Gene therapy has entered clinical trials for the treatment of neurodegenerative disorders and chronic pain, and has shown promise in preclinical animal models for the treatment of spinal cord injury, spinal muscular atrophy, and amyotrophic lateral sclerosis. Gene therapy directed to the central nervous system could realize its full potential upon the development of safe and effective delivery methods capable of targeting gene transfer to the desired location non-invasively.

Both the blood–brain barrier (BBB) and the blood–spinal cord barrier (BSCB) are characterized by the presence of tight junctions and reduced active transport. Large molecules (>500 Da) of low lipid solubility, and with no active transporter, do not readily pass the BBB and BSCB. The development of non-invasive approaches to increase the delivery of therapeutics from the blood to the brain and spinal cord has been an area of great research interest. Transcranial focused ultrasound (FUS), when used in conjunction with systemically circulating microbubbles, has the ability to transiently increase the permeability of the BBB. This modulation of the BBB is observed in conjunction with a downregulation of tight-junctional proteins (for example, zona occludens-1, claudin-1, claudin-5 and occludin), and an upregulation of active transport proteins such as caveolin-1. The BBB permeabilization is transient, lasting for ~4–6 h after sonication. FUS-mediated BBB modulation has been used to deliver large agents such as antibodies (~150 kDa), viral vectors (~20 nm), and stem cells (~8–10 μm) to targeted brain areas. Positive therapeutic response to agents delivered using FUS BBB modulation has been observed in mouse models of cancer and neurodegenerative diseases.

In addition, previous studies show that microbubble-mediated FUS treatment alone increases adult neurogenesis and dendritic plasticity. FUS-mediated BSCB increase in permeabilization has the potential to facilitate drug, cell and gene therapies for spinal cord ailments such as tumors, injury or diseases like amyotrophic lateral sclerosis. However, ultrasound can be scattered by heterogeneous materials such as bone, and the complexity of the vertebrae poses a challenge for the translation of FUS-mediated BSCB permeabilization to the spinal cord. Advances in the field have led to a preliminary investigation demonstrating the feasibility of transient increase in permeabilization of the BSCB. Here, we demonstrate FUS-mediated BSCB permeabilization in a rat model under magnetic resonance imaging (MRI) guidance and its application for gene delivery using self-complementary adeno-associated virus serotype 9 (scAAV9).

**RESULTS**

FUS treatments were performed with an ultrasound transducer located below the animal placed in dorsal recumbency, enhancing BSCB permeability at the level of the cervical spine (Figures 1a and b). scAAV9 encoding green fluorescent protein (scAAV9-GFP) was injected intravenously at doses of $4 \times 10^8$, $2 \times 10^9$ and $7 \times 10^9$ vector genomes per gram (VG g$^{-1}$). FUS-mediated BSCB increase in permeabilization leads to robust GFP expression in FUS-targeted regions of the spinal cord. At a dose of $2 \times 10^9$ VG g$^{-1}$, GFP expression was found in 36% of oligodendrocytes, and in 87% of neurons in FUS-treated areas. FUS applications to the spinal cord could address a long-term goal of gene therapy: delivering vectors from the circulation to diseased areas in a non-invasive manner.

**Non-invasive delivery across the blood–spinal cord barrier (BSCB) remains a challenge for treatment of spinal cord injury and disease. Here, we demonstrate the use of magnetic resonance image-guided focused ultrasound (MRIgFUS) to mediate non-surgical gene delivery to the spinal cord using systemically injected adenovirus serotype 9 (scAAV9). scAAV9 encoding green fluorescent protein (GFP) was injected intravenously in rats at three dosages: $4 \times 10^8$, $2 \times 10^9$ and $7 \times 10^9$ vector genomes per gram (VG g$^{-1}$). MRIgFUS allowed for transient, targeted permeabilization of the BSCB through the interaction of focused ultrasound (FUS) with systemically injected Definity lipido-shealed microbubbles. Viral delivery at $2 \times 10^9$ VG g$^{-1}$ leads to robust GFP expression in FUS-targeted regions of the spinal cord. At a dose of $2 \times 10^9$ VG g$^{-1}$, GFP expression was found in 36% of oligodendrocytes, and in 87% of neurons in FUS-treated areas. FUS applications to the spinal cord could address a long-term goal of gene therapy: delivering vectors from the circulation to diseased areas in a non-invasive manner.**
and $7 \times 10^9 \text{VG g}^{-1}$ to the targeted region of the spinal cord (Figure 2). This resulted in GFP expression in oligodendrocytes (Figure 3) and neurons (Figure 4). At a dose of $2 \times 10^9 \text{VG g}^{-1}$ scAAV9-GFP, 36% of oligodendrocytes and 87% of neurons expressed GFP in FUS-targeted areas of the spinal cord. GFP expression was evident in the liver, minimal in the heart and marginally detected in the muscle (Figure 5).

