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Protocol for the sonogenetic stimulation of mouse brain by non-invasive ultrasound

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HIGHLIGHTS

Protocol for MscL-G22s-mediated sonogenetics

Tips on how to perform sonogenetic stimulation in mice

EMG and immunofluorescent assays for measuring the effects of sonogenetic stimulation

Manipulating specific neural activity by targeted ultrasound intervention is a powerful method to gain causal insight into brain functions and treat brain disorders. The technique of sonogenetics enables controlling of cells that are genetically modulated with ultrasound-sensitive ion channels. Here, we detail the preparations, surgical procedures, ultrasound stimulation process, and simultaneous electromyogram (EMG) measurement necessary for successful sonogenetic stimulation in mice.

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Protocol for the sonogenetic stimulation of mouse brain by non-invasive ultrasound

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SUMMARY

Manipulating specific neural activity by targeted ultrasound intervention is a powerful method to gain causal insight into brain functions and treat brain disorders. The technique of sonogenetics enables controlling of cells that are genetically modulated with ultrasound-sensitive ion channels. Here, we detail the preparations, surgical procedures, ultrasound stimulation process, and simultaneous electromyogram (EMG) measurement necessary for successful sonogenetic stimulation in mice.
For complete details on the use and execution of this protocol, please refer to Qiu et al. (2020).

BEFORE YOU BEGIN

There are many neuromodulation methods currently available, including electrical, magnetic, optogenetics and chemogenetics methods. However, these methods are either invasive or lack sufficient spatial resolution.

The technique of sonogenetics, proposed by Ibsen et al (Ibsen et al., 2015), is relatively less invasive while retaining sufficient spatial resolution as the activation of cells is controlled by ultrasound. Ultrasound is defined as sound waves with frequencies higher than the upper audible limit of human hearing (>20 KHz). The acoustic waves can be utilized to excite or inhibit neural responses. To modulate neural activity selectively and non-invasively, we used mechanosensitive ion channels of large conductance (MscL-G22S) to enhance mechanosensitivity in targeted regions to ultrasound stimulation, whose plasmid was integrated into an AAV9 viral vector with an EYFP tag for fluorescent imaging.

Surgical preparation

⊙ Timing: 1 h

1. Repackage and prepare the virus.
   a. Take a tube of viral vector out from −80°C freezer. Thaw the virus on the ice.
   b. Aliquot 1–2 μL of the virus in each 200 μL autoclaved tube.
   c. Store the virus in a freezer at −80°C.
   d. Dilute the virus with saline. The dilution outlined is based on the titer of virus which is provided by virus manufacturer. The final virus concentration is around 2–3 × 10^{12} viral genomes GC/ml.

    e. Mix the saline and AAV vectors with a pipette thoroughly before use.
2. Preparation of surgical instruments.
    a. Prepare scissors, blunt-end forceps, a needle holder, scalpel and cotton swabs. Additionally, obtain 10%, 70% ethyl alcohol, saline, paraffin oil, absorbable sutures, a heater, eye ointment, drill, a drill bit, disposable syringes, an injection micro syringe, injection glass capillaries, anesthetic (ketamine, xylazine), gloves, and a stereotactic apparatus with injection pump.
3. Setup the stereotaxic apparatus.
    a. Fill the injection glass electrode and 10 μL micro syringe with paraffin oil.
    b. Connect the electrode filled with paraffin oil and the micro syringe filled with paraffin oil with hot melt glue.
    c. Install the micro syringe on the stereotaxic apparatus connector.

> ⚠ CRITICAL: Ensure that the virus is always kept on ice and never warmed to 22°C–24°C during the preparation process. Ensure that no air bubbles are present inside the electrode and micro syringe when filling with paraffin oil. Air bubbles can be avoided by keeping the tip of the electrode under the oil surface.

Viral injection

    ⏱ Timing: 3 h

4. Setup mouse for surgery.
    a. Anesthetize the 6–8 weeks old C57BL/6 mice of both sexes with 100 mg/kg ketamine and 10 mg/kg xylazine in saline via an intraperitoneal (IP) injection with a disposable syringe.
    b. Put the mouse back into its cage until fully anesthetized, characterized by absent tail and foot pinch reflexes.

