode and the reference wire into the head-stage of the amplifier.

(xi) Align the microelectrode over the site of the craniotomy.

(xii) Insert the reference wire into the brain through the second craniotomy site.

(xiii) Slowly lower the electrode down to the surface of the brain using the stereotactic manipulator arm.
▲ **CRITICAL STEP** Ensure that there is enough travel in the *z* axis arm to reach the desired depth. In addition, to obtain the best estimate of electrode depth, observe lowering the electrode with a stereomicroscope so that the starting point on the brain surface (depth = 0 mm) can be easily monitored.

(xiv) Fill an acoustic collimator (**Fig. 2s**) with US gel while using extreme caution to avoid introducing bubbles into the collimating tube.
▲ **CRITICAL STEP** You will want to use an acoustic collimator to transmit US waveforms to restricted brain regions; this is also necessary because of geometrical space constraints imposed by the transducer and recording electrodes. There are many different ways to construct an acoustic collimator. The acoustic collimator is essentially a tube filled with US gel. We have found that one of the easiest ways to construct a collimator is by using a 1-ml syringe that has had the tip cut off (**Fig. 2s,** right). Further, the tip can be cut at an angle (~45°) to permit a flush contact on the mouse skull (**Figs 2t** and **3c**).

(xv) Affix the collimator at an angle such that the US transmission line is targeted to the recording area using a Flexbar (or gooseneck positioning arm) connected on a magnetic base.

(xvi) Set up function generators and RF amplifier as previously described in Steps 7–14.

(xvii) With US gel completely covering the face of the transducer, couple the transducer to the acoustic collimator. Fix the transducer in place with another flexible positioning arm.
▲ **CRITICAL STEP** When using a collimator, do not leave any portion of the US transducer surface uncovered by US gel, as this can cause damage to the transducer face.

(xviii) Begin acquiring data while lowering the electrode to monitor electrophysiological activity. It is recommended to lower the electrode into the brain using an approach angle of ~45°. Such an approach will allow you to target the region of interest

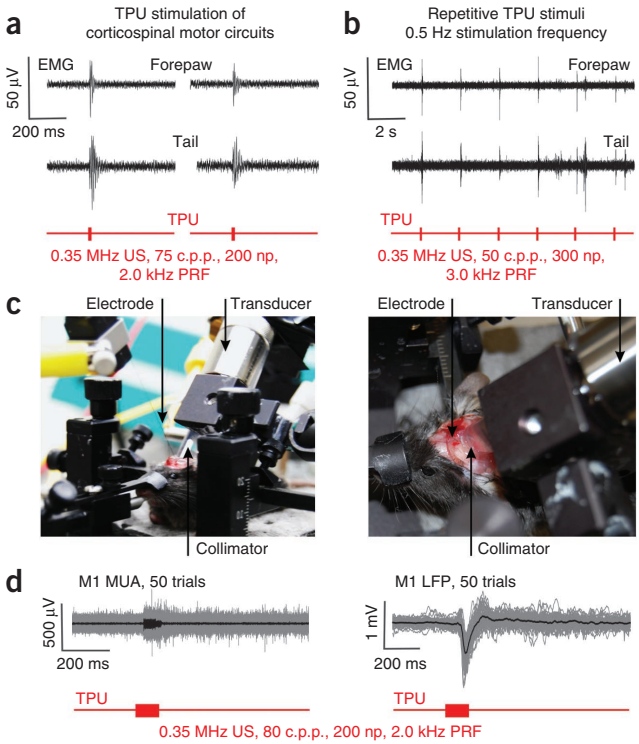

Figure 3 | Electrophysiological recordings in response to brain stimulation with transcranial pulsed ultrasound. (**a**) EMG traces (black) illustrated at a high temporal gain show forepaw (top) and tail (bottom) motor responses produced by two consecutive trials of brain stimulation with the TPU waveform indicated (red). (**b**) Six consecutive EMG responses are illustrated at a lower temporal gain compared with panel **a**, showing the repeatability of brain stimulation evoked with TPU delivered at a 0.5 Hz stimulus frequency. (**c**) Photographs illustrate an extracellular recording configuration used for monitoring *in vivo* neuronal activity in response to TPU stimulus waveforms delivered to the brain. Note that for *in vivo* brain recording experiments, it is highly recommended that an acoustic collimator be used for transmitting TPU through the skull to the extracellular recording site as illustrated. (**d**) Fifty representative individual traces (gray) and average traces (black) of multiunit activity (MUA; left) and local field potentials (LFP; right) recorded in response to brain stimulation with the TPU waveform indicated (red).

by positioning the transducer and collimator at an angle approximately equal and opposite to that of the electrode, as illustrated in **Figure 2t** and **Figure 3c**.