### MRI enhancement and BSCB permeability

1.5-T MRI was used to visualize and target the cervical area of the spinal cord (Figure 1c). Following FUS treatment, BSCB permeabilization was confirmed by the entry of MRI contrast agent in localized areas of the spinal cord (Figure 1d, arrows). Using these FUS parameters, enhancement in the soft tissue surrounding the targeted spinal cord was occasionally noted (Figure 1d). In order to assess long-term safety of MRIgFUS treatment, two animals were reimaged using 7-T MRI 13 days post treatment (Figure 1e). No indications of fluid retention or structural abnormalities were observed on MRI (Supplementary Figure 1a).

In 11 out of the 12 rats treated, tissue collected 13 days post treatment showed no signs of red blood cell infiltration, damage or other histopathological abnormalities at the gross anatomy level (Supplementary Figure 1b). The temperature of liquid-lipid suspension used to form the microbubbles through mechanical agitation has been shown to affect bubble size distribution, and at lower temperatures results in the formation of larger bubbles. We found that in one animal treated at the same FUS parameters, but with microbubbles preactivated at ~4 °C, as opposed to room temperature, resulted in damage of the spinal cord. After FUS treatment this animal demonstrated front limb paresis, and 7-T MRI imaging performed 13 days post treatment showed significant structural abnormalities in the spinal cord tissue (Supplementary Figure 1c). Hematoxylin and eosin-stained sections from the FUS-treated area of this animal showed histopathological abnormalities (Supplementary Figure 1d). There were no clinical symptoms in the remaining 11 animals post treatment with microbubbles at room temperature.

### Gene expression following targeted MRIgFUS scAAV9-GFP delivery to the spinal cord

This experiment aimed to establish that MRIgFUS could be used to target one side of the cervical spinal cord in adult rats and deliver systemically injected scAAV9-GFP to that FUS-treated location, where GFP would then be expressed.

At the lowest dose of scAAV9-GFP ($4 \times 10^8 \text{VG g}^{-1}$), low levels of GFP expression were detected as illustrated in a longitudinal...
spinal cord section (Figure 2a), and transverse section from within the targeted area (Figures 2b and c). GFP expression was below detection levels on the contralateral side (Figure 2d). At the middle dose of scAAV9-GFP (2 × 10^8 VG g\(^{-1}\)), strong GFP expression was observed in longitudinal (Figure 2e) and transverse sections of the spinal cord within the FUS-treated area (Figure 2f). At higher magnification, GFP expression is evident on the treated side (Figure 2g), but not the contralateral side (Figure 2h). At the highest dose of scAAV9-GFP (7 × 10^9 VG g\(^{-1}\)), the longitudinal (Figure 2i) and transverse views (Figure 2j) of the FUS-treated area of the spinal cord showed the most abundant GFP expression compared to the middle and low doses. At higher magnification, GFP is more prominent on the treated side (Figure 2k) than on the contralateral side (Figure 2l).

scAAV9-mediated GFP expression by oligodendrocytes

In order to assess which cell types within the spinal cord express GFP after delivery of scAAV9-GFP with MRigFUS, tissue samples were stained with oligodendrocyte lineage transcription factor 2 (Olig2). At the lowest dose of scAAV9-GFP (4 × 10^8 VG g\(^{-1}\)), GFP expression in Olig2-positive cells was not detected (Figures 3a–c). At the middle dose (2 × 10^9 VG g\(^{-1}\)), GFP expression was abundant (Figure 3d) and present in some Olig2-positive cells (Figures 3e and f, arrow). At this dose, 36 ± 3% (mean ± s.d., n = 4) of Olig2-positive cells in FUS-treated regions of the spinal cord were GFP positive. At the highest dose (7 × 10^9 VG g\(^{-1}\)), GFP expression in the targeted area of the spinal cord was increased compared to the low and middle dose (Figures 3g–i).

scAAV9-mediated GFP expression by neurons

For assessment of GFP expression in neurons, tissue sections were stained with antibodies against the neuronal nuclear antigen (NeuN) and choline acetyltransferase (ChAT). Images were taken within the ventral horn of transverse sections from the treated cervical spine. At the lowest dose of scAAV9-GFP (4 × 10^8 VG g\(^{-1}\)), GFP expression was below detection levels in NeuN-positive (blue) and ChAT-positive (red) neurons (Figures 4a–e). Conversely, at the middle (2 × 10^9 VG g\(^{-1}\); Figures 4f–j) and high (7 × 10^9 VG g\(^{-1}\); Figures 4k–o) doses of scAAV9-GFP, ChAT- and NeuN-positive cells were found to express GFP (Figures 4h–j and m–o, arrows). At 2 × 10^9 VG g\(^{-1}\) scAAV9-GFP, 87 ± 4% (mean ± s.d., n = 4) of NeuN-positive cells were GFP positive in FUS-treated areas. Few cells positive for NeuN and weakly stained for ChAT did not express significant levels of GFP (Figures 4m–o, arrowheads). GFP expression was predominantly detected on the FUS-treated side at 2 × 10^9 and 7 × 10^9 VG g\(^{-1}\), and under the same imaging parameters no background was detected on the contralateral side with no FUS treatment (Figures 4a–c, f–h, k–m).

