Safe and stable noninvasive focal gene delivery to the mammalian brain following focused ultrasound

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OBJECTIVE Surgical infusion of gene therapy vectors has provided opportunities for biological manipulation of specific brain circuits in both animal models and human patients. Transient focal opening of the blood-brain barrier (BBB) by MR-guided focused ultrasound (MRgFUS) raises the possibility of noninvasive CNS gene therapy to target precise brain regions. However, variable efficiency and short follow-up of studies to date, along with recent suggestions of the potential for immune reactions following MRgFUS BBB disruption, all raise questions regarding the viability of this approach for clinical translation. The objective of the current study was to evaluate the efficiency, safety, and long-term stability of MRgFUS-mediated noninvasive gene therapy in the mammalian brain.

METHODS Focused ultrasound under the control of MRI, in combination with microbubbles consisting of albumin-coated gas microspheres, was applied to rat striatum, followed by intravenous infusion of an adeno-associated virus serotype 1/2 (AAV1/2) vector expressing green fluorescent protein (GFP) as a marker. Following recovery, animals were followed from several hours up to 15 months. Immunostaining for GFP quantified transduction efficiency and stability of expression. Quantification of neuronal markers was used to determine histological safety over time, while inflammatory markers were examined for evidence of immune responses.

RESULTS Transitory disruption of the BBB by MRgFUS resulted in efficient delivery of the AAV1/2 vector to the targeted rodent striatum, with 50%–75% of striatal neurons transduced on average. GFP transgene expression appeared to be stable over extended periods of time, from 2 weeks to 6 months, with evidence of ongoing stable expression as long as 16 months in a smaller cohort of animals. No evidence of substantial toxicity, tissue injury, or neuronal loss was observed. While transient inflammation from BBB disruption alone was noted for the first few days, consistent with prior observations, no evidence of brain inflammation was observed from 2 weeks to 6 months following MRgFUS BBB opening, despite delivery of a virus and expression of a foreign protein in target neurons.

CONCLUSIONS This study demonstrates that transitory BBB disruption using MRgFUS can be a safe and efficient method for site-specific delivery of viral vectors to the brain, raising the potential for noninvasive focal human gene therapy for neurological disorders.

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KEYWORDS adeno-associated virus; gene therapy; focused ultrasound; blood-brain barrier; inflammation; rodent

GENE therapy has long held promise as a potentially groundbreaking method for improving a variety of complex disorders. The brain has been a major focus of translational gene therapy research, with several human clinical trials showing safety and evidence of efficacy for Parkinson's disease,^{19,22,24,25} Alzheimer's disease,^{40,46,47} and a variety of neurogenetic disorders. Due to the pres-

ence of the blood-brain barrier (BBB) and its highly selective permeability,^{1,27} the only current means for efficient delivery of viral vectors to specific regions in the human brain have been through invasive direct injection. Not only does this method carry the attendant risks of invasive surgery, but also efficient distribution of gene therapy agents throughout a target area can be difficult to confirm with

ABBREVIATIONS AAV = adeno-associated virus; AAV1/2 = AAV serotype 1/2; BBB = blood-brain barrier; BSA = bovine serum albumin; DAB = 3,3'-diaminobenzidine; DAPI = 4',6-diamino-2-phenylindole; Gd-DTPA = gadopentetate dimeglumine; GFP = green fluorescent protein; MRgFUS = MR-guided focused ultrasound; PBS = phosphate-buffered saline; rAAV = recombinant AAV; RF = radiofrequency; TBST = Tris-buffered saline with Triton.

