ASIC1a is required for neuronal activation via low-intensity ultrasound
stimulation in mouse brain

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SIGNIFICANCE

CNS neurons have no sensory function, protected by the skull. For this reason, brain neuromodulation by ultrasound were either done at a high intensity or through auditory nerves. We demonstrate in this study CNS neurons react to ultrasound stimulation at an intensity (5 mW/cm^2) far lower than typical therapeutic ultrasound ($>30 \text{ mW/cm}^2$). Using micropipette ultrasound in calcium imaging, we show the reactions of CNS neurons to ultrasound is through ASIC1a channels, pointing to the molecular basis for direct ultrasound neuromodulation at low intensity. Furthermore, we also show evidence of neurogenesis with the same ultrasound stimulation, suggesting potential therapeutic translation.

ABSTRACT

Accumulating evidence has shown transcranial low-intensity ultrasound can be potentially a non-invasive neural modulation tool to treat brain diseases. However, the underlying mechanism remains elusive, because the majority of studies on animal models applying rather high-intensity ultrasound that cannot be safely used in humans. Here we showed low-intensity ultrasound was able to activate neurons in the mouse brain and repeated ultrasound stimulation resulted in adult neurogenesis in specific brain regions. *In vitro* calcium imaging studies showed that a specific ultrasound stimulation mode, which combined with both ultrasound-induced pressure and acoustic streaming mechanotransduction, is required to activate cultured cortical neurons. ASIC1a and the tether-mode mechanotransduction were involved in the low-intensity ultrasound-mediated mechanotransduction and cultured neuron activation, which was inhibited by ASIC1a blockade and cytoskeleton-modified agents. In contrast, the inhibition of mechanical sensitive channels involved in bilayer-model mechanotransduction like Piezo or TRP proteins did not affect the ultrasound-mediated neuronal activation.

Introduction

Transcranial ultrasound such as opening blood-brain barrier (BBB) (1) for localized drug release and modulating neural activity (2-4) has been used for therapeutic treatments of various brain diseases. Many *in vivo* animal experiments and human clinical trials (*SI Appendix,* Table 1) proved the clinical potential of transcranial ultrasound stimulation. With the increased interest of this technique, the mechanisms underlying ultrasound-mediated neural modulation has also recently been learned. A study showed high-intensity transcranial ultrasound can elicit a startle-like motor response via an indirect auditory mechanism (5). Emerging sonogenetics in worm model also identified and engineered TRP-4 channels as a sensor for the ultrasound stimulus to activate neurons in living organisms at pressure level above 0.5 MPa (6), and ultrasound at 0.1 MPa acoustic pressure was found to activate neurons via Piezo 1 mechanosensitive ion channel (7). Nonetheless, the energy intensity or acoustic pressure of most clinical trials or basic researches used for BBB opening or neuromodulation are both high, and safety issue of this technique in clinical application remains a concern.

In this study, a much lower intensity ultrasound at the order lower than 10 mW/cm^2 is proposed to activate neurons via mechanosensitive ion channels in mammals' brain for potential clinical application. Mechanosensitve ion channels are mainly grouped into bilayer model, such as PIEZO and TRP channels gated by membrane tension change, and extracellular matrix tethered model such as acid sensing ion channels (ASICs) (8-10). Here we aim to identify possible mechanical sensors in mouse brain that can directly respond to low-intensity ultrasound.

Results

Transcranial ultrasound induced p-ERK in the cortex, hippocampus and amygdala of

mouse brain. We kept ultrasound exposure to below 10 mW/cm^2 (I$_{SATA}$) in our

experiments to ensure safe therapeutic applicability. The phosphorylation of

extracellular-signal-regulated kinase (p-ERK), an established indicator of immediate

neuronal activation (11), was used to evaluate whether transcranial low-intensity

ultrasound can stimulate neuronal activity in mouse brain. Mice with 1-minute

ultrasound exposure (Fig. 1 *A*) had shown significant increase of p-ERK positive cells in

certain brain regions, such as the cortex (Fig. 1 *B-D*), hippocampus (Fig. 1 *E-G*), and

amygdala (Fig. 1 *H-J*) as compared with those received sham treatment (*SI Appendix,*

Table 2). More specifically, increased p-ERK expression generally occurred upon

ultrasound stimulation in the visual, somatosensory, auditory, temporal associations,

retrosplenial, piriform, and entorhinal areas of mouse cortex (*SI Appendix,* Fig. S1 and

