Supporting information

Sonogenetic modulation of cellular activities using an engineered auditory-sensing protein

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The file includes:

Other Supporting Material for this manuscript includes the following:

Movie S1. mPrestin(N7T, N308S) enables ultrasound-evoked calcium response.

Movie S2. mPrestin(N7T, N308S)-positive puncta oscillated upon FUS stimulation.

Supplementary figures

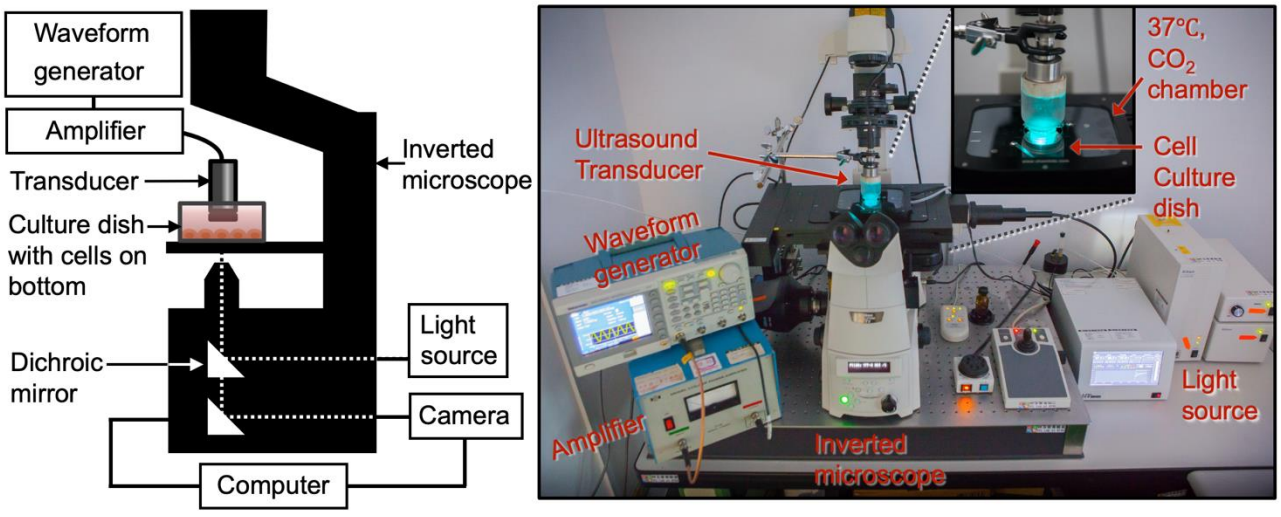

Figure S1. Our computer-controlled live-cell imaging and ultrasound-exposure system. An ultrasound transducer connected to an amplifier and waveform generator was placed in the medium of a culture dish containing a monolayer of cells for FUS excitation. The behavior of cells upon FUS stimulation in real time was observed through an inverted microscope.