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traditional infusion methods. Newer approaches have been tested that permit monitoring of contrast spread during infusion as a surrogate for viral vector distribution utilizing specialized catheter systems with intraoperative MRI methodology.^{9,41}

To reduce surgical risks and avoid the complexities of direct infusion, noninvasive approaches have been explored to permit intravenous delivery of viral vectors into the brain. Use of an osmotic agent such as mannitol has long been known to transiently open the BBB, permitting delivery of a variety of agents to the brain, including viral vectors.^{5,34,39,42} However, systemic administration of mannitol induces widespread opening of the BBB, precluding target-specific gene expression. Selective intra-arterial delivery of BBB disruption agents could provide more targeted gene delivery and cover larger brain areas, but variability in the vascular supply of various important deep-brain structures creates challenges for reproducible delivery between individuals.¹⁰

An alternative to chemical delivery is mechanical disruption of the BBB. One approach that has recently gained increasing interest is MR-guided focused ultrasound (MRgFUS).^{15,16,29} This approach involves focused delivery of ultrasound to a target region, and high-frequency MRgFUS has been used in human patients to create targeted brain lesions to treat essential tremor and pain.^{6,7,17} Use of MRgFUS at lower frequency, in combination with microbubble-mediated cavitation, has been shown to focally open the BBB to facilitate transfer of drugs,⁴⁵ antibodies,¹⁸ and nanoparticles³² from the blood stream to the brain parenchyma. This has also been used for noninvasive gene delivery to the rodent brain.^{2,13,14,43,50} These reports have shown successful transfer of adeno-associated virus (AAV) vectors from the blood stream to the brain following MRgFUS-mediated BBB opening with variable efficiencies and with transgene expression evaluated for relatively short periods following delivery.^{2,13,43,50} Because the goal of gene delivery is long-term neuronal modification, long-term expression following MRgFUS-mediated gene delivery remains to be confirmed. This is particularly important because potential immune-mediated loss of gene expression or transduced cells, due to exposure of the brain to the immune system following BBB disruption, might not fully manifest until later time points, as has been observed in some gene transfer studies outside of the brain.^{3,4,23,30,48} Furthermore, the potential for provoking inflammation in brain parenchyma following the application of focused ultrasound has only been examined up to 2 weeks following either MRgFUS BBB disruption alone^{18,21} or delivery of AAV vectors.^{43,49} The potential consequences of long-term brain exposure to a potential immunogenic viral vector following BBB disruption remain unknown.

In this study we report that MRgFUS-mediated BBB disruption can lead to efficient delivery and wide distribution of AAV vectors to the intended brain target in rodents. We further demonstrate that gene expression is stable over extended periods of time (6–16 months), comparable to what we have historically observed with direct infusion. Finally, while we observed a mild initial inflammatory response for the first 2 days following BBB disruption, we

did not observe evidence of inflammation over the long term, and there was no evidence of behavioral or histological toxicity at any time point.

Methods

Recombinant AAV Vectors

AAV serotype 1/2 (AAV1/2) hybrid vector stocks, encoding the reporter gene green fluorescent protein (GFP) under the control of the CAG promoter, were prepared by packaging the plasmids into AAV particles containing capsid proteins for both AAV1 and AAV2 to create AAV1/2.GFP using a helper-free plasmid transfection system that we have described previously.^{20,31} Vectors were purified using heparin affinity chromatography and dialyzed against phosphate-buffered saline (PBS). Recombinant AAV (rAAV) titers were determined by quantitative polymerase chain reaction using cytomegalovirus enhancer-specific primers and adjusted to 10^9 vector genomic particles (vg) per microliter.

Animal Preparation and Experimental Design

All animal procedures were approved by the Institutional Animal Care and Use Committee of Weill Cornell Medical College and followed NIH guidelines. Ten-week-old male Sprague Dawley rats (250–300 g; Charles River Laboratories) were used in all studies. Four rats were used to assess the efficiency and safety of unilateral MRgFUS-mediated AAV1/2.GFP delivery to the striatum at 3 weeks after sonication. Another group of 15 rats underwent a similar procedure but were killed at different time points: 2 weeks ($n = 3$), 2 months ($n = 4$), 6 months ($n = 6$), and 16 months ($n = 2$). The brains and several organs (liver, lung, and heart) were then harvested and processed for histological analysis (Supplemental Fig. 1).

