Utilization of focused ultrasound for opening of the blood-nerve barrier

Daniel Umansky, Chenchen Bing, Tak Ho Chu, Saud Alzahrani, Jeff F. Dunn, Samuel Pichardo and Rajiv Midha

1 Division of Neurosurgery, Department of Clinical Neurosciences, Calgary Zone, Alberta Health Services, Canada
2 Clinical Neurosciences, Hotchkiss Brain Institute, Cumming School of Medicine, University of Calgary, Alberta, Canada
3 Department of Radiology, Image Science Division, University of Calgary, Alberta, Canada

* Author to whom any correspondence should be addressed.
E-mail: dumansky@gmail.com

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Abstract

Objective. Focused ultrasound (FUS) use with and without microbubbles (MB) for investigation of the blood-nerve barrier (BNB) within the peripheral nervous system (PNS) has been performed in this study. We evaluate the feasibility of BNB opening in a rodent sciatic nerve model by direct vision FUS treatment and provide preliminary results of magnetic resonance guided FUS (MRgFUS). Approach. Twenty rodent bilateral sciatic nerves were investigated. Rodents were treated using a benchtop FUS system to directly visualize nerve FUS studies. Definity MB, Evans blue dye (EB) and latex micro beads were injected during studies. Selected animals underwent further compound muscle action potential (CMAP) studies. Sonication peak pressure (MPa), width, duty-cycle and duration as well as MB concentration were varied to investigate effective pressure threshold. Further preliminary MRgFUS studies were performed on selected animals. Immunohistochemistry and histological analysis under fluorescent microscopy were performed at termination of experiments to verify treatment outcomes.

Main results. Three ultrasound pressures and three microbubble concentrations at a single sonication frequency (476.5 kHz) were performed under direct open targeting. Histological analysis demonstrated nerve internal architecture disruption at 1.2 MPa with 166.7 μl kg⁻¹ while 0.3 MPa, with 40 μl kg⁻¹ MB concentration was the lower threshold for consistently observed disruption of the BNB without anatomical microarchitecture disruption. EB leakage was confirmed at the target region in histological evaluation of nerve following MB injection and FUS sonication. Supra-harmonic emissions were detected during FUS exposures following MB injection but not at baseline reference, indicating effective MB response and stable cavitation. CMAP amplitudes showed delayed onset latency and lower amplitudes in sonicated nerves compared to control nerves without evidence of complete conduction block, suggesting a transient BNB disruption, while at lower limit pressure subtle conduction changes were observed. In MRgFUS, targeted nerves demonstrated further contrast agent leak as well as supra-harmonic frequency detection. Significance. Opening of the BNB in the PNS was achieved using FUS and MB in a rodent model. Ongoing work aims to refine FUS parameters for drug delivery into the nerve after experimental transient BNB disruption.

Introduction

Peripheral nerve injury (PNI) following trauma or neuropathies entail severe long-term disability, rehabilitation, and costs. Over 200 000 trauma-related nerve injuries per year occur in the United States alone (Noble et al 1998) and over 20 million people are estimated to be affected by peripheral neuropathies (Kubiak et al 2018); the annual incidence approximates 45 cases per 100 000 similar to the incidence of
epilepsy (Simon et al 2016). Surgical nerve repair, while feasible, leads to an often-unpredictable process in which recovery and outcome depend on the injury mechanism itself and nerve microarchitecture involvement as well as the technical aspects of the repair used (Umansky and Midha 2021). Regenerating axons need to overcome injury gaps and distal denervated nerve environment in which the chronically denervated Schwann cells cannot support axon degeneration optimally (Zhai et al 2003, Walsh and Midha 2009). Targeted delivery of neurotrophic drugs, vectors and cells into the injured and denervated nerve environment are proposed as therapy to improve nerve regeneration and outcomes (Piotrowicz and Shoichet 2006, Kemp et al 2011, Langert and Brey 2018, Liu et al 2018, Nishihara et al 2018). However, the presence of a blood–nerve barrier (BNB) impedes therapeutic delivery and its temporary targeted disruption would be advantageous to advance care of PNI (Kanda 2013, Peltonen et al 2013).

Magnetic Resonance (MR) guided focused ultrasound (MRgFUS) for disruption of both the blood–brain-barrier (BBB) (Hynynen et al 2001, Burgess et al 2011, MacDonell et al 2018) and blood–spinal cord-barrier (BSCB) (Weber-Adrian et al 2015, Payne et al 2017, O’Reilly et al 2018) has been gaining significant scientific interest in recent years. The aim of these studies are to develop a controlled, temporal disruption of the microvascular lumen for possible central nervous system (CNS) drug delivery, viral therapies and stem cell treatment, in addition to ablative therapies to both native and pathological tissue (Hynynen et al 2006, Yosef et al 2010, Burgess et al 2011, Weber-Adrian et al 2015, Ventre et al 2016, O’Reilly et al 2018). Similar to the brain and spinal cord, an ultrastructural and physiological barrier, termed the BNB, is present within the peripheral nervous system (PNS). In the peripheral nerve, the innermost nerve layer—the endoneurium, is a highly regulated microenvironment consisting of loose collagen arrays in which myelinated and unmyelinated axons and non-fenestrated tight junction-forming microveessels reside. The microenvironment of the endoneurium is regulated by two blood–nerve interfaces possessing non-fenestrated tight junctions (TJ) (Yosef et al 2010); the BNB, made up of the innermost perineurial layer and the endoneurial microveessels. Endothelial cells of endoneural microveessels, which are in direct contact with circulating blood are considered the true interface forming the BNB (Smith et al 2001, Reina et al 2003, Yosef et al 2010) (Ubogo et al 2013).

