

Ultrasound neuromodulation through nanobubble-actuated sonogenetics

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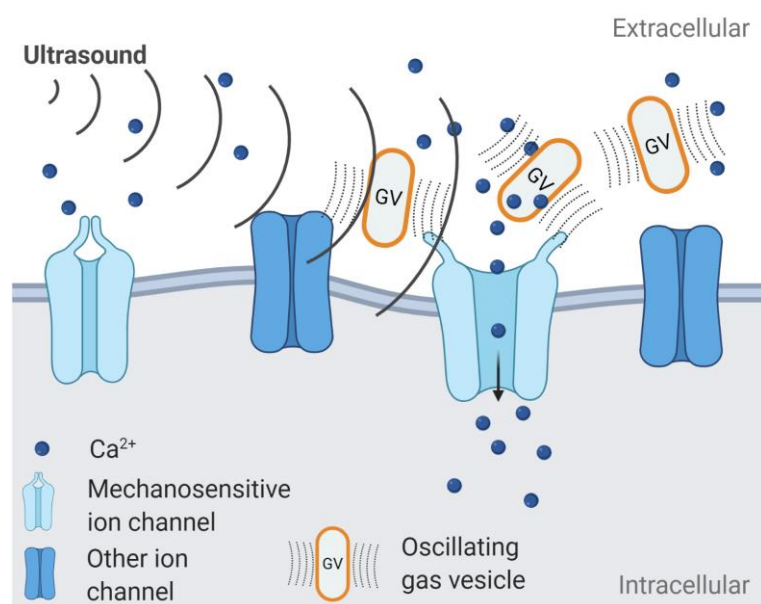
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Abstract

Ultrasound neuromodulation is a promising new method to manipulate brain activity noninvasively. Here, we detail a neurostimulation scheme using gas-filled nanostructures, gas vesicles (GVs), as actuators for improving the efficacy and precision of ultrasound stimuli. Sonicated primary neurons displayed dose-dependent, repeatable Ca^{2+} responses, closely synced to stimuli, and increased nuclear expression of the activation marker c-Fos only in the presence of GVs but not without. We identified mechanosensitive ion channels as important mediators of this effect, and neurons heterologously expressing the mechanosensitive MscL-G22S channel showed greater activation at lower acoustic pressure. This treatment scheme was also found not to induce significant cytotoxicity, apoptosis or membrane poration in treated cells. Altogether, we demonstrate a simple and effective method to achieve enhanced and more selective ultrasound neurostimulation.

Graphical abstract



KEYWORDS

Ultrasonic neuromodulation; sonogenetics; selective neuron activation; nano gas vesicles; mechanosensitive ion channels; MscL-G22S

1 **Introduction**

2 Neuromodulation techniques have expanded greatly over the past decade and have been used to
3 probe neural systems and to treat neurological disorders. Aside from the individual modalities that have
4 been shown to be capable of this, another rapidly-advancing facet of research has been the development
5 of nanoparticles to augment their effects. Such companion nanoparticles have been used as mediators to
6 improve the reach, temporal resolution and targeting of various techniques, or to decrease the
7 invasiveness of the treatment's scheme. Noteworthy approaches that have been demonstrated include
8 upconversion nanoparticle-mediated (UCNP) near-infrared optogenetics¹, gold nanoparticle-assisted
9 photothermal stimulation², and magnetic nanoparticle-based magnetothermal/magnetomechanical
10 stimulation^{3,4,5}. Insofar as the goal is to move towards treatments that have high temporal resolution and
11 are as minimally-invasive as achievable, nanoparticle mediators have helped in this regard.

12 Ultrasound is mechanical energy, able to non-invasively target deep-seated regions in the brain and
13 be focused to spots a few millimeters size. It is also an emerging neuromodulation technique which can
14 be used to modulate brain activity safely^{6,7}. Experiments in many different animal species showed
15 successful stimulation of various brain regions, such as rodents⁸, rabbits⁹, pigs¹⁰, sheep¹¹, non-human
16 primates¹². Low-intensity ultrasound has been used to stimulate various brain regions in the human,
17 including the thalamus¹³, the prefrontal, visual¹⁴, motor¹⁵ and somatosensory^{16,17,18,19} cortices. It is also
18 under study as a possible treatment for a range of neurological disorders, such as Alzheimer's disease^{16,}
19 ^{17,18,19}, Parkinson's disease^{20,21}, epilepsy²², depression²³ and amyotrophic lateral sclerosis²⁴. Ultrasound
20 has thus shown the ability to affect the functioning of the central nervous system without significant
21 accompanying thermal damage. One mechanism through which ultrasound is understood to exert such
22 effects is by activating mechanosensitive ion channels present in the cell membranes, whether
23 endogenous or externally-introduced^{25,26,25,26}. There is also understood to be some endogenous level of
24 mechanosensitivity in most cells, including those of the brain²⁷. In light of this, it would also be helpful

25 to be able to limit the effects of ultrasound to a desired area and cell-type which could help minimize
26 side-effects and increase treatment efficacy. It would, hence, be useful to be able to increase the
27 targetability of ultrasound stimulation.

28 A candidate for such a tool is nano-sized bubbles extracted from cyanobacteria, called gas vesicles
29 (GVs). GVs produce robust ultrasound contrast signals and have been applied to serve as reporters for
30 specific genes, molecules and cellular activities²⁸. The non-linear signals thus generated come from
31 ultrasound driven buckling effects which are frequency-independent. The oscillations can also generate
32 mechanical perturbations to the surrounding environment²⁹. Given these unique acoustic properties, we
33 hypothesized that GVs could oscillate in a low-frequency ultrasound field and serve as actuators to
34 activate mechanosensitive ion channels to induce neurostimulation effects.

35 In the present study we demonstrate a GV-actuated strategy to achieve controllable ultrasound
36 neuronal stimulation. We were able to stimulate primary neurons with low intensity ultrasound in the
37 presence of GVs to induce Ca²⁺ influx, but not otherwise. The neuronal responses were dose-dependent
38 and reversible, as well as closely temporally-tied to the ultrasound stimuli. The combination of GVs and
39 ultrasound (referred to as ‘GVs+US’) also significantly increased the expression of c-Fos in the nuclei
40 of neurons, a further indication of activation. We also show that the stimulation effect was mediated in
41 large part by the activation of mechanosensitive ion channels. Building on this finding, we induced
42 heterologous expression of a mechanosensitive ion channel in neurons, and we were able to reduce the
43 acoustic intensity level required to induce calcium response and neuronal c-Fos expression. The
44 stimulatory effects were also found to be mostly limited to the neurons expressing the channels. The
45 combination of US+GVs was safe and non-harmful to the treated cells. Thus, we provide evidence for
46 an enhanced ultrasound *in vitro* neuromodulation method capable of increasing response to ultrasound
47 and detail how the treatment may be made more targeted through the mechanism of mechanosensitive
48 ion channels.

49 **Results**

50 **Characterization of GVs' properties**

51 GVs were prepared from *Anabaena flos-aquae* through tonic cell lysis and centrifugally-assisted
52 flotation³⁰. They were found to typically be 50 - 100 nm in width and 100 - 500 nm long (Fig. 1A-B).
53 The zeta potential of GVs was -40 ± 5 mV, indicating a suitable surface charge for colloidal stability
54 (Fig. 1C)³¹. We also found that our prepared GVs were not cytotoxic on their own to primary neurons in
55 culture (Fig. 1D). In all, our prepared GVs were found to be nano-sized, stable and non-cytotoxic .

56 Ultrasound is known to induce both stable and inertial cavitation³². Generally, stable cavitation
57 occurs at relatively low ultrasound intensities, caused by size changes of gas-filled bubbles in a sustained,
58 periodic manner. Inertial cavitation usually occurs at high ultrasound intensities, when gas bubbles
59 collapse, generating a shock wave that could cause significant cell damage. We wanted to control
60 ultrasound intensity such that it would enable the GVs to generate robust stable cavitation but not inertial
61 cavitation, which required characterizing the GVs' responses in an ultrasound field. Hence, we performed
62 passive cavitation detection using a setup in a tank of degassed water, where GVs suspensions were
63 exposed to pulsed ultrasound at 0.28 MPa peak negative pressure (PNP) (schematically illustrated in
64 Supplementary Fig. 1A). We observed the backscattered signals in the time- and frequency-domains to
65 monitor the patterns of cavitation produced. We found no broadband signal and only the appearance of
66 1st - 17th harmonic signals (Fig. 1D-E), indicating that no inertial cavitation occurred when the GVs were
67 sonicated in our setup. Crucially, 0.28 MPa was the highest acoustic pressure used in the entire study,
68 making inertial cavitation unlikely at the range of intensities used in the various following experiments.