scAAV9-mediated GFP expression in peripheral organs

GFP expression was evaluated in peripheral organs and in the brain after tail-vein injection of scAAV9-GFP, paired with MRigFUS targeted to the spinal cord. GFP expression within the liver, heart, and muscle was evaluated (Figure 5). The highest level of GFP expression was seen in the liver (Figure 5a), whereas the fluorescence signal in the heart (Figure 5b) using the same confocal parameters was considerably lower. GFP expression in the muscle tissue (Figure 5c) when evaluated at these same confocal parameters was undetectable (data not shown). For this reason, the GFP signal in muscle tissue was enhanced using a GFP antibody coupled to Cyanine 3 (Cy3), and confocal settings were optimized for the detection of this fluorochrome (Figure 5c). GFP expression was not detected in the kidney or brain, despite signal enhancement with GFP antibody (data not shown). Topro3 iodide (Invitrogen, Eugene, OR, USA) was used as a nuclear stain.

DISCUSSION

Direct injection of therapeutic agents into the spinal cord carries the risks of complication due to needle placement, infection, nerve trauma, air embolism, disc entry, hematoma and...
hypersensitivity reaction. By using MRIgFUS, delivery of therapeutic agents to the spinal cord can be carried out non-surgically, greatly reducing the risks associated with direct injection. The current study is a proof of concept that MRIgFUS can be used for non-invasive gene delivery to the spinal cord. Briefly, a viral vector carrying a reporter protein (scAAV9-GFP) was injected intravenously, and MRIgFUS was used to target one side of the cervical spinal cord in adult rats, delivering the transgene across the BSCB at that location.

Systemic injection of AAV9 is an efficient method for gene delivery to the spinal cord in neonate rodents. This is due to the properties of the neonatal BSCB, which does not fully mature until ~14 days after birth. In animals with a mature BSCB, the dose of AAV9 injected intravenously needs to be much higher to effectively permeate the BSCB and reach the spinal cord (for example, 4 × 10^{12} DNase-resistant particles, and 1 × 10^{11} VG g^{-1}). Here, we show that the use of MRIgFUS as a means to increase BSCB permeability decreased the dose required for gene delivery to the spinal cord to 2 × 10^9 VG g^{-1} representing a 50-fold reduction from the previously reported minimum values. FUS-mediated gene delivery could therefore provide an alternative or complement to current intrathecal and intravascular strategies.

As observed in the mouse brain, FUS-mediated gene delivery to the spinal cord did not influence the tropism of scAAV9 toward a particular cell type. scAAV9-GFP injected intravenously at 2 and 7 × 10^9 VG g^{-1} and combined with MRIgFUS resulted in GFP expression in neurons including ChAT-positive motor neurons, and oligodendrocytes. Previous studies using higher dosages of intravenous AAV9, but without FUS, have shown gene transfer in a maximum of 28% of motor neurons, and 7% of oligodendrocytes. In addition, intravenous delivery of scAAV9 was shown to result in GFP expression in cells that were neither NeuN positive nor Olig2 positive. Evidence suggests that these cells may be astrocyte perivascular endfeet, or endothelial cells, which the virus would have been exposed to while crossing the BSCB. Identification of other GFP-positive cell types will be evaluated in future studies.

