Intravenous administration of AAV2/9 to the fetal and neonatal mouse leads to differential targeting of CNS cell types and extensive transduction of the nervous system

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ABSTRACT Several diseases of the nervous system are characterized by neurodegeneration and death in childhood. Conventional medicine is ineffective, but fetal or neonatal gene therapy may provide an alternative route to treatment. We evaluated the ability of single-stranded and self-complementary adeno-associated virus pseudotype 2/9 (AAV2/9) to transduce the nervous system and target gene expression to specific neural cell types following intravenous injection into fetal and neonatal mice, using control uninjected age-matched mice. Fetal and neonatal administration produced global delivery to the central (brain, spinal cord, and all layers of the retina) and peripheral (myenteric plexus and innervating nerves) nervous system but with different expression profiles within the brain; fetal and neonatal administration resulted in expression in neurons and protoplasmic astrocytes, respectively. Neither single-stranded nor self-complementary AAV2/9 triggered a microglia-mediated immune response following either administration. In summary, intravenous AAV2/9 targets gene expression to specific neural cell types dependent on developmental stage. This represents a powerful tool for studying nervous system development and disease. Furthermore, it may provide a therapeutic strategy for treatment of early lethal genetic diseases, such as Gaucher disease, and for disabling neuropathies, such as preterm brain injury.

Key Words: targeted gene expression · perinatal gene therapy · neurodegenerative disease · peripheral nerves

The efficient and widespread delivery of genetic material to the developing fetal and neonatal nervous system is highly desirable. As a research tool, it would allow the overexpression of proteins of interest and aid in the investigation of their role in disease and development. Conversely, the levels of an endogenous protein could be reduced by gene knockdown using RNAi sequences. However, there is also the significant potential to deliver therapeutic genetic material to the developing fetus following prenatal diagnosis of a disease. The concept of fetal gene therapy is especially appealing for treating neonatal or perinatal lethal monogenic disorders. The opportunity of a therapeutic window during gestation is arguably most significant in the cases of neonatal lethal neurodegenerative diseases; pathology begins in the developing fetus, and because of the inability of neurons of the central nervous system (CNS) to regenerate, neonatal intervention may be too late. One example of this is Type II Gaucher disease (GD), in which brain pathology is detectable during gestation (1) and results in death before 2 yr of age (2). Although enzyme replacement therapy is effective in treating the visceral manifestations of GD, the blood-brain barrier (BBB) prevents recombinant enzyme from entering the CNS. As such, no clinical treatment for this disease is currently available. On the other end of the scale, up to 10% of all pregnancies are preterm (3), and the risk of developing cerebral palsy is 70 times greater in infants born at <28 wk compared with term.
birth (4). As such, there is a compelling case to investigate new means of preventing or treating the neurological sequelae associated with preterm labor-associated brain injury.

The BBB also prevents gene delivery vectors (viral and nonviral) from crossing into the CNS. Therefore, intravenous administration of vectors has resulted in poor gene delivery to the brain, and it has been necessary to use direct intracranial injection instead. However, recently it has been shown that self-complementary (sc) adeno-associated virus 9 (AAV9) has the ability to cross the BBB following intravenous administration to adult and neonatal mice, cats, and nonhuman primates, resulting in efficient gene delivery to cells of the CNS (5–7). It has subsequently been shown that a mouse model of spinal muscular atrophy could be corrected following intravenous administration of AAV9-expressing survival motor neuron protein (7).

These encouraging initial results may have a major effect on the practical administration of fetal gene therapy as a treatment for diseases of the CNS. We have previously shown that intracranial delivery of viral vectors leads to efficient and widespread gene expression in the fetal mouse brain (8). However, the potential for translation of such a technique to the clinic would be difficult due to the invasiveness of the procedure and the level of surgical precision required. An intravenous route of administration to the fetus or neonate would overcome these procedural concerns. Indeed, routine clinical techniques, such as those developed for fetal blood transfusions, could be used to safely administer vector to the fetal circulation. Fetal gene therapy uniquely offers a number of potential advantages, such as development of immune tolerance to expressed foreign proteins and increased vector-to-cell ratio. The potential of this concept has been reviewed elsewhere (9–11).

Although scAAV9 has been delivered to neonatal and adult animal models, nothing is known of its properties in the fetus, an important consideration taking into account developmental differences in the CNS, including formation of the BBB. These same differences may provide an opportunity to target gene expression to specific neural cell types at different developmental stages. This is attractive as both a research tool and a means of answering questions more relevant to therapeutic application e.g., should we be delivering therapeutic genes directly to neurons, or to the more abundant glial cells and relying on cross-correction by soluble enzymes? Furthermore, the use of the single-stranded (ss) form of the virus is yet to be tested in a fetal model. The natural wild-type form of AAV has an ssDNA genome that relies on synthesis of a complementary strand. Advances in vector technology have produced an sc form of AAV that is thought to facilitate more efficient gene expression (12). However, in doing so, there is a compromise in the amount of genetic material that can be inserted into the sc vector. This includes larger genes and promoters but also sequences that can enhance gene expression, such as the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE). This tripartite sequence is known to enhance gene expression when included in the 3’ untranslated region of a gene expressed in an AAV vector (13). A direct comparison between expression mediated by scAAV2/9 that lacks a WPRE sequence and an ssAAV2/9 that includes one within the fetal and neonatal CNS would be interesting.

In this study, we present data showing that intravenous administration of both ss- and scAAV2/9 to fetal mice leads to robust, extensive, and widespread gene delivery to various parts of the CNS with evidence of peripheral nervous system (PNS) transduction. We also demonstrate that gene expression can be targeted to specific cell types within the brain and that this is developmental age dependent. Furthermore, no evidence of a microglia-mediated innate immune response was detected using the different forms of the AAV2/9 vectors following intravenous administration to either the fetus or neonate.

