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Sonogenetics: Recent advances and future directions

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ABSTRACT

Sonogenetics refers to the use of genetically encoded, ultrasound-responsive mediators for noninvasive and selective control of neural activity. It is a promising tool for studying neural circuits. However, due to its infancy, basic studies and developments are still underway, including gauging key *in vivo* performance metrics such as spatiotemporal resolution, selectivity, specificity, and safety. In this paper, we summarize recent findings on sonogenetics to highlight technical hurdles that have been cleared, challenges that remain, and future directions for optimization.

1. Introduction

The ultimate goal of neuroscience is to probe brain function and treat dysfunctions [1–3]. In order to achieve the goal, it is essential to have a tool capable of manipulation of neural activities on demand. In addition to being safe and efficacious in humans, the ideal tool for such task would have high spatiotemporal resolution, noninvasive access to the whole brain, and cell-type selective control. Optogenetics, or the use of light to activate target neurons or other cell types defined by artificially expressed photosensitive ion channels, has allowed neuroscientists to dissect previously unexplored neural microcircuits in mice [2,4]. Despite its potential, it is not ideal for use in humans for neuromodulation, because it requires implantation of optical fiber for delivery of light to the target region within the highly scattering brain tissue. Therefore, further efforts have been devoted to developing a non-invasive cell-type selective stimulation method. Researchers have explored changing the stimulation modality to something that is less limited in penetration than light (X-ray, magnetic wave, small molecules, and ultrasound) and also other more sensitive optogenetic mediators (red opsins, upconversion nanoparticle-based excitation) [5–9]. The ultrasound-based strategy, sonogenetics, in particular has gained a lot of attention in recent years [10]. An analogue of optogenetics, sonogenetics utilizes ultrasound wave to open exogenous ultrasound sensitive mediators/actuator in target neurons or other cell types to control their activity. Sonogenetics involves two key components: 1) ultrasound wave and 2) ultrasonic mediator/actuator. For simplicity, we will use mediator to refer mediator/actuator hereafter.

The first component, ultrasound, is a form of mechanical wave that can traverse biological tissues at sub-MHz to several MHz frequencies. Ultrasound has long been used for imaging as it is safe and cost-effective with great spatiotemporal resolution. Over the years, more and more evidence has been emerging that low-intensity ultrasound results in various bio-effects [11,12], paving way for new applications such as cancer treatment, bone healing, gene and drug delivery, and neuromodulation [11,13]. Ultrasound acoustic waves generated by piezoelectric material driven at a certain frequency and voltage can be propagated through an elastoviscous medium through particle displacement up to ~20 nm. The propagation can actively or passively form an acoustic pressure gradient based on standing waves, absorption, and tissue acoustic property gradients and introduce a non-zero net force to the medium known as ultrasound radiation force [13]. Concurrently, the particle oscillation may disperse the mechanical energy through heat generation. In some cases, the negative pressure phase of the

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ultrasound waveform may have an additional bio-effect that results in extraction of dissolved gas from tissue-a process known as cavitation [14]. Along with the aforementioned major physical effects, other effects like microstreaming [15–17], sonochemical effects [18,19], and recently proposed capacitance change induced by off-phase oscillation of lipid bilayer cell membrane [20,21] could come into play depending on the ultrasound wave used and the surrounding media. As of now, there is no clear knowledge on which sets of parameters can be used to reliably generate each of those effects, which in turn makes it challenging to clarify the exact mechanism involved in observed bio-effects. Yet, the advantages of using the resulting bio-effects to activate neurons are clear: 1) it is safe to use ultrasound in biological systems, 2) the propagation of ultrasound wave is fast and easily controlled, especially with an array system, in which the ultrasound focus can be programmed to create a specific spatiotemporal pattern on demand. Ultrasound can serve as a unique excitation source to control neural activity across the whole brain with a defined spatiotemporal pattern.

The second component of sonogenetics is the mediator. It was first found in 2008 that ultrasound can induce synaptic transmission by activating membrane channels [22]. Leading hypotheses for the putative effects of ultrasound on neurons is that pressure can either deform the cell membrane to indirectly produce conformational changes in channel structure or act directly on the channel constituents to create ion gradients across the membrane [23–25]. A recent study showed that when ultrasound deflects the cell membrane, it induces neural depolarizations by activating mechanosensitive ion channels [26]. A comprehensive summary about ultrasound's effects on ion channels can be found in previous review papers [11]. Recently, a considerable number of channel types that are preferentially activated by pressure changes, such as the two-pore potassium (K2P) family (TREK-1, TREK-2 and TRAAK) [27], the Piezo family (Piezo1 [17,28–30] and Piezo2 [31]), and TRP family (TRPA1 [32], TRPC1 [33], TRPP2 [33], TRPM4 [33] and TRPV1 [34]), have been shown to involved in ultrasound neuromodulation *in vitro* and *in vivo*. Although which channels play the dominant role is unknown, these findings set a solid foundation for developing sonogenetics using mechanosensitive ion channels as mediators.

The control of ultrasound beam has been extensively studied in the ultrasound neuromodulation field while the investigation on mediator responses is extremely limited. We are not going to cover this aspect but we will focus more on recent developments in sonogenetics from our research groups and others to demonstrate how some obstacles have been surpassed to give way to new scientific insights.

2. Working mechanism

Sonogenetics induces cell-type specific neuromodulation by genetically inserting exogenous mediators that make target neurons hypersensitive to ultrasound [10,35]. Ultrasound's effect on current dynamics can be described by the Hodgkin-Huxley model as in eq (1) and eq (2). V_m is the resting membrane potential, and C_m is the membrane capacitance.

$$C_m\frac{dV_m}{dt} = I_{ionic} + I_{stim} \tag{1}$$

$$I_{stim} = I_{ex} + I_{end} \tag{2}$$

$$I_{end} = N_{end}g_{end}p_{end}(u)(V_m - E_m) \tag{3}$$

$$I_{ex} = N_{ex}g_{ex}p_{ex}(u)(V_m - E_m) \tag{4}$$

I_{ionic} is the current for all endogenous channels that are not ultrasound sensitive, while I_{stim} is the current induced by ultrasound which include endogenous ultrasound responsive component I_{end} and exogenous ultrasound responsive component I_{ex} as expressed with eq (2). The I_{end} and I_{ex} can be expressed as eq (3) and eq (4). E_m is the rebound membrane potential, and N_{end} and N_{ex} are number of endogenous and exogenous ion channels expressed in the target neuron. g_{end} and g_{ex} are the single channel conductance of the endogenous and exogenous ion channels. p_{end} and p_{ex} stand for probability of the ion channels staying open. The ultrasound induced current dynamic can be rewritten as eq (5)

$$C_m\frac{dV_m}{dt} = I_{ionic} + (N_{end}g_{ex}p_{ex}(u) + N_{ex}g_{end}p_{end}(u))(V_m - E_m) \tag{5}$$