> *Note:* After 5 min, if the mouse still responds to the tail or foot pinch, then administer more anesthetic agent.

    c. Place ointment on the eyes of the mouse to prevent drying.
    d. Shave the fur above cortical region of interest and the surrounding area using scissors or a razor.

> *Note:* In the referenced paper (Qiu et al., 2020), we shaved the area corresponding to the right M1 for some mice and the dorsomedial striatum (DMS) area for others.

    e. Install the mouse into the stereotaxic apparatus. Lock the front teeth on the lower front clamp, then lower the upper front clamp and tighten it so that the jaws are completely fixed.

> ⚠ CRITICAL: Make sure the mouse's respiratory airways are clear – pull the tongue slightly to the side if the mouse cannot breathe.

    f. Insert the ear bar into the ear canal to completely stabilize the head.

> ⚠ CRITICAL: Be careful not to insert the bar too far, otherwise the inner ear may be damaged. When finished, the mouse's body should be able to move slightly without disrupting the position of the head.

> *Note:* Place a heater near the mouse in order to modulate body temperature throughout the whole surgical procedure.

    g. Rub 10% povidone iodine on the surgical region, moving outward from the middle of the surgical site. Then, disinfect the surgical site with 70% ethyl alcohol in the same fashion.

5. Creating an incision.

a. Take the scalpel and blunt forceps. Use the blunt forceps to gently hold the skin of the mouse and make an incision with the scalpel. Start the incision at the midpoint between two ears and extend the incision to about 0.8 cm vertically down the middle of the mouse's head to expose the bregma.
b. Use a cotton tip to gently remove any blood covering the surface of the skull.
c. Clean the surface of the skull with 0.9% saline and dry with a cotton tip. The bregma and lambda will then be exposed.

6. Viral injection.
a. Infuse 5 μL of paraffin oil from the electrode.
b. Withdraw 2.5 μL of virus vector at the appropriate speed. Make sure the tip of the electrode is below the surface of the virus solution to prevent air bubbles.

   Note: Mark the boundary between the oil and virus with a pen. This is to ensure the virus level decreases during injection correctly.
c. Slowly lower the syringe until it is just above the surface of the skull so that the tip of the syringe needle is at the bregma. Set this point to zero as reference for future coordinates.
d. Move the needle of the syringe to the coordinates of interest. Lower the needle until it is right above the skull – this is where the hole needs to be drilled.

   Note: The coordinates used for the cortical region were: AP 0.25 mm, ML-1.50 mm, DV 1.00 mm. The coordinates used for DMS injection were: AP 0.50 mm, ML -1.50 mm, DV -3.00 mm.
e. Lift the glass electrode slightly, take the drill and place it at a 45-degree angle directly above the target drill position. Start drilling and continue until the skull gives way (detected by a drop in resistance).

   Note: A 0.5 mm hole should be drilled in the targeted site. To prevent heat generation during the drilling process, add 22°C–24°C saline appropriately on the drill site every 3–4 s and use cotton to suck the liquid and continue to drill until the skull gives way.

   ⚠ CRITICAL: Be careful not to drill too quickly, or it may enter the brain and cause permanent cortical damage.
f. Remove any blood from the hole with a cotton tip.
g. Low the syringe onto the surface of the brain. Find the targeted site with the syringe based on coordinates.

   Note: Recalibrate the glass electrode after drilling. According to the brain atlas coordinates, lower the syringe to the desired depth. Cortical regions are typically at DV -1.00 mm, while DMS regions are at DV -3.00 mm.
h. Mice were assigned to one of five conditions. They were either given 1 μL of rAAV/9-hSyn-MscL-G22S-EYFP-pA, 1 μL of rAAV/9-hSyn-EYFP-pA virus, 1 μL of pAOV/CaMKIIa-MscL-G22S-EYFP-3FLAG, 1 μL of pAOV/CaMKIIa-EYFP-3FLAG viral particles (2–3 × 10^{12} GC/ml) or 1 μL of saline (for sham experiments) at 0.1 μL/min.

   Note: During the infusion period, apply one or two drops of saline on the injection site to keep the brain moist.
i. After the injection is finished, wait about 10 min to ensure that any residual virus has been absorbed.
j. Slowly raise the syringe and use a cotton tip to remove the blood and liquid from the injection area.

