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Ultrasound neuro-modulation chip: activation of sensory neurons in *Caenorhabditis elegans* by surface acoustic waves†‡

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Received 17th February 2017,
Accepted 12th April 2017

DOI: 10.1039/c7lc00163k

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Ultrasound neuro-modulation has gained increasing attention as a non-invasive method. In this paper, we present an ultrasound neuro-modulation chip, capable of initiating reversal behaviour and activating neurons of *C. elegans* under the stimulation of a single-shot, short-pulsed ultrasound. About 85.29% ± 6.17% of worms respond to the ultrasound stimulation exhibiting reversal behaviour. Furthermore, the worms can adapt to the ultrasound stimulation with a lower acoustic pulse duration of stimulation. *In vivo* calcium imaging shows that the activity of ASH, a polymodal sensory neuron in *C. elegans*, can be directly evoked by the ultrasound stimulation. On the other hand, AFD, a thermal sensitive neuron, cannot be activated by the ultrasound stimulation using the same parameter and the temperature elevation during the stimulation process is relatively small. Consistent with the calcium imaging results, the *tax-4* mutants, which are insensitive to temperature increase, do not show a significant difference in avoidance probability compared to the wild type. Therefore, the mechanical effects induced by ultrasound are the main reason for neural and behavioural modulation of *C. elegans*. With the advantages of confined acoustic energy on the surface, compatible with standard calcium imaging, this neuro-modulation chip could be a powerful tool for revealing the molecular mechanisms of ultrasound neuro-modulation.

Introduction

The incidence of neurological and psychiatric disorders, such as Parkinson's disease, epilepsy and depression, is rapidly increasing around the world.[1,2] Treatment of these neuropsychiatric diseases is one of the greatest challenges in modern medicine and is getting increasing attention from neuroscientists. Unlike cancer therapy, it is difficult to treat these neurological diseases by surgery. Stimulation and modulation of the brain nucleus and neural circuit are the most fundamental strategies in the treatment of neurological and psychiatric disorders.[3,4] The invasiveness of traditional deep brain stimulation techniques involving electric and optogenetic properties has limited their utility in the neural circuit stimulation associated with the deeply located subcortical nucleus.[5–7] Transcranial magnetic stimulation (TMS) is an emerging technology for the non-invasive stimulation of the brain and has potential therapeutic applications in cognitive neuroscience, neurophysiology, psychiatry, and neurology.[8,9] However, the applicability of TMS is hampered due to its spatial resolution and the depth of stimulation.

Ultrasound is a kind of mechanical wave which is capable of delivering acoustic energy into the deep brain nuclei non-invasively.[10,11] Our previous work has shown that ultrasound can successfully activate the brain circuits in mice, inducing various movement responses.[12] Yoo *et al.* demonstrated the feasibility of using ultrasound pulses to transiently modulate the function of regional brain tissue in rabbits.[13] A recent publication by Legon *et al.* indicated that transcranial ultrasound could modulate sensory-evoked brain activity and cortical function in humans.[14] So far, the work on ultrasound neuro-modulation is based on bulk waves generated by a conventional ultrasound transducer. The vibration of bulk

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† L. J. K., L. M. and H. R. Z. designed the experiments; W. Z., J. J. W., K. Y. W., L. L. N., X. Y. Z. and B. H. performed the experiments; F. L., F. Y. C., X. L., Y. C. and H. K. C. contributed to data analysis; W. Z., F. L., F. Y. C., Y. C. and L. M. fabricated the ultrasound neuro-modulation chip; J. J. W., K. Y. W., H. K. C. and L. J. K. prepared and provided *C. elegans*; L. M. and H. R. Z. wrote the paper with contributions from all authors.

‡ Electronic supplementary information (ESI) available. See DOI: 10.1039/c7lc00163k

§ These authors contributed equally to this work.

piezoelectric ceramics (PZT), however, results in acoustic energy dissipation and temperature elevation. Thus, the desired ultrasonic device should localize acoustic energy and stimulate animals with a small input power.

The nematode *Caenorhabditis elegans* (*C. elegans*) is an excellent model organism in neurobiology research due to its small nervous system consisting of just 302 neurons. More than sixty percent of the *C. elegans* genes have human homologs, and in many cases these genes are involved in mediating conserved processes. *C. elegans*, therefore, have been widely used in the neurobiology research.[15–20] Recently, Ibsen *et al.* demonstrated that wild-type *C. elegans* initiated reversal behaviour in response to the oscillation and destruction of microbubbles within an acoustic field.[21] *C. elegans* were insensitive to the acoustic field without microbubbles, limiting the use of ultrasound in neruo-modulation.