Extensive work has been performed in the field of targeted ablation and neuromodulation in both the CNS and the PNS since the early and mid-20th century (Fry et al 1950, Takagi et al 1960, Lele 1963, Raymond et al 2008, Kim et al 2012, Ting et al 2012, Ventre et al 2016). More recent technological development in both targeted imaging guidance modalities and focused ultrasound (FUS) systems (Tung et al 2010, Gavrilov and Tsirulnikov 2012, Muratore and Vaitkenas 2012, Kamimura et al 2016) have heralded the precise and graded application of FUS to permit neuromodulation of the peripheral nerve, with a focus on nerve stimulation to generate action potential responses in vitro (Tsui et al 2005, Colucci et al 2009, Lee et al 2015) and in vivo (Tung et al 2010, Downs et al 2018, Kim et al 2020). Moreover, Cullion et al (2018) took this a step further with microbubbles (MB) utilization, to demonstrate that peripheral nerve action potentials could be blocked when FUS and MB were delivered in conjunction with systemic delivery of a hydrophilic drug, Tetrodotoxin (TTX), which normally would be impeded by entry into the nerve microenvironment by an intact BNB. This work thereby indirectly implicated BNB breakdown, at least transiently. Circulating MB have been shown to follow a cavitation process when exposed to FUS by encountering acoustic-actuated harmonic expansion thus providing physical stimulation to the endothelial cells followed by temporal TJ disruption in the CNS (Kissling et al 2014, Goertz 2015). Furthermore, real-time acoustic feedback can be used to measure cavitation activity levels as potential tissue damage indicator (Tung et al 2010). This body of work has allowed us to propose a possible real-time physiological response of the BNB to acoustic energy. We seek to explicitly utilize FUS and MB to focally disrupt the BNB, yet not injure the nerve.

Herein, we compared FUS utilization to our well established and reproducible rodent crush-injury model (Alant et al 2012) for a study on the local effect of FUS and MB on the BNB. Specifically, we conducted a set of exploratory studies on rodent sciatic nerve using a direct targeting open FUS system to first determine suprathereshold injurious doses of FUS energy at 1.2 MPa with 166.7 μl kg⁻¹ MB concentration and then identified minimal and reproducible acoustic energy thresholds to open the BNB, without producing nerve injury using 0.3 MPa with 40 μl kg⁻¹ MB concentrations. The current work has therefore successfully targeted rodent sciatic nerves and has established for the first time a protocol for successful manipulation of the BNB using dose responsive FUS.

Materials and methods

Direct under vision targeting and FUS sonication of rat hind limbs was performed (supplementary table 1 (available online at stacks.iop.org/PMB/67/205003/mmedia)). In addition, a proof-of-concept preliminary study has targeted FUS using magnetic resonance guidance (MRgFUS) on both mice and rat hind limbs.
Animals

All animal work and handling were carried under an approved protocol in accordance with the guidelines and regulations of the University of Calgary’s Animal Care Committee Protocol. Surgeries, MR imaging, FUS investigations and electrophysiological readings were carried out under 2%–2.5% isoflurane inhalation anesthetic. All studies were performed on hind limbs following shaving, depilation cream application along with 70% ethanol aseptic preparation. Animals were kept on top heating pads to maintain their body temperature until termination of procedures. Surgical procedures were performed under a Leica M651 operating microscope and intraoperative images captured with a microscope camera (Infinity1, Lumenera, Canada). Prior to assigned procedure for each rodent group, selected control and FUS groups were prepared using 24-gauge intravenous (IV) catheters placed into the lateral tail veins using a transcutaneous technique and flushed with 0.9% normal saline via 3 directional valves to preserve patent IV access. At pre-determined experimental point, animals were injected with 3% Evans blue (EB- Sigma-Aldrich, USA) at same interval timepoint of the experiment. Free EB in the blood circulation is expected to exit the intravascular space via fenestrated endothelial cells and rapidly enter extracellular space binding to cells and matrix which have a higher affinity for EB than Albumin (Hoogenboezem and Duvall 2018), serving as a quantification marker for protein leakage (Uyama et al 1988, Yang et al 2007). However, EB will not leak from endoneurial blood vessels into surrounding tissue unless the BNB is disrupted or underdeveloped (as in young but not adults rodents) (Smith et al 2001). Finally, fluorescent latex micro beads with 0.03–1 μm diameter (100 μl 1:10 Sigma-Aldrich, USA) were injected 20 min prior to animal euthanasia. Micro beads have been shown to follow vascular rheological transport as well as CNS transport and provide target labeling if encountered (Katz and Iarovici 1990, Hoover and Durkovic 1992). Animals were euthanized with lethal dose of sodium pentobarbital (Euthanyl).

