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Applications of focused ultrasound-mediated blood-brain barrier opening

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Abstract

The blood brain barrier (BBB) plays a critically important role in the regulation of central nervous system (CNS) homeostasis, but also represents a major limitation to treatments of brain pathologies. In recent years, focused ultrasound (FUS) in conjunction with gas-filled microbubble contrast agents has emerged as a powerful tool for transiently and non-invasively disrupting the BBB in a targeted and image-guided manner, allowing for localized delivery of drugs, genes, or other therapeutic agents. Beyond the delivery of known therapeutics, FUS-mediated BBB opening also demonstrates the potential for use in neuromodulation and the stimulation of a range of cell- and tissue-level physiological responses that may prove beneficial in disease contexts. Clinical trials investigating the safety and efficacy of FUS-mediated BBB opening are well underway, and offer promising non-surgical approaches to treatment of devastating pathologies. This article reviews a range of pre-clinical and clinical studies demonstrating the tremendous potential of FUS to fundamentally change the paradigm of treatment for CNS diseases.

1. Introduction

The blood brain barrier (BBB) is the product of distinct physiologic characteristics of the microvasculature within the central nervous system (CNS) [1-5]. The defining features of the BBB include tight junctions that seal the extracellular space between adjacent endothelial cells (ECs), expression of efflux transporters on the luminal membrane of ECs, dense pericyte coverage, downregulation of transcytosis and transcytosis-associated proteins, and ensheathment of the capillaries by astrocyte end feet [1,3-10] (Fig. 1). Altogether, these features produce a dynamic and highly selective physiological barrier, which exists to

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maintain homeostasis of the neural microenvironment by tightly regulating bidirectional flux of blood-borne neuroactive solutes from the systemic circulation to the CNS and vice versa [1-3,5-7].

Movement of substances across the BBB occurs via several highly controlled mechanisms: paracellular transport, passive diffusion, solute carriers, and adsorptive or receptor mediated transcytosis [2,5]. These transport avenues serve to shield the CNS from potentially harmful endogenous and exogenous species, while simultaneously permitting entry of required metabolites. However, in the context of treatment of CNS disease, the BBB also poses a significant challenge to drug and gene delivery, as it blocks transport of 100 % of large and 98 % of small systemically administered neurotherapeutics [7]. Thus, many of the current treatment options for CNS diseases require invasive procedures that are often associated with substantial risks and long recovery times.

Focused ultrasound (FUS) applied in conjunction with intravenously administered micron-scale microbubbles (MBs) has emerged as an effective modality to non-invasively and reversibly circumvent the transport limitations of the BBB in a targeted and localized manner [10-14]. The technique was first pioneered by Hynynen et al. in 2001, where the combination of FUS and MBs was shown to transiently permeabilize the cerebral vasculature of rabbits [10-13,15]. Mechanistically, ultrasound is a sinusoidal pressure wave, and FUS is the convergence of many ultrasound waves to a small volume, producing an amplified effect [11]. At low acoustic pressures, at its focal point, FUS induces consistent volumetric oscillations of the gas-filled MBs, known as stable cavitation, within the capillary lumen (Fig. 1A). The alternating expansion and contraction of the MBs generates mechanical forces on the surrounding capillary walls [10,12,14,16,17]. The resultant stresses lead to the local permeabilization of the BBB through disruption of the tight junctions between ECs (Fig. 1D), sonoporation of ECs (Fig. 1C), and upregulation of transcytosis (Fig. 1B) [10,11,15]. These impacts are transient and typically reverse themselves within 24 h [10,11,15,18].

Increases in acoustic pressure are correlated with increases in BBB permeability. While this can be beneficial, high acoustic pressures also lead to larger volumetric oscillations of the MBs and eventual MB collapse [16,19]. This phenomenon, known as inertial cavitation, produces additional mechanical stresses on the capillaries in the form of micro-jets that can damage the surrounding capillary and brain parenchyma [14,16]. Under certain circumstances, such as the delivery of therapeutics in the size range of 500–2000 kDa or within a tumor, the increased permeability of inertial cavitation may outweigh its damaging consequences [14]. Regardless of the cavitation specifics, FUS and MBs have provided a method to safely permeabilize the BBB in a localized and transient manner, offering the possibility of noninvasive delivery of molecular species to targeted regions of the CNS [7,11,15,18] (Fig. 1C).

Henceforth, we will refer to FUS-mediated BBB opening with MBs as “BBBO”. While BBBO presents a new way to deliver therapeutics to the CNS, there are also several novel safety considerations, such as the need for accurate targeting, the risk of tissue heating, and the ability to monitor *in vivo* MB cavitation [11,15,18]. Minimizing off-target

application of FUS is commonly addressed by integrating high resolution three-dimensional magnetic resonance imaging (MRI) with the FUS system. This approach, denoted as magnetic resonance guided focused ultrasound (MRgFUS), ensures accurate placement of the focal spot at the intended treatment site [11,13,15]. An alternative approach is neuronavigation-guided FUS, whereby treatment planning on MR images is used to co-localize FUS delivery with a single-element transducer, aided by surface-attached fiducial markers [20,21]. Heating occurs from the temporal summing of energy deposited into the tissue by the ultrasonic waves [17]. This phenomenon has been exploited for procedures such as thermal ablation, where FUS is applied continuously to induce large temperature changes in the targeted tissue [15,17]. In the context of BBB opening, however, large temperature changes are undesirable due to the risk of causing damage to non-diseased neural tissue. Therefore, FUS is applied with lower pressure intensities and in a pulsed manner to reduce energy deposition while still allowing for MB modulation [11,15,17].

Lastly, MB cavitation must be monitored to ascertain whether stable or inertial cavitation is occurring. Traditionally, this is done by monitoring the acoustic emissions of the oscillating MBs in real-time. The presence of harmonics, subharmonics, or ultraharmonics of the original FUS wave in the acoustic emission profiles provides feedback about the *in vivo* behavior of the MBs [14-16]. Therefore, cavitation monitoring allows for real-time adjustments of MB activity and can be used to set FUS pressure thresholds to avoid inertial cavitation [14,16,17].

This review seeks to discuss the current applications of FUS and MB-mediated BBB opening (BBBO) and potential future applications. This includes the delivery of therapeutic agents to treat neurological disorders (Alzheimer's disease, Parkinson's disease, and glioblastoma), the secondary effects of BBB opening/permeabilization, pre-clinical neuroscience tools and a review of current clinical trials. In contrast to other reviews on similar topics, we take advantage of the Advanced Drug and Delivery Reviews format to provide a very comprehensive summary of clinical and preclinical studies within the field, as well as offering our forward-looking thoughts on emerging new applications and future possibilities for the field.

2. Pre-Clinical delivery of therapeutic agents

As described previously, the physiology of the brain causes many challenges to the delivery of therapeutic agents for neurological pathologies. Promising candidates such as genes, peptides, or other pharmacological agents are frequently stymied by the structure of the BBB. Some of these physiological challenges, which have slowed the progression of clinical options for CNS diseases, may be overcome with FUS-mediated BBBO [22-24]. These CNS diseases, including neurodegenerative conditions, primary and secondary brain tumors, and psychiatric disorders, affect many millions of people worldwide and place a tremendous strain on healthcare systems [25]. For these reasons, there have been many preclinical studies on the efficacy of FUS-mediated BBBO to facilitate the delivery of therapeutics in animal models of CNS diseases, some of which will be highlighted below. The molecular and cellular targets of the various therapies delivered are shown in Fig. 2. A summary of the FUS parameters for each study is provided in Table 1.

2.1. Alzheimer's disease

FUS-mediated BBBO has been used to deliver a wide variety of therapeutic agents (i.e. drugs, antibodies, nanoparticles, gene therapies) for the treatment of Alzheimer's Disease (AD) in preclinical studies. The studies to date have investigated the effect of FUS delivery of these therapeutics on amyloid and tau burden, behavioral impairments, inflammatory responses, and neuronal health. It should be noted that a multitude of studies have indicated that FUS-mediated BBBO alone (i.e. without therapeutic delivery) is sufficient to reduce plaque burden and rescue behavioral impairments; however, this phenomenon will be discussed in detail in the later section entitled "Pre-Clinical Secondary Effects of BBB Opening".

2.1.1. Amyloid beta—Several investigations have been conducted using FUS-mediated BBBO specifically in amyloidosis models of AD. Delivery of an inhibitor to glycogen synthase kinase 3 (GSK3), which is thought to be dysregulated in the neurons of the AD brain [26], has been investigated to reduce synthesis of amyloid- β plaque [27]. In this study, the GSK3 inhibitor AR-A014418 was delivered unilaterally to the hippocampus in APP^{swe}/PSEN1-de9 transgenic mice for 5 weekly treatments. Not only was GSK3 activity reduced by over 60 % following their treatment regime, but amyloid- β plaque was found to decrease significantly by over 30 % as well. FUS-mediated drug delivery in amyloidosis models has also sought to combat cholinergic neuron degeneration, which is a known consequence of AD contributing to decreased cognition. Xhima et al. explored FUS-mediated BBBO and delivery of the TrkA selective agonist D3 to rescue neurotrophin signaling in an amyloidosis model [28]. With a single treatment targeting the bilateral basal forebrain in TgCRND8 mice, the selective TrkA agonist enhanced cholinergic transmission and rescued neurotrophin signaling. The group went on to investigate 3 repeat treatments, performed weekly, targeting the bilateral forebrain, hippocampus, and cortex [29]. This treatment regime not only attenuated cholinergic degeneration but also reduced amyloid plaque burden and increased hippocampal neurogenesis.

Several studies have also investigated antibody delivery for amyloid- β reduction. One group investigated FUS-mediated delivery of intravenous immunoglobulin (IVIg) containing human polyclonal antibodies in TgCRND8 mice [30]. FUS + IVIg treatment targeted towards the bilateral dorsal hippocampus resulted in reduced amyloid plaque pathology, increased neurogenesis, and decreased TNF α in the hippocampus. Another study investigated delivery of an anti-pGlu3 amyloid- β monoclonal antibody, which targets the pathogenic form of amyloid- β [31]. In APP^{swe}/PS1dE9 mice, FUS was applied to the hippocampus for 3 weekly treatments, resulting in reduced hippocampal plaque burden, increased hippocampal synaptic proteins, and improved spatial learning and memory. Additionally, monocyte infiltration and recruitment to plaque was augmented with this treatment.

To our knowledge, just one group has utilized FUS-mediated BBBO to deliver nanoparticles for AD. This group used microbubble-embedded sulfur nanoparticles, which have been shown to reduce neurotoxicity and ROS production, modified with quercetin to increase stability [32]. Their delivery to APP^{swe}/PS1dE9 mice via FUS BBBO targeted to the

right hemisphere of the brain for 5 weekly treatments resulted in reduced amyloid- β deposition and neuron loss, as well as improved learning and memory. *In vitro* studies indicated that these nanoparticles reduced neuronal apoptosis, inflammatory response, calcium homeostasis imbalance, and oxidative stress.

FUS-mediated gene therapy has also been demonstrated in mouse models of amyloidosis. One study outlines a strategy for increasing transgene expression near amyloid plaques by using the astrocytic GFAP promoter to drive recombinant adeno-associated virus mosaic serotype 1/2 (rAAV1/2) expression of green fluorescent protein (GFP) [33]. Targeted delivery to the cortex and hippocampus in TgCRND8 mice augmented GFP expression near amyloid- β plaques, though expression was also significant in the liver. FUS-mediated delivery of newer generations of rAAVs in amyloidosis mice has also been explored. One study compared FUS-mediated delivery of the older generation rAAVs (rAAV9) to the newer generation (rAAV-PHP.B) [34]. The same dose was needed for both generations to achieve delivery. However, unlike rAAV9, FUS and MBs did not decrease the rAAV-PHP.B dosage needed to transduce brain cells in TgCRND8 mice. FUS-mediated delivery of both generations induced major histocompatibility complex class II expression, complement system and microglial activation, and T cell infiltration.