To induce specific stimulation of the target neurons while keeping the other neurons silent, the ultrasound induced endogenous and exogenous currents should meet the requirement defined by eq (6) and eq (7).

$$(N_{ex}g_{ex}p_{ex}(u) + N_{end}g_{end}p_{end}(u))(V_m - E_m) > I_{th} \tag{6}$$

$$N_{end}g_{end}p_{end}(u)(V_m - E_m) < I_{th} \tag{7}$$

From these two equations, it is clear that the selectivity is determined by the number ion channels, individual channel conductance, and opening probability of the exogenous ion channels in the targeted neurons. The opening probability p_{end} and p_{ex} increases with ultrasound stimulation which can determined from patch clamp studies. I_{th} is the threshold for activating action potential, which is dependent on the resting membrane potential. V_m and E_m are the electrophysiological properties could vary across different neural types.

3. Mediators of sonogenetics

A breakthrough on mediators came from a proof-of concept study on *Caenorhabditis elegans* (*C. elegans*) by Ibsen et al. (2015). They showed that low-frequency ultrasound at 2 MHz can activate neurons in *C. elegans* and induce behavioral changes in the presence of microbubbles [35]. It was found that TRP-4, a pore-forming subunit of a mechanotransduction channel, is required to mediate the neuromodulatory effects. Artificially overexpressing TRP-4 in ASH and AWC neurons could make those neurons more sensitive to ultrasound and induce large reversal behaviors [35]. Because microbubbles are needed to amplify the mechanical effects of ultrasound on TRP-4, its use was not translated to mammalian models. Efforts have been made to identify a more sensitive genetic mediator for sonogenetic stimulation that does not require the use of microbubbles. In the following sesstion, we summarized the major work on developing Sonogenetics *in vivo.* We grouped them according to the nature of the mediator used and their order appeared in our discussion are based partially on the time of the work published.

a. Large Conductance Mechanosensitive Ion Channel (MscL) channels for mediating sonogenetics

The Large Conductance Mechanosensitive Ion Channel (MscL) family consists of pore-forming membrane proteins that can translate physical forces applied to cell membranes into electrophysiological activities. MscL channels have a relatively large conductance, 3 nS, making them permeable to ions, water, and small proteins when opened [36,37] (Fig. 1A). MscL acts as stretch-

activated osmotic release valve in response to osmotic shock [38,39]. In 2018, Ye et al., showed that MscL-I92L, a MscL mutant with higher mechanosensitivity, can successfully mediate sonogenetic stimulation with high frequency surface acoustic waves *in vitro* in primary cultured neurons [40]. They used patch clamping to demonstrate that neurons with MscL-I92L fire in response to acoustic wave stimulation. The use of surface acoustic waves successfully eliminated the confound induced by the glass pipette vibration and enabled high temporal resolution. The results convincingly demonstrated that sound waves can elicit optogenetics-like responses at 1–5 Hz range. Later on, Qiu et al. (2020) provided complementary evidence with calcium imaging. Low frequency longitudinal ultrasound waves (with deep tissue penetration) activated *in vitro* neurons with MscL-G22s [10], another mutant of MscL with comparable mechanosensitivity to MscL-I92L and less spontaneous channel opening (Fig. 1B). Furthermore, it was shown that overexpression of MscL-G22s can sensitize the neurons in motor cortex to ultrasound as shown by electromyography (EMG) [10,41]. At low acoustic pressures (Fig. 1C), ultrasound can specifically activate MscL-G22s expressing neurons, while the response becomes unspecific with higher acoustic pressures (>0.45 MPa). More importantly, whole brain *c-fos* (a widely used neural activation marker) staining results showed that only MscL-G22s expressing brain regions had significant neural activation, while neurons in other regions do not have clear neural activation (Fig. 1D). These studies demonstrated the feasibility of MscL-sonogenetics.

More recently, a follow-up study by the same research group showed that MscL-G22s used fiber photometry to show that sonogenetic stimulation can effectively induce neural activation in deeper brain regions [42]. Ultrasound triggered activity in MscL-expressing neurons in the dorsal striatum without increased activation in the neighboring regions and increased locomotion in freely-moving mice. Stimulating the mesolimbic pathway using MscL-G22s sonogenetics can trigger dopamine release in nucleus accumbens and modulate appetite conditioning. It was shown that MscL-G22s sonogenetics is circuit-specific, and the stimulation is sufficient to induce defined behavior changes. At the same time, Cadoni et al. utilized the same mediator MscL-G22s to make retinal ganglion cells and visual cortex neurons sensitive to ultrasound stimulation. The study confirmed the feasibility of MscL-G22s sonogenetics with low frequency ultrasound [43]. In addition, the results showed that higher frequency ultrasound at 2.2 MHz and 15 MHz can also be used to improve the spatial resolution. It was further shown that this technique is compatible with millisecond pattern presentation task for visual restoration at the cortical level. It suggested that MscL-G22s sonogenetics is a promising tool for vision restoration and treatment of other brain diseases.

A) Structural of MscL [44].
B) Schematic diagram of sonogenetics [10].
C) GCaMP6s fluorescence change in WT neurons and MscL-G22s expressing neurons induced by ultrasound with different acoustic pressure [10].
D) Ultrasound can activate neurons expressing MscL-G22s in deep brain regions but not other regions [10].