**MATERIALS AND METHODS**

**AAV vectors**

Both ss- and scAAV2/9 viral vectors were supplied by the University of Pennsylvania Vector Core facility (Philadelphia, PA, USA). The vectors contained the green fluorescent protein (GFP) gene driven by the CMV promoter. The ssAAV2/9 expression cassette also included the WPRE sequence downstream of the GFP gene. Both forms of the AAV2/9 were titer matched to $1 \times 10^{13}$ genome copies (GC)/ml.

**In utero and neonatal administrations of AAV2/9 vectors and stereoscopic fluorescence microscopy**

AAV2/9 vector (20 μl; $2\times10^{11}$ GC) was intravenously injected into embryonic day 15 (E15) MF1 fetal mice via vitelline vessels, as described previously (14), where $n = 3$ for both ss- and scAAV2/9. Three mice per dam were treated and marked with colloidal carbon to identify injected animals. The E15 time point for fetal administration was selected on procedural grounds. The vitelline vessels were relatively accessible and developed sufficiently to tolerate intravenous injection of the vector. Injections at later time points during gestation, although feasible, were not conducted because of the increased risk of preterm labor and subsequent cannibalization. Neona
tes at 1 day postgestation (P1) were subject to hypothermic anesthesia. They were then treated with 40 μl ($4\times10^{10}$ GC) of AAV2/9 vector via the superficial temporal vein, where $n = 3$ for both ss- and scAAV2/9. The P1 time point was selected on the basis of demonstrating gene delivery to neonates at the earliest possible time after birth and as a model for preventing neonatal lethal neurodegenerative disease. In addition, the superficial temporal vein becomes less accessible as the mouse ages. For both administration techniques, a 33-gauge needle (Hamilton, Reno, NV, USA) was used. The mice were allowed to recover and return to normal temperature before being placed back into the cage with the mother.

At 1 mo postinjection, both injected and control un.injected mice were subject to terminal exsanguination and perfusion with heparinized PBS followed by 1% paraformaldehyde. This provided the opportunity for examination of
the mice for GFP expression using a stereoscopic fluorescence microscope (MZ16F; Leica, Wetzlar, Germany). Representative images were captured using a digital microscope camera (DFC420; Leica Microsystems, Milton Keynes, UK) and software (Image Analysis; Leica Microsystems).

Free-floating and paraffin-embedded immunohistochemistry

Following removal of nervous system organs, the tissue was fixed in 4% paraformaldehyde. The brains were cryoprotected in 30% sucrose in 50 mM TBS prior to cryosectioning at 40-μm-thick sections using a Microm freezing microtome (Carl Zeiss, Welwyn Garden City, UK). Immunohistochemistry was then conducted on the free-floating sections using anti-GFP and CD68 antibodies and 3,3′-diaminobenzidine (DAB) to visualize staining, as described previously (8), carefully controlling the time for which sections were batch stained together.

Immunohistochemical staining for GFP on paraffin-embedded eye and spinal cord sections was conducted by dewaxing sections twice in xylene for 5 min, followed by 2 changes of 100% industrial methylated spirit (IMS). The sections were washed with deionized water, and antigen retrieval was performed by boiling the sections for 10 min in 0.01 M citrate buffer (pH 6). When cool, the sections were treated with 1% H2O2 in TBS for 30 min to deplete endogeneous peroxidase activity. Following rinsing in TBS, the sections were blocked with TBS containing 0.5% Triton X-100 (TBS-T)/15% normal goat serum (NGS; Vector Laboratories, Peterborough, UK) for 30 min. Rabbit anti-GFP antibodies (1:1000; Abcam, Cambridge, UK) in TBS-T/10% NGS were applied to the sections and left overnight at 4°C. The sections were washed in TBS and incubated with goat anti-rabbit IgG (1:200) in TBS-T/10% NGS for 2 h. Following rinsing in TBS, the sections were incubated for 2 h with Vectastain avidin-biotin solution (1:200; Vector Laboratories) made up in TBS-T/10% NGS (TBS-T)/15% normal goat serum (NGS; Vector Laboratories, Peterborough, UK) for 30 min prior to use. After washes in TBS, a 0.05% DAB solution containing 0.01% H2O2 was applied to visualize immunoreactivity. Washing the section twice in ice-cold TBS stopped the reaction. The sections were dehydrated, cleared, and coverslips were mounted, as described above.

All DAB-stained sections were viewed using an Axioskop 2 Mot microscope (Carl Zeiss), and representative images were captured using an Axiocam HR camera and Axiosvision 4.2 software (Carl Zeiss).

Free-floating and paraffin-embedded immunofluorescence and scanning confocal microscopy

To assess vector cell tropism, free-floating brain sections were double labeled with neuronal and astrocytic markers. The sections were blocked in TBS-T/15% NGS and then incubated overnight with rabbit anti-GFP (1:4000) and either mouse anti-NeuN (1:500; Millipore, Billerica, MA, USA), S100β (1:500; Dako, Ely, UK), or glial fibrillary acidic protein (GFAP) antibodies (1:500; Millipore) made up in TBS-T/10% NGS. Following rinses with TBS, the sections were then incubated for 2 h with goat anti-rabbit Alexa 488 and goat anti-mouse Alexa 546 (1:1000; Invitrogen, Paisley, UK). After further rinsing with TBS, sections were incubated with TO-PRO-3 (1:1000; Invitrogen) or DAPI (1:2000; Invitrogen) and then mounted on chrome-gelatin-coated slides, and coverslips were mounted with Fluoromount G (Southern Biotech, Birmingham, AL, USA). Using the same methodology, a chicken anti-GFP (1:1000; Abcam) was used together with rabbit anti-SOX2 (1:1000; Millipore) and detected with goat anti-chicken Alexa-488 (1:1000) and goat anti-rabbit Alexa 546 (1:1000), and nuclei were labeled with TO-PRO-3 (1:1000). Similarly, rat anti-myelin basic protein (MBP) antibodies (1:1000; Abcam) were also used together with the chicken anti-GFP antibodies and secondary goat anti-chicken Alexa-488, goat anti-rat Alexa 546, and TO-PRO-3. However, Triton X-100 was omitted from all steps of the protocol involving MBP antibodies.

