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Pulsed ultrasound expands the extracellular and perivascular spaces of the brain

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Abstract

Diffusion within the extracellular and perivascular spaces of the brain plays an important role in biological processes, therapeutic delivery, and clearance mechanisms within the central nervous system. Recently, ultrasound has been used to enhance the dispersion of locally administered molecules and particles within the brain, but ultrasound-mediated effects on the brain parenchyma remain poorly understood. We combined an electron microscopy-based ultrastructural analysis with high-resolution tracking of non-adhesive nanoparticles in order to probe changes in the extracellular and perivascular spaces of the brain following a non-destructive pulsed ultrasound regimen known to alter diffusivity in other tissues. Freshly obtained rat brain neocortical slices underwent sham treatment or pulsed, low intensity ultrasound for 5 minutes at 1 MHz.

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Transmission electron microscopy revealed intact cells and blood vessels and evidence of enlarged spaces, particularly adjacent to blood vessels, in ultrasound-treated brain slices. Additionally, ultrasound significantly increased the diffusion rate of 100 nm, 200 nm, and 500 nm nanoparticles that were injected into the brain slices, while 2000 nm particles were unaffected. In ultrasound-treated slices, 91.6% of the 100 nm particles, 20.7% of the 200 nm particles, 13.8% of the 500 nm particles, and 0% of the 2000 nm particles exhibited diffusive motion. Thus, pulsed ultrasound can have meaningful structural effects on the brain extracellular and perivascular spaces without evidence of tissue disruption. Keywords: Ultrasound, Extracellular space, Nanoparticle, Diffusion

1. Introduction

Approximately one-fifth of the total volume of the brain consists of an anisotropic, electrostatically charged extracellular space (ECS) that interconnects the cellular components of the central nervous system (CNS) through a highly tortuous network of fluid-filled pores. Transmission electron microscopy (TEM) studies of the brain ECS were initially skewed by tissue ischemia and processing artifacts, resulting in inaccurate measurements of the diameter of the ECS (d_{ECS}). *in vivo* imaging has therefore been used to measure the spread of ions, fluorescent molecules, and particles within the ECS. Applying restricted diffusion theory (also known as the hydrodynamic theory for hindered diffusion) to the measured diffusion data initially predicted a brain ECS width of 38–64 nm (Thorne and Nicholson, 2006). More recently, real-time high-resolution microscopy of non-adhesive nanoparticles revealed many of the pores in the brain ECS to be > 100 nm in width (Nance et al., 2012; Schneider et al., 2015). A distinct component of the ECS with larger diameters (5–10 μ m or larger) has also been identified in perivascular regions (Wolak and Thorne, 2013). The perivascular space (PVS) is a key component of the glial-lymphatic system (GLS), which channels the clearance of interstitial solutes, particles, and waste products from the brain through the exchange of cerebrospinal fluid (CSF) and interstitial fluid (Foley et al., 2012; Iliff et al., 2012; Iliff et al., 2013; Rennels et al., 1985).

The movement of physiologic, therapeutic, and pathological entities within the brain is mediated in part by diffusion within the brain ECS, as well as by bulk flow through interstitial and paravascular spaces. The structure and dynamics of the brain ECS and PVS therefore have broad implications for numerous processes, including embryogenesis (Thorne et al., 2004), neuronal signaling (Coggan et al., 2005; Matsui et al., 2005; Vargova and Sykova, 2008), drug delivery (Nance et al., 2012; Wolak and Thorne, 2013), and neurodegeneration (Kyrtos and Baras, 2015). In particular, the brain ECS affects the dispersion and clearance of therapeutic agents that have crossed or bypassed the blood-brain barrier (BBB). Additionally, the impaired clearance of substances through the GLS has been implicated in aging (Kress et al., 2014), Alzheimer's disease (Hawkes et al., 2012; Iliff et al., 2012; Kyrtos and Baras, 2015; Tarasoff-Conway et al., 2015), traumatic brain injury (Iliff et al., 2014), and neurodegenerative disease (Mendelsohn and Larrick, 2013).

Strategies to modify the structure of the brain ECS have included the use of enzymes to degrade the extracellular matrix (Neeves et al., 2007) and hyperosmotic solutions to dilate the ECS (Kume-Kick et al., 2002; Neeves et al., 2007) in an effort to optimize the local

delivery of therapeutic agents in the brain. Recently, ultrasound has also been used to increase the dispersion of locally delivered dyes and tracers (Lewis et al., 2012; Liu et al., 2010; Mano et al., 2015; Olbricht et al., 2013). Ultrasound can be applied with varying parameters (frequency, duration, power, focused vs. non-focused, continuous vs. pulsed exposures) to generate thermal, mechanical, or combined effects (Frenkel, 2008; Jolesz and McDannold, 2014). Pulsed ultrasound exposures with short duty cycles minimize heat generation and allow the non-thermal, mechanical effects of ultrasound to predominate. Notably, pulsed ultrasound has been used to modulate brain tissue in selected cortical and subcortical regions without evidence of neurological damage or significant histopathologic injury (Downs et al., 2015; McDannold et al., 2012). In addition, pulsed ultrasound can safely produce mechanical changes in the ECS of various tissues including fish epidermis (Frenkel et al., 2000b; Frenkel et al., 2001), murine flank muscle (Hancock et al., 2009), and squamous cell carcinoma xenografts (Ziadloo et al., 2013), resulting in increased tissue permeability. The structural changes in the ECS resulted in larger distributions of systemically and locally delivered nanoparticles, and enhanced the efficacy of a locally administered gene therapy in a solid tumor model (Ziadloo et al., 2013). Pulsed ultrasound exposures are thought to enlarge the interstitial spaces within the tissue through non-thermal mechanisms. We hypothesized that pulsed ultrasound would affect the brain interstitium through a similar mechanism.