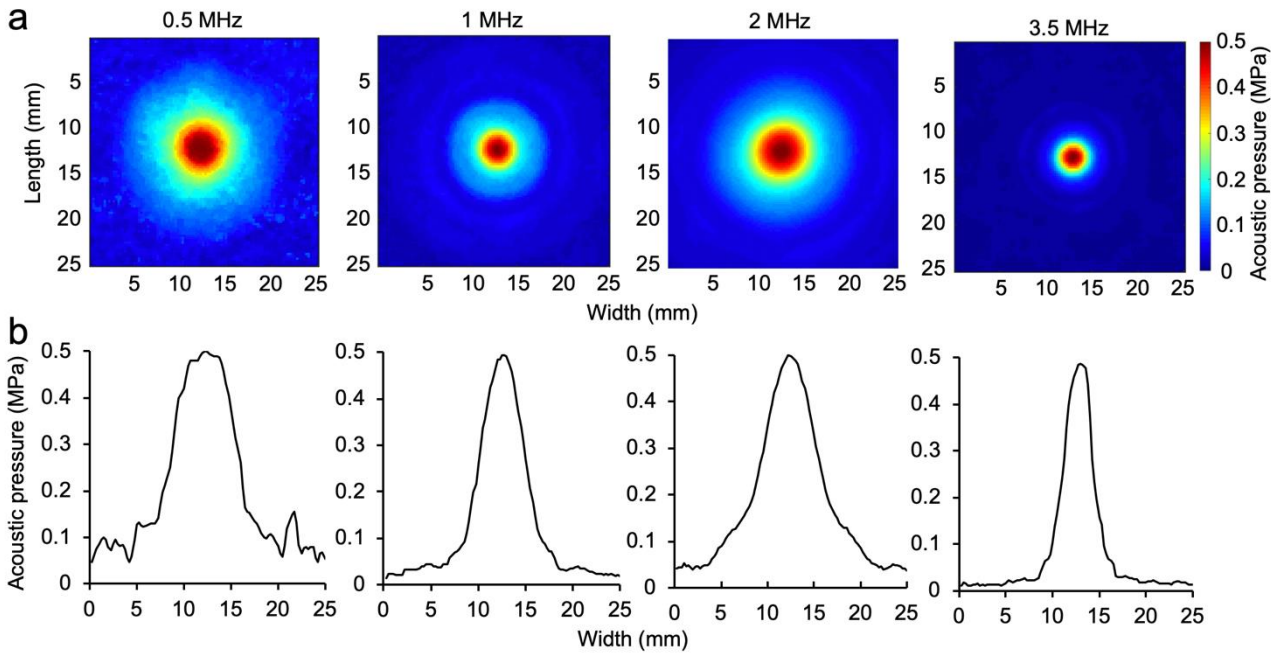

Figure S2. Calibration of acoustic pressure of ultrasound transducers. (a) The acoustic pressure maps of 0.5 MHz, 1 MHz, 2 MHz, and 3.5 MHz ultrasound transducers. (b) The corresponding lateral profiles show that the width at -6 dB area of 0.5 MHz, 1 MHz, 2 MHz, and 3.5 MHz ultrasound transducers are 7.5 mm, 5 mm, 6 mm, and 4 mm, respectively.

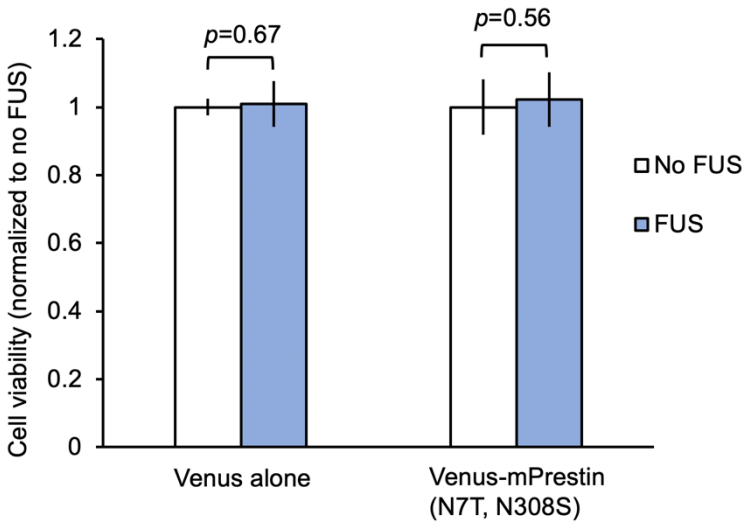

Figure S3. Cell viability after ultrasound stimulation. 293T cells stably expressing Venus alone or Venus-mPrestin(N7T, N308S) were excited by a pulse of 0.5 MHz FUS (3 sec duration, 2000 cycles, 10 Hz PRF, 0.5 MPa). At 24 h after FUS stimulation, the cell viability was determined by measuring the optical density of CCK-8 at 450 nm. Data are shown as the mean ± SD for 3 independent experiments.