Adult Sprague-Dawley rats (250–350 grams; n = 16) were investigated. From this group, adult female transgenic Thy1-GFP rats on a Sprague-Dawley background (n = 10) and adult wild-type Sprague-Dawley (n = 6) were used. Thy-1-GFP rats express green fluorescent protein (GFP) in neural tissue. Importantly, this allows to identify axonal and endoneurial microstructure and demonstrate loss and reappearance of fluorescence upon injury and regeneration, respectively (Magill et al 2010, Moore et al 2012). In addition, C57/BL6 brown mice (30–35 grams; n = 4) were used for MRgFUS protocol development. Direct visualization of nerve fiber anatomy and disruption under fluorescence microscopy, where the sciatic nerve was surgically exposed for precise target localization, was performed. Surgical technique and crush injury methods have been validated in previous work by our group (Alant et al 2012) and are presented briefly in the following sections.

Crush injury group

(n = 1 SD rat, n = 3 C57/BL6 brown mice). Similar surgical procedures were carried out for both rat and mice. This group served as a positive (for nerve injury) control. Exposure of right sciatic nerve was performed via a longitudinal lateral thigh incision. Connective tissue between the superficial gluteus muscle and the biceps femoris muscle is cleared by micro-scissors. Splitting of the biceps femoris muscle in an avascular plane allows us to visualize the sciatic nerve under a small protecting deposit of fat (Gonzalez et al 2014). Extreme care is taken to avoid disrupting microvasculature to the sciatic nerve which is unharmed and remains in its in situ position. After splitting the biceps femoris muscle, the sciatic nerve was exposed from its emergence from the sciatic notch to the trifurcation at the popliteal fossa. Injury sites were repeated at equidistance 9 mm proximal to the sciatic nerve trifurcation point. Double crush injuries were performed repeatedly in all control animals using #5 jewelers’ forceps applying constant force in a vertical direction for 30 s followed by horizontal compression for an additional 30 s, a paradigm which reliably reproduces at least a Sunderland Grade 2, nerve in-continuity injury (Alant et al 2012). Visual verification of crushed nerve took place and after cotton pads were removed, care was taken to avoid nerve and surrounding tissue injury. Following irrigation with normal saline, the retractors were removed and biceps femoris muscle approximated. Skin incision was closed with single horizontal 6–0 Prolene sutures.

Definity MB activation and sonication verification

Definity MB (Lantheus Medical Imaging, MA, USA) 2 ml vials were activated by manufactures VialMix benchtop mechanical mixer as to contain a maximum of 1.2 × 10^10 perlfluten lipid microspheres and about 150 μl ml^-1 (1.1 mg ml^-1) octafluoropropane, per manufactures description Definity Perlfluten Lipid Microsphere (no date). 10 μl kg^-1 MB initial dosages were adjusted to individual rodent weights. Phantom injections along intended tubing and connectors were performed prior to animal injection.

Sciatic nerve FUS BNB investigation (n = 10)

Experimental set up is illustrated in figure 1. Surgical exposure of the right sciatic nerve was carried as described above for crush injury group. The small animal FUS system RK30 benchtop system (FUS Instruments, Toronto,
ON, Canada) was used. Following exposure and external neurolysis of the sciatic nerve, the nerve was placed on top of a normal saline-wetted cotton pad. Biceps femoris muscle was sutured to overlying skin incision using 4/0 silk suture to allow maximal exposure and surface proximity to FUS detector and to facilitate later electrophysiological investigations. Upon completion of target registration, the pointing probe was replaced with the ultrasound transducer. A degassed water chamber was placed directly over the exposed nerve, and the transducer focus was navigated in three planes allowing a 0.5–1 mm distance for visual targeting (Figure 1). 0.3 ml of 3% EB was injected immediately prior to FUS sonication exposure and 5 min were allowed for circulation. Definity MB (Lantheus Medical Imaging, Billerica, MA, USA) chosen doses were 20 µl kg−1, 40 µl kg−1 and 166.7 µl kg−1 and injected at the beginning of FUS application and 5 min following EB injection. Pulsed FUS exposures were delivered immediately after MB injection using the benchtop FUS system at 476.5 kHz. Pulse width was 10 ms with 1 Hz repetition frequency (duty cycle of 1%). Total exposure duration was 120 s. Peak pressure varied from 0.3, 0.5 and 1.2 MPa (n = 4/1 for 0.3/0.5 MPa and 1.2 MPa for n = 4) to investigate the effective pressure threshold. Acoustic emissions during the exposures were collected via the passive cavitation detector (PCD) connected to an oscilloscope (Picoscope 5000, Picotech, UK). Finally, latex microbeads (100 µl 1:10 of 0.03 µm and 0.1 µm diameter beads) were injected within 20 min following sonication and imaging and prior to animal euthanasia.

Electrophysiological evaluation
Compound muscle action potential (CMAP) amplitudes were measured (Cadwell 6200A, Cadwell Laboratories, WA, USA) during nerve exposure of both control and selected FUS treated animals before treatment and 15–20 min following sonication based on previous reports (Takagi et al 1960, Lele 1963, Stratton et al 2018). Both intra-animal (comparing experimental limb to contralateral normal limb) and inter-animal studies were performed and analyzed. Motor latencies, conduction velocities and amplitudes were recorded. Bipolar hook electrode was used for stimulation of the proximal sciatic nerve just above the sciatic notch, and evoked electromyogram activity was recorded from the lateral gastrocnemius muscle. Delayed onset latency and increase of CMAP amplitudes were deemed as a good surrogate marker for acute nerve physiological alteration (Takagi et al 1960, Lele 1963, Downs et al 2018). Here, stimulating current was gradually increased until no increment in CMAP amplitude is observed (supramaximal stimulation of 2 mA) and the largest response is obtained (Raynor et al 1995, Mallik and Weir 2005). Latency was determined from time of stimulus artefact to onset of response which is biphasic with an upward followed by a downward deflection. CMAP amplitude is measured from baseline to negative peak (upward deflection) (Mallik and Weir 2005, Pollari et al 2018).

