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● *Original Contribution*

UNMYELINATED PERIPHERAL NERVES CAN BE STIMULATED *IN VITRO* USING PULSED ULTRASOUND

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Abstract—Appreciation for the medical and research potential of ultrasound neuromodulation is growing rapidly, with potential applications in non-invasive treatment of neurodegenerative disease and functional brain mapping spurring recent progress. However, little progress has been made in our understanding of the ultrasound–tissue interaction. The current study tackles this issue by measuring compound action potentials (CAPs) from an *ex vivo* crab walking leg nerve bundle and analysing the acoustic nature of successful stimuli using a passive cavitation detector (PCD). An unimpeded ultrasound path, new acoustic analysis techniques and simple biological targets are used to detect different modes of cavitation and narrow down the candidate biological effectors with high sensitivity. In the present case, the constituents of unmyelinated axonal tissue alone are found to be sufficient to generate *de novo* action potentials under ultrasound, the stimulation of which is significantly correlated to the presence of inertial cavitation and is never observed in its absence. (E-mail: ucemcjw@ucl.ac.uk) **© 2017 Published by Elsevier Inc. on behalf of World Federation for Ultrasound in Medicine & Biology Printed in the USA.**

Key Words: **Neurostimulation, Neuromodulation, *In vitro*, Peripheral nerves, Therapeutic ultrasound, Cavitation, Axons.**

INTRODUCTION

Diseases and dysfunction of the nervous system, both central and peripheral, are common causes of morbidity and mortality around the world. Despite huge investment into pharmaceutical solutions for some of the more prevalent problems, progress has been slow. For a few of these diseases, successful new treatments have been found in neurostimulatory medical devices. Examples include deep brain stimulation (DBS) for Parkinson's disease (Bronstein et al. 2011) and vagus nerve stimulation (VNS) for epilepsy and depression (Groves and Brown 2005), as well as sacral neuromodulation for incontinence (Thaha et al. 2015). The gold standard for all of these is implantable electrodes, which themselves are associated with much morbidity from the need for highly invasive surgery, regular battery replacements and immunosuppression.

Though implants are improving, techniques that allow non-invasive neurostimulation, such as transcranial magnetic stimulation (TMS) (Lee et al. 2012) and direct current stimulation (DCS) (Nitsche et al. 2009), are gaining favour because they avoid the complications mentioned above. However, neither of these techniques can replicate the location specificity or stimulation of deep structures that implants can achieve.

Ultrasound (US), through the development of high-intensity focused ultrasound (HIFU) for ablative surgery and blood–brain barrier disruption, has proven its ability to overcome both of these targeting issues, reaching anywhere in the brain and other body areas with millimetre precision. Its application to elicit neuromodulation at lower intensities is still relatively new, but is rapidly gaining momentum.

Examples of the neuromodulatory effect of US were first reported as early as 1929 (Harvey 1929), but surfaced only occasionally until the last decade. Almost all of these early, pre-2008 exploratory studies focused on examining effects on peripheral nerves (Dalecki et al. 1995; Foley et al. 2008; Fry 1968; Gavrilov et al. 1977; Lele 1963; Mihran et al. 1990; Sheltawy and Dawson 1966; Tsui et al. 2005; Wright et al. 2002, 2015; Younan et al. 2013), with a few targeting central nervous structures (Tsirulnikov et al. 1988; Wall et al. 1953). This preference shifted dramatically toward