In this paper, we present an ultrasound neuro-modulation chip on which *C. elegans* could initiate a backward movement induced by ultrasound stimulation without the need for microbubbles. Fig. 1(a) shows the schematic of the ultrasound stimulation of *C. elegans* on a neuro-modulation device. The worms were placed on an agar surface and were challenged with ultrasound stimulation, and their locomotion patterns were recorded. The ultrasound used in this study were surface acoustic waves (SAWs) which have been widely used in the study of particle manipulation,[22–27] droplet actuation[28–31] and ultrasound bioeffects.[32–34] SAWs are generated by interdigital transducers (IDTs) on a piezoelectric substrate and propagate along its surface with their energy strongly confined to the surface of the piezoelectric substrate.[35,36] The ultrasound neuro-modulation chip is fabricated using the standard microelectromechanical system (MEMS) technology, ensuring the stability and repeatability. Furthermore, the small size and excellent optical transparency of the chip make it possible to record the *C. elegans* behaviours and neuronal activity in a real-time manner, providing a powerful tool for investigating the mechanism of ultrasound neuro-modulation.

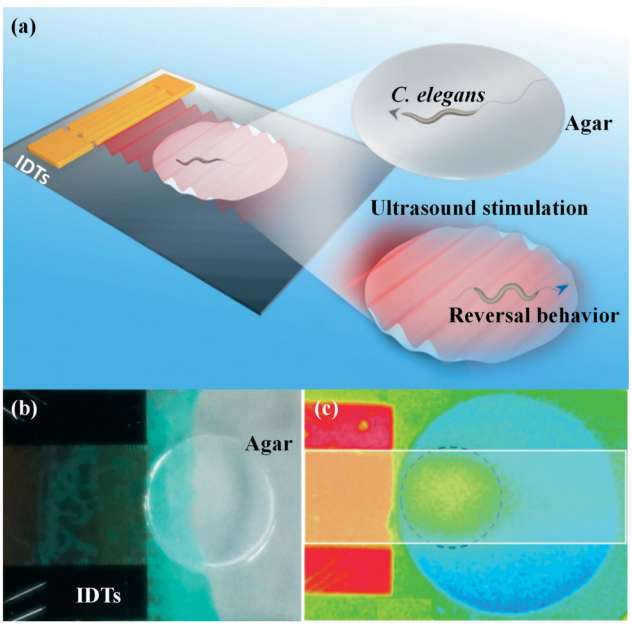

Fig. 1 (a) Schematic of the ultrasound neuro-modulation chip and the reversal behaviour of *C. elegans* evoked by ultrasound stimulation. (b) Photograph of the ultrasound neuro-modulation chip consisting of interdigital transducers (IDTs) and an agar plate. (c) Thermal image shows the aperture of the IDTs and the beam width of the SAWs.

Materials and methods

Ultrasound neuro-modulation chip fabrication

The ultrasound neuro-modulation chip consists of IDTs and an agar plate (Fig. 1(b)). The fabrication of the stimulation device is a standard lift-off process. Aluminium electrodes with a thickness of 200 nm were sputtered to a 1 mm-thick 128° YX-$LiNbO_3$ substrate. The straight electrode widths and spacing gaps were held at 40 μm. The straight IDTs generated an *a* plane wave with a wavelength of 160 μm and the resonant frequency of the IDTs was approximately 28.11 MHz, measured by a network analyzer (ZVA40, Rohde and Schwarz, Germany). Fig. 1(c) shows the beam width of the SAWs and the position of the agar plate (dotted line circle) relative to the IDTs by an infrared thermal imaging method.[37] The distribution of the *a* plane-wave acoustic field was relatively uniform, generated by the straight IDTs. The worms, thus, on the agar surface were subjected approximately to the same stimulation intensity. An agar plate with a diameter of 5 mm was isolated from the bulk agar using a puncher (Harris Uni-Core, Jed Pella, Inc.). As the agar plate was largely composed of water (98% in weight), it had a good ultrasound transmission performance. Most of the ultrasound energy could be delivered to the agar surface effectively.

