Targeted Delivery of Self-Complementary Adeno-Associated Virus Serotype 9 to the Brain, Using Magnetic Resonance Imaging-Guided Focused Ultrasound

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Abstract

Noninvasive drug delivery to the brain remains a major challenge for the treatment of neurological disorders. Transcranial focused ultrasound combined with lipid-coated gas microspheres injected into the bloodstream has been shown to increase the permeability of the blood–brain barrier locally and transiently. Coupled with magnetic resonance imaging, ultrasound can be guided to allow therapeutics administered in the blood to reach brain regions of interest. Using this approach, we perform gene transfer from the blood to specific regions of the mouse brain. Focused ultrasound was targeted to the right hemisphere, at multiple foci, or restricted to one focal point of the hippocampus or the striatum. Doses from 5×10^8 to 1.25×10^{10} vector genomes per gram (VG/g) of self-complementary adeno-associated virus serotype 9 carrying the green fluorescent protein were injected into the tail vein. A dose of 2.5×10^9 VG/g was optimal to express the transgene, 12 days later, in neurons, astrocytes, and oligodendrocytes in brain regions targeted with ultrasound, while minimizing the infection of peripheral organs. In the hippocampus and striatum, predominantly neurons and astrocytes were infected, respectively. Transcranial focused ultrasound applications could fulfill a long-term goal of gene therapy: delivering vectors to diseased brain areas directly from the circulation, in a noninvasive manner.

Introduction

THE USE OF adeno-associated virus (AAV)-based vectors for gene therapy to the CNS shows great promise (Lentz *et al.*, 2012). AAV-dependent gene transfer to the brain results in strong transgene expression in mice (Harding *et al.*, 2006; Carty *et al.*, 2008) and nonhuman primates (Kells *et al.*, 2009; Salegio *et al.*, 2011), and in clinical trials in patients with Parkinson's disease (Kaplitt *et al.*, 2007) and Canavan's disease (McPhee *et al.*, 2006). To date, targeting the delivery of viral vectors to specific areas of the brain can be done only by invasive intracranial injections, with the associated risks of surgical procedures. Ideally, gene delivery to particular brain regions would be done without the need for invasive surgery.

Transcranial focused ultrasound (FUS) in conjunction with peripheral administration of a clinically approved ultrasound contrast agent, such as DEFINITY (perflutren lipid-

coated gas microspheres; Lantheus Medical Imaging, North Billerica, MA), has been shown to increase the permeability of the blood–brain barrier (BBB) locally and transiently, for up to 6 hr (Hynynen *et al.*, 2001). The concentrated acoustic energy can be focused in spot(s) approximately 2 mm in diameter to target specific brain region(s) that can be accurately localized by magnetic resonance imaging (MRI). In a mouse model of Alzheimer's disease, we previously demonstrated that MRI-guided FUS (MRIgFUS) allows peripherally injected antibodies against amyloid β peptides ($A\beta$) to enter the brain and reduce plaque load in FUS-targeted cortical areas (Jordão *et al.*, 2010).

The use of MRIgFUS to noninvasively deliver therapeutics to the brain is especially attractive for treatments such as cell therapy and gene transfer, which can have long-lasting beneficial effects. The current study addresses the potential of MRIgFUS for targeted gene delivery to the brain, using a peripherally injected AAV-based vector.

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At relatively high concentrations administered peripherally, the AAV9 serotype has the ability to cross the BBB and infect neural cells, especially at early developmental stages (Cearley and Wolfe, 2006; Manfredsson *et al.*, 2009; Foust *et al.*, 2010; Gray *et al.*, 2011). Previous studies have shown that peripheral administration of AAV9 vectors carrying therapeutic transgenes successfully rescued postnatal mice from spinal muscular atrophy (Foust *et al.*, 2010), and adult mice from mucopolysaccharidosis IIIB (Fu *et al.*, 2011). Although these studies demonstrated the potential of AAV9 to treat neuropathological disorders, they also highlighted some of the limitations of administering AAV-based vectors peripherally to target the CNS. For example, in adult mice, studies have shown that the self-complementary AAV9 (scAAV9) vector needs to be administered at relatively large doses systemically (10^{10} to 10^{11} vector genomes [VG]/g) to infect cells of the CNS, with levels of glial and neuronal infections varying in different brain regions (Duque *et al.*, 2009; Foust *et al.*, 2009; Gray *et al.*, 2011). At these intravenous doses, peripheral organs such as the liver (Pulicherla *et al.*, 2011) and the heart (Pacak and Byrne, 2011) become significantly infected with viral particles, which in some paradigms can cause unwanted side effects and irreversible toxicity.

Here, we evaluated the potential of MRIGFUS for gene delivery to targeted areas of the brain. scAAV9 was injected intravenously at various doses and MRIGFUS was targeted to a full hemisphere or limited to the striatum or hippocampus to assess (1) the entry of scAAV9 in MRIGFUS-targeted compared with nontargeted brain regions; (2) the infection of neurons, astrocytes, and oligodendrocytes after MRIGFUS delivery of scAAV9 to targeted brain regions; and (3) the levels of scAAV9 infection in the peripheral organs.