S2). In hippocampus, CA1 and CA2 were dramatically lightened up with p-ERK signals

in response to ultrasound whereas CA3 and dentate gyrus showed sparsely stimulated

(*SI Appendix,* Fig. S1 and S2). In amygdala, the central amygdala nucleus showed the

strongest p-ERK signals, while medial and basolateral also obviously increased in p-ERK

signals (*SI Appendix,* Fig. S1 and S2). We also observed a consistently unchanged p-ERK

staining in the paraventricular nucleus of hypothalamus (PVH) (*SI Appendix,* Fig. S3),

revealing an intriguing regional specificity of the ultrasound response.

Micropipette guided ultrasound as the mechanical stimuli with combined ultrasound

and acoustic streaming. To determine whether low-intensity ultrasound can directly

activate neurons mechanically, we next used in vitro calcium-imaging approach to

probe the possible ion channels responding to ultrasound mechanical stimulus in

cultured cortical neurons. A micropipette was used to guide ultrasound to cultured

cells (Fig. 2 *A*). The device can generate either an ultrasound induced pressure

predominant condition (2000 mVpp, DF 0.05%, measured peak pressure versus the

distance to the pipette tip shown in *SI Appendix,* Fig. S4 *A*) or an acoustic streaming

predominant condition (100 mVpp, DF 100%, flow pattern for the acoustic streaming

shown in *SI Appendix,* Fig. S4 *A* and Movie S1), or a mixed loading condition (700 mVpp,

DF 20%) depending on duty cycle applied. Ultrasound pressure predominant

conditions (up to 2000 mVpp, DF 0.05%) generated compressional stress on cells that

were not able to elevate calcium responses (Fig. 2 *B* and *SI Appendix,* Movie S2), while

acoustic streaming predominant conditions (up to 100 mVpp, DF 100%) invoked shear

stress that could only activate little calcium responses in neurons in the maximal

energy level applied (Fig. 2 *B*); whereas a mixed loading condition (700 mVpp, 20% DF)

effectively yielded much higher calcium responses (Fig. 2 *B* and *SI Appendix,* Movie S3).

The ultrasound predominant mode with only compressional stress cannot induce a

response even when the stimulation was extended to 10 seconds (*SI Appendix,* Fig. S5

A). Similarly, the prolong stimulation of acoustic streaming invoking shear stresses also

only elevated mildly the amplitude of calcium response (*SI Appendix,* Fig. S5 *B*). On the

other hand, the combined compression stress with acoustic streaming can

reproducibly elevated calcium response even in 1.5 second stimulation (Fig. 2 *B* and *SI*

Appendix, Movie S3, Fig. S5 *C*). The ultrasound-induced calcium responses were dose-

dependent with a threshold of 400 mVpp (8 kPa) and EC_{50} of 700 mVpp (12kPa) (Fig. 2

C and *SI Appendix* Fig. S5 *D-K*). The corresponding stress levels of ultrasound at 400

mVpp, 500 mVpp, 700 mVpp or 900 mVpp were 8 kPa, 8.72 kPa, 12 kPa and 15.3 kPa,

respectively.

Neuronal calcium signal upon ultrasound simulation suppressed by ASIC channels

inhibitors. The micropipette ultrasound mechanotransduction was pharmacologically

tested with selective or non-selective blockers of mechanosensitive ion channels for

Piezo, TRP, and ASICs. First, the treatment with Gadolinium (10 μM), a non-selective

blocker of mechanically sensitive ion channels, partially suppressed the ultrasound-

induced calcium signals (Fig. 2 *D*). Treating the cells with a selective Piezo inhibitor,