Paraffin-embedded spinal cord sections were dewaxed, treated for antigen retrieval, blocked, and incubated with rabbit anti-GFP antibodies, as described previously. The sections were rinsed in TBS before being incubated with goat anti-rabbit Alexa 488 (1:200, Invitrogen) for 2 h. After rinsing in TBS, the sections were incubated with DAPI and washed, and then coverslips were mounted using Fluoromount G.

Immunofluorescently labeled slides were examined, and Z stacks were captured using a laser-scanning confocal microscope (Leica SP5; Leica Microsystems).

Quantitative analysis of immunohistological staining

A quantitative analysis of GFP and CD68 staining was carried out using thresholding analysis, as described previously (15, 16). Forty nonoverlapping RGB images were captured from 4 consecutive sections through the caudate putamen (CPu), the CA1 region of the hippocampus (CA1), primary motor cortex (M1), piriform cortex (Pir), medial septal nucleus (MS), the ventral posterior lateral nucleus and ventral posterior medial nucleus of the thalamus (VPL/VPM), and the 10Ch region of the cerebellum (10Ch). The images were captured using a live video camera (JVC, KY-F55B; JVC, Yokohama, Japan) mounted onto a Zeiss Axiosplan microscope using the ×40 objective lens. All camera and microscope settings and calibrations were kept constant during the image-capture period. Images were analyzed for optimal segmentation, and the immunoreactive profiles were determined using Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA). Foreground immunostaining was accurately defined according to averaging of the highest and lowest immunoreactivities within the sample population for a given immunohistochemical marker (per color/filter channel selected) and measured on a scale from 0 (100% transmitted light) to 255 (0% transmitted light) for each pixel. This threshold setting was kept constant for all subsequent images analyzed for the antigen used. The immunoreactive profiles were discriminated in this way to determine the specific immunoreactive area (the mean gray value obtained by subtracting the total mean gray value from nonimmunoreactive value per defined field). Macros were recorded for transfer of the data to a spreadsheet for statistical analysis. The data were plotted as the mean ± SE percentage area of immunoreactivity per field for each region.

The quantification of transduced neurons and protoplasmic astrocytes was done using StereoInvestigator software (MBF Bioscience, Williston, VT, USA) on a Zeiss Axioskop 2 (Carl Zeiss) microscope linked to a DAGE-MTI CCD-100 camera (DAGE-MTI, Michigan City, IN, USA). The boundaries of the S1BF region were delineated according to Franklin and Paxinos (17) in 3 consecutive sections stained for transduced cells via GFP immunoreactivity. Using the ×40 objective lens, the total numbers of neurons and protoplasmic astrocytes were counted within the S1BF region. Quantification was carried out by an individual masked to the time and type of AAV2/9 vector administration. The data were plotted as the total ± SE number of cells counted for the S1BF region.

Statistical analysis

Thresholding data, shown as percentages and therefore following a binomial distribution, were converted to a normal...
distribution by applying an inverse sine transformation $P = \arcsin(P^{1/2})$. One-way ANOVA was performed on the 4 groups of transformed data. (Minitab 16; Minitab, State College, PA, USA). Multiple pairwise comparisons were performed using the Fisher method using individual 95% confidence intervals.

RESULTS

Extensive CNS and PNS transduction following intravenous administration of AAV2/9 vectors to fetal and neonatal mice

Both ssAAV2/9 and scAAV2/9 expressing GFP driven by the CMV promoter were used in this study. The vectors were titrated at $1 \times 10^{13}$ GC/ml. The ssAAV2/9 differed from scAAV2/9 due to the inclusion of a WPRE downstream of the GFP gene. The vectors were injected singly into E15 fetuses via the vitelline vessels or into P1 neonates via the superficial temporal vein. At 1 mo postinjection, the mice were sacrificed, together with age-matched uninjected mice. Diffusion tensor microimaging studies have shown, using various anatomical landmarks, that significant increase in brain volume growth ends at approximately this time point (18). The skin was removed for direct visualization under a fluorescence stereoscopic microscope, using an uninjected control mouse as a negative control for tissue autofluorescence. Extensive GFP expression was seen throughout the bodies of mice that received fetal and neonatal injections with either ss- or scAAV2/9 (Fig. 1A). Gross dissection of the mice allowed for closer examination of GFP expression. Representative images were taken of various areas of the CNS following fetal administration of ssAAV2/9, including the surface of the cerebellum (Fig. 1B), the surface of the cerebrum (Fig. 1C), spinal cord (Fig. 1D), dorsal root ganglia (DRG; Fig. 1E), eye (Fig. 1F), and optic nerve (Fig. 1G). Similar transduction patterns were also seen following neonatal administration of ssAAV2/9, and representative images were taken of areas such as the optic chiasma (Fig. 1H).

Figure 1. Nervous system transduction following fetal and neonatal administration of ss- and scAAV2/9. Following fetal and neonatal administration of ss- and scAAV2/9, mice were harvested at 1 mo postinjection. A) Widespread and robust GFP expression was clearly visible. B–G) Representative images from mice that received fetal injections of ssAAV2/9. B) Surface of the cerebellum. C) Surface of the and cerebrum. D) Spinal cord. E) Dorsal root ganglia. F) Eye. G) Optic nerve. H–J) Representative images of tissue harvested from mice that received neonatal administration of ssAAV2/9. H) Optic chiasma. I) Myenteric plexus along the surface of the gut. J) Nerves (arrows) that innervate the muscles (M) associated with the ear (E).
vous system transduction was visible by GFP expression within the myenteric plexus along the surface of the gut (Fig. 1J). Extensive peripheral nerve transduction was also seen throughout the body. Figure 1J shows an example where removal of skin exposed GFP-positive nerves (highlighted by arrows) innervating the muscles (M) of the ear (E). Macroscopically, both vectors appeared to transduce similar regions of the nervous system, although the intensity of GFP signal was visibly higher in tissue taken from mice injected with ssAAV2/9.