Materials and Methods

Animals

Male and female 12-week-old C57BL/6 mice (Charles River, Sherbrooke, QC, Canada) were used. Of a total of 23 mice, the number of animals per MRIGFUS-treated group was as follows: 4 mice were injected with trypan blue, 2 mice received 1.25×10^{10} VG/g, 12 mice received 2.5×10^9 VG/g, and 5 mice received 5×10^8 VG/g. All injections were administered via the tail vein, as described in the section MRIGFUS (below).

Animal procedures were conducted with the approval of the Animal Care Committee of Sunnybrook Health Sciences Centre (Toronto, ON, Canada) and in compliance with the guidelines established by the Canadian Council on Animal Care and the Animals for Research Act of Ontario.

Virus

scAAV9 was produced by transient transfection procedures, using a double-stranded AAV2-ITR-based CB-GFP vector, with a plasmid encoding Rep2Cap9 sequence as previously described along with an adenoviral helper plasmid pHelper (Stratagene, Santa Clara, CA) in 293 cells. Our serotype 9 sequence was verified by sequencing and was identical to that previously described. Virus was purified by two cesium chloride density gradient purification steps, dialyzed against phosphate-buffered saline (PBS), and formulated with 0.001% Pluronic-F68 to prevent virus aggregation

and stored at 4°C (Foust *et al.*, 2009). Vector preparations were titrated by quantitative PCR, using TaqMan technology. The purity of vectors was assessed by 4–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and silver staining (Invitrogen, Carlsbad, CA).

MRIGFUS

Mice were anesthetized with ketamine (150 mg/kg) and xylazine (10 mg/kg). An angiocatheter was inserted into each mouse via the tail vein, and the mouse was secured in a supine position. The FUS system was placed inside a 1.5-T MRI for positioning of the target locations, using a radio-frequency (RF) coil with inner dimensions of 3.2×4.2 cm. An FUS beam, guided by MRI using T2-weighted images (fast spin echo [FSE], time to echo [TE]=61.7 msec, time to repetition [TR]=2000 msec, echo train length [ETL]=4, field of view [FOV]= 6×6 cm, slice thickness=1 mm, 128×128) to select the targets, was generated with two different piezoelectric transducers. The first transducer was an in-house constructed piezoceramic (diameter, 10 cm; radius of curvature, 8 cm) driven at 0.558 MHz using an external matching circuit and used to sonicate four locations in the right hemisphere. The second transducer was a wideband-composite eight-sector array (diameter, 10 cm; radius of curvature, 8 cm; Imasonic, Voray-sur-l'Ognon, France) driven as a single element at 1.18 MHz. The second transducer was used to create more localized effects at single sonication locations in either the striatum or the hippocampus. The transducers were positioned inside a degassed water tank, using an MRI-compatible three-axis motorized system. For right hemisphere treatment, four spots, 1.5 mm apart, along the hemisphere were targeted with ultrasound (0.558 MHz, 0.3 MPa, 120 sec, 10-msec bursts/Hz). For striatum- or hippocampus-targeted treatments, ultrasound was focused on a single spot within the striatum or hippocampus of the right hemisphere and treated at 1.18 MHz, 0.53–0.6 MPa using a pulse sequence (10-msec bursts composed of single excitation cycles separated by 6- μ sec delays, 1-Hz burst repetition frequency, 120-sec duration) designed to eliminate standing waves in the skull cavity (O'Reilly *et al.*, 2010). DEFINITY microspheres (mean size, 1–2 μ m; 40–80 μ l/kg; Lantheus Medical Imaging) and MRI contrast agent Gadovist (gadobutrol, 0.2 ml/kg; Schering, Berlin, Germany) were delivered through the tail vein at the time ultrasound was applied.

Five minutes after ultrasound treatment, mice received an injection of 7.5 μ l of viral solution or 2% trypan blue solution in saline per gram of animal, via the tail vein catheter. After this, a 100- μ l bolus of saline solution was used to flush the catheter of any residual virus, to ensure transfer into the bloodstream. The three doses used for this study were 5×10^8 , 2.5×10^9 , and 1.25×10^{10} viral genomes of scAAV9-CB-GFP per gram (VG/g) of treated animal. After recovery, mice were returned to their individual cages for 12 days with the exception of mice that received trypan blue solution, which were killed 20 min after injection of the dye.

Brain perfusion and processing

Twelve days after treatment, mice were deeply anesthetized with ketamine (250 mg/kg) and xylazine (17 mg/kg) and then transcardially perfused first with 0.9% saline followed by 4% paraformaldehyde (PFA)–0.1 M PO_4 . Brain,

heart, kidney, quadriceps muscle, and liver were collected and immersed in 4% PFA solution for 24 hr and stored at 4°C in 30% sucrose–0.1 M PO₄ solution. For sectioning, organs were mounted onto a sliding microtome with Tissue-Tek O.C.T. compound (Sakura Finetek USA, Torrance, CA) and frozen with dry ice. Forty-micrometer-thick sections were cut for histological analysis. For trypan blue analysis, 250- μ m brain sections were prepared. Tissues were placed in cryoprotective solution and transferred to –20°C.

