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Non-invasive peripheral nerve stimulation via focused ultrasound *in vivo*

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Abstract

Focused ultrasound (FUS) has been employed on a wide range of clinical applications to safely and non-invasively achieve desired effects that have previously required invasive and lengthy procedures with conventional methods. Conventional electrical neuromodulation therapies that are applied to the peripheral nervous system (PNS) are invasive and/or non-specific. Recently, focused ultrasound has demonstrated the ability to modulate the central nervous system and *ex vivo* peripheral neurons. Here, for the first time, noninvasive stimulation of the sciatic nerve eliciting a physiological response *in vivo* is demonstrated with FUS. FUS was applied on the sciatic nerve in mice with simultaneous electromyography (EMG) on the tibialis anterior muscle. EMG signals were detected during or directly after ultrasound stimulation along with observable muscle contraction of the hind limb. Transecting the sciatic nerve downstream of FUS stimulation eliminated EMG activity during FUS stimulation. Peak-to-peak EMG response amplitudes and latency were found to be comparable to conventional electrical stimulation methods. Histology along with behavioral and thermal testing did not indicate damage to the nerve or surrounding regions. The findings presented herein demonstrate that FUS can serve as a targeted, safe and non-invasive alternative to conventional peripheral nervous system stimulation to treat peripheral neuropathic diseases in the clinic.

Introduction

Whether in the laboratory or the clinic, few techniques are as versatile as ultrasound. Ultrasound imaging is a widespread technique for monitoring fetal development or cardiac abnormalities, but many recent research advances are employing ultrasound as a therapeutic treatment for procedures that require non-invasive, target specific, and temporally efficient procedures. These techniques utilize the ability of the ultrasound to have thermal, mechanical or a combined thermal/mechanical effect.

Over the past decade a subset of therapeutic ultrasound utilizing focused ultrasound (FUS) has been shown to be effective at stimulating, or inhibiting neuronal activity in both the central nervous system (CNS; Defieux *et al* 2013, Hameroff *et al* 2013, Legon *et al* 2014, Lee *et al* 2015, Kamimura *et al* 2016) and peripheral nervous system (PNS; Mihran *et al* 1990, Gavrilov *et al* 1996, Tsui *et al* 2005, Juan *et al* 2014, Downs *et al* 2015, Wright *et al* 2015, Kubanek *et al* 2016). Some current studies utilizing FUS to stimulate peripheral nerves *ex vivo* suggest thermal effects responsible for the block of action potentials (Tsui *et al* 2005, Colucci *et al* 2009). The application of FUS increased the temperature of the solution surrounding the *ex vivo* nerve, first reducing the peak-to-peak response, and eventually blocked the action potential from propagating throughout the axon. Other *ex vivo* studies indicated a mechanical effect during FUS stimulation (Mihran *et al* 1990, Gavrilov *et al* 1996, Wright *et al* 2015, Kubanek *et al* 2016). Kubanek *et al* demonstrated activation of mechanosensitive ion channels with specific FUS parameters during *in vitro* stimulation of the *xenopus* oocyte system. Other work revealed FUS stimulation targeting the axon of the sciatic nerve elicits compound action potentials *ex* vivo

(Mihran *et al* 1990, Wright *et al* 2015). One study investigated the effects of FUS application *in vivo* targeting the vagus nerve, but only recorded the effect ultrasound had on the compound action potential while electrically stimulating the nerve (Juan *et al* 2014). The only *in vivo* studies examining physiological effects of FUS stimulation has been targeting various structures in the CNS (Deffieux *et al* 2013, Hameroff *et al* 2013, Legon *et al* 2014, Lee *et al* 2015, Kamimura *et al* 2016). Results from stimulating specific brain regions resulted in the delay of antisaccade motion in monkeys and limb twitching in mice, to enhanced tactile discrimination and generation of phosphenes in humans. All these studies showing stimulation of the PNS or CNS demonstrate that FUS can have an excitatory or inhibitory effect on neurons, with different potential mechanisms of action depending on the ultrasound parameters employed. Thus far, it has not been shown if FUS stimulation of the PNS is sufficient to elicit physiological effects *in vivo*.

Successful modulation of the PNS with FUS *in vivo* would provide physicians with an important complementary tool to treat diseases and conditions such as neuropathic pain. Current methods of treatment involve drug therapy, electrical stimulation and surgical interventions, yet each of those methods have drawbacks (National Institute of Neurological Disorders and Stroke 2014). Following drug therapy, which is spatially untargeted, the next most common therapy is electrical stimulation. Electrical stimulation is either invasive and targets the specific damaged peripheral nerve, or non-invasive and non-specific, targeting the region around the damaged peripheral nerve (Adigüzel *et al* 2016). Alternatively, therapeutic ultrasound can be a noninvasive, and targeted approach for treating peripheral nerve damage. This would eliminate the potential side effects from drug therapies and the need for invasive surgery. Additionally, as FUS systems are inexpensive and highly portable, they allow clinicians to treat a larger patient population.

In this study, we demonstrate for the first time FUS stimulation of peripheral nerves *in vivo* can elicit a physiological response. The sciatic nerve in anesthetized mice was stimulated via FUS while EMG signals were recorded through needle electrodes placed into the tibialis anterior muscle. Successful stimulation of the sciatic nerve rather than the surrounding muscle tissue was verified through transecting the nerve downstream of the FUS targeted region, which completely eliminated the electromyography (EMG) signal. These findings indicate that FUS can be used for the excitation of peripheral nerves noninvasively and safely resulting in the desired physiological response.

