Transduction of the Central Nervous System After Intracerebroventricular Injection of Adeno-Associated Viral Vectors in Neonatal and Juvenile Mice

Shervin Gholizadeh,¹ Sujeenthatharmalingam,¹ Margarita E. MacAldaz,¹ and David R. Hampson^{1,2}

Abstract

Several neurodevelopmental and neurodegenerative disorders affecting the central nervous system are potentially treatable via viral vector-mediated gene transfer. Adeno-associated viral (AAV) vectors have been used in clinical trials because of their desirable properties including a high degree of safety, efficacy, and stability. Major factors affecting tropism, expression level, and cell type specificity of AAV-mediated transgenes include encapsidation of different AAV serotypes, promoter selection, and the timing of vector administration. In this study, we evaluated the ability of single-stranded AAV2 vectors pseudotyped with viral capsids from serotype 9 (AAV2/9) to transduce the brain and target gene expression to specific cell types after intracerebroventricular injection into mice. Titer-matched AAV2/9 vectors encoding the enhanced green fluorescent protein (eGFP) reporter, driven by the cytomegalovirus (CMV) promoter, or the neuron-specific synapsin-1 promoter, were injected bilaterally into the lateral ventricles of C57/BL6 mice on postnatal day 5 (neonatal) or 21 (juvenile). Brain sections were analyzed 25 days after injection, using immunocytochemistry and confocal microscopy. eGFP immunohistochemistry after neonatal and juvenile administration of viral vectors revealed transduction throughout the brain including the striatum, hippocampus, cerebral cortex, and cerebellum, but with different patterns of cell-specific gene expression. eGFP expression was seen in astrocytes after treatment on postnatal day 5 with vectors carrying the CMV promoter, expanding the usefulness of AAVs for modeling and treating diseases involving glial cell pathology. In contrast, injection of AAV2/9-CMV-eGFP on postnatal day 21 resulted in preferential transduction of neurons. Administration of AAV2/9-eGFP with the synapsin-1 promoter on either postnatal day 5 or 21 resulted in widespread neuronal transduction. These results outline efficient methods and tools for gene delivery to the nervous system by direct, early postnatal administration of AAV vectors. Our findings highlight the importance of promoter selection and age of administration on the intensity, distribution, and cell type specificity of AAV transduction in the brain.

Introduction

ADVANCEMENTS IN VIRAL VECTOR TECHNOLOGY have been encouraging for those wanting to study and develop treatments for disorders of the CNS. In the context of biological therapeutic drug delivery, diseases in which a relatively small area of the brain is affected are more amenable to treatment compared with diseases that affect all or most of the CNS. An example of the former is Parkinson's disease, in which there is a loss of dopaminergic neurons in a small discrete region of the brain (the substantia nigra). More challenging indications for CNS-directed gene therapy are those disorders that affect multiple brain regions. Examples

include fragile X syndrome (Zeier *et al.*, 2009), spinal muscular atrophy, Rett syndrome (Gadalla *et al.*, 2013), and Huntington's disease (Ramaswamy and Kordower, 2012; Zuleta *et al.*, 2012). These are diseases or disorders that could potentially be treated by globally replacing a missing or defective gene or, in the case of Huntington's disease, by knocking down or otherwise removing toxic aggregates formed by the mutant protein. The objective in these cases is to use a vector and delivery approach to broadly deliver the therapeutic reagent to enough of the CNS to impact the course of the disease (Hampson *et al.*, 2012).

Among the various vector options available for treating brain disorders, adeno-associated virus (AAV)-based vectors

¹Department of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, and ²Department of Pharmacology, Faculty of Medicine, University of Toronto, Toronto, ON, Canada M5S 3M2.

are attractive because they can transduce nondividing cells and they have the ability to confer long-term, stable transgene expression with little or no associated inflammation or toxicity (Goncalves, 2005). One key parameter for developing CNS gene delivery strategies is the choice of AAV serotype. The most common modification of AAV vectors to modulate their tropism is to use capsids from different serotypes to package the genome. Many AAV variants have been identified, each with potentially different cell tropism; these provide a broad toolkit of vectors for optimized biological therapeutic drug delivery to target cells and tissues (Wu *et al.*, 2006).

