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● *Original Contribution*

ULTRASOUND CAN MODULATE NEURONAL DEVELOPMENT: IMPACT ON NEURITE GROWTH AND CELL BODY MORPHOLOGY

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Abstract—Neuronal development is known to be a dynamic process that can be modulated by presenting guidance cues to neuronal cells. We show that ultrasound, when applied at pulsed settings and with intensities slightly greater than clinical diagnosis levels, can potentially act as a repulsive cue for modulating neuronal growth dynamics. Using differentiated Neuro-2a cells as the model, we have examined *in vitro* how neuronal development can change during and after exposure to 1-MHz ultrasound for different acoustic settings. Neurite retraction and cell body shrinkage were found in neuronal cells over a 10-min exposure period with 1.168 W/cm^2 spatial-peak, time-averaged intensity (based on 0.84 MPa peak acoustic pressure, 100-cycle pulse duration, and 500-Hz pulse repetition frequency). These effects were found to result in instances of neuronal cell body displacement. The extent of the effects was dependent on acoustic intensity, with peak acoustic pressure being a more important contributing factor compared with pulse duration. The morphological changes were found to be non-destructive, in that post-exposure neurite outgrowth and neuritogenesis were respectively observed in neurite-bearing and neurite-less neuronal cells. Our results also showed that mechanotransduction might be involved in mediating ultrasound-neuron interactions, as the morphological changes were suppressed if stretch-activated ion channels were blocked or if calcium messenger ions were chelated. Overall, these findings suggest that ultrasound can potentially influence how neuronal cells develop through modifying their cytomechanical characteristics. (E-mail: alfred.yu@hku.hk and jmfwan@hku.hk) © **2013 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Ultrasound, Neuronal development, Repulsive cue, Neurite retraction, Cell body shrinkage, Cell body displacement, Mechanotransduction.**

INTRODUCTION

Neuronal development is known to be a highly dynamic course of action that involves establishing an interconnected network of neurons to form the structural basis for functional neuronal circuits (van Ooyen 2011). A core event in facilitating such development is the outgrowth of neurite branches (or processes) from a post-mitotic neuron's cell body (also known as soma) where the nucleus and various organelles are located (Arimura and Kaibuchi 2007; Da Silva and Dotti 2002). It is well recognized that the development of neuronal cells is intrinsically regulated by various cytoplasmic signaling cascades (Goldberg 2004; Rossi et al. 2007) and cytomechanical factors (Ayali 2010; Suter and Miller 2011). To facilitate their growth, developing neurons and their neurites would continually probe their surrounding environment to identify local conditions that favor their development and avoid unfavorable ones (Franze and Guck 2010). As such, by presenting external guidance cues to the extracellular environment, neuronal development can potentially be modulated.

A few types of extracellular cues have been determined as being capable of regulating the growth of developing neurons and in turn modulating the morphogenesis of neuronal networks. One of them is the biochemical approach whereby molecular gradients of netrin, ephrin or similar signaling proteins are introduced to the extracellular environment to modify neuronal growth dynamics (Guan and Rao 2003; Song and Poo 2001). Another approach is to introduce patterned topologies on the attachment substrate to guide neuronal navigation (Hoffman-Kim et al. 2010). Physical cues have also been considered, and they involve the application of electrical (Ming et al. 2001), optical (Ehrlicher et al. 2002) or mechanical forces (Franze et al. 2009; Smith 2009) to

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stimulate neurite extension or retraction. From an application standpoint, these types of cues can significantly benefit neural tissue engineering under cell culture settings (Norman et al. 2008; Schmidt and Leach 2003). Nevertheless, their direct application to neural treatments (*e.g.*, as a cure for neuronal development disorders) is perhaps not a trivial task because targeted, non-invasive delivery of these cues *in vivo* might not be straightforward, especially for topological cues that are delivered based on grafting mechanisms.