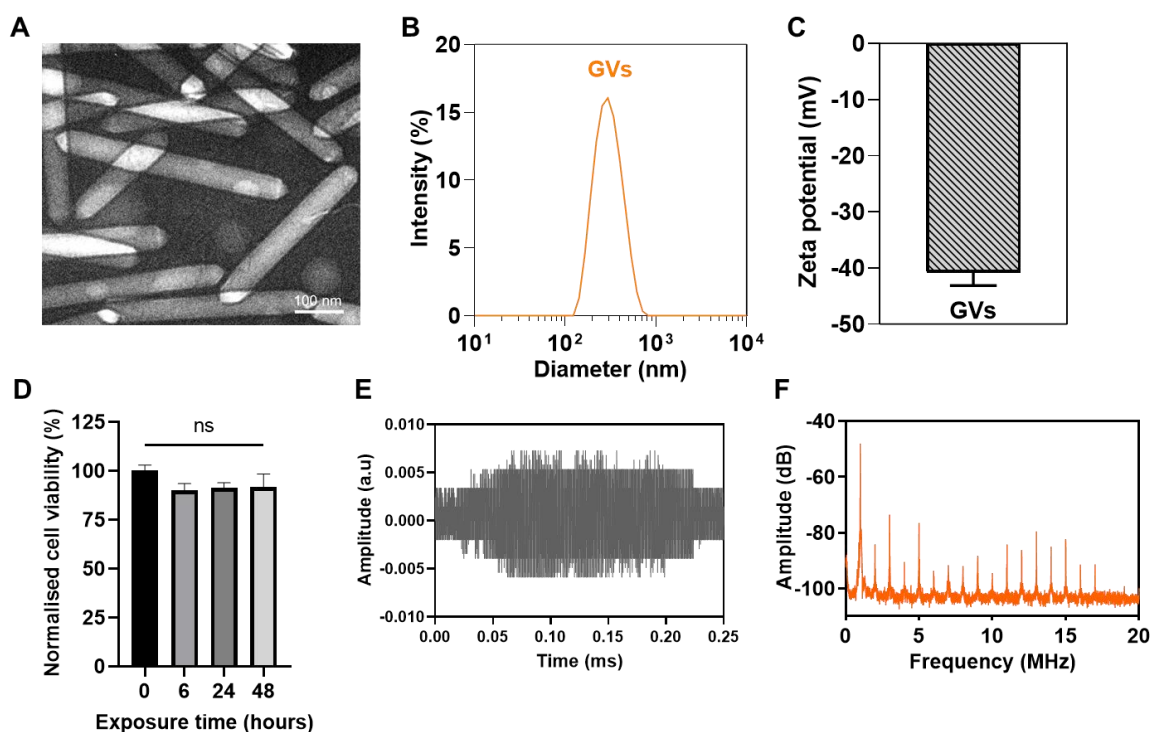


Fig. 1. Basic characterization of the prepared GVs. **a**, Transmission electron microscopy (TEM) image of the prepared GVs. Scale bar represents 100 nm. **b**, Number-averaged diameter of GVs in deionized (DI) H₂O as measured by Dynamic Light Scattering (DLS). Data represent the mean of 3 independent experiments. **c**, Zeta potential of GVs in DI H₂O. Bar represents mean \pm SD of 3 independent experiments. **d**, Cytotoxicity of GVs (0.8 nM), as measured by an MTT test. Primary neurons were exposed to GVs in medium for the stated amounts of time. Bars represent the mean \pm SEM of 3 independent experiments. No significant differences were found by one-way ANOVA. **e**, Representative time-domain waveform of backscattered signals from a purified GV suspension (0.8 nM) sonicated by a 1.0 MHz tone burst sinusoidal wave at 0.28 MPa PNP, after one burst interval (300 cycles). **f**, Averaged frequency spectrum of backscattered signals from purified GVs suspension under the same sonicating conditions as in **(e)**.

70 **Customized *in vitro* ultrasound stimulation setup**

71 For the present study, we used a customized system which facilitated ultrasound stimulation and
72 calcium imaging simultaneously (Fig. 2A). Briefly, the ultrasound stimulation system was aligned with
73 a calcium imaging system and the calcium responses of the stimulated neurons were monitored.
74 Ultrasound was delivered through a waveguide filled with degassed water that was attached to the
75 ultrasound transducer assembly. Cells were cultured on glass coverslips placed inside a culture dish, and
76 GVs were added to the medium and gently mixed just before stimulation. Prior to cellular stimulation,
77 we tested the acoustic pressure and field produced by this setup using a hydrophone, and found that it
78 provided a relative homogeneous ultrasound field in the central region (Supplementary Fig. 1B). Each
79 stimulus was composed of 300 tone burst pulses at a center frequency of 1.0 MHz, 10% duty cycle, pulse
80 repetition frequency (PRF) of 1 kHz, at low acoustic intensities (0.03 - 0.20 MPa). These parameters
81 amounted to ultrasound being delivered in very short bursts, minimize thermal effects. For experiments
82 not involving real-time imaging, cells were treated inside a standard cell culture incubator, as described
83 in our previous study³³. Parameters for stimulation in these experiments was the same as mentioned
84 above, with a slightly different range of acoustic pressures (0.14 - 0.28 MPa) and a treatment time of 15
85 minutes.

86 **GVs enable efficient neuromodulation by low-intensity ultrasound**

87 We first observed the Ca^{2+} response in rat primary cortical neurons when stimulated with
88 ultrasound and GVs (1.0 MHz center frequency, 0.20 MPa ultrasound and 0.8 nM GVs unless otherwise
89 indicated). Neurons were made to express the genetically-encoded calcium sensor GCaMP6s under the
90 human synapsin promoter using AAVs, and we monitored its fluorescence upon stimulation. We found
91 that GCaMP6s fluorescence increased quickly and dramatically when stimulated with an ultrasound
92 pulse in the presence of GVs, but not without them, and the fluorescence gradually returned to the

93 baseline without further stimulation (Fig. 2B-D). Next we tested whether neuronal activation could be
94 induced repeatedly. Five 300 ms pulses were delivered to cells at intervals of 3, 5, 10, 20, 30 or 60
95 seconds in the presence of GVs and the temporal profiles of the cells' Ca^{2+} response was charted. Stable
96 and reversible calcium transients were seen to quickly follow each pulse, and the neurons were able to
97 recover after each pulse when given enough time (max $\Delta\text{F}/\text{F} = 46 \pm 1.8\%$, five pulses) (Fig. 2E &
98 Supplementary Video 1). Aside from primary neurons, we also observed the same pattern of responses,
99 albeit at lower amplitudes, in the mouse hippocampal cell line mHippoE-18 (referred to as 'CLU199' in
100 this manuscript). In the presence of GVs, ultrasound triggered robust, repeatable, and rapid calcium
101 responses from cells after which calcium levels would gradually recover, while no response was seen
102 without GVs (Supplementary Fig. 2A-D). Furthermore, the cellular response was found to vary with,
103 both, the concentration of GVs and the acoustic pressure applied, both in primary neurons (Fig. 2F-G)
104 and in CLU199 cells (Supplementary Fig. 2E-F). These data help to establish that the responses observed
105 were indeed caused by the US+GVs treatment, and also reveal how such a combination treatment can
106 easily be tweaked to suit the degree of response desired. A GVs concentration as low as 0.1 nM was
107 sufficient to induce a significantly higher response than without GVs, showing that GVs could indeed
108 lower the acoustic pressure threshold needed for Ca^{2+} response, which would otherwise require increased
109 ultrasound intensity⁷. Finally, when treated with US+GVs primary neurons showed approximately
110 double the nuclear c-Fos expression, a marker of neuronal activity downstream of Ca^{2+} influx³⁴, than
111 when they were untreated or exposed to only GVs or ultrasound (Fig. 2H-I). Thus we were able to use
112 GVs to efficiently activate primary neurons with short bursts of low-intensity ultrasound.