**Differential gene expression in neural and glial cells of the brain following fetal and neonatal administration of AAV2/9 vectors**

The brains from injected and control uninjected mice were sectioned and examined by immunohistochemistry using antibodies against GFP and detected using DAB. The stained sections were evaluated using light microscopy and revealed extensive transduction. A systematic examination of the sections moving from rostral to caudal areas of the brain revealed expression throughout both hemispheres of the CNS. Representative images were taken at the levels of the prefrontal cortex, lateral ventricles, hippocampus, Pir, and cerebellum (Fig. 2A). Equivalent areas of the brain from uninjected mice showed no specific staining and an absence of background staining. The staining intensity of sections from brains taken from mice injected with ssAAV2/9 was noticeably higher than that seen in brain sections from scAAV2/9-injected mice, and this was the case for both fetal and neonatal administration.

A semiquantitative analysis of staining within discrete areas of the brain was conducted by thresholding analysis. The staining intensity was measured in the CA1, CPu, M1, MS, Pir, VPL/VPM, and 10Cb (Fig. 2B). This analysis showed that ssAAV2/9 resulted in increased GFP staining when administered either to fetuses or to neonates in the CA1, CPu, MS, and VPL/VPM when compared to scAAV2/9. In addition, *in utero* delivery of ssAAV2/9 resulted in greater staining intensity when compared to neonatal delivery in the CA1, CPu, MS, and Pir.

Interestingly, examination of brain sections under higher magnification revealed that the morphology of GFP-expressing cells was markedly different when AAV2/9 vectors were administered to fetuses compared to neonates. Figure 3A shows this using ssAAV2/9, where *in utero* delivery resulted in gene expression in cells with neuronal morphology. In contrast, neonatal delivery of the same vector resulted in gene expression within fewer neurons but predominantly in many larger and morphologically distinct protoplasmic astrocytes. This pattern was consistent in all regions inspected, including the M1, S1BF, CPu, CA2-CA3, and VPL/VPM (Supplemental Fig. S1). The only region where no distinct difference was seen between prenatal and neonatal injections was in the 10Cb lobe of the cerebellum, where AAV2/9 vectors transduced cells with Purkinje cell morphology regardless of the age of administration. On the basis of the distinct morphological differences between neurons and protoplasmic astrocytes (Fig. 3B; shown in high magnification), quantification of the two cell types was carried out in the S1BF region of the cortex. Of the total cells counted, *in utero* delivery of ssAAV2/9 resulted in 98% of GFP-positive cells being neurons and only 2% being astrocytes. Similarly, *in utero* delivery of scAAV2/9 resulted in 90.8% of GFP-positive cells being neurons and 9.2% being astrocytes. However, following neonatal delivery of ssAAV2/9, the pattern of gene expression was significantly altered, resulting in just 28.3% of GFP-positive cells being neurons and 71.7% being astrocytes. This was also the case using scAAV2/9, where 24.8% of GFP-positive cells were neurons, and 75.2% were astrocytes.

**Figure 2.** Widespread and efficient gene delivery to the brain following fetal and neonatal administration of AAV2/9 vectors. Brains of mice injected *in utero* or neonatally and control uninjected mice were harvested and sectioned. Immunohistochemistry for GFP revealed extensive and widespread gene delivery throughout the brain. A) Representative images at various rostrocaudal levels, including the prefrontal cortex, lateral ventricles, hippocampus, Pir, and cerebellum. B) Quantitative measurement of GFP staining in discrete areas of the brain, as indicated, was carried out using thresholding analysis. Data are plotted as mean ± se percentage area of immunoreactivity per field for each region (n=5).
Figure 3. Differential developmental stage-dependent gene expression profiles in cells of the brain following prenatal and neonatal administration of AAV2/9 vectors. Immunohistochemistry on brain sections from prenatally and neonatally treated mice revealed pronounced differences in the distribution and cell-type specificity of gene expression. A) Light microscopy revealed gene expression in neurons when AAV2/9 vectors were administered in utero. However, neonatal administration resulted in gene expression in markedly fewer neurons and predominantly in protoplasmic astrocytes. This was most clearly seen in the cerebral cortex. B) Cell counts of GFP-positive cell types revealed significantly more neurons compared to astrocytes following in utero administration with either ss- or scAAV2/9. High-magnification images of the two cell types highlight their distinct morphologies. Conversely, neonatal administration resulted in significantly more GFP-positive protoplasmic astrocytes compared to neurons, again using either ss- or scAAV2/9. Data are plotted as mean ± SD cell count, and the calculated percentage is shown (n = 3). *P < 0.05 vs. other cell type.

Confirmation of the phenotype of GFP-expressing cell types following in utero and neonatal administration of AAV2/9 vectors