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central nervous targets after 2008 when Tyler's group reported that hippocampal slices could be stimulated at intensities much lower than those used on peripheral nerves (Tyler et al. 2008). Furthermore, a comparison of threshold neuromodulation intensities in studies on peripheral or central nervous tissue reveals the same large difference: peripheral nervous system (PNS) mean threshold = 59 W/cm^2, σ = 68 (Colucci 2009; Dalecki et al. 1995; Dickey et al. 2011; Foley et al. 2008; Fry et al. 1950; Gavrilov et al. 1977; Hu et al. 2014; Kim et al. 2012; Lee et al. 2014; Legon et al. 2012; Lele 1963; Tsui et al. 2005; Tych et al. 2013; Wright and Davies 1989); central nervous system (CNS) mean threshold = 3 W/cm^2, σ = 3 (Deffieux et al. 2013; Hameroff et al. 2013; Kim et al. 2014a, 2014b, 2015; King et al. 2014; Legon et al. 2014; Lee et al. 2015; Min et al. 2011a, 2011b; Moore et al. 2015; Tufail et al. 2010; Tyler et al. 2008; Yang et al. 2012; Yoo et al. 2011; Younan et al. 2013). Subsequent to 2008, studies on the effects of low-intensity US in the living brain have yielded a range of exciting results, such as stimulating motor activity (Tufail et al. 2010), affecting GABA release (Yang et al. 2012), reversibly inhibiting epileptic activity (Min et al. 2011a) and eliciting somatosensory sensations (Lee et al. 2015).

Despite recent progress in the application of the technique, still very little is known about the mechanism at work behind the observations. Understanding in this regard has been hampered by poor characterisation of the ultrasound field, especially in small animal models in which small cranial volumes make reflections and standing waves a significant problem (Young and Henneman 1961). Combined with the biological complexity of brain tissue and the variety of models used, very little consensus has been achieved on successful US parameters, exemplified by occasional directly conflicting or negative findings (Colucci 2009; Gavrilov and Tsirulnikov 2012).

There is at least consensus that ultrasound stimulates nervous tissue through a mechanical effect, not a thermal one. The field is far from united on the nature of this mechanical interaction, but the leading two theories for the key mechanism involve either acoustic radiation force or cavitation.

Cavitation is most often brushed aside as a potential mechanism in the CNS stimulation literature because of the low intensities used to elicit neurostimulation (Deffieux et al. 2013; Lee et al. 2015; Tufail et al. 2010; Yoo et al. 2011), below the U.S. Food and Drug Administration (FDA)-recommended mechanical index (MI) limits for soft tissue ultrasound (Duck 2007). The limitations with this claim, however, are that the MI limit was formulated from observations of bubbles in free water, is concerned only with preventing inertial cavitation of sufficiently large bubbles to cause significant damage and MI is only a guide and cannot be used to truly predict the occurrence of cavitation as this will depend on the tissue type, bubble nuclei, dissolved gas content and other factors. Though some studies have reported very high pressure thresholds for *in vivo* cavitation in the brain (Gateau et al. 2011), others have found significant non-inertial cavitation at much lower intensities (240 mW/cm^2) (ter Haar et al. 1982, 1986). Though these two studies had exposures of much longer duration, over a minute, the finding does indicate that bubble nuclei can be affected in some way by low intensities over much shorter durations.

In this study, a controlled *in vitro* environment is used, simplifying both the biological and the acoustic environments so that insight can be gained into the mechanism by which mechanical forces are transduced into propagating electrical activity in axons. Given this goal, it was decided that the best first course of action was to isolate and understand the direct stimulation phenomena observed previously by the authors in the crab walking leg nerve axon (Wright et al. 2015). To this end, a test setup was designed with several key capabilities:

- Ultrasonic stimulation of a nerve bundle with known exposure parameters.
- Electrical stimulation of the bundle, providing saturated control measurements of the compound action potential (CAP) before each US stimulus.
- Measurement of cavitational activity at the US stimulus site.
- Measurement of electrical CAPs at a distal site, resulting from either stimulus modality.

By use of this experimental approach combined with modelling of ultrasonic radiation forces at various stimulus parameters, the likely stimulus mechanism was determined by calculating the correlation of radiation force or cavitation activity with successful stimulation. Other features of successful US stimuli, such as response latency and response reliability, were also investigated to determine the responsible force mechanism.

METHODS

Experimental setup

The equipment used in the current setup illustrated in Figure 1 is detailed here. The US stimulus waveform was produced by two function generators (Agilent 33220A, Agilent, Santa Clara, CA, USA), one gated by the other to produce the pulsed protocol, which was then amplified by a class AB linear power amplifier with 55-dBm gain (E&I 1020L 200 W, E&I, Rochester, NY, USA). The three US stimulus transducers and the transducer used as a passive cavitation detector (PCD) are detailed in Table 1. The signal of the PCD was