7. Finishing the procedure.

a. Take out the sterile suture and needle from the package and clamp the needle with a needle holder. Gently push the suture into the skin and use blunt forceps to grasp the skin on the other side of the incision. Pull the suture material out until approximately 0.75 inches remains and make a knot. Repeat until 3 knots are made, then cut off any excess suture.

b. Repeat the previous step 3 or 4 times, or until the incision has been completely closed with an interrupted knot.

c. Gently remove the mouse from the surgical table and keep the mouse on a heating pad until it wakes up. Put the mouse back in the cage when it wakes up for recovery.

d. The virus will express for around 4 weeks, so conduct any experiments before then.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
c-Fos (9F6) monoclonal primary antibody	Cell Signaling Technology	Cat. # 2250; RRID: AB_2247211
Goat anti-rabbit IgG (H+L), Alexa Fluor 555	Invitrogen	Cat. # A-21428; RRID: AB_2535849
Bacterial and virus strains		
rAAV/9-hSyn:EYFP-WPRE-pA	BrainVTA (Wuhan) Co. Ltd	Cat. # PT-0102
rAAV/9-hSyn:MscL-G22S-F2A-EYFP-WPRE-pA	BrainVTA (Wuhan) Co. Ltd	Cat. # PT-0280
pAOV/CaMKIIa-EYFP-3FLAG	OBiO Technology, Shanghai	Cat. # AOV016
pAOV/CaMKIIa-MscL-G22S-EYFP-3FLAG	OBiO Technology, Shanghai	Cat. # H15130
Experimental models: organisms/strains		
Mouse: C57BL/6J	The Jackson Laboratory	JAX: 000664
Chemicals, peptides, and recombinant proteins		
Paraformaldehyde, 4%	Solarbio	Cat. # P-1110
Saline	Kelun Pharmaceutical	N/A
Paraffin oil	Sangon Biotechnogy	Cat. # A630217-0100
Ketamine (10%)	Alfasan International B.V., Holland	Cat. # HK-37715
Xylazine (2%)	Alfasan International B.V., Holland	Cat. # HK-56179
Normal goat serum	Abcam	Cat. # Ab7481
BSA	Sigma	Cat. # A3059
Triton	Sigma	Cat. # T8787
PBS	Gibco	Cat. # 70011-044
Software and algorithms		
ImageJ	National Institutes of Health	https://imagej.nih.gov/ij
GraphPad Prism	GraphPad Software, CA, USA	https://www.graphpad.com
EMG software	Bio-Signal Technologies	Cat. # Medusa 1.06.01
MATLAB	The MathWorks, Inc., MA, USA	https://www.mathworks.com/products/matlab.html
Other		
Prolong Diamond Antifade Mountant with DAPI	Invitrogen	Cat. # P36971
Scissors	RWD	Cat. # S12005-10
Blunt-end forceps	RWD	Cat. # F21001-12
Scalpel	RWD	Cat. # S32003-12
Povidone iodine	Allbond	Cat. # 20172640200
Absorbable sutures	Hangzhou Huawei Medical Instruments Co., Ltd	Cat. # 20143021851
Heater	PHILIPS	Cat. # PR3120
Eye ointment	Forgood	Cat. # H19983011
Drill	RWD	Cat. # 78001

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
A drill bit	Meisinger	Lot: B28624
Injection glass capillaries	World Precision Instruments	Cat. # 1B150F-4
Injection micro syringe	Trajan Scientific and Medical	Cat. # P/N 002000
Stereotactic apparatus	RWD	Cat. # G1020604-001
Injection pump	KD Scientific	Cat. # 78-8130
500 kHz transducer	Olympus	Cat. # I7-0012-P-SU
Function generator	Tektronix	Cat. # AFG251
Power amplifier	Electronics & Innovation Ltd	Cat. # A075
Ultrasound gel	Aquasonic Clear	Cat. # BT-025-0039N
Vibratome	Leica	Cat. # VT1200S
Confocal microscope	Leica	Cat. # TCS SP8
Recording electrodes	Bio-Signal Technologies	N/A
Ground wire	Bio-Signal Technologies	N/A
Multichannel signal acquisition system	Bio-Signal Technologies	# Medusa
Heating pad	N/A	N/A
Isoflurane	JD Medical	# Attane™
Isoflurane system	Smiths Medical	# Classic T3™

## STEP-BY-STEP METHOD DETAILS
Ultrasound stimulation and brain slice collection

🕓 Timing: 3 days

This step outlines how to determine the effectiveness of ultrasound stimulation in the targeted region through brain slice staining. If the experiment is successful, significantly more neurons should be activated and fluorescently labeled in the ultrasound stimulation group compared to the control group in the region of interest.