MRgFUS and Viral Vector Delivery

Animals were anesthetized using a ketamine (90 mg/kg) and xylazine (4 mg/kg) cocktail. A 22-G intravenous catheter (BD InsyteAutoguard) was inserted into the lateral tail vein for substance administration during experiments. After scalp shaving, animals were secured in a supine position on the focused ultrasound system and the head was coupled with the degassed water tank holding the transducer. The spherically focused transducer (7-cm diameter, $f\# = 0.8$) was driven by a computer-controlled function generator (33220A Agilent Function/Arbitrary 20 MHz waveform generator; Agilent Technologies) and a 43-dB radiofrequency (RF) power amplifier (FUS Instruments).

Before sonication, MRI was performed with a 3.0-T GE scanner, using a 4×7 -cm RF surface coil. T2-weighted axial images (10 slices) perpendicular to the direction of the ultrasound beam propagation were acquired before sonication to calculate the coordinates of the target. The transducer was then moved to the desired position using a motorized 3-axis positioning system (FUS Instruments). The striatum was sonicated in 4 points, 1.5 mm apart. Assuming a 49% loss of ultrasound power due to attenuation through the rat skull,⁴⁴ an estimated *in situ* rarefactional pressure of 0.97 MPa was applied at the sonication points, with a 1-Hz pulse repetition frequency, 10-msec burst

length, and 200-second total sonication time. The cocktail of rAAV and Optison microspheres (Perflutren Protein-Type A microspheres, mean size between 3 and 4.5 μm , dose between 0.4×10^8 microbubbles/kg and -0.64×10^8 microbubbles/kg; GE Healthcare Life Sciences) was administered simultaneously with sonication through the tail vein catheter, followed by MRI contrast agent Magnevist (gadopentetate dimeglumine [Gd-DTPA], 0.4 ml/kg; Bayer). T1-weighted images (7 slices) were collected at the conclusion of sonication to monitor the degree of the BBB opening based on contrast extravasation. The slice thickness was 0.8 mm, with a spacing of 0.2 mm.

Immunohistochemistry and Histology

Rats were deeply anesthetized with sodium pentobarbital (150 mg/kg) and transcardially perfused with ice-cold 0.1 M heparinized PBS (pH 7.4) followed by 4% (weight/volume) buffered paraformaldehyde solution. The brains, liver, heart, and lungs were removed, postfixed in the same fixative solution for 24 hours, and subsequently immersed in 30% (weight/volume) sucrose cryoprotective solution at 4°C. The brains were frozen and sectioned serially (6 series per brain) into 40- μm -thick sections in the coronal plane on an AO Spencer 860 sliding microtome. The organs were embedded in agar solution and 40- μm -thick sections were cut on a Leica VT1200 vibratome for histological analysis.

To determine the extent of the area transduced by GFP, an entire series of brain coronal sections per each animal was rinsed in Tris-buffered saline with 0.1% Triton (TBST). Following the quenching of endogenous peroxides with a 0.3% solution of hydrogen peroxide in TBST, sections were incubated in blocking solution (3% bovine serum albumin [BSA] and 2% goat serum in TBST) for 1 hour at room temperature and then for 24 hours at 4°C with a rabbit polyclonal anti-GFP antibody (Abcam, ab290, 1:4000). The following day, sections were rinsed in TBST before a 1-hour incubation with biotinylated secondary antibodies. Following several washes, sections were incubated with Vectastain Elite ABC kit (1:500) in TBST for 1 hour. Staining was visualized using a 3,3'-diaminobenzidine (DAB) peroxidase substrate solution.