The AAV serotypes most commonly used for CNS applications include AAV1, AAV2, AAV4, AAV5, AAV6, AAV8, and AAV9 (Weinberg *et al.*, 2013). AAV2 has been widely studied during the early development of AAV vectors and was subsequently used in several clinical trials (Marks *et al.*, 2010; LeWitt *et al.*, 2011; Mittermeyer *et al.*, 2012). However, in the context of gene delivery to the CNS, AAV2 may not perform as well as more recently characterized AAV serotypes (Bockstael *et al.*, 2011; McCown, 2011). Notably, AAV9 (and to a lesser extent AAV8) can cross the blood–brain barrier after intravenous administration to transduce neurons and glia within the brain and spinal cord (Foust *et al.*, 2009; Gray *et al.*, 2011; Rahim *et al.*, 2011; Zhang *et al.*, 2011). Moreover, AAV9 undergoes efficient axonal transport within the brain (Cearley and Wolfe, 2006).

In this study, we compared the efficiency of AAV delivery after neonatal or juvenile administration of AAV2/9 and AAV2/5 vectors expressing enhanced green fluorescent protein (eGFP) driven by three different promoters. We show that intracerebroventricular administration of single-stranded AAV2/9-eGFP driven by the cytomegalovirus (CMV) or synapsin-1 (SYN) promoter to neonatal or juvenile mice leads to robust widespread gene delivery to multiple regions of the brain. Our results demonstrate that gene expression can be targeted preferentially to neurons and that this is dependent on the gene promoter used and developmental age at which the vector is administered.

Materials and Methods

Animals

All injections were carried out with wild-type C57/BL6 mice on postnatal day (PND) 5 or 21. All procedures were approved by the University of Toronto (Toronto, ON, Canada) Animal Care Committee and were carried out in compliance with the Canadian Council on Animal Care guidelines.

AAV vectors

Three different single-stranded AAV vectors were compared for their ability to transduce the brain (Fig. 1A). Single-stranded AAV-serotype 2/9 vectors (AAV2/9) were supplied by the University of Pennsylvania Vector Core facility (Philadelphia, PA). The vectors carried the eGFP gene driven by either the CMV or the human synapsin-1 (SYN) promoter. The AAV2/9 expression cassettes also included a woodchuck hepatitis posttranscriptional regulatory element (WPRE) sequence, downstream of the eGFP gene, to enhance transcription (Loeb *et al.*, 1999; Cederfjäll *et al.*, 2012) (Fig. 1A).

FIG. 1. (A) Schematic representation of the three different AAV vector constructs studied. The vectors were single-stranded, contained ITR elements from AAV serotype 2, and were packaged in either serotype 5 or serotype 9 capsids. A WPRE was inserted into the AAV2/9 vectors downstream of the eGFP cDNA to enhance transcription. (B) Schematic diagram depicting bilateral intracerebroventricular injections on postnatal day 5 and postnatal day 21. Twenty-five days after AAV administrations (i.e., on postnatal day 30 or 46), animals were killed and brains were analyzed for GFP immunoreactivity. AAV, adeno-associated viral; CBA, chicken β -actin; CMV, cytomegalovirus; i.c.v., intracerebroventricular; ITR, inverted terminal repeat; WPRE, woodchuck hepatitis post-transcriptional regulatory element; eGFP, enhanced green fluorescent protein; hSynapsin, human synapsin-1.

Both forms of the AAV2/9 vector were titer matched to 1×10^{13} genomes/ml. Single-stranded AAV-serotype 2/5 (AAV2/5) vectors were constructed and packaged at the University of Florida Powell Gene Therapy Center (Gainesville, FL). The AAV2/5 vector carries an eGFP reporter gene driven by the chicken β -actin (CBA) promoter, and the titer was 1.24×10^{13} genomes/ml. All AAV vectors were suspended in sterile phosphate-buffered saline (PBS).