⚠ CRITICAL: The procedure should be done in a quiet environment to prevent excessive auditory cortex activation.

1. Setup the ultrasound system.
   a. Make a water tube. Prepare a 50 mL centrifuge tube, shorten the sharp side of the tube until the diameter of the aperture is 6 mm. Shorten the tube until it is 4 cm long (Figure 1A).
   b. Seal the aperture with plastic wrap (Sinya), then fix the plastic wrap with sealing film.
   c. Fill the prepared tube with water that has been standing more than 12 h, to reduce the presence of air bubbles. Pour with care to minimize formation of new air bubbles.
   d. Affix the water tube to the 500 kHz transducer (I7-0012-P-SU, Olympus) slowly, prevent formation of air gap or bubbles between the transducer and water (one may fully fill the tube in step c to get this done more easily). Use tissue to dry any overflowing water.
   e. Use sealing film to fix the transducer to the water tube.
   f. Screw the integrated ultrasound transducer onto a stand.
   g. Connect the output of the function generator (AFG251, Tektronix) to the input of the power amplifier (A075, Electronics & Innovation Ltd) with a BNC wiring, then connect the output of the amplifier to the transducer (Figure 1B).
2. Anesthetize the mouse with ketamine and xylazine (100 mg/kg and 10 mg/kg respectively) via IP injection with a disposable syringe and place it back to its original cage.
3. Judge the mouse's level of consciousness by tail and foot pinch reflexes. After confirming that the mouse is deeply anesthetized, place ointment on the mouse's eyes to prevent drying.
4. Shave the fur around the skull and sterilize the shaved regions with 70% alcohol.
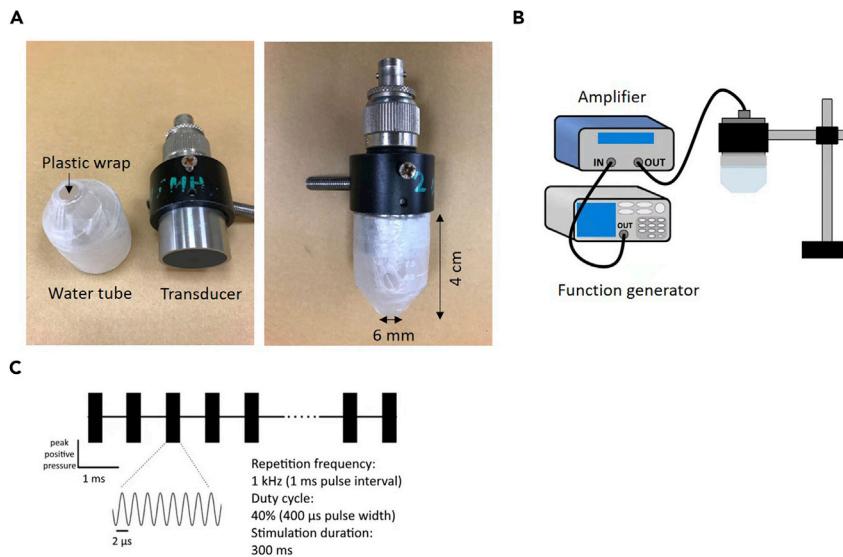
Figure 1. .Technical details of the ultrasound system
(A) Diagram of the ultrasound transducer with a transducer cone as wave guide.
(B) A schematic of ultrasound system installment.
(C) An illustration of the ultrasound temporal profile used in our experiments with a typical intensity of 0.3 MPa.
Figure 1C reprinted with permission from Qiu et al. (2020).