Identification of the neurons transduced by GFP was performed using an immunofluorescence protocol. Sections were incubated in blocking solution (3% BSA and 2% goat serum in TBST) for 1 hour at room temperature and then for 24 hours at 4°C with a rabbit polyclonal anti-GFP (Abcam, ab290, 1:4000), and mouse monoclonal anti-NeuN (neuronal marker beta-tubulin III; Abcam, AB104224, 1:1000) antibodies. The following day, sections were rinsed and incubated in goat anti-rabbit Alexa Fluor 488 and goat anti-mouse Alexa Fluor 594-conjugated secondary antibodies (Life Technologies), and nuclei were stained with 4',6-diamino-2-phenylindole (DAPI; Invitrogen, 1:10,000). Hematoxylin and eosin staining was used to evaluate intact cells and tissue integrity.

For detection of inflammatory markers, sections were incubated with mouse monoclonal anti-Iba1 (Millipore, MABN92, 1:500) and rabbit monoclonal anti-GFAP (Abcam, ab7260, 1:1000) antibodies and the staining was visualized with goat anti-mouse Alexa Fluor 488 and goat

anti-rabbit Alexa Fluor 594-conjugated secondary antibodies (Life Technologies). The nuclei were stained with DAPI (Invitrogen, 1:10,000).

Image Analysis and Quantitative Analysis of GFP-Positive Area

Quantitative analysis of the GFP-transduced striatum was performed using ImageJ Fiji software. Z-stacks from 4 sections per animal were collected at 10 $\times$ magnification using an Olympus BX61 upright microscope, and cells were quantified from 20 random fields taken from a pre-defined region of interest within the sonicated striatum ($100 \times 100 \times 40$ - μm depth). The region of interest was kept constant between animals to permit between-animal comparison. To determine the proportion of GFP-transduced neuronal and nonneuronal cells, colocalization of GFP and NeuN was analyzed using immunofluorescence microscopy. Neuronal cell transduction rate was calculated by expressing GFP-positive neuronal cells as a percentage of the total number of neurons in the analyzed area. To quantify the distribution of neuronal and nonneuronal cells among GFP-positive cells, both GFP-positive neuronal cells and GFP-positive nonneuronal cells were expressed as a percentage of total GFP-transduced cells.

Statistical Analysis

A 2-tailed t-test was used for statistical comparison of all groups. All data are expressed as the mean value with standard error of the mean (SEM). When the p value was < 0.05, the difference was considered statistically significant.

Results

MRgFUS-Facilitated, AAV1/2-Mediated GFP Gene Transduction of Rat Striatum

To first evaluate the efficiency of our system to safely and transiently disrupt the BBB in the striatum, 3 rats underwent unilateral striatal MRI-guided 4-point sonication. A postsonication T1-weighted MR image demonstrated local extravasation of Gd-DTPA into the local brain tissue, and confirmed BBB disruption (Fig. 1A). Gd-DTPA extravasation overlapped the striatal area selected on the T2-weighted presonation MRI. Three weeks later, immunohistological analysis confirmed GFP transgene expression in the sonicated striatum, in both neuronal and nonneuronal cells, while no signal was detected on the contralateral side of the brain (Fig. 1B and C, Supplemental Fig. 2). DAB chromogenic staining of GFP transgene in a series of sections showed an extensive and efficient anteroposterior and dorsomedial GFP transduction of the targeted striatum within the volume of tissue subject to sonication (Fig. 1C). In addition, hematoxylin and eosin staining revealed no evidence of tissue damage in the sonicated area (Supplemental Fig. 2).