It must be noted that the large opening pore of MscL channels could be a potential issue, as it not only passes the ions and water molecules, but also some proteins. Cellular toxicity must be tested carefully [45]. Additionally, these channels can interact with other ion channels or with themselves. For example, MscL-G22s may form clusters with reduced sensitivity to protect the cells from long lasting mechanical stimulation [38]. Qiu et al. (2020) showed that the *in vivo* expression of MscL-G22s in neurons mostly form clusters [10]. The role of the cluster is still unclear. More studies are needed to understand the full extent of MscL's actions.

b. Ultrasound responsive proteins/nanoparticles for localized response amplification

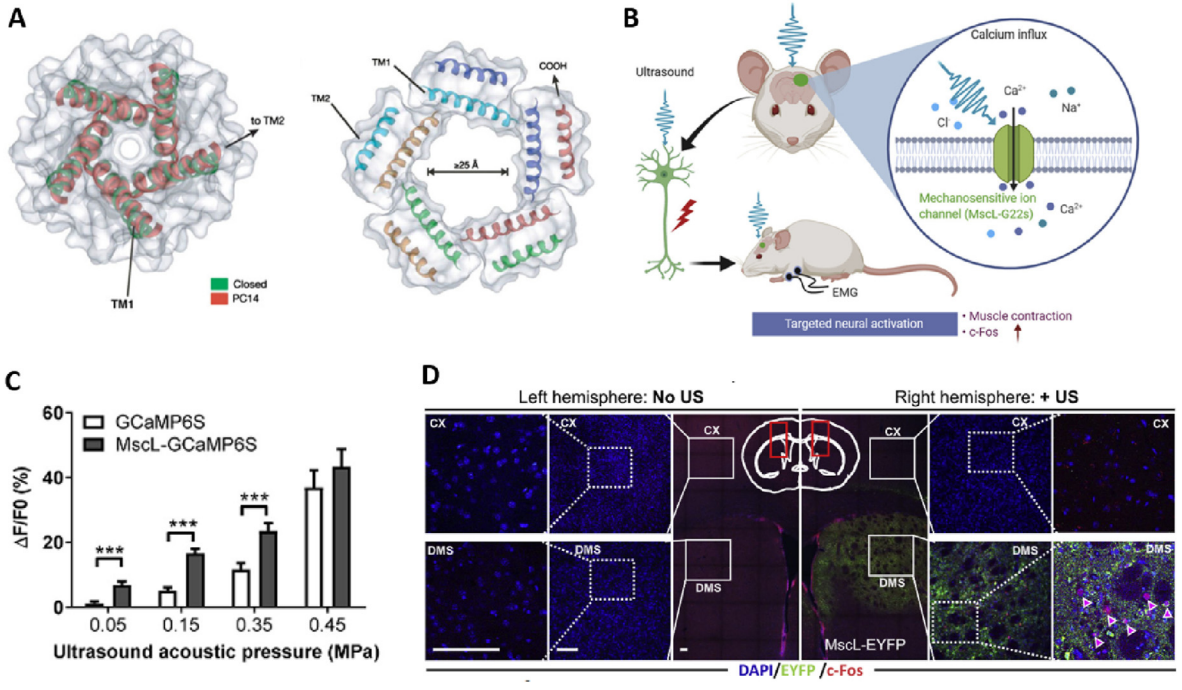

Fig. 1. MscL-G22s mediated sonogenetics.

Alternatively, an engineered prestin, an auditory-sensing protein, can be used to mediate cell-type-specific ultrasound neuromodulation in the mouse brain [46]. Huang et al. (2019) first screened for ultrasound effects on prestin in six nonecholocating and eight echolocating species. They confirmed that mPrestin mutants N7T and N308S containing two ultrasound sensing substitutions are evolutionally conserved in echolocating species and can effectively sensitize cells to ultrasound *in vitro* as shown using calcium imaging. They further expressed mPrestin (N7T, N308S) in ventral tegmental area neurons *in vivo* and stimulated the neurons with ultrasound. The *c*-fos expression results showed that the mPrestin (N7T, N308S) can sensitize neurons to ultrasound. More importantly, it was found that the activation of was specifically efficient at 0.5 MHz and no other frequencies often used in neuromodulation studies (Fig. 2B). The reason why mPrestin is sensitive to 500 kHz ultrasound is not well understood. In their follow up study, they used this approach to treat Parkinson's disease (PD). When mPrestin (N7T, N308S) was expressed in dopaminergic neurons, ultrasound was shown to induce neurotrophin expression in PD mice, thereby improving PD-related symptom [47].

Prestin amplifies the deformation of cell membranes, and in turn amplifies endogenous mechanosensitive ion channels activation leading to neural firing. It serves as an amplifier/actuator for activating endogenous mechanosensitive ion channels which provides a plausible explanation for the abovementioned 500 kHz sensitivity as it is widely observed *in vivo* that ultrasound neuromodulation seems to work better at 500 kHz than other frequencies [48,49]. There are two challenges to using prestin as a sonogenetic mediator. Firstly, because it is not an ion channel, the results of prestin-based sonogenetic stimulation depend on the expression profile of endogenous mechanosensitive ion channels, which is not well-understood. A possible solution to this problem is co-expressing prestin with exogenous mechanosenstive ion channels such as MscL-G22s, making it less dependent on intrinsic properties. Secondly, prestin can mediate the electromotility in the cells. As shown in Fig. 2(C-E) from Refs. [50,51], expressing prestin in HEK 293t cells can make the cells gain electromotility. As neurons can fire spontaneously with quick membrane potential changes from −70 mV to 40 mV. Therefore, the existence of prestin can induce the cell membrane defamation with large cell volume change without ultrasound stimulation.

A) A schematic of prestin based sonogenetics [46].
B) Prestin sonogenetics is specific to 0.5 MHz ultrasound [46].
C) Structure of and the proposed mechanism of electromotility of prestin [51].
D) Dolphin prestin mediated cell electromotility shown in HEK 293t cells [51].
E) Membrane voltage dependence of electromotility of prestin in HEK 293t cells [51].

Hou et al. (2021) developed a similar strategy for targeted deep ultrasonic brain stimulation using biogenic gas vesicles (GVs) as an actuator (Fig. 3A) [52]. GVs are nano-sized protein structures extracted from cyanobacteria (Fig. 3B). They have hollow protein shells with an impressive ability to enhance ultrasound contrast, making them a unique nanosized ultrasound contrast agent [53]. Their oscillation behavior in acoustic field can be used to induce mechanical perturbation in the surrounding tissue. It was shown that GV-ultrasound stimulation can activate neurons robustly *in vitro*, and the neural responses were directly related to the stimulation frequency, indicating fine temporal resolution (Fig. 3C and D). A potential mechanism is activation of mechanosensitive ion channels, which consequently stimulate neurons *in vivo* as shown by *c*-fos staining and fiber photometry recordings [52]. Overexpression of exogenous mechanosensitive ion channels (MscL-G22s) in neurons can further enhance the response *in vitro*, suggesting a potential dual strategy for cell-type-specific neural stimulation.