Further confirmation of the differential gene expression profiles in specific neural cell types following in utero or neonatal administration of AAV2/9 was conducted. Sections from uninjected and mice injected either in utero or neonatally with ssAAV2/9 were simultaneously stained, using immunofluorescence, for GFP and cell-specific phenotypic markers and examined using multichannel scanning confocal microscopy. Sections from uninjected mice were stained with TO-PRO-3 to label cell nuclei (Fig. 4A), NeuN to label neurons (Fig. 4B) and anti-GFP antibodies to label any transduced cells (Fig. 4C), and the S1BF region of the cerebral cortex was examined. As expected, no GFP-expressing cells could be detected, and merging of the signals did not produce any colocalization within neurons (Fig. 4D). Sections from mice injected with ssAAV2/9 in utero were labeled with TO-PRO-3 (Fig. 4E), NeuN (Fig. 4F), and anti-GFP antibodies (Fig. 4G). GFP-expressing cells were detected with the same homogeneous morphology observed previously using immunoperoxidase staining (Fig. 2B). Unlike immunoperoxidase staining, no avidin-biotin-mediated amplification of signal was used for immunofluorescence detection of GFP. Therefore, it was more clearly observed that some cells expressed higher levels of GFP, while others expressed lower levels. Merging of signals revealed colocalization of this GFP signal with NeuN, most distinctly seen in those cells expressing higher levels of GFP (Fig. 4H, arrows) and confirming neuronal transduction. Sections from mice administered as neonates were stained with TO-PRO-3 (Fig. 4I), NeuN, (Fig. 4J) and anti-GFP antibodies. GFP-expressing cells were detected with both neuronal and, predominantly, astrocyte-like morphologies (Fig. 4K), as observed in previous immunoperoxidase staining. Merging of the signals revealed colocalization of a subpopulation of transduced cells with NeuN (Fig. 4L, arrows). To confirm our hypothesis that the more numerous GFP-positive cells were protoplasmic astrocytes, sections were also stained with TO-PRO-3 (Fig. 4M), anti-protoplasmic astrocyte marker S100β (Fig. 4N), and anti-GFP antibodies (Fig. 4O). Merging of the signals demonstrated colocalization of GFP- and S100β-labeled cells (Fig. 4P, arrows), confirming the larger population of GFP-positive cells as protoplasmic astrocytes. The same patterns of gene expression were also seen in other areas of the brain examined, including the hippocampus (Supplemental Fig. S2). Sections from neonatally treated mice were also stained with antibodies against GFAP to label fibrous astrocytes (Supplemental Fig. S2), but no evidence of GFP expression could be detected in this subset of astrocytes in these mice. Furthermore, sections from mice treated in utero or neonatally were stained with antibodies against the oligodendrocyte marker myelin basic protein. Representative images were captured that included the CA2 region of the hippocampus and the corpus callosum. However, there was no evidence of colocalization between myelin basic protein-labeled cells and GFP expression (Supplemental Fig. S2).

To further investigate the cell specificity of gene expression, sections from the cerebellum of brains injected in utero with ssAAV2/9 were stained with TO-PRO-3 (Fig. 4Q), the Purkinje cell marker calbindin (Fig. 4R) and anti-GFP antibodies (Fig. 4S). Extensive GFP expression was visible on examination of the 10Cb region of the cerebellum. Merging of the signals revealed that GFP expression was localized within Purkinje cells (Fig. 4T, arrows). Equivalent sections taken from mice injected neonatally were also stained with Topro (Fig. 4U), calbindin (Fig. 4V) and anti-GFP antibodies (Fig. 4W). Merging of the signals confirmed that GFP expression was localized within Purkinje cells (Fig. 4X) and, unlike in the cortical mantle, gene expression was seen in neurons (Fig. 4Y, arrows).
expression within the cerebellum appeared identical to that seen in sections from mice injected in utero.

To evaluate any AAV2/9-mediated gene expression in multipotent neural stem cells, brain sections from mice administered ssAAV2/9 prenatally or neonatally were stained with Topro and antibodies against SOX2 and GFP. Examination of the subventricular zone revealed no colocalization between SOX2-stained cells and GFP expression in brain sections from either prenatally or neonatally treated mice (Supplemental Fig. S2).

**Evaluation of microglia-mediated innate immune response to fetal and neonatal administration of AAV2/9**

To investigate the ability of either the vector or of GFP expression to produce an immune response following in utero or neonatal administration of AAV2/9, brain sections from uninjected and injected mice were analyzed for microglial activation using antibodies against CD68. As a positive control, brains were taken from palmitoyl-protein thioesterase 1-deficient (Ppt1−/−) mice that exhibit profound microglial activation as part of their infantile neuronal ceroid lipofuscinosis phenotype (16). Examination of the M1 region of the cerebral cortex by light microscopy revealed extensive activation and engorgement of microglia in brain sections taken from Ppt1−/− mice (Fig. 5A). However, no evidence for microglial activation was evident in sections from mice injected prenatally or neonatally or uninjected mice. A semiquantitative evaluation using thresholding analysis to measure staining intensity confirmed these observations (Fig. 5B). ANOVA revealed a significant difference between groups (P=0.026 using pooled sd of 0.01). Compared to uninjected controls, there was a significant reduction in CD68-positive cells in those mice treated in utero, but no such reduction was detected in those injected after birth.

**Gene delivery to the spinal cord**

The spinal cords of both uninjected and injected mice were harvested and sectioned. As in other brain regions, GFP expression was examined by immunohistochemistry in transverse sections through the spinal cords. Both in utero and neonatal administration of either ss- or scAAV2/9 resulted in GFP staining within the spinal cord. Representative images were taken of stained sections from prenatally treated mice using ssAAV2/9 with low-power (Fig. 6B) and higher-power (Fig. 6C) light microscopy. Further examination of the sections revealed extensive gene expression in the DRG (Fig. 6D). Expression in the spinal cord and DRG was also observed in sections taken from mice injected neonatally with ssAAV2/9 (Fig. 6E).

To confirm gene expression within neurons of the spinal cord, sections from neonatal mice administered ssAAV2/9 were immunofluorescently stained with DAPI to label cell nuclei (Fig. 6F), anti-neurofilament M to label neurons (Fig. 6G), and anti-GFP antibodies to label transduced cells (Fig. 6H). Merging of the channels confirmed GFP expression within neurons (Fig. 6I). Sections of the DRG were also stained with DAPI (Fig. 6J), neurofilament M (Fig. 6K), and anti-GFP antibodies (Fig. 6L), with merged channels again confirming the expression of GFP within neurons (Fig. 6M).