5. Pad the mouse's lower jaw with a few pieces of paper towel to ensure that the mouse's head is in a flat position (Figure 2B).
6. Use 70% alcohol to sterilize the shaved region again.
7. Centrifuge the ultrasound gel at 1000 g for 10 min before doing the experiment. Put some ultrasound gel on the surface of the shaved region. Make sure air bubbles are not present in the gel.
8. Place the ultrasound transducer on the surface of the ultrasound gel atop the targeted regions. The distance between the water tube and the surface of the mouse brain is around 2 mm. Try to position the middle of transducer on the injection site.
9. Stimulate the mouse with ultrasound (500 kHz ultrasound, 400 μs pulse width, 1 ms pulse interval, 300 ms stimulation duration and 0.3 MPa) for 40 min with a 10 s inter stimulus interval (Figure 1C).
10. After stimulation, return the mouse to its original cage.
11. 90 min after ultrasound treatment, perfuse the mouse with PBS, followed by 4% paraformaldehyde (PFA) in PBS. A full perfusion protocol is described by Gage et al (Gage et al., 2012).
12. Keep the isolated brain in 4% PFA about 12–16 h at 4°C. After that, wash the brain with 1×PBS 3 times, 15 min each time.
13. Vibratome slice the brain, obtaining individual sections that are spaced 40 μm apart.

    Note: Fill the vibratome with PBS and attach perfused brain to the pad with superglue. After each slice, extract the slice and place it in an individual well filled with PBS to maintain its structural integrity.

    Note: Coronal sections were prepared from brain regions spanning +0.1 mm to −0.8 mm of Bregma in mouse brains for cortical sections and spanning +0.20 mm to 1.0 mm of Bregma for DMS sections.

14. c-Fos staining.
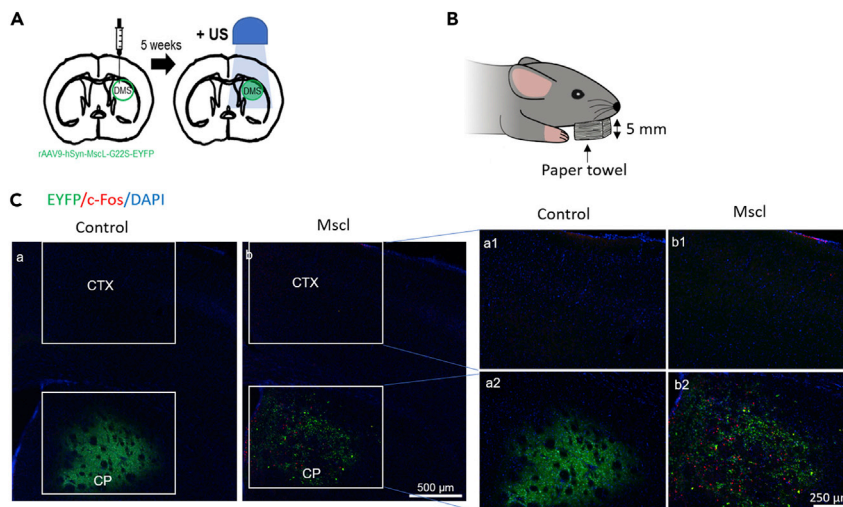
Figure 2. .c-Fos staining in brain slices

(A) Schematic illustration of our in vivo neuron sensitization and ultrasound stimulation plan.

(B) A diagram of the tissue platform.

(C) Representative images of the right DMS and the regions of cerebral cortex directly above it, expressing DAPI, EYFP and c-Fos. The scale bars in the b panel and b2 panel represent 500 μm and 250 μm, respectively. Figure 2A reprinted with permission from Qiu et al. (2020).

a. In order to prevent nonspecific antibody binding, slices are blocked in the blocking buffer (10% normal goat serum + 5% BSA + 0.3% Triton in 1×PBS) for 2 h at 22°C–24°C.

b. The primary antibody (1: 500; Cell Signaling Technology, 2250) is diluted with blocking buffer. Slices are incubated with primary antibody for 16–20 h at 4°C.

c. Wash the slices with 1× PBS 3 times, 5 min each time.

d. Incubate with secondary antibody, goat anti-rabbit IgG (H+L), Alexa Fluor 555 (A-21428, Invitrogen, dilution 1:1,000) diluted in blocking buffer for 2 h at 22°C–24°C.

e. Wash slices for 5 min 3 times.

f. Remove the brain slice from storage and place on the glass slide with a brush or other thin utensil. Multiple slices can be arranged on a single slide.

g. Let the slide dry for 5 min. Administer small drops of Prolong Diamond Antifade Mountant with DAPI, then place cover slide on top of the glass slide.

h. Image the brain slices (Figure 2) and count c-Fos signals.