In addition to reducing the required effective dose of scAAV9 for gene delivery, MRIgFUS is able to target a localized area or preferential side of the spinal cord. This is of significant value toward the design of therapeutic approaches for spinal cord disorders or injury. Also, the ability to target one side of the spinal cord could eventually be used for the treatment of hemisection lesions, such as in Brown-Sequard syndrome, in that a therapeutic agent could be non-invasively delivered to only the injured side of the spinal cord. The non-FUS-targeted areas of the spinal cord in our study showed no GFP expression, allowing us to conclude that the delivery of the vector was mediated by MRIgFUS and not by the innate ability of scAAV9 to cross the BSCB. Furthermore, the lack of GFP expression in the brain...
scAAV9-mediated GFP expression by neurons at the lowest dose of scAAV9-GFP. GFP expression within the treated area of the spinal cord was below detection levels in ChAT (red)- and NeuN (blue)-positive cells (a-e). Images of the untreated side of the spinal cord also show no detectable GFP expression (a′-e′). At the middle dose, GFP expression was observed in ChAT- and NeuN-positive cells (f and g; h-j, arrow). On the contralateral side of the spinal cord, no GFP background expression was detected (f′-j′). At this dose and within the FUS-treated area of the spinal cord, 87 ± 4% (mean ± s.d., n = 4) of NeuN-positive cells expressed GFP. At the highest dose, most (k and l; m-o, arrow), but not all (m-o, arrowhead), ChAT- and NeuN-positive cells, expressed GFP. GFP expression on the untreated side was below detection levels (k′-o′). Scale bar (a, f, k), 50 μm. Scale bar (b-e, g-j, l-o), 20 μm.
indicates that scAAV9, at the doses tested, did not cross the BBB in an appreciable amount.

In order to assess gene expression in peripheral organs, the liver, kidney, quadriceps muscle and heart tissue were analyzed, with only the liver, heart and muscle showing GFP expression. These results are in agreement with previous studies in rats that found systemic scAAV9 delivery resulted in gene expression in the liver43 and heart.31,45 Studies investigating the systemic delivery of scAAV9 in mice also found abundant gene expression in skeletal muscle with some expression in the kidney.20,36,37 Our study is consistent with the findings that scAAV9 delivery does not result in significant gene expression in the rat kidney.43 This variance in gene expression by peripheral organs in mice and rat studies can be due to the dosages, preparations of scAAV9 and potential interspecies differences in cell surface expression of terminal N-linked galactose, which is the primary receptor for AAV9.45 One strategy that could be implemented to decrease peripheral gene expression would be the use of neural-specific promoters. In the case of scAAV9, there have been significant differences observed in motor neuron transgene expression after intrathecal injection in mice, when the promoter was varied between a cytomegalovirus promoter or a chicken-β-actin promoter, with scAAV9-cytomegalovirus inducing the greatest amount of gene expression in motor neurons.44 By varying the promoter, further regulation of gene expression after MRIgFUS-mediated gene delivery may be possible.

With regard to the animal that experienced post-treatment spinal cord injury, it has been shown that preactivation via temperature affects microbubble size distribution.31 Definity (Lantheus Medical Imaging, North Billerica, MA, USA) microbubbles are activated by mechanical agitation, which generates bubbles from a vial containing an aqueous solution of lipids with octafluoropropane gas; vials activated at a colder temperature produce a greater number of larger bubbles.31 Larger microbubbles can result in greater barrier permeability.45 The histology results of the remaining 11 animals, treated at the same FUS parameters, showed no signs of tissue damage, which further suggests that the injury seen in the first animal was caused by larger microbubbles, generated through activation of the vial at a lower temperature. A recent study that used FUS to intentionally generate a spinal cord injury model in rats demonstrated that FUS applied at an acoustic power of at least 1.3–1.6 W in the presence of microbubbles would result in such damage.46 When the acoustic power is limited to 0.73 W, as it was here, there was no evidence of spinal cord damage, when using microbubbles brought to room temperature before activation. The MRI enhancement in soft tissue immediately surrounding the FUS-treated area of the spinal cord is due to the small size of the animal relative to the focal characteristics of the transducer used in this study. In a clinical setting, a wider aperture transducer could be used, which would allow for a tighter focal spot.47 To provide additional safety to FUS-mediated BSCB permeabilization, real-time monitoring techniques developed to tune the exposure parameters for a controlled BBB permeabilization in transcranial FUS treatment48,49 could be adapted for application to the spinal cord.

Finally, it is known that FUS alone in other regions of the central nervous system can enhance neurogenesis, dendritic complexity26,27 and glial activation.25 Therefore, it will be of interest to evaluate the impact of FUS alone on neuronal and glial plasticity in the spinal cord, and assess the long-term benefits or risks of such potential remodeling.

In summary, an intravenous dose of 2 × 10^9 VG g⁻¹ was sufficient for targeted delivery of scAAV9-GFP to the rat cervical spinal cord resulting in significant gene expression in 87% of neurons and 36% of oligodendrocytes in the FUS-targeted region. MRIgFUS has the potential to be further developed for targeted, non-invasive gene therapy to the spinal cord.

**Materials and Methods**

**Animals**

This experiment used 12 ~ 300 g male Wistar rats (Charles River Laboratories, Portage, MI, USA). All of the animal procedures were carried out in compliance with the Canadian Council on Animal Care and the Animals for Research Act of Ontario guidelines, and with the approval of the Sunnybrook Research Institute Animal Care Committee.