Intracerebroventricular injections into neonatal mice

For injection of PND 5 mouse neonates, pups were immobilized via cryo-anesthesia for 2 min; each immobilized pup was then grasped by the skin behind the head and placed on a fiber-optic light to illuminate the midline and transverse sutures, which were used as a guide for injections. A 30-gauge needle attached to a 5-ml Hamilton syringe (Hamilton, Reno, NV) through long polyethylene tubing was used. The needle was inserted 2 mm deep, perpendicular to the skull surface, at a location approximately 0.25 mm lateral

to the sagittal suture and 0.50–0.75 mm rostral to the neonatal coronary suture. One microliter of vector or vehicle (0.9% NaCl, pH 7.4) was injected, using a syringe pump at a rate of 1 μ l/min, into each lateral ventricle. The needle was left in place for 1 min after discontinuation of plunger movement to prevent backflow. The pups were allowed to recover in a warmed container in order to return to normal temperature and were placed back into the cage with the dam after normal movement and general responsiveness were restored.

Stereotaxic injections in juvenile mice

Juvenile PND 21 animals were anesthetized with isoflurane and secured in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). The skull was exposed and holes the size of the 30-gauge injection needle were drilled into the skull. Injections were done bilaterally into the lateral ventricles, with 1 μ l of vector or vehicle per side at the rate of 0.5 μ l/min. Coordinates for injections were 0.22 mm caudal to bregma, $\pm$ 1 mm lateral to the midline, and 1 mm ventral to the pial surface. After infusion, the needle was left in place for 5 min before being slowly retracted from the brain. The incision was cleaned with sterile saline and closed with taper point nonabsorbable 5-0 surgical sutures (Syneture, Mansfield, MA). After surgery, the mice were housed individually and were monitored every day for signs of stress or infection until euthanasia.

Tissue collection and preparation

At 25 days postinjection, both eGFP vector-injected and saline-injected control mice were anesthetized with a mixture of ketamine and xylazine (intraperitoneal, 80 and 8 mg/kg, respectively) and perfused transcardially with a solution of PBS (pH 7.4) followed by 4% paraformaldehyde (pH 7.4). Perfused brains were then removed and placed in 4% paraformaldehyde overnight at 4°C, and then transferred to 30% sucrose for cryoprotection. Once the brains sank in the sucrose, they were mounted in optimum cutting temperature solution (Sakura, Torrance, CA) and frozen at –20°C until sectioning. Serial coronal sectioning of the entire brain was performed at a thickness of 25 μ m, using a cryostat (Leica Microsystems, Wetzlar, Germany).

Immunocytochemistry and confocal microscopy

Free-floating sections were rinsed once with Tris-buffered saline (TBS) for 5 min at room temperature before blocking for 1 hr with TBS containing 5% goat serum and 0.2% Triton X-100, after which sections were washed four times in TBS for 5 min with gentle rocking. Sections were then incubated overnight at 4°C in primary antibodies diluted in TBS containing 5% goat serum. To access cell type specificity of transduction, free-floating coronal brain sections were double labeled with anti-GFP and either an antibody specific for a neuronal marker (NeuN), or an astrocyte marker (GFAP). Polyclonal rabbit anti-GFP (diluted 1:2000; Abcam, Cambridge, UK) was used along with each of the following monoclonal mouse primary antibodies: neuronal nuclear antigen (NeuN, diluted 1:200; Millipore, Temecula, CA) to mark neurons and anti-S100 β (diluted 1:7500; Sigma-Aldrich, St. Louis, MO) to mark astrocytes. Cerebellar sections were