MRgFUS-Facilitated, AAV1/2-Mediated GFP Expression: Efficiency and Stability Over an Extended Period

Although long-term stability of AAV-mediated gene expression has been well established in the brain following

FIG. 1. MRgFUS facilitates AAV-mediated gene delivery to the brain. **A:** Gd-DTPA-enhanced T1-weighted images collected after sonication showed disruption of the BBB and Gd-DTPA extravasation in brain parenchyma (*dashed line*). **B:** The brain tissue was harvested 3 weeks after sonication and the colocalization of GFP and NeuN was visualized using immunostaining. Bar = 50 μm. **C:** DAB visualization of GFP transduction from serial sections centered on the targeted area (serial sections through the center of the targeted points).

direct infusion, factors such as exposure to the immune system following BBB disruption could influence longevity of gene expression and survival of transduced neurons. GFP immunostaining with DAB-peroxidase substrate performed in rat tissue collected 6 months after sonication revealed long-term expression of GFP transgene through extended brain parenchyma (Video 1).

VIDEO 1. Clip showing a 3D model of an MRgFUS-mediated GFP-transduced rat brain. This 3D model was created from brain sections immunostained for GFP expression and harvested 6 months after MRgFUS in an animal that underwent 4-point sonication. The 3D reconstructed rat brain revealed the volume of Gd-DTPA extravasated into brain parenchyma following the BBB opening. Copyright Michael Kaplitt. Published with permission. Click here to view.

Triple immunolabeling for the GFP transgene with NeuN and DAPI allowed us to quantify transduced neurons and nonneuronal cells from 2 weeks to 6 months following MRgFUS-facilitated AAV1/2.GFP delivery (Fig. 2). Quantification of GFP-positive neurons from the total number of neurons revealed transduction of between 50% and 74% of neurons within the analyzed region. A lower rate of transduction was observed at 2 weeks (50%), with higher transduction rates noted at 2 months (74%) and 6 months (63%; Fig. 2B). This could be consistent with the known pattern of AAV-mediated transduction, which generally increases over several weeks following delivery, but the relatively small number of animals in the 2-week group could also have influenced this variation. Analysis of the type of GFP-transduced cells revealed that neurons represented 86%–98% of total GFP-expressing cells (Fig. 2C), again consistent with our previous experience with

direct infusion of this particular AAV serotype. Immunohistological analysis of GFP-NeuN colocalization of 2 animals that were sacrificed at 16 months after MRgFUS-facilitated AAV-mediated GFP delivery revealed a similar prevalence of GFP neurons in the transduced striatum (Fig. 3, Supplemental Fig. 4). These two subjects were not part of the cohort of animals included in the time course study, but had been treated much earlier during the pilot period, and therefore it is difficult to directly compare cell numbers in these 2 animals compared with the rest of the time course cohort. Nonetheless, these data show that there is demonstrable ongoing expression 16 months after MRgFUS-mediated gene delivery. While the focus of our transducer was on the striatum, the nature of the conical ultrasound beam from the single curved transducer resulted in considerable BBB disruption in the overlying cortex. Transduction of the overlying cortex was similar to what we observed with the striatum, suggesting that MRgFUS is capable of efficient and stable gene delivery to different brain regions (Supplemental Figs. 5 and 6). Finally, neuronal quantification revealed no evidence of neuronal loss over time in either the sonicated striatum or cortex and no difference in these regions compared with the corresponding nonsonicated hemisphere (Supplemental Fig. 6).

Transient Peripheral MRgFUS-Facilitated GFP Gene Transduction

To noninvasively deliver AAV into the brain via MRgFUS, vectors were infused intravenously, which could also result in transduction of peripheral organs depending on the tropism of the serotype used. To determine the

FIG. 2. MRgFUS facilitates stable, long-term GFP transduction. **A:** High-magnification immunostaining for GFP and NeuN reveals a dominantly neuronal population of GFP-transduced cells in the striatum. Bar = 50 μ m. **B:** Bar graph showing that quantification of striatal GFP transduction is stable over time. GFP-positive neurons are expressed as a percentage of the total number of striatal neurons per 20 hpf per animal (see *Methods*). **C:** Bar graph demonstrating that the MRgFUS-mediated GFP transduction is restricted mainly to neurons.

extent of peripheral gene expression, the heart, lung, and liver were harvested at the different time points following unilateral striatal MRgFUS and immunostained for GFP expression. While GFP was detected in the liver 2 weeks after sonication, no signal was detected at later time points (6 months and 16 months), consistent with a likely immune-mediated loss of gene expression as observed in other studies of foreign transgenes in this organ.^{3,23} Heart and lungs did not test positive for GFP at any time point (Fig. 4). As a negative control, we harvested these same organs from animals in which AAV1/2.GFP was delivered by direct infusion into striatum, with no evidence of gene expression in any organ.

