Bioeffective Ultrasound at Very Low Doses: Reversible Manipulation of Neuronal Cell Morphology and Function in Vitro

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Abstract. Direct and safe manipulation of neurons by external means is an increasingly studied therapeutic modality with the potential to treat many neurological diseases. Anticipating such future applications, we investigated reversible bioeffects of very low dose focused ultrasound on neuronal cell morphology and function in vitro. To test morphological changes, undifferentiated PC12 cells were serum-cultured. The culture plates were placed on an inverted optical microscope. An f/1.1 ultrasound transducer with a water-filled coupling cone was focused on the culture and excited with 30-ms 4.67-MHz 100-kPa pulses. To test functional changes, rat hippocampal slices were cultured and individually transferred to the well of a 60-channel multi electrode array. An f/2.1 ultrasound transducer with a water-filled coupling cone was focused on a culture and excited with 100-μs 4.04-MHz 77-kPa pulses. The culture was stimulated before and after the ultrasonic stimulus with a 100-μs 100-μA biphasic electrical stimulus. Optical microscopy of PC12 cultures under insonification revealed that cells that were clustered near the ultrasound focal region elongated by approximately 2 μm during insonification and returned to approximately their original shapes following insonification. We conclude that the acoustic radiation force is capable of reversibly deforming cultured cells. In the rat hippocampal cultures, the ultrasonically and electrically evoked responses exhibited similar biphasic waveforms. In addition, robust electrically evoked responses following insonification indicated that the insonified cultures remained viable. We conclude that low-dose ultrasound can stimulate neurons; the mechanism is currently under investigation.

Keywords: ultrasonics, bioeffects, neuroscience, hippocampus, electrophysiology
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INTRODUCTION

Ultrasound attenuation imparts momentum to the attenuating medium [1]. The attenuating region of interest (e.g., bulk tissue, individual cells, or subcellular components) can exhibit three possible structural responses: translation, rotation, or

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deformation. Functionally, these responses can trigger surface and internal receptors (e.g., integrin-mediated and cytoskeletal responses) [2,3].

A considerable body of work exists on the use of acoustic tweezers for translation and rotation of cells [4-6]. Gavrilov and co-workers demonstrated a functional response in peripheral nerves from an acoustic radiation force stimulus [7-9]. Changes in electrically evoked neuronal responses in vitro and in vivo in response to ultrasonic stimuli have been studied also [10-12].

Sub-ablation therapeutic ultrasound has the potential to manipulate tissues remotely and safely. Possible neuroscience applications include non-invasive brain stimulus, plasticity studies [13], and the study of brain injury mechanisms [14]. In anticipation of such future applications, this study investigated reversible bioeffects of very low dose focused ultrasound on neuronal cell morphology and function in vitro.

METHODS

Structural Studies

Nondifferentiated PC12 cells [15] were serum-cultured in DMEM/F12 with 15% horse serum and 2.5% newborn calf serum on poly-L-lysine-coated polystyrene plates. The culture plates were placed on an inverted microscope (model IX71, Olympus America, Inc., Center Valley PA USA). An 80-mm diameter, 90-mm focal length f/1.1 PZT-4 spherical cap ultrasound transducer (model CST-100, Sonocare, Inc., Upper Saddle River NJ USA) [16] with a water-filled coupling cone, sealed at the distal end with a latex membrane, was focused on the culture plate at an approximately 45° angle-of-incidence, and excited with 30-ms 4.67-MHz pulses from a waveform generator (model 33250A, Agilent Technologies, Inc., Santa Clara CA USA) amplified by a radio-frequency amplifier (model 2100L, ENI, Rochester NY USA). Streaming was blocked by the latex membrane and by an intervening acetate sheet placed within 1 mm of the cell culture. The pressure within the focal region was estimated to be approximately 100 kPa, based on measurements with a needle hydrophone (model HNA-0400, Onda Corp., Sunnyvale CA USA). Brightfield digital images were recorded before, during, and after insonification with a 12-bit monochrome camera (model Photometrics CoolSNAP ES, Roper Industries, Inc., Sarasota FL USA) under the control of MetaMorph software (version 6.3r7, Molecular Devices, Downingtown PA USA).