This strategy shares the same issues as prestin, which can be overcome by co-expression of exogenous mechanosensitive ion channels. In addition, the GVs must be transcranially injected into the brain region. To make it widely accessible to the neuroscience community, it is important to express it in neurons or non-invasively deliver to the brain region via blood-brain barrier opening. It is still challenging to genetically express gas vesicles in mammalian cells since multiple genes are involved in its formation.

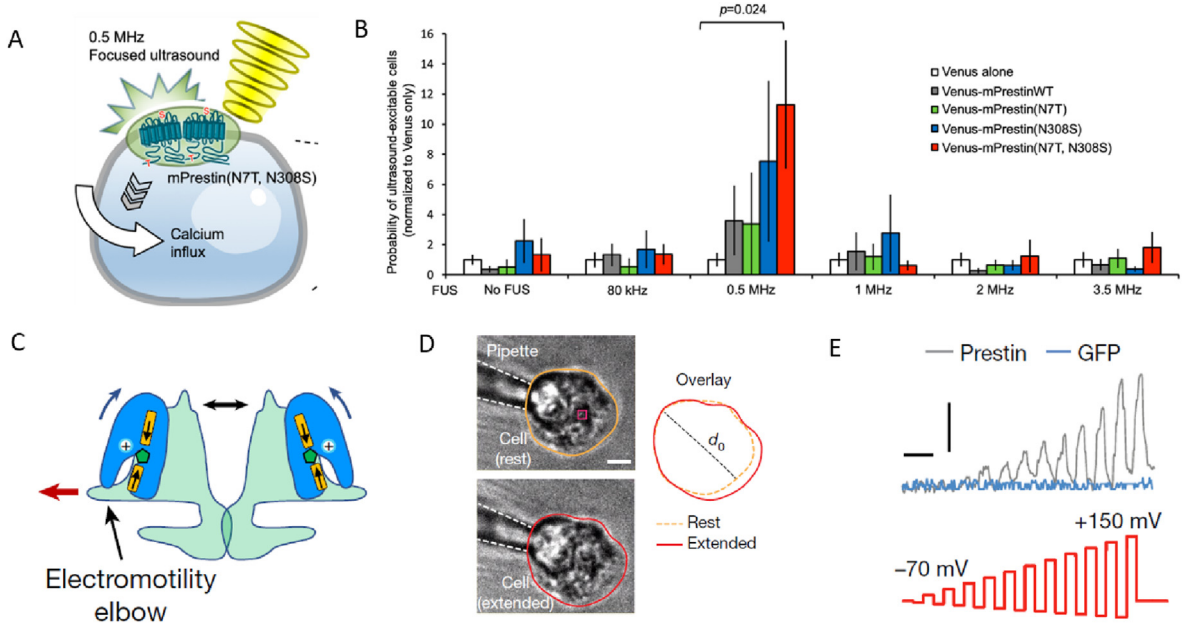

Fig. 2. Prestin sonogenetics.

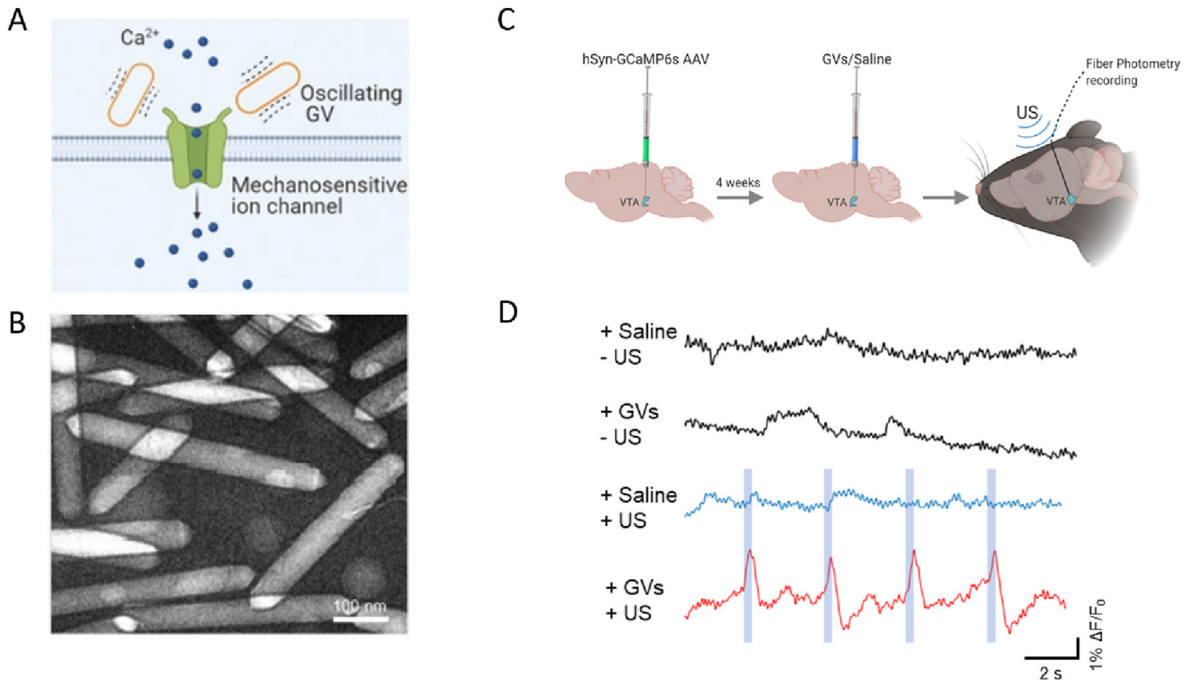

Fig. 3. Gas vesicle mediated stimulation [54].

It is worth to explore the possibility of delivery of GVs through BBB opening via vein injection.