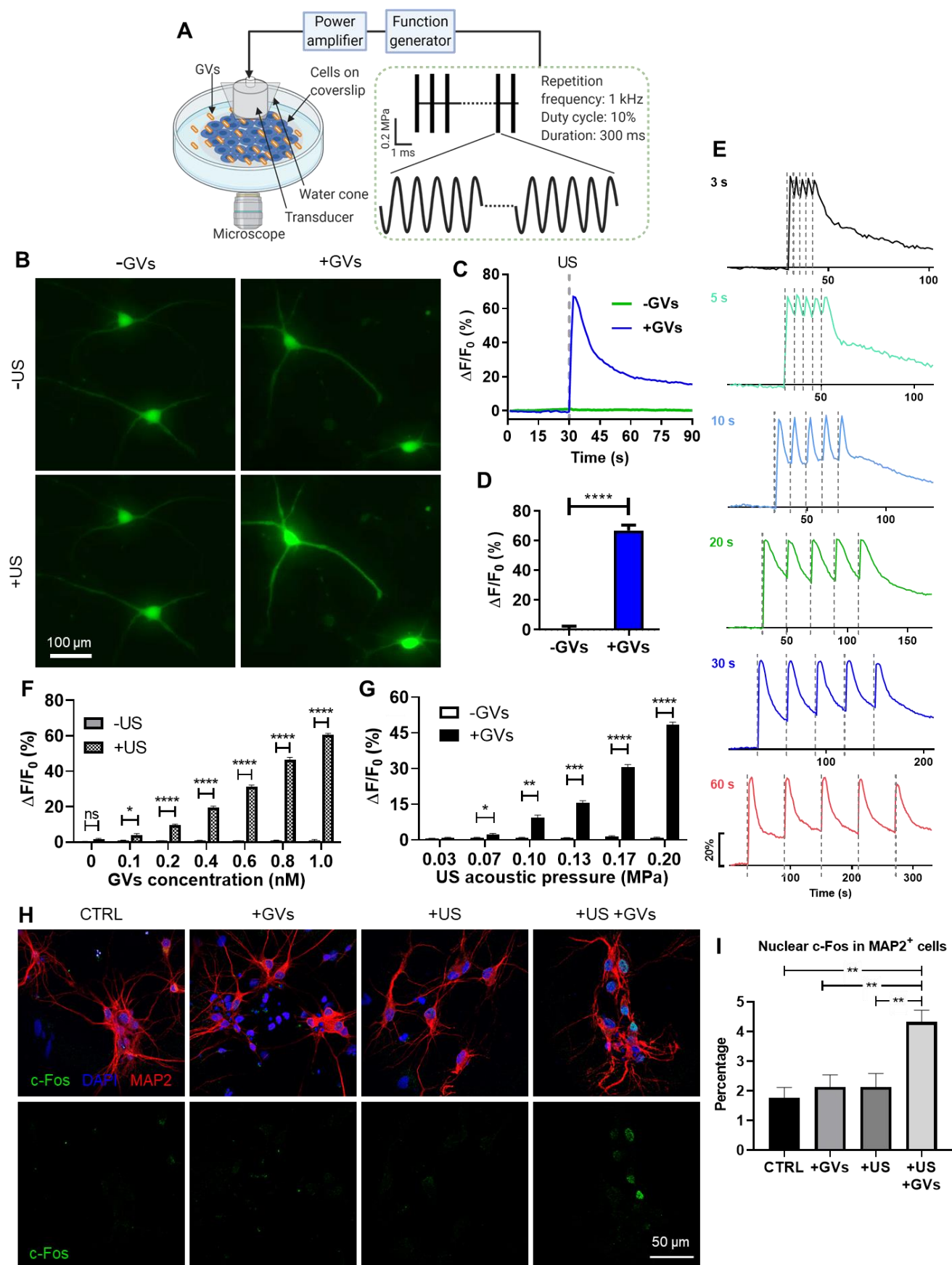


Fig. 2. GVs enable low-intensity ultrasound to stimulate activity in primary neurons. **a**, Schematic illustration of the GV-mediated ultrasound setup for recording cells. GVs were mixed into cell culture medium. Cellular response upon US+GVs stimulation was observed in real time. **b**, Representative images of GCaMP6s fluorescence in primary neurons with or without GVs, before and after 0.20 MPa ultrasound. **c**, Ca²⁺ imaging time course of neurons in **(b)**. **d**, Ca²⁺ response of neurons to stimulation by 0.20 MPa ultrasound. Bars represent mean \pm SD from 3 independent experiments. ****, $p < 0.0001$, two-tailed unpaired *t*-test. **e**, Time-resolved Ca²⁺ responses of neurons stimulated by 5 ultrasound pulses at varying intervals. **f**, Ca²⁺ response of cells to varying ultrasound intensities, 0.8 nM GVs. Bars represent mean \pm SEM of 3 independent experiments. *, $p < 0.05$, **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, two-tailed unpaired *t*-test with Holm-Sidak correction. **g**, Ca²⁺ response of cells to varying GV concentrations, 0.20 MPa ultrasound. Bars represent mean \pm SEM of 3 independent experiments. *, $p < 0.05$; **, $p < 0.0001$, two-tailed unpaired *t*-test with Holm-Sidak correction. **h**, Representative IF images of c-Fos and MAP2 staining in untreated cells, and cells treated with the indicated combinations of ultrasound (0.28 MPa) or GVs (0.8 nM). **i**, Quantified results of nuclear c-Fos staining in MAP2⁺ cells after various treatments, as in **(h)**. Bars represent mean \pm SEM from 4 independent experiments. **, $p < 0.01$, one-way ANOVA with post-hoc Tukey test.

113 **GV-mediated ultrasound stimulates cells by activating mechanosensitive ion channels in the cell**
114 **membrane**

115 A possible confounding factor in our experiments was that sonoporation, of the kind that is typically
116 induced by ultrasound in the presence of microbubbles, is known to play a role in initiating Ca^{2+}
117 response^{37,38}. Although only stable cavitation was detected in our system, that evidence could not address
118 whether our treatment was causing pore formation in the membrane. Thus, we performed a membrane
119 integrity assay to see whether sonoporation was involved in the Ca^{2+} responses to the GV-mediated
120 ultrasound treatment. We used the membrane impermeable dye propidium iodide (PI) and observed
121 whether it could penetrate the cell membrane during the stimulation. Insonated primary neurons in the
122 presence of GVs evoked Ca^{2+} influx but no PI could be detected inside the cells; brightfield imaging also
123 showed that the cells maintained their morphology following the treatment (Fig. 3A, Supplementary Fig.
124 3A & Supplementary Video 3). To contrast, Triton X-100 was used as a positive control for membrane
125 permeation³⁵, and PI influx was seen within 30 seconds of its addition and continued to increase for the
126 remainder of the assay, while the intracellular calcium signal decreased and the cell was visibly damaged
127 (Fig. 3A & Supplementary Fig. 3A). In general, neither ultrasound alone nor the +GVs condition used in
128 our experiments were seen to trigger PI influx in primary neurons or CLU199 cells (Supplementary Fig.
129 3B-C). Further, we did not observe obvious cytotoxicity or apoptosis in primary neurons following the
130 treatments (Supplementary Fig. 3 D-E). Thus, we concluded that our US+GVs treatment could trigger
131 calcium responses in cells with negligible loss of membrane integrity, which is consistent with stable
132 cavitation hypothesis.

133 We next tried to address whether calcium influx by mechanosensitive ion channels was in fact
134 responsible for the observed effects of the treatment. We used Ruthenium Red (RR, 20 μM), a blocker
135 for a wide range of mechanosensitive ion channels³⁶, to see if the response to US+GVs would be altered.
136 Calcium responses to ultrasound pulses were found to be significantly suppressed in the presence of RR,

137 and the responses recovered when it was washed away (Fig. 3C-D). We observed a similar suppression
138 of Ca^{2+} influx in the presence of RR in CLU199 cells (Supplementary Fig. 2A-C). We then tried to
139 identify the main source of Ca^{2+} responses by treating cells with US+GVs in EGTA-chelated medium,
140 or pre-treating cells with Thapsigargin (TG, 3 μM) to deplete intracellular calcium stores³⁷. Compared to
141 normal conditions, cells in Ca^{2+} -free medium showed much reduced calcium influx (~75% reduction),
142 but the reduction in TG-treated cells was much lesser (~ 25-30% reduction) (Fig. 3D-E & Supplementary
143 Fig. 2A-C). We thus found that while intracellular Ca^{2+} release played some role in the US+GVs response,
144 calcium influx from the external medium had a much larger contribution to the observed outcomes.
145 Putting our evidence together, with no significant sonoporation being observed, cellular response being
146 significantly depressed when treated with RR or in Ca^{2+} -free medium but not in TG-treated cells, we
147 inferred that activation of mechanosensitive ion channels was an important mechanism of GV-mediated
148 ultrasound stimulation.