(xix) Once electrode placement in the appropriate brain location has been achieved, begin stimulating with US and record UNMOD-evoked responses (**Fig. 3d**).

▲ **CRITICAL STEP** It is important to mark when UNMOD stimuli are delivered in relation to the electrophysiological data being acquired. This can be easily achieved by sending a TTL trigger out from the acquisition software and ADC board via a BNC to FG1, which should then be set to an external trigger mode as described in the option for Step 8.

? TROUBLESHOOTING

(C) UNMOD for the translational study and treatment of animal models of epilepsy ● TIMING >1 h

(i) Weigh each mouse and calculate the volume of KA solution to be injected in order to achieve a dose of 15–20 per kg body weight. Inject KA systemically (i.p.) every 30 min or until a desired level of seizure activity is observed.

▲ **CRITICAL STEP** Because of the variability in the onset of seizure activity across animals when using KA, mice need to be under constant surveillance after they are first injected.

In conscious animals, we use a modified Racine scale to assess seizure activity and typically perform UNMOD experiments when an animal has reached stage 3 or higher: stage 1 = behavioral arrest with muse/facial movement; stage 2 = head nodding; stage 3 = forelimb clonus; stage 4 = rearing; stage 5 = rearing and falling (**Supplementary Video 1**); and stage 6 = loss of posture and generalized convulsive activity[77].

▲ **CRITICAL STEP** The number of KA injections and the time it takes for a particular animal to reach any given Racine stage are highly variable. This variability will depend on the age and strain of the mice used in your experiments and will need to be adjusted accordingly. Regardless of the strain, we typically use juvenile mice (postnatal day 35–50) for experiments involving the KA induction of seizure activity. You may decide whether or not to anesthetize mice depending on your experimental situation. Anesthetizing mice will facilitate the placement and maintenance of animals in a stereotax (**Fig. 4**). We have found that animals anesthetized with ketamine/xylazine proceed through the development of seizure activity, albeit slower than conscious animals. It should be further noted that the Racine scale assessment of seizure activity is not valid for anesthetized animals. Thus, we often use EMG recordings (**Box 3**) in combination with observations of forepaw activity to gauge seizure severity in anesthetized animals (**Fig. 4b** and **Supplementary Video 2**).

(ii) Once mice reach the desired level of seizure activity following KA injection, apply US coupling gel to the head and begin delivering UNMOD waveforms to the brain using either CW US or pulsed US waveforms (**Box 1**). Either of these options can be used for terminating seizure activity following systemic KA administration and can be applied to anesthetized head-fixed mice or to conscious mice restrained manually for coupling the US transducers to the head (**Supplementary Video 1**).

! CAUTION If you choose to manually restrain mice for applying transcranial US to conscious epileptic mice, then be sure to take proper safety precautions to avoid bite-related injuries when handling laboratory rodents. In addition, it should be noted that the US transducers and brain stimulation parameters used, including the duration of stimulation, will affect the outcome of experiments.

