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Research report

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 min at 1 MHz. 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.

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–

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; PVS, perivascular space; PDI, polydispersity index; PEG, polyethylene glycol; PS, polystyrene; PVS, perivascular space; TEM, transmission electron microscopy; TTC, triphenyltetrazolium chloride

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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 $\geq$100 nm in width (Nance et al., 2012; Schneider et al., 2015). A distinct component of the ECS with larger diameters (5–10 μm 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 (Kyrtsos 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; Kyrtsos 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 similar mechanisms.

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 indicated that despite these trends, the differences in the perivascular regions and extracellular spaces between the two groups were not statistically significant ($U_{PVS}=81$, $n_{sham}=9$, $n_{ultrasound}=21$, $p_{PVS}=0.56$ and $U_{ECS}=252.5$, $n_{sham}=15$, $n_{ultrasound}=49$, $p_{ECS}=0.07$, 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 mean square displacement (MSDs) of

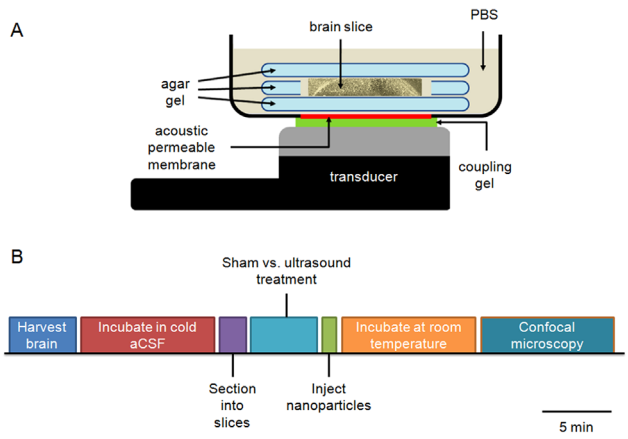

Fig. 1. Experimental setup for sonication of ex vivo rat brain slices. (A) The brain slice was held in place by agar gels and placed in a custom built chamber. An acoustic permeable membrane, coupling gel, and agar gels were used to couple the transducer to the brain slice. (B) A schematic of the workflow during the particle-tracking experiments (aCSF, artificial cerebrospinal fluid). (Color is available online only.).