2.1.2. Tau protein—Fewer studies have been conducted in tau-based models of AD. Nevertheless, promising studies have been conducted with FUS-mediated antibody delivery regimens. Nisbet et al. investigated the delivery of a single chain antibody fragment against the 2 N tau isoform (RN2N) [35]. Scanning focused ultrasound (with MBs) over the entire forebrain in pR5 mice expressing 2N4R tau, in conjunction with RN2N delivery, increased uptake by neurons in the targeted brain region, reduced phosphorylated tau levels, and reduced anxiety-like behaviors. This group went on to investigate the fragment antigen-binding versus full-sized immunoglobulin format of RN2N with scanning focused ultrasound [36]. The study indicates that full-sized RN2N is better retained and has higher concentrations in the brain compared to the smaller formats; meanwhile, uptake and distribution remain similar between the two groups. Overall, several studies have now robustly indicated the utility of FUS BBBO to enhance local therapeutic delivery to the brain in AD animal models. A multitude of therapeutic agents are effectively delivered and demonstrate improved cognitive effects and reduced amyloid and tau burdens.

2.2. Parkinson's disease

Parkinson's disease (PD) pathology is largely marked by overexpression of alpha-synuclein (α -syn) and neuron degeneration leading to impaired motor function and memory [37,38]. Thus, many PD therapies focus on delivering molecules to repair these dysregulations.

2.2.1. Alpha-Synuclein— α -syn is a neuronal protein that is misfolded and overexpressed in PD. This overexpression and misfolding allows for permissive templating of neighboring, healthy α -syn, leading to spreading of diseased tissue to multiple brain regions [37]. Xhima et al. used FUS-mediated BBBO to deliver recombinant adeno-associated virus serotype 9 (AAV9) expressing human α -syn shRNA to silence α -syn expression in transgenic mice expressing wild-type human α -syn. The construct was

delivered to the hippocampus, substantia nigra (SN), olfactory bulbs, and dorsal motor nucleus of the vagus, all of which are brain regions in which α -syn aggregates. One month following treatment, α -syn expression, measured by α -syn immunoreactivity, was suppressed ~ 60 % across all brain regions [37]. However, behavioral changes post-treatment were not observed in this study. Another study performed by Yan et al. investigated the therapeutic impact of delivering curcumin, a neuroprotective agent that has been shown to inhibit and remove α -syn proteins [39]. An early stage MTPT PD model was exposed to FUS-mediated BBBO and curcumin treatments targeting deep-seated diseased brain regions [39]. Behavioral tests indicated improved neuromuscular coordination and motor abilities. While biological changes in α -syn expression were not observed, it can be postulated that the improved motor abilities result from a reduction of α -syn, given the anti- α -syn nature of curcumin [39], although further tests will need to be conducted to confirm this. This treatment regimen is a promising therapy for early-stage PD. However, most cases of PD are not diagnosed in the early stages due to late onset of symptoms [38]. It would be interesting to note the efficacy of this therapy on later-stage PD models.

In addition to using FUS for PD therapeutic developments, BBBO has also been used to create murine PD models more closely mimicking human PD pathology. Lin et al. used FUS to deliver α -syn plasmids to C57BL/6 mice resulting in increased α -syn expression, increased distribution of plasmid DNA *in vivo*, and human PD-like aggregation patterns of α -syn as compared to direct injection of the same plasmid [38]. Not only does this technique demonstrate the potential of BBBO to generate more accurate PD models, but it can also be used as a platform to enhance plasmid DNA delivery to brain tissue for therapeutic applications, such as knocking down α -syn protein expression or targeting other PD pathological implications.

2.2.2. Neurotrophic factors—Another component of PD pathology is loss of dopaminergic (DA) neurons and depletion of dopamine, which leads to motor activity degeneration [40-45]. Delivery of neurotrophic factors has been proven to elicit neuroprotective and neurotrophic effects [40,41,44,45], which may slow or reverse the effects of the disease.

The delivery of genetic material through viral vectors with BBBO provides strong and long-lasting transgene expression, minimal to no off-target effects, and a large reduction in therapeutic viral dose [46]. Karakatsani et al. used AAV1 to deliver glial-derived neurotrophic factor (GDNF), which can promote DA neuron regeneration and prevent disease progression [40,41,43,45,46], targeting the SN and striatum. This treatment resulted in increased optical densities of dopaminergic dendrites coupled with improved behavioral responses [46]. While the viral dose was significantly decreased with BBBO, viral dosage may be further reduced by engineering AAVs to possess cell-specific capsids or cell-specific gene promoters, limiting delivery to non-target organs [46].

Although efficient at delivering gene therapies to tissue, viral vectors can elicit an immune response depending on the frequency and dosage of administration [45]. To overcome challenges associated with viral vectors, several non-viral methods of delivering genetic materials have been attempted for PD therapies. Many groups have exploited the positive

charge of cationic MBs and negative charge of plasmid DNA to electrostatically load plasmids onto MBs to protect the DNA from enzyme-mediated degradation and enhance localization of the therapeutic to the treatment site [41,42,45]. GDNF plasmid (pGDNF)-MB complexes delivered to the SN and striatum of a 6-OHDA rat PD model with FUS BBBO resulted in enhanced transfection efficiency and duration as opposed to delivery of free plasmid DNA and MBs [41]. Furthermore, pGDNF-MB complexes displayed neuroprotective effects in PD, as evidenced by restoration of GDNF concentration, increased DA, and improved motor skills in rats [41].

Electrostatic interactions between DNA and cationic MBs can be transient, so alternative methods of conjugation between plasmids and MBs may ensure better colocalization at the FUS-targeted region [40,44]. Liposomes containing GDNF plasmids were complexed to MBs via biotin-avidin linkages and delivered to the SN of two different PD models after BBBO: an MPTP-induced murine model [44] and a 6-OHDA rat model [40]. In both models, DA levels increased after FUS-mediated treatment with the MB complexes, indicated by increased levels of GDNF, DAT (dopamine transporter) and TH-positive neurons (a marker for dopaminergic activity) [40,44]. Additionally, both models exhibited improvements in behavior and motor skills. While neither PD model exactly recapitulates human PD, it is promising that two different PD models respond to the therapy similarly.

Although the BBB markedly hinders the passage of therapeutics to brain tissue, once across the BBB, the transport of such therapeutics is further limited by the dense, negatively charged extracellular matrix (ECM) [43]. Recently, brain penetrating nanoparticles (BPNs), which are coated with a dense layer of neutral, hydrophilic PEG, and are less than 114 nm in size, have been shown to overcome the limitations of the ECM [43]. pGDNF encapsulating BPNs were delivered to the striatum of a 6-OHDA rat PD model after BBBO, yielding increased GDNF expression, increased DA levels, and increased TH-positive neurons up to 12 weeks post-treatment, indicating the regimen's neuroprotective abilities. In addition, motor skills were significantly improved in treated rats. While nanoparticle-facilitated delivery of therapeutics to the brain helps overcome therapeutic limitations resulting from the brain microenvironment, the nanoparticles can accumulate in off-target tissue, thus resulting in off-target effects [45]. To further improve gene delivery and enhance therapeutic efficacy of neurotrophic factors, nanoparticles can be formulated with magnetic materials to allow for magnetic navigation to target tissues in addition to BBBO with FUS [45].

As an alternative to manipulating neurotrophic factor expression with gene therapy, some groups used FUS-mediated BBBO to facilitate localized delivery of intranasally administered neurotrophic factor proteins [47,48]. Brain-derived neurotrophic factor (BDNF), a key regulator of neuron growth and function [47], was successfully delivered to the striatum and SN, marked by on-target BDNF accumulation [48] and upregulation of TH expression [47], indicating the therapy's potential to improve PD treatments.

While this section highlights therapies focusing on common PD targets, like α -syn and neurotrophic factors, other targets have also shown promising anti-PD results. One group delivered nuclear factor E2-related factor 2 (Nrf2) plasmids to the SN of a 6-OHDA rat PD model to regulate ROS, which play a role in PD [42]. Delivery of Nrf2 following BBBO

significantly improved ROS regulation and increased expression of DAT and TH, suggesting neuroprotection [42]. In summary, FUS-induced BBBO is a promising supplement to current PD treatments as it improves localized therapeutic accumulation and treatment efficacy.

2.3. Glioblastoma Multiforme (GBM)

Glioblastoma Multiforme (GBM) is the most commonly diagnosed primary brain neoplasm, often treated with surgical resection of the tumor and concurrent chemoradiation therapy (CRT). However, even with intervention, patients are faced with the grim survival expectancy of only ~ 15 months post-diagnosis [49]. The low survival outcome is attributed in part to the BBB, as it limits the delivery of therapeutics to tumors and their surrounding infiltrating rim. As such, infiltrating tumor cells that are undetectable via conventional imaging modalities and unresectable with surgery are protected from cytotoxic therapies, allowing for tumor recurrence. Within the tumor, the BBB is often heterogeneously disrupted due to aberrant angiogenic signals, creating a 'leaky' blood-tumor barrier (BTB) [49,50]. Despite the irregular leakiness of these blood vessels, delivery of therapeutics is limited and would benefit from FUS BBBO to create a more evenly permeable network of capillaries.

2.3.1. Chemotherapy—FUS is a safe, noninvasive tool that can potentiate targeted chemotherapy in pre-clinical animal models of GBM. It is worth noting that, due to inherent physiological differences between the tumor tissue and healthy brain, such as higher cerebral blood volume in high grade GBM and a baseline irregularity and leakiness of the tumor capillaries, effort should be taken to tune safe FUS parameters for GBM applications. Recently, studies investigating systemic carboplatin and etoposide administration with FUS BBBO have been conducted. In one study, BBBO increased carboplatin brain penetration 4.2-fold and significantly increased survival and growth control in U87 xenograft and patient derived (PDX) orthotopic GBM models [51]. In another, FUS BBBO enhanced etoposide penetration and therapeutic efficacy in an orthotopic MGPP3 (Pdgf+, Pten-/-, P53-/-) mouse model of GBM [52]. These results showcase the therapeutic promise of FUS-mediated BBBO in conjunction with chemotherapies to control tumor growth and increase survival; however, systemic administration of chemotherapy can cause adverse effects [53], and FUS-increased penetrance of cytotoxic agents into the brain causes concern for increased neurotoxicity [52]. Recent studies have aimed to address side effects and neurotoxicity concerns. One such study leveraged liposome-encapsulated TMZ (TMZ-lipo) with FUS BBBO in an orthotopic C6 GBM rat model to address the side effects caused by high dosage requirements of systemic temozolomide (TMZ) [54]. TMZ-lipo + FUS enhanced local therapeutic delivery and significantly increased survival and tumor growth control. Another study assessed distribution, toxicity, and efficacy of FUS-mediated delivery of two clinically approved formulations of paclitaxel (PTX), albumin-bound PTX (ABX) and PTX dissolved in cremophor (CrEL-PTX) [55]. ABX increased survival in a murine PDX model when compared to untreated control and CrEL-PTX treated mice. Further, while repeated FUS administration of ABX was well tolerated, ultrasound-mediated delivery of CrEL-PTX and CrEL alone were both lethal in > 35 % of mice treated. Together, these results emphasize that, while FUS is clearly able to enhance chemotherapeutic drug delivery to GBM, therapeutic formulation (and the clinical approval status of novel

drug formulations) and toxicities will remain a key consideration when developing novel chemotherapeutic strategies against GBM.

In addition to increasing the therapeutic delivery of packaged chemotherapies, FUS BBBO has been used to enhance the delivery of gene-bearing nanoparticles for gene therapy. FUS with MBs has been shown to modulate intratumoral interstitial fluid flow and convective transport within the tumor that results in enhanced nanoparticle dispersion and tumor cell transfection in murine orthotopic models of GBM and melanoma [56]. Additionally, MB-conjugated lipid-polymer hybrid nanoparticles loaded with CRISPR/Cas9 plasmids targeting the MGMT gene, which confers TMZ resistance, enhanced the efficacy of TMZ, inhibited tumor growth, and increased survival in a murine T98G xenograft model of GBM upon activation with FUS [57].