A) A schematic of gas vesicle mediated ultrasound neuromodulation.
B) Structure of gas vesicles measured by EM.
C) *in vivo* paradigm for testing the efficacy of gas vesicle mediated ultrasound neuromodulation.
D) GCaMP6s fluorescence change induced by ultrasound only and gas vesicles mediated ultrasound neuromodulation.
c. Transient receptor potential (TRP) channels for mechano-thermal genetics

TRP channels play an important role in many important physiological processes, including pain, touch, and thermal sensation. TRPV1, a well-characterized channel with thermal sensitivity, has been artificially overexpressed in neurons to mediate targeted neural stimulation by photothermal [55], magnetothermal [56–58], and most recently, ultrasonic thermal means [34]. When a tissue absorbs ultrasound, it may convert some mechanical energy to heat. There is concomitant temperature rise, especially with high frequency and long duration ultrasound as suggested by a simulation study [59]. A study demonstrated that ultrasound neuromodulation at 3.2 MHz can induce a 2 °C temperature rise in deep brain regions, as measured by ultrasound thermometry [60]. The ultrasound frequency used in that study (3.2 MHz) is higher than what is normally used for neuromodulation studies (~500 kHz), resulting in more deposited energy in the targeted region. Yang et al. (2021) demonstrated that the local heating in mouse brains induced by ultrasound can be visualized by magnetic resonance thermometry and is controllable by changing the ultrasound frequency and pulse length. They showed that the well-defined ultrasound thermal effects can effectively activate neurons expressing TRPV1 *in vitro* and *in vivo* in mice as shown by calcium imaging using continuous wave ultrasound (1.7 MHz, 1.3 MPa, 1 s) that induces a 1 °C temperature rise [34,61]. This approach with high cell-type specificity, millimeter spatial resolution, and temporal resolution within several seconds (<5 s) is sufficient to be used to control locomotion of freely-moving animals.

In the same vein, Duque et al. (2022) screened for ultrasound responsive proteins from 191 candidate proteins. It was shown that mammalian TRPA1 channels respond dramatically to ultrasound stimulus at 6.9 MHz [62,63]. Interestingly, they found that the human homolog was the most effective candidate while mouse homolog was only a third as responsive as *hs*TRPA1. The TRPA1 N-terminal tip domain might be crucial for its ultrasound sensitivity. When they overexpressed *hs*TRPA1 in neurons *in vitro*, they showed significantly higher ultrasonic sensitivity compares to wildtype neurons. An *in vivo* experiment confirmed that expression of *hs*TRPA1 in the motor cortex can sensitize neurons to ultrasound, as shown by more muscle contractions on contralateral sites. A *c*-fos staining also confirmed that the neural activation by *hs*TRPA1-sonogenetics is well confined in *hs*TRPA1-expressing neurons.

As summarized in Table 1, these studies suggested that sonogenetics is feasible across various acoustic parameters with different types mediators. Their efficacies have been tested from *in vitro* calcium imaging, *in vitro* MEA, *in vivo* fiber photometry, *in vivo* two photon microscopy, c fos immunostaning, and behavior test *in vivo*. In the following session, we discussed their key performance including selectivity, specificity, spatial and temporal resolution.

4. Selectivity and specificity of sonogenetics

The selectivity of sonogenetic neural activation mainly relies on genetic manipulations of promoters, enhancers of the specific targeting sequences of the chosen sonogenetic mediator-encoding DNA to manipulate specific types of neurons through the use of Cre-driver lines. The proof of concept studies usually used simple promoter such as hSyn and CamKIIa. The selectivity was validated by comparing the responses between selected cells expressing mediators and control cells. It is worth to note that Cre-LoxP system may have leakage effects in untargeted cells depending on viral concentration used [66,67]. It is suggested to characterize the

Table 1
Mediators for Sonogenetics *in vivo*.

Mediator	Nature of the mediator	Frequency (MHz)	Acoustic pressure (MPa)	Promoter	Validation method	References
MscL-G22s	Mechanosensitive ion channel	0.5, 2.25, 15	>0.3	hSyn, CaMKII, SNCG,	Cellular calcium imaging, EMG, fiber photometry, MEA, EcoCG, Behaviors	[10,41,64,65]
TRPV1	Thermal sensitive ion channel	1.5, 1.7	>0.9	CaMKII	In vitro calcium imaging, *in vivo* two photon calcium imaging, Place preference behaviors	[34]
*m*Prestin	Membrane protein for electromobility in hair cell in ear	0.5	>0.5	hSyn	Cellular calcium imaging *in vitro*, c fos staining	[46]
*hs*TRPA1	Ion channel	7	>1.05	hSyn DIO, hSyn. Cre	EMG, c-fos staining, Behaviors	[63]
Gas vesicles	Nano-sized protein structure	1	0.2	N.A.	fiber photometry, EMG, c fos staining	[54]

effects of viral concentration for the actual neuroscience applications [68]. On the other hand, the specificity of sonogenetic neural activation defined by eq (6) and eq (7) mainly relies on how the off-target effects were suppressed. Qiu et al. (2020) and Cadoni et al. (2021), showed that MscL-G22s based Sonogenetics are specific to targeted cells with 500 kHz ultrasound stimulation below 0.45 MPa [10,64]. Increasing acoustic pressure can induce unspecific activation in control cells. Interestingly, Cadoni et al. (2021) showed that sonogenetics with 2.25 MHz ultrasound dose have minimal stimulatory effects on WT neurons compares to 0.5 MHz and 15 MHz ultrasound in the ultrasound pressure range they tested [64]. Similarly, the *hs*TRPA1 based showed unspecific neural activation *in vitro*, while the *in vivo* EMG response is specific to sonogenetic stimulation [63]. The TRPV1 based Sonogenetics using ultrasound at 1.7 MHz showed highest specificity both *in vitro* and *in vivo* [34]. It is crucial to further improve the selectivity and specificity of Sonogenetics for the future applications.

5. Spatial and temporal resolution of sonogenetics

Spatial resolution of Sonogenetics can be turned by: 1) spatial resolution of ultrasound focusing, 2) spatial distribution of sonogenetic mediators and their combination [69]. Cadoni et al. (2021) nicely showed that the spatial resolution of Sonogenetics can be adjusted up to by using focused ultrasound at different frequencies (up to 0.59 mm at 15 MHz). The holographic acoustic lens, transducer arrays can be used to improving the spatial resolution for sonogenetic stimulation. Temporal resolution is another crucial factor for neuroscience application as neurons may firing in their own rhythms to give rise to brain functions. The standard method for characterizing the temporal responses of neural firing is patch clamping [17,70]. To facilitate the use of patch clamping technique, high frequency ultrasound (>40 MHz) or surface acoustic wave must be used. An *in vitro* patch clamping study showed that ultrasound (43 MHz) can activate Piezo1 in HEK 293T cells with instant inward current. On the other hand, MscL-I92L sonogenetic stimulation at 5Hz achieved a neural stimulation success rate of 50%. Alternatively, calcium imaging and behavior readouts can be used to obtain results with sub-second resolution. An *in vivo* study showed that the latency of muscle contraction induced by MscL-G22s sonogenetic stimulation of the motor cortex is about 200 ms (Fig. 4B). A recent study tested the direct neural responses by measuring the calcium dynamic, dopamine release (Fig. 4C), and electrocochleography (Fig. 4D) in retinal ganglion cells, visual cortex, and sub-cortical regions. These metrics also had high temporal resolution that adequately assessed the effect of MscL-G22s sonogenetics. Also, place preference behavior task had a sub-second temporal resolution (Fig. 4E). TRPA1 sonogenetics has also been assessed using sub-second resolution. These studies suggest that sonogenetic approaches are capable of neural stimulation at high temporal resolution [71]. On the other hand, the results of sonothermogenetics has only been assessed at several seconds resolution [34]. The temporal resolution of sonogenetics covers a wide range from sub-second to seconds, depending on the strategy used. This is a great advantage compares to current available non-invasive cell type selective neuromodulation approach chemogenetics which utilizes vein injected small molecules to activate selected neurons is a widely used non-invasive cell type selective whose temporal resolution is in the range of hours which is passively determined by the pharmacokinetics of the small molecules used [6,72].

