**Title: Mechanistic study of optoacoustic neuromodulation**

**Abstract**

Towards understanding the biomolecular and cellular mechanisms of optoacoustic cell modulation, in this work, we show that optoacoustic excites primary cortical neurons through specific calcium-selective mechanosensitive ion channels with the assistant of calcium amplifier channel and voltage-gated channels. Pharmacological inhibition of specific ion channels leads to reduced responses, while over-expressing these channels results in stronger stimulation. These results shed new insights into the mechanism of ultrasound neurostimulation.

**Introduction**

The biophysical and cellular mechanism of optoacoustic neuromodulation also remains largely unknown. Since optoacoustic devices generates acoustic wave in the ultrasonic range, it is conceived that that the optoacoustic neurostimulation shares the same mechanisms as ultrasound neurostimulation. To date, several hypotheses on cellular mechanism has been proposed for ultrasound stimulation, including local temperature increase[1], transient sonoporation[2, 3], intramembrane cavitation[4, 5], and activation of the mechanosensitive ion channels[8-10].

Ultrasonic heating was considered as the primary mechanism for high intensity focused ultrasound modulation[1]. However, more recent studies using low intensity ultrasound have shown a minimal temperature increase of less than 0.1 ℃[11-13], much less than the thermal threshold for activation. In optoacoustic neuromodulation, the pressures and frequencies used are within the range of conditions used by ultrasound neuromodulation, however, optoacoustic pulses are delivered with less than 0.1% duty cycle. Single pulses have been demonstrated for successful neural activation[14], in which the accumulated heat effect is minimal, evident from a temperature increase less than 0.1 °C.

Sonoporation involves transient and reversible disruption of membrane integrity due to the mechanical force exerted by the acoustic wave and allows ion exchange across the neuronal membrane and depolarizes the membrane potential. Sonoporation has been demonstrated in fiber and thin film based optoacoustic devices for applications including drug delivery[3], gene transfection[15]. Notably, but its contribution in optoacoustic neuromodulation remains unknown. Future investigations of model membrane system, combining optoacoustic stimulation and whole cell electrophysiology could shed light to this hypothesis.

Another pathway recently reported is the astrocytes signaling[17]. In this systematic study, Oh et al. showed that the low frequency low intensity ultrasound triggered the opening of mechanosensitive TRPA1 (transient receptor potential cation channel, subfamily A, member 1) channel in astrocytes. Then the calcium influx caused the release of glutamate via the Best1 (Bestrophin-1) channel, which ultimately activated the NMDA (N-methyl-D-aspartate) receptors in surrounding neurons. Since the TRPA1 is expressed in astrocytes of the hippocampus, cortex, and trigeminal caudal nucleus, serving as a calcium regulator, it is likely that this pathway is involved in ultrasound modulation in vivo. The only thing that would have made this work better is if they patch clamp was performed under the real ultrasound treatment. Oh et al. encountered a difficulty in measuring the channel current because low intensity low frequency focused ultrasound (LILFU) disrupted the whole-cell patch clamp, which led them to change the stimulation strategy from ultrasound into three different ways: a piezo-electrically driven fast exerted mechanical stimulation, a fast-exerted negative pressure under cell-attached configuration, and a slow exerted poking with glass pipette. Although the pipette poking strategy gave a repeatable channel current, the mechanical perturb differs from ultrasound, which intrinsically consists of positive and negative pressure within a cycle on the time scale of ＜50 µs. Thus, this pathway needs to be further validated through reliable patch clamp study under ultrasound treatment.

