Foamy Virus Vectors Transduce Visceral Organs and Hippocampal Structures following In Vivo Delivery to Neonatal Mice

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Viral vectors are rapidly being developed for a range of applications in research and gene therapy. Prototype foamy virus (PFV) vectors have been described for gene therapy, although their use has mainly been restricted to ex vivo stem cell modification. Here we report direct in vivo transgene delivery with PFV vectors carrying reporter gene constructs. In our investigations, systemic PFV vector delivery to neonatal mice gave transgene expression in the heart, xiphisternum, liver, pancreas, and gut, whereas intracranial administration produced brain expression until animals were euthanized 49 days post-transduction. Immunostaining and confocal microscopy analysis of injected brains showed that transgene expression was highly localized to hippocampal architecture despite vector delivery being administered to the lateral ventricle. This was compared with intracranial biodistribution of lentiviral vectors and adeno-associated viruses (AAVs). LV vectors have been widely used for their ability to integrate into target cell genomes, which has been key to their recent success in stem cell gene therapy.8–12 AAV vectors have shown burgeoning potential for in vivo gene therapy, demonstrating safe and efficient transduction of the human brain by intracranial administration.13 Furthermore, systemic AAV delivery can deliver body-wide gene expression in humans14,15 and many animal models, with some vector serotypes able to cross the blood-brain barrier.16,17

Notwithstanding the recent successes of AAVs and LVs, these vectors suffer drawbacks that mitigate continued exploration of alternative systems. AAVs are restricted to transgene payloads below 5.2 kb,18–20 which restricts fine-tuning of therapeutic cassettes with additional regulatory genetic sequences, such as promoters, enhancers, and microRNA recognition sequences. Additionally, AAV vectors do not commonly integrate into target cell genomes; thus, vector copies are lost after repeated cell divisions.21 LV vectors can package considerably larger genes than AAVs and efficiently integrate their DNA into host chromosomes, which has made them a popular choice for stem cell

INTRODUCTION

Since the mid-1980s, recombinant DNA technology and the nascent field of viral vectorology have been developed as therapeutic tools for the treatment of inherited genetic diseases.1–6 Many viral vector systems have been explored, with each providing a unique set of characteristics that can be exploited for a specific purpose.7

Two of the most widely used viral vectors in research and clinical gene therapy are based on lentiviruses (LVs) and adeno-associated viruses (AAVs). LV vectors have been widely used for their ability to integrate into target cell genomes, which has been key to their recent success in stem cell gene therapy.8–12 AAV vectors have shown burgeoning potential for in vivo gene therapy, demonstrating safe and efficient transduction of the human brain by intracranial administration.13 Furthermore, systemic AAV delivery can deliver body-wide gene expression in humans14,15 and many animal models, with some vector serotypes able to cross the blood-brain barrier.16,17

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The use of PFV vectors in vivo has recently been demonstrated in gene transfer to the regenerating limb tissue of salamanders and for transduction of juvenile pig liver by hydrodynamic injection. Additionally, PFV vectors have been used to deliver their genomic RNA as mRNA in vivo. However, broader use of PFV vectors for permanent in vivo transgene delivery remains largely unexplored in mammals.

Here we investigated the in vivo transduction characteristics of PFV vectors by delivering transgenes to neonatal mice via intracranial, intravenous, intraperitoneal, and subcutaneous routes of administration. Systemic PFV vector delivery gave expression in a range of visceral organs, whereas intracranial administration gave a region-specific expression profile localized to hippocampal architecture. This hippocampal expression pattern was not observed in mice that received intracranial LV and AAV vectors via the same route of delivery, even when administered at lower doses than PFV vectors. Thus, our data introduce PFV vectors as unique gene transfer agents for use in research and gene therapy, and their discrete brain expression profile provides a novel approach for accurate manipulation of brain function.

RESULTS

PFV vectors were packaged with either EGFP or luciferase (Luc) driven by the cytomegalovirus (CMV) promoter. PFV-EGFP and PFV-luciferase vectors were delivered to neonatal outbred CD1 mice by intraperitoneal (i.p.), intravenous (i.v.), subcutaneous (s.c.), and intracranial (i.c.) injection on the day of birth.