Behavioural assay system

An experimental system was set up to characterize the behaviour of the worms in response to ultrasound stimulation. Radio frequency (RF) signals at 28.11 MHz were generated by an arbitrary waveform generator (AFG 3102, Tektronix, Beaverton, Oregon), amplified (ZHL-20 W-13+, Mini-Circuits, Brooklyn, NY, USA), and then applied to the single IDTs. The electric power applied to the device was approximately 5 W, measured by a power sensor (PWR-4GHS, Mini-circuits, Brooklyn, NY, USA) with a cold 30 dB attenuator. The device was mounted on a stereomicroscope (Leica M205 C, Leica, Germany) and the behaviour of the worms was recorded by a high-speed CCD camera (MC1310, Mikrotron, Germany) with a frame rate of 30 fps. Data were analysed using ImageJ software (National Institutes of Health, Bethesda, Maryland). A LED synchronized with a triggered pulse of ultrasound was used to indicate the generation of the ultrasound. Once the ultrasound was generated by IDTs, the light was turned on.

Worm strains and maintenance

All nematode strains were grown on NG agar plates seeded with OP50 *Escherichia coli* bacteria at 20 °C using standard

methods.[38] Adult day 1 worms were used for all experiments. The wild type N_2, *tax-4 (ks11)* strains, and worms expressing the calcium indicator GCaMP5.0 in ASH and AFD neurons, under the *sra-6* and *gcy-8* promoters, respectively, were used in this study.[39]

Calcium imaging

Day 1 adult worms were glued to the substrate by surgical glue as previously described[40] (Gluture Topical Tissue Adhesive, Abbott Laboratories). Ultrasound stimulation was subsequently delivered toward the worms and a CCD camera (OptiMOS, QImaging, Canada) equipped with a fluorescence microscope (D-35578 Wetzlar, Leica, Germany) was used to record the neuronal activity.

Results and discussion

Ultrasound stimulation evokes avoidance responses of *C. elegans*

To test whether *C. elegans* respond to ultrasound stimulation without the oscillation of microbubbles, we transferred worms from standard culture plates to the surface of the agar without OP50 bacteria mounted on the $LiNbO_3$ substrate, and recorded their locomotion behaviour with a frame rate of 30 fps. Initially, the worms crawled freely on the agar surface for 5 min to adapt to the new environment. As shown in Fig. 2(a), the worms initiated backward locomotion and reversal immediately when stimulated with a single radio frequency (RF) ultrasound pulse (28.11 MHz frequency, 6.40 ms pulse duration, and 5 W electric input power). Video S1 in the ESI‡ shows the response of a worm to the single pulsed ultrasound stimulation on the agar surface. To avoid their adaptation to the ultrasound, each worm was subjected to a single stimulus. In the control group, the worms were only exposed to the LED light without ultrasound stimulation. As shown in Fig. 2(b), 85.29% ± 6.17% of worms displayed reversal upon ultrasound stimulation whereas a few worms showed avoidance behaviours in the control group. Furthermore, the proportion of worms showing reversal behaviour was dependent on the pulse duration. Upon decreasing the pulse duration, the proportion of worms responding to the ultrasound stimulation decreased correspondingly (Fig. 2(c)). A longer pulse duration (>6.40 ms) was not employed in the experiments to avoid the temperature elevation induced by ultrasound. These results demonstrated that ultrasound stimulation induced avoidance responses of worms.

We further stimulated different positions of the worm body to determine which part was essential for ultrasound-sensing. The boundary of the acoustic field was determined by the width of the aperture[41] and the head, head plus middle, and the whole body of worms were stimulated by ultrasound as shown in Fig. 3(a). Our results showed that the proportion of worms displaying reversal in response to an ultrasound stimulus on the head plus middle (86.96%) was apparently more than that of on the head only (56%) and approximately equal to that of on the whole body (85.29%). These observations implied that both the head and mid-body of the worms are sensitive to ultrasound stimulation (Fig. 3(b)).