Functional Studies

Hippocampal slices were prepared from 8-day-postnatal Sprague Dawley rats that had been sacrificed for other purposes under IACUC guidelines. The 400-μm thick slices were cultured for 6 days at 37°C on cellulose ester filter membranes (Millipore Corp., Billerica MA USA) in Neurobasal culture medium [17]. The mature slices were transferred to a glass-well 60-channel multi electrode array (Multi Channel Systems GmbH, Reutlingen Germany), secured by a stainless alloy ring with parallel wires spaced at 1-mm intervals, and irrigated with artificial cerebrospinal fluid saturated

with a 0.95 O_2, 0.05 CO_2 gas mixture [18]. The location of the slice with respect to the electrodes was determined by optical microscopy. A custom-manufactured 42-mm diameter, 90-mm focal length f/2.1 PZT-4 spherical cap ultrasound transducer with a water-filled coupling cone, sealed at the distal end with a latex membrane, was focused on the culture plate at an approximately 45° angle-of-incidence (Figure 1), and excited with 100-μs 4.04-MHz pulses. The pressure within the focal region was estimated to be 77 kPa. Alternately, the slice culture was excited with a 100-μA 100-μs biphasic electrical stimulus applied across two adjacent electrodes. Waveforms from all electrodes were digitized at 20 kHz and recorded under the control of MC_Rack software (version 3.5.1.0, Multi Channel Systems); the recording period ranged from 100 ms pre-stimulus to 200 ms post-stimulus (excluding a 300-μs blanking period beginning with the stimulus onset).

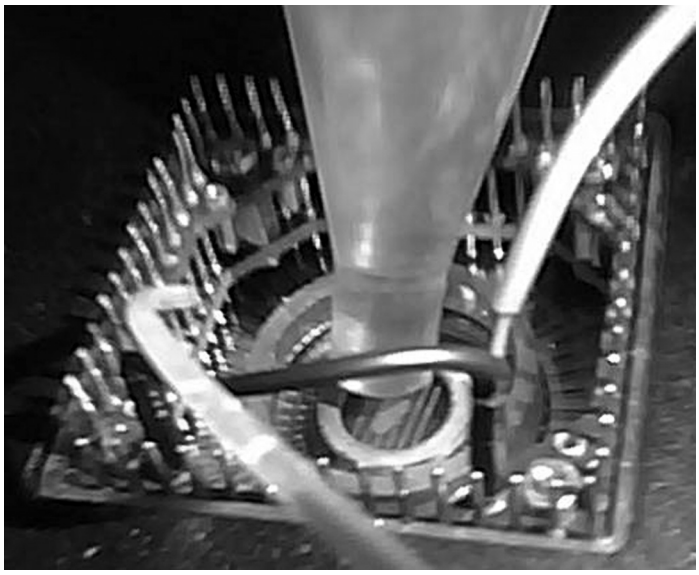

FIGURE 1. Apparatus for functional studies. The tip of the ultrasound coupling cone is immersed in a multi electrode array well. A rat hippocampal slice culture is just below the cone tip. The parallel wires securing the culture are spaced by 1 mm.

RESULTS

Optical microscopy of PC12 cultures under insonification revealed three cell populations: those which were stationary (apparently outside the effective force field region), those (seen in Figure 2) which elongated about 2 μm under radiation force and returned to approximately their original shapes when the force was removed (apparently adhered to the substrate), and those which moved about 50 μm with each pulse and did not return (apparently free-floating).

Multi electrode recordings of hippocampal cultures (Figure 3) demonstrated that ultrasonic stimuli elicited responses that were similar in their biphasic waveform to the electrically evoked responses. Typical amplitudes for electrically evoked responses before insonification were 500 μV; typical amplitudes for ultrasonically evoked

responses were 100 μV. Post-insonification, electrically evoked responses exhibited waveforms similar to the ultrasonically evoked responses, with 1000-μV amplitudes.