also coincubated with a polyclonal rabbit anti-calbindin antibody (diluted 1:10,000; Swant, Marly, Switzerland) to label Purkinje neurons, and a monoclonal mouse anti-GFP (diluted 1:1000; UC Davis/NIH NeuroMab Facility, Antibodies Incorporated, Davis, CA). After overnight incubation, five washes for 10 min each were carried out in TBS before the secondary antibodies (diluted in TBS containing 5% goat serum) were applied to the brain sections for 2 hr at room temperature in the dark, with gentle shaking. The sections incubated with anti-calbindin were labeled with goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 594 (diluted 1:1000; Jackson ImmunoResearch Laboratories, West Grove, PA). Goat anti-mouse Alexa Fluor 595 and goat anti-rabbit Alexa Fluor 488 (diluted 1:1000; Jackson ImmunoResearch Laboratories) were used for all other antibody combinations. The sections were then washed in TBS (five times, 10 min each) with minimal exposure to light, mounted on glass slides, air dried, and then coverslipped with Prolong Gold antifade mounting medium (Invitrogen, Carlsbad, CA). Representative tissue sections covering discrete brain regions including prefrontal cortex, striatum, cingulate cortex, hippocampus, retrosplenial cortex, piriform cortex, and cerebellum were examined with a laser-scanning confocal microscope (Nikon A1; Nikon Instruments, Tokyo, Japan). The images were captured at $\times$ 10 and $\times$ 40 magnifications and analyzed with NIS-Elements software (Nikon Instruments).

Semiquantitative and quantitative analysis of eGFP transduction in the brain

For comparative analysis of the transduction pattern in the brain, two scoring methods were used: a semiquantitative scoring system to analyze transduction in different brain regions and a quantitative cell count of transduced cells in the retrosplenial cortex. We first used a semiquantitative scoring system to estimate the transduction efficiency of various AAV vectors in discrete regions of the mouse brain including the prefrontal cortex, striatum, cingulate cortex, hippocampus, retrosplenial cortex, piriform cortex, and cerebellum (Table 1). Briefly, regions with no eGFP-positive cells were marked as (–). Regions with few eGFP-positive cells were scored (+), regions with a moderate number of eGFP-positive cells were ranked as (++) , regions with many eGFP-positive cells were scored as (+++), and regions filled with eGFP-positive cells were scored as (++++) .

Because individual cells were most easily resolved and distinguished in the retrosplenial cortex, this brain region was targeted for quantitative analysis of neuronal versus astroglial virus transduction (Table 2). Photomicrographs made with the $\times$ 10 objective lens were used to assess the total number of neurons and astrocytes within the retrosplenial cortex. The boundaries of the retrosplenial cortex were delineated according to the Paxinos atlas of the mouse brain (Paxinos and Franklin, 2013) in three consecutive 25- μ m sections stained for transduced cells via GFP immunoreactivity. Quantification was carried out by an individual masked to the time and type of AAV vector administration. The total number of eGFP-positive cells in each group as well as the percentage of eGFP-positive cells that colocalized with the neuronal marker NeuN or the astrocytic marker S100 β in the retrosplenial cortex was recorded.

TABLE 1. SEMI-QUANTITATIVE ANALYSIS OF VECTOR TRANSDUCTION PATTERNS IN DISCRETE AREAS OF THE BRAIN

	PND 5			PND 21		
	AAV2/5-CBA-eGFP	AAV2/9-CMV-eGFP	AAV2/9-SYN-eGFP	AAV2/5-CBA-eGFP	AAV2/9-CMV-eGFP	AAV2/9-SYN-eGFP
No.	3	5	3	2	3	3
Prefrontal cortex	–	+++	+++	+	++	++
Striatum	+	++++	++++	++	++++	++++
Cingulate cortex	+	++	++++	+	++	++++
Hippocampus	–	++++	++++	+	++++	++++
Retrosplenial cortex	–	++	++++	+	++	++++
Piriform cortex	–	+++	+++	–	+	+
Cerebellum	–	++	+++	–	–	–

Note: The levels of transduction were graded as follows: (–) no transduction, (+) a few transduced cells, (++) a moderate number of transduced cells, (+++) many transduced cells, and (++++ a region that was saturated with transduced cells.

Note: Light-colored squares, predominant astrocytic transduction; dark-colored squares, predominant neuronal transduction.

AAV, adeno-associated virus; CBA, chicken β actin; CMV, cytomegalovirus; eGFP, enhanced green fluorescent protein; PND, postnatal day; SYN, synapsin-1.