Materials and methods

All procedures with mice were approved by the Institutional Animal Care and Use Committee of Columbia University and ACURO. Male C57BL/6J mice weighing between 22 to 28 g were used in all experiments ($n = 42$). Mice were housed in rooms with 12 h light/dark cycles and provided food and water ad lib. Mice were anesthetized with 50 mg kg^{-1} pentobarbital for all FUS and electrical stimulation experiments. A heating pad was used to maintain proper body temperature throughout the experiments. For non-survival studies (histology, electrical stimulation, thermocouple) mice were sacrificed by cervical dislocation before harvesting the hind limbs.

All FUS experiments were conducted with a HIFU transducer with a 3.57 MHz center frequency (0.46 × 3.55 mm focal area, 35 mm focal depth; SU-107, SonicConcepts, Seattle, WA, USA). This frequency was chosen as the focal diameter is comparable to that of a mouse's sciatic nerve (50 μm). The driving signal was derived from a function generator (33220A, Keysight Technologies Inc., Santa Rosa, CA, USA) and amplified through a 150 W amplifier (A150, Electronics & Innovation, Ltd Rochester, NY, USA). The transducer was calibrated with a fiber optic hydrophone (HFO-690, ONDA, Sunnyvale, CA, USA) which did not display any ringing due to the transducer after the transducer was turned off. The sciatic nerve was targeted as it innervates the leg muscles branching into the peroneal and tibial nerves. FUS parameters employed during the experiments ranged from 1.1–8.3 MPa peak negative pressure, 4 ms–1 s stimulation duration, 15–100% duty cycle, 1 kHz PRF. Accounting for the pressure attenuation through the muscle to reach the sciatic nerve, delivered pressures ranged from 0.7 to 5.4 MPa (Marquet *et al* 2011). Changes for each parameter were modified as such: Duty cycle 15, 35, 50, 90, 100%, Pressure 0.6 MPa increments and duration 100 ms increments, unless under 10 ms, then by 1 ms increments. Each combination of parameter was tried $n = 5$ times for a total of 2000 trials. The sciatic nerve was targeted for FUS experiments through B-mode imaging with a L22-14V imaging probe (128 elements, linear array, 18.5 MHz, Verasonics, Kirkland, WA, USA). Coupling gel was used for both B-mode imaging and FUS stimulation. A focusing cone filled with degassed water was used with the FUS stimulation. A mechanical positioning system was utilized for placement of both B-mode and stimulating transducers with submillimeter resolution (Velmex, Bloomfield, NY, USA). Stimulation and imaging were controlled and recorded through in-house developed Matlab code (Mathworks, Natick, MA, USA). EMG recordings were acquired with two stainless steel needle electrodes placed in the Tibialis Anterior muscle (figure 1(A)) and recorded at 5 MHz (Biopac, Goleta, CA, USA). A radiation force balance was used to determine radiation force generated by the transducer (Maruvada *et al* 2007). Video recordings were acquired simultaneously with stimulation to archive muscle activation along with EMG activity.

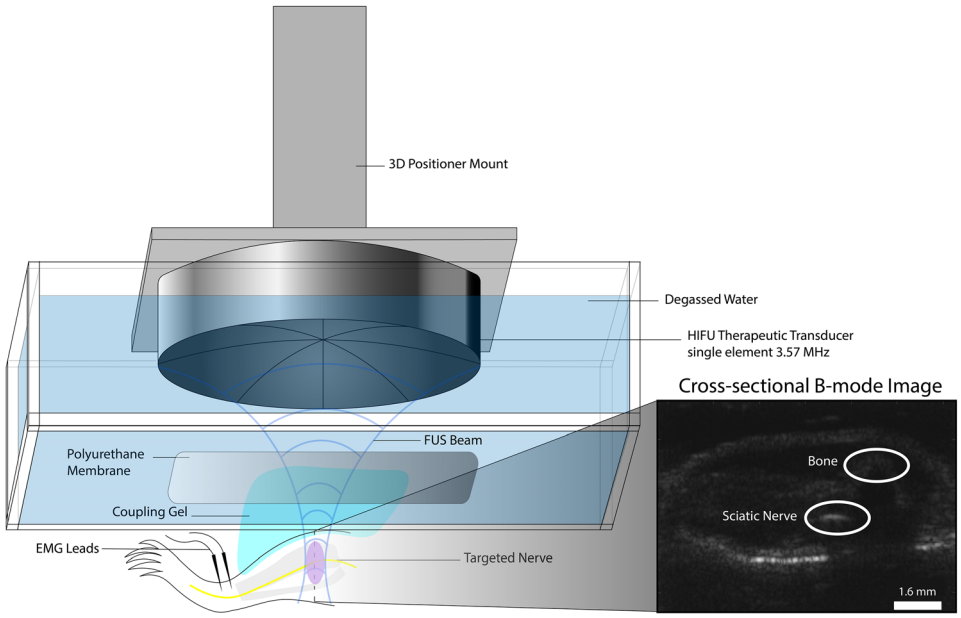

Figure 1. FUS Stimulation setup. Targeting and positioning for both the stimulation and imaging transducer. Shows the targeting (lilac focal area) of the ultrasound transducers on the sciatic nerve. The position of the mouse leg under the water bath and stimulation transducer. B-mode images are acquired initially by switching out the transducer probe on the 3D positioner mount. The B-mode images are used to target the sciatic nerve with nerve and bone highlighted.

Electrical stimulation experiments ($n = 9$) were conducted with a S48 single channel stimulator (Grass, Warwick, RI, USA). A small incision was made through the skin and thigh muscle then the sciatic nerve was teased apart from the surrounding connective tissue and muscle. Platinum electrodes were coiled around the sciatic nerve. The following parameters were explored: 1–10 V, 1–10 mA, 200–500 μs with 1 V, 1 mA and 50 μs step sizes based on parameters employed in prior electrical stimulation studies treating peripheral neuropathy (Kılınç *et al* 2014, Vance *et al* 2015). EMG recordings were the same as outlined above. Between stimulations 0.9% saline solution was used to hydrate the nerve and exposed tissue.