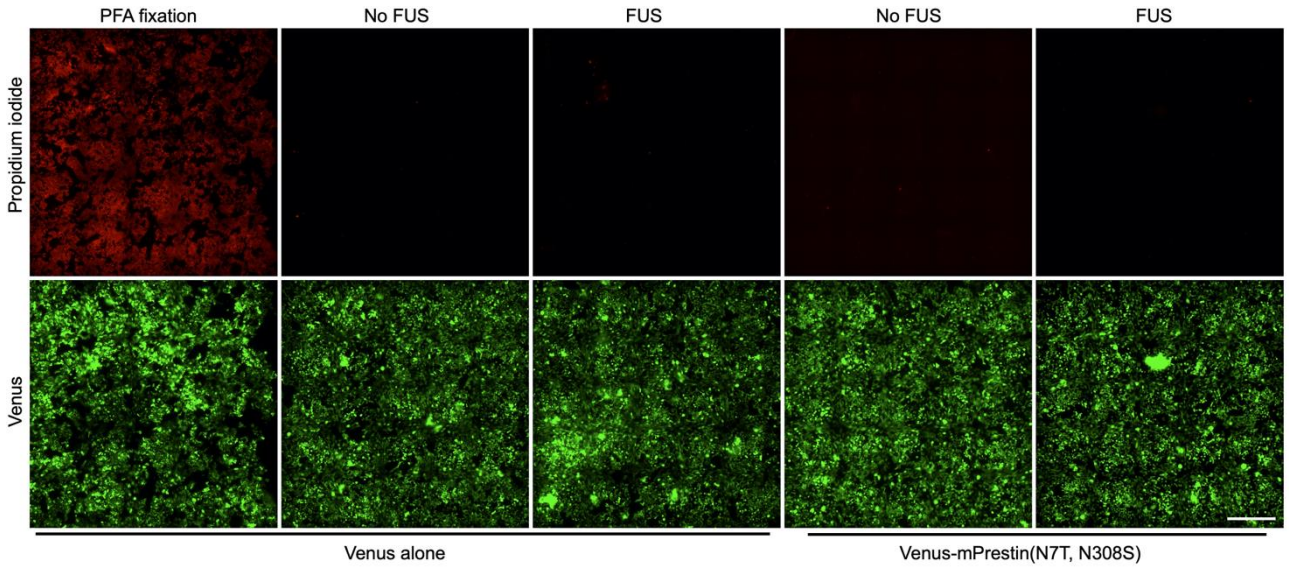

Figure S4. Ultrasound stimulation does not affect the membrane integrity of cells. Cells stably expressing Venus and Venus-mPrestin(N7T, N308S), respectively, were excited by a pulse of 0.5 MHz FUS (3 sec duration, 2000 cycles, 10 Hz PRF, 0.5 MPa) in the presence of a membrane impermeable dye, Propidium iodide (PI, 1 μM). At 1 h after FUS stimulation, the cells that lose membrane integrity were labeled by PI. Cells pretreated with 4% paraformaldehyde were included as a positive control for PI staining. Scale bar, 500 μm.

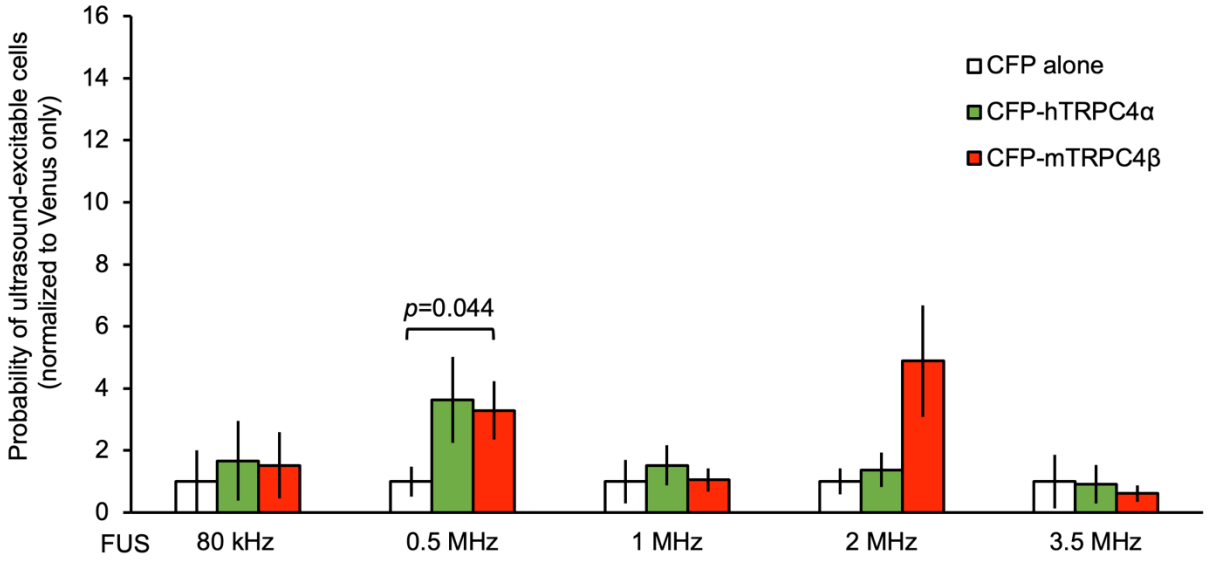

Figure S5. mTRPC4β enables a weak ultrasound-evoked calcium response in a frequency-specific manner. HEK293 cells transfected with one of the indicated DNA constructs were bathed in PBS and stimulated with ultrasound of different frequencies (3 sec duration, 2000 cycles, 10 Hz PRF, 0.5 MPa). Data are presented as the relative number of cells in each group (expressed as fold-probability) that were excitable by ultrasound after normalization to that of cells expressing only Venus that were stimulated at the same frequency. The absolute number of cells in each group was 1209, 768, 889, 1634, 1665, 1736, 1054, 1035, 1116, 1012, 1000, 960, 1168, 857, and 909 cells from left to right. Data are shown as the mean ± s.e.m. for 7–17 independent experiments. *P* values > 0.05 are not shown.