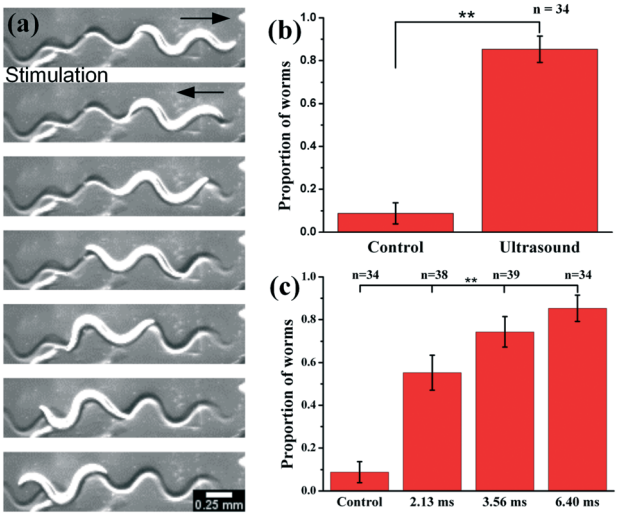

Fig. 2 (a) Image sequence showing a worm initiating backward locomotion and reversal immediately at the stimulation of a single-shot, short-pulsed ultrasound (28.11 MHz frequency, 6.40 ms pulse duration, and 5 W electric input power). (b) The proportion of worms displaying reversal is 85.29% ± 6.17% for the ultrasound stimulation group while that of the control group is less than 10% (n = 34). All behavioural data were analysed using the Fisher's exact test, with error bars showing the standard error of the proportion. The difference between the two groups is statistically significant ($p < 0.01$). (c) Reversal response of worms to different pulse durations. The proportion of worms displaying reversal response decreased with the reduction in the pulse duration.

Adaptation of *C. elegans* to ultrasound stimulation

Upon prolonged or repetitive stimulation, most sensory neurons display decreased sensitivity to the stimulation, a phenomenon called adaptation, which is ubiquitously present in the animal kingdom to prevent the saturation of the sensory transduction machinery.[42] To investigate the adaptation of the worms to ultrasound stimulation, periodic pulsed

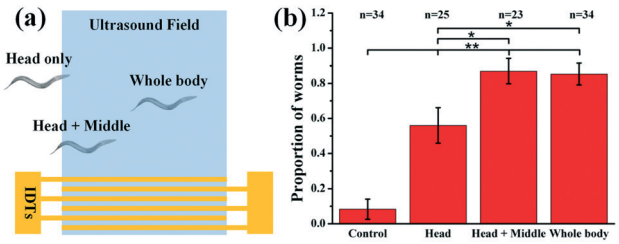

Fig. 3 Response of the worms at different stimulation positions. (a) The schematic of ultrasound stimulation on the head, head plus middle, and the whole body. Due to the apparent boundary of the acoustic field, spatial stimulation at various positions can be achieved. The blue area represents the acoustic field generated by the IDTs. (b) The proportion of the reversal behaviour of response to an acoustic stimulus on the head (n = 25), head plus middle (n = 23), and whole body (n = 34) is 56% ± 10.13%, 86.96% ± 7.18% and 85.29% ± 6.17%, respectively.

ultrasound was generated at various ultrasound pulse durations. The normalized results were equal to the ratio of the proportion of avoidance probability of worms at different times to that at the first time. Fig. 4(a) shows the avoidance probability of the worms after repeated stimuli with a 10 s interval with different pulse durations. No significant adaptation was observed when the worms were subjected to repeated ultrasound stimuli with a 6.40 ms pulse duration. Notably, when the pulse duration was reduced to 2.13 ms, the percent initial response of worms declined by 70% after 16 applications of stimuli. There was a significant difference among the three groups with different pulse durations (p = 0.001). The avoidance probabilities at various stimulation intervals were also investigated when the pulse duration was held at 2.13 ms. As displayed in Fig. 4(b), the worms adapted to ultrasound stimulation when the stimulation interval was 10 s and 15 s and there was no significant difference. These results suggested that worms adapt to a lower pulse duration of ultrasound stimulation.

Ultrasound stimulation activates the ASH neurons

Intracellular Ca^{2+} events are hallmarks of neuronal activity. To investigate whether ultrasound could modulate neuronal activity, we transgenically expressed the calcium indicator GCaMP5.0 in the ASH neurons under the *sra-6* promoter.[43] The Ca^{2+}-insensitive red fluorescent protein mCherry was co-expressed with GCaMP5.0 as a reference. The ASH neuron is a polymodal sensory neuron in *C. elegans* and mediates the avoidance responses when a broad range of aversive stimuli are applied, including harmful repellents,[44] high osmolarity,[45] and touch to the nose.[46] *C. elegans* was glued to the substrate