Histology samples of both hind limbs were collected ($n = 8$ FUS stimulation, $n = 8$ negative control, $n = 1$ positive control), fixed in 0.4% PFA, 70% EtOH rinse and embedded in paraffin. Stimulation parameters were as follows: 4.5 MPa, 90% DC, 1 kHz PRF, 4.5–9 ms stimulation duration. Samples were sectioned coronally acquiring 5 μm slices with 200 μm interstice gaps and affixed to slides. Samples were stained with H&E. A blinded study was then conducted determining damage to the tissue as follows: inflammation/abnormal cell morphology, red blood cell extravasation, and cell membrane rupture.

Open field tests were conducted in a 30 cm^3 opaque white box ($n = 4$ FUS stimulation, $n = 4$ control). FUS stimulation parameters were as follows: 4.5 MPa, 90% DC, 1 kHz PRF, 4.5–9 ms stimulation duration. Only one of the hindlimbs were stimulated 20 times and verified with video recording as EMG electrodes would have introduced damage to the limbs and may have generated false positives. Behavioral testing was recorded on days −1, 1, 2, and 3 with day 0 being the day FUS stimulation was applied. Behavioral testing was recorded and analyzed using the EthoVision behavioral analysis suite (Noldus, Wageningen, The Netherlands). Total distance traveled, number of rotations to ipsilateral side of FUS stimulation and time spent in center/along the walls of the cube (Center = 15 × 15cm square in middle of cube, along walls = remaining area between center square and walls).

Thermocouple experiments were conducted with the FUS transducer and parameters as follows: 0.7–5.4 MPa peak negative pressure, 5 ms stimulation duration, 100% duty cycle, 1 kHz PRF. Wire thermocouples (Omega, Norwalk, CT, USA) were embedded in *ex vivo* hind limb tissue along the sciatic nerve. The thermocouple was placed in the *z*-axis, 50 mm away from the HIFU transducer (focal length) and a 2D raster scan was performed. Raster scans of a 5 mm^2 area were acquired with 0.5 mm step sizes ($n = 5$) with a temperature sampling rate of 20 Hz. Samples were kept at an average 21 °C throughout experiments.

Radiation force experiments utilized a radiation force balance to measure the acoustic power of the transducer (Maruvada *et al* 2007, Canney *et al* 2008, Han *et al* 2015). A brush absorber was placed in a tank and filled with DI water before degassing the entire setup. Once degassed, this tank was placed on a weight balance and set to zero. The transducer was positioned above the brush absorber with the focal area targeting within the brush. Voltages ranging from 0.1 to 0.9 V were applied three times to find an average increased weight (g) displayed from the radiation force exerted on the weight balance. To determine the acoustic power from the measurements the following equation was used:

$$\text{Acoustic Power} = \frac{2Mgc}{\left(1+\cos\left(\arcsin\left(\frac{a}{F}\right)\right)\right)} \times e^{2\alpha d}$$

where M is the mass reading from the weight balance, g is gravity, c is speed of sound in water at room temperature, a is half the length of the transducer aperture, F is the focal length, α is the acoustic attenuation of water and d is the distance of the transducer the acoustic absorber. With this acoustic power we can find the radiation force as:

$$\text{Radiation Force} = \frac{2\alpha I}{c}$$

where F is a volumetric force (N m^{-3}), α is the tissue absorption coefficient (m^{-1}), I is the temporal average acoustic intensity (W m^{-2}), and c is the speed of sound (m s^{-1}). Deformation of the tissue was found with young's modulus:

$$\varepsilon = \frac{\sigma}{E}$$

assuming a young's modulus of 576 kPa (Han *et al* 2015).

All analysis of data was done through functions (student's *t*-test, 1-way ANOVA, linear regression) provided within Matlab.

Results

Targeting of the sciatic nerve in anesthetized mice was done with an 18.5 MHz imaging probe, and subsequently stimulated with a 3.57 MHz stimulation transducer (figure 1). Initially, a range of FUS parameters (0.7–5.7 MPa peak-negative-pressure (PNP), 15–100% duty cycle (DC), 1 kHz pulse repetition frequency (PRF), 0.8 ms–1 s stimulation duration) were employed to determine those that were efficacious in stimulating the sciatic nerve. Reported pressures throughout the paper for *in vivo* experiments account for skin and muscle attenuation (Marquet *et al* 2011). The range of these initial FUS parameters were determined from prior PNS and CNS stimulation studies (Gavrilov *et al* 1996, Juan *et al* 2014, Kamimura *et al* 2016, Kubanek *et al* 2016). Lower pressures and duty cycles were investigated first, but EMG activity and visible muscle activation was only detected once pressures and duty cycles were above 3.2 MPa and 35% respectively. From these preliminary experiments, a set of parameters were found to successfully elicit EMG results: 3.2–5.7 MPa, 35–100% DC, 1 kHz PRF, 0.8–10.5 ms stimulation duration. For evaluation, the data was divided into two groups. The first pertaining to FUS stimulations with a total stimulation time of 0.8 ms, and a second with stimulation times between 1 to 10.5 ms. The varied stimulation times between 1 to 10.5 ms did not have a significant effect on EMG response delay (time between onset of FUS stimulation and EMG response) or peak-to-peak EMG signals (1-way ANOVA, $p = 0.6934$, $p = 0.5961$ respectively). While excitation of the sciatic nerve was possible at higher pressures/longer duration (>100 ms), gross examination of skin and surrounding muscle revealed tissue damage (change of color, consistency) in that range without the need for histological evaluation. The excitation associated with the visible damage was not reproducible after the first EMG response regardless of intra-trial pauses. The damage generated would be irreversible as shown in the positive control for histology (see Results: Histology). Damage was detected after stimulating the limb > 30 ms at pressures of 5.7 MPa at 90% DC. No damage was detected at pressures that were unsuccessful at eliciting excitation for stimulation durations (2.0–3.8 at 35% DC) for up to 1 s.