Histological and microscopical evaluation
Following animals’ euthanasia, treated area was visually identified for EB leakage and sciatic nerve was resected proximal to its distal emergence from the spinal canal and distal to its trifurcation to the tibial, peroneal, and sural branches. Nerves were cautiously harvested from anterior of the hamstring branch to posterior of the distal

Figure 1. Benchtop focused ultrasound system and the experiment setup. (a) The sciatic nerve was surgically exposed, and the ultrasound focus was registered at targeted nerve using the stereotaxic system. The right panel shows the experiment workflow. After preparation and targeting, 3% Evans blue dye was injected via the tail vein to visualize blood-nerve barrier disruption. FUS exposure was enabled simultaneously with the injection of microbubbles. Followed by the FUS exposure, EMG signals were acquired and recorded, and the animal was sacrificed for histology evaluation. (b) Right Sciatic nerve targeting. The pointing probe is directed within the water chamber under vision in three planes to reach ideal position 0.5–1 mm over the nerve target. EMG—Electromyography; FUS—Focused Ultrasound; V—Ventral, D—Dorsal, R—Rostral, C—Caudal.
trifurcation after crush injury and FUS treatment verifying no additional physical manipulation inadvertently occurred. The nerve was fixed with 4% paraformaldehyde in 0.1 M phosphate buffer overnight at 4 °C and transferred to 30% sucrose for cryosectioning. Serial longitudinal sections of 8 μm were cut with a cryostat (Leica CM1900; Leica Microsystems Inc.) and collected on Superfrost Plus slides (VWR, USA). Slides were store at −20 °C freezer for later investigation under fluorescence microscope.

Immunohistochemistry
Selected treatment animal slides were warmed to room temperature and washed in 0.01 M phosphate buffered saline (PBS) twice for five minutes. Sections were blocked in 5% normal goat serum in diluent for 1 h at room temperature. Primary antibodies rabbit anti-Glut1 (1:1000, Sigma-Aldrich, USA) was used to label perineurium (Takata et al. 1997, Tserentsodol et al. 1998); chicken anti-neurofilament (NF200 1:500 Sigma-Aldrich, St Louis, MO) was used to identify axonal profile and Alexa 647 conjugated CD31 was used to label blood vessel/TJ investigations. After blocking solution removal, the primary antibodies were left overnight at 4 °C. After three-time washes with PBS with 0.05% tween 20 (PBST), secondary antibodies including rat anti-Rabbit (568), goat anti-chicken (594) and were applied and incubated for 1 h at room temperature. Slides were then washed with PBST three times for five minutes each, followed by PBS for the last wash. Finally, sections were counterstained with DAPI nuclear staining (1:5000) for 2 min and slides were covered with fluorescent mounting medium (PermaFluor, Thermo Scientific, USA) and coverslips followed by nail polish application at coverslip edges. Sections were examined in a blinded fashion initially with a fluorescence microscope (Olympus BX51; Olympus, Japan) and imaged with slide scanner (Olympus VS110-S5 Slidescanner, Japan). Selected sections underwent Hematoxylin and Eosin (H&E) staining and were further investigated under fluorescence microscopy. H&E staining was used to rule-out possible nerve and or brain targeted field injury hallmarks (hemorrhage and extravasation of red-blood-cells (RBCs), edema, nuclear condensation, inflammatory cell infiltrate and rarefaction) (Bing et al. 2014, Ventre et al. 2016, Huang et al. 2017).

MRgFUS Sciatic nerve targeting
A preliminary series of experiments were also performed prior to the benchtop experiments. C57BL/6 (n = 4) brown adult mice were selected to evaluate the feasibility of using MR guidance together with FUS (MRgFUS). Mice were chosen for MR guided targeting due to coil dimension restriction for larger rodents and were placed on a custom designed saddle coil. MRI was performed using a 9.4T/21 cm horizontal bore magnet (Magnex, UK) equipped with an Avance II console (Bruker, Germany) using ParaVision (v5.1) and a Bruker BGA-S Gradient Insert with 116 mm inner diameter and 720 mT m−1 gradient strength. MRI parameters were: (supplementary table 2) (1) Rapid Acquisition with Relaxation Enhancement (RARE) FatSat T2W, (2) Fast Low Angle Shot (FLASH) FatSat T1W, (3) 3D Gradient Echo with Flow Compensation (GEFC) T1. Unilateral targeted nerves were sonicated with an MRgFUS system (Image Guided Therapy, Bordeaux, France) that uses a 7-element concentric ring transducer (focal length of 20 mm and θ-number of 0.8) operating at 1.5 MHz plus a PCD lodged at the center of the transducer. FUS beam has a −3 dB axial length of 2 mm with an axial length of 5 mm. Ultrasound parameters were 5 ms pulse duration and 95 ms of pausing (5% duty cycle), with 1 MPa acoustic peak pressure for 5 min total. Driving parameters to achieve 1 MPa were established based on calibration information provided by the manufacturer. The choice of a longer duty cycle of 5% served part of the exploratory nature of these preliminary experiments based on reports on the effects of duty cycle from a safe BBB calibration information provided by the manufacturer. The choice of a longer duty cycle of 5% served part of the exploratory nature of these preliminary experiments based on reports on the effects of duty cycle from a safe BBB