Here, we studied the effects of pulsed ultrasound on the structure of the extracellular and perivascular spaces of the brain using TEM and high-resolution confocal microscopy. TEM was used to visualize the effects of ultrasound on the structure of the brain ECS and PVS. To further investigate the influence of ultrasound on the spaces within the brain interstitium, we performed high-resolution, real-time multiple particle tracking (MPT) of non-adhesive nanoparticle probes in freshly obtained rat brain slices. We observed that ultrasound can alter the ECS pore size as well as the PVS within the brain parenchyma, without evidence of tissue disruption.

2. Results

2.1. Effect of ultrasound on brain slice ultrastructure

Freshly obtained rat brain slices were sham- or ultrasound-treated using a custom-built chamber (Fig. 1). TEM analysis of the brain slices revealed an anisotropic, densely packed ECS in the cortices of the sham-treated slices, with enlarged spaces in the perivascular regions (Fig. 2A, C). In the ultrasound-treated slices, however, the enlarged regions were more extensive in size and number, occurring predominantly adjacent to blood vessels, and to a lesser degree within the parenchyma (Fig. 2B, D). Vestiges of membrane-delimited structures could be observed within the enlarged regions. Endothelial cell membranes, however, remained intact, as did the organelles in the adjacent regions.

Image processing was used to measure the ECS and PVS in each electron micrograph (Fig. 3). Sonication produced geographically heterogeneous effects, with some fields of view containing a larger percentage of extracellular and perivascular space than in sham-treated brain slices. On average, enlarged perivascular regions accounted for 3.7% of the field of view in ultrasound-treated slices vs. 1.5% in sham-treated slices. Enlarged extracellular

spaces within the brain parenchyma, on the other hand, accounted for 2.1% of the field of view in ultrasound- treated slices vs. 1.1% in sham-treated slices. A Wilcoxon rank sum test suggested that despite these trends, the differences in the perivascular regions and extracellular spaces between the two groups were not statistically significant ($U_{PVS} = 81$, $n_{\text{sham}} = 9$, $n_{\text{ultrasound}} = 21$, $p_{PVS} = 0.56$ and $U_{ECS} = 252.5$, $n_{\text{sham}} = 15$, $n_{\text{ultrasound}} = 49$, $p_{ECS} = 0.07$, respectively).

2.2. Nanoparticle diffusion and dispersion in brain slices

Non-adhesive nanoparticles of different sizes were used to probe the ECS in freshly obtained brain slices. The physicochemical properties of the nanoparticles are shown in Table 1. High- resolution MPT was used to analyze the trajectories and movement of individual particles that were injected in sham- or ultrasound-treated brain slices (Fig. 4A). Pretreatment with ultrasound significantly increased the MSDs of 100 nm, 200 nm, and 500 nm diameter particles by at least one order of magnitude (all corresponding *p-values* are < 0.0001). In contrast, the movement of 2000 nm nanoparticles was unaffected by ultrasound. Statistical analysis was performed using a linear mixed-effects model (Boisgontier and Cheval, 2016); the complete results are shown in Supplementary Table 1. An inverse relationship between nanoparticle size and movement following ultrasound was observed. In sham-treated brain slices, however, all nanoparticles > 200 nm in diameter demonstrated similar, non-diffusive movement.

The distribution of individual particle MSDs was examined at 1 s. In ultrasound-treated brain slices, 91.6% of the 100 nm particles exhibited diffusive motion (defined by MSDs larger than the particle diameter), demonstrating a second, distinct population relative to the same particles in sham-treated slices (Fig. 4B). Additionally, whereas none of the 200 nm or 500 nm particles were diffusive in sham-treated slices, 20.7% of the 200 nm particles and 13.8% of the 500 nm particles were diffusive in ultrasound-treated slices. However, 100% of the 2000 nm particle MSDs were immobilized or strongly hindered (defined by MSDs below the resolution of the microscope or smaller than the particle diameter, respectively) in both ultrasound-treated and sham-treated brain slices.