Intravenous PFV Administration to Neonatal Mice Produces Expression in Visceral Organs

Whole-body bioluminescent images were captured and quantified 13 days and 49 days after neonatal intraportal, intravenous, and subcutaneous administration of PFV-Luc (Figure 1). For intraportal delivery, the mean total flux was calculated as $2.6 \pm 1.3 \times 10^8$ photons/s 13 days post-injection. Expression from intraportal delivery remained detectable 49 days post-injection, at which point mean flux was detected at $4.0 \pm 6.2 \times 10^7$. Subcutaneously-injected animals gave a mean flux of $1.1 \pm 0.7 \times 10^7$ on day 13 and $1.7 \pm 1.1 \times 10^6$ on day 49. Intravenous injection was poorly tolerated; three of the four PFV-luciferase cohorts died before the first imaging time point. The remaining animal gave a mean luciferase reading of $8.4 \times 10^7$ on day 13, which was detected at $1.3 \times 10^6$ on day 49.

PFV-EGFP-injected animals were dissected after 11 days to investigate tissue tropism in greater detail (Figure 2). Intravenous delivery gave expression in the heart, liver, lung, and spleen, whereas intraportal injection transduced the xiphisternum, liver, pancreas, and gut.

Intracranial PFV Administration to Neonatal Mice Gives Discrete Hippocampal Transgene Expression That Is Not Seen with LV and AAV Vector Technologies

Neonatal mice received a unilateral intracranial injection of PFV-luciferase, with bioluminescence quantified at two time points (Figure 3A). 11 days post-injection, we detected luciferase expression in...
the brains of all animals, with some expression detectable in the spinal cord (mean value of 5.1 ± 1.8 \times 10^6 \text{ photons/s}). Subsequent imaging 49 days post-injection showed that luciferase expression remained in the brain but was no longer detectable in the spinal cord (mean value of 6.2 ± 2.3 \times 10^5 \text{ photons/s}). Brain expression produced 8.2-fold less bioluminescence signal at the later time point (p = 0.0495 by Kruskal-Wallis test) (Figure 3B).

We suspected that the higher expression level on day 11 may have been produced by transient expression from plasmid DNA carried over from PFV vector production. We investigated this by injecting 1.5 \mu g (equivalent to total plasmid used to transfect a whole 10-cm dish of producer cells) of naked plasmid DNA expressing a luciferase expression cassette, but the resulting bioluminescence was indiscernible from uninjected controls 11 days post-injection (Figure S3).

To investigate the intracranial localization of PFV expression, we administered PFV-EGFP to the brains of newborn mice on post-natal day 1, with unilateral intracranial injections aimed toward the anterior horn of the lateral ventricle on the left side of the brain. Post-mortem analysis 11 days post-injection showed EGFP fluorescence through the top of the skull to the left of bregma, which was the approximate site of injection (Figure 3C). Dissected brains were further analyzed by immunostaining to investigate intracranial PFV biodistribution (Figures S1A and S2). Focused evaluation of the stained brain regions revealed that EGFP expression was localized to the hippocampus (Figure 4A). Further magnification revealed dense expression localized to the dentate gyrus, with staining detected throughout the associated architecture.

LV and AAV vectors are commonly used for brain gene therapy, having demonstrated widespread transduction of the mouse brain in a variety of applications.\textsuperscript{16,17,48-50} Thus, we sought to compare the intracranial biodistribution of PFV-EGFP vectors with AAV and LV vector technologies.

LV-EGFP vectors were delivered at a lower dose (4.2 \times 10^6 \text{ genome copies}) than PFV-EGFP vectors (2.5 \times 10^7 \text{ genome copies}), but LV
clearly showed greater diffusion from the lateral ventricle injection site, with staining detected in a variety of brain structures (Figure 4B; Figure S1B). However, administration of LV-EGFP to adult mice limited vector diffusion through the brain, with expression restricted to the hippocampus, piriform cortex, and olfactory bulb at lower levels than observed after neonatal injection (Figure S4).

AAV-EGFP was initially delivered to mice at a dose of $2.5 \times 10^{10}$ total vector genomes, producing widespread expression that migrated extensively from the injection site (Figure S1C). However, to account for the 3 orders of magnitude difference between PFV and AAV vector doses, we additionally administered AAV-EGFP at a lower dose ($2.5 \times 10^6$ vector genome copies) to interrogate the importance of vector genome copy number in the hippocampal localization of PFV-EGFP. Surprisingly, the lower dose of AAV-EGFP continued to exhibit broader expression than PFV-EGFP, suggesting that vector genome copy number alone was not the exclusive reason for intracranial biodistribution (Figure 4C).