MRgFUS and Transitory Local Inflammatory Response

The opening of the BBB, even when transient, could permit passage of various components from the blood stream into brain parenchyma. Previous studies have reported evidence of inflammation in the targeted brain soon after MRgFUS. No astrogliosis or microgliosis was detected in either sonicated or nonsonicated striatum at 2 weeks, 2 months, and 6 months after MRgFUS BBB disruption and AAV delivery (Fig. 5). No evidence of inflammatory response or tissue damage in the sonicated area was detected at any time point in both striatum and cortex (Fig. 5; Supplemental Figs. 3, 6, and 7). Given prior reports of transient inflammation following MRgFUS-mediated BBB disruption alone without delivering any agent to the brain,^{18,21} we performed striatal MRgFUS BBB opening

alone in additional animals, killed at 3, 24, and 48 hours, and confirmed (as expected) an increase in both Iba1 and GFAP staining at these early time points in both striatum and cortex (Supplemental Figs. 8 and 9). Finally, while we did not conduct formal behavioral testing, regular gross observation revealed no evidence of abnormal behavior, and periodic monitoring of body weights and food intake showed no evidence of gross metabolic abnormality to suggest poor health.

Discussion

Minimally invasive or noninvasive therapies are increasingly attractive for diseases traditionally treated with invasive neurosurgical procedures. This is highlighted by the increasing application of radiosurgery for tumors and functional diseases, endovascular therapies for vascular diseases, and the recent interest in MRgFUS thalamotomy for essential tremor. Gene therapy in the nervous system remains experimental, but translation of human gene therapy has been led by the neurosurgical community with promising results from human studies. To date, all human CNS gene therapy studies have required direct surgical infusion due to the size of the viral particles and the presence of an intact BBB, which precludes efficient transfer of viral vectors from the blood to the brain. However, an advantage of direct infusion that would be lost with widespread delivery is the ability to manipulate only a defined population of neurons while avoiding altering off-target

FIG. 3. Detection of striatal MRgFUS-facilitated AAV-mediated GFP transduction 16 months after sonication. Gd-DTPA-enhanced T1-weighted images collected after sonication showed disruption of the BBB and Gd-DTPA extravasation in brain parenchyma (*dashed line*). Histological analysis of the brain harvested 16 months after sonication showed GFP transduction mostly in neurons. Bar = 50 μ m.

cells, which could cause adverse or unintended effects. Previous studies have used MRgFUS BBB disruption to deliver viral vectors to the brain, but the efficiency, long-term stability of gene expression, and long-term safety of this approach remain unknown. Our results demonstrate that microbubble-facilitated MRgFUS successfully mediated passage of an AAV1/2 viral vector from the blood stream following intravenous administration into the targeted striatum without transduction of cells outside the zone of ultrasound delivery. This resulted in efficient and long-term GFP transduction within the targeted striatal neurons for more than 1 year and caused no obvious toxicity, neuronal loss, or long-term inflammatory response as measured by an absence of sustained astrocytic or microglial proliferation at various time points.