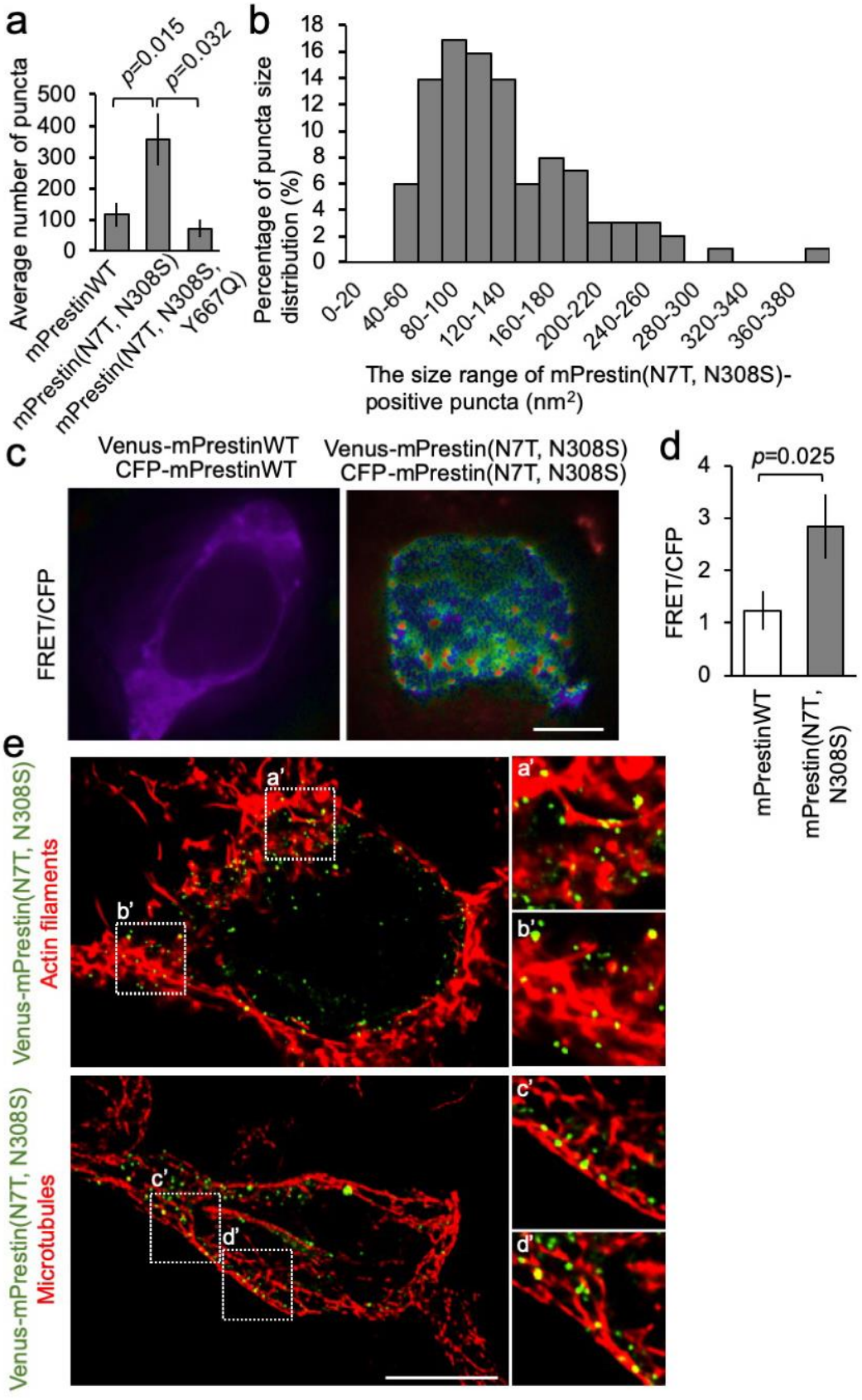
a
Average number of puncta
p=0.015
p=0.032
500
400
300
200
100
0
mPrestinWT
mPrestin(N7T, N308S)
mPrestin(N7T, N308S, Y667Q)
b
Percentage of puncta size distribution (%)
18
16
14
12
10
8
6
4
2
0
0-20
40-60
80-100
120-140
160-180
200-220
240-260
280-300
320-340
360-380
The size range of mPrestin(N7T, N308S)-positive puncta (nm²)
c
Venus-mPrestinWT
CFP-mPrestinWT
Venus-mPrestin(N7T, N308S)
CFP-mPrestin(N7T, N308S)
FRET/CFP
d
p=0.025
FRET/CFP
4
3
2
1
0
mPrestinWT
mPrestin(N7T, N308S)
e
Venus-mPrestin(N7T, N308S)
Actin filaments
a'
b'
Venus-mPrestin(N7T, N308S)
Microtubules
c'
d'