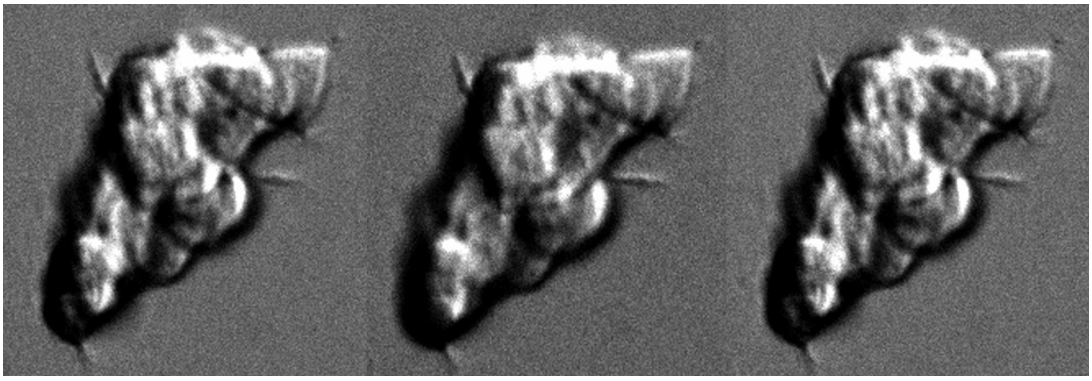

FIGURE 2. Optical microscopy image of a cluster of PC12 cells. The vertical field of view is 76 μm. (Left) Before insonification. (Center) During 30-ms insonification at 4.67 MHz, 100 kPa. The radiation force was applied diagonally from the upper right corner. (Right) After insonification. The cells reverted to their original positions.

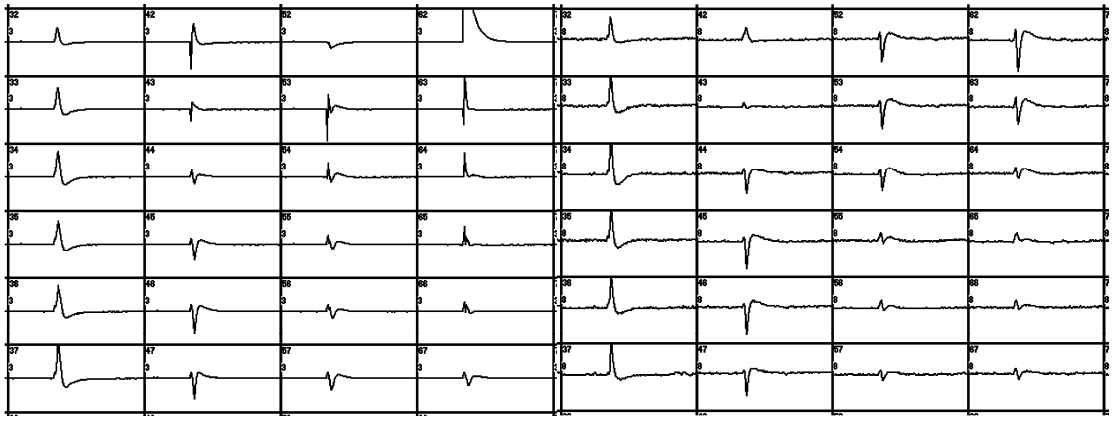

FIGURE 3. Multi electrode array recordings of evoked responses of rat hippocampal culture. Of the 60 channels recorded, the central 24 electrode recordings are presented here as average responses over multiple stimuli; the remaining channels exhibited similar behavior. The DG region (Dentate Gyrus, composed of granular cells) was located near the lower right electrodes, and the CA1 region (Cornu Ammonis, composed of pyramidal cells) was located near the central and upper left electrodes. (Left) Response to a 100-μA 100-μs biphasic electrical stimulus applied across the electrodes whose waveforms are represented by the two upper right plots. Individual waveform plots are 1 mV high and 300 ms wide. (Right) Response to a 100-μs 4.04-MHz 77-kPa ultrasonic stimulus, applied from the lower right. Individual waveform plots are 0.2 mV high and 300 ms wide.