**Virus**

scAAV9 was produced using a transient transfection procedure and a double-stranded AAV2-inverted terminal repeats based CB promoter-GFP vector, with a plasmid encoding the Rep2Cap9 gene sequence as previously described,46 as well as an adenoviral helper plasmid, pHelper (Stratagene, Santa Clara, CA, USA). The vector was then sequenced to verify that it was identical to that of the previously described AAV9 serotype 9. Vector purification was performed with two cesium chloride density gradient purification steps, dialylation against phosphate-buffered saline (PBS) and formulation with 0.001% Pluronic F-68 (Life Technologies, Burlington, ON, Canada) to prevent virus aggregation, before storage at 4 °C.47 Viral preparations were titered via quantitative PCR and TaqMan technology (Life Technologies). The vector purity was analyzed via 4–12% sodium dodeyl sulfate-acrylamide gel electrophoresis and silver staining (Invitrogen, Carlsbad, CA, USA).

The three dosages of scAAV9-GFP used for this study were 4 × 10^8 (n = 4), 2 × 10^9 (n = 6) and 7 × 10^9 (n = 2) VG g⁻¹ of treated animal.

**Magnetic resonance imaging-guided focused ultrasound**

Rats were anesthetized with isofluorane, and then with an additional mixture of ketamine (40–50 mg kg⁻¹) and xylazine (10 mg kg⁻¹) delivered via intramuscular injection. The hair was removed from the neck and back using an electric razor followed by depilatory cream. A 22-G angiocatheter was placed in the tail vein to facilitate delivery of microbubbles and the MRI contrast agent. Animals were placed in dorsal recumbency on the top plate of a three-axis positioner, operationally similar to that described by Chopra et al.,50 with their necks contacting a water bath. The ultrasound transducer was located on a positioning arm below the animal (Figure 1a).

Animals were imaged using a 1.5-T MRI (1.5T Signa, General Electric, Milwaukee, WI, USA). Pretreatment T2-weighted images were used for selecting the ultrasound targets. Contrast-enhanced baseline T1-weighted images were captured before sonication (Figure 1c). The cervical spine was selected for targeting to minimize MRI artifacts from heart motion. Ultrasound was generated using a 1.114-MHz spherically focused transducer (aperture: 7 cm, F-number: 0.8), driven using a function generator and radio frequency power amplifier. Sonications consisted of 10-ms ultrasound bursts at a repetition rate of 0.5 Hz, for a total of 5 min, targeted to the cervical spine. The acoustic power during the burst was kept below inertial cavitation threshold (based on earlier experiments) and set to 0.73 W. Definity microbubbles (0.02 ml kg⁻¹; Lantheus Medical Imaging) were injected into the tail-vein catheter at the start of sonication, followed by 0.5 ml saline. During each sonication, six spots were sonicated at 1-mm spacing to produce a band of BSCB permeabilization in the cervical spine (Figure 1b). Gadodiamide (0.2 ml kg⁻¹; Omniscan, GE Healthcare Canada, Missauga, ON, Canada) contrast-enhanced T1-weighted images were captured post FUS to assess the permeability of the BSCB (Figure 1d). Some animals received a second sonication if the first did not result in BSCB permeabilization, as assessed by MRI enhancement. Five to 30 min following ultrasound treatment, rats received an injection of 0.3 ml of viral solution via tail-vein catheter followed by 0.5 ml of saline to clear the catheter of any residual virus and ensure transfer into the bloodstream. After recovery, rats were returned to individual cages for 13 days.

**High-resolution MRI**

Images were acquired using a 7T Bruker Biospin (Bruker BioSpin MRI GmbH, Erltingen, Germany) post FUS at a resolution of 0.15 × 0.15 mm per pixel and 1-mm slice thickness in order to more precisely localize BSCB permeabilization. Contrast-enhanced axial images were acquired using a two-dimensional fast low angle shot MRI sequence (TE 2.47 ms, TR 200 ms, 10 averages, fractional anisotropy 65°, resolution 0.15 × 0.15 mm per pixel and 1-mm slice thickness). Six of the animals were imaged immediately following ultrasound treatment (Figure 1e), and two of these
animals were imaged 13 days post treatment to confirm that the BSCB was no longer permeable (Supplementary Figure 1a).