TABLE 2. QUANTITATIVE ANALYSIS OF eGFP TRANSDUCTION IN RETROSPLENIAL CORTEX AFTER INTRACEREBROVENTRICULAR INJECTION OF AAV2/9 VECTORS ON PND 5 OR PND 21

AAV vector	Age at administration	eGFP ⁺ /mm ² (mean $\pm$ SEM)	% eGFP ⁺ /NeuN ⁺ (mean $\pm$ SEM)	% eGFP ⁺ /S100 β ⁺ (mean $\pm$ SEM)
AAV2/9-CMV-eGFP	PND 5	261 $\pm$ 91	ND	89 $\pm$ 1
AAV2/9-SYN-eGFP	PND 5	1027 $\pm$ 91	95 $\pm$ 1	ND
AAV2/9-CMV-eGFP	PND 21	195 $\pm$ 23	81 $\pm$ 6	ND
AAV2/9-SYN-eGFP	PND 21	856 $\pm$ 85	92 $\pm$ 2	ND

Note: The total number of eGFP-positive cells in both hemispheres was counted in three sections from each brain and reported as the average number of cells per square millimeter. Percentages of neuronal and astrocytic transductions were calculated on the basis of the number of eGFP-positive cells colocalized with antibodies to NeuN and S100 β .

AAV, adeno-associated virus; CMV, cytomegalovirus; eGFP, enhanced green fluorescent protein; GFP, green fluorescent protein; ND, not determined; NeuN, neuronal nuclear antigen; PND, postnatal day; SYN, synapsin-1.

Results

Intracerebroventricular administration of AAV2/9-CMV-eGFP preferentially targets astrocytes in neonatal mice but neurons in juvenile mice

To evaluate virus transduction of the brain at early stages before CNS maturation, we investigated transgene expression after intracerebroventricular injections in neonatal mice on PND 5, or juvenile mice on PND 21. One group of mice received intracerebroventricular injections of 1×10^{13} particles of a single-stranded AAV2/9-CMV-eGFP (Fig. 1A). The animals were killed 25 days postinjection, and brains were evaluated for transgene expression.

We observed remarkable differences between PND 5 and PND 21 injections both in the distribution and cell type specificity of transductions. Injection of AAV-CMV-eGFP on PND 5 resulted in extensive distribution in all brain regions examined: prefrontal cortex, striatum, hippocampus, piriform cortex, cingulate cortex, retrosplenial cortex, and cerebellum. The transduction was observed primarily in astrocytes in several of the forebrain regions examined including the striatum (Fig. 2); retrosplenial cortex (Fig. 3); and prefrontal, piriform, and cingulate cortices (results not shown), as assessed by double labeling with anti-GFP and anti-S100 β antibodies. In contrast, in the hippocampus transduction was mainly neuronal, as assessed by double labeling with anti-

GFP and anti-NeuN antibodies (Fig. 4A–F). In the cerebellum, transgene expression was present predominantly in the dendrites and cell bodies of Purkinje neurons as determined by double labeling of the sections with anti-GFP and anti-calbindin antibodies (Fig. 5A–F).

In contrast, intracerebroventricular injections of titer-matched AAV2/9-CMV-eGFP vector on PND 21 resulted in almost exclusively neuronal transduction but with a more restricted distribution compared with neonatal injections on PND 5. Robust eGFP expression was found in many regions in the forebrain including the prefrontal cortex, striatum, and hippocampus (Table 1). Colabeling for NeuN and GFP expression in the striatum (Fig. 2D–F), retrosplenial cortex (Fig. 3D–F), hippocampus (Fig. 4D–F), and cingulate cortex (data not shown) revealed many NeuN-positive cells expressing GFP throughout all examined sections, indicating widespread neuronal transduction. Unlike PND 5 injections, which resulted in transduction of the cerebellum, PND 21 administration of AAV2/9-CMV-eGFP produced virtually no GFP-positive cells in the cerebellum and showed limited transduction in the piriform cortex (Table 1), suggesting lower vector diffusion after injection on PND 21 compared with PND 5.