2.3. Brain slice viability following sonication

We used 2-3-2-triphenyltetrazolium chloride (TTC) staining and histological analysis to: 1) confirm the integrity of the brain slice cytoarchitecture during the timeframe in which the particle-tracking experiments were conducted, and 2) assess for indications of short-term structural damage in ultrasound-treated brain slices. Sham- and pulsed ultrasound-treated brain slices were stained red following TTC incubation, indicating cell viability as assessed by mitochondrial dehydrogenase activity (Fig. 5A and 5B). The brain slices exposed to continuous ultrasound, our positive control for tissue damage, remained white (Fig. 5C). Additionally, hematoxylin and eosin (H&E) staining of representative sham- and pulsed ultrasound-treated slices appeared similar, without immediate evidence of tissue disruption (Fig. 5D, E, G, H). In contrast, the positive control sample demonstrated large areas of disrupted tissue and pyknotic nuclei (Fig. 5F, I).

3. Discussion

In this study, we examined the structural effects of pulsed ultrasound within the brain parenchyma. Specifically, we investigated changes in the effective pore size within the brain ECS. Unexpectedly, we observed that many of these effects occurred adjacent to blood vessels, in the PVS. Using fluorescent nanoparticle probes, we tracked the movement of the particles in sham- or ultrasound-treated brain tissue to assess the steric limits of the ECS. Our results show that pulsed ultrasound produces heterogeneous effects within the brain parenchyma, generating enlarged ECS pores of various sizes, some as large as 500 nm, and many localized in perivascular regions. Importantly, these ultrasound exposures did not appear to cause tissue disruption within the brain slices. These findings demonstrate that pulsed ultrasound can expand the brain ECS and PVS.

To date, preclinical studies exploring the non-destructive structural effects of ultrasound on the brain have concentrated on reversible opening of the BBB for the targeted extravasation of systemically administered agents (Hynynen et al., 2005; McDannold et al., 2010; Treat et al., 2012; Wei et al., 2013). While BBB disruption offers one potential direction for the use of ultrasound in the brain, more recently ultrasound has been used to enhance the dispersion of locally delivered dyes (e.g. Evans blue dye), H-labeled molecules, and drugs (e.g. BCNU) within the brain parenchyma (Lewis et al., 2008; Lewis et al., 2012; Liu et al., 2010; Mano et al., 2015; Olbricht et al., 2013). In each case, ultrasound was applied concurrently with the infusion of the administered agents, and acoustic streaming likely played a major role by contributing to the convective flow of the infusion. This possibility is supported by a study in which the administration of microbubbles improved the volume of distribution of Evans blue dye (Olbricht et al., 2013). Microbubbles increase the attenuation of the ultrasound energy (Sokka et al., 2003) and lead to increased streaming, which has previously been shown to enhance convection-based mass transfer of both small and large particles (Frenkel et al., 2000a; Frenkel et al., 2001). In contrast with these earlier studies, we sought to isolate the effects of ultrasound on the brain parenchyma by applying ultrasound prior to injecting nanoparticle probes.

Through a TEM-based analysis of the brain parenchyma, we established that ultrasound has a structural effect on the extracellular and perivascular spaces of the brain. Due to the heterogeneous effect of ultrasound on the tissue and the variability within the sham- and ultrasound-treated groups, the effects of ultrasound did not reach statistical significance. However, due to the limitations of measuring d_{ECS} directly with TEM, we then used variably sized fluorescent particle probes to further study the properties of the brain ECS following sonication. This strategy has been used by others to fit measured diffusion data to Amsden's obstruction scaling model (Amsden, 1999) in order to estimate the effective pore size of the brain ECS (Nance et al., 2012; Nance et al., 2014). Real-time MPT revealed that nanoparticles with diameters > 200 nm were immobilized or strongly hindered in sham-treated brain slices, supporting prior evidence that while 28% of the pores in the brain ECS are larger than 100 nm, few are larger than 200 nm (Nance et al., 2012). In ultrasound-treated brain slices, however, nanoparticles as large as 500 nm exhibited significantly increased movement relative to the sham-treated group. Upon examining the diffusion rates of individual particles, we found that among 100 nm, 200 nm, and 500 nm particles, only a

subpopulation of nanoparticles demonstrated diffusive movement. With increasing particle size, progressively fewer particles were diffusive. One possible explanation for these findings is that the nanoparticles themselves were non-uniform, containing a subpopulation of “sticky” or adhesive particles. However, we found that the particle preparations had low polydispersity indices, indicating a homogenous particle population. An alternative explanation, supported by our TEM findings, is that ultrasound has a heterogeneous effect on the brain parenchyma, generating gaps of various sizes. At least some of the enlarged pores were observed to be greater than 500 nm in size, though smaller than 2 microns.