2.3.2. Radiotherapy—While FUS BBBO is widely used to increase chemotherapy delivery to GBM, there have been fewer pre-clinical studies on the use of FUS in conjunction with radiotherapy (RT). One study explored FUS BBBO to increase the penetration of sodium borocaptate and BSH to potentiate boron neutron capture therapy (BNCT)[58]. Their therapeutic strategy coupled self-assembled boron-containing polyanion nanoparticles with cationic microbubble (B-MB) mediated FUS treatment of orthotopic murine GL261 tumor bearing mice. When coupled with FUS, the B-MB group had increased boron uptake by the tumor directly after sonication, suggesting that B-MBs increased delivery efficiency. However, future studies are needed to optimize this strategy, as the tumor-to-blood boron ratio did not reach a therapeutic threshold. Another study stressed the need to consider the impact of prior therapy on FUS BBBO, as GBM patients will likely have had previous RT, chemotherapy, and surgery prior to FUS sonication [59]. White et al. assessed the impact of full brain 3 Gy irradiation for 10 days on trypan blue dye penetration into mouse brains treated with FUS alone or FUS with prior irradiation. FUS + prior irradiation resulted in increased trypan blue when compared to irradiation or FUS alone, suggesting that radiation may be a sensitizing factor for FUS BBBO. While the brains of irradiated mice had hemorrhagic petechiae, they were outside of the sonication region, indicating that they are likely a direct result of radiation therapy and not FUS BBBO [59]. Together, these studies demonstrate that there is much left to investigate in how FUS BBBO can be used to potentiate RT and how previous RT may impact the safety and efficacy of FUS BBBO.

2.3.3. Antibody delivery—In addition to chemotherapy, FUS has been widely used to increase the delivery of immunotherapeutics. A recent study aimed to address intratumoral delivery of an anti-CD47 (mCD47) monoclonal antibody, assessing both dosing strategy and therapeutic efficacy [60]. Zirconium 89 (^{89}Zr) labeled mCD47 was used to leverage positron emission tomography (immuno-PET) to non-invasively monitor mCD47 kinetics in an orthotopic GL261 murine GBM model. Two dosing strategies were used; IV administration directly prior to FUS BBBO (FUS-pre) or IV administration 15 min post-FUS BBBO (FUS-post). FUS-post administration significantly increased intratumoral antibody penetration and enhanced animal survival, suggesting that timing of administration is a key parameter to consider. Another study employed immuno-PET and contrast-enhanced

MRI to monitor the [^{89}Zr] anti-EphA2 receptor kinetics in a PDX WK1 xenograft model of non-enhancing high-grade glioma (HGG) [61]. This study investigated FUS BBBO in an infiltrating tumor model characterized by high tumor burden protected by intact BBB. FUS significantly increased antibody uptake in previously non-enhancing tumor regions. Under experimental conditions, FUS BBBO was not sufficient to increase antibody delivery across already-disrupted BTB but was able to improve delivery to infiltrating tumor cells. Both studies underscore the value of using MRI and PET imaging in pre-clinical FUS BBBO studies and punctuate the need to probe how therapeutic administration, dosing strategy, FUS parameters, and model impact therapeutic efficacy and antibody delivery.

2.3.4. Liquid biopsy—In addition to FUS BBBO for drug delivery, groups are evaluating how FUS BBBO opens “two-way trafficking” from blood to brain and vice versa [62]. Two recent studies from Zhu and colleagues underscore the promise of FUS-mediated liquid biopsy (FUS-LBx), by assessing release of eGFP mRNA from the tumor into the bloodstream at different FUS pressures [62,63]. Peak negative pressures ranging from 0.59 MPa to 1.58 MPa were sufficient to enable FUS-LBx detection of eGFP mRNA in the plasma. Another study investigated FUS-LBx to detect brain-specific biomarkers in a non-tumor bearing porcine model after FUS-LBx [64]. Both studies, however, have some limitations. For example, the first only detected eGFP, which is not natively expressed by tumor cells. Further, this study lacked investigation on how eGFP signal changes over time. Meanwhile, the second study did not aim to detect tumor or disease specific biomarkers. The addition of such elements to future studies will further support the promise of FUS-mediated liquid biopsy for GBM.

2.4. Other applications

As demonstrated in the studies summarized above, FUS-mediated BBBO shows tremendous promise in pre-clinical studies to facilitate delivery of known therapeutic agents, including antibodies, proteins, gene therapy constructs, and small molecule drugs, into specific diseased structures of the brain. While we have primarily focused on the areas of Alzheimer’s disease, Parkinson’s disease, and GBM, it should be noted that FUS for BBBO and drug delivery is an active area of pre-clinical research in other neurological conditions as well. For example, FUS has been used in animal studies of temporal lobe epilepsy to deliver a neurotoxin called quinolinic acid to specific regions of excess electrical activity, to offer a non-invasive “surgical” option to resolve seizures [65-67]. There is also significant interest in using FUS to help ease the symptoms of psychiatric disorders, including depression [68,69]. In addition to delivering small molecules and proteins, there is ongoing research investigating the use of FUS-mediated BBBO to deliver stem cells to the brain, for a variety of CNS applications [70-73]. As pre-clinical research into FUS-mediated BBBO for therapeutic delivery advances, so too does advancement in the safety of such approaches, including with the use of chemical agents like vasculotide to restore BBB function more quickly after FUS [74], and via acoustic cavitation monitoring to assess microbubble behavior in real time [14].

3. Pre-Clinical secondary effects of BBB opening

Beyond the increased delivery of therapeutic molecules facilitated by FUS-mediated BBBO, there is increasing evidence for several secondary effects of BBB disruption with FUS, including activation of inflammatory pathways, stimulation of neurogenesis and angiogenesis, increased clearance of amyloid beta plaques and tau protein tangles, and cell-specific activation. The timeline of these secondary effects is summarized in Fig. 3.

3.1. Neurogenesis and angiogenesis

3.1.1. Neurogenesis—Interesting data has supported a role for FUS-mediated BBBO in facilitating the development of new neurons, known as neurogenesis. There has been a major focus on inducing neurogenesis in the dentate gyrus (DG) of the hippocampus since this is the region of the brain involved in learning and memory [75]. If FUS could increase neurogenesis in the hippocampus of patients with Alzheimer's disease or other neurological diseases, this could represent a therapeutic strategy for slowing progression of the disease and improving cognition. Gene set enrichment analyses have suggested that neurogenesis, by way of extracellular matrix organization and regulation of ERK signaling cascades, is upregulated shortly after FUS BBBO [76]. In mouse models, FUS results in an increase in proliferation of neurons in the hippocampus as evidenced by increased BrdU staining in NeuN + cells [75]. Other cell types, including astrocytes, were unaffected by the treatment, suggesting that FUS may elicit neuron-specific growth responses in the hippocampus.

More recently, a study was conducted to elucidate the FUS parameters necessary to induce neurogenesis. Only when FUS was applied at 1 Hz burst repetition frequency in 10-ms bursts for 120-s at 0.78 MPa alongside MBs, did BrdU + NeuN + cells increase in the DG [77]. A pressure of 0.39 MPa with MBs, or 1.56–3 MPa without MBs, did not result in BBBO and therefore did not show increased neurogenesis in the hippocampus. Taken together, these data suggest that BBBO and thus exchange of components from the vascular environment are required for neurogenesis to occur.

Traditionally, cholinergic neurons release acetylcholinesterase (AChE) and choline acetyltransferase (ChAT) which aid in adult neurogenesis in the hippocampus. However, in Alzheimer's disease, these cholinergic neurons can be dysfunctional [78]. While FUS BBBO has been shown to increase adult neurogenesis in Alzheimer's models in which these cholinergic neurons are functional, the effect of FUS on neurogenesis in cholinergic-deficient models remained elusive. Cholinergic degeneration rat models were generated by administering a selective immunotoxin to induce lesions in the basal forebrain cholinergic neurons. Following FUS BBBO, AChE activity was increased significantly by 18 days post-treatment. BDNF protein expression, which is necessary for neurogenesis, also increased significantly as soon as 24 h post-treatment. Neuronal plasticity marker EGR1 was also upregulated in the cholinergic-deficient model following FUS BBBO treatment, while gliosis remained unaffected [78]. These data suggest that FUS with MBs may be an effective form of treatment for patients with dysfunctional cholinergic neurons in Alzheimer's by promoting neurogenesis.

3.1.2. Angiogenesis—While FUS-mediated BBBO transiently increases the permeability of the cerebral microvasculature, there is evidence that BBBO can remodel brain vasculature long-term via stimulation of angiogenesis. Gene set enrichment analyses following FUS show upregulated expression of genes associated with endothelial development, regulation of vasculature development, and regulation of angiogenesis [76]. In rat models using MRgFUS, the density of new endothelial cells increased, suggesting that FUS results in increased generation of new blood vessels within the brain [79]. Additionally, BBBO limits the neurovascular response by impairing vessel dilation for increased blood flow in the region of sonication, though the underlying mechanism of how this occurs still needs to be investigated [80]. Thus, while many effects of FUS BBBO for drug delivery are transient, long-term secondary effects on the brain vasculature may also exist.

3.2. Amyloid- β

3.2.1. Plaque reduction—The pathological hallmark of Alzheimer's disease (AD) is the presence of amyloid- β (A β) peptide aggregates that form amyloid plaques, which result from the inability to clear the peptide [81,82]. Recent treatment advances for AD make use of FUS BBBO [81]. In clinical AD cases, amyloid plaques are frequently found in small arteries, known as cerebral amyloid angiopathy [83]. Cerebral amyloid angiopathy results in reduced stability of the BBB because tight junctions are reduced. As a result, there exist concerns with using FUS BBBO in this pathology due to the risk of permanent vessel damage. To address these concerns, TgCRND8 mice were treated with MRgFUS and BBB dysfunction was evaluated for leakage probability using a fluorescently labeled dextran [83]. As acoustic pressure increased, as expected, the probability of BBB dysfunction increased as well. A peak negative pressure of 0.4 MPa in AD mice showed a 60 % probability of BBB dysfunction. While this study suggests that FUS does not worsen the dysfunction of amyloid-plaque containing vessels, future studies need to be conducted to establish if amyloid plaques impact the closure kinetics of the BBB[84,85].

Scanning ultrasound (SUS) with lipid-shelled MBs has proven effective in reducing A β plaques in APP23 A β -plaque forming transgenic mice [81]. However, in the absence of MBs, ultrasound alone is insufficient to clear A β deposits [86]. In TgCRND8 AD mice treated with FUS and commercially available MBs, A β plaques were reduced in size and surface area [87]. Data-driven approaches have also supported A β plaque clearance via gene set enrichment analyses [76].

3.2.2. Glial activation—In addition to plaque reduction, studies have shown that microglia can be activated as a result of BBBO in AD models. SUS-treated APP23 AD mice have shown increased A β present within the lysosomal compartment of CD68 positive microglia, suggesting that SUS is driving phagocytosis of A β by microglia and subsequent clearance of plaques [81]. 5xFAD mice treated with MBs and FUS for 1 h per day for 5 days show increased microglial activation that colocalized with A β relative to sham treated mice [88]. Iba1 staining marking microglia increased in TgCRND8 AD mice as soon as 4 h post-FUS [89]. Astrocytes, marked by GFAP, also increased starting 4 days post-treatment. Though staining increased, this was not a result of a greater number of Iba1 and GFAP-positive cells, but rather an increase in the volume and surface area of the cells

themselves. Confocal imaging revealed a significant increase in the internalization of A β peptide within microglia and astrocytes [89].

3.2.3. Improved cognition—Reduced plaque burden has been frequently shown to improve memory and cognition in AD. In the APP23 AD mouse model, FUS BBBO treatment led to improved spatial learning as evaluated by the active place avoidance (APA) test and the novel object recognition (NOR) test [81]. Even clearance of A β from the brain parenchyma into the CSF led to improved cognition in AD mouse models [90].