Prussian blue staining

Staining for red blood cell extravasation was performed by Perl's method (Prussian blue) (Perl and Good, 1992). Sections were incubated for 15 min with 1% potassium ferrocyanide in 1% hydrochloric acid, washed, and counterstained with nuclear fast red.

Immunohistochemistry

Coronal and sagittal floating brain sections were incubated in blocking solution (PBS, 10% donkey serum, 0.4% Triton X-100) for 1 hr at room temperature, and then for 72 hr at 4°C with goat anti-GFP (green fluorescent protein) antibody (cat. no. AB3080, diluted 1:500; Millipore, Bedford, MA) diluted in blocking solution. Sections were washed three times with PBS and then incubated for in secondary antibody (cat. no. 705-065-147, diluted 1:100; donkey anti-goat IgG biotin) for 2 hr. For 3,3'-diaminobenzidine tetrahydrochloride (DAB) staining, sections were processed as described in the VECTASTAIN ABC Elite kit (cat. no. PK-6100; Vector Laboratories, Burlingame, CA) and the DAB peroxidase substrate kit (cat. no. SK-4100; Vector Laboratories). For immunofluorescent staining sections were incubated with cyanine-3 (Cy3)-conjugated streptavidin (cat. no. 016-160-084, diluted 1:200; Jackson ImmunoResearch) for 1 hr at room temperature. For high-magnification colabeling studies, sections were stained with rabbit anti-GFAP (glial fibrillary acidic protein) (diluted 1:1000; Dako, Mississauga, ON, Canada) or rabbit anti-Olig2 (oligodendrocyte transcription factor-2) (cat. no. AB9610, diluted 1:500; Millipore) and mouse anti-NeuN (neuronal nuclear antigen) (cat. no. MAB377, diluted 1:1000; Millipore) as primary antibodies and with Cy3-conjugated anti-rabbit secondary antibody (cat. no. 711-165-152, diluted 1:200; Jackson ImmunoResearch) and Cy5-conjugated anti-mouse secondary antibody (cat. no. 715-175-150, diluted 1:200; Jackson ImmunoResearch), respectively.

For peripheral tissue, 40- μ m-thick sections were blocked and permeabilized in PBS containing 0.4% Triton X-100 and 10% donkey serum for 1 hr at room temperature. Sections were then incubated for 2 hr at room temperature in blocking solution containing TO-PRO-3 iodide (cat. no. T3605, diluted 1:500; Invitrogen) and rinsed three times (10 min each) in PBS with 0.4% Triton X-100 before mounting. For quadriceps muscles, sections were incubated for 48 hr at 4°C with rabbit anti-GFP (cat. no. AB3080, diluted 1:500; Millipore) diluted in blocking solution. After three washings in PBS, sections were then incubated for 2 hr with Cy3-conjugated anti-rabbit (cat. no. 711-165-152, diluted 1:200; Jackson ImmunoResearch) and TO-PRO-3 iodide (cat. no. T3605, diluted 1:500; Invitrogen) for 2 hr at room temperature and rinsed three times (10 min each) in PBS with 0.4% Triton X-100 before mounting.

Imaging and quantification

MRI images were captured to determine the extent of BBB disruption, based on the increase in signal intensity. BBB disruption was confirmed via contrast-enhanced T1-weighted images (FSE, TE = 10 msec, TR = 500 msec).

For trypan blue fluorescence imaging, confocal microscopy was done with an LSM 510/Zeiss Axiovert 100 (Carl Zeiss, Toronto, ON, Canada) at an excitation wavelength of 488 nm. An emitted wavelength of 545 nm, corresponding to trypan blue fluorescence, was detected.

For Prussian blue and DAB staining, images were obtained in bright-field with an Axioplan 2 microscope (Carl Zeiss). Differential interference contrast (DIC) images were captured at $\times 20$ magnification.

Entire brain sections were acquired with the Virtual Slice module of StereoInvestigator software (MBF Bioscience, Williston, VT) combined with an Axioplan 2 microscope (Carl Zeiss). Delineation of the brain was processed with a $\times 4$ objective, and multiple images were captured with a $\times 10$ objective and assembled into one mosaic.