To confirm the phenotype of EGFP-expressing cells, brain sections were stained for EGFP and the neuron-specific marker NeuN, with z stacks captured by confocal microscopy. Confocal images showed that PFV-treated sections were localized to neurons of the dentate gyrus, aside from some non-specific labeling of blood vessels because of suboptimal perfusion (Figure S5).

**DISCUSSION**

Viral vectors are rapidly being developed as gene therapy agents for an increasing range of diseases. This naturally brings a growing demand for broader functionality and diversity of viral vectors and a need to thoroughly examine novel and existing vector technologies to fully understand their capabilities. Here we have shown that PFV vectors are capable tools for direct *in vivo* gene delivery, particularly in the developing neonatal hippocampus, which adds a new dimension to their role in the growing field of gene therapy.

The ability of PFV vectors to integrate into the host genome makes them an appropriate choice for *ex vivo* gene therapy. PFV vectors have been assessed extensively for *ex vivo* manipulation of hematopoietic stem cells and engraftment in disease models in rodent and canine models. Our study provides a comprehensive examination of their biodistribution and efficacy following *in vivo* gene delivery to neonatal mice.

Our investigations were not only designed to show proof of concept for neonatal gene therapy with PFV vector technology but also to investigate the behavior of these vectors in postnatal tissues for physiological research purposes. It has been reported that PFV vectors absolutely require mitosis for efficient transduction of target cells, but these vectors can form a stable transduction intermediate in quiescent (G0) cells. In our investigations, we restricted our studies to newborn mice because we expected that murine organs would retain some degree of mitotic activity during postnatal development.

Systemic injection of PFV vectors gave transgene expression in several visceral organs, with luciferase expression detectable until termination of the experiments 49 days post-injection. However, our imaging data showed that EGFP-positive cells were sparsely distributed throughout the transduced organs, suggesting inefficient transduction. This was potentially due to the unique transduction characteristics of PFV vectors and their requirement for mitosis.
It is possible that the EGFP-positive cells were those that remained mitotically active in the early postnatal period. This has important implications for therapeutic use of systemically delivered PFV vectors because transduction efficiency may not reach the levels required for gene restoration, even when administered to neonates. But PFV may alternatively possess a useful property in its ability to target cells that remain mitotically active during postnatal development.

PFV vector administration to the neonatal mouse brain gave CNS expression on day 13, which fell 8.2-fold by day 49 post-injection. Direct PFV injection into the CNS has previously been compared with LV vectors in adult rats, where it has been reported that PFV vector transduction was less stable than LVs, potentially because of restrictions on PFV transduction of post-mitotic tissues. The expression pattern we observed in dorsal brain regions and the spinal cord were most likely due to vector migration through cerebrospinal fluid. The comparatively low level of spinal cord expression on day 13 potentially explains the absence of detectable expression in this region on day 49, given that vector expression in other regions fell substantially across this period.

The fall in expression seen in all tissues between days 13 and 49 could be attributed to a number of factors. Bioluminescent signaling regularly falls after neonatal luciferase gene transfer, potentially because of expansion of non-transduced cells, restricting bioluminescence from deeper transduced tissues. Additionally, subgenomic RNA copies are known to be packaged into PFV vector particles during production, which may have contributed to higher expression levels at the early time point. But our experiments showed that intracranial administration of naked plasmid DNA gave expression that was indistinguishable from uninjected controls, indicating that early expression was primarily derived from the contents of PFV vector particles.

The discrete hippocampal expression profile is an interesting point for more detailed discussion. An intracerebroventricular injection into neonatal mice would normally permit vector spread to distant brain regions. Indeed, we observed broad intracranial diffusion of LV and AAV vectors following neonatal intracranial injection, even when administering these vectors at lower doses than PFVs. It is likely that the biodistribution of LV vectors in the neonatal brain was partly influenced by age-related differences in brain structure, given that LV diffusion was clearly more restricted when administered to adult mouse brains.

However, despite neonatal brain architecture being somewhat permissive to vector diffusion, our data showed that PFV vector expression was highly localized to hippocampal structures, particularly the dentate gyrus. When rationalizing a potential mechanism for this expression pattern, it is important to note that PFV vector transduction has shown dependence on the target cell cycle. In quiescent cells, PFV capsids pause at the centrosome, and uncoating
After 48 hr, the supernatant was harvested, sodium butyrate was added for 8 hr to boost cellular transcription. mids pcoPE, pcoPG4, and pcoPP. 24 hr after transfection, 10 mM sodium). The supernatant was centrifuged in a Surespin 630 rotor pore), and layered onto 6 mL of a sucrose cushion (20% in me-

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Additionally, our immunostaining images show signs of EGFP expression in microglia and choroid plexus epithelial cells. This observation, along with evidence for PFV transduction of mesenchymal stem cells, suggests that the postnatal hippocampal expression pattern is attributed to transduction of multiple cell types in vivo.