3.3. Tau

3.3.1. Clearance—Tau, a microtubule-associated protein, can get hyperphosphorylated at particular serine and threonine residues which can cause the normally unfolded highly soluble protein to form aggregates. While A β deposits are the primary pathology associated with Alzheimer's disease, tau pathology is also frequently detected. In Alzheimer's disease, tau forms what are known as neurofibrillary tangles (NFTs) within neurons. SUS-mediated BBBO of K3691 tau transgenic mice has shown promise in reducing the phosphorylation of tau proteins as well as NFTs [91]. Tau is traditionally cleared by the proteasome, though in its aggregated form, some studies have suggested that clearance is mediated by the autophagosome [91,92]. Increased LC3 and decreased mTOR activity supported the role of autophagy in the clearance of tau aggregates in the neurons of SUS-treated tau transgenic mice.

Increased phosphorylation of tau is correlated with impaired neuronal activity and memory. In rTg4510 mice, which depict early stages of tau pathology, FUS with MBs was able to reduce the levels of phosphorylated tau in the hippocampus relative to non-treated controls [93]. FUS BBBO had no effect on the density of NeuN + neuronal cells nor the density of neuronal processes, suggesting that the FUS regimen does not result in neuronal death or hyperproliferation. CD68 + activated immune cells were greatly increased following treatment as was colocalization of microglia with tau deposits.

3.3.2. Improved cognition—Following SUS-mediated BBBO of K3691 tau transgenic mice, reduction in NFTs within neurons also improved motor function. This was assessed with the Rotarod performance test, in which mice are placed on a rotating cylinder for as long as they can balance. Mice were significantly less likely to fall from the Rotarod and had better grip strength in both their fore and hindlegs [91]. Additionally, the K3691 mice treated with SUS showed improved spatial memory as evaluated by the Y-maze and general memory function according to the NOR test.

4. Pre-Clinical neuroscience tools

The tunability of FUS parameters and the ability to induce different bioeffects in conjunction with different co-administered agents has resulted in the development of a number of tools for neuroscience research at the pre-clinical level. These tools, including neuromodulation and sonogenetics, are summarized in Fig. 4.

4.1. Neuromodulation

Neuromodulation is the alteration or regulation of nerve activity via electrical or pharmacological agents. Many brain diseases affect neurotransmitter communication, thus neuromodulation via deep brain stimulation (DBS) and drug therapy are commonly employed. However, DBS is an invasive procedure and is limited by critical brain structures, and drug therapy can result in cytotoxicity from off-target effects [94]. FUS is a non-invasive alternative to neuromodulation. Herein, we discuss the use of neuromodulation using FUS for (1) BBBO to allow delivery of non-permeable pharmaceuticals and (2) spatiotemporal uncaging of brain permeable agents.

4.1.1. BBBO for impermeable agents—As described previously in Section 2, extensive pre-clinical studies have examined FUS-mediated BBBO to facilitate drug and gene delivery in a variety of disease contexts. This approach to non-invasive delivery can also be used to allow for delivery of otherwise impermeable agents that permit neuromodulation of specific brain regions. In one such study, FUS was targeted to the right hindlimb somatosensory cortex in conjunction with intravenous gas-filled microbubbles. After FUS BBBO, animals received a bolus of the inhibitory neurotransmitter GABA. This elicited a reduced response to electrical stimulation of the right hind paw (but not the left), with a greater degree of inhibition than was observed with FUS BBBO alone or GABA alone [95]. A similar study was conducted to deliver GABA across the BBB in non-human primates, but targeting the visual cortex. The combination of FUS-mediated BBBO and GABA injection resulted in a significant inhibition in neuronal response to a visual stimulus [96]. FUS-mediated BBBO has also been used to permit the delivery of intravenously-administered glutamate-loaded nanoclusters to a specific structure in the brain. These nanoclusters can then be later activated by FUS to release their glutamate payload, stimulating regional neuronal activation [97].

4.1.2. Uncaging for permeable agents—Another strategy is to use chemical “caging” of BBB-permeable agents to conceal their effects systemically (i.e. periphery and whole brain) and FUS-induced “uncaging” to reveal their effect only to the targeted brain region. One group has demonstrated this through the caging of the readily BBB-permeable anesthetic agent propofol. Through nanoemulsion formulation and loading of propofol, the molecule is only released upon FUS sonication which transforms the nanoemulsion’s liquid core to its gaseous state, effectively ejecting the anesthetic agent. This technique has been demonstrated useful for the silencing of seizures in a rat model [98] as well as non-invasive mapping of brain network connectivity [99]. This strategy can prove useful for a variety of applications where it is desirable for only select brain areas to receive BBB-permeable agents.

4.1.3. FUS BBBO alone—It has been noted that FUS-induced BBBO alone is sufficient to elicit neuromodulation and the intensity and duration of its effects correlate with the magnitude of BBB disruption [100]. Changes induced by FUS neuromodulation were analyzed by measuring somatosensory evoked potentials (SSEPs) and blood-oxygen-level-dependent (BOLD) responses, which are commonly measured neuromodulation parameters [96,100]. Importantly, effects from repetitive BBBO treatments did not compound,

suggesting the safety of repetitive treatments [100]. Although significant, one study found that BBBO-induced neuromodulation was found to be ~ 8.7 times less than effects from GABA inhibitor delivery [96]. Still, neuromodulatory effects resulting from BBBO should be a consideration in all treatment designs. Importantly, clinical trials are currently underway to test repetitive BBBO in humans indicating that BBBO-neuromodulation may be an option for near-future therapies [101].

4.2. Sonogenetics

Through the use of gas-filled MBs or vesicles, the mechanical bioeffects of FUS can be amplified for targeted, non-invasive modulation of neurons deep within the brain. An emerging and elegant technique for neuromodulation is sonogenetics, an approach deriving from opto- and chemogenetics, that targets specific neuronal populations through the use of sound waves activating genetically overexpressed mechanically or thermally sensitive ion channels. The first application of sonogenetic neuromodulation identified TRP-4 as a low-pressure ultrasound-sensitive subunit of a mechanosensitive ion channel. In a transgenic *Caenorhabditis elegans* model, FUS and MB activation of TRP-4 induced reversible behavioral outputs [102]. Other mechanosensitive channels have been identified in *in vitro* studies [17] and recent preliminary studies have highlighted the promise of sonogenetics in *in vivo* neuromodulation applications. One such study identified mPrestin, a transmembrane mechanosensitive protein, as a target to induce ultrasound sensitivity in an *in vivo* transgenic mouse model [103]. FUS stimulation (without MBs) of ventral tegmental area neurons overexpressing modified ultrasound-sensitive mPrestin induced a significant increase in c-Fos expression and calcium influx. Most preliminary studies leverage mechanical FUS; however, with recent advances in MR thermometry and image-guided FUS, additional sonogenetic platforms can be explored. One study developed sonothermogenetics using the thermosensitive ion channel TRPV1 to induce non-invasive FUS heating for neuron-specific neuromodulation *in vivo* [104]. Virally transfected TRPV1 neurons in the somatosensory cortex of wild-type mice were activated with FUS stimulation in the absence of MBs, while TRPV1-negative neurons were not—emphasizing the utility of sonothermogenetics to target specific deep brain neurons. Additionally, FUS stimulation induced locomotor behaviors consistent with previous studies using other established methods (i.e., optogenetics and chemogenetics) to activate similar brain regions. Going forward, future studies will have to be conducted to optimize and identify ultrasound-sensitive proteins and investigate their utility *in vivo*.

4.3. Acoustic control of biomolecular therapeutics

As the therapeutic ultrasound domain becomes increasingly interdisciplinary, approaches resting at the nexus of synthetic biology and FUS are showing increasing promise for the utility of FUS as a remote, localizable acoustic switch for activation of engineered materials and genetic circuits [105].

4.3.1. Liposomes—Liposomes have been explored as drug delivery vehicles across multiple facets of nanomedicine. However, they can be limited in their capacity to release payloads in a controlled manner [106,107]. Recent work has demonstrated that low intensity FUS (LIFU) can achieve protein-membrane disruption and pore formation, leading to efflux

of payloads. Specifically, acoustic intensity and duration of LIFU stimulation appear to impact protein-membrane channel disruption [107]. One study demonstrated the therapeutic potential of liposome stimulation via FUS mechanical disruption of the lipid bilayer. The liposomes generated had either NAK2K F92A potassium channel, KvAP potassium channel, mechanosensitive channel of large conductance (MscL), or no channel present. Encapsulated in the liposome was a self-quenching calcein dye, which limits fluorescent intensity when enclosed in the liposome. Through an *in-vitro* proteoliposome model, it was suggested that MscL, when stimulated with LIFU, can cause an increase in calcein efflux. This study also found that LIFU stimulation can even cause efflux of calcein with voltagegated channels (i.e. NaK2K F92A and KvAP) - suggesting that the mechanism underpinning this efflux is lipid bilayer perturbations rather than channel disruption [107].

4.3.2. Gene vectors—Many neurological and psychiatric pathologies are underscored by the dysfunction of neuronal networks. Acoustically targeted chemogenetics (ATAC) is an emerging neuromodulation technique that seeks to enable non-invasive spatial, cell-specific, and temporal control of neural networks via combination of FUS BBBO, adeno-associated virus (AAV) vector delivery, and engineered chemogenetic receptors [108-110]. In a transgenic Cre murine model, this strategy was effective in targeting and inhibiting neurons in the hippocampus. AAVs encoding chemogenetic designer receptor activated exclusively by designer drugs (DREADDs) were delivered across the BBB with FUS and MBs, resulting in the selective inhibition or excitement of neurons following introduction of clozapine-*N*-oxide (CNO). DREADDs are a commonly used mechanism that confer targeted neuronal control and are discriminately responsive to CNO. The behavioral impact of this approach was demonstrated via a fear conditioning protocol, wherein CNO-recipient mice saw a reduction in fear memory formation [108-111].

4.3.3. Engineered T cells—Genetically engineered T cells, such as chimeric antigen receptor (CAR)-expressing T cells, hold tremendous promise for cancer immunotherapy. However, off-tumor, on-target toxicities remain a critical challenge for these therapies [105,112-114]. It has been demonstrated that the remote acoustic control of engineered T cells with FUS may serve to curb these off-tumor toxicities by enabling localized control of thermally responsive T cells [112-115]. One recent *in vitro* study highlighting the development of genetically engineered thermally activatable T cells, through the incorporation of heat shock promoter (pHSP), showed that FUS-induced hyperthermia (up to 42°C) enabled regulation of key T cell functions, such as targeting of cancer cells through CAR recognition, as well as the production and efflux of cytokines [112]. A separate study extended this work to the *in vivo* setting, showcasing the ability of these acoustically responsive CAR-T cells to target solid tumors (Nalm6 leukemia and PC3 prostate cancer bilateral tumor models) with reduced non-specific off-tumor toxicity. Results demonstrated that FUS hyperthermia (43°C) enabled activation of CAR-T cells locally within the targeted tumor [113]. Akin to the aforementioned *in vitro* study, another also investigated exploiting Piezo1 ion channel through FUS as a mechanism for selective and modular activation of the CAR in engineered T cells. Specifically, this study utilized the Piezo1 ion channel as a mechanical sensor through which sonification could lead to transcriptional activation of anti-CD19 CAR expression, leading to recognition and eradication of tumor cells [114].

Through the advancement of acoustogenetic technology, CAR-T cells can be remotely controlled to exert their effects within confined target regions of solid tumors. This strategy will vastly aid in the reduction of broad off-target toxicities and further the advancement of this promising branch of immunotherapy. CAR-T cell delivery across the BBB with FUS is in its nascency, but we expect that future studies will aim to deliver acoustogenetically controllable CAR-T cells to brain tumors with FUS.

5. Clinical trials of FUS-Mediated BBB disruption

With rapid advances seen in the pre-clinical setting, more recent focus has been placed on translating FUS-mediated BBBO into the clinic. Given the brain's vital role in cognition, motor control and maintaining homeostasis, even a slight disturbance to its function can have devastating consequences on an individual's physical and mental wellbeing. Globally, between 1990 and 2019, neurological disorders have been associated with an increase of over 70 percent in disability-adjusted life years (DALYs) and more than double the proportion of deaths [25]. Moreover, by 2030, it is projected that neurological disorders will contribute to 103 million DALYs [116]. Therefore, non-invasive tools that can be precisely targeted to open the BBB, such as transcranial FUS, will accelerate new systemic treatment options for a range of neurological disorders.