To characterize cell types infected in the brain, confocal microscopy was done with an LSM 510/Zeiss Axiovert 100 (Carl Zeiss) at excitation wavelengths of 488 nm for GFP, 561 nm for GFAP and Olig2, and 633 nm for NeuN. The percentage of GFP-positive cells expressing neuronal (NeuN) and astrocytic (GFAP) markers was quantified in seven animals treated with MRIGFUS and scAAV9-CB-GFP (2.5×10^9 VG/g), using three sections per animal. For each section, four z-stacks were collected at $\times 20$ magnification ($512 \times 512 \times 15 \mu\text{m}$ depth), for a total volume of 0.047 mm^3 per animal in the treated region. Colocalization was quantified with three-dimensional imaging reconstruction software (Imaris; Bitplane, Zurich, Switzerland). Briefly, scAAV9-CB-GFP-positive staining was used as a mask, and its colocalization within cells coexpressing NeuN or GFAP was quantified. A total of 5349 scAAV9-CB-GFP-positive cells were evaluated for coexpression with NeuN or GFAP.

For evaluation of infection in peripheral organs, laser intensities (488 nm for GFP and 633 nm for TO-PRO-3) were optimized for a viral concentration of 2.5×10^9 VG/g for each tissue type and images for 5×10^8 and 1.25×10^{10} VG/g were taken, using the same parameters. The number of z projection(s), which were combined to obtain enough fluorescence signal for each organ, was as follows: liver, 1; heart, kidney, and muscle, 3 ($\sim 3.4 \mu\text{m}$ stack). Producing the final montage of the figures was performed with Adobe Photoshop CS5 (Adobe Systems, San Jose, CA).

Statistical analysis

Statistical analyses were performed with GraphPad Prism software (GraphPad Software, San Diego, CA). The statistical significance of differences between cell types within FUS-treated regions was assessed by one-way analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test.

Results

MRIGFUS increases permeability of BBB in targeted brain areas

To assess the capacity of FUS to increase the delivery of viral vectors from the bloodstream to the brain, C57BL/6

FIG. 1. MRI-guided focused ultrasound and opening of the blood–brain barrier. Anesthetized mice were placed in a supine position over an MRI-compatible focused ultrasound (FUS) transducer (a). MRI images were taken to accurately target FUS waves upward to the right hemisphere (b), striatum (c), or hippocampus (d). DEFINITY microspheres and MRI gadolinium contrast agent were delivered through the tail vein when FUS was applied. Opening of the blood–brain barrier was confirmed by MRI with the entry of gadolinium (white arrow) approximately 2 min after FUS (e).

mice were anesthetized and placed on a small-animal MRIGFUS positioning system (Fig. 1a), similar to previous studies (Chopra *et al.*, 2009; Jordão *et al.*, 2010). MRIGFUS was used to target a large region of the brain (Fig. 1b, right hemisphere) or specific brain regions, such as the striatum (Fig. 1c) or the hippocampus (Fig. 1d). The permeability of the BBB within the treated area was evaluated by MRI, visualizing the leakage of the gadolinium-based contrast agent (Gadovist) into the brain (Fig. 1e). For example, when targeting the hippocampus with FUS, an influx of gadolinium was visualized, approximately 2 min after treatment, demonstrating that the BBB was permeabilized within a region of 2 mm in diameter viewed in a horizontal plane (Fig. 1e, top, white arrow). The opening of the BBB was confirmed in coronal and sagittal views (Fig. 1e, middle and bottom, respectively, white arrows).

The area targeted by MRIGFUS was identified histologically with trypan blue dye, which normally does not cross the BBB. Trypan blue was injected into the bloodstream 10 min after BBB disruption, and brains were collected 30 min later. On brain retrieval and sectioning, a blue spot was observed in MRIGFUS-targeted regions such as the striatum (Fig. 2a and b, white arrow) and hippocampus (data not shown), indicating that the dye crossed the BBB in FUS-targeted areas. Brain sections were imaged by confocal microscopy to visualize trypan blue (excitation, 488 nm; emission, 650 nm). After striatal targeting with FUS (Fig. 2a–d), trypan blue was observed predominantly in the right striatum

(Fig. 2c, white arrows) compared with non-FUS-targeted regions such as the hippocampus (Fig. 2d). When FUS was targeted to the right hippocampus, trypan blue was detected primarily at the level of the right hippocampus (Fig. 2f, white arrows) compared with the level of the striatum (Fig. 2e). Trypan blue was observed in blood vessels on both sides of the brain (Fig. 2c–f, white arrowheads), confirming that the dye was distributed throughout the brain vasculature, crossing the BBB only in FUS-treated regions.

Perl's method was used to detect the presence of ferric iron in the brain parenchyma, which is indicative of red blood cell infiltration and microhemorrhages. Only 2 of 10 animals had small iron clusters detected on the FUS-treated side, 12 days after treatment (Fig. 2g–j).

MRIGFUS gene delivery: targeting specific brain regions with scAAV9

MRIGFUS gene delivery to the brain was done with an scAAV9 vector carrying the gene encoding GFP under the control of the chicken β -actin hybrid promoter (CB). All mice received scAAV9-CB-GFP by tail vein injection within 10 min of FUS treatment and they were killed 12 days later. GFP expression was detected directly by confocal microscopy, and it was enhanced for optimal visualization after immunostaining with an antibody against GFP and processing for bright-field and confocal microscopy (e.g., see Fig. 4).