Our findings are supported by prior studies which used similar ultrasound exposures to safely and reversibly generate intercellular space widening in a variety of tissues outside of the CNS. Early work identified desmosome disruption following sonication in fish epidermis (Frenkel et al., 2000a; Frenkel et al., 2000b). These effects were subsequently found to enable greater penetration of electron-dense nanoparticles in the tissue, as well as 5- to 6-fold increases in the effective diffusion rates through the epidermal ECS (Frenkel et al., 2000a). These tissue effects have been attributed to the formation of a “bilayer sonophore” where ultrasound energy is absorbed and transformed into expansions and contractions of the intramembrane space. The dynamic stretch and release of the cell membrane and the cytoskeleton are induced by the cyclic variations in the ultrasound pressure field. The resulting deformations in the cell membrane may be accompanied by the activation of mechano-sensitive signaling cascades and/or increases in membrane permeability (Krasovitski et al., 2011). Similar ultrasound-induced interstitial effects have been identified in murine flank muscle and squamous cell carcinoma xenografts, as well. Pulsed ultrasound transiently disrupted the connective tissue linking the muscle fiber bundles, producing gaps identified on histology (Hancock et al., 2009). In ultrasound-treated squamous cell carcinoma xenografts, gaps between the tumors cells, sometimes microns in size, were detected by TEM (Ziadloo et al., 2013). Three dimensional intensity field simulations, as well as experimental acoustic radiation force impulse (ARFI) measurements and tissue displacement data, indicated that repetitive pulses of ultrasound generate non-uniform tissue displacements (Hancock et al., 2009). The resulting shear strain can mechanically disrupt the structures at cellular interfaces, such as the connective tissue joining muscle fiber bundles, or in our case, components of the brain extracellular matrix.

Alterations in the structure of the extracellular and perivascular spaces of the brain are likely to impact various processes. Currently, the tortuosity and narrow width of the brain ECS are key obstacles to drug penetration throughout the brain. Studies have shown that even substances that are characterized as minimally or non-adhesive must, on average, be less than 100 nm in size in order to diffuse within the ECS (Nance et al., 2012; Nance et al., 2014). This size limitation limits the drug payload and release kinetics of drug carriers, further reducing the likelihood of treatment efficacy (Langer, 1998). Our findings suggest that following pulsed ultrasound, relatively large particles could be delivered to broader regions of the brain parenchyma. In the context of drug delivery, however, this should be interpreted with caution, as simultaneous enlargement of the perivascular space, as we observed, may also enhance the clearance of large particles via the GLS (Foley et al., 2012). Conversely, in the setting of neurodegenerative disease, increased clearance via the GLS may have beneficial effects by reducing tau or amyloid- beta accumulation within the brain

(Iliff et al., 2014; Tarasoff-Conway et al., 2015). This was effectively demonstrated in a mouse model of Alzheimer's disease, in which transcranial ultrasound reduced the amyloid-beta plaque burden after a single treatment (Jordao et al., 2013).

Our results show that the application of ultrasound has a substantial effect on the structure of the brain parenchyma, specifically the extracellular and perivascular spaces. Transcranial ultrasound is gaining widespread use at the clinical level, and has been applied in patients for the creation of focal ablative lesions. A recently completed multi-center, randomized, placebo- controlled clinical trial studying its effects in Essential Tremor (NCT: NCT01827904), and ongoing trials in Parkinson's disease (NCT: NCT01772693, NCT02246374, NCT02263885, and NCT02347254) and chronic neuropathic pain (NCT: NCT01699477) are demonstrating the safety and efficacy of this approach. It should be noted that these clinical studies use much higher rates of energy deposition than we have used here. Recently, transcranial ultrasound was also used to open the BBB for the first time in a patient (NCT: NCT02343991). However, our findings show that ultrasound may not only affect the blood vessels in that region, but the extracellular and perivascular spaces as well. Our work supports further investigations into the impact of ultrasound-induced structural changes on diffusion, clearance, and physiologic processes of the CNS *in vivo*.

4. Experimental Procedures

4.1. Preparation and sonication of rat brain slices

All animal experiments were carried out using 6-8 week old Sprague Dawley rats (Envigo RMS, Indianapolis, IN) in accordance with local Institutional Animal Care and Use Committee approval. Twelve rats were used. Rat brain neocortical slices (3-4 slices per animal) were prepared as previously described (Nance et al., 2012; Schneider et al., 2015). Briefly, animals were anesthetized with isoflurane and euthanized via craniocervical dislocation followed by decapitation. Following euthanasia the brain was rapidly harvested and incubated for 10 min in chilled artificial CSF (aCSF; Tocris Bioscience, Bristol UK) supplemented with 10% glucose. The brain was subsequently sliced into 2 mm coronal sections with a brain matrix slicer (Zivic Instruments, Pittsburgh, PA). Brain slices were randomly assigned to sham or ultrasound treatment. Brain slices were maintained in aCSF on ice until selected for treatment, at which time they were sandwiched in 1% agar gels 2 mm thick and placed in a custom-built chamber filled with chilled phosphate-buffered saline (PBS) for 5 min. All brain slices were treated within 1 hour of harvesting.

All experiments used an Intellect Transport therapeutic ultrasound system (Encore Medical Tech., Los Angeles, CA). Brain slices in the “ultrasound” group were exposed to ultrasound for 5 min at a spatial average, temporal peak intensity (I_{SATP}) of 2 W cm⁻² and frequency of 1 MHz. Exposures were applied in pulses, using a duty cycle of 10% and a pulse repetition frequency of 1 Hz (100 ms ON / 900 ms OFF). Those in the “sham” (i.e. negative control) group were placed in the chamber for 5 min and exposed to an intensity of 0 W cm⁻². Preliminary experiments included a second negative control group that was not placed in the chamber, in order to reveal any potential handling artifacts. Results were similar to the sham group (data not shown) and therefore only sham-treated brain slices were used in further experiments. In the brain slice viability experiments, a “positive control” for assessing tissue

damage was obtained by exposing brain slices to continuous ultrasound for 30 min in the absence of agar gels at an I_{SATP} of 2 W cm⁻² and frequency of 1 MHz.