5.1. Glioma and brain metastases

To date, the majority of clinical studies of FUS-mediated BBBO have been targeted to the treatment of malignant brain tumors, particularly glioblastoma (GBM). This group of diseases is ideal for study due to the availability of multiple active chemotherapeutic agents. It is known that malignant brain tumors alter the BBB as they infiltrate the brain, forming a blood-tumor barrier (BTB). Despite the loss of the protective BBB, the BTB has been shown to be heterogeneously permeable and still restricts drug ingress, leading to poor therapeutic efficacy [117,118].

One of the earliest published studies investigating FUS-mediated BTB disruption in recurrent GBM was performed using an implantable ultrasound device system, SonoCloud-1 (Carthera, France). In this dose escalating phase I/2a study, Carpentier et al. showed that repeated sonications of MBs at up to 1.1 MPa acoustic pressure could be applied safely in conjunction with a systemically administered cytotoxic chemotherapy without long-lasting adverse effects in any patient [119]. In their follow-up publication, Ibdaih et al. reported that dose-limiting toxicity was not reached with escalating sonication pressures and that a trend towards longer progression-free survival and overall survival was observed in sonicated patients who had clear BTB opening compared to those without BTB opening [120].

Mainprize et al. performed a window of opportunity study in five patients with confirmed or suspected high grade glioma. These patients received MBs and transcranial FUS delivered by a helmet-shaped transducer array, ExAblate Neuro (InSightec Tirat Carmel, Israel), coupled with real-time MR imaging guidance. Cytotoxic chemotherapy was systemically delivered concomitantly with MRgFUS-induced BTB opening, before tumor resection. Liquid chromatography-mass spectrometry analysis of peritumor tissue samples showed a trend towards elevated concentration in the "sonicated" versus "unsonicated" tissue

[121]. However, the small number of quantifiable samples and the use of a chemotherapy (Temozolomide) that is known to penetrate the BBB precluded any solid conclusions from being drawn on the extent to which FUS-mediated BTB disruption impacts on drug delivery to a brain tumor.

More recently, a study in patients with Her2-positive breast cancer brain metastases has provided more definitive evidence of the therapeutic potential of FUS-mediated BTB opening with MBs. In a pilot study, Meng et al. selectively targeted intracranial lesions with MRgFUS before systemic administration of an anti-Her2 monoclonal antibody, Trastuzumab [122]. Using radiolabeled Trastuzumab and single-photon emission computed tomography imaging, the study group showed that drug penetrance into the tumor was enhanced following MRgFUS activation of MBs, particularly at a delayed (48 h) timepoint. Moreover, volumetric assessment of the treated tumors demonstrated that they were stable or reduced on size on MRI follow up compared to baseline measurements [122].

Considerable efforts remain in establishing the parameters for safe FUS-mediated BTB disruption. Anastasiadis et al. showed, in a phase 0 clinical study, that the acoustic energy required for sonication can greatly vary between patients, highlighting the importance of individual patient monitoring and dose titration. Furthermore, they observed that radiological and histological changes associated with BTB opening were positively correlated with the MB acoustic emissions, or harmonic dose, opening the possibility of tailoring the treatment regimen according to the desired effect [123]. Since chemotherapy treatment of brain tumors typically depends on multi-dose regimens, it is vital that repeated FUS and MB application does not cause any major toxicities. To that end, Park et al. demonstrated that, in patients with resected GBM on adjuvant chemotherapy, repeated and multiple treatments with MRgFUS and MBs on a four week cycle led to transient BTB opening that was well-tolerated [124]. In their follow up study, Park et al. found that none of the enrolled patients suffered from lasting sequelae arising from BTB opening [101].

Although much of the focus on FUS-mediated BTB opening has been on drug delivery, other novel applications have also been tested. One exciting development has been the use of FUS-induced opening of the BTB to promote release of brain-derived biomarkers from GBM, providing proof-of-concept in its potential utility as an aid for liquid biopsy in neuro-oncology [125]. Another potential application in the field of brain tumors is to stimulate an anti-tumor immune response, which is particularly relevant with the growing interest in cancer immunotherapy. Chen et al. investigated whether a neuronavigation-guided single element transducer, NaviFUS (NaviFUS Inc), for FUS delivery with MBs to open the BTB in recurrent GBM patients might lead to an enhanced immunological response. Although it was found that transient BTB opening was induced in a dose-dependent manner, no histological changes were seen pointing to increased immune infiltration into the GBM or surrounding brain parenchyma [21]. Subsequent data in a rat model confirmed that increased tumor lymphocyte infiltration could be achieved with a mechanical index of 0.81, raising the possibility that this effect could be replicated in humans, albeit with higher acoustic energy deposition levels [21].

5.2. Alzheimer's disease

As previously discussed in Section 2, much excitement has been generated from pre-clinical models demonstrating the potential for MRgFUS-mediated BBBO for beta-amyloid clearance. In the first published study investigating this clinical effect in humans, Lipsman et al. conducted a phase I study in five patients with early to moderate AD. No significant reduction in A β was detected by [^{18}F]-Florbetaben positron emission tomography (FBB PET) following BBBO [126]. Nonetheless, this pilot study yielded important information regarding the safety of transient BBBO in amyloid-positive AD subjects with no associated serious adverse events or deleterious effects on cognitive function. Similarly, Rezai et al. showed that focal BBBO by MRgFUS targeted to the hippocampus and entorhinal cortex in patients with AD was safe [18]. In a follow up study, the same group showed that there was an average reduction in A β plaque of roughly 5 percent following FUS-mediated BBBO [87].

To assess the impact of focal BBBO on fluid flow in early AD patients, Mehta et al. conducted a prospective, phase II clinical study exploring multiple MR imaging parameters after FUS BBBO. Besides parenchymal enhancement seen at the site of FUS targeting post-gadolinium contrast, indicating selective BBBO, the authors also observed enhancement along the draining venous structures immediately after sonication and 24 h later, after radiological signs of BBBO had resolved. This observation has been interpreted as blood-meningeal barrier permeabilization as a side effect of BBBO, which may have an additional role in immune cell trafficking [127].

Since the neuropathological changes associated with AD can be extensive, Park et al. investigated whether large volume ($>20\text{ cm}^3$) FUS treatment could be delivered repeatedly in the brain. This proof-of-concept study demonstrated that extensive BBBO in the frontal lobe of patients, with AD and significant brain A β deposits, was well-tolerated. Secondly, it was found on FBB PET that there was a significant reduction in A β deposits at targeted regions post-sonication; however, there was no overall improvement in neurocognitive assessments within the timeframe of the study [82].

5.3. Parkinson's disease and amyotrophic lateral sclerosis

Though the bulk of published clinical studies has been targeted at brain tumors and AD, work is underway to establish FUS-mediated BBBO as a therapy across a much wider range of neurological disorders. In Parkinson's disease (PD), FUS and MBs have already been shown to be effective at managing the motor manifestations of PD through targeted thermal ablation of deep brain structures, such as the subthalamus [128]. In the context of BBBO, FUS has been tested in patients with evidence of PD-associated dementia. Gasca-Salas et al. showed that repeated transient BBBO in the right parieto-occipito-temporal cortex was safe, though there was no significant change in radiological signs of A β volume or metabolic function in the posterior cortex [129]. In another example, Abrahao et al. investigated the safety of FUS-induced BBBO in patients with amyotrophic lateral sclerosis. Uniquely, this study targeted sonication to the primary motor cortex using functional MRI mapping and demonstrated that BBBO could be safely applied with minimal side effects [130].

5.4. Conclusions

In conclusion, the recent stream of published clinical studies is a positive sign that FUS-mediated BBB disruption is becoming a viable treatment tool for patients with neurological disorders. Given the possibility of delivering drugs previously excluded by the BBB, FUS has the potential to dramatically alter the treatment options for these challenging diseases. Though meaningful conclusions regarding therapeutic efficacy cannot be answered due to the small group sizes in most published studies, they all point to FUS-mediated BBBO as being safe, with the capability to treat eloquent regions of the cortex, to deliver treatment over repeated sessions and over a large brain volume. Therefore, these trials provide strong justification for further study in larger cohorts of patients. Table 2 outlines all currently registered clinical trials that have been completed or are in progress.

A number of unresolved issues remain on the path to translating FUS-mediated BBBO into new clinical treatment paradigms. Since many neurological diseases follow a chronic or indolent course, consideration will need to be made regarding frequency of sonications, the total number that can be safely administered, and the long-term effects/risk of repeated BBB disruption. Continued close collaboration between the bench and bedside will be necessary to select rational drug candidates for combining with FUS-mediated BBBO. Several competing FUS systems have been described and it will be interesting to see whether a dominant system will emerge or if unique niches will develop dependent on clinical indication. Finally, it is evident that FUS is a versatile tool that can be safely titrated to achieve a range of effects on the BBB. Therefore, careful consideration of the desired outcomes according to the clinical indication and personalization of treatment protocols to the patient will be necessary in future studies.

6. Future approaches and indications of focused ultrasound in the CNS

Thus far, this review has covered previous studies that emphasize the use of FUS for modulation of BBB permeability. Most preclinical and clinical applications have centered on deploying this approach for neuromodulation and/or image-guided drug and gene delivery to treat many common CNS pathologies. In this final section, we take a more forward-looking stance, offering perspectives on how, in the future, focused ultrasound might be rationally tuned to (1) further augment therapeutic efficacy via modulation of interstitial fluid transport, (2) achieve transfection of the BBB itself as a tool for neuroscience discovery and therapy, and (3) treat pathologies of the CNS wherein FUS-mediated BBBO has not yet been considered an option, but could still be quite promising.

6.1. Modulation of interstitial and glymphatic fluid flow

The ability of pulsed ultrasonic energy to augment interstitial tumor pressure, leading to the enhanced delivery of therapeutics from the bloodstream via enhanced convective transport, is known and fairly well-studied. Recently, an excellent review of the literature in this space was provided by Keller and Averkiou [131], and we refer the interested reader to that article for more detailed information. Here, we submit that this literature, as well as other key sources, suggest that FUS modulation of interstitial fluid flow could be deployed for therapeutic purposes in the CNS, both with and without concomitant BBBO. Indeed,

consistent with generation of augmented interstitial fluid flow and studies performed in extracranial tumors, opening the BBB with FUS and MBs has been shown to enhance the dispersion of therapeutic agents through tissue [132-134]. In further support, a recent study used an MRI-based approach to demonstrate that BTB opening with FUS and MBs causes an approximate doubling of interstitial flow and marked alterations in fluid flow directions, which led to the enhanced dispersion of nanoparticles throughout the tumor [56]. These concepts have also been extended to consideration of changes in glymphatic transport due to FUS-mediated BBBO. Indeed, recent observations from clinical trials wherein the BBB was opened with FUS and MBs in Alzheimer's disease and ALS patients provide compelling evidence that, at least in some patients, enhanced glymphatic transport of contrast agent from the brain to subarachnoid spaces occurs [135].

In all, these studies raise the intriguing possibility of developing FUS sequences that, in conjunction with BBBO for enhanced therapeutic delivery, may be able to augment and/or guide the transport of delivered payloads through other mechanisms. For example, "pre-conditioning" rat brain with pulsed FUS in the absence of MBs before seeding of subsequent BBBO with i.v.-injected MBs has been shown to augment brain tissue transfection magnitude with non-viral nanoparticles [136]. Although the mechanism of this effect is unknown, it appears to be primarily mechanical in nature and is likely related to the aforementioned effect of pulsed FUS on interstitial tissue structure and/or hemodynamics. Moreover, broadly along these same lines, important new research has shown that the penetration of intrathecally-injected agents into the brain can be augmented by scanning [137] or pulsed [138] ultrasound (without MBs or BBBO). The exciting potential to modulate glymphatic flow with FUS opens the door to the development of immunotherapeutic approaches that control the exposure of immune cells in the meninges and draining lymphatics to antigen and/or delivered immune-stimulatory molecules. Indeed, there is emerging evidence that immune cells in the meninges do respond, albeit modestly, to gentle BTB opening with FUS [139], indicating that such communication exists. Going forward, we are hopeful that acquiring a more mechanistic understanding of fluid interactions between the FUS-treated brain, meninges, and lymphatics will lead to exciting new methods to control such responses.