Figure S6. Characterization of mPrestin(N7T, N308S)-positive puncta. (a) The average number of mPrestin-positive puncta in cells expressing the indicated constructs. The number of cells counted in each group are 7, 3, and 3 cells from 3 independent experiments. Data are shown as mean ± s.e.m. (b) Size distribution of mPrestin(N7T, N308S)-positive puncta. n = 101 puncta from five cells expressing mPrestin(N7T, N308S). (c) HEK293 cells transfected with the indicated DNA constructs were imaged by fluorescence resonance energy transfer (FRET). Scale bars, 10 μm. (d) Quantification of the FRET/CFP ratios for cells expressing the indicated DNA constructs. The numbers of cells were 25 (mPrestinWT) and 21 (mPrestin(N7T, N308S). Data are shown as the mean ± s.e.m. for two independent experiments. (e) HEK293T cells expressing Venus-mPrestin(N7T, N308S) were processed for immunofluorescence with phalloidin (actin filaments) or anti-**α**-tubulin antibody (microtubules). For each field, a maximal z projection was created from 15 stacks separated by 0.3 μm. Scale bar, 10 μm.

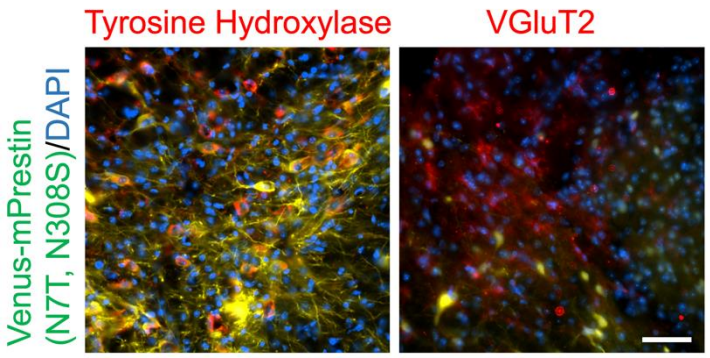

Figure S7. The mPrestin(N7T, N308S) expression was detected mainly in Tyrosine hydroxylase-positive VTA neurons and rarely in VGluT2-positive glutamatergic neurons after virus infection. Representative images of mouse brain sections after injection of AAV-encoding Venus-mPrestin(N7T, N308S) and immunostaining with anti-tyrosine hydroxylase antibody (a dopaminergic neuron marker) or anti-VGluT2 antibody (a glutamatergic neuron marker), respectively. Scale bar, 50 μm.

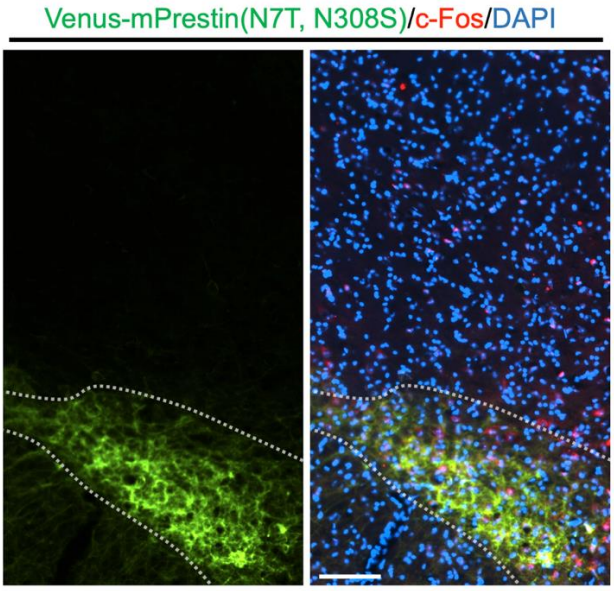

Figure S8. Ultrasound stimulation activates neurons expressing Venus-mPrestin(N7T, N308S) with limited induction in non-transfected cells of mouse brains. The representative image of mouse brain sections with local expression of Venus-mPrestin(N7T, N308S) (yellow) in VTA region (a dotted region). Extensive FUS-driven c-Fos (red) expression was detected in cells expressing Venus-mPrestin(N7T, N308S) but not in neighboring regions after FUS stimulation (0.5 MHz, 0.5 MPa, 10 Hz PRF, 2000 cycles, 3 sec duration). Scale bar, 100 μm.