DISCUSSION

The observed magnitude of motion of the cultured cells under insonification is consistent with earlier studies of bulk tissue motion under acoustic radiation force [19,20]. Stress-softening or -hardening of actin filaments [21], if present, did not mask

the structural effects of the acoustic radiation force. The deformation of the cells and the minimization of streaming suggest that the acoustic radiation force is the mechanical cause of the observed structural changes. It is less clear that the acoustic radiation force is the mechanical cause of the observed functional effects. However, there is no evidence for competing mechanisms: stray electrical charge was not observed, and the heat deposited in the tissue by the 100-μs ultrasonic pulse is probably insignificant; further tests are planned.

CONCLUSIONS

The acoustic radiation force is capable of reversibly deforming cultured cells. Low-dose ultrasound can stimulate neurons in a rat hippocampal culture; the insonified cultures remain viable.

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REFERENCES

1. H. Starritt, F. A. Duck et al., *Phys. Med. Biol.* **36**, 1465-1474 (1991).
2. F. G. Giancotti and E. Ruoslahti, *Science* **285**, 1028-1032 (1999).
3. D. E. Ingber, *J. Cell Sci.* **116**, 1397-1408 (2003).
4. J. R. Wu, *J. Acoust. Soc. Am.* **89**, 2140-2143 (1991).
5. A. Haake, A. Neild et al., *Ultrasound Med. Biol.* **31**, 857-864 (2005).
6. J. Lee and K. K. Shung, *Ultrasound Med. Biol.* **32**, 1575-1583 (2006).
7. L. R. Gavrilov, E. M. Tsirulnikov and I. A. Davies, *Ultrasound Med. Biol.* **22**, 179-192 (1996).
8. L. R. Gavrilov, J. W. Hand and E. M. Tsirulnikov, *J. Acoust. Soc. Am.* **123**, 3790-3791 (2008).
9. I. ab Ithel Davies, L. R. Gavrilov and E. M. Tsirulnikov, *Pain* **67**, 17-27 (1996).
10. P.C.. Rinaldi, J. P. Jones et al., *Brain Res*. **558**, 36-42 (1991).
11. M. R. Bachtold, P. C. Rinaldi et al., *Ultrasound Med. Biol.* **24**, 557-565 (1998).
12. N. I. Vykhodtseva and V. I. Koroleva, "Steady Potential Changes and Spreading Depression in Rat Brains Produced by Focused Ultrasound" in *Therapeutic Ultrasound: 5th International Symposium on Therapeutic Ultrasound*, edited by G. T. Clement et al., AIP Conference Proceedings 829, American Institute of Physics, Melville, NY, 2005; pp. 59-63.
13. M. O'Toole, P. Lamoureux and K. E. Miller, *Biophys. J.* **94**, 2610-2620 (2008).
14. B. Morrison III, H. L. Cater et al., *Stapp Car Crash J.* **47**, 93-105 (2003).
15. L. A. Greene and A. S. Tischler, *Proc. Natl. Acad. Sci. USA* **73**, 2424-2428 (1976).
16. R. Muratore, "A history of the Sonocare CST-100: The first FDA-approved HIFU device" in: *Therapeutic Ultrasound: 5th International Symposium on Therapeutic Ultrasound*, edited by G. T. Clement et al., AIP Conference Proceedings 829, American Institute of Physics, Melville, NY, 2005; pp. 508-512.
17. G. J. Brewer, J. R. Torricelli et al., *J. Neurosci. Res.* **35**, 567-576 (1993).
18. Z. Yu , T. E. McKnight et al., *Nano Lett.* **7**, 2188-2195 (2007).
19. F. L. Lizzi, R. Muratore et al., *Ultrasound Med. Biol.* **29**, 1593-1605 (2003).
20. K. Nightingale, R. Bentley and G. Trahey, Ultrasonic Imaging **24**, 129-138 (2002).
21. O. Chaudhuri, S. H. Parekh and D. A. Fletcher, *Nature* **445**, 295-298 (2007).