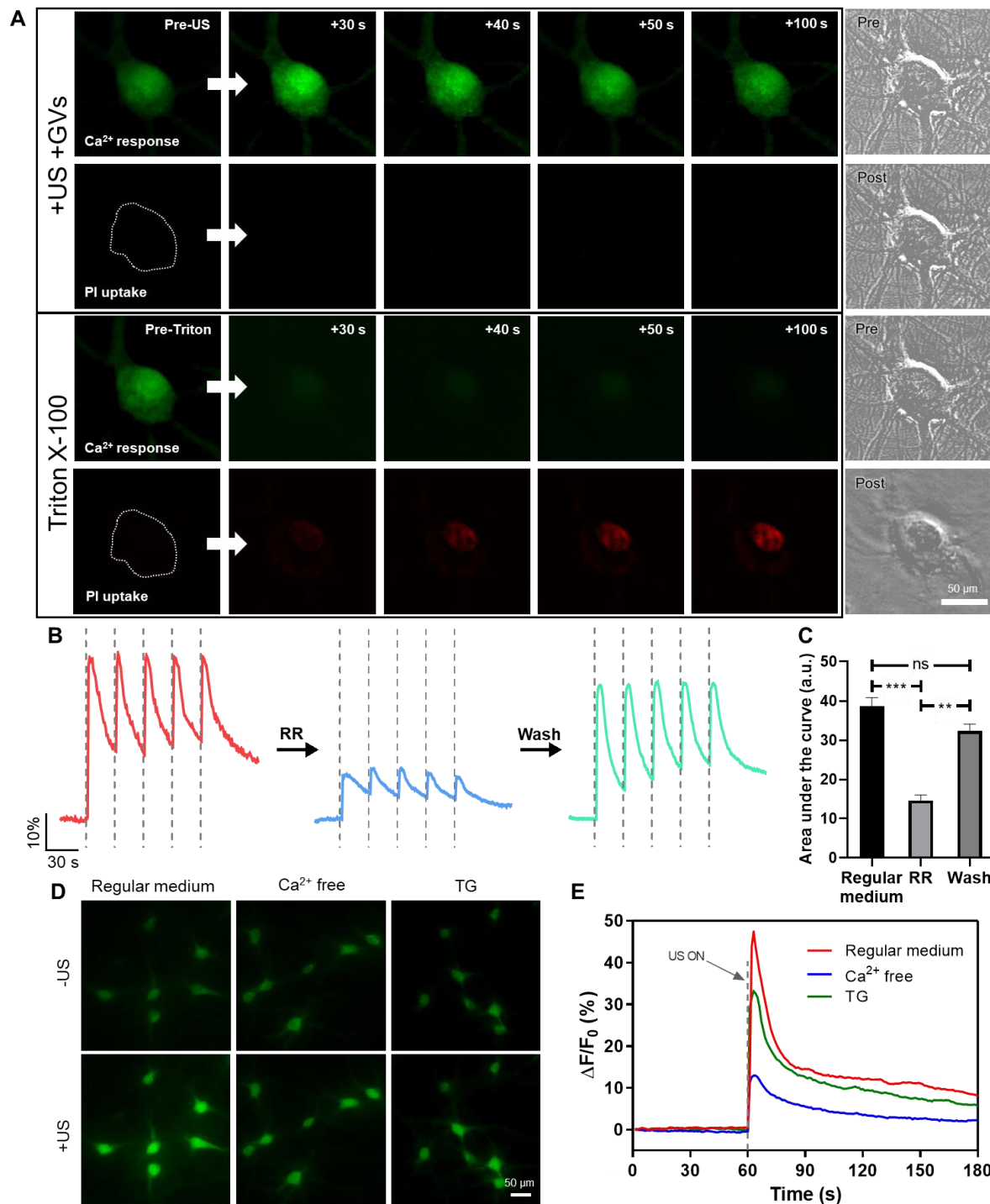


Fig. 3. Mechanosensitive ion channels are an important mechanism for GV-mediated ultrasound stimulation. **a**, Upper: Calcium response and PI uptake (indicating membrane integrity) during US+GVs stimulation. Lower: Calcium response and PI uptake (indicating membrane integrity) following addition of 0.2 mM Triton X-100 as a positive control for loss of membrane integrity. Right: Brightfield images of the images cells before and after ultrasound + GV or Triton X-100. All images shown in this panel are representative. **b**, Time-resolved calcium responses of neurons during US+GVs stimulation, first as normal, then in the presence of mechanosensitive ion channel blocker ruthenium red (RR), and then after RR was washed away. **c**, Quantification of area under the curve before, during and after RR treatment as shown in **(b)**. Bars represents the mean \pm SEM of 3 independent experiments. ** $p < 0.01$, *** $p < 0.001$, one-way ANOVA with post-hoc Tukey test. **d**, Representative images of calcium responses of neurons in regular medium, Ca²⁺-free solution and Thapsigargin (TG). **e**, Time-course calcium imaging of cells before and after ultrasound + GV stimulation in the three solutions indicated in **(d)**.

150 **Increased levels of mechanosensitive ion channels improve selectivity of GV-mediated ultrasound**
151 **stimulation**

152 Having seen the important role mechanosensitive ion channels play in GV-mediated ultrasound,
153 we surmised that one way of increasing the sensitivity and efficiency of our treatment would be to
154 increase expression of mechanosensitive ion channels in desired cells. Furthermore, since methods of
155 inducing expression of a desired protein (such as AAVs) offer the option of cell-type selectivity, we
156 hypothesized that we could also improve the targeting of the stimulation to neurons. Our general idea
157 was to induce the expression of a mechanosensitive ion channel in primary neurons, which would
158 sensitize them to GV-mediated ultrasound stimulation at lowered intensities (illustrated schematically in
159 Fig. 4A).

160 We chose a mutant version of the well-studied bacterial channel large conductance
161 mechanosensitive ion channel (MscL-G22S)⁴². In our previous work, we induced neurons in certain
162 mouse brain region to express this channel and found that we were able to both sensitize neurons to
163 ultrasound alone, and target brain regions using this method³⁸. We used human synapsin-promoted AAVs
164 encoding for MscL-G22S-EYFP (called ‘MscL-EYFP’), or EYFP alone as a control, to transduce
165 primary neurons. EYFP fluorescence was used to identify cells that were transduced successfully, as
166 described in our previous study³⁸. To evaluate the ability of MscL to sensitize neurons to acoustic
167 radiation force, we treated them with lowered acoustic intensities (0.07 to 0.17 MPa). For calcium
168 imaging experiments, the GV concentration was also halved to 0.4 nM to more clearly observe the effects
169 of greater neuronal mechanosensitivity without saturating our imaging system. We found that the
170 MscL+US+GVs condition showed a rapid and strong response to one pulse of 0.13 MPa ultrasound, with
171 all other conditions showing much lower or no response (Fig. 4B & Supplementary Video 3). Crucially,
172 we found that cells expressing MscL-EYFP showed a significantly higher response to ultrasound
173 stimulation than did cells without EYFP in the same dish (Supplementary Fig. 4A). MscL+US+GVs also

174 showed the strongest response to 3 out of 4 ultrasound intensities tested, and significantly higher response
175 than EYFP+US+GVs at the 2 intermediate intensities (Fig. 4C). The lowest ultrasound intensity at which
176 a differential response between the +GVs and -GVs groups could be observed was 0.10 MPa. The EYFP
177 group did not show obvious responses at any of the tested intensities, while the MscL and EYFP+GVs
178 groups showed responses with increasing ultrasound intensity, indicating the sensitizing role of both
179 MscL expression and GV. Interestingly, as in our previous study, we found again that as ultrasound
180 intensity is increased, the strength of the EYFP and MscL-EYFP conditions' responses converge until
181 they do not differ significantly³⁸. We also found that when stimulated by 0.14 MPa ultrasound and 0.8
182 nM GV, MscL+US+GVs neurons showed significantly higher nuclear c-Fos expression than all other
183 groups tested, including EYFP+GVs (Fig. 4D-E & Supplementary Fig. 4B). We also compared the cells
184 expressing MscL-EYFP vs those that did not in the same dish, and found that the EYFP⁺ cells showed
185 significantly higher nuclear c-Fos levels (Fig. 4F). Thus, we demonstrate that increasing the expression
186 of mechanosensitive ion channels in desired cells can increase their sensitivity to ultrasound, and that
187 their preferential expression in specific cell-types is an effective way to target the effects of GV-mediated
188 ultrasound stimulation.