Results
Confirmation of direct FUS targeted sonication
Macroscopic and microscopical histological evaluations were performed in all nerves. Initial investigations were carried by an experienced PhD Research Associate and a board-certified Neurosurgeon who were blinded to specific treatment conditions. Caution during harvesting and handling of nerve and surrounding tissue was
taken as to not cause any injury that can mimic or confound results. Localization of crush and sonication targets were confirmed by topographical positioning on top of cotton pads along x, y and z axis registration and pinpointing of the transducer, respectively.

Naïve nerves did not show any macroscopic or microscopic evidence of EB leakage nor RBCs extravasation in H&E sections (figures 2(a)–(c)) supporting that surgical exposure (sham operation) and tissue harvesting by itself does not affect nerve architecture.

Crush injured nerves demonstrated expected focal disruption of green fluorescent protein (GFP) positive axons, in addition to both morphological endoneurial and perineurial disruption (figures 2(d)–(f)).

1.2 MPa high pressure sonication with 166.7 μl kg⁻¹ and 40 μl kg⁻¹ MB concentration consistently demonstrated significant axonal architecture disorganization, extensive vacuolization and edema, suggesting supra-threshold sonication and disruption of targeted nerves. No latex micro beads were visible along sectioned nerve but were accumulated at targeted sonication site inside larger caliber endoneurial and along epineurial blood vessels suggesting possible clotting. Here, in sampled slides undergoing immunohistochemistry analysis, perineurium layer (labeled by Glut1) was observed to be intact (figure 2(h)), whereas loss of both axonal microarchitecture (labeled by NF200 staining) and blood microvessels (with tight junction targeted CD31 immunolabeling) integrity were evident (figures 2(g)–(i)).
adjusted lower peak negative pressure sonications with varying MB concentration were used (20, 40, 166.7 μl kg⁻¹). Either one of 20 μl kg⁻¹ and 40 μl kg⁻¹ MB concentration provided desirable target alteration supported by EB extravasation out of the microvasculature and into the endoneurial environment, confirming BNB disruption—without evidence of axonal architecture disorganization nor vacuolization or edema (Smith et al 2001). The latter findings however were evident when using 166.7 μl kg⁻¹ MB concentration. The combination of 0.5 MPa peak negative pressure together with 20 and 40 μl kg⁻¹ of MB dosage demonstrated repeated BNB disruption. Yet, even when adjusting to a lower peak negative pressure of 0.3 MPa with 40 μl kg⁻¹ MB concentration we demonstrated clear BNB disruption without evidence of axonal architecture disorganization nor vacuolization or edema as seen in figures 3(b) and (c). Furthermore, no RBCs extravasation was evident in H&E sections using this later combination (figures 3(d), (e)). Additional reassuring results were evident during acoustic emission detection when using 0.3 MPa peak negative pressure combined with 20 μl kg⁻¹ MB concentration (figure 4) detecting supra-harmonic spectrum component at 715 kHz (fundamental frequency was 476.5 kHz), indicating stable cavitation and disruption effect. Yet, while supra-harmonic component detection was reproduced when combining 0.3 MPa peak negative pressure with 20 μl kg⁻¹ MB concentration, we observed inconsistent and subtle histological tissue changes, suggesting that this dosage combination was sub-threshold (data not shown).

We conclude that 0.3 MPa positive peak pressure in conjunction with 40 μl kg⁻¹ MB concentration is the lowest limit to reliably achieve anatomical BNB disruption through sonication utilization, yet it does not produce axonal injury or disruption.

**Electrophysiological evaluation**

FUS targeted hind limb were compared to contralateral untreated sides. Bipolar stimulation ranged from 0.5 mA to supra-threshold 3 mA. CMAP recording on sonicated nerve compared to contralateral healthy nerve
demonstrated delayed onset latency and lower CMAP amplitudes in the sonication targeted group at 1.2 MPa pressure and 166.7 μl kg⁻¹ MB concentration compared to the non-treated group, yet complete conduction block whether transient or temporary was not observed (figure 5). Under reduced pressure of 0.3 MPa and 40 μl kg⁻¹ MB concentration, CMAP recording 20 min before and after sonication of the same leg demonstrated

**Figure 4.** Frequency spectrum of single 10 ms ultrasound pulse targeting at rat sciatic nerve using benchtop system. (a) Baseline frequency prior to micro bubble injection. No acoustic emission is detected at ultra-harmonic (around 715 kHz, dashed red rectangle) frequency. (b) Frequency spectrum following microbubble injection. Focal pressure estimated at 0.3 MPa, ultrasound transmitting frequency at 476.5 kHz. Ultra-harmonic emission is detected following microbubble injection (red arrow) indicating potential stable cavitation and BNB opening effect. BNB—Blood-nerve barrier.