**Gene delivery to the eye**

Eyes were harvested from mice treated prenatally or neonatally with ss- and scAAV2/9 and from control uninjected mice. Direct visualization of GFP expression using a fluorescence stereoscopic microscope revealed no visible GFP expression in eyes from uninjected mice, but intense GFP expression was clearly seen in eyes from mice injected with ssAAV2/9 in utero (Figs. 7A and 7F) and as neonates (Fig. 7B). Removal and examination of the lens from eyes of mice treated as neonates also revealed strong GFP expression (Fig. 7C). Immuno-peroxidase staining for GFP was carried out on sections of eyes from mice injected with scAAV2/9 to visualize transduced cells. The retina and cornea were examined by light microscopy, and representative images were captured. No specific or background staining was present in the retina or cornea of control uninjected mice (Fig. 7D, E, respectively). Interestingly, extensive gene expression in the retina was visible in eyes taken from prenatally and neonatally injected mice (Fig. 7F, H, respectively). Staining was present in multiple layers comprising the retina, including the ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, photoreceptors, and retinal pigment epithelia. Examination of the cornea revealed staining within the corneal endothelium, stroma, and corneal epithelium in both prenatally (Fig. 7G) and neonatally (Fig. 7I) treated mice. The staining was noticeably more intense in sections taken from the eyes of mice treated in utero with scAAV2/9.

**DISCUSSION**

Gene therapy has shown great potential for treating a number of monogenic neurodegenerative diseases in both animal models (7, 19) and patients (20). For early onset genetic diseases, it has been proposed that it may be preferable to perform gene transfer to the fetus or neonate (21). This is based on the rationale that reconstitution of the defective or missing protein early in life may prevent manifestation of disease pathology rather than reversing it, and the spread of viral vector is likely to be greater in a smaller immature brain rather than in the fully developed larger brain. This first point is crucial when addressing neurodegenerative diseases, with the postmitotic nature of neurons and the limited capacity of the CNS to regenerate meaning that once disease pathology has already set in, the damage is irreversible. The second point is also vital, since these
diseases often affect the entire brain, and glial expression would be required for the therapy to be most effective. A number of studies have provided data supporting the case for neonatal over adult intervention in extending the life span of animal models of neurodegenerative diseases (22, 23).

Although neonatal gene therapy could be ideal for treating late-childhood lethal diseases, there are a number of neurodegenerative diseases that result in neonatal lethality. This is primarily because disease pathology is already manifest in the developing fetus, so in such cases, neonatal intervention could be too late. Examples of this are the congenital form of neuronal ceroid lipofuscinosis (NCL) and type II GD, where death occurs within the first few days of life and before 2 yr of age, respectively (2, 24–26). The concept of fetal gene therapy for treating such diseases has been well reviewed (9–11), and we, and others, have demonstrated efficient gene delivery to the fetal mouse brain via intracranial injection (8, 27). This has proved to be a very useful research tool, but any potential for translational application into the clinic has been thought difficult because of the technical issues involved. This is perhaps best exemplified in a gene therapy clinical trial for late infantile NCL (28), where intracranial administration of vector into 12 sites of the brain in children was achieved, but proved technically and surgically challenging (29). These difficulties would only be magnified in the context of in utero surgery, making intracranial delivery of vector to the human fetal brain a challenge with current technologies.

Recently a number of studies have shown that intravenous injection of AAV9 into neonatal and adult mice, cats, and monkeys crosses the BBB and mediates extensive gene delivery to the CNS (5–7). These important studies have now opened the way for potentially non-invasive methods of treating diseases of the CNS. This has recently been demonstrated by correction of a mouse model of spinal muscular atrophy (7). However, these advances also offer the opportunity for overcoming the procedural concerns associated with fetal gene therapy for CNS disorders. One may now consider a

Figure 4. Confirming gene expression in specific cell types following in utero and neonatal administration by immunofluorescence and scanning confocal microscopy. Brain sections from injected and control uninjected mice were used to confirm the cell types expressing GFP following prenatal and neonatal administration of ssAAV2/9. A–C) Sections from uninjected mice were immunofluorescently labeled using TO-PRO-3 to label cell nuclei (A), neuron-specific NeuN antibodies (B) and anti-GFP antibodies (C). As expected, no GFP could be detected. D) Merging of the signals produced no colocalization. E–G) Sections of brains taken from mice injected in utero were stained with TO-PRO-3 (E), NeuN (F), and anti-GFP antibodies (G). H) GFP-positive cells of neuronal morphology were detected using anti-GFP antibodies, confirmed by merging of the channels (arrows). I–K) Sections of brains from neonatally treated mice were labeled using TO-PRO-3 (I), NeuN (J), and anti-GFP antibodies (K). Two populations of morphologically distinct cells expressing GFP were observed. L) Merging of the channels identified one population of cells to be neurons (yellow; arrows). M–O) To identify the second population, sections were labeled with TO-PRO-5 (M), the protoplasmic astrocyte-specific S100β antibodies (N), and anti-GFP antibodies (O). P) Merging of the channels confirmed the second transduced population of cells as protoplasmic astrocytes (arrows). Q–S) Sections of the cerebellum from prenatally injected mice were stained with TO-PRO-3 (Q), the Purkinje cell-specific calbindin antibody (R), and anti-GFP antibodies (S). T) Merging of the signals confirmed expression in Purkinje cells (arrows). U–W) Similarly, sections of the cerebellum from neonatally injected mice were labeled with TO-PRO-3 (U), calbindin (V), and anti-GFP antibodies (W). X) Merging of the signals produced colocalization with the Purkinje cells (arrows). Insets: cells of interest at higher magnification.

Figure 5. Evaluation of microglia-mediated immune response. Sections of brains from untreated, prenatally treated, and neonatally treated mice were analyzed for microglia activation by immunohistochemistry using antibodies against CD68 and DAB for visualization. Sections of brains taken from Ppt1−/− mice were also included as a positive control. A) Representative images from the M1 region. Extensive microglia activation and engulfment are clearly seen in brain sections from Ppt1−/− mice, whereas no increase in staining intensity or cell morphology can be observed in any of the brains from mice administered AAV2/9 when compared to uninjected mice. B) Quantitative analysis of staining was conducted by thresholding analysis. Data are plotted as mean ± sd percentage area of immunoreactivity per field. Statistical analysis revealed a significant decrease in CD68 immunoreactivity in sections from prenatally treated ss- and scAAV2/9 mice. *P < 0.05 vs. uninjected control.
minimally invasive intravenous injection of AAV9 into the fetal circulation using established clinical protocols as practically feasible. An example of such a protocol is percutaneous ultrasound-guided fetal blood transfusion, a routinely utilized clinical procedure in which intravenous access of the fetal circulation is achieved for transfusion of a severely anemic fetus. This would be a very attractive approach but, as yet, no evaluation of fetal gene delivery using intravenously delivered AAV9 has been conducted.