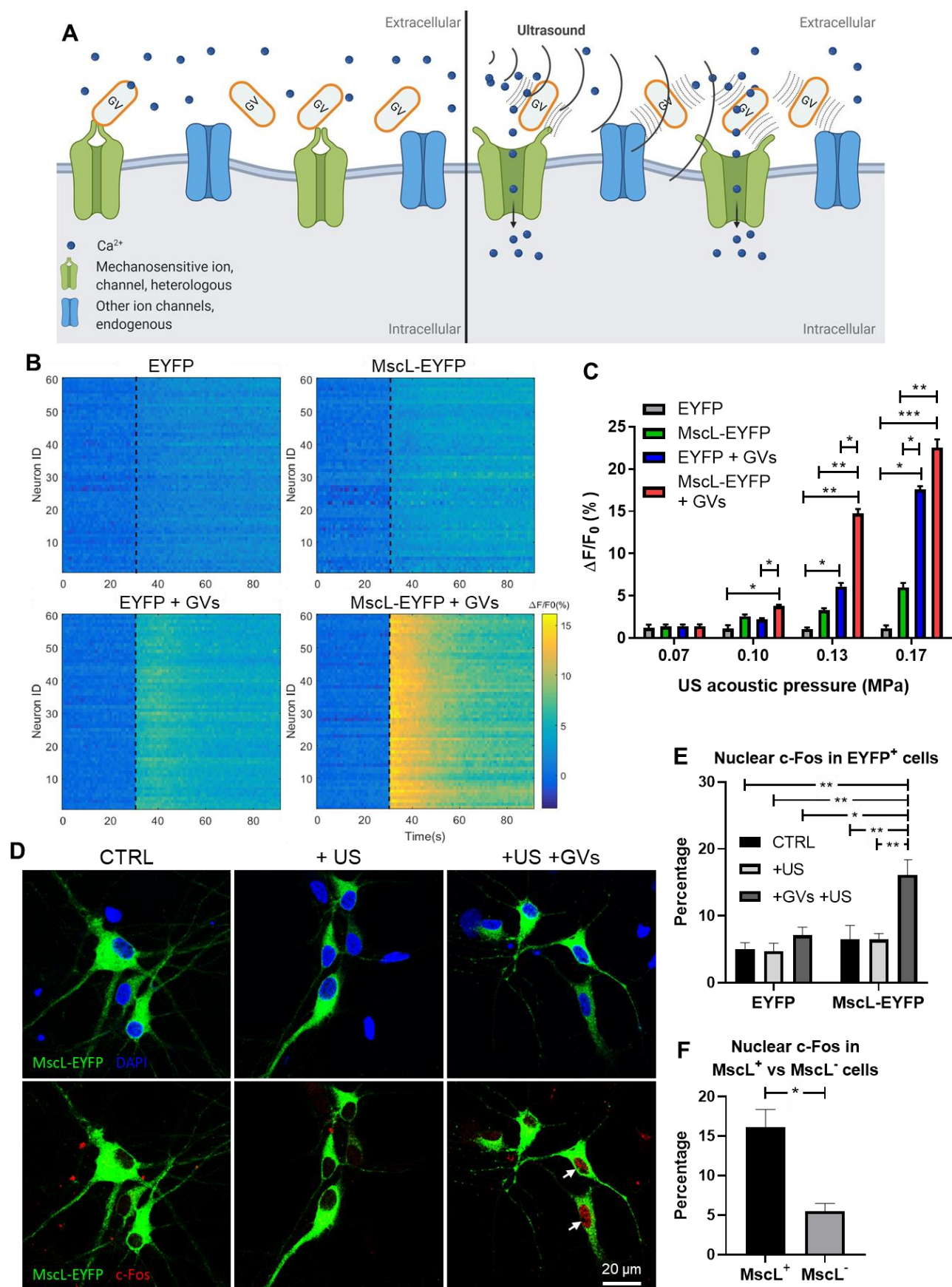


Fig. 4. Increased expression of a mechanosensitive ion channel increases neurons' sensitivity to GV-mediated ultrasound stimulation. **a**, Schematic illustration of the sonogenetic experimental scheme. Briefly, a heterologous mechanosensitive channel, MscL-G22S is expressed preferentially in primary neurons, which increases their sensitivity to ultrasound + GVs treatment, enabling cell-type specific activation. **b**, Temporal raster plots of fluorescence changes in neurons, transduced with EYFP or MscL-G22S-EYFP AAVs, stimulated by a single 300 ms ultrasound pulse (0.13 MPa, black dashed line) with or without GVs. **c**, Calcium responses in AAV-transduced neurons after ultrasound stimulation with varying acoustic pressures, with or without GVs. Bar chart represents means \pm SEM of 3 independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$, two-way ANOVA with post-hoc Tukey test. **d**, Representative images of neuronal c-Fos expression in cells transduced with MscL-EYFP in untreated cells, or cells treated with ultrasound alone or ultrasound + GVs. **e**, Quantified results of nuclear c-Fos staining in MscL-EYFP⁺ cells after treatments, as in **(d)**. Bars represent mean \pm SEM from 3 independent experiments. **, $p < 0.01$; one-way ANOVA with post-hoc Tukey test. **f**, Quantified results of nuclear c-Fos staining in cells with and without MscL-EYFP in the same dish, following ultrasound + GVs stimulation. Bars represent means \pm SEM of 3 independent experiments. *, $p < 0.05$, two-tailed unpaired *t*-test.

189 **Discussion**

190 Non-invasive neuromodulation technologies with cell-type selectivity hold great potential for
191 studying neural circuits and treating neurological conditions. Here we present a GV-mediated
192 sonogenetic toolkit for selective neuronal activation, comparable to previously-demonstrated
193 magnetothermal and magnetomechanical approaches. We employed a 1.0 MHz transducer and
194 demonstrated that GVs can serve as localized acoustic amplifiers to decrease the threshold of ultrasound
195 intensity for neurostimulation. We achieved controllable calcium signaling in both primary neurons and
196 a neuronal cell line, and increased c-Fos expression in primary neurons with low-intensity ultrasound
197 stimulation in the presence of GVs through activation of endogenously-expressed mechanosensitive ion
198 channels. We also showed that combining increased mechanosensitivity (through expression of MscL in
199 neurons) with GVs can significantly increase cells' ability to respond to lowered acoustic pressures, and
200 that the stimulation can potentially be limited to the targeted cells. These data suggest that the GV-
201 mediated ultrasound reliably and reversibly activates cells *in vitro* by opening mechanosensitive ion
202 channels, and that the treatment is generally not harmful to cells.

203 A limitation of this study is that it lacks information about ion-channel dynamics, which could be
204 obtained by patch clamping during ultrasound stimulation. We found this to be unachievable during the
205 present study as we could not eliminate vibration of the patch pipettes when ultrasound was turned on,
206 which has been reported in other studies as well^{39, 40}. Indeed, it would be useful to know the specific
207 parameters required to trigger channel opening in GV-mediated ultrasound stimulation. However, we
208 were able to collect evidence of the role of mechanosensitive ion channels through calcium imaging by
209 using various blockers and calcium-free imaging. We also showed that the cells' calcium influx through
210 ion channels response was independent of, and indeed greater than, intracellular calcium release or
211 calcium influx through sonoporation. The expression of MscL in neurons was seen to be an effective
212 method of increasing cells' sensitivity to ultrasound, even when the concentration of GVs was lowered.

213 We also showed that cells expressing MscL showed significantly higher calcium response and nuclear c-
214 Fos than other cells in the same dish that did not, which indicates that US+GVs stimulation does act on
215 mechanosensitive ion channels. Thus, we provide alternative data for the activation of mechanosensitive
216 ion channels by tracking effects downstream of channel opening.

217 Consistent with our previous study, we found the expression of the MscL channel in neurons to be
218 effective in increasing cellular sensitivity to ultrasound³⁸. We detected low Ca²⁺ response from cells
219 without MscL, but obvious and significantly greater responses from MscL-expressing cells when
220 stimulated with low-intensity US+GVs. The observed MscL-dependence of these effects in our
221 experiments pinpoints the channel as the key source of improved sensitivity to stimulation. The
222 performance of this approach may be further improved by using other ultrasound-sensitive ion channels
223 or using different or novel mutants of the MscL channel. By the same logic, our scheme may be applied
224 to identify natural problematic situations in which cells show increased expression of mechanosensitive
225 ion channels, such as in aging brains⁴¹ or in the progression of some cancers or infectious diseases^{42, 43}.
226 Thus, the ability of GV-mediated ultrasound stimulation to act upon mechanosensitive ion channels could
227 be applied in fields beyond neurostimulation.

228 A recent study from our group demonstrated that surface-modified GVs can escape clearance from
229 the reticuloendothelial system and penetrate tumor vasculature through enhanced permeability and
230 retention (EPR) effects⁴⁴. In addition, expressing GVs as acoustic reporter genes (ARGs) in mammalian
231 cells to enable ultrasound imaging of mammalian gene expression have been reported⁴⁵. Such application
232 of ARGs could be a milestone development for ultrasound imaging, almost analogous to the role of green
233 fluorescent protein (GFP) in optical imaging. Alternatively, GVs can also be delivered to targeted regions,
234 since it is nano-sized. Microbubble-mediated ultrasonic bio-effects have been widely explored and
235 utilized to open cell membranes and the blood-brain barrier^{46, 47, 48, 49, 50}. However, the micrometer size
236 of microbubbles limits spatial resolution and they are restricted to use in blood vessels due to their size

237 and have a short half-life in vivo (<5 min in the blood)^{51, 52}. In contrast, GVs are gas-filled protein-
238 shelled nanostructures and these protein shells exclude water but permit gas to freely diffuse in and out
239 from the surrounding media, making them physically stable despite their nanometer size. Therefore,
240 US+GVs could even be developed to have a more theranostic role in the brain.

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249 **Author contributions:**

250 Conceptualization, X.H., Z.Q. and L.S.; Methodology, X.H., Z.Q., S.K., J.G. and L.S.;
251 Investigation, X.H., S.K., K.F.W., T.Z. and M.Y.; Data Analysis, X.H., Z.Q., S.K., J.Z. and Q.X.;
252 Manuscript Preparation, H.X, Q.Z., S.K. and L.S.; Supervision, J.G. and L.S.; Funding Acquisition, L.S.

253 **Declaration of interests:**

254 The authors have submitted a patent application titled “A non-invasive method for selective neural
255 stimulation by ultrasound” with the U.S. Patent and Trade Office, dated April 10, 2018, assigned
256 application number 15/949,991. The authors declare no further financial interests.

257 **Materials and methods**

258 **Gas vesicle preparation**

259 *Anabaena flos-aquae* was cultured in sterile BG-11 medium at 25 °C under fluorescent lighting
260 with 14 hours/10 hours light/dark cycle. GVs were isolated by hypertonic lysis to release GVs by quickly
261 adding sucrose solution to a final concentration of 25%. GVs were isolated by centrifugation at 400 x g
262 for 3 hours after lysis. To purify GVs, the solution was washed by the same centrifugation process 3
263 times and stored in PBS at 4 °C. The GVs' concentration was measured by optical density at 500 nm
264 (OD500) by UV-Visible spectrophotometer³².

265 **Passive cavitation detection**

266 Acoustic spectroscopy on GV suspensions were performed in a custom-built chamber, the 1 MHz
267 flat transducer and hydrophone (HGL-0200, Onda) were perpendicularly aligned and immersed in a tank
268 of deionized, degassed water (Fig S1A). A rectangular agarose (3%) chamber of wall thickness 5 mm
269 and cavity 15×15 mm was placed in the middle, with the center point 17.5 mm away from both the
270 transducer and the hydrophone. 1 MHz sinusoidal trains of burst width 200 μs and burst interval 2 ms
271 were generated by a function generator (AFG251, Tektronix), amplified by a radio frequency (RF)
272 amplifier (A075, Electronics & Innovation Ltd.), to drive the emitting transducer, producing acoustic
273 output with 0.28 MPa peak negative pressure. Signals received by the hydrophone were amplified (AH-
274 2010, Onda) and digitized (CSE1222, GaGe) before analysis. 20 sections of 200 μs digitized signal in 20
275 separate bursts were processed with fast Fourier transform (FFT) using MATLAB and the resulting
276 frequency spectra were averaged.

277 **Cell culture**

278 All cells were grown inside a standard humidified cell culture incubator at 37 °C with 5% CO₂.

279 CLU199 cells were routinely maintained in DMEM culture medium supplemented with 10% FBS and
280 1% Pen-Strep (all from Gibco) and seeded on PLL-coated glass coverslips as needed, allowed to grow
281 overnight and used for experiments thereafter.

282 **Viruses**

283 We purchased high-titer viruses from BrainVTA (Wuhan) Co. Ltd, viruses were aliquoted and
284 stored at -80°C prior to use. We used an rAAV-9 vector, with a human synapsin (hSyn) promoter,
285 which enabled preferential transduction of neurons. The MscL-G22S sequence was fused with either
286 the fluorescent reporter EYFP or the Ca^{2+} sensor protein GCaMP6S, and a concluding polyA tag. We
287 also used vector controls in addition to the MscL-containing viruses. Viruses used in this study were
288 rAAV/9-hSyn:EYFP-WPRE-pA, rAAV/9-hSyn:MscL-G22S-WPRE-EYFP-pA and rAAV/9-
289 hSyn:MscL-G22S-GCaMP6S-WPRE-pA, rAAV/9-hSyn:GCaMP6S-WPRE-pA.