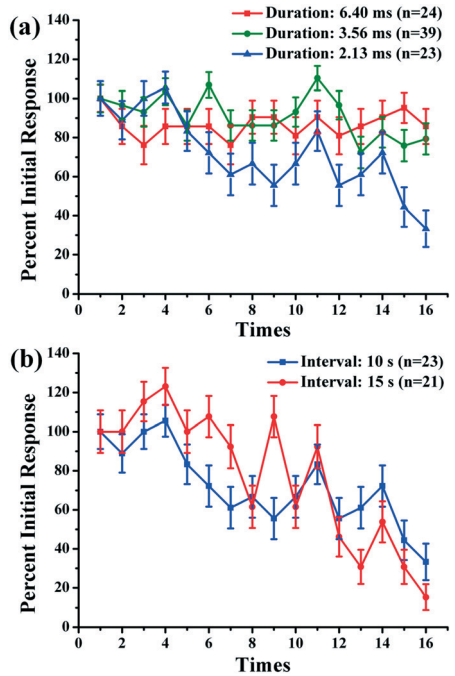

Fig. 4 (a) Avoidance probability of the worms after repeated stimuli with different pulse durations at a constant interval of 10 s. The worms are more likely to adapt to the stimulation with a lower pulse duration (p = 0.001). (b) The avoidance probability of the worms after repeated stimuli with different intervals when the pulse duration was held at 2.13 ms. The results show that the worms adapted to ultrasound stimulation when the stimulation interval was 10 s and 15 s.

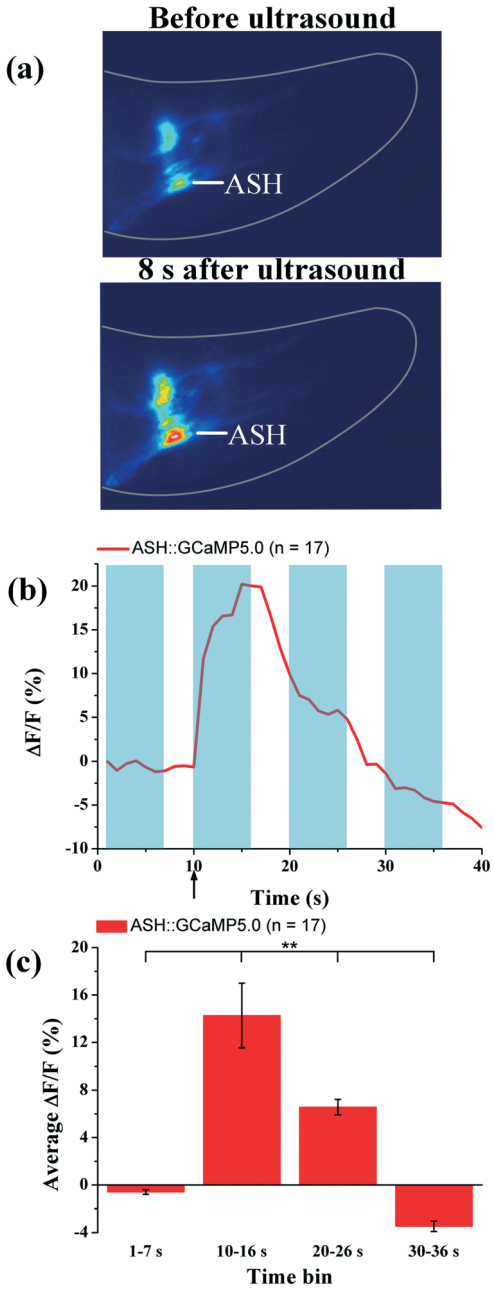

Fig. 5 ASH neurons are sensitive to ultrasound stimulation. (a) Fluorescence in the ASH cell body. False-color images showing the changes in GCaMP5.0 fluorescence in ASH neurons responding to the ultrasound stimulation. Warmer colours indicate Ca^{2+} elevations in ASH neurons. (b) Average ratio change of fluorescence intensity in ASH under ultrasound stimulation at t = 10 s (n = 17). The peak of the fluorescence intensity in ASH occurred at 8 s after ultrasound stimulation. (c) Average responses binned by distinct times for ASH::GCaMP5.0. The average increase in the intensity of GCaMP5.0 fluorescence was approximately 14.27% ± 2.72% (n = 17).