Histological processing
Thirteen days after FUS treatment, rats were deeply anesthetized with ketamine (75 mg kg\(^{-1}\)) and xylazine (10 mg kg\(^{-1}\)), and transcardially perfused with 0.9% saline solution, followed by 4% paraformaldehyde in 0.1 M PO\(_4\). The spinal cord, brain and organs including heart, kidney, quadriceps muscle and liver were collected and post fixed in 4% paraformaldehyde solution for 24 h and stored at 4 °C in 30% sucrose solution. Spinal cord sections were cut in either transverse or longitudinal orientation on a vibratome at room temperature. The distance to the treatment area was estimated, in millimeters from the brain stem, using the MRI enhancement images and ImageJ software (National Institutes of Health, Bethesda, MD, USA). For transverse sections, each spine was first sectioned into eight, 3-mm-thick pieces with the fourth piece (P4) representing the treatment target area (Figure 1f). Then 12, 50-μm thick sections were cut from the top of each piece for analysis. The bottom of each piece was marked with blue tissue dye (Davidson Marking System, Bradley Products, 1013-5, Bloomington, MN, USA) to indicate orientation. The brain and peripheral organs were mounted onto a sliding microtome with Tissue-Tek OCT (Sakura, Torrance, CA, USA) and frozen with dry ice for cutting into 40-μm-thick sections. Sections were stored at −20 °C in cryoprotective glycerol solution.

Histology
Hematoxylin and eosin staining was used on 50-μm-thick (\(n=11\)) and 5-μm-thick (\(n=7\)) spinal cord sections as previously described\(^5\), free-floating sections were mounted on paraffin-coated microscope slides and allowed to adhere for 72 h, before staining by standard hematoxylin and eosin procedures. Tissue integrity of the spinal cord in 11 animals was assessed from both within and outside the targeted region.

Immunohistochemistry
Free-floating spinal cord sections were washed three times for 10 min in PBS, and incubated for 1 h in blocking solution containing PBS with 10% donkey serum and 0.4% Triton X-100 (Sigma-Aldrich Canada, Oakville, ON, Canada) (PBS++). Sections were then incubated for 72 h at 4 °C in rabbit anti-GFP (1:500; Millipore, AB3080, Bedford, MA, USA), mouse anti-Olig2 (1:500; Millipore, MABN50), mouse anti-NeuN (1:500; Millipore, MAB377) or goat anti-ChAT (1:100; Millipore, AB144P) diluted in PBS++. The sections were then washed three times for 10 min in PBS. This was followed by incubation at room temperature for 1 h in the following secondary antibodies in PBS++: biotin-conjugated donkey anti-rabbit IgG (1:80; Jackson ImmunoResearch, 711-065-152, West Grove, PA, USA), Cy3-conjugated donkey anti-goat IgG (1:200; Jackson ImmunoResearch, 705-165-147) and Cy5-conjugated donkey anti-mouse IgG (1:200; Jackson ImmunoResearch, 715-175-150). After washing the sections twice for 10 min in PBS, they were incubated at room temperature in PBS++ with Alexa 488-conjugated streptavidin (1:200; Jackson ImmunoResearch, 016-540-084) for 2 h. After washing three times in PBS, sections were rinsed in 0.1 M PO\(_4\) and mounted on a microscope slide with polyvinyl alcohol (Sigma-Aldrich, St Louis, MO, USA) and 1,4-diazabicyclo(2.2.2)octane (Sigma-Aldrich) (PVA-DABCO) and a coverslip.

Brain, heart, and quadriceps muscle sections, cut at a thickness of 40 μm, were washed three times in Tris-buffered saline (TBS) (Tris HCl, Tris Base, and sodium chloride purchased from Sigma-Aldrich) for 10 min, before a 1-h incubation in blocking solution (TBS, donkey serum 7.5% and Triton X-100 (Sigma-Aldrich) 0.5%) at room temperature. Sections were incubated

Figure 5. scAAV9-mediated GFP expression in peripheral organs. scAAV9-mediated GFP expression in the liver (a), heart (b) and muscle (c). GFP detection in the muscle was enhanced using an antibody against GFP coupled to Cy3 (red). Topro3 iodide nuclear stain is shown here in blue. Scale bar, 50 μm.
Figure 5. (Continued).
in rabbit anti-GFP antibody (1:500; AB3080, Millipore) in blocking solution overnight at 4 °C. Sections were washed two times with TBS for 15 min before a 2-h incubation with donkey anti-rabbit IgG biotin (1:500; Jackson Immunoresearch, 711-065-152), diluted in blocking solution. After two 15-min washes in TBS, the sections were incubated with Cy3-conjugated streptavidin (1:500, Jackson Immunoresearch, 016-160-084) and Topro3 iodide (1:500; Invitrogen, T3605, Eugene, OR, USA) for 2 h at room temperature. Sections were then rinsed three times in TBS, and then once in 0.1 M PO4 before mounting on a microscope slide with PVA-DABCO and a coverslip.

Liver and kidney sections of 40-μm thickness were washed three times for 10 min in TBS, and then incubated for 1 h in blocking solution at room temperature. Sections were then incubated with Topro3 iodide (1:500; Invitrogen, T3605) in blocking solution at room temperature for 2 h. The sections were rinsed three times in TBS and once in 0.1 M PO4 before mounting on a microscope slide with PVA-DABCO and a coverslip.