? TROUBLESHOOTING

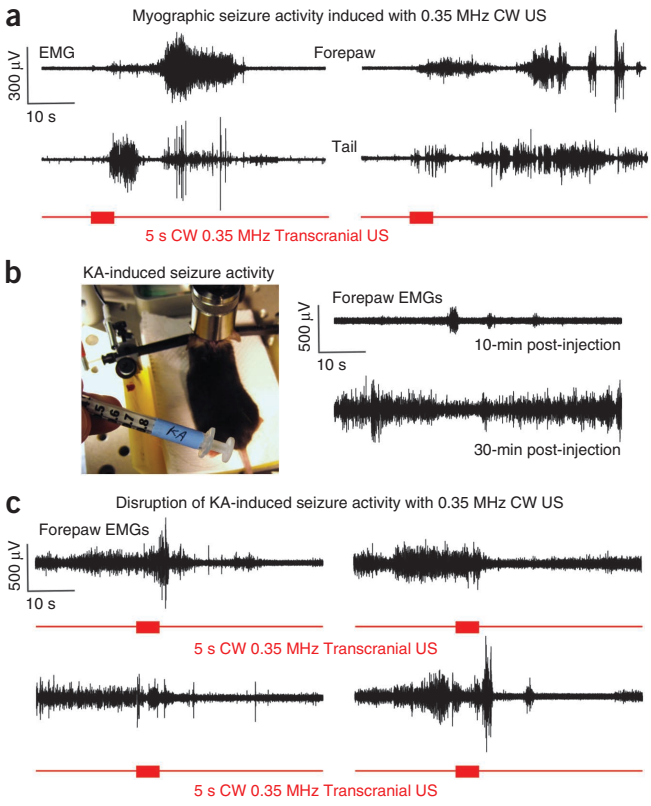

Figure 4 | Induction and disruption of electrographic seizure activity using UNMOD. (**a**) EMG recording traces of forepaw and tail motor responses produced by the transcranial delivery of a 5-s continuous wave (CW) US (0.35 MHz) stimulus waveform to an intact mouse brain. Note that brain circuit activity evoked with CW waveforms lasts tens of seconds compared with those evoked with pulsed US waveforms, which last tens of milliseconds, as illustrated in **Figure 3a,d**. (**b**) Left, photograph showing a mouse immediately after an i.p. injection with kainic acid (KA) to induce seizure activity. Right, example EMG recording traces illustrate typical spontaneous activity patterns as a mouse develops pharmacologically induced seizures. The top EMG trace shows forepaw limb activity 10 min after KA injection, whereas the bottom trace clearly depicts electromyographic seizure activity 30 min following systemic KA administration. (**c**) KA-induced seizure activity can be disrupted using responsive UNMOD as illustrated by the EMG-recording traces from four representative trials of brain stimulation with US in epileptic mice (also see **Supplementary Video 2**). The EMG recordings obtained from a forepaw limb clearly show the attenuation of seizure activity in response to a 5 s CW US stimulus waveform transmitted to the brain. In addition to CW US, seizure activity can also be attenuated using TPU as shown in **Supplementary Video 1**.

(iii) In some investigations, it may be desirable to recover mice following bouts of seizure activity and UNMOD treatment for a variety of experimental design considerations. To eliminate recurrent seizure activity in KA-injected mice, administer an i.p. injection of the $GABA_A$ receptor agonist diazepam (10 mg per kg body weight). If mice do not respond to the initial dose within 10 min, administer a second supplemental i.p. injection of diazepam (5 mg per kg body weight).

▲ **CRITICAL STEP** Recovery of animals should be closely monitored as seizure may re-emerge after the initial injection of diazepam. In addition, mice should be recovered on an isothermal heating pad and hydrated with 0.3-ml s.c. injections of 4% (wt/vol) glucose in 0.18% (wt/vol) saline solution administered every 30 min to 1 h during the recovery period of a few hours.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
16	Voltage traces do not appear on oscilloscope	Faulty connections	Check cables and connections
		FGs are not in the correct trigger mode	Check FG trigger mode status as described in Steps 7 and 12
		Oscilloscope is trigger level not set to an appropriate threshold	Check trigger levels for both channels, depending on which channel is being used as a trigger. It is best to trigger off CH1 on the oscilloscope, which corresponds to the FG1 output. Also check the *x* axis (time) scale on the oscilloscope. If scale is not appropriate the oscilloscope may not trigger. Also check the mode of the oscilloscope. The oscilloscope should be in the 'Run' mode

(continued)

TABLE 2 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
27A(v), 27B(xix), 27C(ii)	Mouse does not show a motor or physiological response to US stimuli	Anesthesia	Wait an additional 15–30 min for the mouse to further metabolize the anesthetic. It can also be useful to trigger US stimulus waveforms using an external trigger (TTL) to drive FG1 at 0.1 Hz during this time. If this is done, be sure to set the trigger mode of FG1 to the external trigger mode. Robust US waveforms can also be delivered periodically (once every 45–60 s) to monitor for responses. This can be achieved by delivering US in a CW mode by touching the 'Burst' button on FG1 and then pushing it again after 2–3 s to terminate the CW waveform. Take care not to drive the transducer in CW mode for more than a few seconds, as it can damage the transducer.
		Poor transducer or collimator placement	Reposition the transducer or collimator angle in relation to the head. You may also change the distance between the transducer and the head
		Faulty connections or no power	Ensure that all cables are properly connected. Be sure power is on for all FGs and RF amplifier. Be sure FGs are in appropriate trigger modes
		Bad transducer	Check US transducer output with hydrophone, according to Steps 16–21 above
		Mouse placement	Allow limbs to hang freely (e.g., raise the mouse by placing gauze squares or other padding underneath its thoracic and abdominal cavities or by incrementally raising its head using the adjustable towers on the stereotactic frame)