290 **Primary cortical neuron culture**

291 Cultured neurons from rat embryos at embryonic day 18 were obtained as previously described⁵³.
292 Briefly, cortices were dissected and treated with 0.25% trypsin for 15 min at 37°C , followed by gentle
293 mixing. The digestion was stopped with Neurobasal medium (Gibco) with 10% fetal bovine serum and
294 1% penicillin-streptomycin. The cells were resuspended in medium and gently mechanically triturated
295 with a pipette, and then allowed to stand for 15 minutes. The resultant supernatant was discarded, and
296 the cells were resuspended in the abovementioned medium and plated at 1×10^5 cells/cm² in 35 mm
297 dishes with poly-L-lysine-coated (PLL, Gibco) coverslips or PLL-coated glass-bottomed confocal dishes.
298 After 24 hours, the medium was changed to Neurobasal + 2% B27 + 0.25% L-Glutamine + 1% Penicillin-
299 Streptomycin (all from Gibco). Half of the medium was replaced every 2-3 days. Cultured neurons were
300 transduced with AAVs on day 7 and were used in experiments between DIV 10-12 (3-4 days post-
301 infection). All animal studies and experimental procedures were approved by the Animal Subjects Ethics

302 Sub-Committee (ASESC) of the Hong Kong Polytechnic University, and were performed in compliance
303 with the guidelines of the Department of Health - Animals (Control of Experiments) of the Hong Kong
304 S.A.R. government.

305 **Characterization of ultrasound setup for Ca²⁺ imaging**

306 A flat transducer with center frequency 1.0 MHz (A303S, Olympus) was employed in this study.
307 Ultrasonic pulses were generated using function generator (AFG251, Tektronix) and power amplifier
308 (A075, Electronics & Innovation Ltd.). For ultrasound stimulation, the planar transducer with a diameter
309 of 1.0 cm was fixed perpendicularly downward facing. Cells were grown on glass coverslips, which were
310 held 1.5 cm away from the transducer coupled by plastic wrap encasing degassed deionized water at
311 25 °C. Acoustic intensity profile was characterized by a hydrophone.

312 **Cell treatments for calcium imaging**

313 Culture medium was replaced with Fluo-4 AM (5 μM) or X-Rhod-1 AM (10 μM) (both from
314 Invitrogen) working solution in Ca²⁺ solution (pH 7.4), and the cells were incubated at 37 °C in the dark
315 for 30 min. Subsequently, fresh Ca²⁺ solution was used to flush away excess dye before ultrasound
316 stimulation. In mechanistic studies, several different media were used. To remove extracellular Ca²⁺, the
317 coverslip was placed Ca²⁺ free solution with 0.5 mM EGTA to ensure that residual Ca²⁺ was completely
318 chelated. To monitor concurrent cell membrane sonoporation during Ca²⁺ response measurement, the
319 coverslip was perfused with PI solution (100 μg/mL in Ca²⁺ solution, Invitrogen). RR solution (20 μM
320 RR in Ca²⁺ solution, Tocris Bioscience) into the culture medium to evaluate the effect of
321 mechanosensitive ion channels on US+GVs-elicited Ca²⁺ response. 0.20 mM Triton X-100 was added to
322 cells as a positive control of membrane permeability.

323 **GV-mediated ultrasound stimulation and optical imaging**

324 Briefly, the calcium imaging was done with a modified inverted epifluorescence microscope. The
325 excitation light was generated by a dual-color LED, filtered and delivered to the sample to illuminate the
326 calcium sensor. To minimize phototoxicity, the LEDs were triggered at 1 Hz and synchronized with
327 sCMOS time-lapse imaging. Coverslips with dye-loaded or GCaMP6s-expressing cells were placed
328 above the objective, and GVs were distributed into the media directly before ultrasound stimulation. A
329 camera was used to record the intracellular Fluo-4 AM/X-Rhod-1 AM images with defined time intervals
330 from a function generator at excitation wavelengths of 494 nm for Fluo-4 AM or 580 nm for X-Rhod-1
331 AM. A bright field image was taken to register the morphology of the cell immediately before and after
332 the GVs mediated ultrasound stimulation. We used software to communicate and coordinate the operation
333 sequence between the microscope and monochromator.

334 **Evaluation of cytotoxic effects and apoptotic effects**

335 MTT assays was used to evaluate cytotoxicity at different concentration of GVs mediated
336 ultrasound stimulation in the treated CLU199 cells. Cells were treated with GVs alone, or US+GVs in
337 96-well or 24-well plates. After the indicated treatments and incubations, cells were incubated with 0.5
338 mg/ml MTT in medium for 3-4 hours at 37°C, solubilized with DMSO and 15 minutes' shaking, and the
339 solutions' absorbance at 570 nm was read using an LEDTect 96 microplate reader.

340 **Western Blot**

341 The treatments' apoptotic effects were evaluated by a WB of caspase-3. Cells were treated inside
342 an incubator for 15 minutes, allowed to incubate overnight, and protein was collected using RIPA buffer
343 supplemented with 1X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific). Cells were
344 run on an 4-20% Tris-Glycine SDS-PAGE gel, transferred to activated PVDF membrane (Millipore), and
345 incubated overnight with caspase-3 primary antibody (Cell Signaling #9662) diluted 1:1,000 or α -tubulin
346 primary antibody (Proteintech # 66031-1-Ig) diluted 1:2,500 in 5% milk + TBST. Membranes were

347 washed with TBST, and incubated at room temperature with Goat anti-Rabbit IgG (H+L) superclonal
348 secondary (Invitrogen #A27022) or Rabbit anti-Mouse IgG (H+L) superclonal secondary antibody
349 (Invitrogen #A27033), diluted at 1:10,000 in 5% milk + TBST. Signals were developed using
350 SuperSignal West Pico PLUS Chemiluminescent Substrate and visualized on a ChemiDoc MP imaging
351 system (Bio-Rad). Proteins were quantified using image densitometry and normalized to the α -tubulin
352 expression levels with ImageJ.

353 **Immunocytochemical fluorescent staining**

354 Cells were treated, allowed to incubate for 90 minutes and fixed using 4% paraformaldehyde +
355 PBS and permeabilized using 0.1% Triton X-100 + PBS, and washes were done with 1X PBS or 1X
356 PBS+Tween-20 (PBST) (after permeabilization). Cells were blocked with 2% BSA + 0.3M Glycine +
357 PBST, and incubated overnight with primary antibodies in 2% BSA + PBST. The next day, cells were
358 washed and incubated with secondary antibodies in 2% BSA + PBST, then washed and mounted with
359 Fluoroshield Mounting Medium with DAPI (Abcam). Stained cells were imaged on a Leica TCS SP8
360 confocal microscope. All steps from secondary antibody incubation onwards were performed in the dark.

361 Primary antibodies used were c-Fos (Cell Signaling #2250) at a dilution of 1: 3,000, and MAP2
362 (PA1-10005, Invitrogen) at a dilution of 1:2,500. Secondary antibodies, used at a dilution of 1:1,000,
363 were Goat anti-Rabbit IgG Alexa Fluor Plus 488 (#A32731), Goat anti-Chicken IgY Alexa Fluor Plus
364 555(#A32932) or Goat anti-Rabbit IgG Alexa Fluor Plus 555 (#A32732), all from Invitrogen.

365 **c-Fos counting**

366 The number of c-Fos⁺ cells in primary neurons was determined by counting the number of neuronal
367 nuclei showing c-Fos expression 90 minutes after stimulation. For non-transduced cells, nuclear c-Fos
368 was counted in cells staining positive for MAP2. For transduced cells, nuclear c-Fos was counted in

369 cells showing EYFP expression. The percentage of cells showing c-Fos among the cells identified was
370 then calculated per experiment. We also calculated the number of EYFP⁺ and EYFP⁻ cells with nuclear
371 c-Fos expression in MscL-transduced dishes. Each experiment had a minimum of 10 photographed
372 FOVs and minimum of 50 total cells counted per condition.