4.2. TEM analysis

Rat brain slices were prepared as described above. Sham- and ultrasound-treated brain slices (n = 3 per group) were immediately fixed with 2% paraformaldehyde, 2.5% glutaraldehyde, 2 mM CaCl₂, and 0.1 M PIPES buffer (pH 7.2) at 4°C overnight. Several 1 mm diameter biopsy punches were randomly collected from the cortex (n = 3 per brain slice), quenched with 50 mM glycine in 0.1 M PIPES buffer, washed and post-fixed with 1% osmium tetroxide and 0.25% potassium ferrocyanide in 0.1 M PIPES buffer for 1 hour at 4°C. After washing, the specimens were en-bloc stained with 1% uranyl acetate in water for 60 min at 4°C, washed, and dehydrated using increasingly concentrated ethanol (30%, 50%; 70%; 85%; 90% and 100%, for 10 min each). Specimens were stained with 1% phosphotungstic acid in 100% ethanol (ethanolic PTA) for 30 min, washed with 100% ethanol, then 100% acetone, and embedded in Durcupan resin (Electron Microscopy Sciences, Hatfield, PA, USA) following the manufacturer's recommendation. Ultrathin sections (~70 nm thick) were cut with a Leica UC6 ultramicrotome (Leica Microsystems, Inc., IL), collected onto copper grids and examined in a FEI Tecnai T12 TEM microscope (FEI, Hillsboro, OR) operated at 80 kV.

Digital images were acquired by using an AMT bottom mount CCD camera and AMT600 software. Sixty four fields of view were randomly selected and were analyzed by one of the authors (B.A.N.) who was blinded to the treatment status of the corresponding brain slice. The digital images were loaded into MATLAB R2016a (MathWorks, Natick, MA) using the `imread()` function. The Image Segmenter app was then used to identify regions of interest, defined as any visible gaps between cells. The “draw,” “flood fill,” and “fill holes” commands were used to generate a mask representing the PVS and parenchymal ECS within each TEM field of view. A custom script was used to calculate the area within the PVS and ECS for each field of view. The area was then normalized to the total area of the field of view.

4.3. Nanoparticle preparation and characterization

Non-adhesive nanoparticles were prepared as described previously (Nance et al., 2012; Nance et al., 2014; Schneider et al., 2015). Briefly, the surface of red fluorescent COOH-modified polystyrene (PS) nanoparticles (100 nm, 200 nm, 500 nm, and 2000 nm) were PEGylated using methoxy-PEG5k-amine (Creative PEGWorks, Winston Salem, NC) and a carboxyl-amine reaction. A Zetasizer NanoZS instrument (Malvern Instruments, Southborough, MA) was used to measure the hydrodynamic diameter, ζ potential, and polydispersity index (PDI) of the nanoparticles via dynamic light scattering and laser Doppler anemometry.

4.4. Analysis of nanoparticle diffusion in rat brain slices

Rat brain slices were exposed to sham or ultrasound treatments as described above. The brain slices were then placed in a custom microscope slide chamber and either 100 nm, 200 nm, 500 nm, or 2000 nm nanoparticles (0.5 μ L) were injected into the cortex via a Hamilton

syringe. Slices were incubated at room temperature for 10 min prior to undergoing imaging at a frame rate of 20 frames/s for a total of 400 frames (20 s) using an LSM5 Duo slit scanning confocal microscope (Zeiss, Thornwood, NY) with a 63x Plan-Apo/1.4 NA oil-immersion objective. For each brain slice, the injection site was identified and images were obtained of distinct regions that were 1-2 fields of view away from the injection site in each of 4 directions (medial, lateral, caudal, and rostral relative to the injection site). High-resolution MPT was performed using a custom written MATLAB tracking code described previously (Schneider et al., 2015; Schuster et al., 2013). Eight brain slices (4 sham-treated slices and 4 ultrasound-treated slices) were imaged per nanoparticle size, with at least 100 nanoparticles tracked per sample.

4.5. Tetrazolium staining and histopathological analysis

In order to assess the effect of ultrasound on rat brain slice viability, a tetrazolium-based assay was used as previously described (Joshi et al., 2004). Sham, pulsed ultrasound-treated, and continuous ultrasound-treated brain slices ($n = 3$ per group) were incubated in a 0.05% solution of TTC for 30 min. Brain slices from a second cohort of rats were preserved in formalin immediately following sham, pulsed ultrasound, or continuous ultrasound treatment ($n = 3$ per group). After 24 hours, the slices were transferred to a 70% ethanol solution until paraffin embedding, sectioning, and H&E staining. The brain slices were studied for histological evidence of damage by a neuropathologist who was blinded to the treatment status of the corresponding brain slice.