FIG. 2. Opening of the blood-brain barrier visualized with trypan blue. After FUS treatment of the right striatum and intravenous injection of trypan blue, a blue coloration was observed at the surface of the targeted region of the mouse brain (a, white arrow). On sectioning, the blue coloration was visible in coronal planes at the level of the right striatum (b, white arrow). Trypan blue dye, detected by confocal microscopy (excitation, 480 nm; emission, 650 nm), was found in FUS-treated striatum (c, white arrows) and not in non-FUS-treated areas, such as the hippocampus (d). In another animal in which FUS targeted the right hippocampus (e and f), trypan blue was absent in non-FUS-treated areas such as the striatum (e), and it was detected in the right hippocampus (f, white arrows). White arrowheads indicate the presence of trypan blue in blood vessels on both sides of the brain (c–f). Twelve days after treatment, Prussian blue staining (g–j) reveals rare iron clusters in the striatum (g and h) and the hippocampus (i and j), only on the treated side for 2 animals of 10 tested. Barely detectable at low magnification ($\times 4$, bright field; g and i), a low density of clusters (black arrows) appears at higher magnification ($\times 20$, DIC; h and j). CPu, striatum (caudate-putamen); LSI, intermediate lateral septum; Hc, hippocampus; cc, corpus callosum; Th, thalamus; LV, lateral ventricle. Color images available online at www.liebertpub.com/hum

In all mice treated with FUS, increased GFP expression was found in the brain regions targeted. The most significant difference between FUS-treated and nontreated regions was observed at an scAAV9-CB-GFP dose of 2.5×10^9 VG/g. This relatively low dose of scAAV9-CB-GFP, injected into the periphery, is usually ineffective at reaching the brain (Gray *et al.*, 2011). In mice receiving four FUS spots to cover the right hemisphere (Fig. 1b), delivery of the *gfp* gene led to high GFP expression in the cortex, striatum, hippocampus, and thalamus (Fig. 3a). Gene delivery to specific brain regions was performed by focusing the ultrasound to one spot in the striatum (Fig. 3b) or one spot in the hippocampus (Fig. 3c). FUS-targeted striatum (Fig. 3b and d) and hippocampus (Fig. 3c and e) had high levels of GFP expression compared with non-FUS-targeted brain regions.

At a dose of 2.5×10^9 VG/g, scAAV9-CB-GFP gene transfer was found predominantly in FUS-targeted areas (Fig. 3). We then compared this dose with amounts of scAAV9-CB-GFP that were 5-fold lower (5×10^8 VG/g) or 5-fold higher (1.25×10^{10} VG/g), using mice in which the striatum or hippocampus was specifically targeted by FUS. As expected at 2.5×10^9 VG/g, GFP-positive cells were abundant in FUS-treated areas (i.e., striatum [Fig. 4a] or hippocampus [Fig. 4c, right hemisphere] and rarely found in non-FUS-treated areas (Fig. 4a and c, left hemisphere). At a lower dose of 5×10^8 VG/g, GFP expression in the brain was barely detected, even in FUS-treated areas (Fig. 4b). At a

higher dose of 1.25×10^{10} VG/g, GFP expression was strong in FUS-targeted areas and significant in some non-FUS-targeted areas (Fig. 4d).

MRlgFUS scAAV9-CB-GFP gene transfer to neurons, astrocytes, and oligodendrocytes

After MRlgFUS delivery of scAAV9-CB-GFP (2.5×10^9 VG/g) to the whole hemisphere, GFP-positive cells were found to colocalize with cells expressing markers for neurons (NeuN-positive cells), astrocytes (GFAP-positive cells), and oligodendrocytes (Olig2-positive cells) in the hippocampus and the striatum, as well as in cortical and thalamic regions (data not shown). In the FUS-targeted hippocampus (Fig. 5a), the proportion of GFP/NeuN-positive cells (58%) was significantly higher compared with GFP/GFAP-positive cells (37%) ($p < 0.05$; Fig. 5d and e). In the striatal region treated with FUS (Fig. 5b), GFP expression was found mainly in astrocytes (GFP/GFAP-positive cells, 63%) compared with neurons (GFP/NeuN-positive cells, 18%) ($**p < 0.005$; Fig. 5d and e). In all brain regions, a proportion of GFP-expressing cells did not express NeuN or GFAP (Fig. 5d and e), and some of these were found to be oligodendrocytes (Olig2-positive; Fig. 5c). We did not find GFP-positive cells expressing the ionizing calcium binding adaptor molecule-1 (Iba1), a marker of microglia (data not shown). FUS-treated areas did not demonstrate a noticeable change in Iba1 level (data not shown).

FIG. 3. MRIGFUS gene delivery to targeted brain regions. Mice were treated with transcranial MRIGFUS and they received AAV9-CB-GFP intravenously. Twelve days later, GFP was detected in brain sections by immunohistochemistry and DAB, predominantly in FUS-treated areas, namely the right hemisphere (a), the striatum (b and d), and the hippocampus (c and e). Ctx, cortex; CPu, striatum (caudate-putamen); lsi, intermediate lateral septum; Hc, hippocampus; Cb, cerebellum; Th, thalamus.