373 **Statistical analyses**

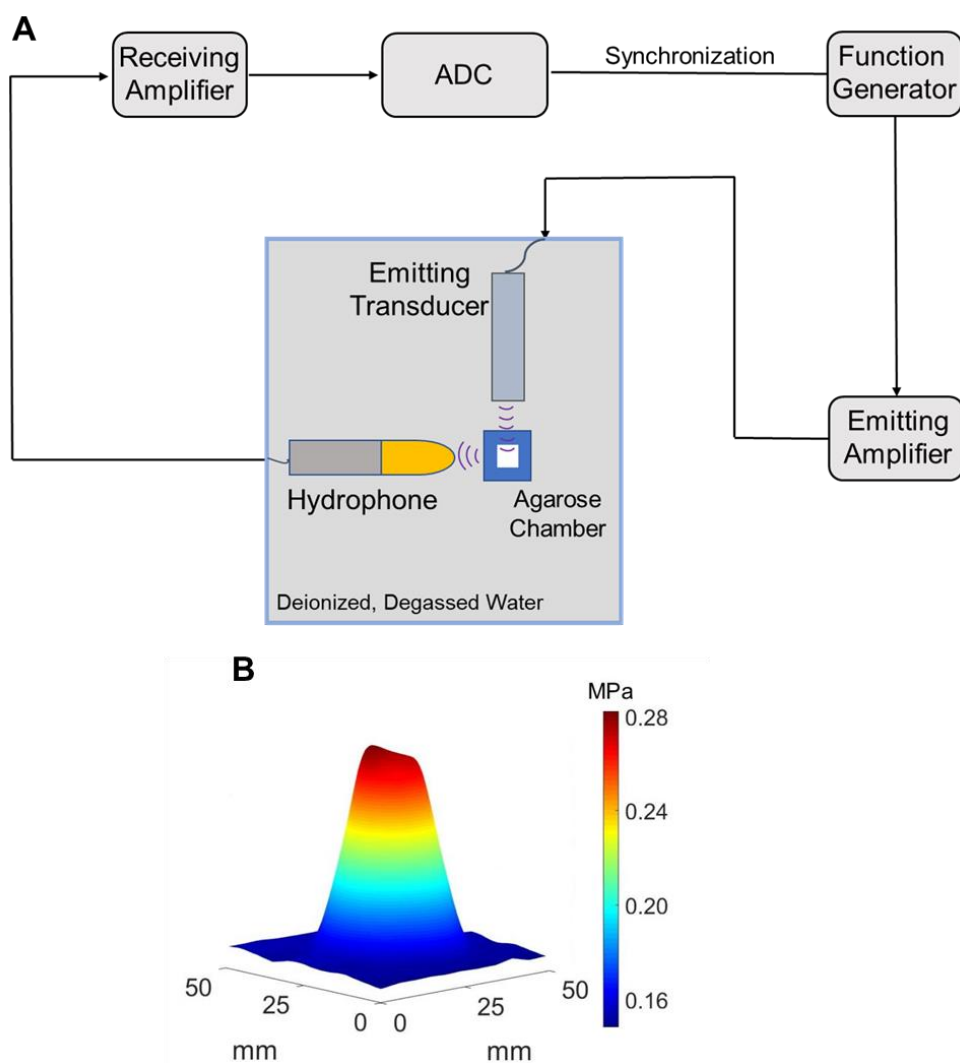
374 A minimum of 3 independent experiments were performed for all experiments shown, meaning at
375 least 3 separate ‘rounds’ of cell preparations, transfections or primary neuron harvests that were used for
376 various experiments. Wherever possible, multiple plates from each round were evaluated. The data were
377 collected into GraphPad Prism sheets for statistical analysis and graph preparation. Two-tailed unpaired
378 *t*-tests or one-way or two-way ANOVA were performed to determine statistical significance, with post-
379 hoc tests or corrections applied where appropriate. P values below 0.05 were considered significant.