4.6. Statistical analysis

The Wilcoxon rank sum test was used to compare the percentage of PVS and ECS in the sham- and ultrasound-treated brain slices. A linear mixed-effects model was used to estimate and compare the average MSD for sham- and ultrasound-treated groups over time (Boisgontier and Cheval, 2016). MSD values were log transformed (base 10) in order to ensure an approximate normality. The model included the fixed effects of nanoparticle size, sham vs. ultrasound treatment, time, individual brain slice, and their interactions. As there was no effect of the individual brain slice on MSD, this factor was excluded from the fixed effects in the final model. Random effects of individual brain slice and treatment interactions were also included. The reported p -values correspond to the tests for overall difference between the average MSD for sham- and ultrasound-treated groups over time for each nanoparticle size. All tests were performed at the 0.05 level of significance. Statistical analyses were performed using JMP Pro 10.0.2 (SAS Institute Inc., Cary, NC) and SAS 9.4 (SAS Institute Inc., Cary, NC).

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References

- Amsden B. An obstruction-scaling model for diffusion in homogeneous hydrogels. *Macromolecules*. 1999; 32:874–879.
- Boisgontier MP, Cheval B. The anova to mixed model transition. *Neurosci Biobehav Rev*. 2016 Epub ahead of print.
- Coggan JS, et al. Evidence for ectopic neurotransmission at a neuronal synapse. *Science*. 2005; 309:446–51. [PubMed: 16020730]
- Downs ME, et al. Blood-brain barrier opening in behaving non-human primates via focused ultrasound with systemically administered microbubbles. *Sci Rep*. 2015; 5:15076. [PubMed: 26496829]
- Foley CP, et al. Real-time imaging of perivascular transport of nanoparticles during convection-enhanced delivery in the rat cortex. *Annals of biomedical engineering*. 2012; 40:292–303. [PubMed: 22009318]
- Frenkel V, Kimmel E, Iger Y. Ultrasound-facilitated transport of silver chloride (AgCl) particles in fish skin. *Journal of controlled release : official journal of the Controlled Release Society*. 2000a; 68:251–61. [PubMed: 10925133]
- Frenkel V, Kimmel E, Iger Y. Ultrasound-induced intercellular space widening in fish epidermis. *Ultrasound in medicine & biology*. 2000b; 26:473–80. [PubMed: 10773379]
- Frenkel V, et al. Preliminary investigations of ultrasound induced acoustic streaming using particle image velocimetry. *Ultrasonics*. 2001; 39:153–6. [PubMed: 11349995]
- Frenkel V. Ultrasound mediated delivery of drugs and genes to solid tumors. *Adv Drug Deliv Rev*. 2008; 60:1193–208. [PubMed: 18474406]
- Hancock HA, et al. Investigations into pulsed high-intensity focused ultrasound- enhanced delivery: preliminary evidence for a novel mechanism. *Ultrasound in medicine & biology*. 2009; 35:1722–36. [PubMed: 19616368]
- Hawkes CA, et al. Disruption of arterial perivascular drainage of amyloid-beta from the brains of mice expressing the human APOE epsilon4 allele. *PLoS One*. 2012; 7:e41636. [PubMed: 22848551]
- Hynynen K, et al. Local and reversible blood-brain barrier disruption by noninvasive focused ultrasound at frequencies suitable for trans-skull sonications. *Neuroimage*. 2005; 24:12–20. [PubMed: 15588592]
- Iliff JJ, et al. A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta. *Science translational medicine*. 2012; 4:147r. a111.
- Iliff JJ, et al. Brain-wide pathway for waste clearance captured by contrast-enhanced MRI. *The Journal of clinical investigation*. 2013; 123:1299–309. [PubMed: 23434588]
- Iliff JJ, et al. Impairment of glymphatic pathway function promotes tau pathology after traumatic brain injury. *J Neurosci*. 2014; 34:16180–93. [PubMed: 25471560]
- Jolesz FA, McDannold NJ. Magnetic resonance-guided focused ultrasound: a new technology for clinical neurosciences. *Neurol Clin*. 2014; 32:253–69. [PubMed: 24287394]
- Jordao JF, et al. Amyloid-beta plaque reduction, endogenous antibody delivery and glial activation by brain-targeted, transcranial focused ultrasound. *Exp Neurol*. 2013; 248:16–29. [PubMed: 23707300]
- Joshi CN, Jain SK, Murthy PS. An optimized triphenyltetrazolium chloride method for identification of cerebral infarcts. *Brain research Brain research protocols*. 2004; 13:11–7. [PubMed: 15063836]
- Krasovitski B, et al. Intramembrane cavitation as a unifying mechanism for ultrasound- induced bioeffects. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:3258–63. [PubMed: 21300891]