Reducing infection levels in peripheral organs, using MRIGFUS to deliver genes to brain

Systemic administration of scAAV9 at doses required to reach the brain without FUS results in significant infection in organs such as the liver and the heart (Zincarelli *et al.*, 2008; Gray *et al.*, 2011). In our study, at all scAAV9 doses tested, GFP expression was stronger in the liver compared with other organs examined, that is, heart, kidney, and quadriceps muscles (e.g., Fig. 6a–d). At a high dose of scAAV9-CB-GFP (1.25×10^{10} VG/g), liver cells were greatly infected and strongly expressed GFP (Fig. 6a, bottom). The level of infection in the liver was significantly reduced when the concentration of scAAV9-CB-GFP was decreased 5-fold (2.5×10^9 VG/g; Fig. 6a, middle), and it was barely detectable at a concentration of 5×10^8 VG/g (Fig. 6a, top). GFP-positive cells in the heart, kidney, and muscle were also found mainly at the highest concentration (1.25×10^{10} VG/g) of scAAV9-CB-GFP injected into the bloodstream (Fig. 6b–d, bottom) compared with the intermediate (Fig. 6b–d, middle) and lower (Fig. 6b–d, top) concentrations of scAAV9.

Discussion

Several AAV-based gene therapies have been developed over the last two decades (Murphy *et al.*, 2009). For gene delivery to the CNS, AAV has emerged as the preferred viral vector (Lentz *et al.*, 2012). Advances in the AAV field have led to the selection of appropriate serotypes, exhibiting tropism for various organs (Pulicherla *et al.*, 2011), and to the characterization of conditions that are important for infection efficiency (Gray *et al.*, 2011; Dayton *et al.*, 2012; Rapti *et al.*, 2012). Targeting specific brain regions, reducing the amount of viral particles needed for gene transfer to the CNS, and limiting the infection of peripheral organs remain important challenges to overcome.

Chemical alterations of the BBB to increase its permeability with hyperosmotic agents or vasodilators can facilitate gene delivery from the bloodstream to the CNS (Seyfried *et al.*, 2008; Carty *et al.*, 2010). Studies have evaluated intravenously injected AAV for CNS targeting after kainic acid-induced seizures (Gray *et al.*, 2010) or mannitol treatment (Gray *et al.*, 2011), both of which are known to affect the permeability of the BBB. Using these approaches, the extent

FIG. 4. MRigFUS gene delivery to the brain at different doses of intravenous scAAV9. Mice were injected with scAAV9-CB-GFP via the tail vein after MRigFUS treatment to the right striatum (**a**) or the right hippocampus (**b–e**). Twelve days later, GFP was detected in brain sections by immunohistochemistry and bright-field microscopy (**a–d**) and directly by confocal microscopy (**e**). Greater GFP expression was observed in FUS-treated (**a**, **c**, **d**, and **e**, *right*) compared with untreated (**a**, **c**, and **d**, *left*) areas at 2.5×10^9 and 1.25×10^{10} VG/g but not at 5×10^8 VG/g (**b**, **e**). Ctx, cortex; CPu, striatum (caudate-putamen); lsi, intermediate lateral septum; DG, dentate gyrus; CA1, cornu ammonis 1; cc, corpus callosum; Th, thalamus; LV, lateral ventricle.

and duration of BBB disruption are difficult to control and targeting specific brain region(s) is not possible. To date, gene delivery to specific brain areas could be performed only by using stereotaxic intracranial injections. For example, in a mouse model of Huntington's disease, AAV gene transfer of brain-derived neurotrophic factor (BDNF) and glial cell-derived neurotrophic factor (GDNF) was done by injections

into the striatum, where it induced neuronal protection (Kells *et al.*, 2004). In a mouse model of Alzheimer's disease, AAV-based vectors carrying transgenes encoding cholesterol 24-hydroxylase (Hudry *et al.*, 2010) and endothelin-converting enzyme (Carty *et al.*, 2008) were injected into the cortex and hippocampus, leading to significant reductions in amyloid plaque load. Stereotaxic injections are site specific but they

FIG. 5. Gene transfer to neurons, astrocytes, and oligodendrocytes after MRIGFUS. Immunohistochemistry was used to detect GFP expression in hippocampus for (a) NeuN-positive cells (neurons, white arrows), and striatum for (b) GFAP-positive cells (astrocytes, white arrows) and (c) Olig2-positive cells (oligodendrocytes, white arrow). Using Imaris software, GFP-positive cells were quantified and classified as NeuN⁺/GFAP⁻ (neuron), NeuN⁻/GFAP⁺ (astrocyte), or NeuN⁻/GFAP⁻ (undefined) cell types (d). Three regions of 0.016 mm³ have been analyzed per animal for a total of seven animals. Data are presented as cells per cubic millimeter. Significant differences between number of infected neurons and infected astrocytes are found in striatum and hippocampus. ANOVA, followed by Bonferroni post hoc test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001; ns, *p* > 0.05.

are invasive and tedious, especially when numerous injections are required over time or to cover large or multiple brain regions. Another drawback of intracranial injections is that the targeting accuracy can be confirmed only on histological analysis of brain sections.