6.2. Selective transfection of Blood-Brain barrier endothelium with focused ultrasound

Another exciting new direction for FUS, in the context of both neuroscience research and therapeutic development for CNS pathologies, is the targeted genetic modulation of the endothelial layer of the BBB [76,140]. In essence, it has been determined that, when relatively low peak-negative pressure FUS is applied to plasmid-bearing cationic MBs, gene transfer to the endothelium in the BBB may be achieved without opening the BBB and without the need for an endothelial cell-specific promoter (Fig. 5). In this so-called "sonoselective" low peak-negative pressure FUS regime, consistent acoustic harmonic emissions signatures are generated and may be leveraged for control of transfection in the future. Because the integrity of the BBB is retained during transfection, this approach could be especially useful for conditions wherein even the modest elicitation of sterile inflammation could be too detrimental. Further, given the flexibility inherent in this system, manipulations centered on genome editing the BBB, as well as those facilitating controlled

BBBO through chemogenetics (or similar approaches), may be operative. Indications potentially benefiting from (1) the recruitment of specific immune and/or progenitor cells from the circulation to FUS-targeted pathologies, (2) the expansion of the microvasculature via stimulation of angiogenesis, and/or (3) the long-term, spatially-controlled, modulation of BBB-function may all be germane to this sonoselective transfection strategy.

6.3. Potential future clinical applications

Understandably, most pre-clinical and clinical research to date in the field of FUS-mediated BBBO has revolved around a handful of pathological indications that are pervasive, generate high mortality and morbidity, and are generally agreed upon to have a sufficient safety profile for FUS-mediated BBBO. In particular, as previously highlighted in this review, investigations on brain tumors (primary and metastatic) and neurodegenerative diseases (Parkinson's disease, Alzheimer's disease, and ALS) have dominated the field to date. Nonetheless, given the great success and safety enjoyed by the field, as well as our improved understanding of the side effects of FUS-mediated BBBO, it may be time to consider the potential use of FUS-mediated BBBO for conditions that are either more challenging from a safety perspective and/or less common. We close this review by highlighting two such applications, ischemic stroke and cerebral cavernous malformations (CCMs), that we submit could benefit markedly from the development of FUS-based therapies, despite limited (ischemic stroke) or no (CCMs) progress to date with regard to the development of therapies deploying FUS-mediated BBB modulation.

Broadly speaking, FUS treatment options for ischemic stroke have been under investigation for some time; however, most approaches have centered on the use of FUS for clot dissolution (i.e. "sonothrombolysis") [141]. Relatively fewer studies have tested the use of FUS-mediated BBBO for therapeutic delivery. This is certainly understandable given the delicate state of ischemic neural tissue, as well as the likelihood of limited microbubble access due to poor perfusion. Nonetheless, a couple of preclinical studies offer promise for the use of FUS in treating stroke beyond sonothrombolysis. In one such investigation, the neuroprotective agent erythropoietin was delivered to the infarct zone of rats exposed to middle cerebral artery occlusion, eliciting reduced infarct volume and neuronal loss, as well as improved long-term behavior [142]. And, more recently, successful subcortical stroke treatment, as assessed by improved functional recovery and fiber tract connectivity, was achieved by delivering brain-derived neurotrophic factor (BDNF) to the brain with FUS and MBs [143]. Notably, despite the already inflamed tissue microenvironment, neither study reported significant adverse effects.

In a similar vein, we posit that CCMs could also represent an attractive application for drug and gene delivery via FUS-mediated BBB modulation. CCMs are hemorrhagic vascular lesions in the central nervous system, affecting ~ 0.5 % of the population [144-146]. CCM patients can experience weakness, numbness, severe headaches, vision changes, difficulty speaking and understanding, stroke, seizures, and paralysis [147], and in some patients, neurological issues progressively worsen due to recurrent hemorrhages [148]. The primary treatment for symptomatic CCM is surgical removal, which presents high risk. Notably, given that CCMs are characterized by endothelial cell proliferation and neuroinflammation,

many existing drug and gene therapies (i.e. anti-angiogenesis and anti-inflammatory agents) could be successfully deployed to control CCMs and their sequelae. Because CCMs present as discrete lesions on MRI, it is reasonable to hypothesize that they may be successfully targeted with FUS under MR image guidance. Furthermore, vascular access to the CCM microenvironment exists via surrounding microvasculature, the permeability of which could be enhanced via FUS and MBs to facilitate drug and gene delivery. Of course, given the altered endothelial cell phenotypes that are present in organisms harboring CCMs, BBB-opening FUS thresholds may be different than in brains without lesions. Additionally, excessive iron deposition within the lesions could result in altered acoustic properties and potentially increased heating upon application of FUS. For these reasons, careful safety studies would need to be performed, including MR thermometry. However, given the field's increasing refinement of acoustic emissions-based feedback control for BBBO with FUS, we are hopeful it may eventually be possible to safely control BBB opening in this challenging microenvironment to provide new treatment options for patients suffering from CCMs.

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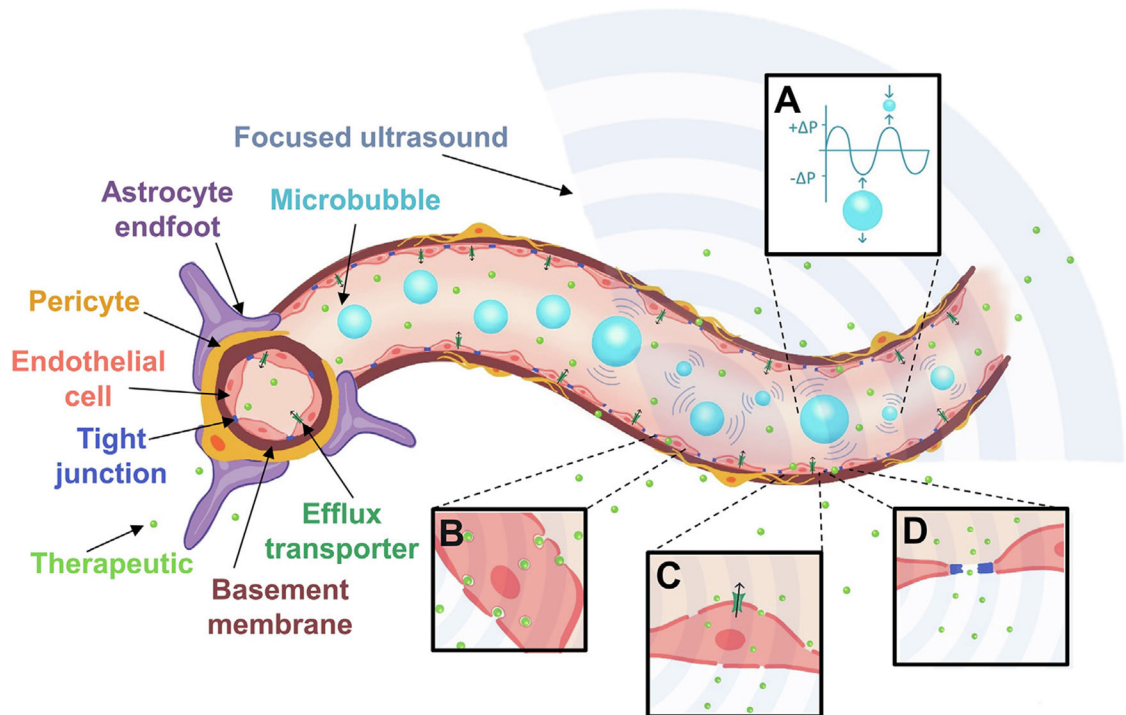


Fig. 1.

The physiology of the blood brain barrier, and the permeabilization of the blood brain barrier with microbubbles and focused ultrasound. (A) Focused ultrasound induced volumetric oscillations of microbubbles. (B) Transcytosis of a therapeutic through an endothelial cell. (C) Sonoporation of an endothelial cell. (D) Disruption of tight junctions between adjacent endothelial cells.

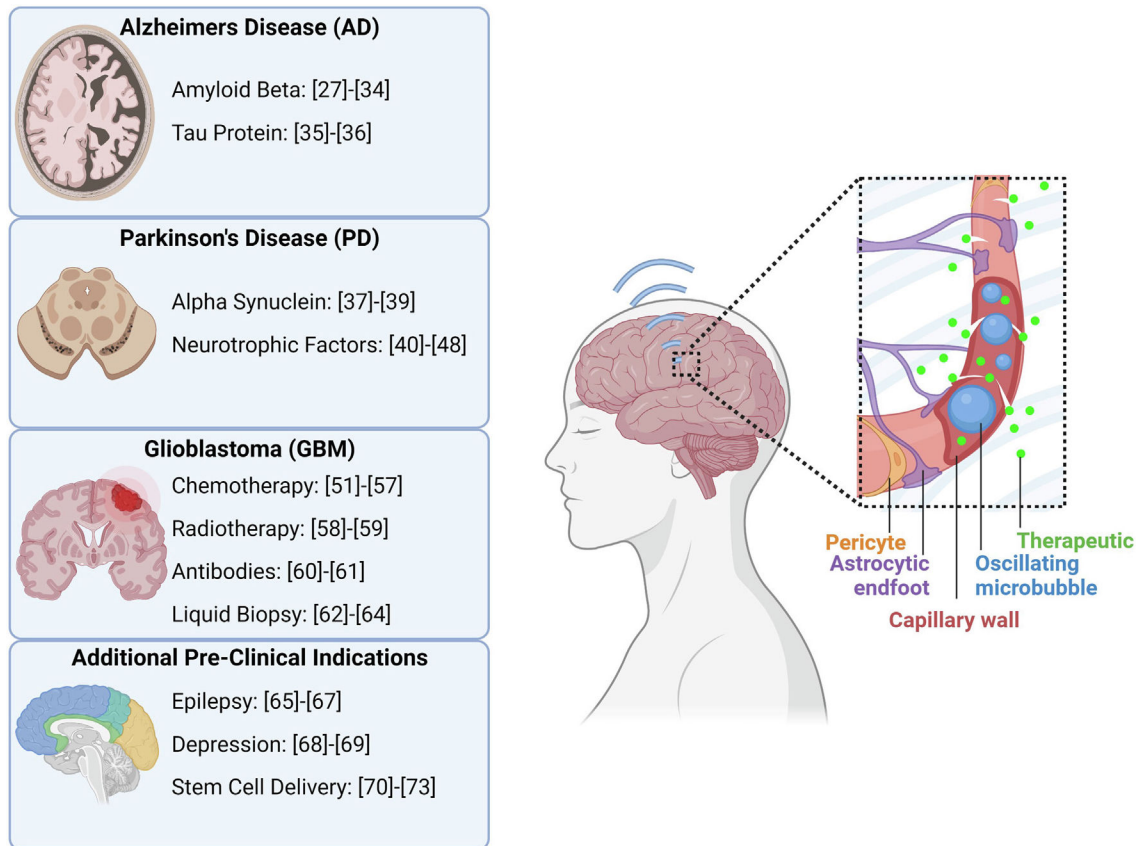
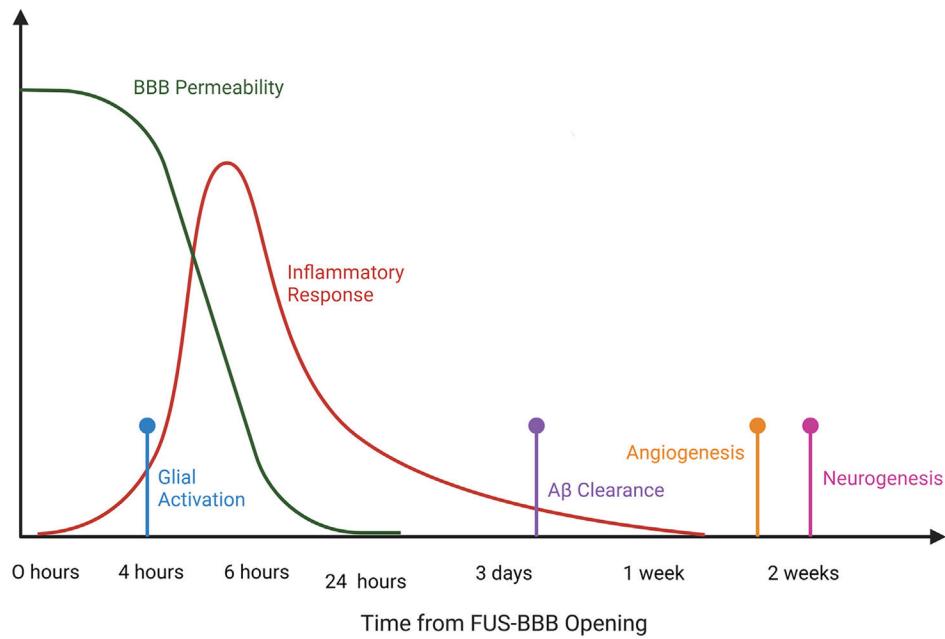


Fig. 2.