Note: All brain slices were imaged using a confocal microscope (TCS SP8, Leica) in the University Research Facility in Life Sciences (ULS) facilities in The Hong Kong Polytechnic University. The number of cells showing c-Fos signals (red) and DAPI (blue) were counted using ImageJ, and the number of c-Fos$^+$ cells per 733 × 733 μm slice were calculated. The counting of c-Fos$^+$ cells was single-blinded, performed by an experimenter who did not know the experimental groups beforehand.

Electromyogram (EMG) recording

⊙ Timing: 3 h

The purpose of this step is to test whether ultrasound stimulation can induce motoric responses. If the region of interest is in a motor-related area, spikes in EMG signals should be observed simultaneously with ultrasound stimulation at a higher amplitude than control. If the region is not motor-related, ultrasound stimulation should have no correlation with EMG signal spikes.

15. The EMG system used consists of two recording electrodes, 1 ground wire and a multi-channel signal acquisition system (Medusa, Bio-Signal Technologies). The surgical instruments and reagents necessary are an isoflurane system, a heating pad, tissue, scissors, scalpel, cotton tip, 70% alcohol and saline.

   Note: The mice models used in our protocol were either injected with CaMKII-EYFP or Cam-KII-MscL-G22S viruses in their M1 regions 4 weeks prior. See "before you begin" for the viral injection procedure.

16. Setup the EMG and ultrasound system. Connect the ultrasound system to the EMG system in order to synchronize the ultrasonic signals to the EMG recording software.
17. Hold the mice in the procedure room for 30 min before beginning the experiment.
18. Anesthetize the mouse with 2% isoflurane for 5 min in the isoflurane box.
19. Put the mouse flat on the operation table. Spread all four limbs so that they are easily accessible. Connect the tube that outputs isoflurane and oxygen to the mouse's nose. Keep the concentration of isoflurane as above. Add a 37°C heating pad under the mouse's body during the whole experiment if necessary.

   Note: Consistently check on the mouse's condition by observing its heartbeat. If its vitals are weak, decrease the isoflurane concentration.