MRIGFUS, combined with lipid-coated gas microspheres, builds on the strength of MRI to target the treatment to specific brain regions and the advantages of nonsurgical transcranial FUS to transiently increase the permeability of the BBB in a controlled manner. Here, we demonstrate that

FIG. 6. Reducing the dose of sAAV9 minimized infection levels in peripheral organs. Shown is GFP expression in peripheral organs after MRIGFUS and injections of sAAV9-CB-GFP into the tail vein at 5×10^8 , 2.5×10^9 , and 1.25×10^{10} VG/g. Confocal images compare the expression of GFP in liver (a), heart (b), kidney (c), and muscle (d). TO-PRO-3 (blue) was used to visualize the total number of cell nuclei.

by using MRIGFUS, BBB opening and gene delivery can cover large areas of the brain (e.g., the right hemisphere) or be restricted to a 2-mm-diameter region of interest, for example, targeting the striatum or the hippocampus. To date, MRIGFUS has been used for gene transfer to the brain in one study in which naked plasmid DNA carrying BDNF was loaded in microspheres and delivered to the striatal region of the mouse brain (Huang *et al.*, 2012). This study highlighted the feasibility of using MRIGFUS combined with lipid-coated gas microspheres to carry and deliver BDNF to the striatum. This approach involves the modification of lipid-coated gas microspheres to load them with naked plasmid DNA before

intravenous injection (Huang *et al.*, 2012). In general, the use of naked plasmid DNA for gene transfer requires repeat doses for efficient and prolonged expression of the transgene (Shi *et al.*, 2003; Hughes *et al.*, 2009). The long-term expression of BDNF (48 hr was the longest time point evaluated; Huang *et al.*, 2012) and the type of cells expressing the transgene remain to be established.

In our study, we delivered sAAV9-CB-GFP from the blood to the brain, without the need to alter readily available commercial microspheres. On the basis of previous studies using AAV9-based vectors (Fu *et al.*, 2011; Gray *et al.*, 2011) and our expertise in MRIGFUS delivery of therapeutics to the

brain (Treat *et al.*, 2007; Jordão *et al.*, 2010; Burgess *et al.*, 2011), we predicted that significant gene transfer in MRIG-FUS-targeted brain regions could be achieved with 10–50 times fewer viral particles than are usually required after peripheral injections without MRIG-FUS. At a dose of 5×10^8 VG/g, the infection level was low in both FUS-treated and nontreated sides. Peripheral doses of 2.5×10^9 VG/g or greater resulted in significant GFP expression in FUS-treated, compared with nontreated, brain areas. To our knowledge, it is the first work describing this level of infection to the brain after intravascular injection of AAV-based vectors at only 2.5×10^9 VG/g. For potential therapeutic application, specific transgenes are likely to require different expression levels in relevant brain regions for optimal treatment efficacy. It is clear that the rate of infection is related to the dose of virus injected and to the application of FUS (Fig. 4). To optimize treatment efficacy, dose–response studies will be required in preclinical animal models, modulating the doses of AAV and varying the brain areas targeted by FUS.

We found that scAAV9-CB-GFP delivered to the brain by MRIG-FUS infected neurons, astrocytes, and oligodendrocytes, which expressed high levels of the transgene for at least 12 days. Other studies have shown that one intraparenchymal administration of AAV can lead to long-term transgene expression, lasting for more than 6 months in the brain and up to 6 years in other organs (see Lentz *et al.*, 2012, for review). After systemic administration of an AAV9-based vector to adult mice, transgene expression was observed in the brain from 6 to 9 months (Fu *et al.*, 2011) and for up to 18 months in the brain of mice injected postnatally (Miyake *et al.*, 2011). We anticipate that FUS delivery of transgene to the brain will result in similar long-term expression compared with intraparenchymal and systemic injections. Future studies will be carried out to evaluate the length of expression of specific therapeutic transgenes in the brain after FUS delivery in preclinical animal models.