Lastly, activation of mechanosensitive ion channels has been the most studied hypothesis for acoustic neuromodulation. In an oocyte membrane system, Kubanek et al. recorded ion channel current from mechanosensitive ion channels including TREK-1, TREK-2 and TRAAK and Nav1.5[8]. Kubanek et al. identified MEC-4, an ion channel for a touch sensation was required for ultrasound modulated response in Caenorhabditis elegans[9]. In addition, overexpression of TRP-4, a TRPN family channel has been shown to enhance ultrasound modulation in Caenorhabditis elegans as well[18]. Using calcium imaging, Gaub et al. investigated the neuronal response to pure mechanical stimuli using atomic force microscope cantilever and identified force and pressure required for transient and sustained activation[19]. The contribution of various mechanosensitive ion channels has also been studied using pharmacological manipulation. Using calcium imaging, Yoo et al. looked at activation of various mechanosensitive ion channels by ultrasound stimulation, and identified the key contribution of three ion channels including TRPP2, TRPC1 and Piezo1[10]. Calcium amplification by TRPM4 and voltage gated calcium channels was proposed to be the downstream molecular pathway.

While sharing many similarities, optoacoustic neuromodulation and ultrasound neuromodulation do have some key differences. For ultrasound neuromodulation, continuous wave sonication has been shown to stimulate brain activity in select studies[20, 21], and the pulsed delivery paradigm with tone burst consist of tens of or hundreds of acoustic wave cycles[13] is more favorable due to a lower risk of tissue heating and lower thresholds for neural activation[20]. Ultra-short ultrasound pulses with pulse widths up to a few ten microseconds have been reported. Specifically, Kim et al.[22] applied a 2.1 µs ultrasound pulse at a repetition rate of 1.16 kHz with total duration of ~63 s to evoke spiking. Weinreb et al.[23] used a single ultrasound burst of 4 µs for neuron stimulation in vitro. Tyler et al.[24] used ultrasound burst with total duration of 22.7 µs to evoke single action potential ex vivo. In comparison, optoacoustic stimulation can be successful under a single or multiple acoustic pulse and each pulse has ~1 µs duration. Optoacoustic wave often has a broad bandwidth, ranging from 1-20 MHz, compared to single frequency acoustic wave from a piezo based ultrasonic transducer of millisecond time scale, often at sub-MHz frequency for high transcranial efficiency. These differences could lead to important changes in ion channel activation dynamics, threshold, frequency dependent response and cell type specificity (if any).

To identify the ion channels involved during the optoacoustic stimulation while excluding the thermal effect, in the present work, we designed a tapered fiber optoacoustic emitter with minimal thermal toxicity. Meanwhile, neurons overexpressing ion channels were tested to study the channel involvement. Pharmacological treatments were applied to manipulate cellular pathways towards understanding the cellular mechanisms of optoacoustic neuromodulation.

**Results**

**Design TFOE with minimal thermal toxicity for neuron modulation**

The optoacoustic effect is known to be associated with photothermal effect. To minimize the thermal toxicity, in the present work, several strategies are developed and evaluated. First, the optoacoustic conversion efficiency is maximized by choosing CNT and PDMS mixture as the photoacoustic coating, as they have large light absorption coefficient and thermal expansion coefficient and have shown improved photoacoustic conversion compared to other material combinations. The mixture of CNT and PDMS was coated on the tapered fiber distal end to generate optoacoustic signal as preciously described. Second, to avoid the thermal accumulation during a long burst treatment, short laser burst treatment was applied. The temperature increase under these bursts was evaluated. As shown in Figure 1 b-c, while applying a laser burst of 500 ms with 24 mW power, the GCaMP6f fluorescence intensity in neurons drops upon the optoacoustic treatment. The corresponding temperature was measured using a miniaturized ultrafast thermal probe, showing the temperature increase up to 2.5 ℃. The GCaMP6f shows a linear relationship between the fluorescence intensity drop and temperature change (Fig 1 e). This property is similar to rhodamine B and other thermal sensitive fluorescent dye, due to the fact the quantum yield is decreasing with elevated temperature, which is widely used for investigating temperature changes in various systems[25]. Although the temperature increases are all below the previously reported threshold for thermal induced neural activation (ΔT˃5 ℃), the possibility of the thermal effect on the cell membrane capacitance change, thermal sensitive ion channels (e.g., TRPV1) activation and subsequently membrane depolarization cannot be excluded. Thus, in order to minimize the thermal effect, a 5 ms short laser burst with 52.4 mW laser power was used. As shown in Figure 1f-h, this dosage allows for successful neurostimulation with minor temperature increase, which makes it a good candidate for mechanism study of optoacoustic neuromodulation in the following sections.