GsMTx-4, led to a marginal (not significant) inhibition of the ultrasound-induced

calcium elevation as compared with the vehicle control (Fig. 2 *E*). Instead, the

treatment with a TRP blocker ruthenium red (1-10 μM) partially suppressed the

calcium signals (Fig. 2 *F*), whereas the non-selective ASIC inhibitor, amiloride, totally

abolished the calcium signals by micropipette ultrasound (Fig. 2 *G*). Above results

suggested ASICs might be the major channels involved in micropipette ultrasound

mechanotransduction. To narrow down the specific candidate of ASICs, ASIC1a

inhibitor, PcTx1 (50nM) was tested. PcTx1 (50 nM) significantly inhibited the calcium

response by micropipette ultrasound (Fig. 2 *H* and *SI Appendix,* Fig. S5 *C*). The relative

inhibitions of above channel blockers were summarized in Fig. 2*I*. We further tested

the dose-dependent inhibition of PcTx1 on micropipette ultrasound and determined

an IC50 of 0.2nM, suggesting a homotrimeric ASIC1a is the mechanoreceptor in

action (Fig. 3 *A* and *SI Appendix,* Fig. S6 *A-F*). To validate how ASIC1a activation would

lead to calcium responses to ultrasound, we treated the cells with calcium chelating

agent, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (1-

5mM) to block the extracellular calcium. The results showed that calcium influx was

absolutely essential (Fig. 3 *B* and *SI Appendix,* Fig. S6 *G* and *H*). To examine whether

endoplasmic reticulum calcium was involved in calcium signaling, we found the

calcium surge of cells treated with the RyR inhibitor, JTV519 fumarate (10 μM) (*SI*

Appendix, Fig. S6 *G* and *I*) was partially inhibited, while as the IP3R inhibitor, (-)-

Xestonspongin C (1 μM) was most inhibited (Fig. 3 *B*).

ASIC1a mechano-response required cytoskeletal dynamics. Previous studies have

shown ASICs are involved in tether mode mechanotransduction, which relies on

intact cytoskeletal structures (8, 12). We likewise treated the cells with either actin

polymerization inhibitor, cytochalasin D (5-10 μg/ml) or microtubule assembly

inhibitor, nocodazole (5-10 μg/ml). Indeed, inhibition of cytoskeletal dynamics could

dose dependently and significantly suppress the calcium response stimulated by

micropipette ultrasound (Fig. 3 *C-E*). The collated data revealed a novel mode of

ultrasound mechanotransduction with a combination of compression force and shear

force that activates ASIC1a channels in mouse neurons via tether mode

mechanotransduction (Fig. 3 *F*).

Transcranial ultrasound treatments promoted neurogenesis in dentate gyrus. We

next investigated whether the low-intensity ultrasound stimulation in mouse brain

could lead to a favorable outcome in terms of adult neurogenesis. We selected

doublecortin (DCX) as a marker for neurogenesis in dentate gyrus (13-15). Compared

to the non-treated controls, after 3 consecutive days of 5 minute ultrasound

treatments (5 mW/cm^2), DCX staining in dentate gyrus at day 4 and day 7 showed a

significant 2-fold increase (Fig. 4). The results indicated that repeated stimulation of

low-intensity ultrasound on mouse brain might achieve beneficial neural modulation

and lead to neurogenesis in dentate gyrus.

Discussion

Accumulating evidence has shown ASICs are involved in different types of

mechanotransduction in the sensory nervous system, including nociception,

barorecpetion, proprioception, and hearing (8, 16, 17). However, the

mechanosensitive role of ASICs in the brain is still not known, although ASIC1a is a

predominant acid sensor modulating neural activity in physiological and pathological

conditions (18, 19). Here we demonstrated low-intensity ultrasound could modulate

neural activity in mouse brain and directly activate neurons (Fig. 1) via an ASIC1a-

depenent manner (Fig. 2-3). Moreover, repeated transcranial low-intensity

ultrasound stimulations are safe and able to elicit adult neurogenesis in mouse brains

(Fig. 4).

Although ASIC1a was determined as the molecular determinant involved in low-

intensity ultrasound mechanotransduction, non-selective mechanosensor inhibitors

such as gadolinium and ruthenium red were partially suppressing the calcium

response triggered by micropipette ultrasound (Fig. 2 *G* and *H*). Of note gadolinium

also blocked ASICs in µM to mM ranges. However, we cannot rule out a role of TRP

channels in the micropipette ultrasound mechanotransduction, because there is no

evidence showing ruthenium can also inhibit ASICs. Previous studies have proposed a

role of TRP for ultrasound-mediated mechanotransduction while high-intensity

ultrasound was applied. More studies are required to validate the role of TRP in low-

intensity ultrasound mechanotransdcution or the unexpected role of ruthenium red

in neuronal ASIC1a signaling pathways.

ASIC1a is widely expressed in the brain and could form as homotrimeric and

heterotrimeric channels with different sensitivity to PcTx1 inhibition [20, 21].