Imaging

Images of spinal cord in Figures 2a and b, e, f, i, j were acquired using an Axiosoplan 2 imaging system (Carl Zeiss, Toronto, ON, Canada). Series of adjacent images were captured using a ×10 objective, which were then assembled into a single mosaic with Virtual Slide (Stereo Investigator, MBF Bioscience, Williston, VT, USA).

All other images were taken using either a Zeiss Axiostar 100/LSM 510, Zeiss AxiObserver Z1/LSM 700 or a Zeiss Z1 Observer/Yokogawa spinning disk (Carl Zeiss) microscope. Excitation wavelengths of 488 nm (GFP), 561 nm (Cy3) and 633 nm (Cy5) were used. Spinal cord images are shown as single confocal plane. Liver, muscle, and heart images as shown are a projection of five 1.13-μm Z-stacks. Image montages were prepared with Adobe Photoshop CS5 (Adobe Systems Incorporated, San Jose, CA, USA).

Cell counts

The percentages of Olig2-positive and NeuN-positive cells expressing GFP were quantified using stereology-based principles using Stereo Investigator (MBF Bioscience) on a Zeiss AxioImager M2 microscope (Carl Zeiss, Dublin, CA, USA). Three representative sections at the FUS-treated level of the spinal cord from animals receiving 2 × 10^6 Vg g^-1 (n = 4) were used. Quantification was performed with a ×63 oil objective using the optical fractionator probe from z-stacks collected following systematic random sampling. Regions of interest were at the FUS-treated level and included visible areas of GFP expression in the spinal cord at ×5 magnification. The average coefficient of error (Gundersen m = 1) was 0.04 for the number of Olig2-positive cells expressing GFP and 0.06 for the number of NeuN-positive cells expressing GFP.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the Centre for Spinal Trauma, Sunnybrook Health Sciences Centre, OHR FRN 93603 (A), NIH grant R01-EBOO328 (KH) and the Canada Research Chair Program (KH). We thank Dr Paul Nagy for helping to edit this manuscript. We also thank Dr Julie Korich and MBF Bioscience for their counsel in regard to cell counting.

REFERENCES

1 Goins WF, Cohen JB, Glorioso JC. Gene therapy for the treatment of chronic peripheral nervous system pain. Neurobiol Dis 2012; 48: 255–270.

2 Romero MJ, Rangappa N, Garry MG, Smith GM. Functional regeneration of chronically injured sensory afferents into adult spinal cord after neurotrophin gene therapy. J Neurosci 2001; 21: 8408–8416.

3 Nagahara AH, Tusuzynski MH. Potential therapeutic uses of BDNF in neurological and psychiatric disorders. Nat Rev Drug Discov 2011; 10: 209–219.

4 Passini MA, Bu J, Roskelley EM, Richards AM, Sardi SP, O’Riordan CR et al. CNS-targeted gene therapy improves survival and motor function in a mouse model of spinal muscular atrophy. J Clin Invest 2010; 120: 1253–1264.

5 Foust KD, Wang X, McGovern VL, Braun L, Bevan AK, Haidet AM et al. Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. Nat Biotechnol 2010; 28: 271–274.

6 Bevan AK, Duque S, Foust KD, Morales PR, Braun L, Schmelzer L et al. Systemic gene delivery in large species for targeting spinal cord, brain, and peripheral tissues for pediatric disorders. Mol Ther 2011; 19: 1971–1980.

7 Meyer K, Ferraulo L, Schmelzer L, Braun L, McGovern V, Likhite S et al. Improving single injection CSF delivery of AAV9-mediated gene therapy for SMA: a dose–response study in mice and nonhuman primates. Mol Ther 2013; 21: 477–487.

8 Lepore AC, Haenggeli C, Gasmì M, Bishop KM, Bartus RT, Maragakis NJ et al. Intraperineal spinal cord delivery of adeno-associated virus IGF-1 is protective in the SOD1G93A model of ALS. Brain Res 2007; 1185: 256–265.

9 Reese TS, Karnovsky MJ. Fine structural localization of a blood-brain barrier to exogenous peroxidase. J Cell Biol 1967; 34: 207–217.

10 Partridge WM. Blood-brain barrier delivery. Drug Discov Today 2007; 12: 54–61.

11 Hynynen K, McDonnell N, Vyakhotskova N, Jolesz FA. Noninvasive MR imaging-guided focal opening of the blood-brain barrier in rabbits. Radiology 2001; 220: 640–646.