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**Figure 1**. TFOE allows for non-thermal modulation. a. Schematic and picture of TFOE. Scale bar: xxx b-e Neural fluorescence signal suppressed by using TFOE with 500 ms laser of 24 mW. d Temperature change during the TFOE treatment. e Dependence of fluorescence with temperature change. f-h Neural stimulation by using TFOE with 5 ms laser of 52.4 mW. g-h Fluorescence signal and temperature upon TFOE treatment.

**Synaptic transmission is involved in the TFOE neurostimulation**

In previous studies using focused ultrasound for neuron modulation, the synaptic transmission was not well understood. Since the focal area is normally larger than the field of view, it was challenging to separate the direct ultrasound stimulation with the synaptic transmission induced activation. By virtue of the highly confined optoacoustic field from the TFOE, we investigated how synaptic inputs affects stimulation outcome. We infused synaptic blockers, a cocktail of NBQX (AMPA receptor antagonist), gabazine (GABA receptor blocker) and D-APV (NMDA receptor antagonist), which have been shown to be effective in blocking glutamate (excitatory) and GABA (inhibitory) synaptic transmission. As shown in Figure 2 a-c, with the presence of synaptic blockers (10 µM NBQX, 10 µM gabazine, and 50 µM DL-AP5), the number of activated neurons shows a significant reduction from 30 to 8 compared to the control group.

Notably, two types of responses were detected as shown in Figure 2 d-f. The transient response spikes upon the stimulation and recovers quickly, while the prolonged response taking a longer time for the recovery. In order to quantify the two response types, we fitted the decay of the response curves exponentially y = y0 + A1\*exp(-(x-x0)/t1) and defined a time constant when they decreased by a factor of 1/e (e = 0.368) from the peak fluorescence intensity as previously reported[26]. The transient activation is defined with a decay time constants shorter than 5 s, while the prolonged activation is defined with a time constants of 5 s and beyond. The percentage of the two types among the activated neurons are compared (Fig 2g). With the absence of synaptic blockers, 28% of the neurons were observed showing the transient responses. While 91% of the neurons showed transient responses with the presence of synaptic blockers. These results suggest that the transient activation is likely originating from direct TFOE-mediated stimulation, while the prolonged activation indicates a train of action potentials coming from the activation of surrounding neural networks via synaptic transmission. Collectively, the results indicate that TFOE-triggered neural activities involve synaptic transmission.

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Figure 2. TFOE induced neuron stimulation involving synaptic transmission. a-b Max ΔF/F contrast image of TFOE evoked neuron activity without/with synaptic blocker (S.B.). Dash line: location of TFOE. c Analysis of number of activated neurons. Error bars denote mean ± SD. d-e Colormaps of fluorescence changes of neurons stimulated by TFOE without/with S.B. f Averaged Calcium traces of neurons upon TFOE stimulation. Shaded area: mean ± SD. g Analysis of percentage for transient and prolonged calcium traces. Error bars denote mean ± SD.

**Mechanosensitive ion channels are responsible for optoacoustic stimulation**

Our hypothesis is that optoacoustic induces activation of mechanosensitive channels, which accumulates ion current to form membrane depolarization, and subsequently activates voltage gated sodium channels to fire action potentials. To test this hypothesis, contribution of mechanosensitive channels and voltage sensitive sodium channels in neuromodulation are assessed by pharmacological inhibition of channel activity.