- Kress BT, et al. Impairment of paravascular clearance pathways in the aging brain. *Ann Neurol*. 2014; 76:845–61. [PubMed: 25204284]
- Kume-Kick J, et al. Independence of extracellular tortuosity and volume fraction during osmotic challenge in rat neocortex. *The Journal of physiology*. 2002; 542:515–27. [PubMed: 12122149]
- Kyrtos CR, Baras JS. Modeling the role of the glymphatic pathway and cerebral blood vessel properties in Alzheimer's Disease pathogenesis. *PLoS One*. 2015; 10:e0139574. [PubMed: 26448331]
- Langer R. Drug delivery and targeting. *Nature*. 1998; 392:5–10. [PubMed: 9579855]
- Lewis GK Jr, Olbricht WL, Lewis GK Sr. Acoustically enhanced Evans blue dye perfusion in neurological tissues. *The Journal of the Acoustical Society of America*. 2008; 2:20001–200017. [PubMed: 21197390]
- Lewis GK Jr, et al. Ultrasound-assisted convection-enhanced delivery to the brain in vivo with a novel transducer cannula assembly: laboratory investigation. *Journal of neurosurgery*. 2012; 117:1128–40. [PubMed: 22998056]
- Liu Y, et al. Ultrasound-enhanced drug transport and distribution in the brain. *AAPS PharmSciTech*. 2010; 11:1005–17. [PubMed: 20532711]
- Mano Y, et al. Intraparenchymal ultrasound application and improved distribution of infusate with convection-enhanced delivery in rodent and nonhuman primate brain. *J Neurosurg*. 2015:1–11.
- Matsui K, Jahr CE, Rubio ME. High-concentration rapid transients of glutamate mediate neural-glia communication via ectopic release. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2005; 25:7538–47. [PubMed: 16107641]
- McDannold N, et al. Transcranial magnetic resonance imaging- guided focused ultrasound surgery of brain tumors: initial findings in 3 patients. *Neurosurgery*. 2010; 66:323–32. discussion 332. [PubMed: 20087132]
- McDannold N, et al. Temporary disruption of the blood-brain barrier by use of ultrasound and microbubbles: safety and efficacy evaluation in rhesus macaques. *Cancer Res*. 2012; 72:3652–63. [PubMed: 22552291]
- Mendelsohn AR, Larrick JW. Sleep facilitates clearance of metabolites from the brain: glymphatic function in aging and neurodegenerative diseases. *Rejuvenation Res*. 2013; 16:518–23. [PubMed: 24199995]
- Nance EA, et al. A dense poly(ethylene glycol) coating improves penetration of large polymeric nanoparticles within brain tissue. *Science translational medicine*. 2012; 4:149ra119.
- Nance EA, et al. Non-invasive delivery of stealth, brain-penetrating nanoparticles across the blood-brain barrier using MRI-guided focused ultrasound. *Journal of controlled release : official journal of the Controlled Release Society*. 2014; 189:123–32. [PubMed: 24979210]
- Neeves KB, et al. Dilation and degradation of the brain extracellular matrix enhances penetration of infused polymer nanoparticles. *Brain research*. 2007; 1180:121–32. [PubMed: 17920047]
- Olbricht W, et al. Time-reversal acoustics and ultrasound-assisted convection-enhanced drug delivery to the brain. *The Journal of the Acoustical Society of America*. 2013; 134:1569–75. [PubMed: 23927197]
- Rennels ML, et al. Evidence for a 'paravascular' fluid circulation in the mammalian central nervous system, provided by the rapid distribution of tracer protein throughout the brain from the subarachnoid space. *Brain Res*. 1985; 326:47–63. [PubMed: 3971148]
- Schneider CS, et al. Minimizing the non-specific binding of nanoparticles to the brain enables active targeting of Fn14-positive glioblastoma cells. *Biomaterials*. 2015; 42:42–51. [PubMed: 25542792]
- Schuster BS, et al. Nanoparticle diffusion in respiratory mucus from humans without lung disease. *Biomaterials*. 2013; 34:3439–46. [PubMed: 23384790]
- Sokka SD, King R, Hynynen K. MRI-guided gas bubble enhanced ultrasound heating in in vivo rabbit thigh. *Phys Med Biol*. 2003; 48:223–41. [PubMed: 12587906]
- Tarasoff-Conway JM, et al. Clearance systems in the brain-implications for Alzheimer disease. *Nat Rev Neurol*. 2015; 11:457–70. [PubMed: 26195256]
- Thorne RG, Hrabetova S, Nicholson C. Diffusion of epidermal growth factor in rat brain extracellular space measured by integrative optical imaging. *Journal of neurophysiology*. 2004; 92:3471–81. [PubMed: 15269225]