Supporting information

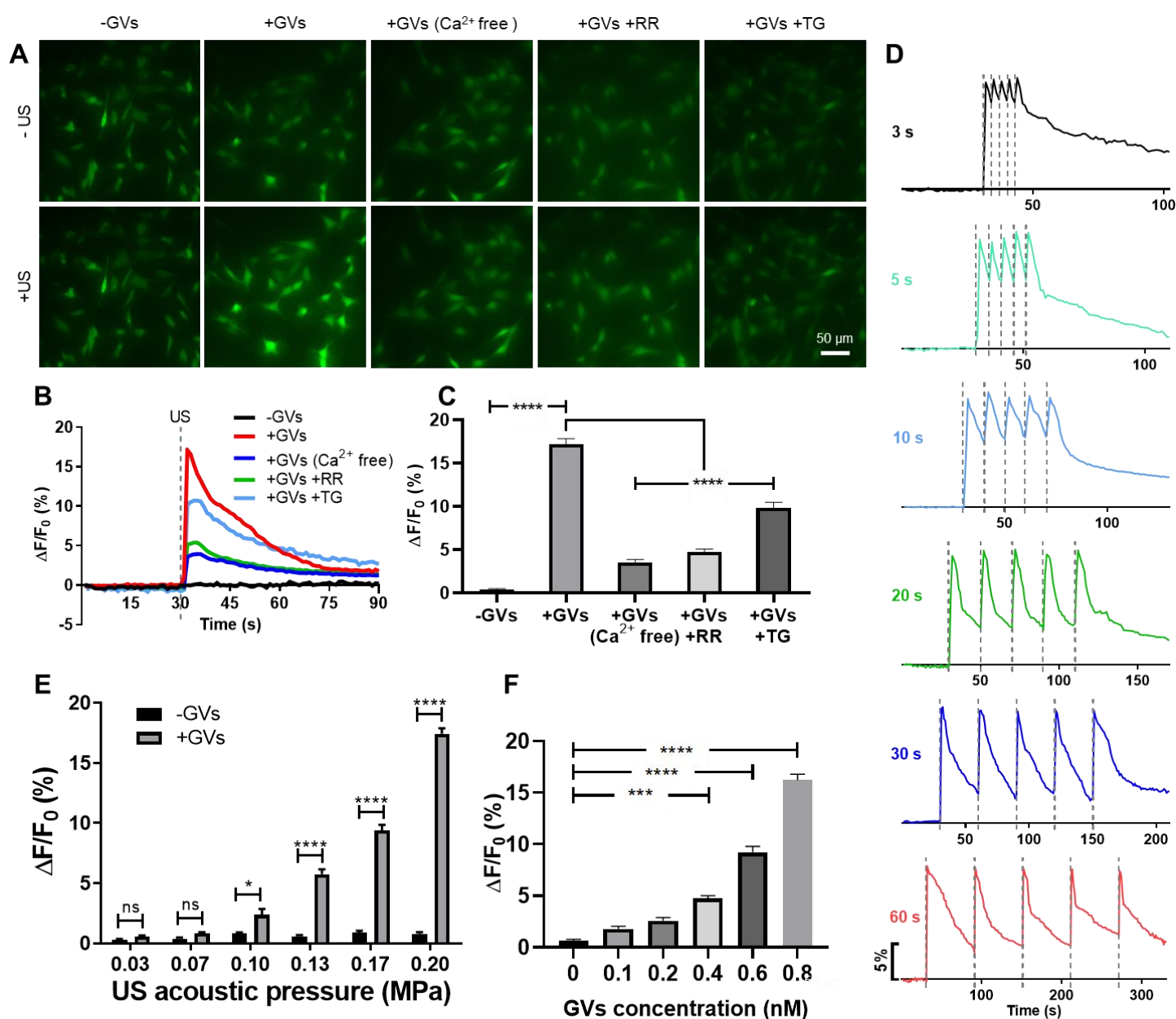
Supplementary Figs. 1 – 4

Figure Legends for Supplementary Videos 1 – 3

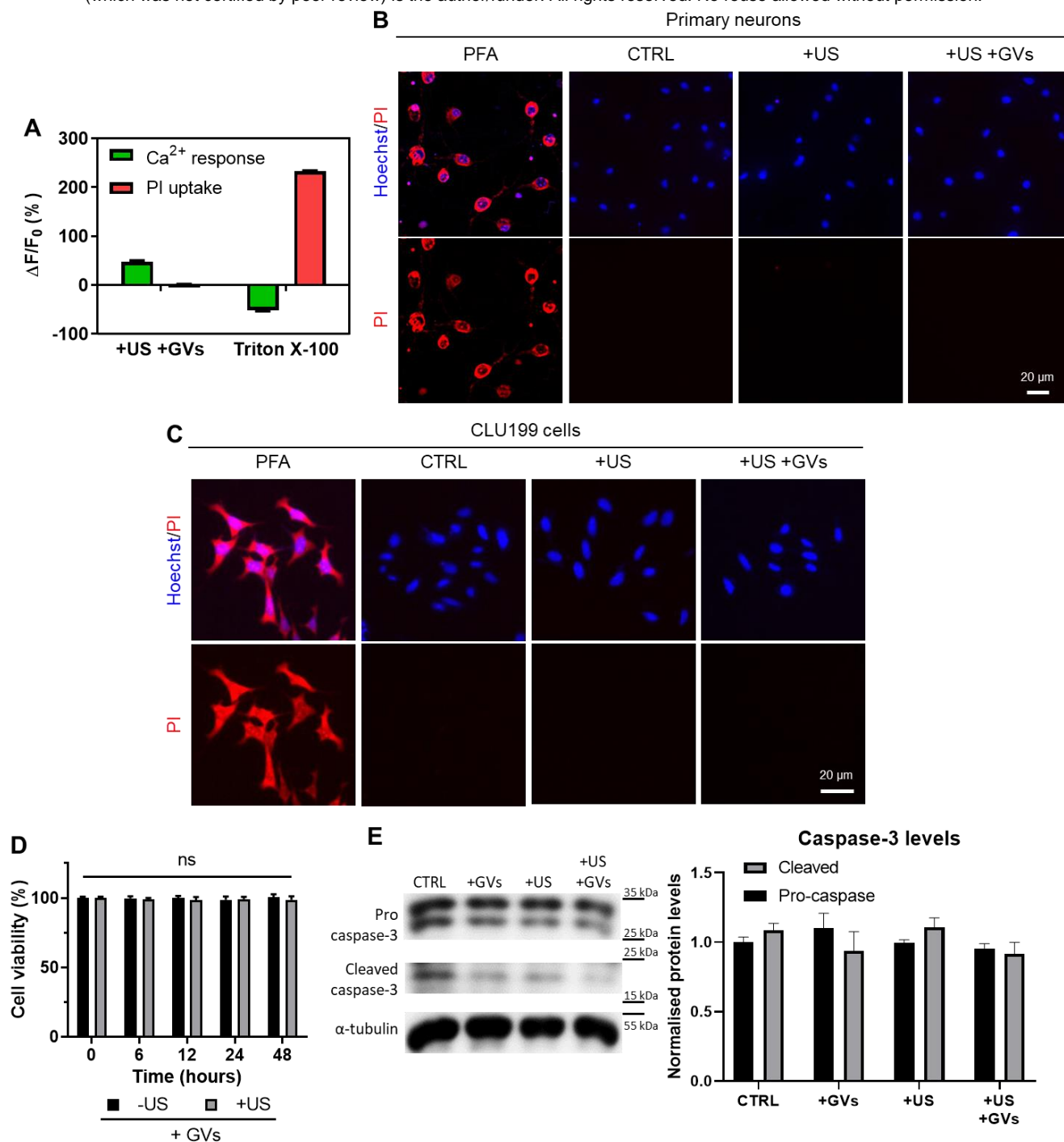
Supplementary Videos 1 – 3



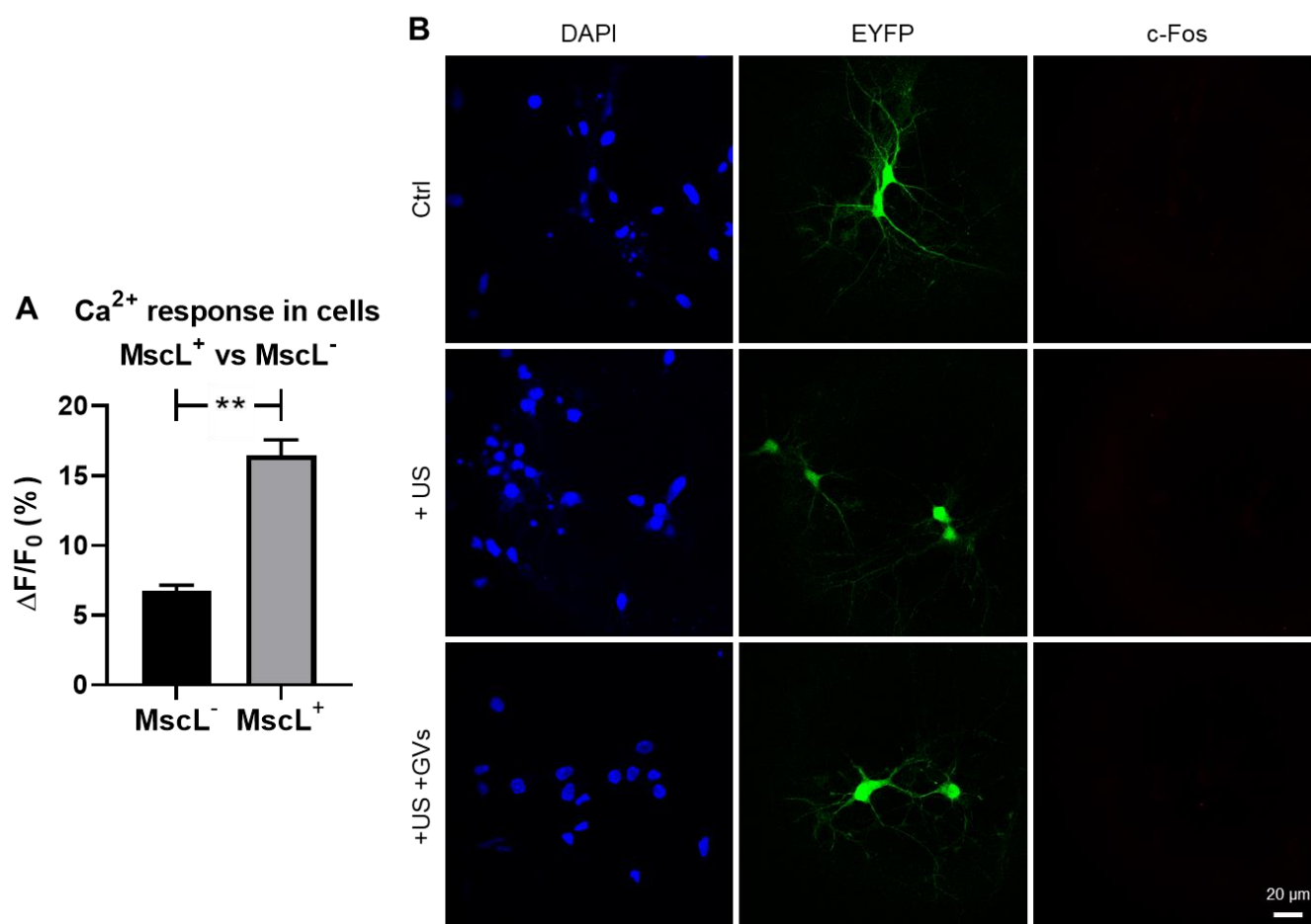
Supplementary Fig. 1. Cavitation detection system and acoustic field characterization. **a**, The *in vitro* passive cavitation detection system used to measure the backscattered acoustic signals of cavitation in response to GVs + ultrasound stimulation. **b**, Acoustic field characterization of our stimulation setup, with a spatial PNP of 0.28 MPa.



Supplementary Fig. 2. Calcium imaging of ultrasound + GV stimulation performed in the neuronal cell line, CLU199. **a**, Representative images of the typical Ca²⁺ response seen in CLU199 cells before and after 0.20 MPa ultrasound stimulation with or without GVs, in calcium-free medium, with the broad-spectrum mechanosensitive ion channel blocker ruthenium red (RR) or with the internal calcium chelator Thapsigargin (TG). **b**, Time-course of the imaging results depicted in **(a)**. **c**, Quantification of the fluorescence intensity changes shown in **(a)** and **(b)**. Bars represent mean ± SEM of 3 independent experiments. ****, *p* < 0.0001 compared only to the +GVs condition, unpaired one-way ANOVA with post-hoc Dunnett test. **d**, Time-resolved Ca²⁺ responses of CLU199 cells stimulated by 5 ultrasound pulses at varying intervals. **e**, Ca²⁺ response of cells to varying ultrasound intensities, 0.8 nM GV. Bars represent the mean ± SEM of 3 independent experiments. *, *p* < 0.05; ****, *p* < 0.0001; two-way ANOVA with Sidak correction. **f**, Ca²⁺ response of cells to varying GV concentrations, 0.20 MPa ultrasound. Bars represent the mean ± SEM of 3 independent experiments. *, *p* < 0.05; *p* < 0.0001 compared only to the 0 nM GVs condition, unpaired one-way ANOVA with Dunnett correction.



Supplementary Fig. 3. Evidence for the non-cytotoxicity of our GV-mediated ultrasound treatment. a, Quantified fluorescence changes of neuronal Ca^{2+} response and propidium iodide (PI) uptake of the representative images shown in Fig. 3A. Bars represent mean \pm SD from 3 independent experiments. **b**, Intracellular uptake of PI by primary neurons when untreated, treated with ultrasound-alone or with ultrasound + GV (0.20 MPa, 10 second interval, 10% duty cycle, 0.8 nM GV). Primary neurons treated with 4% paraformaldehyde (PFA) are shown here as a positive control for membrane permeation and PI staining. **c**, Intracellular uptake of PI by CLU199 cells. All treatment conditions were the same as in **(b)**. **d**, Cell viability following US+GV treatments. CLU199 cells were treated with either GV alone or US+GV for 15 minutes, and their cell viability at various times post-treatment was determined using an MTT assay. Bars represent the mean \pm SEM of 3 independent experiments. No significant differences found, multiple two-tailed *t*-tests with Holm-Sidak correction. **e**, Caspase-3 levels in primary neurons following various treatments. Primary neurons were exposed to either GV, ultrasound or ultrasound + GV for 15 minutes, proteins were collected after overnight incubation and a WB was performed to observe levels of pro- and cleaved caspase-3. Only upper bands were quantified for pro caspase-3. Bars represent the mean \pm SEM of 3 independent experiments. No significant differences found, two-way ANOVA with post-hoc Tukey test.



Supplementary Fig. 4. Further Ca^{2+} imaging and c-Fos staining data from transduced neurons. a, Quantified fluorescence intensity changes in primary neurons in a dish transduced with MscL-EYFP virus, as shown in Supplementary Video 3. Ca^{2+} intensities of cells in the same dish were quantified and grouped according to whether the cells expressed EYFP ('MscL⁺') or did not express EYFP ('MscL⁻'). **, $p < 0.01$, two-tailed unpaired t -test. **b,** Representative images of neuronal c-Fos expression in cells transduced with EYFP AAVs in untreated cells, or cells treated with ultrasound alone or US+GVs.

Figure Legends for Supplementary Videos 1 – 3

Video 1. Primary neurons show rapid and reversible calcium influx in response to each ultrasound pulse (0.20 MPa) when GVs (0.8 nM) are present (8 pulses delivered).

Video 2. GV-mediated ultrasound stimulation (0.20 MPa) triggers calcium influx into primary neurons, without allowing PI to enter cells (showing harmful membrane permeation).

Video 3. Primary neurons expressing the mechanosensitive ion channel MscL-G22S-EYFP display stronger responses to US+GVs (0.13 MPa, 0.4 nM) than EYFP⁻ cells.

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