**Figure 5.** Representative nerve conduction study tracing. Control tracing alongside 0.3 and 1.2 MPa sonicated nerves. Latency is determined by the delay from stimulation to the onset (O) of CMAP response. Amplitude is measured from baseline to negative peak (P). Note CMAP amplitudes in 0.3 MPa sonicated nerve (16.5 mV) and 1.2 MPa sonicated nerve (10.3 mV) compared to control nerve (19.9 mV). Also note similar onset latency between 0.3 MPa sonicated nerve (2.1 ms) and control nerve (2.3 ms). CMAP—Compound Muscle Action Potential; mV—millivolt; ms—millisecond.
more subtle changes in nerve conduction without any delayed onset latency, while a trend towards lower CMAP amplitudes were apparent (figure 5).

**Confirmation of MR guided FUS**

Real-time *in vivo* FUS targeting and sonication (figure 6) in mice revealed positive backscattering detection of MB at target following injection, demonstrating supra-harmonic oscillations at 3 MHz (2× from baseline). 9.4T MRI T1 and T2 weighted fat suppressed images demonstrate signal intensity change and contrast enhancement...
suggesting disruption of targeted nerve and subsequent Gadolinium enhancement. No measurable change of temperature over baseline was observed during MRI-based thermometry.

Discussion

Seminal work studying the ultrasonic effect on peripheral nerves in both mammalian and non-mammalian models including ex vivo and in vivo studies have provided better understanding of axonal firing, CMAP properties and ablative action of high intensity FUS (Takagi et al 1960, Lele 1963, Colucci et al 2009, Kim et al 2012, Downs et al 2018). Harvey and Loomis in their first reported ultrasonic experiments already demonstrated direct depolarization of nerve, but not muscles, resulting in cardiac muscle contraction (Harvey and Loomis 1928). Subsequent experiments by Fry et al measured acoustic waveform parameters along with cavitation and thermal effect on neural tissue including studies for controlled reversibility of ultrasonic application on the CNS (Fry et al 1950, Fry et al 1958). Yet, none of these pioneers’ work addressed the possible role of reversible opening of the BNB once disrupted.

Recent work for neuromodulation of specific signaling pathways using ultrasound targeting and subsequent FUS sonication in several organ targets demonstrated site-selective neuromodulation for distinct physiological pathway function regulation (Naor et al 2016). Cotero et al (2019) demonstrated modulation of the anti-inflammatory response in a rat model of lipopolysaccharide-induced inflammation, and their companion work (Zachs et al 2019) demonstrated modulation of the splenocytes involved in the anti-inflammatory pathway through an arthritis mice model. Both works further demonstrate the potential power of using this treatment approach and rest on previous approaches for ultrasound focusing. Notably, these include studies with stimulation of ex vivo nerves with demonstrable action potentials (Wright et al 2017), including suprathereshold sonication with potential nerve and surrounding tissue damage (Colucci et al 2009). Others performed in vivo studies through direct nerve activation causing inhibition only results (Juan et al 2014), or indirectly observed the effects of nerve alteration through behavioral observations and electromyography (EMG) recordings (Kim et al 2012, Downs et al 2018).

Inertial cavitation, occurring under intense oscillated acoustic pressure—can cause a gas pocket to eventually implode—thus its utilization in selective destruction of tumors and lithotripsy (Ziskin 1993, Ventre et al 2016). Inertial and stable cavitation are further differentiated by a PCD (Chu et al 2016). TJ opening created under shear stresses of stable cavitation are reversible and temporary but can increase vascular permeability and eventually red blood cell extravasation. Controlling for ultrasonic stimulation parameters allows a physical change leading to non-inertial stable cavitation. This phenomenon, allows us to utilize MB which amplify cavitation effects on the cellular surface and membrane permeability in an acoustic intensity-dependent manner (Ibsen et al 2015). Indeed, Rwei et al demonstrated administration of local liposome based hydrophilic sodium channel blocker (TTX) anesthetic using ultrasonic triggering in both in vitro and in vivo (sciatic nerve) targeting (Rwei et al 2017). Their ultrasound parameters consisted of 1 MHz and a 100% duty cycle for 10 min, inducing an initial nerve block of well over several hours (8.3 ± 4.7 h) along with thermal latency effects (time for the animal to withdraw its hind paw from a hot plate). Unfortunately, 50% of the in vivo sciatic nerve group experimental animals died 3–10 h after injection with an additional two thirds of animals experiencing prolonged latency in the contralateral non-injected extremity. While developing a novel pain relief delivery system this model, albeit using similar sonication pressures as our study, used significantly higher duty cycles over longer cycles and further suggested inducing inertial cavitation and implosion of ultrasound induced cavitation bubbles generating reactive oxygen species as a possible detrimental effect for extended nerve blocking, thermal latencies and toxicities. Our selected sonication parameters as well as MB utilization were therefore adjusted to avoid inertial cavitation undesirable outcome especially when operating at high-pressure value of 1.2 MPa.

Following previously published CNS BBB disruption literature (Tung et al 2010, Aryal et al 2014, Bing et al 2014, McDannold et al 2020) we expected similar results in the PNS BNB despite several physiological differences between the two. The BNB comprising the perineurium and the endoneurial microvessels endothelial cells has variation in permeability between species, nerves and location (Smith et al 2001). Herein, cellular response to ultrasonic energy is expected to vary. For example, transmembrane ion channels initiating action potentials probably undergo subtle change effect in response to FUS but their significance is unclear (Hu et al 2013); Mechanosensitive ion channel type and concentration differences may explain different sensitivity threshold between the CNS and the PNS (Legon et al 2012); Direct membrane disruption and cavitation effects secondary to MB activation (Ibsen et al 2015) as well as vesicular fusion within the pre-synaptic membrane (Siechen et al 2009) are physiologically different between the two systems and their role is still, mostly, unclear (Wright et al 2015). Finally, increased vascular permeability to plasma protein observed in regenerating PNS
axons facilitating growth factor transport, following nerve injury models (Mellick and Cavanagh 1968), is not observed in the CNS (Kiernan 1985, Smith et al 2001).