First, to alter the mechanical properties of the neurons, cytochalasin D was added to the neurons to inhibit the membrane ruffling by depolymerizing the actin cytoskeleton[27]. The max ΔF/F decreased by approximately 33%, indicating the elastic modulus is important during the acoustic induced membrane distortion.

To evaluate the optoacoustic effect on the thermal sensitive ion channels, Ruthenium red was tested on the neurons to block the pores of TRPV1, TRPV2 and TRPV4 channels[28]. As shown in Figure 3, no significant difference was observed compared to the control group, indicating the thermal effect from the TFOE was negligible and the thermal sensitive channels were not involved.

Gadolinium(III) was tested on the neurons, which had been reported as a nonspecific agent that blocks mechano-gated channels via changing the deformability of the lipid bilayer [29]. As shown in Figure 3, the TFOE induced calcium activities were significantly inhibited, indicating the involvement of mechanosensitive ion channels.

Next, peptide inhibitor GsMTx4, which blocks Piezo1 and TRPC1 channel was used. The max ΔF/F of neurons showed a decrease of 42% in response to the TFOE stimulation, meaning the Piezo1 and or TRPC1 channels were playing a key role during this process.

Besides, L-type, N-type, T-type, and P-type Calcium channels have been shown to be mechanically sensitive under various conditions[30, 31]. Recently, voltage-gated T-type calcium channels have been reported as the downstream amplifiers for ultrasound neuromodulation[10]. To validate that, we treated the cells with the selective pore blocker TTA-P2, which suppressed the max ΔF/F by 62%. Thus, voltage-gated T-type calcium channels are demonstrated to be activated during the TFOE stimulation process.

Beyond ion channels, G protein-coupled receptors (GPCRs) are sensory molecules, which had been reported important for mechano-transduction in vasculature as shear stress sensors[32]. To investigate the whether the GPCRs were activated, suramin was used to block the GPCR signaling. The max ΔF/F decreased by 54%, suggesting the shear stress promoted the signaling of GPCRs for cell activation. It’s also worth noting that, in the recent work using focused ultrasound for neurostimulation, the GPCRs were evidenced to be not involved in the stimulation process[10]. This discrepancy might originate from the difference in acoustic wave propagation. In the FUS work, the focal area with 5 mm diameter covered the entire cell culture, which could be regarded as a planar wave. While in the TFOE work, the acoustic source with 100 µm diameter was propagating omnidirectionally, denoting a point source. Thus, shear stress was more likely to be present in the TFOE wave propagation.

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Figure 3 Pharmacologically blocking specific channels/membrane integrity inhibited neuron responses. a-f, Calcium traces of neurons treated with blockers (Blue) and control group (Red) upon TFOE stimulation. Shaded area: mean ±SD. g, statistics of the max ΔF/F under varied treatments.

**Ion channels including TRPC1/TRPP2/TRPM4 increased the neuron mechanic sensitivity and boosted the calcium signal upon TFOE stimulation**

According to the established hypothesis that ultrasound excites neurons via the activation of endogenous mechanosensitive ion channels, including TRPP2 and TRPC1, as well as calcium-dependent amplifier TRPM4 channel[10], we overexpressed three of the ion channels to identify their roles in optoacoustic stimulation. The gene constructs for TRPC1, TRPP2, and TRPM4 ion channels were overexpressed in GCaMP6f neurons under a hSyn promoter (Fig 4a). The fluorescent protein mCherry was co-expressed as an expression indicator (Fig 4b). To further quantify the overexpression of ion channels, immunofluorescent labeling was performed in control group and overexpression group. As shown in Figure 4.6c, the signals for TRPC1, TRPP2 and TRPM4 channels in the overexpression groups are 33±8%, 30±15%, 32±14% higher than the wild type groups, respectively.