Figure 2(A) shows a typical single spike EMG response to FUS stimulation of the sciatic nerve with a PRF of 1 kHz and an 8 ms stimulation duration. These were the most common responses observed ($n = 63$) with the set of parameters defined above. Occasionally, a second EMG signal ($n = 18$) was observe following the FUS stimulus as shown in figure 2(B). 83% of such secondary signals occurred when a 50% DC was utilized during FUS stimulation. When stimulating the sciatic nerve with a 35% DC, only this secondary EMG response was observed after stimulation had occurred ($n = 7$). As seen in figures 2(A) and (B) these EMG responses during FUS stimulation were accompanied by an electromagnetic field (EMF) artifact. The EMF produced from the transducer generated various artifacts such as signal depression, which can appear as a false positive response (figures 3(A) and (B)). Additionally, stimulation of the skin and muscle tissue at light planes of anesthesia did result in compound EMG activity shown in figure 3(C) ($n = 80$), but never single spikes as observed with stimulation of the sciatic nerve. Reducing the stimulation duration to 0.8 ms with 100% DC (continuous wave), we were able to elicit single EMG responses with reduced EMF noise as shown in figure 2(C) ($n = 57$). Multiple EMG responses are shown in supplemental figure 1 (stacks.iop.org/PMB/63/035011/mmedia). No EMG signals or observable muscle activation was detected using a DC less than 100% for the 0.8 ms stimulation group. There were no significant changes in peak-to-peak EMG responses with changes in stimulation duration for the 1–10.5 ms group (1-way ANOVA, $p = 0.5961$). On average the EMG responses for the 0.8 ms stimulation group were not significantly different from the EMG responses from the 1–10.5 ms stimulation group per each pressure (Student's *t*-test, $p = 0.1044$). The success of stimulation for each parameter group is shown in figure 4 (Data is found in

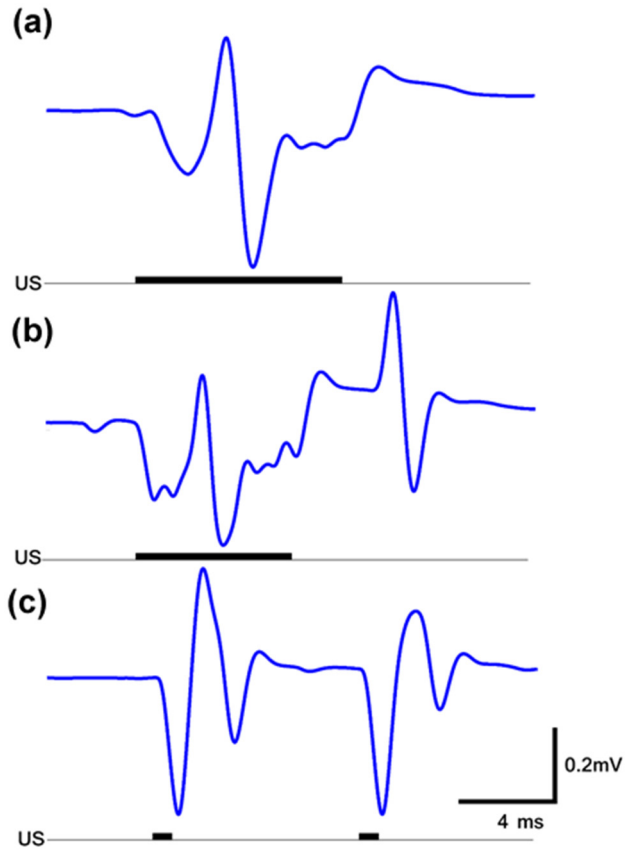

Figure 2. Typical EMG responses to FUS stimulation. The blue line is the recorded EMG signal. The thick black bars indicate when ultrasound is being applied. (a) shows a single spike EMG response to FUS stimulation of the long duration group (1–10 ms stimulation duration). (b) shows a double spike EMG response to FUS stimulation of the long duration group. (c) shows two EMG spikes for the short FUS stimulation duration group (0.8 ms stimulation duration).

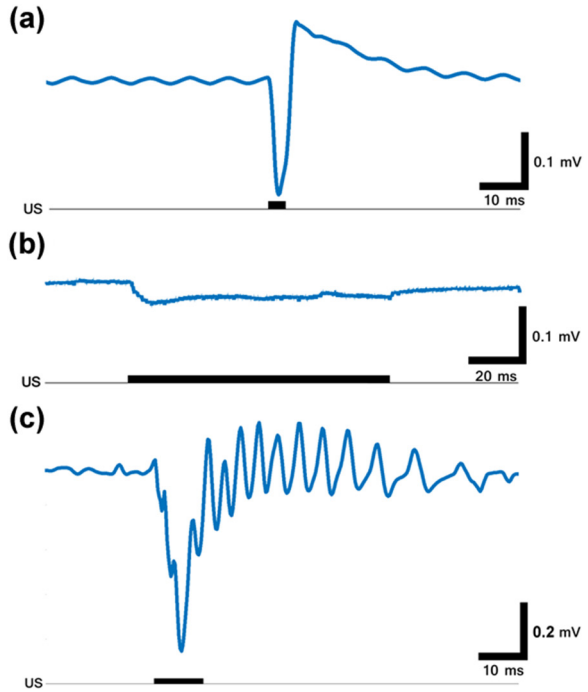

Figure 3. EMG artifact responses. The blue lines are traces from EMG recordings. The black bars indicate when ultrasound was being applied. (a) Artifacts from EMF noise. The blue lines are traces from EMG recordings. The black bars indicate when ultrasound was being applied. The signal observed in (A) and (B) is EMF noise generated by power being applied to the transducer. The response in (c) is observed while stimulating the skin and muscle, not the nerve. (a) 138 W for 8 ms (b) 3.2 W for 1 s (c) 138 W for 9 ms.