Still, targeting peripheral nerves with FUS minimizes thermal effect observed in both cranial and spinal experiments and treatment protocols due to lack of cranial vault and posterior spinal bony elements (Ventre et al 2016, MacDonell et al 2018). This effect is believed to take place due to the nerves’ soft tissue characteristics, allowing only propagation of longitudinal waves as opposed to both longitudinal and transverse wave propagation through bone (Ziskin 1993, Tufail et al 2010). We therefore attempted to optimize imaging and FUS parameters for BNB disruption using a higher frequency to reduce the divergence within the emitted ultrasound beam, thus producing a smaller focal beam allowing increase in spatial resolution and reduction of attenuated energy (Ventre et al 2016). This, along with an acoustic coupling gel and degassed water bath to decrease acoustic impedance mismatch and minimize ultrasonic reflection (Ziskin 1993, Ventre et al 2016). However, our results indicate that the minimal MB dosage required for BNB disruption is actually higher than that used for BBB disruption, supported by previous work (Rossini et al 2015, Wright et al 2015). We believe that very subtle changes occurring in targeted nerves when low power sonication and MB concentrations was used, not evident in published cranial sonication work, further supports the possible barriers (i.e. dura, calvaria) role (King et al 2013) in the latter.

Importantly, results when operating at these pressure levels—considered a high intensity focused ultrasound (HIFU)—can be dominated by thermal and cavitation (gas bubble) effects (Tufail et al 2010) and can create ablative like results as has been shown in epileptic foci lesioning (Foley et al 2007) and evidenced by our initial studies with 1.2 MPa. However, we have later adjusted our sonication peak pressure accordingly along with a lower duty cycle (<10%) to overcome these effects in our proposed treatment parameters.

Free EB in the blood circulation is expected to rapidly enter extracellular space binding to cells and matrix which have a higher affinity for EB than Albumin (Uyama et al 1988, Yang et al 2007, Hoogenboezem and Duvall 2018) thus potentially masking leakage. Furthermore, current literature supports concentration of EB in the CNS is synonymous with penetration of albumin across the BBB (Smith et al 2001, Shin et al 2018) while rodents are not expected to leak albumin into the endoneurium under normal conditions when transitioning from young to adult animals (Jacobs 1977, Olsson 1986, 1990, Smith et al 2001). Our findings of EB extravasation into the endoneurial environment, therefore, confirms BNB disruption at the target site has indeed occurred in our suggested optimal parameters of 0.3 MPa pressure with 40 μl kg⁻¹ MB concentration. Furthermore, no evidence of axonal architecture disorganization, vacuolization, edema nor RBCs extravasation, which would have been expected to be identified through either microscopical or histological studies (Uyama et al 1988, Smith et al 2001, Yang et al 2007, Bing et al 2014, Ventre et al 2016, Huang et al 2017, Hoogenboezem and Duvall 2018), were evident.

Latex micro beads ranging from 0.03 to 1 μm are expected to be visualized through disrupted blood vessels and following BNB disruption were expected to be visualized throughout the epineurium as communication between the anatomically distinct layers has been disrupted. Our finding of beads within the endoneurial blood vessels as well as along the external epineural layer support preservation of nerve microarchitecture, and we have not observed beads within axonal layers, disrupted endoneurium or perineurium. This finding confirms micro beads resistance to fading under florescent illumination (Katz and Jarovici 1990) and further suggests their size is too large to be facilitated through intercellular fluid flow and through TJ opening—supporting previous TJ morphological size observations (Smith et al 2001, Reina et al 2003). Finally, no macroscopically or microscopically EB leakage or microbead detection was evident when rodents were sonicated in preferential energy without MB adjunct injection, supporting MB enhanced cavitation effect (Ventre et al 2016).

Our preliminary CMAP results performed in 1.2 MPa and 0.3 MPa peak pressures sonicated animals, point to a possible reversible disruption effect. Yet, ongoing CMAP investigations are currently being investigated, and we regard our results with caution. Delayed onset latencies were observed in high pressure sonicated nerve when compared to non-sonicated studies, along with lower CMAP amplitudes, indicating a trend to nerve conduction difference (Colucci et al 2009) (figure 5). Furthermore, when optimizing sonication pressure to lower limits of possible BBB disruption (0.3 MPa) (Shin et al 2018), we did not observe any delayed onset latencies, thus suggesting that a possible threshold—reversible conduction disruption occurred. Certain physiological parameters including body temperature, depth of anesthesia, size of muscle recorded (Harrigan et al 2019) and recording needle placement (Bromberg and Spiegelberg 1997) can have a confounding effect on electrophysiological studies and must be taken under consideration when interpreting these results. Still, these initial finding can support early ultrasonic stimulative effect observations on myelinated nerves by Takagi et al suggesting a repeatedly threshold stimulation factor occurring over several minutes (Takagi et al 1960). Later works suggested a possible BBB reversible closure over anywhere from 1 to 48 h post treatment (Hynynen et al 2001, 2003) and most recent studies support 2–3 h closure (Chu et al 2016) including multiple weekly FUS exposures needed for safety in glioma treatment (Tsai et al 2018), leaving us without any firm literature support.
We have not, however, observed any myelin breakdown in our histological sections which could, if occurring, be evident through electron microscopy (Crisci and Ferreira 2002, Foley, Vaezy and Crum 2007).