A summary of the pre-clinical therapeutic targets for which drugs and genes are being delivered with FUS to the brain for various disease applications. References correspond to studies investigating that therapeutic target with FUS BBBO. Created with [Biorender.com](https://www.biorender.com).

Pre-Clinical Secondary Effects of BBB Opening**Fig. 3.**

A summary of the timeline of various secondary physiological effects of FUS-mediated BBBO. Adapted from a figure in Todd et al., 2020 [91]. Made with [Biorender.com](https://biorender.com).

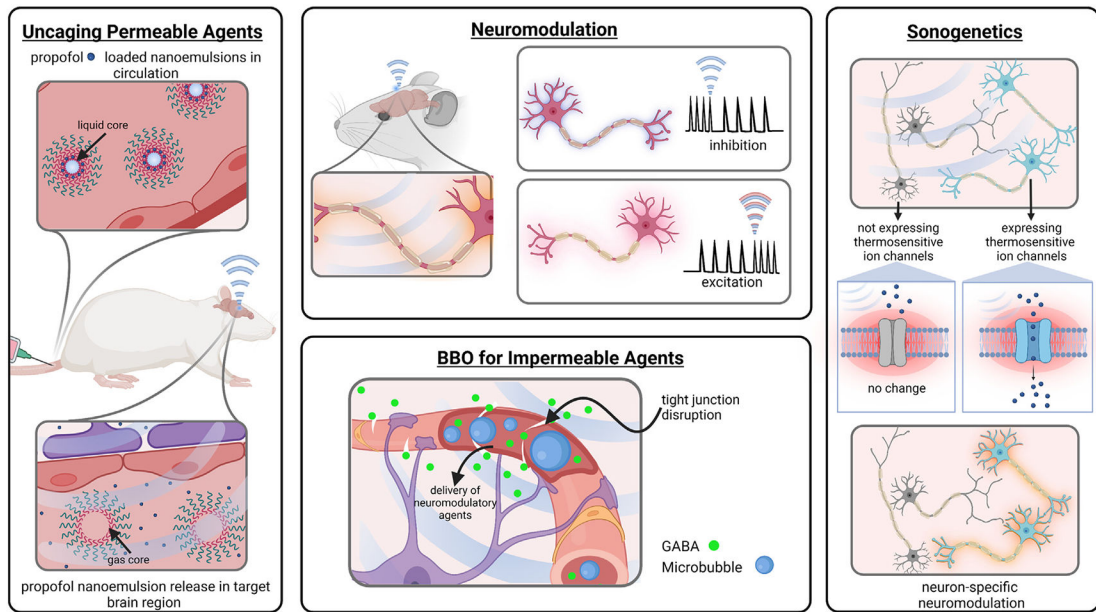


Fig. 4. A summary of the neuroscience tools being studied at the pre-clinical level which utilize FUS for various neurological applications. Created with [Biorender.com](https://www.biorender.com).

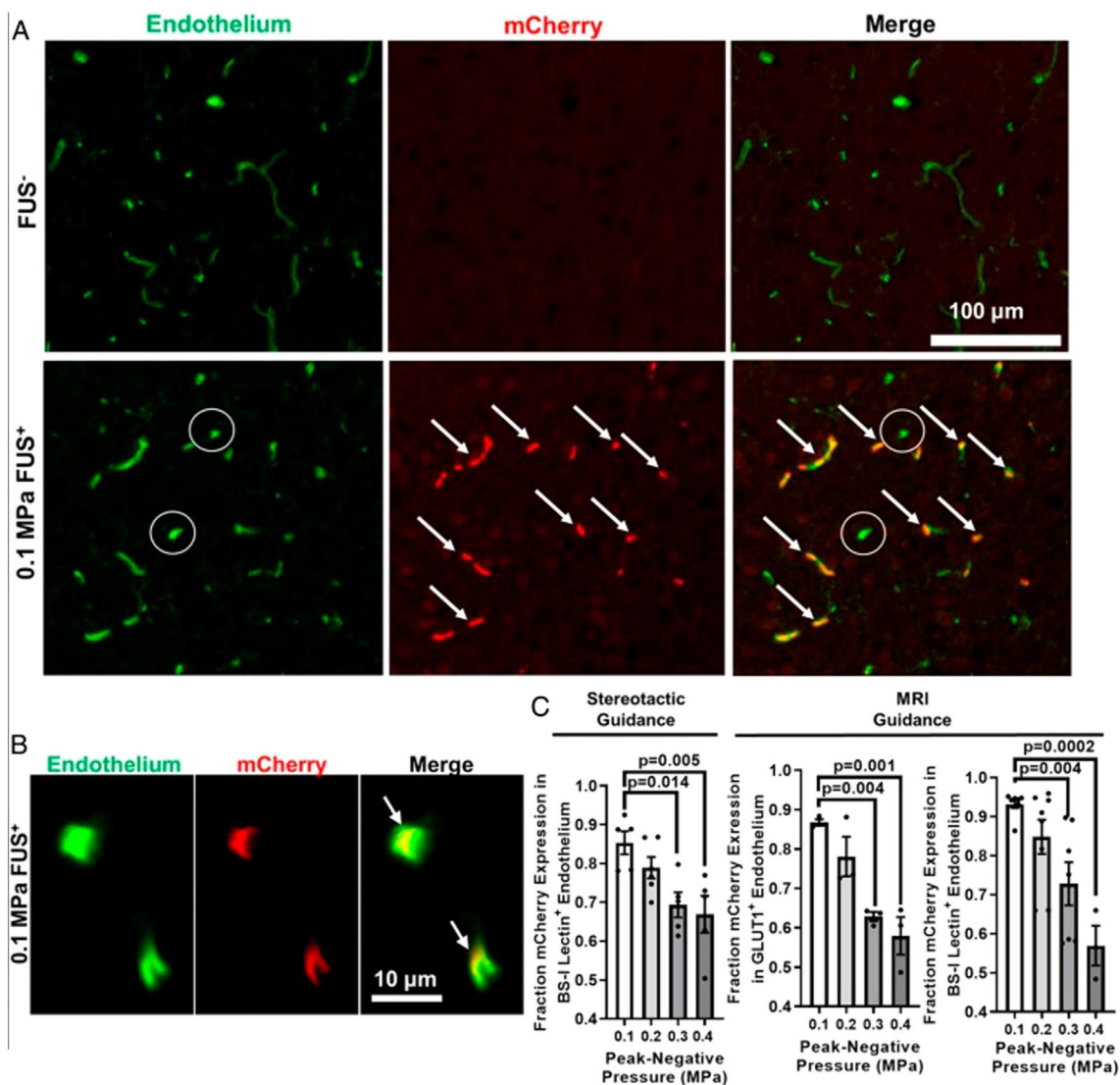


Fig. 5. FUS peak-negative acoustic pressure (PNP) may be tuned to yield sonoselective cerebrovascular endothelial transfection. (A and B) Confocal images of FUS⁺ (0.1 MPa) and contralateral FUS⁻ brain tissue showing expression of mCherry reporter gene (red) with respect to endothelial cells (BS-I lectin, green). Arrows denote mCherry colocalization with endothelium. (C) Bar graphs of fraction of mCherry expression in cerebrovascular endothelium as a function of PNP. Highly selective endothelial transfection is observed at low PNPs (i.e., 0.1 MPa and 0.2 MPa). One-way ANOVAs followed by Dunnett's multiple comparisons tests. [140].

Table 1

Summary of FUS parameters utilized and therapeutic payload delivered in the studies covered in the Pre-Clinical Delivery of Therapeutic Agents section. It should be noted that the studies provide different levels of detail in their methodology, including FUS pulsing parameters and MB dosage, composition, and size distribution. Here, we have highlighted as many FUS parameters as could be determined from the studies. MBs can be formulated with a wide variety of shell materials, gas cores, charges, functional group attachments, and concentrations. These features may differentially impact the bioeffects of the MBs upon FUS application. It is difficult to draw definitive conclusions on their relative contributions due to a lack of standardization within the field with regards to reporting and controlling for these characteristics.

Disease	Ref.	Animal model	Delivered agent	FUS Parameters f = frequency; dc = duty cycle; BL = burst length; PRF = pulse repetition frequency; PNP = peak negative pressure; I = Intensity t = time	MB type
<i>Alzheimer's Disease (AD)</i>	[27]	APPswe/PSEN1-dE9 transgenic mice	GSK-3 inhibitor	f = 0.4 MHz; BL = 10 ms; PRF = 1 Hz; PNP = 0.41 MPa; t = 60 s	SonoVue® SF6-filled
	[28]	TgCRND8 transgenic mice	TrkA-agonist	f = 1.68 MHz; BL = 10 ms; PRF = 1 Hz; PNP = 1.29 MPa; t = 120 s	Definity
	[29]	TgCRND8 transgenic mice	TrkA-agonist	f = 1.68 MHz; BL = 10 ms; PRF = 1 Hz; PNP = 1.29 MPa; t = 120 s	Definity
	[30]	TgCRND8 Transgenic mice	Intravenous immunoglobulin (IVIg)	f = 1.68 MHz; BL = 10 ms; PRF = 1 Hz; t = 120 s	Definity
	[31]	APPswe/PSdE9 transgenic mice	anti-pGlu3 Aβ mAb	f = 0.835 MHz; BL = 10 ms; PRF = 2 Hz; PNP = 0.33 MPa; t = 100 s	Optison™
	[32]	APP/PS1 transgenic mice	Quercetin-modified sulfur nanoparticles	PNP = 1 MPa; t = 600 s	Poly(α-cyanoacrylate nbutyl acrylate)
	[33]	TgCRND8 Transgenic mice	rAAV1/2	f = 1.68 MHz; BL = 10 ms; PRF = 1 Hz; t = 120 s	Definity
	[34]	TgCRND8 transgenic mice	rAAV9	f = 1.68–1.78 MHz; BL = 10 ms; PRF = 1 Hz; t = 120 s	Definity
	[35]	pR5 tau transgenic mice	Single chain antibody fragment RN2N	f = 1 MHz; dc = 10 %; BL = 10 ms; PRF = 10 Hz; PNP = 0.7 MPa; t = 6 s/spot	Phospholipid shell, octafluoropropane gas core
	[36]	pR5 tau transgenic mice	RN2N IgG	f = 1 MHz; dc = 10 %; BL = 10 ms; PRF = 10 Hz; PNP = 0.6 MPa; t = 60 s	Phospholipid shell, octafluoropropane gas core
<i>Parkinson's Disease (PD)</i>	[37]	Human α-syn expressing transgenic mice	rAAV9	f = 1.68 MHz; BL = 10 ms; PRF = 1 Hz; PNP = 1.0–1.1 MPa; t = 120 s	Definity
	[38]	C57BL/6 mice	α-syn plasmid	f = 0.4 MHz; BL = 10 ms; PRF = 1 Hz; PNP = 0.3 MPa; t = 60 s	SonoVue® SF6-filled
	[39]	MPTP mice	Curcumin	f = 1 MHz; BL = 200 ms; PRF = 1 Hz; PNP = 0.24–0.45 MPa; t = 60 s	Lipid-PLGA nanobubbles