MRgFUS follows previous approaches to gene delivery through intravascular administration of viral vectors. The most common has been systemic use of an osmotic agent such as mannitol, which has been shown for many years to transiently open the BBB and has been used successfully in human patients for drug delivery.^{33,36–38} Several studies have evaluated the role of mannitol in facilitating global distribution, broad dispersion, and AAV-mediated gene expression in the brain.^{11,12,26} In addition, intraarterial administration of mannitol with an AAV vector allowed limited BBB opening followed by target-specific gene expression. Real-time MRI visualization of Gd after BBB disruption was observed immediately upon intraarterial mannitol administration.¹⁰ However, there are two caveats regarding the use of mannitol for transient BBB disruption. First,

systemic administration of mannitol induces BBB opening throughout the brain. This could be highly desirable for widespread diseases, such as pediatric genetic disorders or infiltrative cancers,^{10,28,35} but could be problematic when delivering genes that could be therapeutic in one brain region but harmful in other areas, such as the glutamic acid decarboxylase gene for Parkinson's disease.^{8,19,22} Site-specific delivery of mannitol via an intraarterial catheter could overcome this problem, but the variability in vascular supply to specific brain regions and the lower limit of the caliber of vessels that can currently be accessed could make reliable transduction of defined targets between individuals with limited off-target transduction more difficult.

One possible concern about MRgFUS BBB disruption is the potential for causing inflammation and tissue damage following brain exposure to the systemic immune system. This could be of particular concern when delivering a potential immunogen, such as a viral vector. Earlier studies in rodents have shown evidence of inflammatory reactions following FUS BBB disruption alone, without delivery of any agents. One report, using the same inflammatory markers that we used here, showed increased microglial activation several hours after BBB disruption, with an astrocytic response observed several days later.¹⁸ A recent study has more extensively examined both cellular and humoral mediators of inflammation from 1 to 24 hours following MRgFUS BBB disruption and reported evidence of a sterile inflammatory response.²¹ The goal of our study was to explore long-term consequences of MRgFUS delivery of gene therapy agents to the brain to determine the potential translational therapeutic relevance of this approach, so we did not examine inflammatory responses in such fine detail in the first 24–48 hours following delivery. Nonetheless, we did see some evidence of early microgliosis and astrogliosis between 3 and 48 hours following MRgFUS BBB disruption alone, consistent with these earlier reports.²¹ However, this appeared to resolve over a period of days, and was not evident at our 2-week through 6-month time points. We also saw no evidence of gross tissue damage or neuronal loss, nor any obvious behavioral abnormalities, to suggest significant toxicity from MRgFUS-mediated delivery of AAV vectors to the striatum under the conditions used in our study. It should be noted that immune reactions are generally more profound after subsequent exposures, and one potential advantage of gene therapy is the ability to provide long-term benefits from a single treatment. However, should more than one treatment become necessary for particular applications of MRgFUS BBB disruption, regardless of whether this is used for gene transduction or delivery of any other agent, the transient inflammatory reactions observed in both our study and earlier reports could represent a greater concern and should be studied carefully prior to considering clinical applications.

Another issue highlighted by our study is the problem of transduction of systemic organs with intravenous viral vector administration, even if MRgFUS restricts delivery within the brain. This could not only lead to production of potentially bioactive molecules from the gene of interest in undesirable organs, leading to adverse consequences, but also provoke inflammatory reactions that could result in organ tissue damage. In our study, a dose of 10^9

FIG. 4. MRgFUS-facilitated AAV-mediated gene delivery in peripheral organs is present in the short term but not the long term. Analysis of high-power immunofluorescent images of tissue collected from animals with MRgFUS with AAV1/2.GFP, and animals with AAV1/2.GFP stereotactically administered in the striatum shows no long-term GFP transgene expression in the liver, heart, and lungs. Bar = 100 μ m. FUS = MRgFUS.