The exploratory MRgFUS results indicate the potential of performing these procedures using a mouse model that can allow MR visualization of nerve and tissue disruption (figure 6). This can be used to ensure correct alignment and sonication effect on targeted tissue. However, additional testing needs to be performed to establish if this MRgFUS approach is equivalent to the results obtained using the rat model with parameters used with the benchtop system to support previous works published (Hynnen et al 2001, Shin et al 2018).

Our experiments have been performed in a rodent model using direct vision targeted FUS operating at a frequency of 476.5 kHz coupled with a commercial microbubble agent. We have established acoustic parameters and qualitative histological confirmation of BNB disruption. Minimal threshold dose—optimal parameters of 0.3 MPa pressure with 40 μl kg⁻¹ MB concentration—demonstrated consistent BNB opening without nerve microarchitecture disruption while supra-maximal dose of focused ultrasound energy at 1.2 MPa coupled with 166.7 μl kg⁻¹ MB concentration produced harmful microarchitecture damage and were deemed the upper cavitation threshold. These preliminary pilot study results indicate that selected MB dosage coupled with preferential FUS sonication power allows establishment of both minimal as well as maximal dose-response strategy for treatment, supported by earlier work of Takagi et al (1960), Foley et al (2007). We believe that using 0.3 MPa, our lowest selected sonication pressure, along with a MB dosage concentration of 40 μl kg⁻¹ can reliably achieve anatomical BNB disruption through sonication utilization, without producing axonal injury or disruption as previously observed by Foley et al (2007) and Crisci and Ferreira (2002) and support out histological results as the strongest representable data of this work. Furthermore, we have not observed inadvertent tissue blanching—to suggest coagulative necrosis nor indicated demyelination of axons and Schwann cell necrosis on histological preparations in these preferential settings. Still, our results of a temporary or permanent conduction block in higher energy treatments (1.2 MPa combined with 166.7 μl kg⁻¹ microbubble dosage), suggest that disorganization and disruption of myelin has indeed occurred (Foley et al 2007) at supra-threshold sonication.

Study limitations

Having the nature of a pilot study, we are hesitant to report any statistical power in this pilot study as we have used a relatively small number of animals with very heterogeneous conditions. Several optimizations and future investigations have yet to been carried out centering on dose-response, controlled, reversible BNB opening under CMAP confirmation in both short- and longer-term survival experiments coupled with electron microscopy confirmation. Nonetheless, combining FUS with MR guidance or possible real-time non-invasive ultrasound imaging guidance (Downs et al 2018) will establish a non-surgical, minimally invasive treatment approach for BNB opening, and facilitate translation of our model for clinical implication and future application. Utilizing low duty cycles makes thermal damage difficult to anticipate, as our thermometry test did not show any measurable change of temperature, yet software optimization is underway. Finally, it is worth mentioning that the central frequency in the MR-guided system is roughly triple the frequency of the benchtop system (1.5 MHz versus 476.5 kHz, respectively). The cavitation response depends on both the central frequency and MB size distribution, which in this work is mainly distributed with an average size of 2.7 ± 2.0 μm and resonates ideally at higher frequencies (>3 MHz) (Talu et al 2007). Consequently, the results obtained at 1.5 MHz are not completely comparable when sonicating at 476.5 kHz when using similar pressures. The highest pressure tested here was 1.2 MPa for 476.5 kHz, corresponding to a mechanical index \( \left( \frac{\text{Negative peak pressure (MPa)}}{\sqrt{\text{Frequency (MHz)}}} \right) \) of 1.7, which is at the upper limit of medical imaging safety threshold and above the recommend threshold of 0.8 for Definity MB (Tsai et al 2018). However, analysis of the backscattered signal showed supra-harmonic emissions, characteristics of stable cavitation. Nonetheless, it is possible the pressure still implied bubble oscillations large enough to produce the observed damage associated to 1.2 MPa and bubble concentration of 166.7 μl kg⁻¹. This indicates, if using this frequency, that these conditions should be considered as upper limits to not reach in future studies.

Conclusion

Targeted and temporary opening of the BNB is possible in rodent sciatic nerve using a 476.5 kHz FUS frequency with MB. The finding of a minimal threshold sonication pressure of 0.3 MPa coupled with 40 μl kg⁻¹ MB dosing was required for reliable BNB disruption. These results will allow future experiments, including evaluating the possibility of spatially precise and targeted drug or viral vector delivery to the PNS.
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Ethical statement

All animal work and handling were carried under an approved protocol in accordance with the guidelines and regulations of the University of Calgary’s Animal Care Committee Protocol.

Author disclosures

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ORCID iDs

Daniel Umansky  https://orcid.org/0000-0001-5274-6793
Saud Alzahrani  https://orcid.org/0000-0003-4853-3271
Samuel Pichardo  https://orcid.org/0000-0002-7919-8587

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