and then *in vivo* calcium imaging was carried out to study the fluorescence variations of ASH under ultrasound stimulation. Consistent with our behavioural data, robust Ca^{2+} elevations in the ASH neurons were observed under ultrasound stimulation (Fig. 5(a)). As shown in Fig. 5(b), a significant increase of Ca^{2+} elevation in ASH neurons could be achieved at 8 s after ultrasound stimulation which lasted for 20 s (n = 17). Furthermore, Fig. 5(c) indicates that the average increase in the intensity of GCaMP5.0 fluorescence was approximately 14.27% ± 2.72% (n = 17) at a single pulsed ultrasound stimulus (6.40 ms pulse duration, and 5 W input power). The fluorescence intensity of mCherry, on the other hand, remained at the same level regardless of the application of ultrasound. As the ASH neuron is sensitive to the mechanical stimulation,[46] the mechanical effects induced by ultrasound are likely associated with the neuronal calcium activation. These results indicate that the activity of the mechano-sensory neurons can be evoked by ultrasound stimulation, leading to the behavioural responses of *C. elegans*. Video S2 in the ESI‡ shows the variation of fluorescence intensity in ASH neurons under the stimulation of ultrasound.

Ultrasound stimulation-induced temperature variation

Ultrasound stimulation may induce both mechanical and thermal effects.[47] We next sought to determine whether thermal effects induced by ultrasound are involved in our study. We visualized the temperature profile of the surface of the agar under pulsed ultrasound stimulation using a thermal infrared imager (R300, NEC Avio, Tokyo, Japan). Fig. 6(b) shows the transient temperature profile of the agar induced by ultrasound stimulation (6.40 ms pulse duration and 5 W input power). Short pulsed ultrasound did not cause significant transient temperature elevation, which was less than 0.5 °C, compared to the control group (Fig. 6(a)); the temperature of the agar surface was also stable after 16 rounds of ultrasound stimulation with a time interval of 10 s (Fig. 6(c)). These results indicate that the thermal accumulation was relatively small, ensuring the viability of *C. elegans*. No significant change was observed in the velocity and pattern of movement of worms. Furthermore, the lifetime of the stimulated worms was about 18 days with normal reproductive capacity which was similar to the unexposed worms. The results demonstrate that ultrasound stimulation causes no visible adverse impact on *C. elegans* or apparent long-term developmental change.

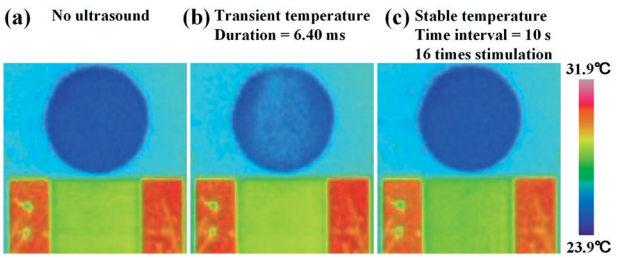

Fig. 6 Temperature distribution and elevation of the agar surface by ultrasound stimulation. (a) No ultrasound was applied. (b) Transient temperature of the agar surface by ultrasound stimulation (6.40 ms pulse duration and 5 W input power). The temperature elevation was less than 0.5 °C. (c) Stable temperature of the agar surface after 16 times ultrasound stimulation (10 s time interval, 6.40 ms pulse duration, and 5 W input power). No obvious increment of the temperature can be detected.