Disease	Ref.	Animal model	Delivered agent	FUS Parameters f = frequency; dc = duty cycle; BL = burst length; PRF = pulse repetition frequency; PNP = peak negative pressure; I = Intensity t = time	MB type
	[40]	MPTP mice	GDNF plasmid liposome	$f = 0.5 \text{ MHz}; BL = 10 \text{ ms}; PRF = 1 \text{ Hz}; PNP = 0.3\text{--}0.8 \text{ MPa}; t = 60 \text{ s}$	Avidinylated MBs conjugated with LpDNA
	[41]	6-OHDA rats	GDNF plasmid	$f = 1 \text{ MHz}; \text{cycle number} = 5,000; PRF = 1 \text{ Hz}; PNP = 0.7 \text{ MPa}$	GDNFp-loaded cationic microbubbles
	[42]	6-OHDA rats	Nrf2	$f = 0.5 \text{ MHz}; t = 30 \text{ s}$	Nrf2-loaded cationic nanomicrobubbles
	[43]	6-OHDA rats	GDNF-loaded BPNs	$f = 1.15 \text{ MHz}; BL = 10 \text{ ms}; PRF = 0.5 \text{ Hz}; PNP = 0.6 \text{ MPa}; t = 120 \text{ s}$	Albumin-shelled, octofluoropropane gas core
	[44]	6-OHDA rats	GDNF-loaded PEGylated liposome	$f = 1 \text{ MHz}; dc = 20\%; I = 2 \text{ W/cm}^2$	Avidinylated biotinylated lipid-shelled
	[45]	MitoPark mice	polyethylenimine superparamagnetic iron oxide plasmid DNA	$f = 1 \text{ MHz}; \text{cycle number} = 5000; PRF = 1 \text{ Hz}; PNP = 0.3 \text{ MPa}; t = 60 \text{ s}$	Lipid-shelled
	[46]	MPTP mice	AAV-GDNF and neurturin (NTN)	$f = 1.5 \text{ MHz}; PRF = 10 \text{ Hz}; PNP = 0.45 \text{ MPa}; t = 60 \text{ s}$	In-house polydisperse
	[47]	MPTP mice	BDNF	$f = 1.5 \text{ MHz}; BL = 6.7 \text{ ms}; PRF = 10 \text{ Hz}; PNP = 0.45 \text{ MPa}; t = 60 \text{ s}$	Size-isolated lipid-shelled
	[48]	C57BL/6 mice	BDNF	$f = 1.5 \text{ MHz}; BL = 6.7 \text{ ms}; PRF = 5 \text{ Hz}; PNP = 0.45 \text{ MPa}; t = 60 \text{ s}$	Lipid-shelled, perfluorobutane core
<i>Glioblastoma Multiforme (GBM)</i>	[51]	U87-luc and patient-derived 6240-luc Mice	Carboplatin	$f = 1.05 \text{ MHz}; BL = 23.8 \text{ ms}; PRF = 1 \text{ Hz}; PNP = 0.3 \text{ MPa}; t = 120 \text{ s}$	SonoVue®
	[52]	MGPP3 (Pdgfr ^{+/+} /Pten ^{-/-} /P53 ^{-/-}) Mice	Etoposide	$f = 1.5 \text{ MHz}; BL = 1 \text{ ms}; PRF = 5 \text{ Hz}; PNP = 0.7 \text{ MPa}; t = 30 \text{ s}$	In-house polydisperse, lipid-shelled
	[54]	C6 Rats	Liposomal temozolomide nanoparticles	$f = 1 \text{ MHz}; \text{cycle number} = 10,000; PRF = 1 \text{ Hz}; PNP = 0.8 \text{ MPa}; t = 60 \text{ s}$	In-house lipid-shelled, perfluoropropane gas core
	[55]	Patient-derived glioma Mice	Paclitaxel	$f = 1 \text{ MHz}; BL = 12 \text{ ms}; PRF = 1 \text{ Hz}; PNP = 0.3 \text{ MPa}; t = 120 \text{ s}$	Lumason
	[56]	U87 and B16F10va Mice	ZsGreen BPNs and Luc-BPNs	$f = 1.1 \text{ MHz}; BL = 10 \text{ ms}; PRF = 0.5 \text{ Hz}; PNP = 0.55 \text{ MPa}; t = 120 \text{ s}$	Albumin-shelled
	[57]	T98G Mice	pCas9/MGMT-loaded cRGD Lipid-polymer hybrid nanoparticles (LPHNs)	$I = 1.84 \text{ W}$	In-house avidinylated biotinylated lipid-shelled conjugated to LPHNs-cRGD
	[58]	GL261 Mice	PEG- <i>b</i> -PMBSH nanoparticles	$f = 1 \text{ MHz}; dc = 0.5\%; \text{cycle number} = 5,000; PRF = 1 \text{ Hz}; PNP = 0.3\text{--}0.7 \text{ MPa}; t = 60 \text{ s}$	In-house cationic lipid-shelled

Disease	Ref.	Animal model	Delivered agent	FUS Parameters f = frequency; dc = duty cycle; BL = burst length; PRF = pulse repetition frequency; PNP = peak negative pressure; I = Intensity t = time	MB type
	[59]	3-Gy irradiated Mice	Trypan blue dye solution	$f = 0.69 \text{ MHz}; BL = 10 \text{ ms}; PRF = 2 \text{ Hz}; PNP = 0.26\text{--}0.27 \text{ MPa}; t = 95 \text{ s}$	Optison
	[60]	GL261 Mice	mCD47	$f = 1.1 \text{ MHz}; dc = 0.5 \%; PNP = 0.4 \text{ MPa}; t = 120 \text{ s}$	Albumin-shelled
	[61]	Patient-derived glioma Mice	^{89}Zr -radiolabelled antibody targeting EphA2 receptors	$f = 1.1 \text{ MHz}; BL = 10 \text{ ms}; PRF = 0.4 \text{ Hz}; PNP = 0.85 \text{ MPa}; t = 120 \text{ s}$	Definity
	[62]	eGFP-transduced U87 and GL261 Mice	eGFP mRNA	$f = 1.44 \text{ MHz}; dc = 1 \%; BL = 10 \text{ ms}; PRF = 1 \text{ Hz}; PNP = 3.82 \text{ MPa}; t = 120 \text{ s}$	In-house lipid-shelled, perfluorobutane core
	[63]	eGFP-transduced U87 and GL261 Mice	eGFP mRNA	$f = 1.44 \text{ MHz}; dc = 1 \%; BL = 10 \text{ ms}; PRF = 1 \text{ Hz}; PNP = 3.82 \text{ MPa} (U87) \text{ and } 1.52, 2.74, 3.53 \text{ MPa} (GL261); t = 120 \text{ s}$	In-house lipid-shelled, perfluorobutane core
	[64]	Porcine model Normal brain tissue	GFAP and myelin basic protein release	$f = 0.65 \text{ MHz}; BL = 10 \text{ ms}; PRF = 1 \text{ Hz}; PNP = 1.5 \text{ MPa}; t = 180 \text{ s}$	Definity
Temporal Lobe Epilepsy	[65]	Rat model pilocarpine	N/A	$f = 0.65 \text{ MHz}; dc = 2 \%; BL = 20 \text{ ms}; PRF = 1 \text{ Hz}; PNP = 0.4 \text{ MPa}; t = 90 \text{ s}$	Definity
	[66]	CD-1 mice	Quinolinic acid	$f = 1.5 \text{ MHz}; dc = 2 \%; BL = 20 \text{ ms}; PRF = 1 \text{ Hz}; PNP = 0.33, 0.5, 0.67 \text{ MPa}; t = 120 \text{ s}$	Definity
	[67]	Sprague Dawley rats	Quinolinic acid	$f = 1.5 \text{ MHz}; dc = 2 \%; BL = 20 \text{ ms}; PRF = 1 \text{ Hz}; PNP = 0.69 \text{ MPa}; t = 120 \text{ s}$	In-house PEG lipid-shelled, decafluorobutane gas core
Stem Cell Delivery for CNS Disorders	[70]	Sprague Dawley rats	Iron labeled GFP neural stem cells	$f = 0.558 \text{ MHz}; BL = 10 \text{ ms}; PRF = 1 \text{ Hz}; PNP = 0.24 \text{ MPa}; t = 120 \text{ s}$	Definity
	[72]	Sprague-Dawley rats	SPION-loaded hNPCs	$f = 1.5 \text{ MHz}; BL = 10 \text{ ms}; PRF = 10 \text{ Hz}; PNP = 0.45 \text{ MPa}; t = 60 \text{ s}$	monodisperse microbubbles (SIMB4-5)
	[73]	Sprague-Dawley rats	Mesenchymal stem cells	$f = 0.515 \text{ MHz}; BL = 10 \text{ ms}; PRF = 1 \text{ Hz}; PNP = 0.25 \text{ MPa}; t = 300 \text{ s}$	Definity

Summary of all currently-registered clinical trials of FUS-mediated BBB opening in human patients, separated by disease condition.

Table 2

Disease / condition	Study ID	No. of patients	Outcome measures	FUS device	Complete?
<i>Alzheimer's disease</i>	NCT04118764	6	Efficacy of BBBO No. of safety events Change in amyloid uptake Change in cognitive function	Neuronavigation-guided system	No
	NCT04526262	6	Adverse events Efficacy of BBBO	ExAblate 4000 Type 2	No
	NCT02986932	6	Efficacy of BBBO Change in amyloid uptake Adverse events Change in cognitive function	ExAblate 4000 Type 2	Yes
	NCT03739905	30	Adverse events Efficacy of BBBO Change in cognitive function Change in amyloid uptake	ExAblate 4000 Type 2	No
	NCT04526262	6	Adverse events Efficacy of BBBO	ExAblate 4000 Type 2	Yes
	NCT03608553	10	Adverse events Feasibility of BBBO	ExAblate 4000 Type 2	No
<i>Parkinson's disease</i>	NCT04370665	4	Efficacy of BBBO Adverse events Efficacy of Cerezyme delivery	ExAblate 4000 Type 2	No
	NCT02343991	10	Adverse events	ExAblate 4000 Type 2	Yes
<i>Brain tumors</i>	NCT03551249	20	Adverse events Feasibility of repeated BBBO	ExAblate 4000 Type 2	No
	NCT04063514	15	Adverse events Functional magnetic resonance imaging	DWL or Brainsomix	No
	NCT04804709	3	Adverse events Survival Efficacy of BBBO	Neuronavigation-guided system	No
	NCT03616860	20	Adverse events Feasibility of repeated BBBO Efficacy of BBBO	ExAblate 4000 Type 2	No
	NCT04998864	5	Adverse events Feasibility of repeated BBBO	ExAblate 4000 Type 2	No
	NCT03714243	10	Adverse events Efficacy of BBBO	ExAblate 4000 Type 2	No
	NCT04440358	50	Adverse events Efficacy of BBBO	ExAblate 4000 Type 2	No

Disease / condition	Study ID	No. of patients	Outcome measures	FUS device	Complete?
	NCT04417088	30	Adverse events Efficacy of BBBO	ExAblate 4000 Type 2	No
	NCT03712293	10	Adverse events Efficacy of BBBO	ExAblate 4000 Type 2	No
	NCT03626896	6	Adverse events Dose limiting toxicity Efficacy of BBBO	NavifUS	Yes
	NCT04446416	10	Adverse events Survival Clinical response Efficacy of BBBO	NavifUS	No
	NCT03744026	33	Dose limiting toxicity Efficacy of BBBO	SonoCloud-9	Yes
<i>Amyotrophic lateral sclerosis</i>	NCT03321487	8	Adverse events Efficacy of BBBO	ExAblate 4000 Type 2	Yes