vg/g of AAV1/2.GFP administered via a tail vein catheter was sufficient to efficiently transduce the sonicated area in the brain. Under these conditions, we did not observe GFP expression in the heart or lungs at any time point. However, the liver revealed GFP expression at 2 weeks, which was lost at later time points. This could represent either a loss of gene expression, loss of cells, or both, and extensive analyses of the causes of this observation in the liver are beyond the scope of our current study. However, previous reports have examined the phenomenon of loss of expression in peripheral organs several weeks after AAV transduction. Often this appears to be an immune-mediated effect.^{3,4,23} While GFP is a foreign gene, which could provoke immune responses that might not be observed with delivery of genes encoding native proteins, immune reactions against the AAV vector used to transduce the

cells can also cause loss of expression even after transduction has successfully occurred.^{3,23} In fact, the loss of GFP expression in the liver several weeks following gene delivery without loss of expression in the brain further supports the likelihood that restoration of brain immune privilege following closure of the BBB was achieved prior to onset of GFP expression. Nonetheless, to both limit off-target effects of gene expression in peripheral organs and prevent possible immune-mediated tissue injury, development of methods to restrict viral vector transduction of peripheral organs following intravenous delivery may be very important for successful clinical translation of MRgFUS-mediated gene delivery for many CNS disorders.

There are several limitations of our study, which could be addressed in the future. As a pilot study to determine the safety and stability of gene expression, we included a

FIG. 5. No evidence of long-term inflammatory response induced by unilateral striatal MRgFUS-mediated gene transfer to the brain. Both Iba1 (microglia marker) and GFAP (astrocytic marker) returned to baseline levels by week 2, suggesting that local inflammatory response is transitory. Bar = 50 μ m.

relatively small number of animals in each group, particularly at the 16-month time point, although there was consistency among animals at each time point. Therefore, more infrequent complications may not have been detected here. Furthermore, the rodent device that we have employed uses a single transducer element, so the area of focus is rather large compared with what might be achieved with an array of transducers, such as the system currently in use for human lesioning. The rodent immune system also may not accurately reflect the human condition, and ultimately it will be important to replicate our findings in other species to gain sufficient confidence prior to considering human translation. It would also be interesting to compare the relative transduction efficiencies of direct injection and systemic delivery from MRgFUS to the same structures, but the current study was not designed to address this. Finally, while we did not observe significant long-term inflammation, cell death, or loss of expression, it is possible that use of other AAV serotypes or different viral vectors or other transgenes, as well as different ultrasound parameters, could lead to different results. While our results do not guarantee that all parameters and viral vectors will be equivalent without additional testing, our data do support the potential of MRgFUS as a safe and efficient means for noninvasive, stable, focal gene delivery in the mammalian brain.

Conclusions

Our study demonstrates that BBB disruption using MRgFUS can be a safe and efficient method for site-specific delivery of viral vectors to the brain, suggesting the potential for noninvasive human gene therapy. While further study is necessary, the long-term safety and stability of gene expression reported here provides a framework for future development of this approach. Since direct infusion of AAV vectors has been safely applied to many human patients for a variety of diseases, and at least one human clinical device was recently FDA approved to perform MRgFUS thalamotomy for essential tremor, all nec-

essary technology is currently available for translation of our findings into human studies. Further work is needed to address remaining questions, including the applicability to the primate brain and potential safety issues from peripheral gene expression and central inflammation, depending on the specific application. Nonetheless, our long-term safety and gene expression data support the continued development of this approach as a potentially viable future option for noninvasive focal CNS gene therapy.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: Kaplitt, Stavarache, Ballon. Acquisition of data: all authors. Analysis and interpretation of data: Kaplitt, Stavarache, Petersen, Ballon. Drafting the article: Kaplitt, Stavarache. Critically revising the article: Kaplitt, Stavarache. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Kaplitt. Statistical analysis: Kaplitt, Stavarache. Administrative/technical/material support: Kaplitt. Study supervision: Kaplitt, Stavarache.

Supplemental Information

Videos

Video 1. <https://vimeo.com/242943981>.

Online-Only Content

Supplemental material is available with the online version of the article.

Supplemental Figs. 1–9. <https://thejns.org/doi/suppl/10.3171/2017.8.JNS17790>.

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