12 Shekow N, McDonnell N, Sharma S, Hynynen K. Effect of focused ultrasound applied with an ultrasound contrast agent on the tight junctional integrity of the brain microvascular endothelium. Ultrasound Med Biol 2008; 34: 1093–1104.

13 Zhang X, Zue Y, Liu Y, Shang X. Additive effect of low-frequency ultrasound and endothelial monocyte-activating polypeptide II on blood-tumor barrier in rats with brain glioma. Neurosci Lett 2010; 481: 21–25.

14 Fan L, Liu Y, Ying H, Xue Y, Zhang Z, Wang P et al. Increasing of blood-tumor barrier permeability through paracellular pathway by low-frequency ultrasound irradiation in vitro. J Mol Neurosci 2011; 43: 541–548.

15 Xia CY, Zhang Z, Xue YX, Wang P, Liu YH. Mechanisms of the increase in the permeability of the blood-tumor barrier obtained by combining low-frequency ultrasound irradiation with small-dose bradykinin. J Neurooncology 2009; 94: 41–50.

16 Deng J, Huang Q, Wang F, Liu Y, Wang Z, Zeng Z et al. The role of caveolin-1 in blood-brain barrier disruption induced by focused ultrasound combined with microbubbles. J Mol Neurosci 2012; 46: 677–687.

17 Hynynen K, McDonnell N, Vyakhotskova N, Raymond S, Weissleder R, Jolesz FA et al. Focal disruption of the blood-brain barrier due to 260-KHz ultrasound bursts: a method for molecular imaging and targeted drug delivery. J Neurosurg 2006; 105: 445–454.

18 Kinoshita M, McDonnell N, Jolesz FA, Hynynen K. Targeted delivery of antibodies through the blood-brain barrier by MR-guided focused ultrasound. Biochem Biophys Res Commun 2006; 340: 1085–1090.

19 Raymond SB, Treat LH, Dewey JD, McDonnell NJ, Hynynen K, Backsider BJ. Ultrasound enhanced delivery of molecular imaging and therapeutic agents in Alzheimer’s disease mouse models. PLoS One 2008; 3: e27175.

20 Jordao JF, Ayala-Grosso CA, Markham K, Huang Y, Chopra R, McLaurin J et al. Antibodies targeted to the brain with image-guided focused ultrasound reduces amyloid-beta plaque load in the TgCRND8 mouse model of Alzheimer’s disease. PLoS One 2010; 5: e10549.

21 Thevenot E, Jordao JF, O’Reilly MA, Markham K, Weng YQ, Foust KD et al. Targeted delivery of self-complementary adeno-associated virus serotype 9 to the brain, using magnetic resonance imaging-guided focused ultrasound. Hum Gene Ther 2012; 23: 1144–1155.

22 Grimm D, Kay MA. From virus evolution to vector revolution: use of naturally occurring serotypes of adeno-associated virus (AAV) as novel vectors for human gene therapy. Curr Gene Ther 2003; 3: 281–304.

23 Burgess A, Ayala-Grosso CA, Ganguly M, Jordao JF, Aubert L, Hynynen K. Targeted delivery of neural stem cells to the brain using MR-guided focused ultrasound to disrupt the blood-brain barrier. PLoS One 2011; 6: e27877.

24 Chen L, Mu Z, Hachem P, Ma CM, Wallentine A, Pollow A. MR-guided focused ultrasound: enhancement of intratumoral uptake of [3H]-docetaxel in vivo. Phys Med Biol 2010; 55: 7399–7410.

25 Jordao JF, Thevenot E, Markham-Coutles K, Scarcelli T, Weng YQ, Xhma K et al. Amyloid-beta plaque reduction, endogenous antibody delivery and glial activation by brain-targeted, transcranial focused ultrasound. Exp Neurol 2013; 248: 16–29.

26 Scarcelli T, Jordao JF, O’Reilly MA, Ellens N, Hynynen K, Aubert I. Stimulation of hippocampal neurogenesis by transcranial focused ultrasound and microbubbles in adult mice. Brain Stimul 2014; 7: 304–307.

27 Burgess A, Dubey S, Yeung S, Hough O, Eteman N, Aubert I et al. Alzheimer’s disease in a mouse model: MR imaging-guided focused ultrasound targeted to the hippocampus opens the blood-brain barrier and improves pathologic abnormalities and behavior. Radiology 2014; 273: 736–745.

28 Fry FJ, Barger JE. Acoustical properties of the human skull. J Acoust Soc Am 1978; 63: 1576–1590.

29 Kaufman JJ, Einhorn TA. Ultrasound assessment of bone. J Bone Miner Res 1993; 8: 517–525.

Gene Therapy (2015) 568 – 577
 Supplementary Information accompanies this paper on Gene Therapy website (http://www.nature.com/gt)