It is often desirable to target discrete brain regions with gene transfer vectors, but we have shown that LV and AAV vectors spread extensively from an injection site and give widespread expression after neonatal injection, meaning that anatomical accuracy is lost without a region-specific promoter. This constitutes a potential advantage of PFV technology in scenarios requiring expression localized to the hippocampus, such as in the recent correction of an Alzheimer’s disease model by LV-mediated peroxisome proliferator-activated receptor gamma (PPARγ) coactivator 1α gene transfer. Of course, AAVs may be engineered to confer brain region specificity with customized promoters and regulatory elements, but restrictions regarding AAV packaging capacity mostly hinder inclusion of extensive non-coding sequences. Thus, PFV has the potential advantage of being able to package large transgenes with multiple reporters while retaining region specificity.

MATERIALS AND METHODS

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initiates when cells undergo mitosis. A study by Caprariello et al. showed that stable PFV vector expression was detected only in proliferating cells, suggesting that PFV vectors require cell division for stable transduction. This is particularly relevant to our study because hippocampal regions, such as the dentate gyrus, generate neurons postnatally because of proliferation and differentiation of neuronal stem cells. This results in a substantial increase in the size of the dentate gyrus during the first 2 postnatal weeks. Thus, there is a possibility that our PFV vector expression pattern relates to unique transduction characteristics, with expression potentially derived from transduction of neuronal progenitors of the dentate gyrus. This would constitute an interesting feature of PFV vector technology because neuronal stem cell pathways could be hijacked and exploited for targeted transgene delivery.

Construction of the AAV-CMV EGFP vector (titer, $1 \times 10^{13}$ genome copies/mL) and the LV-EGFP vector (titer, $8.4 \times 10^8$ genome copies/mL) have been described previously. The LV-EGFP vector that was administered to adult mice was produced by Oxford Genetics (titer, $8.0 \times 10^7$ genome copies/mL). The maps of each vector used in this study are detailed in Figure S6.

Animal Procedures

The outbred CD1 mice used in this study were supplied by Charles River Laboratories. All animal experiments conducted within this study were in agreement with the United Kingdom Home Office guidelines, approved by the ethical review committee, and followed the institutional guidelines at University College London.

Vector Administration

For neonatal intracranial injections, vectors were administered using a 33G needle to deliver 2.5 μL of vector (5 μL in the case of LV vectors) into the left lateral ventricle. On post-natal day 1, non-randomized neonates were subjected to brief hypothermic anesthesia and injected with viral vectors or plasmid DNA via the appropriate route.

For adult intracranial injections, 32-day-old CD1 mice (two females and one male, approximately 20 g body weight) were initially anesthetized with isoflurane and placed in a stereotaxic frame ( Kopf Instruments, USA) over a heat mat. Metacam (0.013 mg/kg) and buprenorphine (0.02 mg/kg) were injected via subcutaneous injection. One burr hole was drilled unilaterally at the following coordinates: medium/lateral (ML), 1.00; anteroposterior, −0.2; dorsoventral, −2.00. The coordinates were adjusted in proportion to the distance between the real bregma and lambda over an ML max of 4.00 mm. 4.0 $\times$ 10^6 vector genome copies were injected into the lateral ventricle using a Hamilton syringe at a speed of 250 nL/min.

Plasmid DNA (Figure S3) was injected at a dose of 1.5 μg. Intravenous injections were delivered via the superficial temporal vein in 20 μL volumes. Intra-peritoneal injections were delivered in 200-μL volumes. Subcutaneous injections were delivered in 2.5-μL volumes administered under the skin of the left flank. Experimental groups were blinded during the course of in vivo investigations. Each pup received unique identification with a subcutaneous footpad tattoo.

All experiments were carried out under United Kingdom Home Office license PPL 70/8030 and approved by the ethical review committee of University College London.