In the following section, we will discuss the challenges and future directions for sonogenetics.

A) Surface acoustic wave ultrasound stimulator is compatible with patch clamping. It can stimulate MscL-I92L expressing neuron *in vitro*. The following frequency is up to 5 Hz [40].
B) MscL-G22s Sonogenetics stimulates neurons in motor cortex and induces well controlled muscle contraction [10].
C) Measured by fiber photometry, MscL-G22s neurons showed higher sensitivity compared to WT neurons. The latency is around 200 ms [42].
D) MscL-G22s sonogenetic stimulation of visual cortex showed millisecond resolution as measured by μEcoGarray [64].
E) Given the high spatiotemporal resolution of MscL-G22s sonogeneitcs, it is feasible for controlling place preference behavior [42].

6. Challenges

Possible off-target effects accompanying sonogenetics.

The peripheral auditory system may be activated inadvertently by transcranial ultrasound, which could result in off-target neural activation confounding the selective neural control by sonogenetics. It has been shown that transcranial ultrasound, despite having a central frequency well above the hearing range, can produce auditory responses in cats [73], mice [74], and guinea pigs [75]. It was hypothesized that the peripheral auditory system activation is due to the rectangular ultrasound signal envelope [76]. In this study, auditory brainstem responses (ABR) were present at the onset and offset of the ultrasound, suggesting that the sharp onsets and offsets with frequency components in the audible range stimulate the peripheral auditory system. Smoothing the onsets and offsets eliminated the auditory brainstem responses. Thus, waveform smoothing should be strongly considered in future sonogenetic studies.

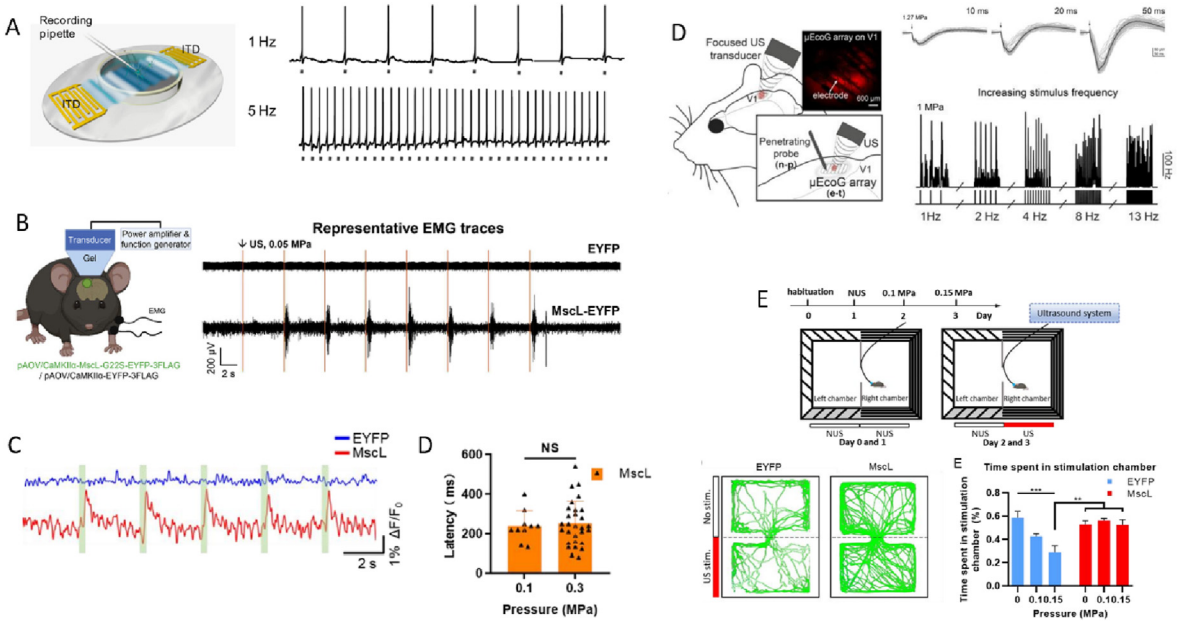

Fig. 4. Temporal characteristics of sonogenetics.

Ultrasound can also activate endogenous mechanosensitive ion channels, such as Piezo1, TRPA1, TRPC1, and TRAAK. Assuming the endogenous neuron's sensitivity to ultrasound is uniform across different brain regions, a deeper brain region could be a better target for sonogenetics. As shown in Fig. 5A, the ultrasound intensity decreases exponentially as it travels through tissue due to scattering. Assuming we have neurons expressing MscL in superficial cortex and deep brain regions as shown in Fig. 5B. The cross-section of ultrasonic effects on ion channels can be expressed by *in situ* ultrasound intensity times ultrasound sensitivity of the neurons at given depth. The cross-session is shown in Fig. 5C. As we can see, there could be background activation comparable to sonogenetic activation at cortical region. As it gets deeper, MscL expressing neurons are more likely to be activated specifically. Hence, focusing the ultrasound beam in the axial direction with transducer arrays, keeping the ultrasound intensity low at cortical region in the ultrasound path, or increasing the sensitivity of the mediators could be a good way to limit the ultrasound effects to target neurons. It is important to note that the actual sensitivity of neurons to ultrasound across brain regions may not be homogeneous. Thus, it is important to characterize sensitivity as well as specificity to ultrasound in different brain regions.