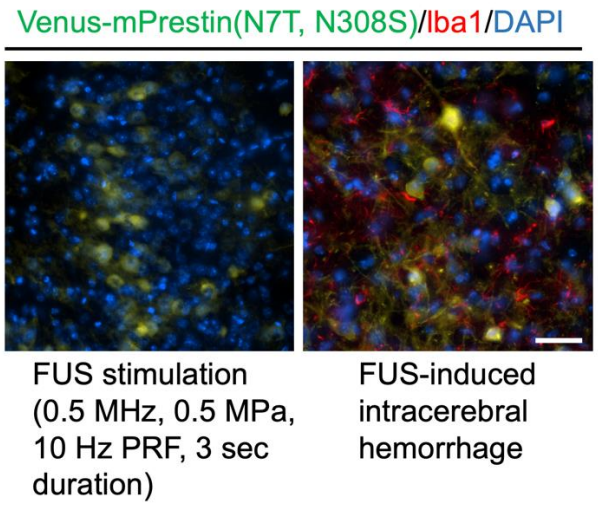

Figure S9. FUS stimulation does not activate microglia in VTA region with Venus-mPrestin(N7T, N308S) expression. Representative images of mouse brain sections after injection of AAV-encoding Venus-mPrestin(N7T, N308S) and immunostaining with anti-lba1 antibody (a microglia marker). The parameters of FUS used to stimulate mPrestin(N7T, N308S)-positive neurons are 0.5 MHz, 0.5 MPa, 10 Hz PRF, and 3 sec duration. FUS-induced intracerebral hemorrhage serves as a positive control of microglia staining. Scale bar, 50 μm.