With a systemic scAAV9-CB-GFP dose of 2.5×10^9 VG/g, only a few GFP-positive cells were found in the left hemisphere, which was not treated with FUS. In FUS-treated areas of the right hemisphere, the expression of GFP and cell tropism were similar to that obtained by Gray and colleagues (2011), where the systemic dose of AAV was 25 times greater (6.4×10^{10} VG/g). Previous studies have found that intracranial injections lead to neuronal cell tropism when using the AAV9 serotype (Cearley *et al.*, 2008; Foust *et al.*, 2009). After systemic administration in adult mice, tropism has been found for neurons and astrocytes in different brain regions (Foust *et al.*, 2009; Fu *et al.*, 2011; Gray *et al.*, 2011; Samaranch *et al.*, 2012). In the hippocampus treated with FUS, GFP-positive cells were 58% neuronal and 36% astrocytic, similar to the percentage of infected neurons and astrocytes observed by Gray and colleagues (2011) after systemic injection of scAAV9/CBh-GFP (1×10^{10} VG/g) in adult mice. In the striatum, our data indicate a preferential expression of the GFP transgene in astrocytes, as previously reported (Foust *et al.*, 2009). Neuronal infection was estimated to be less than 20% in the striatal region targeted by FUS when injecting scAAV9-CB-GFP at 2.5×10^9 VG/g, based on our quantification of GFP-positive cell density and a previous study estimating the total density of neurons in the striatum (Rosen and Williams, 2001). Taken together, FUS-mediated scAAV9 delivery does not modify cell type

tropism compared with other brain delivery methods described for this serotype. The possible cellular mechanisms by which FUS can improve trans-BBB delivery of AAV9 include transcytosis, as well as trans- and interendothelial openings (Sheikov *et al.*, 2004; Deng *et al.*, 2012). In absence of FUS, previous studies suggested active transport mechanisms for AAV-based vector to cross the BBB (Gray *et al.*, 2011). The impact of MRIG-FUS on active and passive transport mechanisms and cell tropism for AAV9 remains to be fully investigated. To date, the use of a cell type-specific gene promoter remains the most attractive option to control transgene expression in a particular cell type, as described by Lawlor and colleagues (2009).

An scAAV9-CB-GFP dose of 2.5×10^9 VG/g was efficient at reaching FUS-treated brain areas, and it resulted in significantly less peripheral infection than the usual systemic dose of 8×10^{10} VG/g or more, required for CNS infection in adult mice without FUS (Gray *et al.*, 2011). Indeed, GFP expression in the liver, heart, kidney, and muscle was notably reduced at an scAAV9-CB-GFP dose of 2.5×10^9 VG/g compared with 1.25×10^{10} VG/g. The liver is a major organ where infection levels need to be controlled and, in addition to lowering the dose, efforts are being concentrated on the development of new variants of the AAV9 serotype for reducing infection of the liver (Pulicherla *et al.*, 2011). Another approach is to incorporate specific neural promoters to optimize transgene transduction in brain cells (Lawlor *et al.*, 2009). Such strategies, combined with FUS and a low dose of AAV9 injected peripherally, could significantly reduce the level of infection in peripheral organs. Furthermore, we have evidence that injections of AAV9 at the time of sonication (instead of after the sonication, as done here) will be more efficient, allowing significant AAV9 delivery to the brain and further reducing peripheral infection.

Minimizing the dose of AAV administered systemically would be more cost-effective and safer for potential translation to the clinic, due to less infection of peripheral organs, and would limit the risk of an adverse immune response (Forsayeth and Bankiewicz, 2011; Gray *et al.*, 2011). The presence of neutralizing antibodies (NAbs) in AAV-based gene therapy can represent a challenge (Gray *et al.*, 2011; Rapti *et al.*, 2012). Neutralizing antibodies, in response to natural exposure affecting humans (Murphy *et al.*, 2009) and laboratory animals (Gray *et al.*, 2011; Rapti *et al.*, 2012), interact with AAV capsid proteins and potentially reduce infection efficiency of the vector. Reducing the amount of viral particles injected may be important to limit the potential AAV-mediated immunological response and allow repeated AAV administration as required. Efforts were made to keep the acoustic pressure and enhancement levels as low as possible to minimize FUS-mediated vascular and parenchymal damage and to avoid edema (O'Reilly *et al.*, 2011). The impact of FUS itself and FUS-mediated AAV gene transfer on inflammation remains to be fully characterized, and this will be of particular interest for future studies using therapeutic transgenes in animal models of disease. AAV-based therapy in patients with hemophilia is currently in clinical trial using viral vectors carrying the factor IX transgene at scAAV8 doses of 6×10^8 to 2×10^9 VG/g (Nathwani *et al.*, 2011). These doses are comparable to the scAAV9-CB-GFP dose of 2.5×10^9 VG/g used in our study. It will be of interest to monitor such clinical trials for safety, immunological

response, and other side effects related to this level of AAV vector administered intravenously.

In summary, MRIgFUS efficiently delivered scAAV9-CB-GFP from the bloodstream to specific brain region(s) targeted by FUS. GFP expression lasted for a minimum of 12 days and was found mainly in neurons and astrocytes in FUS-targeted hippocampus and striatum, respectively. The relatively low dose of scAAV9 required for efficient MRIgFUS gene delivery to the brain produced limited infection of peripheral organs.

Our work highlights the potential of MRIgFUS to significantly improve gene therapy applications to the brain by delivering vectors from the circulation and in a noninvasive manner to specific brain areas that require treatment.

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Author Disclosure Statement

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