● TIMING

Steps 1–4, Connecting basic UNMOD equipment: 30–60 min
Steps 5–16, Configuring UNMOD rig for US waveform generation: 10–15 min
Steps 17–26, Characterizing acoustic pressure fields and measuring US intensity: ~20 min (deeper analyses of US waveforms and intensities may require additional time offline)
Step 27A, Ultrasonic stimulation of intact brain circuits: minutes to hours, depending on the design and purpose of an experiment
Step 27B, *In vivo* monitoring of US-induced cortical activity: 2–3 h for setup and then as long as needed according to the design of the experiment
Step 27C, UNMOD for the translational study and treatment of animal models of epilepsy: >1 h
Box 2, Acoustic intensity calculations: ~30 min
Box 3, EMG monitoring of US brain stimulation of motor cortex: ~1 h

ANTICIPATED RESULTS

This protocol provides the details needed to visually observe, electrophysiologically record and functionally translate US-mediated stimulation of intact mouse brain circuits. We have described how to apply US for brain stimulation using both pulsed and CW stimuli. The specific protocols we provided should enable you to study how different types of UNMOD stimulus waveforms influence brain circuit activity. Further, you should be able to construct and implement a broad set of pulsed US waveforms for brain stimulation by varying key parameters, including A_f, c.p.p., PRF) and np (**Fig. 1d–g**). General guidelines for pulsed UNMOD waveform parameters include A_f from 0.25 to 0.50 MHz, c.p.p. from 50 to 490, PRF from 1 to 3 kHz and np from 250 to 1,000.

When the entire motor cortex is subjected to pulsed UNMOD waveforms, robust motor responses should be observable and/or electrophysiologically recordable using EMG electrodes (**Fig. 3a**,**b** and **Supplementary Video 3**). It should be noted that deeply anesthetized animals are less likely to respond compared with lightly sedated animals as previously noted[5].
The use of acoustic collimators in UNMOD procedures readily supports extracellular recordings of US-evoked brain activity, as geometrical constraints imposed by *in vivo* electrode placement pose fewer problems when transmitting US from remote distances through collimators (**Fig. 3c**). If performed correctly, stimulation of intact brain circuits with pulsed US waveforms will evoke prominent MUA and LFP observable using *in vivo* extracellular recording electrodes (**Fig. 3d**). Stimulation of the motor cortex with TPU can safely and repeatedly evoke MUA, LFP and EMG activity, having response kinetics (**Fig. 3a**,**b**,**d**) similar to those reported using ChR2 and stimulating microelectrodes[34]. Using the protocols described here, we have previously shown that UNMOD is also capable of stimulating intact subcortical circuits, such as the mouse hippocampus[5].

Refer to **Table 2** for troubleshooting advice if UNMOD brain stimulation techniques are not eliciting behaviorally and/or electrophysiologically identifiable responses.

In stark contrast to TPU-evoked brain activity lasting tens of milliseconds (**Fig. 3a**,**b**,**d**), stimulation of motor cortex using CW US waveforms lasting several seconds can induce prolonged seizure activity, which often lasts tens of seconds (**Fig. 4a**). These prolonged and recurrent brain activity patterns generated by CW US can be useful in the experimental generation of acute seizure activity/epilepsy in animals[7,8]. As you can observe upon successful UNMOD implementation, seizure activity produced by CW US does not temporally coincide with the stimulus onset as it does for evoked responses produced by TPU (~20-ms response latency; **Fig. 3a**,**b**,**d**). Rather, seizure activity triggered by CW US emerges with lag lasting a second or more after stimulus onset (**Fig. 4a**) and can last for several tens of seconds. Such stimulation of seizure activity by CW US can be used for mapping brain circuits afflicted by epilepsy or in studies designed to screen how potential pharmacological interventions influence seizure susceptibility as previously indicated[8]. Similar to many other brain stimulation techniques applied to diseased circuits, modulation of neuronal activity with US has also been shown to be capable of terminating chemically induced seizure activity[6,9]. Using the general approaches described here, such applications of UNMOD are useful for disrupting aberrant neuronal activity observed in refractory seizure episodes. If applied correctly after chemically generated seizure emergence, you will be able to observe behavioral and electrophysiological evidence that UNMOD, achieved using either CW or pulsed US waveforms, can attenuate prolonged bouts of epileptic activity (**Fig. 4c**; **Supplementary Videos 1** and **2**).

The general UNMOD approach described here can begin to support studies of normal brain function, as well as provide a platform for the future development of novel therapeutic interventions against pervasive brain diseases and new generations of functional brain-mapping strategies. This protocol provides a necessary starting point for driving such exciting new tools and possibilities to fruition in modern translational neuroscience.

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AUTHOR CONTRIBUTIONS Y.T., A.Y., S.P. and W.J.T. designed and conducted the experimental procedures. Y.T., A.Y., M.M.L. and W.J.T. analyzed and interpreted data from the experiments. Y.T., A.Y., S.P., M.M.L. and W.J.T. wrote the manuscript.

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