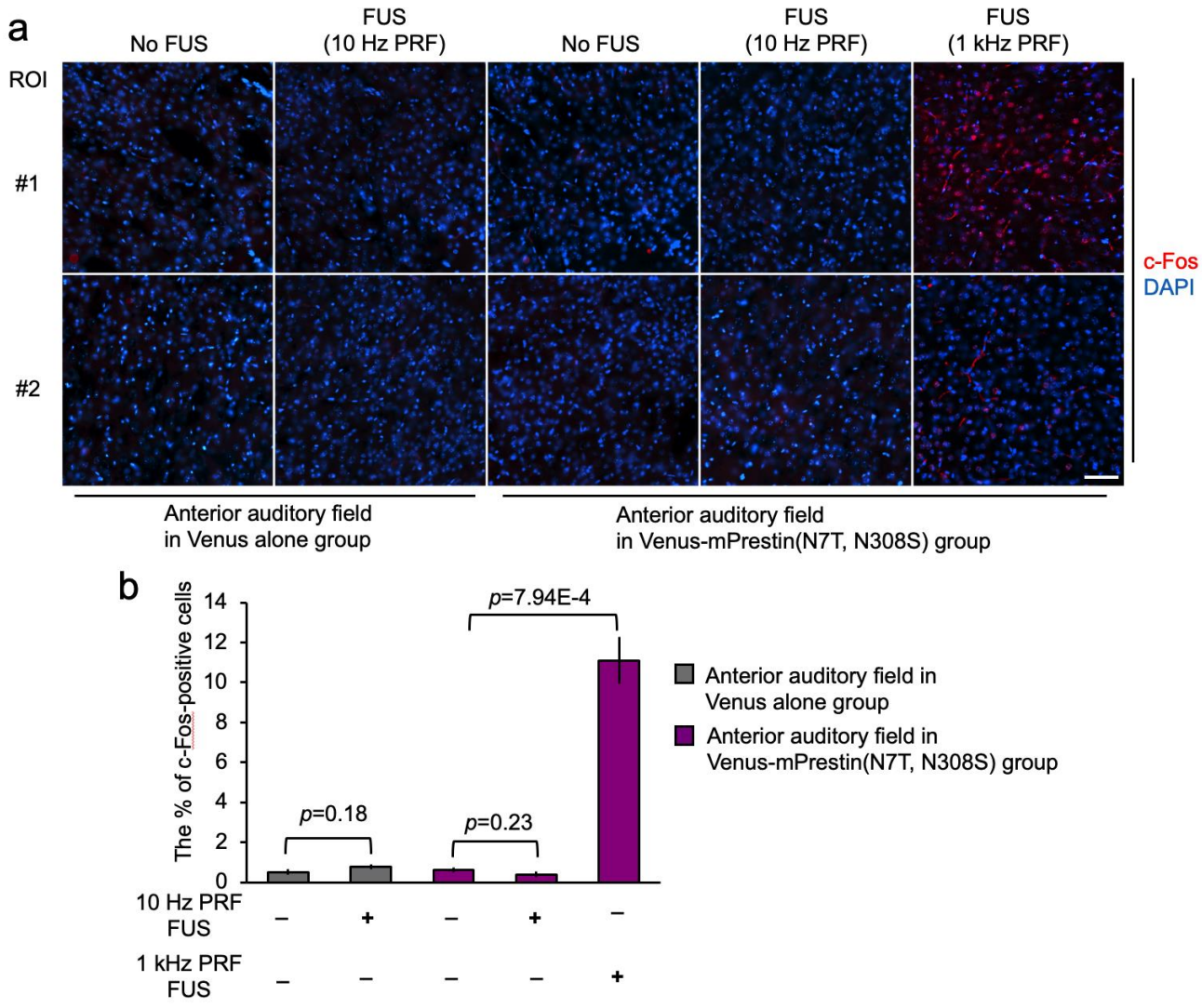

Figure S10. Ultrasound at high pulse repetition frequency activates auditory regions of mouse brains. (a) The representative images of anterior auditory field in the indicated groups presented in figures 4e and f. Extensive FUS-driven c-Fos (red) expression was detected in auditory regions of mouse brains after 1 kHz PRF ultrasound stimulation (0.5 MHz, 0.5 MPa, 1 kHz PRF, 150 cycles, 6 sec duration). 10 Hz PRF ultrasound (0.5 MHz, 0.5 MPa, 10 Hz PRF, 2000 cycles, 3 sec duration) does not significantly activate auditory regions in mice. Two different regions of interest (ROIs) are shown. Scale bar, 50 μm. (b) Percentage of c-Fos-positive neurons in anterior auditory regions of mouse

brains for the indicated conditions. Data are shown as the mean ± s.e.m. for 5~9 different sections from 3 mice per condition.

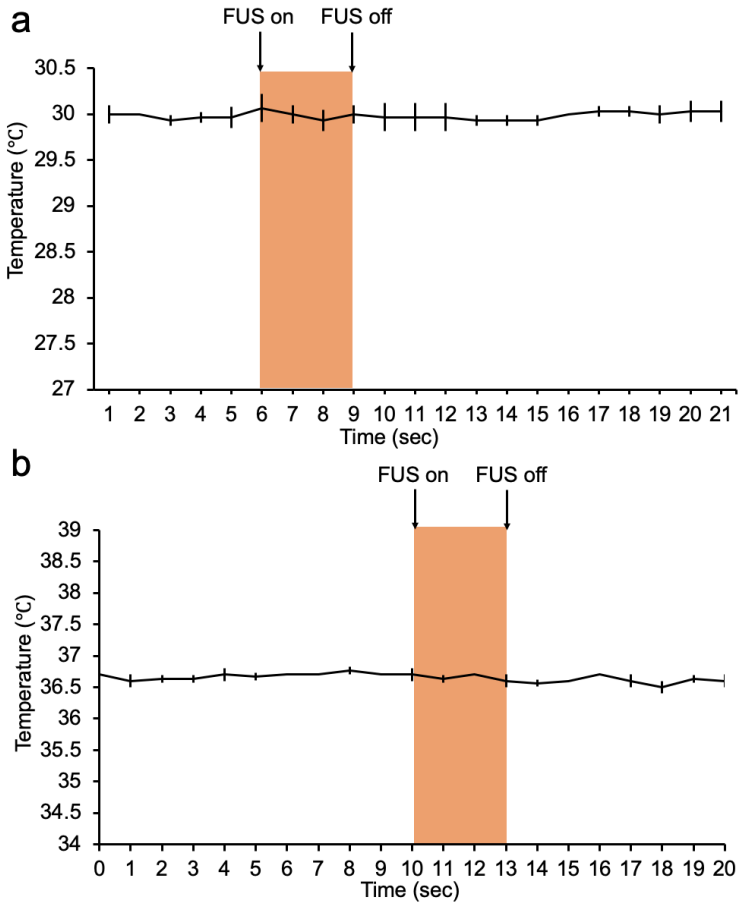

Figure S11. Ultrasound stimulation does not induce a thermal effect *in vitro* and *in vivo***.** A thermocouple probe was placed onto the culture dish (a) or mouse brains (b) for measuring the temperature upon ultrasound stimulation (0.5 MHz, 3 sec duration, 2000 cycles, 10 Hz PRF, 0.5 MPa). Data are shown as the mean ± SD for three independent experiments.