A) Ultrasound intensity or pressure exponentially decays with depth.
B) Sensitivity to ultrasound for wildtype and and MscL neurons, assuming background sensitivity mediated by endogenous mechanosensitive ion channels is relatively homogeneous. There are superficial region and deep region expressing MscL.
C) Sensitivity times ultrasound intensity as a metric for probability of neural activation. The specificity increases with depth.

Optimize ultrasonic sensitive ion channels and inhibitory ion channels.

The specificity can be further improved by using additional ultrasonic-sensitive ion channels or novel mediators. In order to be a suitable mediator, first, it can be activated by ultrasound rapidly and repeatedly. Second, the activation of the candidate mediator should be independent of membrane potential, endogenous ion

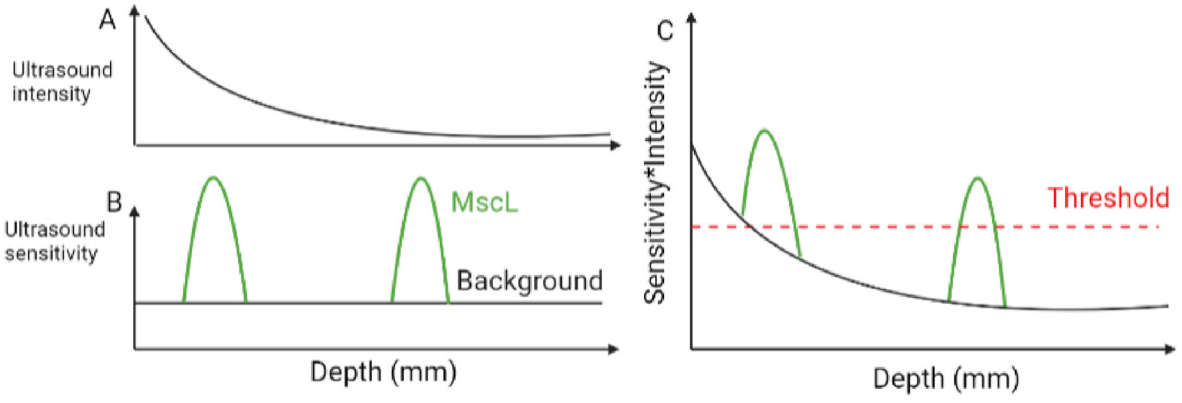

Fig. 5. Depth dependent specificity.

channels or other uncontrollable physiological parameters. For example, the spontaneous neural firing changes the membrane potential from −40 mV to 20 mV, while the expression profile of endogenous mechanosensitive ion channels in the central nervous system is still unclear. Voltage- or other signaling-pathway-dependent mediators could introduce unpredictable outcomes or bias the intrinsic neural networks. Third, it should have a low probability of spontaneous activation, as it could be toxic to the neuron.

To obtain an optimal mediator, two strategies could be used. 1) Top-down screening from a large library of different ion channels and variants. This strategy does not rely on prior knowledge on how ultrasound interacts with ion channels but rather relies on a robust readout to tell whether the channels can be activated by ultrasound or not. The challenge is the patch clamping cannot be used since it is not compatible with low frequency longitudinal ultrasound wave stimulus. Thus, calcium imaging, which could be less sensitive or unable to screen for inhibitory ion channels, has to be used. Developing a voltage imaging-based method or ultrasound-compatible patch clamping is crucial. 2) Bottom-up channel engineering based on prior assumptions or knowledge. For example, based on the data that ultrasonic can activate Piezo1, the Piezo1.1 and Piezo2.1 isoforms with improved sensitivity and enhanced single-channel conductance would be promising candidate mediators for sonogenetics [77]. Ultrasound sensitive potassium or chloride channels are important to look into as well since they can potentially serve as mediators with inhibitory effects, making bidirectional control of neural activity possible. A structured hypothesis-driven development of sonogenetics mediators will not only allow us to engineer novel variants with desired mechanosensitivity and ion selectivity but also offer us the opportunity to gain deep insights into its activation and regulatory mechanisms.

7. Future directions

Computational sonogenetics.

Here, we suggest a new approach we call computational sonogenetics based on the working mechanisms we proposed that studies how different ultrasound pulsing schemes can influence ion channel dynamics and neural firing. It can aid in a quick and comprehensive computational prediction of the behavior of sonogenetic mediators and their influence on a wide range of ion channels, cell types, and tissues. Such simulations can also drive the design and characterization of sonogenetic tools optimized for specific cell/tissue environments. Furthermore, they can assist in developing suitable ultrasound parameters for achieving more selective and specific neural activations.

A) A schematic of the four-state gating mechanism for Piezo1. The arrows indicate transitions between open (O), closed (C) and two inactivated states (I_1 and I_2). The e(p) and a(p) are rate constant transition from I_1, C to O states, respectively, which are modulated by the pressure applied to the plasma membrane, leading to membrane tension. The b and f are rate constant transiting from O, C to I_1 states. The g and h are the transition rate constant between O and I_2 states, while the d and c are the transition rate constant between O and I_1 states, which are constants. (B) Simulated Piezo1 open (black), closed (magenta), inactivation 1 (red) and inactivation 2 (blue) probabilities change in response to 100 ms sonication, and C) the consequent neural activation with firing of action potentials. D) An example of PRF-dependent neural excitation with given number of Piezo1 expression.

Results from our group and other researchers showed that ultrasound can activate Piezo1 in different neurons *in vitro*[6][30]. The Piezo1 current can be expressed as equations (8) and (9).

$$I_{piezo} = N_{piezo} g_{piezo} p_{piezo}(u)(V_m - E_m) \tag{8}$$

$$\dot{p}_{piezo} = a(p)C_{piezo} + I_1 d + I_2 h - (b + c + g)p_{piezo} \tag{9}$$

$\dot{p}_{piezo}$ is open probability of Piezo1 channel. As shown in Fig. 1A, the e(p) and a(p) are rate constant transition from I1, C to O states, respectively, which are modulated by the pressure applied to the plasma membrane, leading to membrane tension. The b and f are rate constants transiting from O, C to I_1 states. The g and h are the transition rate constant between O and I_2 states, while the d and c are the transition rate constant between O and I_1 states, which are constants. Incorporating equation (8)-(9) into the Hodgkin Huxley model gives us a simple sonogenetics model. The opening probability can be described as a function of ultrasound parameters, such as pressure, intensity, pulse length. For Piezo1, the activation is related to pressure and pulse length [30]. As shown in Fig. 6B, with 45 Piezo1 channels expressed in a single neuron, ultrasound can activate peizo1 and robustly induce neural firing. Given the fact that the Piezo1 expression is low, a lower expression condition can be simulated. As shown in Fig. 6C, for a lower expression condition (13 channels expression), the success of neural stimulation is dependent on the pulse repetition frequency. At 200–400 Hz, the opening of Piezo1 can induce neural firing, while below 100 Hz, there is no action potential. At 200 Hz, the latency of action potential is about 100 ms, which is in line with *in vitro* and *in vivo* results [10,33]. It is unclear why ultrasound induces a long latency response, but a leading hypothesis is that it has to do with some electrochemical response.