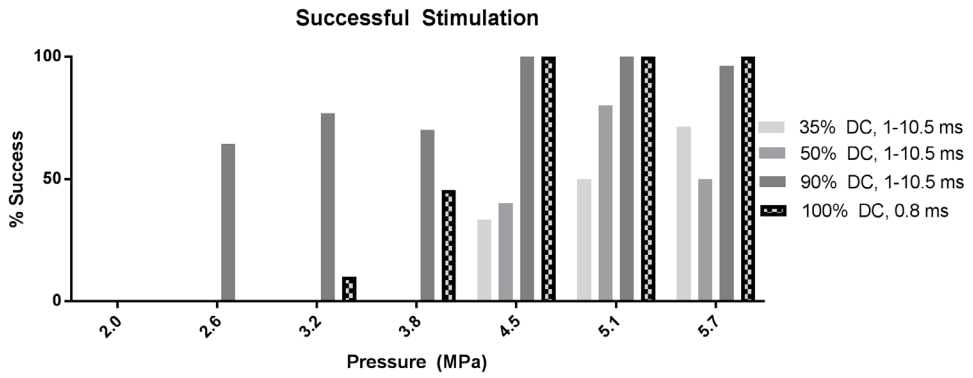

Figure 4. Stimulation success rates. Success rate with pressure and pulse length. The non-patterned bars are from the long stimulation group (1–10.5 ms) while the checkered red bar is from the short stimulation group (0.8 ms).

supplemental table 1). Success was determined as the ability to elicit subsequent EMG responses following initial EMG detection or observable muscle contraction. As shown with both the 0.8 ms and longer duration groups, success rates increased overall with pressure and number of cycles. For both the short and long duration groups, there was a large decrease in success between the 4.5 MPa and 3.8 MPa groups (55 and 32% respectively). Successful stimulation occurred at a higher rate for lower pressures (2.6–3.8 MPa) with the longer duration group, than for the same pressures in the 0.8 ms stimulation duration group. It was observed that following a stimulation without EMG activity (unsuccessful trial), a break period of 20–30 s improved the next stimulation success to 92% ($n = 15$), suggesting a greater latency needed for repolarization after multiple failed stimulations. Moving the FUS focal spot away from the targeted sciatic nerve eliminated both observable muscle activation and single spike EMG activity with fully anesthetized mice.

To verify EMG signals and muscle contraction occurred due to stimulation of the nerve and not the surrounding tissue, nerve transection experiments were conducted. After acquiring multiple ($n = 20$) successful EMG responses, a small incision was made in the thigh muscle exposing the sciatic nerve. The nerve was then clipped downstream of FUS stimulation and the transducer was repositioned at the prior location of successful stimulation. Transection of the sciatic nerve abolished all EMG signals from FUS stimulation as shown in figure 5.

Electrical stimulation of the sciatic nerve was used both as a validation step for placement of the EMG electrodes in the tibialis anterior muscle initially as well as a benchmark for comparing the FUS responses to conventional methods. A range of electrical stimulation parameters were investigated to determine the parameters most similar to FUS stimulation. These parameters were selected from prior studies employing electrical stimulation to treat neuropathy (Kılınç *et al* 2014, Vance *et al* 2015). Figure 6(A) shows that the latency to the EMG signal (average 2.1 ms) was comparable for both the 0.8 ms and long/1–10.5 ms duration stimulation groups, except for the 35% DC subgroup, which was significantly slower than all other groups (student's *t*-test, $p = 6.1321 \times 10^{-36}$). As noted above, the 35% DC subgroup only generated EMG responses after FUS stimulation had ceased. The 0.8 ms stimulation duration group had the most consistent delay and was not significantly different than that of the electrical stimulation group (Student's *t*-test, $p = 0.0593$). EMG responses comparing FUS and electrical stimulation can be seen in figure 6(B). An electrical stimulation of 10 mA, 250 μs stimulation duration and 5 V, generated similar peak-to-peak EMG spikes as that of the FUS stimulation response.

An open field test (30 cm^2 opaque square box) was utilized to assess short term damage to the nerve and surrounding tissue from the FUS stimulation. Mice were recorded 1 d prior and 3 d following FUS stimulation. The total distance traveled and number of rotations to the ipsilateral side of FUS stimulation were monitored as a decrease in distance traveled and ipsilateral rotations would indicate damage. Distance traveled for mice that received FUS stimulation did not significantly change from the control group, nor from their average distance traveled on day −1 (supplemental figure 2, 1-way ANOVA, $p = 0.4533$). Average number of rotations towards the ipsilateral side of FUS stimulation did not significantly change between the days following FUS stimulation nor the control group (supplemental figure 2, 1-way ANOVA, $p = 0.1695$). We also monitored their time spent in the center and along the walls of the open field test as a determinant of their anxiety levels. If the FUS stimulation had caused discomfort, but not to the point of generating detectable damage with the metrics employed above, monitoring their activity relative to their position in the box could be used to determine if they were more anxious following the procedure. Supplemental figure 2 also shows the stimulated group does not significantly differ from the trends of the control group, as the mice spend less time overall within the center of the box and more time in the outside of the box over the time course of the experiment (student's *t*-test, all groups $p > 0.05$). These behavioral results indicate the FUS stimulation parameters we determined to be successful at eliciting EMG responses are safe for short term applications.