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Figure 4. Ion channel overexpression in neurons. a Neurons expressing GCaMP6f as calcium indicator. b Co-expressing of mCherry with the TRPC1/TRPP2/TRPM4 ion channels. Scale bars: 50 µm. Right panel: statistics of the mCherry signal intensity. c Immunostaining of ion channels in wild type (WT) group and overexpression (O/E) group. Scale bars: 20 µm. Right panel: statistics of the immunostaining signal intensity.

Next, the TFOE stimulation of neurons overexpressing specific ion channels are performed. A 3-nanosecond pulsed laser at 1030 nm and 1.7 kHz repetition rate was used to deliver laser pulses of 2 milliseconds duration, corresponding to 3 pulses. Varied laser pulse energy of 12/16/20/24 µJ were applied to test the threshold. As shown in Figure 5, the TFOE stimulation of neurons overexpressing TRPC1 and TRPP2 evoked substantially larger calcium activities compared to the control group with pulse energy of 24 µJ. Meanwhile, even under the pulse energy of 16/20 µJ, the TRPC1 and TRPP2 group can be stimulated, while no activity was observed in the control group, indicating that the overexpressing of TRPC1 and TRPP2 channels increased the mechano-sensitivity of the neurons. Likewise, neurons overexpressing the TRPM4 channel showed increased calcium response and lower stimulation threshold, validating the hypothesis that the TRPM4 serves as the downstream calcium-dependent amplifier even though itself is not mechanosensitive.

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Figure 5 TFOE stimulation of neuron overexpressing ion channels. a Representative max ΔF/F contrast imaging of TFOE stimulation. Dashed line: location of the TFOE. b Analysis of the calcium responses of neurons overexpressing ion channels upon TFOE stimulation with varied laser pulse energy. Error bars: mean ±SD. c-f Calcium traces of neurons overexpressing TRPC1/TRPP2/TRPM4 channels and control group upon TFOE stimulation with varied laser pulse energy. Shaded area: mean ±SD.

**Methods**

*TFOE fabrication and characterization of thermal effect*

To improve the laser energy density, a multimode fiber (FT200EMT, Thorlabs, Inc., NJ, USA) was tapered from 200 µm to 50-100 µm as previously described. The distal end was cut using a carbide fiber scribe (S90C, Thorlabs, Inc., NJ, USA). To assure a maximum optoacoustic conversion efficiency and minimum light leakage in the tapered fiber, CNT in PDMS mixture was prepared. For PDMS, the silicone elastomer (*Sylgard* 184, Dow Corning Corporation, USA) was dispensed directly into a container carefully to minimize air entrapment, followed by mixing with the curing agent in a ratio of 10:1 by weight. Multiwall CNTs (*<*8 nm OD, 2-5 nm ID, Length 0.5-2 µm, VWR, Inc., NY, USA) and isopropyl alcohol (IPA) were added to PDMS. The mixture was sonicated for 5 min followed by degassing in vacuum for 30 min. Considering the evaporation of IPA, the final CNT concentration in PDMS reached to 15%. The coating matrix was then casted on a metal mesh to form a uniform film. After partial evaporation of the IPA at room temperature for 10 min, the fiber was controlled by a 3-D micromanipulator to punch through the film with a layer transferred to the tapered end. The coated fiber was then cured vertically at 100 °C.