In this study, we show that in utero intravenous delivery of both ss- and scAAV2/9 to the fetus results in extensive and widespread gene delivery to the nervous system. This was most prominent in the CNS, although evidence of PNS transduction was also observed, for example, in the myenteric plexus. A comparison with neonatal delivery confirmed the widespread CNS transduction reported by others (5–7) but also revealed stark differences in gene expression profiles in neurons and glia within the brain. In utero delivery resulted in gene expression predominantly in neurons, whereas neonatal delivery switched this profile to preferential expression within protoplasmic astrocytes. A possible explanation for this differential tropism is that astrogenesis only just begins at E17.5 (30), and therefore in utero administration at E15 may understandably result in little or no transduction of astrocytes. Conversely, at E16–E17, active neurogenesis is taking place, and at its peak, in many areas of the brain (31), and therefore neurons are readily available for transduction. The situation following neonatal administration is different, with both astrocytes and neurons readily available for transduction by AAV2/9, although gene expression is more prominent in protoplasmic astrocytes. These contrasts in cell-type-specific gene expression profiles at different stages in development are an important consideration if planning gene therapy studies for neurodegenerative diseases. This is because it presents two very different therapeutic scenarios. Fetal administration would result in the therapeutic protein being produced predominantly in the neurons where it is most critically required; if the protein is soluble and is secreted, then it can be taken up by neighboring neurons, resulting in cross-correction. Neonatal administration would result in the therapeutic protein being produced predominantly in the protoplasmic astrocytes, meaning that a greater reliance on cross-correction would be required for neuronal uptake. This may

Figure 6. Gene delivery to the spinal cord and DRG. Immunohistochemistry for GFP was carried out on sections of spinal cord from uninjected and AAV2/9-treated mice. A) No background staining could be detected in sections taken from uninjected mice. B, C). GFP-expressing cells were detected in spinal cord sections taken from mice injected with ssAAV2/9 in utero, using low-power (B) and higher-power light microscopy (C). D) Extensive staining was also observed in the DRG. E). Examination of sections taken from mice injected with ssAAV2/9 as neonates also revealed extensive GFP expression in the spinal cord and DRG. F–H). To confirm transduction of neurons, sections of spinal cord from neonatally injected mice were immunofluorescently labeled with DAPI to label cell nuclei (F), neurofilament M to label neurons (G), and anti-GFP antibodies to label transduced cells (H). I) Merging of the signals produced colocalization, thus confirming neuronal transduction. Insets: cells of interest at higher magnification. J–L) Sections of DRG were also immunofluorescently labeled with DAPI (J), neurofilament M (K), and anti-GFP antibodies (L). M) Merging of the signals produced colocalization confirming transduction of neurons.
not happen at all if the protein is not soluble or secreted, or the receptors for uptake are not present on neurons. It is unlikely that the functional status of the BBB during the fetal and neonatal period has a major role in the differential cell-type gene expression that we observe. Daneman et al. (32) showed that vascularization of the cortex begins at E11 in mice and similarly at E12 in rats. The same study demonstrated that injection of a tracer dye into embryonic (E15) and neonatal (P1) rats via the bloodstream did not enter the CNS parenchyma, therefore providing evidence of a functional BBB at both time points. The potential influence of a developmentally dependent expression of a cell receptor for AAV2/9 on the pattern of expression must also be considered. Recently, terminal galactose was identified as the primary receptor for AAV9 (33), and investigating the expression of this receptor on neurons and astrocytes at different stages of development is warranted together with the expression of other putative receptors, such as the 37- to 67-kDa laminin receptor (34).

Notably, we have also shown, using CD68 staining, that both prenatal and neonatal administration of both ss- and scAAV2/9 did not trigger a microglia-mediated innate immune response. This is encouraging, given the relatively high doses of vector administered in this study. It is possible that the immature status of the fetal and neonatal immune system has aided in avoiding a microglia-mediated immune response. This point has been recognized as a potential advantage of fetal gene therapy. The reduction of CD68-positive cells at 1 mo after fetal, but not neonatal, AAV administration is surprising, since fetal infection (albeit with live virus rather than vector) is associated with microglial activation (35). However, there is evidence in pigs (36) that prenatal stress results in immune suppression, including reduced thymic weight, up to at least a month after

Figure 7. Extensive and efficient gene expression in the eye. Eyeballs from uninjected and AAV2/9-injected mice were examined for GFP expression. A, B) No GFP expression was visible in eyes from uninjected mice (A); extensive expression was visible in eyes from mice that received prenatal (A) and neonatal (B) administration of ssAAV2/9. C) Removal of the lens from neonatally treated mice revealed robust GFP expression. D–J) Eyeballs from mice that received scAAV2/9 were sectioned. Immunohistochemistry for GFP showed no background staining in layers of the retina in uninjected mice (D), but GFP expression was observed in sections taken from both prenatally injected (F) and neonatally injected mice (H). These layers included the ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), photoreceptors (P) and the retinal pigment epithelium (RPE). Examination of the cornea also showed no background staining in uninjected mice (E) but revealed GFP expression in the corneal endothelium (CEn), stroma (S), and corneal epithelium (CEp) in both prenatally injected (G) and neonatally injected mice (I).
birth. Therefore, maternal stress caused by laparotomy and exteriorization of the uterus may account for our observations. Further studies will also be needed to evaluate the presence of maternal viraemia and trans-placental passage of AAV2/9 to the mother, especially when taking into account the ability of this vector to cross the BBB and vascular endothelium. Furthermore, given the systemic transduction that we observe, it will be important to determine whether the vector (trans-gene or capsid) triggers humoral or cellular immune responses in organs other than the brain. Both these points will be important when evaluating this promising vector’s potential for translation into the clinic.