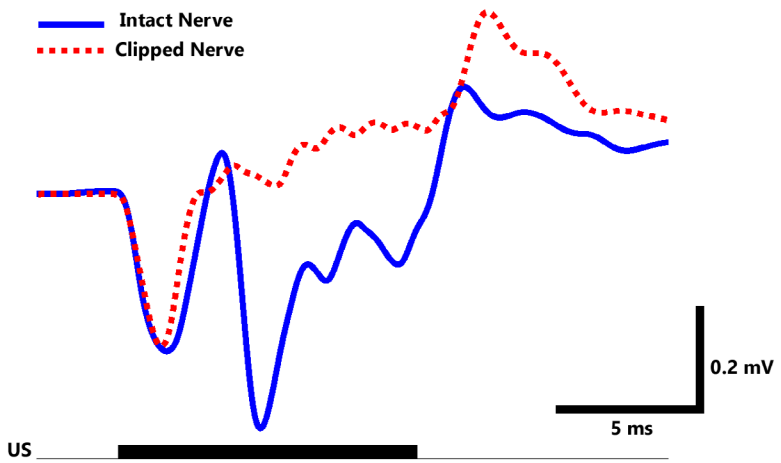

Figure 5. EMG responses before and after nerve clip. The blue line is the recorded EMG signal before nerve transection while the red dashed line is the EMG signal after the nerve had been transected. The black bar indicates when ultrasound was being applied.

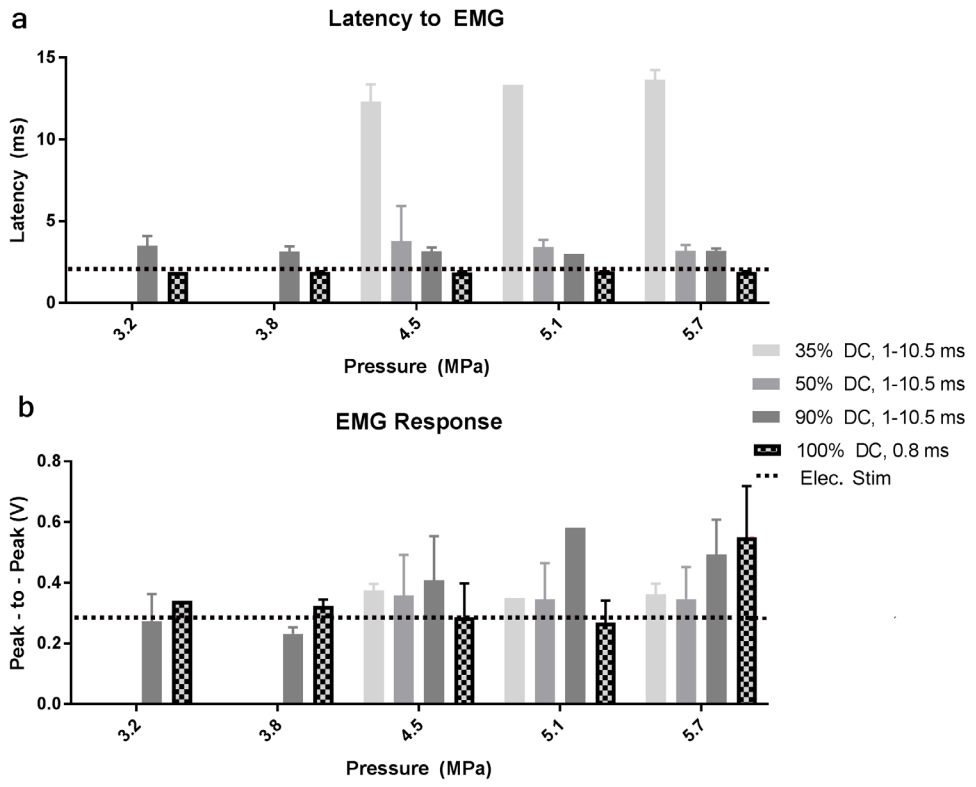

Figure 6. EMG signal comparison between electrical and FUS stimulation. The dotted horizontal bar indicates electrical stimulation response. The non-patterned bars are from the long stimulation group (1–10.5 ms) while the checkered bar is from the short stimulation group (0.8 ms). (a) The average delay from onset of stimulation to EMG signal and standard deviation for each pressure and duty cycle. (b) The average peak-to-peak and standard deviation EMG for electrical stimulation along with ultrasound stimulation each pressure and duty cycle.

H&E staining of the sciatic nerve and surrounding tissue in the FUS targeted area were evaluated with a blinded study for damage ($n = 8$ FUS stimulation, $n = 8$ negative control, $n = 1$ positive control. See SI Methods for full parameters). Damage was defined as red blood cell extravasation, abnormal cell morphology, inflammation and destruction of cellular membranes. Figure 7(A) shows the FUS targeted area when using parameters found successful to elicit EMG responses. No damage was observed for any of stimulated mice samples, nor with the negative controls (no FUS stimulation). Damage was detected for the positive control (5.4 MPa, 90% DC, 1 kHz PRF, 0.5 s stimulation duration) as shown in figure 7(B). Red blood cell extravasation, inflammation as well as cell membrane destruction was found in the stimulated region, while areas neighboring the targeted region were unaffected, demonstrating the target specificity of the FUS stimulation. As with the behavioral results, the FUS parameters found efficacious for eliciting EMG response while stimulating the sciatic nerve were safe.