20. Place ointment on the mouse's eyes to prevent drying.
21. Shave the fur on the mouse's head and around the left forelimb near the triceps muscle with a scissor or razor carefully.
22. Disinfect the surgical region with 70% alcohol, moving outwards from the middle of the surgical site.
23. Take the scalpel and blunt forceps. Use the blunt forceps to gently hold the exposed skin on the left forelimb and make an incision with the scalpel. The incision should be vertical and about 0.5 cm in length to expose the triceps. Separate two sides of skin with cotton tip slightly to expose the triceps. Clean the blood with a cotton tip.
24. Insert two recording electrodes into the triceps 2 mm deep and leave 2–3 mm between the electrodes. Fix the electrodes with a sticker. Connect a ground electrode to the tail.
25. Apply some ultrasound gel on the top of the mouse's head. Make sure no air bubbles are present in the gel.
26. Place the integrated ultrasound transducer mentioned above on the mouse's head, with the middle of transducer on the injection site.
27. Setup the ultrasound system with the following parameters: 500 kHz frequency, 400 μs pulse width, 1 ms pulse interval, 300 ms stimulation duration, 5 s interval. The independent variable is peak positive pressure 0.05–0.5 MPa.
28. After setup, reduce the concentration of isoflurane to 0.5%–0.8%.
29. Allow the mice to rest for 5 min to adapt to the new level of isoflurane.
30. Open the EMG software. Press the input button and check that the EMG signal is present.
31. Press the record button and name the file. Record 1 min of the EMG signal without ultrasound stimulation.
32. Adjust the amplitude of ultrasound to 0.05 MPa. Press the start button in the ultrasound machine. Treat the mouse with 0.05 MPa ultrasound stimulation for 7–10 times.
33. Press the stop button after ultrasound stimulation.
34. Rest for around 60 s.
35. Repeat steps 33–34 for the other acoustic pressure.
36. Press the stop button in the EMG recording software after all acoustic pressure are tested.
37. Adjust the concentration of isoflurane to 2% for 2 min to reduce pain during electrode withdrawal. Remove the recording electrodes from the triceps with tweezers and remove the ground wire from the mouse's tail.
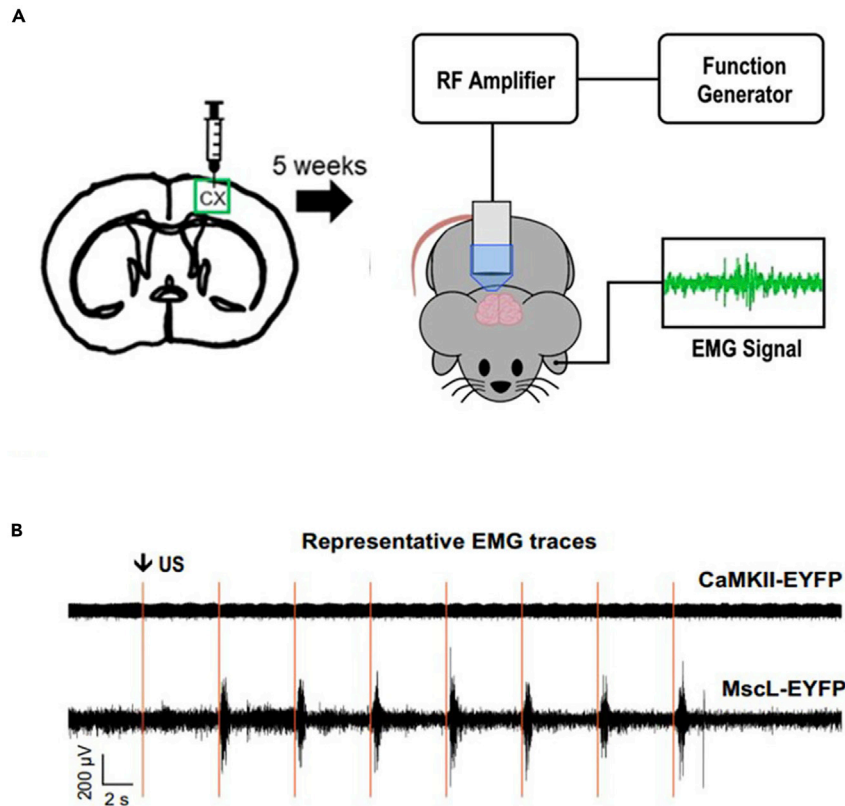
Figure 3. .Work flow of EMG experiment with ultrasound stimulation in mice
(A) A diagram shows the steps of experimental procedures.
(B) EMG traces in muscle activity generated by 0.05 MPa of low-intensity ultrasound stimulation in the control group and mscl group. The orange lines represent events of sync pulses ultrasound stimulation. The black waveforms show the EMG signals corresponding to ultrasound stimulation. Figure 3 reprinted with permission from Qiu et al. (2020).

38. Use an alcohol-soaked cotton tip to disinfect the triceps. Prepare the sterile suture and needle and clamp the needle with a needle holder. Gently push the suture into the skin and use blunt forceps to grasp the skin on the other side of the incision. Pull the suture material out until approximately 0.75 inches remain and make a knot.
39. Repeat the suture step (step 38) 3—5 times or until the incision is fully closed. Use an interrupted knot.
40. Continue to warm the mouse with the heater until the mouse wakes up. Place the mouse into the original cage.

EXPECTED OUTCOMES

This protocol will help to establish the Mscl-G22S expressed and control mice models. Neurons in targeted regions of mice that express Mscl-G22S can be activated with low intensity ultrasound stimulation with evidence from c-fos staining. The application of ultrasound stimulation to mice brain could evoke some background neuronal activity, but the Mscl-G22S expression enhanced such effect significantly. The stimulation of Mscl-G22S expressing mice at low intensities could induce a clear muscular response showing by synchronous EMG signal, with smaller or no response in the control mice (Figure 3B). Ultrasound stimulation could evoke significantly greater responses when stimulating cortical regions expressing MscL-G22S.