- Thorne RG, Nicholson C. In vivo diffusion analysis with quantum dots and dextrans predicts the width of brain extracellular space. *Proceedings of the National Academy of Sciences of the United States of America*. 2006; 103:5567–72. [PubMed: 16567637]
- Treat LH, et al. Improved anti-tumor effect of liposomal doxorubicin after targeted blood-brain barrier disruption by MRI-guided focused ultrasound in rat glioma. *Ultrasound Med Biol*. 2012; 38:1716–25. [PubMed: 22818878]
- Vargova L, Sykova E. Extracellular space diffusion and extrasynaptic transmission. *Physiological research / Academia Scientiarum Bohemoslovaca*. 2008; 57(3):S89–99.
- Wei KC, et al. Focused ultrasound-induced blood-brain barrier opening to enhance temozolomide delivery for glioblastoma treatment: a preclinical study. *PLoS One*. 2013; 8:e58995. [PubMed: 23527068]
- Wolak DJ, Thorne RG. Diffusion of macromolecules in the brain: implications for drug delivery. *Mol Pharm*. 2013; 10:1492–504. [PubMed: 23298378]
- Ziadloo A, Xie J, Frenkel V. Pulsed focused ultrasound exposures enhance locally administered gene therapy in a murine solid tumor model. *The Journal of the Acoustical Society of America*. 2013; 133:1827–34. [PubMed: 23464051]

Abbreviations

aCSF	artificial cerebrospinal fluid
BBB	blood-brain barrier
CSF	cerebrospinal fluid
CNS	central nervous system
D*	effective diffusion coefficient, d_{ECS} , diameter of the extracellular space
ECS	extracellular space
GLS	glial-lymphatic system
I_{satp}	spatial average temporal peak intensity
MPT	multiple particle tracking
PBS	phosphate-buffered saline
PDI	polydispersity index
PEG	polyethylene glycPS, polystyrene
PVS	perivascular space
TEM	transmission electron microscopy
TTC	triphenyltetrazolium chloride

Highlights

- Pulsed ultrasound can enlarge the pore size of the brain extracellular space
- These ultrastructural changes are not associated with widespread tissue disruption
- The effects of ultrasound on the extracellular space are heterogeneous
- These effects may preferentially affect perivascular regions

Figure 1.

Experimental setup for sonication of *ex vivo* rat brain slices. (A) The brain slice was held in place by agar gels and placed in a custom built chamber. An acoustic permeable membrane, coupling gel, and agar gels were used to couple the transducer to the brain slice. (B) A schematic of the workflow during the particle-tracking experiments (aCSF, artificial cerebrospinal fluid). (Color is available online only.)

Figure 2.

Effect of pulsed ultrasound on rat brain slice ultrastructure. Representative transmission electron micrographs of (A, C) sham- and (B, D) ultrasound-treated brain slices are shown (magnification 3200x). Arrows indicate widening of the perivascular (B) and extracellular (D) spaces relative to those in sham-treated brain slices (arrowheads). A red blood cell (asterisk) is visualized within an intact capillary lumen, and adjacent mitochondria appear undamaged (scale bar = 1 μ m).

Figure 3.

Image processing and analysis of transmission electron micrographs. (A) A representative field of view obtained with the transmission electron microscope (magnification 3200x). (B) Image processing was performed to generate a mask depicting the perivascular (PVS, red) and extracellular (ECS, blue) spaces. (C) The percentage of the total area comprised of ECS or PVS in each field of view is represented by the individual ovals. A total of 64 fields of view were analyzed. The mean area and standard error are represented by the long and short horizontal bars, respectively. (Color is available online only.)

Figure 4.

Movement of nanoparticles in rat brain neocortical slices. (A) Ensemble-averaged geometric mean square displacements (MSD) as a function of time scale for 100 nm, 200 nm, 500 nm, and 2000 nm non-adhesive nanoparticles, with (dashed line) or without (solid line) brain slice exposure to ultrasound (2 W cm^{-2} for 5 min). (B) Distributions of individual particle MSDs expressed in logarithmic form for 100 nm, 200 nm, 500 nm, and 2000 nm non-adhesive nanoparticles, with (black) or without (white) brain slice exposure to ultrasound, at a time scale of 1 s. Data represent the average of 4 to 5 independent experiments per group, with $n > 100$ particles per experiment.

Figure 5.

Viability of rat brain slices following exposure to pulsed ultrasound. Representative images of rat brain slices incubated in TTC are shown following exposure to 0 W/cm for 5 min 9 9 (negative control) (A), 2 W/cm (pulsed) for 5 min (B), or 2 W/cm (continuous) for 30 min (positive control for tissue damage) (C). Red staining indicates viable tissue (scale bar = 2.5 mm). (D-E) Low magnification hematoxylin and eosin (H&E) stained cortical sections of the corresponding slice in A-C (scale bar = 600 μm). (G-I) Higher magnification views of the regions depicted by the white boxes in D-F (scale bar = 120 μm). (Color is available online only.)

Table 1
Physicochemical properties of non-adhesive polystyrene nanoparticles

Commercially supplied size (nm)	Particle diameter (nm) ^a	ζ-potential (mV) ^b	Polydispersity index
100	125 ± 1	-2.1 ± 0.2	0.02
200	206 ± 2	-5.4 ± 0.3	0.06
500	572 ± 4	-5.8 ± 0.3	0.03
2000	188638	-6.7 ± 0.2	0.06

^aDiameter (number mean) measured via dynamic light scattering. Data represents the average of 3 independent experiments ± standard error of the mean.

^bMeasured in 25°C in 15x diluted PBS with 10 mM NaCl, pH 7.4. Data represents the average of 3 independent experiments ± standard error of the mean.