We confirmed that in utero delivery results in gene expression in neurons within the spinal cord and the DRG as we, in this study, and others have shown in neonates (5, 7). Interestingly, we observed that extensive expression in the retina and cornea was achieved following both in utero and neonatal administration of AAV2/9. Although AAV2/9 has been shown to transduce layers of the retina following subretinal injection (37) and corneal stroma after topical administration (38), to our knowledge, this is the first report of this vector being able to mediate expression of a protein that is detectable in all layers of the retina and cornea from a single intravenous injection. This observation makes AAV2/9 an especially attractive vector for those neurodegenerative disorders that also affect the eyes, such as infantile NCL. Retinal degeneration in this disease is one of the first signs of CNS deterioration and leads to subsequent blindness prior to brain pathology-related death (39).

Although the titers of vectors used in this study were matched, we unexpectedly observed enhanced gene expression in the brain and eye using ssAAV2/9 when compared to scAAV2/9. The sc format of AAV does not require DNA synthesis of a complementary strand, a rate-limiting step thought to hinder gene expression from the naturally occurring ss form. This has led to the publication of a number of studies in which scAAV has been shown to have the advantage in terms of gene expression (reviewed in ref. 12). An important detail to take into account in this study is the inclusion of the WPRE element in the ssAAV2/9. This is a tripartite post-transcriptional regulatory element that is required for the cytoplasmic accumulation of viral RNA (40). The WPRE is not included in the scAAV2/9 due to reduced cloning capacity, a consequence of including the complementary strand in the vector. It has been known for some time that the WPRE increases gene expression when incorporated into the untranslated 3’ region of a construct, and this has been shown in the context of AAV vectors (13). Our data suggest that an ssAAV2/9 vector that includes a WPRE sequence is more efficient at gene expression than a scAAV2/9 vector that lacks a WPRE, in terms of expression 1 mo later. However, Yokoi et al. (41) demonstrated that a scAAV initiated robust gene delivery in the retina at earlier time points when compared to the ssAAV counterpart. This may be an important consideration when rapid initiation of therapeutic gene expression in the fetus is required before disease pathology can manifest and is particularly important in addressing mouse models of disease. Because our data do not include an ssAAV2/9 that lacks the WPRE sequence, any meaningful comparisons cannot be made, and so further studies designed to specifically address and investigate these issues will be required.

If AAV2/9 vectors are to be developed for use in the clinic, then the correct choice of promoter is important. Our use of the CMV promoter for strong but relatively short-term expression is well suited for the purpose of this study. However, studies have shown that gene expression driven by the CMV promoter is silenced over time, while mammalian tissue-specific promoters allow for long-term expression (42). A realistic treatment for a number of neonatal lethal neurodegenerative diseases will require long-term expression, and so an appropriate promoter will need to be included in the vector. This does raise the question of whether a different promoter could change the cell-type specificity of gene expression that we have observed. Although the focus of this study is on the nervous system, we have observed significant transduction of peripheral tissue and organs, particularly in the musculature, when viewed under the stereoscopic fluorescence microscope. This is expected, given the intravenous route of administration and use of a ubiquitous CMV promoter. If expression of a particular gene is required to be restricted to the nervous system, then a neural specific promoter may be required. It is possible that although overexpression of a therapeutic gene is required in the nervous system, it may be detrimental in the normal development of other unaffected peripheral organs. It will be necessary to examine this on a gene-for-gene basis.

The decision to use AAV2/9 in this study was based on the recent interest and subsequent above-mentioned studies that confirmed its ability to cross the BBB in neonatal and adult animal models (5, 6). Little is known of the relative abilities of other AAV serotypes to mediate trans-BBB gene delivery following fetal intravenous administration. Identifying other vectors that share this property will require a larger and systematic screen of the various serotypes available. The isolation of novel serotypes that cross the BBB following intravenous injection would be useful in terms of “serotype switching” in the event of an antibody-mediated immune response to AAV9 or the presence of preexisting antibodies. These are both important considerations when taking into account the potential translation of this technology to the clinic. However, the likelihood of having preexisting antibodies would be minimized by early intervention, i.e., since the immune system would not yet have had chance to be exposed to viral infections.

Of course, the timing of CNS development in rodents and humans is profoundly different, and it is difficult to extrapolate between these species, depending on what criteria are used, e.g., physiological, ana-
tomical, or immunological differences. For example, if anatomical markers such as the cortical plate and subplate are used, then diffusion sensor magnetic resonance imaging studies have shown that thickening of these areas occurs in the second trimester of human development, and the equivalent occurs later, at E14–E18, in mice (43, 44). Such data highlight the importance of nonhuman primate studies to bridge this species gap. However, we do not believe that this diminishes the value of our observations in small-animal models. A range of genetically modified mice are available, and the ability to target the expression of genes to specific cell types during development will be a powerful tool and will play an important role in proof of concept studies of fetal or early neonatal gene therapy protocols. Furthermore, the widespread gene expression that prenatally and neonatally administered AAV9 mediates throughout the nervous system has potential for generating animal models of nervous system disease through gene overexpression or knockdown. This approach could circumvent the technically difficult and laborious process of generating knockout animal models. This must be weighed against the nonintegrating and therefore potentially transient expression of which AAV vectors are capable in actively dividing cells, but this may not be an issue in postmitotic neurons.

Fetal gene therapy has been shown to be effective in treating monogenic disease in animal models (9, 45). This proof of concept has now raised questions regarding the practical translation of this powerful and promising technology to the clinic to treat CNS disorders. We believe that this study provides evidence for overcoming the practical concerns and circumventing the need for invasive and technically difficult surgical procedures. This could advance the development of a realistic therapy for neonatal lethal genetic diseases of the CNS, such as type II GD and congenital NCL. Moreover this may also provide an alternative means of preventing or treating more common and, often, serious disabling diseases affecting the CNS of the preterm infant, such as cerebral palsy.

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