To characterize the optoacoustic signal, a customized and compact passively Q-switched diode-pumped solid-state laser (1030 nm, 3 ns, 100 µJ, repetition rate of 1.7 kHz, RPMC, Fallon, MO, USA) was used as the excitation source. The laser was connected to an optical fiber through a homemade fiber jumper (SMA-to-SC/PC, ~81% coupling efficiency), then connected to the TFOE with a SubMiniature version A (SMA) connector. To adjust the laser power, fiber optic attenuator sets (multimode, varied gap of 2/4/8/14/26/50 mm, SMA Connector, Thorlabs, Inc., NJ, USA) were used. A needle hydrophone (ID. 40 µm; OD, 300 µm) with a frequency range of 1-30MHz (NH0040, Precision Acoustics Inc., Dorchester, UK) was utilized for the acoustic measurement. The acquired signal was processed with an ultrasonic pre-amplifier (0.2–40MHz, 40 dB gain, Model 5678, Olympus, USA) and a digital oscilloscope (DSO6014A, Agilent Technologies, USA). The distance between the TFOE tip and hydrophone was controlled using a 4-axis micro-manipulator (MC1000e controller with MX7600R motorized manipulator, Siskiyou Corporation, USA) with a controllable motion of 0.2 µm. The distance was measured using a widefield microscope with a 20× objective. The TFOE tip and hydrophone tip were both immersed in degassed water dropped on a cover glass. The pressure values were calculated based on the calibration curve obtained from the hydrophone manufacturer. The frequency data was obtained through the Fast Fourier Transform (FFT) using Origin 2019.

To characterize the thermal profile generated by TFOE in water during acoustic generation, temperature on the fiber tip was measured by a miniaturized ultrafast thermal sensor (DI-245, DataQ, OH, USA) directly in contact with the TFOE tip surface.

*Neuron culture with GCaMP6f expression*

The glass-bottom culture dishes used in the embryonic neuron cell cultures were immersed in 0.01% Poly- D-Lysine (Sigma-Aldrich, MO) for overnight at 4 °C and washed in PBS twice before culture initiation. Primary cortical neurons were obtained from SD rat E15 embryos. Dissociated cells were washed with and triturated in 10% heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals, GA), 5% heat-inactivated horse serum (HS, Atlanta Biologicals, GA), 2 mM Glutamine-Dulbecco’s Modified Eagle Medium (DMEM, Thermo Fisher Scientific Inc., MA), and cultured in cell culture dishes (100 mm diameter) for 30 min at 37 °C to eliminate glial cells and fibroblasts. The supernatant containing neurons was collected and seeded on poly-D-lysine coated cover glass and incubated in a humidified atmosphere containing 5% CO2 at 37 °C with 10% FBS + 5% HS + 2 mM glutamine DMEM. After 16 h, the medium was replaced with Neurobasal medium (Thermo Fisher Scientific Inc., MA) containing 2% B27 (Thermo Fisher Scientific Inc., MA), 1% N2 (Thermo Fisher Scientific Inc., MA), and 2 mM glutamine (Thermo Fisher Scientific Inc., MA). Half of the medium was changed with the fresh medium every 3 days, and neurons were used for ultrasound stimulation experiments after 12–14 days from the seeding. For calcium imaging, Syn-driven GCaMP6f as a calcium sensor was delivered to neurons via AAV9 viral vector transfection (Addgene, pAAV.Syn.GCaMP6f.WPRE.SV40, 1E10 vp/dish) at 4 days in vitro.

*Calcium imaging of TFOE induced neuron stimulation*

Calcium fluorescence imaging was performed on a lab-built wide-field fluorescence microscope. The microscope was based on an Olympus IX71 microscope frame. With a 20 air objective (UPLSAPO20X, 0.75NA, Olympus), illuminated by a 470 nm LED (M470L2, Thorlabs) and a dichroic mirror (DMLP505R, Thorlabs). A 3-D micromanipulator (Thorlabs, Inc., NJ, USA) was used to position the TFOE approaching the cells at an angle of 45°.Image sequences were acquired with a scientific CMOS camera (Zyla 5.5, Andor) at 20 frames per second. The fluorescence intensity analysis and exponential curve fitting were performed using ImageJ (Fiji).