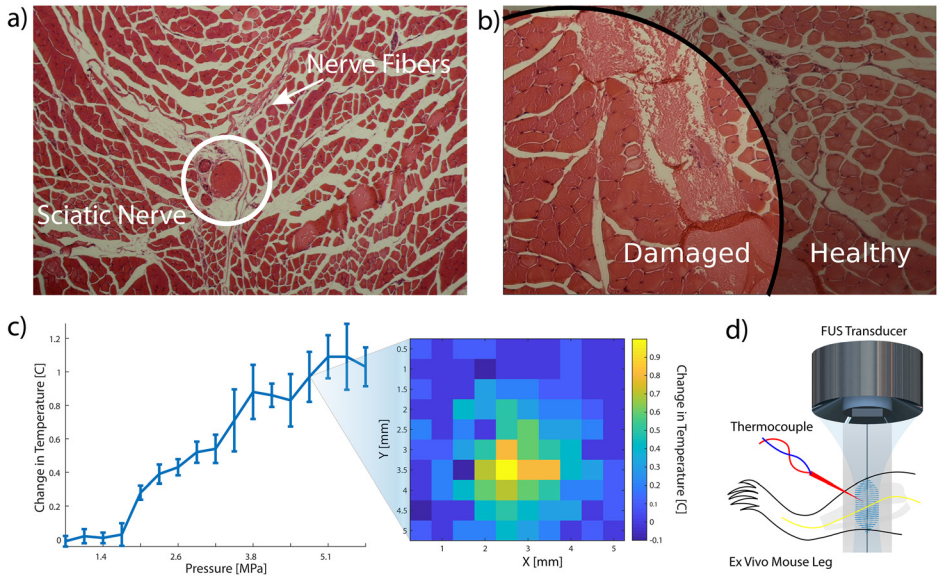

Figure 7. H&E Histology and thermal measurements for FUS Stimulation. (a) shows the sciatic nerve bundle and surrounding neural and muscle tissue for the FUS stimulated group with parameters found for successful EMG and muscle activation. (b) is the positive control group showing damaged areas by applying FUS stimulation for 0.5s continuous wave. (c) Plot of pressure versus average ± s.d. temperature increase in an *ex vivo* mouse hind limb from baseline (room temperature 21 °C). The raster plot of temperature increase in an *ex vivo* mouse hind limb during FUS stimulation at a PNP of 4.5 MPa. (d) Schematic of thermocouple measurements positioning.

To determine if the FUS stimulation elicited a thermal effect, thermocouples were embedded in an *ex vivo* mouse hind limb adjacent to the sciatic nerve. The mouse limb was pinned to a dissection tray filled with degassed water and maintained at room temperature (21 °C). Figure 7(C) shows the average ± s.d. temperature for the following FUS parameters: 0.7–5.3 MPa, 90% duty cycle, 1 kHz PRF. Stimulation at the higher pressures exhibited a 1.09 °C increase in the *ex vivo* limb with an overall range of 0–1.09 °C. The raster of the temperature during FUS stimulation at 4.5 MPa, shows the peak temperature increase was at the focal area, but there was local heating surround the focal area due to the femur being within the raster area. For stimulations >100 ms at a pressure of 5.7 MPa, the temperature increase was >20 °C. These increases in temperature decayed to baseline within 14 ± 2 s on average ($n = 5$). This small change in temperature recorded was significantly lower than prior reported values for inhibition of peripheral neurons during *ex vivo* experiments which required temperature increases of up to 20 °C (Tsui *et al* 2005, Colucci *et al* 2009).

Utilizing the pressures we found successful for eliciting EMG responses, the acoustic radiation force generated from the transducer was capable of deforming the tissue in the targeted area relative to the adjacent region. The acoustic radiation force was measured using a force balance to determine the total power from the transducer and then converted to determine the deformation at the focal region (Xia *et al* 2014, Han *et al* 2015). Assuming a young's modulus of 576 kPa for neural tissue, the deformation varied with applied input voltages from 14 to 422 μm (0.1 V/0.7 MPa, 0.9 V/5.7 MPa respectively) (Borschel *et al* 2003). Table 1 details the power output and deformation for all pressures utilized within the study. The displacement generated by the FUS parameters we employed in this study was large enough to facilitate the firing of the action potential to elicit EMG activity according to prior work (Mihran *et al* 1990, Gavrilov *et al* 1996, Kubanek *et al* 2016).

Discussion

In this study, we showed for the first time successful *in vivo* FUS stimulation of the PNS. Prior conventional techniques such as electrical stimulation or drug therapies are respectively either invasive or untargeted. Drug therapies are the most common treatments, but with all drug approaches there is the possibility for unwanted systemic side effects (Sindrup and Jensen 1999). Throughout the lifetime of implantable electrical stimulation devices, complications can arise from surgery, immune response to implant, and damage to the nerve from repeated electrical stimulation (Agnew and McCreery 1990, Ben-Menachem 2001). FUS stimulation of the PNS is both non-invasive and targeted, reducing the complications for treatment of damaged peripheral nerves and the overall cost of treatment as surgery is not necessary. For at-home transdermal electrical stimulation systems, patients have reported the stimulation could not penetrate deep enough to reach the target area, along with irritation during stimulation (Gladwell *et al* 2016). With a FUS system, these issues would be resolved as the target depth could be tailored using a multi-element array and varying the parameters applied. Since ultrasound

Table 1. Values for power output, theoretical deformation, pulse energy and temperature increase for applied pressures.

Acoustic parameters and effects from FUS					
Pressure (PNP, MPa)	Power (W)	Displacement (μm)	Pulse energy (mJ)	Temperature increase (°C)	Intensity (W cm^{-2} I_{SPPA})
0.7	3.2	8.5	0.5	0.01	0.0245
1.4	12.8	34.1	1.9	0.01	0.0978
2.0	31.0	82.7	4.6	0.28	0.2373
2.6	54.7	146.0	8.1	0.43	1.1176
3.2	85.2	227.1	12.5	0.54	1.7384
3.8	97.8	260.6	14.4	0.88	1.9978
4.5	118.1	314.7	17.4	0.83	2.4089
5.1	138.4	368.7	20.4	1.09	2.8223
5.7	158.6	422.8	23.4	1.01	3.2364

is employed for the targeting of the nerve, an all-in-one system for targeting and stimulation of the peripheral nerve can be used, and with training, an at-home system could be used by patients, allowing for treatment without the need for travel to the clinic. This also reduces the overall financial and temporal costs of the treatment compared to conventional techniques that require multiple tests to determine therapy outcomes.