Specifically, heterotrimeric ASIC2b/ASIC1a can be inhibited 50% by approximately 3

nM [21] PcTx1 and ASIC1a/ASIC2a heterotrimeric can be inhibited by 50 nM[20]

whereas 0.5 nM to 1 nM can inhibit the homotrimeric ASIC1a [22, 23]. Therefore,

since PcTx1 in low-doses effectively inhibited ASIC1a-mediated calcium signal by

micropipette ultrasound, homotrimeric ASIC1a channels may be the predominant

subtype involved in ultrasound mechanotransduction in cortical neurons (Fig. 3 *A* and

SI Appendix, Fig. S6).

To explain how ultrasound could activate ASIC1a via the tether-mode

mechanotransduction, we hypothesized a physical effect of micropipette ultrasound

at cell level; which the acoustic streaming imposes shear stress on cell apical surfaces

while ultrasound exerts compressional stresses throughout the cells. Considering the

tether model *in vitro*, we argue, in a mixed loading condition (Fig. 2 *B* and 3 *F*), the

extracellular domains of ASIC1a are under shear force pulling the protein to the flow

direction while the intracellular domains of the ASIC1a are connected to cortical

actin or other cytoskeleton, which experiences dynamic reorganization coupling with

membrane withdrawals in response to ultrasound (24). The anchor-pulling condition

in vivo on the other hand (*SI Appendix,* Fig. S6 *J*), is accomplished differently. Neurons

are embedded in extracellular matrixes (earthy yellow color) such as laminin, poly-

lysine, or poly-ornithine in the brain. When ultrasound is applied to the brain,

extracellular matrixes anchor the ASIC1a (earthy arrow) while cytoskeletal changes

pull it in a different direction (purple arrow), causing an activation of the cells

manifesting as ERK phosphorylation.

Since DCX has been accepted as a neurogenesis markers in dentate gyrus (14,

25), increased DCX-positive cells in dentate gyrus with a consecutive 3-day of

ultrasound treatment suggests a therapeutic implication. Of note, DCX plays multiple

roles in brain development including hippocampal pyramidal neuronal lamination,

cortical neuronal migration and axonal wiring (13, 26). The use of ultrasound in

developing brains should be extremely cautious.

In conclusion, here we provide evidence that a clinically safely low-intensity

transcranial ultrasound could modulate neuronal activity in mouse brain. The low-

intensity ultrasound can directly activate neurons via tether-mode

mechanotransduction and ASIC1a, which provides a molecular basis for future

development of ultrasound neuromodulation.

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Figure legend

Fig. 1. Transcranial ultrasound induces pERK expression in neurons of the cortex,

hippocampus and amygdala of mouse brain. (*A*) Illustration depicting mouse head

stimulated by 1 MHz transducer which was positioned in between the mouse nasal

process of maxilla and the axis of mouse ear of an anesthetized mouse. (*B*)

Micrograph representing cortical region with basal level of p-ERK staining in sham

control mice (n=5), scale bar 100 μm. Sham control mice were handled with similar

procedures of placing transducer on the head without turning on the ultrasound

function generator. (*C*) Micrograph representing cortical region with p-ERK staining

stimulated by ultrasound (I_{SPTA}=5mW/cm^2, 1 minute) (n=5). (*D*) Quantitative bar

graph of the number of p-ERK stained cells within comparable area of 1.224mm^2

(Length 1275μm, Width 960μm), showing significant difference (p=0.0007) of cell

count by ImageJ. (*E*) Micrograph representing hippocampal region with basal level of

p-ERK staining in sham control mice. (*F*) Micrograph representing hippocampal

region with p-ERK staining in mice stimulated by ultrasound. (*G*) Bar graph showing

quantification of significantly p-ERK different cell count (1.224mm^2) (p=0.0132) in

hippocampal region. (*H*) Micrograph representing amygdala of sham controls. Scale

bar 100 μm (*I*) Micrograph representing amygdala of ultrasound stimulated mice. (*J*)

Quantification of amygdaloid significant difference (p=0.0023) of p-ERK cell count

(1.224mm^2).