*Gene overexpression of ion channels in cultured neurons*

The viral particles expressing TRPV1, TRPP2 and TRPM4 are gifts from Dr. Sangjin Yoo in Dr. Mikhail G. Shapiro’s group at California Institute of Technology. As described in their previous work[10], the mouse TRPV1 (GenBank: AB040873.1), TRPP2 (GenBank: BC053058) and TRPM4 (GenBank: BC096475), human TRPC1 (GenBank: Z73903.1), genes were synthesized commercially (Integrated DNA Technologies) and cloned upstream of an internal ribosome entry site (IRES2) and mScarlet (TRPC1, TRPP2) or mRuby3 (TRPV1, TRPM4) gene. The construct was inserted into the lenti-backbone. The viral particles were added to neurons at 3 days in vitro (1E9 vp/sample) and maintained for 10 days. hSyn-driven mCherry was inserted into the lenti-backbone by Gibson assembly to confirm the gene expression. The viral particles were added to neurons at 3 days in vitro (1E9 vp/sample), whole media was replaced with the fresh media at 4 days in vitro, and the cells were maintained for 10 additional days.

*Immunostaining characterization of ion channel expression levels*

For immunostaining, primary neurons were fixed using ice-cold paraformaldehyde (4% in PBS, VWR) for 10 min at 4 °C, and washed with PBS twice. Nonspecific biding was blocked by 6% bovine serum albumin (Sigma) for 30 min at room temperature and cells were washed in PBS. Primary antibody anti-TRPC1 (1:200, Alomone Labs), anti-TRPM4 (1:200, Alomone Labs) and anti-TRPP2 (1:200, Alomone Labs) were diluted in 1.5% bovine serum albumin, and incubated with cells at 4 °C overnight. After washing with PBS for 3 times, secondary antibodies (Alexa Fluor 488 (1:500, Invitrogen) that were diluted in 1.5% BSA were loaded to neurons for 1 h at 37 °C. Cells were washed with PBS, and imaged using a confocal microscope (FV3000, Olympus).

*Pharmacological treatments with chemical blockers and peptide inhibitors of ion channels*

To identify the cellular pathway involved during the optoacoustic modulation, chemical blockers or peptide inhibitors were used to block specific ion channels or modify cellular properties. To investigate how synaptic inputs affects stimulation outcome, we applied a cocktail of synaptic blockers (final conc: 10 µM NBQX, 10 µM gabazine, and 50 µM DL-AP5, Abcam, MA, USA) to the neuron culture 30 min before imaging. To block voltage-gated sodium channels, tetrodotoxin citrate (ab120055, Abcam, MA, USA) was added to the culture to reach 3 µM final concentration 30 min before Calcium imaging. The following blockers were added to medium and incubated with cells for 4h before stimulation cells with optoacoustic: Actin filaments were depolymerized by their specific inhibitors, cytochalasin D (final conc.: 10 µM). Gadolinium was applied to nonspecifically block the mechanosensitive ion channels (final conc.: 20 µM). Ruthenium red (final conc.: 1 µM) was used before ultrasound stimulation to block TRP channels (TRPV1, 2, 4) and TTA-P2 (final conc.: 3 µM) was added to block T-type calcium channels. To inhibit GPCRs, suramin was added (final conc.: 60 µM). GsMTx4 was added to medium (final conc.: 10 µM) to inhibit Piezo1 and TRPC1 channels.

**Conclusion and Discussion**

In this work, we investigated the biomolecular mechanisms of optoacoustic cell modulation, showing that optoacoustic excites primary cortical neurons through specific calcium-selective mechanosensitive ion channels with the assistant of calcium amplifier channel and voltage-gated channels. First, the synaptic transmission is demonstrated to be involved during the optoacoustic stimulation, while the direct effect on synapses still needs further investigation. In the future work, synaptopHluorin (spH) transfected hippocampal brain slice or neurons can be used. The spH serves as an pH-dependent fluorescence probe of synaptic vesicle exocytosis, which reflects neurotransmitter release through an increase in fluorescence signal when protons are released from synaptic vesicles during the fusion process [24, 33].

As our key findings, pharmacological inhibition of specific ion channels leads to reduced responses, while over-expressing TRPC1, TRPP2 and TRPM4 channels results in stronger stimulation. These results shed new insights into the mechanism of ultrasound neurostimulation.

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