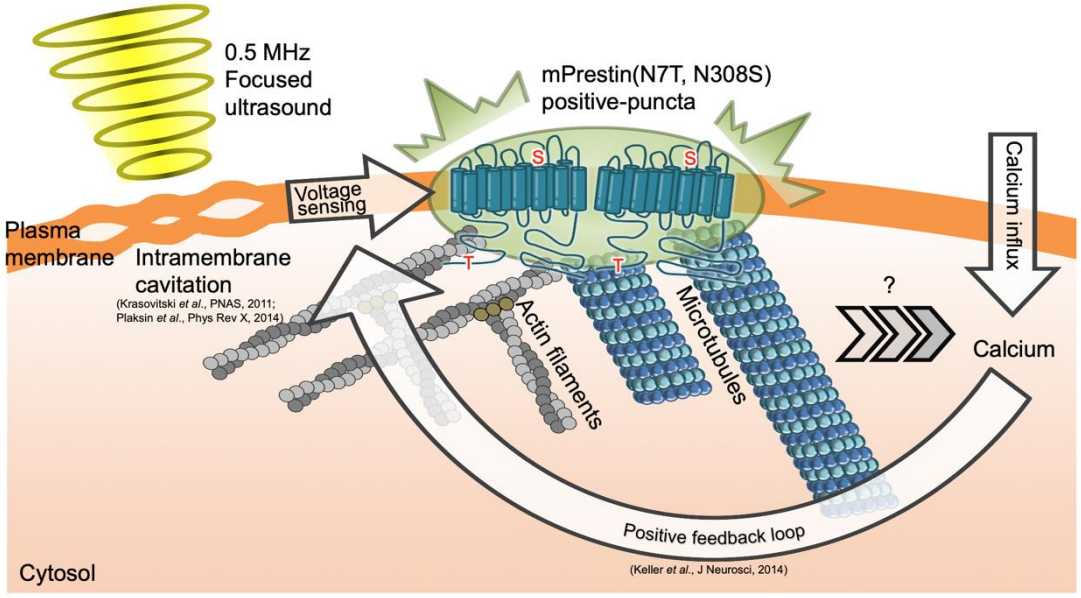

Figure S12. The working model of mPrestin(N7T, N308S)-mediated calcium influx upon ultrasound stimulation. Two evolutionarily conserved mutants N7T and N308S enhance self-assembly of mPrestin in the punctate regions of plasma membrane where they associate with actin filaments and microtubules. 0.5 MHz has been shown to induce temporal intramembrane cavitation which changes membrane thickness or membrane potential.[1–3] The mPrestin(N7T, N308S)-positive puncta detect the change of membrane potential and in turn trigger the observed calcium influx dependent on their electromotility. The calcium influx further activates the electromotility of Prestin that builds up a positive feedback loop for amplifying ultrasound-induced bioeffects.[1]

Supplementary tables

Transducer model	Frequency (MHz)	Focal distance (mm)	Aperture (mm)	Diameter of focal spot (mm)	Attenuation of skull (%)	Vendor
080SR365B	0.08	50	36.5	N/A	N/A	Pro-Wave Electronics Corp.
V301	0.5	50	28	7.0	2.1 ± 0.2%	Olympus
V302	1	55	23	4.0	5.5 ± 0.6%	Olympus
V305	2	55	28	5	11.8 ± 2.8%	Olympus
V380	3.5	55	28	3.0	16.6 ± 2.3%	Olympus

Table S1. Transducer characteristics and operating frequency ranges.

US frequency (MHz)	Peak negative pressure (MPa)	Power density (mW/cm^2)
0.08	N/A	16.4
0.5	0.5	20.0
1.0	0.5	22.6
2.0	0.5	18.1
3.5	0.5	26.7

Table S2. The summary of acoustic peak negative pressure and power density used in this study.

Other supporting files

Movie S1. mPrestin(N7T, N308S) enables ultrasound-evoked calcium response. Excitation of 0.5 MHz FUS evokes calcium response in cells expressing Venus-mPrestin(N7T, N308S) but not in control cells (Venus alone). The cells co-transfected with a calcium biosensor, CFP-R-GECO, and Venus alone or Venus-mPrestin(N7T, N308S), were excited by 0.5 MHz pulsed FUS (3 sec duration, 10 Hz PRF, 2000 cycles, 0.5 MPa). The intensity of R-GECO in cells was monitored by live-cell imaging. Scale bar, 10 μm.

Movie S2. mPrestin(N7T, N308S)-positive puncta oscillated upon FUS stimulation. HEK293T cells were transfected with Venus-mPrestin(N7T, N308S) or Venus-mPrestin(N7T, N308S, V499G, Y501H). Video showing the structural dynamics of mPrestin-positive puncta in cells that had or had not been stimulated with 0.5 MHz FUS. The boundaries of the punctate regions are outlined in white. Scale bar, 0.2 μm.

Supporting references:

(1) Keller, J. P.; Homma, K.; Duan, C.; Zheng, J.; Cheatham, M. A.; Dallos, P. Functional Regulation of the SLC26-Family Protein Prestin by Calcium/Calmodulin. *J. Neurosci.* **2014**, *34* (4), 1325–1332. https://doi.org/10.1523/JNEUROSCI.4020-13.2014.

(2) Krasovitski, B.; Frenkel, V.; Shoham, S.; Kimmel, E. Intramembrane Cavitation as a Unifying Mechanism for Ultrasound-Induced Bioeffects. *Proc. Natl. Acad. Sci.* **2011**, *108* (8), 3258–3263. https://doi.org/10.1073/pnas.1015771108.

(3) Plaksin, M.; Shoham, S.; Kimmel, E. Intramembrane Cavitation as a Predictive Bio-Piezoelectric Mechanism for Ultrasonic Brain Stimulation. *Phys. Rev. X* **2014**, *4* (1), 1–10. https://doi.org/10.1103/PhysRevX.4.011004.