7.1. Noninvasive delivery of sonogenetic mediators

Due to the existence of the blood-brain barrier (BBB), the only feasible route for viral vector delivery is through direct brain infusion. The invasive infusion procedure inevitably causes damage to the brain regions of interest, and the infused viral vectors often experience backflow along the inserted cannula leading to insufficient gene expression. An alternative approach is to use transgenic animal models that express ultrasound-activated protein channels. Albeit it being non-invasive, it costs a lot to develop an animal model with this genome alteration. Recently, a new strategy based on engineered rAAV serotype, rAAV-PhP.eB which can infect neurons efficiently via vein injection, was developed and showed great potential for non-invasive delivery of genes [78]. Viral vectors encoding various light-activated protein channels delivered via this approach for optogenetics has allowed for an entirely non-invasive neural stimulation procedure *in vivo* [78]. Combined with the Cre-loxP strategy, sonogenetic mediators can be expressed in specific neural circuits non-invasively using rAAV-PhP.eB. Another possibility is using ultrasound based non-invasive gene delivery. With recent advancement in transcranial focused ultrasound (FUS) technology, non-invasive and targeted BBB opening has been done in several animal models [79]. This technique involves a systemic injection of a mixture composed of ultrasound contrast agents (lipid-based microbubbles) and molecules to be delivered. Ultrasonic waves can be propagated through the skull and cause the microbubbles to cavitate (to oscillate) within the capillaries in the target brain region. The basic principle is that it transiently loosens the tight junctions between endothelial cells, temporarily opening the BBB. The molecules of interest can then diffuse into the brain parenchyma down the concentration gradient. Taking advantage of the non-invasive nature of the FUS technique, a design and implementation of a FUS-facilitated gene delivery for sonogenetic applications has been reported [80]. More importantly, FUS-facilitated plasmids based gene delivery is also feasible which can

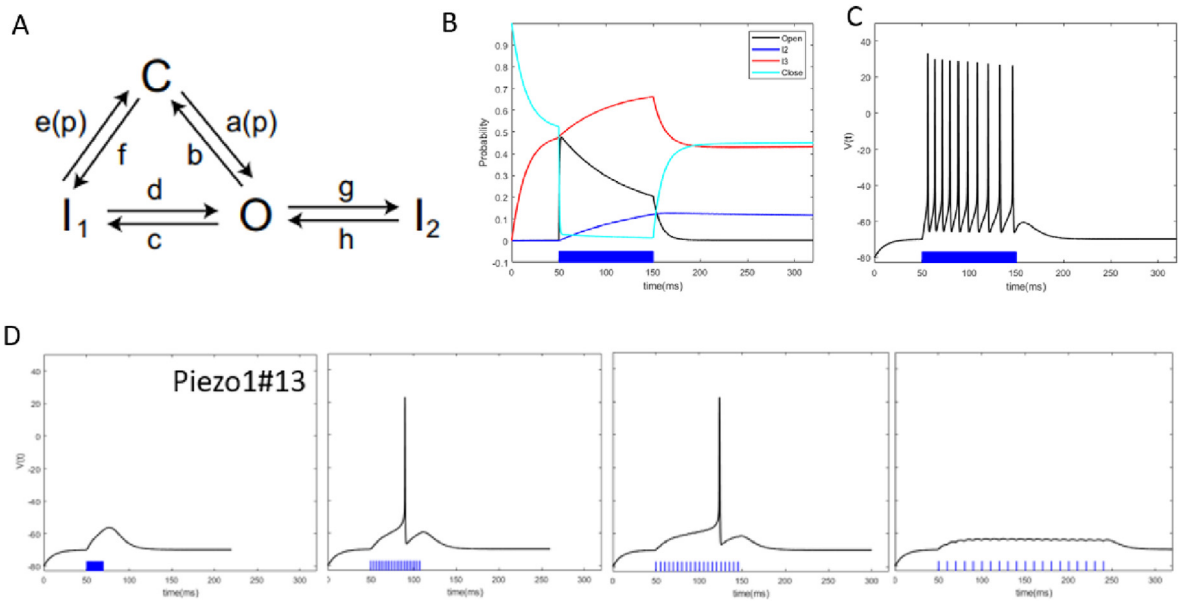

Fig. 6. Computational sonogenetics.

eliminate the ethical concerns about the use of virus during clinical translation of Sonogenetics and Chemogenetics.

7.2. Applications in nonhuman primates and safety

Monkeys are model organisms for neuroscience research. Neural activities in their central nervous system can be recorded and manipulated while they perform complex perceptual, motor, or cognitive tasks. Optogenetics has been used to manipulate neural activity in monkeys [81–83], but they come with unique challenges. A single animal is often used in multiple experiments, and optogenetic manipulations can cause irreversible damage to the brain [82,83], because light is typically delivered via optical fibers that are inserted into and removed from the brain area of interest each day. Sonogenetics allows an experimenter to target different neuronal populations by programming the movement of the ultrasound focus electronically within milliseconds. A hurdle for primate sonogenetics is that vector injections may trigger immune responses that reduce transduction efficiency. There are other issues similar to primate optogenetics, which have been previously reviewed [83].

8. Conclusion and remarks

Sonogenetics has a great potential for dissecting neural circuits and treating brain diseases noninvasively. Current strategies, which use MscL, TRPA1, Prestin and TRPV1 as mediators, proved sonogenetics feasible. We envision that new approaches, including voltage imaging and computational sonogenetics, could help better understand and screen for novel sensors.

Declaration of competing interest

Z.Q., J.G., J. Z., and L.S. have submitted a patent application titled "A Non-invasive method for selective neural stimulation by ultrasound" with the USPTO dated April 10, 2018, assigned application number 15/949,991, which relates to the ultrasound setup used in all the experiments. The authors declare no further financial interests.

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