Fig. 2. Neuronal calcium signals induced by micropipette-guided ultrasound

suppressed by ASIC1a inhibitors. (*A*) A micropipette positioned to the cortical

neurons cultured on a 30mm cover slips mounted to a chamber coupled to

microscope platform. Calcium signals recorded from neurons stained by Invitrogen™

Oregon Green™ 488 BAPTA-1, AM cell permeant. (*B*) Line graphs of averaged calcium

signals in 4 neurons stimulated by micropipette ultrasound for 3 seconds with an

input voltage 2000mVpp, duty factor (DF) 0.05%; or 100mVpp, DF100% or 700mVpp,

DF20%. The red-dotted line denotes start of the stimulation while the yellow-dotted

line denotes the end. (*C*) Calcium responses as a function of micropipette ultrasound

in 20%DF. Dose-dependent (input voltages from 10mVpp, to 900mVpp, DF20%)

responses of ultrasound with an EC50 of 700mVpp is shown (n=5). (*D*) Effects of

gadolinium (120 μM), a non-selective blocker of mechanically sensitive ion channels,

on calcium signals in cortical neurons. (*E*) Effects of GsMTx-4 (500nM), a selective

Piezo inhibitor, on calcium signals in cortical neurons. (*F*) Effects of Ruthenium red

(10 μM), a non-selective TRP inhibitor, on calcium signals in cortical neurons. (*G*)

Effects of amiloride (100 μm), an ASICs family inhibitor, on calcium signals in cortical

neurons. (*H*) Effects of PcTx1 treated (50 nM), a selective ASIC1a inhibitor, on calcium

signals in cortical neurons. (*I*) Statistical analyses of channel blockers on ultrasound-

induced calcium signals in cortical neurons.

Fig. 3. ASIC1a as a mechanoreceptor responsive to mechanical stimuli with combined

ultrasound and acoustic streaming. (*A*) PcTx1 dose-dependent inhibition curve of

calcium responses induced by micropipette ultrasound of 700mVpp, DF20% for 3-

second. (*B*) Whisker plots showing comparison of peak $\Delta F/F_0$ within 3-5 seconds

upon micropipette ultrasound (700mVpp, DF20%, 3-second) stimulation in the

untreated control primary neurons, 2- or 5mM EGTA treated neurons, RyR inhibitor

JTV519 fumarate (10 μM) treated neurons, or IP$_3$R inhibitor (-)-Xestonspongin C (1

μM) treated neurons. Student t-test with *p* value compared to control listed above

the whisker plot. (*C*) Graph showing calcium response of actin polymerization

inhibitor Cytochalasin D (5-10 μg/ml) treated neurons compared to untreated

control. (*D*) Calcium signals showing the effect of the microtubule assembly inhibitor

nocodazole (5-10 μg/ml) on neurons. (*E*) Whisker plots showing comparison of peak

$\Delta F/F_0$ within 3-5 seconds upon micropipette ultrasound (700mVpp, DF20%, 3-

second) stimulation in the untreated control primary neurons, cytochalasin D 5- or 10

μg/ml treated neurons, nocodazole 5- or 10 μg/ml treated neurons. Statistical *p*

values of one-way ANOVA analysis were listed to show the significance of treatment.

(*F*) Cartoon depicting ultrasound stimulating ASIC1a in the cell body of a neuron

under the micropipette ultrasound stimulation. Green arrow represents the pulling

force of acoustic stream and purple arrow represents the compression force of

ultrasound that results in cytoskeletal rearrangement.

Fig. 4. Neurogenesis in dentate gyrus induced by repeated transcranial ultrasound

treatments. (*A*) The mice were treated 3 consecutive days by ultrasound of

4mW/cm^2, 1% for 5 minutes, subsequently perfused fixed at day 4 or day 7 and

brains were dissected from the head and sectioned for immunofluorescence

procedures. The DCX staining in dentate gyrus of treated mice were compared to

control untreated one. (*B*) Cell count with clear DAPI stained nucleus surrounded by

DCX markers compared in control, day 4 and day 7 post-ultrasound treatments.

Statistical analysis: p=0.0013 and F ratio=15.18 in one-way ANOVA (n=4). (*C, D*)

Representative micrograph of untreated mice. The vibratome coronal brain sections

(100 μm) of dentate gyrus region immunofluorescently stained for DCX (green) and

MAP2 (red). Blue color indicates DAPI stained nuclei. Representative micrograph

showing. (*E, F*) Representative micrograph from ultrasound treated mice fixed at day

4. (*G, H*) Representative micrograph from ultrasound treated mice fixed at day