In this work, we seek to investigate whether and how ultrasound can be exploited as another type of guidance cue for neuronal development. We hypothesize that ultrasound, when applied at low intensity and pulsed settings, can possibly serve as a physical cue that operates under principles similar to those based on mechanical force stimulation (Franze et al. 2009; Smith 2009). This hypothesis is formed on the basis that ultrasound, as an acoustic form of radiation, is known to be able to produce mechanical bioeffects on living cells without causing temperature elevation (Ahmadi et al. 2012; Dalecki 2004). The technical advantage of using ultrasound is that it can be delivered remotely to the target area in a non-invasive manner using well-established beam focusing principles. Therefore, ultrasound might have potential in being applied clinically to modulate neuronal development. It is worth noting that the neuromodulation potential of ultrasound has been explored recently in the context of peripheral nerve regeneration (Chang and Hsu 2004; Chen et al. 2010; Crisci and Ferreira 2002) and functional neuronal circuit excitation (Bystritsky et al. 2011; Tufail et al. 2010, 2011; Tyler et al. 2008). Our investigation reported here can serve to complement these ongoing efforts by providing novel insight on ultrasound-neuron interactions from a neuronal development perspective and at a single-cell level.

The focus of this study is on demonstrating that ultrasound can be a repulsive cue for modulating neuronal growth dynamics *in vitro*. In particular, it will be shown that ultrasound may induce various morphological changes in neuronal cells and in turn modify their physical positioning and developmental behavior. We also seek to provide evidence on the cellular mechanism involved in this process. Data will be presented to show that stretch-activated ion channels and calcium ion (Ca^{2+}) signaling are playing a direct role in a neuronal cell's ability to sense pulsed ultrasound stimulation.

MATERIALS AND METHODS

Exposure platform components

Ultrasound transmission device. A customized experimental platform has been developed for this work. As illustrated in Figure 1, the platform sends ultrasound pulses through a piston transducer with a center frequency of 1 MHz and diameter of 25.4 mm (Wuxi Beisheng Technology, Wuxi, Jiangsu, China). The pulse shape was defined using an arbitrary waveform generator (33120A; Agilent Technologies, Santa Clara, CA, USA), and a broadband power amplifier (2100L; Electronics and Innovation, Rochester, NY, USA) was used to boost the electrical signal that drives the ultrasound transducer. To collimate ultrasound energy to the microscope's field of view (*i.e.*, center of the xy-plane), the transducer was mounted onto a custom-made, water-coupled hollow cone waveguide (79 mm total cone height; acrylic casing) with a detachable tip (upper 9 mm of the cone height) that was removed following physical alignment (*i.e.*, after positioning the tip in contact with the center of the field of view). Note that the transducer-waveguide block was clamped to a retort stand. It was elevationally angled at 45 degrees (with respect to the z-axis) using an angle plate, and its rotational angle about the z-axis was 42 degrees (calibrated *in situ*; to be discussed in the Acoustic Field Calibration subsection).

Sample holder. A 100-mm–diameter polystyrene dish (430167; Corning Life Sciences, Tewksbury, MA, USA) was used as the host substrate for neuronal cells. Its plate layer was 0.9 mm thick, and it was slightly elevated by 0.5 mm with respect to the bottom of the cylindrical side casing. During operation, the cell dish was placed upon a larger polystyrene container that was filled with 37°C degassed water (0.8 mm thick; 20 mm high; 180 × 112 mm base dimensions). In turn (Fig. 1, insert), this resulted in the formation of a 2.2-mm–thick multi-layer base (*i.e.*, a water layer sandwiched between two polystyrene layers). Such a configuration was used so that, as confirmed by our hydrophone measurements (see subsection on Acoustic Field Calibration), incident ultrasound pulses impinging on the cell dish at a 45-degree angle would reflect out at a distance away from the incidence point (as an angled reflection from the bottom air interface), and in turn strong *in situ* reflections would be avoided. This helped to maintain acoustic field homogeneity over the microscope's field of view.

Imaging of neuronal cells. To facilitate real-time imaging of neuronal development dynamics during and after ultrasound exposure, our experimental platform was equipped with an inverted phase contrast microscope (Leica DM IL; Leica Microsystems, Wetzlar, Germany) carrying a 10× objective (numerical aperture: 0.20). A color video camera (TK-C9510E; JVC, Yokohoma, Japan) was used to acquire images from the microscope at a frame rate of up to 25 frames per second (fps). The camera images were 720 × 576 pixels, and the field-of-view was 650 × 500 μm (*i.e.*, pixel dimension: 0.90 × 0.87 μm). In each experiment trial, image frames