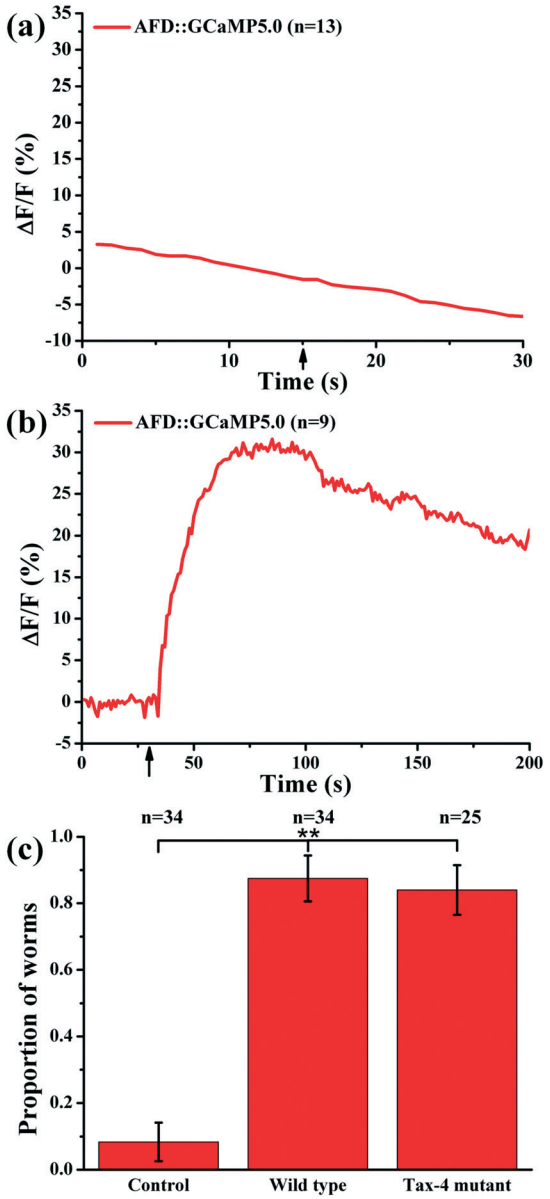

Fig. 7 AFD neurons are not sensitive to ultrasound stimulation. (a) The average fluorescence intensity of AFD in response to the ultrasound stimulation at the parameter used for ASH stimulation (n = 13). There is no significant change in fluorescence intensity under ultrasound stimulation, indicating AFD is not activated. (b) The average fluorescence intensity of AFD in response to temperature elevation. A calcium increase was observed when the surrounding temperature increased to 10 °C at 30 s, suggesting that the AFD neuron could be evoked by the temperature increment. (c) No significant difference was observed in the avoidance probability under ultrasound stimulation between the wild type (n = 34) and *tax-4* mutants (n = 25).

Ultrasound stimulation does not activate AFD neurons

Despite the small temperature rise induced by a single-pulsed ultrasound (Fig. 6(b)), previous work has shown that the worms are very sensitive to temperature variation.[48] It is, therefore, necessary to examine whether the thermal effects of ultrasound could activate the neurons. AFD is a major thermosensory neuron mediating thermotaxis behaviour.[49] Using the transgenic strain *gcy-8::GCaMP5.0*, we investigated whether the AFD neurons could be activated on our ultrasound neuro-modulation chip. Fig. 7(a) shows the average fluorescence intensity of AFD in response to ultrasound stimulation at the parameter used for ASH stimulation (n = 13). No significant calcium increase was observed, indicating that the AFD neuron was not activated by short pulsed ultrasound. For the control group, AFD could be activated when the surrounding temperature increased by 10 °C (Fig. 7(b)). The cyclic nucleotide-gated (CNG) channel subunit TAX-4 is essential for thermotransduction. It has been shown that *tax-4* mutant animals are not responsive to temperature elevation.[50]

Thus, we examined whether *tax-4* mutant animals are sensitive to ultrasound stimulation. Fig. 7(c) shows the avoidance probability of the worms between the wild type and *tax-4* mutants when the pulse duration was 6.40 ms. The results illustrate that the avoidance probability between the two groups was almost the same with no statistically significant difference. Therefore, the mechanical effect of the ultrasound is the main reason leading to the avoidance behaviours.

Conclusions

We have demonstrated that *C. elegans* show dramatic responses to ultrasound stimulation on a neuro-modulation chip. Calcium increases in the ASH neurons can be observed immediately after the stimulation by ultrasound. The thermal images show that the temperature elevation during the stimulation process is relatively small and cannot induce detectable calcium elevation in the thermosensory neuron AFD. Thus, the escape responses of the worms are mainly determined by the mechanical effects of the ultrasound. Compared with traditional ultrasound transducers, the acoustic energy generated by this chip is localized along the surface of the substrate, facilitating the neuronal stimulation with a relatively small input power. Due to the advantages of its transparent and miniature character, the device is readily compatible with functional assays such as electrophysiological recording and calcium imaging, providing a powerful tool for investigating the mechanism of ultrasound neuro-modulation.

Acknowledgements

The work was supported by the 973 Program (Grant No. 2015CB755500), the National Natural Science Foundation of China (Grant No. 11674347, 31600689, 11574341, 11534013, 81527901, 11325420 and 61306145), the China Postdoctoral Science Foundation (Grant No. 2015M572382), the Shenzhen Basic Science Research (JCYJ20160429184552717 and JCYJ20150316144345646) and the Shenzhen Science and Technology Innovation Committee Grant (KQCX20140521115045445).

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