The success of the technique ranged from 16% to 100% depending on the FUS parameters employed, demonstrating the reproducibility of this technique. While some of the success rates are lower than that for electrical stimulation (100% success), the mechanisms are fundamentally different. Electrical stimulation of an axon activates the voltage gated ion channels in the nodes of Ranvier generating an action potential. With ultrasound, the stimulation is fundamentally a mechanical force.

In this study, we hypothesized that the mechanical forces from FUS are being converted into an electrical signal by forcing open the voltage gated ion channels in the nodes of Ranvier. The direct mechanics of this conversion are unknown and are being currently investigated, but the findings presented herein indicate that the generation of the action potential occurs within onset of FUS stimulation. The short 0.8 ms stimulation duration elicited EMG responses with 100% success for pressures ranging from 4.5 to 5.7 MPa. These pressures correspond to a theoretical tissue displacement of up to 422 μm. Prior studies have shown mechanically stimulating a peripheral nerve axon can elicit an action potential with a deformation of only 10–60 μm (Julian and Goldman 1962, Ganot *et al* 1981, Galbraith *et al* 1993). These studies used unmyelinated axons which are easier to stimulate, but here we are deforming the nerve tissue orders of magnitude greater (Gross *et al* 1983). While mechanosensitive ion channels exist on cell bodies, they are not present on the axon of the naïve sciatic nerve (Bearzatto *et al* 2000, Alloui *et al* 2006). Thus, with the lack of FUS activated mechanosensitive ion channels, we may conclude that the deflection from the acoustic radiation force generates displacement of the axon, forcing opening of the voltage-gated ion channels located at the nodes of Ranvier (Mihran *et al* 1990). Prior work by Mihran *et al* demonstrated stimulation of an *ex vivo* frog sciatic nerve by both ultrasound and mechanical stimulation, postulating the initial deformation of the nerve was the impetus of action potential generation. Here we are targeting the sciatic nerve bundle (0.19 mm^2), and not a solitary axon. Thus while the theoretical tissue displacement is large for a single axon, it may be necessary for stimulating multiple axons within a bundle.

Other FUS studies have postulated intra-membrane cavitation and oscillation of bubbles within the membrane could change the membrane capacitance, thus triggering the cell to fire an action potential (Krasovitski *et al* 2011, Rappaport *et al* 2013, Livneh *et al* 2014, Plaksin *et al* 2014, Wright *et al* 2015, 2017). We will be exploring the use of harmonic motion imaging to verify deformation *in vivo* during FUS stimulation as well as the detection of cavitation in future experiments.

Current *ex vivo* and *in vitro* reports on FUS PNS modulation are divided between thermal or mechanical effects driving the neuromodulaton. Thus far, thermal effects are associated with inactivation of the stimulated nerve while mechanical effects are associated with the activation of the nerve (Gavrilov *et al* 1996, Tsui *et al* 2005, Colucci *et al* 2009, Kubanek *et al* 2016). With our thermocouple experiments, we verified our stimulation effects were not due to temperature increase. The maximum temperature increase with the highest pressures / longest durations we found to only have a temperature change of 1.09 °C in *ex vivo* tissue. Temperature increases of 14–20 °C were needed in prior work stimulating excised peripheral nerves before the action potential was inhibited (Colucci *et al* 2009). Even with a thermal decay time with an average of 6 s the increase in temperature at the sciatic nerve *in vivo* with the FUS parameters we found efficacious would not generate a thermal increase of a magnitude required for inhibition. Thus, our *in vivo* experiments agree with the *ex vivo* literature stating excitation of the PNS is a mechanical, not thermal effect.

Comparison of FUS stimulation to conventional electrical stimulation, shows that the latency of the EMG response for the 0.8 ms stimulation duration group was not significantly different than that of the electrical stimulation group. Although the stimulation constitutes a different mechanism, the finding indicate the mechanical stimulation to be as temporally efficient as with electrical stimulation. FUS stimulation responses were strong enough to elicit EMG spikes comparable to that of electrical stimulation and visible muscle contraction was recorded. Our results demonstrate FUS can potentially serve as an alternative or complimentary treatment to various patient conditions that are currently treated with electrical stimulation at peripheral nerve sites like chronic pain and incontinence.

Investigation of the short-term physiological effects of FUS stimulation on the sciatic nerve revealed no detectable damage with either histology or behavioral testing. For many electrical stimulation therapies to treat peripheral nerve damage device implantation and removal can generate damage to the nerve or surrounding tissue (Agnew and McCreery 1990, Ben-Menachem 2001). Our blinded histological examination study did not detect any RBC extravasations, nor changes in cellular morphology of the surrounding tissue for FUS parameters we found successful for stimulation, demonstrating the technique is safe. Open field testing did not indicate any damage to the sonicated limb, nor a change in their behavior as the stimulation results were not significantly different from the control or baseline groups.

Conclusion

Overall, we have demonstrated FUS stimulation of the sciatic nerve *in vivo* for the first time. A range of FUS parameters have been determined to successfully elicit EMG activity downstream of FUS stimulation, as well as the abolishment of EMG signal when the nerve is transected. Safety experiments did not indicate any short-term damage to the nerve or the surrounding tissue. Recorded EMG signals were comparable to those generated using conventional electrical stimulation methods, indicating FUS stimulation can be a non-invasive alternative to electrical stimulation for peripheral nerve excitation. FUS having the ability to both excite and inhibit neuronal activity can be a powerful tool to target multiple nerve types including the vagus, which has the potential to treat multiple diseases such as epilepsy and depression and metabolic disorders. These results support further investigation of FUS-based techniques as a non-invasive and safe alternative to conventional treatment of electrical stimulating peripheral nerve sites.

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