QUANTIFICATION AND STATISTICAL ANALYSIS
EMG data processing

1. Pass the raw EMG data through a 50 Hz notch (frequency of AC power supply) and a 10–150 Hz band-pass filter.
2. Select a 'quiet period' (200 ms at the start of the recording). Calculate the mean and rectify the filtered data about it.
3. Smooth the rectified data (by taking the upper root-mean-square envelope with a 200 ms sliding window).
4. Calculate the mean of the rectified data within the 'quiet period' in step 2, which would be treated as signal baseline.
5. Set a threshold with reference to the signal baseline in step 4 (1.2 times the baseline).
6. Set a minimum duration of 'spike' (40 ms), that any segment of the smoothed data in step 3 rising above the threshold in step 5 would be treated as a 'spike'.
7. Set a post-stimulation window (2.5 s), that if the first 'spike' within this window meets any of the two criteria below, would be treated as a successful ultrasound-induced EMG event.
   a. Having a full-width-at-half-maximum of at least 100 ms.
   b. Having a width above threshold of at least 100 ms.
8. Calculate the success rate, relative amplitude and response latency as listed below to evaluate the data.
   a. Success rate: number of successful ultrasound-induced EMG events, divided by ultrasound stimulation count.
   b. Relative amplitude: the difference between the peak value of the identified EMG event and the signal baseline, divided by the same baseline ($\Delta A/A_{base}$).
   c. Response latency: the time between start of each ultrasound stimulus and the time-point at which the corresponding EMG signal rose above the threshold.
9. To statistical compare data between groups, a two-tailed unpaired t test with Holm-Sidak correction can be used. Note that success rate is a binomial dataset (either 'yes' or 'no') that standard error could be estimated by binomial proportion confidence interval using the normal approximation interval at the 95% confidence level.

All statistical tests in this study were performed using GraphPad Prism 8 for Windows. Details of the statistical analyses were conducted as previously described (Qiu et al., 2020). A p value of < 0.05 or below was considered statistically significant for all experiments.

LIMITATIONS

Due to the different weights of the mice being anesthetized and the constant level of isoflurane administered, there may be inconsistencies in level of anesthesia between mice which could lead to some level of variations in recorded EMG signal. Furthermore, the entire EMG process is conducted when the mouse is anesthetized. This could reduce twitch responses to the ultrasound stimulation. Moreover, limitations of this method lie in reduced temporal resolution in comparison to traditional optogenetic stimulation.

TROUBLESHOOTING
Problem 1
Expression of the mscl-G22S virus is suboptimal in the mouse brain, leading to reduced c-Fos expression and twitch response (step viral injection, 7d).

Potential solution
Increase the dose of virus injection and extend the expression period to over 4 weeks.

Problem 2

There might be some trapped air bubbles or gap between the ultrasound transducer and the skull which may fail to deliver ultrasound energy to the brain (step ultrasound stimulation and brain slice collection, 1d, 7 and 8).

Potential solution

Always pay attention to the transducer coupling. Check whether there are any bubbles in the coupling media.

Problem 3

The triceps can be damaged easily if the process of exposed muscles is mis-operated, which can affect the recording of electrical activity during muscle contraction (step electromyogram recording, 23).

Potential solution

Gently lift the skin of the mouse's arm with a tweezer and make a small incision in the skin with scissors very carefully prevent scissors from damage muscles and causing bleeding. The damage triceps might fail to be activated by ultrasound stimulation.

Problem 4

The EMG recording experiment is sensitive to anesthesia level. Deep anesthesia can abolish the responses. (step electromyogram recording, 28).

Potential solution

Control the isoflurane concentration between 0.5%–0.8%.

Problem 5

The transducer output could be fluctuated which will result in unexpected results (e.g., If the installation of ultrasound instruments is incorrect, it would result in no ultrasonic signal output) (step ultrasound stimulation and brain slice collection, 1 g).

Potential solution

Before ultrasound treatment, make sure the electrical connection is correct.

RESOURCE AVAILABILITY

Lead contact

Further information and request for reagents and resources should be addressed to, and will be fulfilled by, the lead contact, Lei Sun (lei.sun@polyu.edu.hk).

Materials availability

This study did not generate any new reagents.

Data and code availability

This study did not generate datasets.

AUTHOR CONTRIBUTIONS

Experimental design and conception by Z.H., J.G., S.K., Q.X., and L.S.; investigation by Q.X., S.K., J.G., Z.Q., J.Z., X.H., T.Z., and K.F.W.; manuscript preparation by Q.X., S.K., S.G., and L.S. with input from all other authors. L.S. supervised the research and acquired funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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