Whole-Body and Macroscopic Imaging

Mice injected with the PFV-luciferase vector were subsequently imaged 13 and 49 days after injection by whole-body bioluminescence imaging (IVIS) (Caliper Life Sciences, Hopkinton, MA, USA) as described previously. Those that received PFV-EGFP were sacrificed on day 11 and examined for direct EGFP expression using a stereoscopic fluorescence microscope (MZ16F, Leica Microsystems, Wetzlar, Germany) as described previously. Images were captured
using a digital microscope camera (DFC420, Leica Microsystems, Milton Keynes, UK) and software (Image Analysis, Leica Microsystems). Mice that received intracerebral injections of double-stranded DNA (dsDNA) plasmid were imaged 5 days and 11 days post-injection, with luciferase activity normalized to the bioluminescent signal produced by replicate 1 of the uninjected group.

Tissue Preparation
Mice that received neonatal intracranial injections were euthanized by terminal anesthesia 11 days after vector injection before fixing the skinned cranium in 4% paraformaldehyde solution for 24 hr. The brain was carefully excised and fixed for a further 24 hr before transfer to 30% sucrose in 50 mM Tris-buffered saline (TBS).

For each sample, 40-μm frozen sections were cut using a Microm HM 430 freezing microtome (Thermo Fisher Scientific, Loughborough, UK).

Free-Floating Immunohistochemistry
Sections were rinsed with TBS three times for 5 min between each step. Endogenous peroxidase activity was quenched by incubating the sections in 1% hydrogen peroxide in TBS for 30 min. Blocking was carried out for 30 min in a solution of 15% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) in TBS-T (TBS solution containing 0.3% Triton X-100). Sections were incubated overnight at 4°C with rabbit anti-EGFP antibody (1:10,000, ab183734, Abcam, Cambridge, UK), followed by 2 hr with goat anti-rabbit immunoglobulin G (IgG) (Vector Laboratories, PI-1000) at 1:1,000; both antibodies were diluted with 10% NGS in TBS-T. Sections were then incubated for 2 hr in Vectastain ABC (avidin-biotin) solution (ABC, Vector Laboratories, Peterborough, UK) prepared at 1:1000 in TBS 30 min before use. Sections were incubated in the dark in a 0.05% solution of diaminobenzidine (DAB), prepared by dissolving a 10-mg DAB tablet (Sigma, D5905) into 20 mL TBS. After mixing well and filtration through a 0.45-μm syringe filter, 6 μL of 30% hydrogen peroxide was added. Sections were transferred onto gelatin-coated slides in a rostral-caudal order and allowed to dry overnight. They were dehydrated in a series of industrial methylated alcohol and allowed to dry over-night, with PBS-heparin (0.8 mg/mL), followed by 4% paraformaldehyde (PFA) in PBS (Santa Cruz Biotec). The brains were then removed and left in 4% PFA and PBS overnight at 4°C.

Immunofluorescence and Scanning Confocal Microscopy
Free-floating brain sections were subjected to fluorescent immunohistochemistry. Antibodies against EGFP and the neuron-specific marker NeuN were used. Sections were initially blocked for 30 min in TBS-T and 15% NGs and then incubated at 4°C overnight with rabbit anti-EGFP (1:4,000) and mouse anti-NeuN (1:500; ABN91, Millipore, Billerica, MA, USA) antibodies made up in TBS-T and 10% NGs. The sections were rinsed three times for 5 min in TBS and then incubated with goat anti-rabbit Alexa 488 (1:1,000, Thermo Fisher Scientific, A-11008) and goat anti-mouse Alexa 546 (1:1,000, Thermo Fisher Scientific, A-11030) for 2 hr. Sections were again rinsed three times in TBS solution and then incubated with DAPI (1:2,000, Invitrogen). Sections were then mounted onto gelatin-coated slides, and coverslips were mounted with Fluoromount G (Southern Biotech, Birmingham, AL, USA). Slides labeled with immunofluorescence were then analyzed, and Z stacks were captured using a laser-scanning confocal microscope (Leica SP5, Leica Microsystems).

Statistical Analysis
All statistical analyses were carried out using MATLAB 2015a. A two-tailed Welch’s t test was used to compare mean bioluminescence values. This statistical test is robust for datasets without equal variance or sample size. Mouse sample sizes were limited to three or four animals per experimental group for in vivo investigations.

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.07.006.

AUTHOR CONTRIBUTIONS
J.R.C., analysis and review of results and writing of the manuscript; R.K., J.A.D., L.C., T.W., S.M.K.B., S.A., J.N., J.B., R.S., E.L., G.L., F.A., and S.N.W., review and analysis of results and review of the manuscript; R.d.S., D.L., A.R., S.N.W., and S.J.H., planning of experiments, review and analysis of results, and review of the manuscript.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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