In summary, the pattern of eGFP expression observed after intracerebroventricular administration of AAV2/9-CMV-eGFP on PND 5 showed preferential astrocytic transduction

FIG. 2. eGFP expression in the mouse striatum. AAV2/9 vectors were injected on PND 5 (A–C, G–I) or on PND 21 (D–F, J–L). Different patterns of cell-specific gene expression were observed; AAV2/9-CMV-eGFP transduced predominantly protoplasmic astrocytes in PND 5 injections, as assessed by double labeling with anti-GFP and with the protoplasmic astrocyte-specific anti-S100 β antibody (A–C). In contrast, AAV2/9-CMV-eGFP vectors injected on PND 21 and AAV2/9-SYN-eGFP injected on either PND 5 or PND 21 resulted in primarily neuronal transduction (D–L), as confirmed by co-staining with NeuN. *Insets:* Cells of interest at higher magnification. Scale bars: 50 μ m. Color images available online at www.liebertpub.com/hgtb

with extensive distribution in forebrain regions and the cerebellum, whereas injections on PND 21 gave nearly exclusive neuronal transgene expression and a more restricted distribution in forebrain regions and no expression in the cerebellum.

Use of the synapsin-1 promoter provides nearly exclusive neuronal transduction

We next assessed CNS expression after intracerebroventricular injection of single-stranded AAV2/9-SYN-eGFP on PND 5 and PND 21 to compare the cell type specificity and level of transgene expression when using this vector, with the same construct driven by the CMV promoter. The use of the synapsin-1 promoter resulted in extensive neuron-specific transduction regardless of the age at injection. Intracerebroventricular injections of AAV2/9-SYN-eGFP on PND 5 or PND 21 both resulted in robust neuron-specific eGFP expression in the prefrontal and cingulate cortex, striatum (Fig. 2G–L and Supplementary Fig. S2; supplementary data are available online at [http://www.liebertpub](http://www.liebertpub.com/hgtb)

[.com/hgtb](http://www.liebertpub.com/hgtb)), retrosplenial cortex (Fig. 3G–L), and hippocampus (Fig. 4G–L; Supplementary Fig. S1). However, transgene expression in the piriform cortex (Table 1) and cerebellum (Fig. 5G–L) was observed only in mice injected on PND 5. The fact that there was no transgene expression in the cerebellum after intracerebroventricular delivery of vector on PND 21 suggests a wider dispersion of AAV2/9 after intracerebroventricular delivery on PND 5. In the cerebellum, unlike AAV2/9-CMV-eGFP, which resulted in transgene expression only in the Purkinje cells (Fig. 5A–F), transduction of AAV2/9-SYN-eGFP extended to neurons in the granule cell layer as well (Fig. 5G–L).

Low transduction efficiency of AAV2/5-GFP vectors after intracerebroventricular delivery

We also sought to compare AAV2/9 vectors with an AAV2/5 vector. The AAV2/5-eGFP vector contained the chicken β -actin (CBA) promoter. The CBA promoter has previously been used in AAV vectors to transduce neurons (Gray *et al.*, 2010, 2011). We observed, however, that unlike the

FIG. 3. eGFP expression in the retrosplenial cortex. In mice injected with AAV2/9-CMV-eGFP on PND 5, the majority of eGFP-positive cells in the retrosplenial cortex were confirmed to be protoplasmic astrocytes by double labeling with anti-S100 β (A–C). In contrast, AAV2/9-CMV-eGFP injected on PND 21 and AAV2/9-SYN-eGFP injected on either PND 5 or PND 21 resulted in transduction of almost exclusively neurons (D–L), as confirmed by co-staining with anti-NeuN. *Insets:* Cells of interest at higher magnification. Scale bars: 50 μ m. Color images available online at www.liebertpub.com/hgtb

AAV2/9 vectors, intracerebroventricular injections of titer-matched AAV2/5-CBA-eGFP vector on PND 5 resulted in transduction of only a few cells in the striatum, most of which were located close to the lateral ventricles (data not shown). No GFP immunoreactivity was detected in other brain regions in mice injected on PND 5 with AAV2/5-CBA-eGFP. Administration of AAV2/5-GFP on PND 21 resulted in transduction of a few cells in the hippocampus, striatum, prefrontal cortex, cingulate cortex, and retrosplenial cortex, but no transduction in the piriform cortex and cerebellum (Table 1). Taken together, these results indicate that (1) higher transduction efficiency was obtained with AAV2/5-CBA-eGFP injected on PND 21 compared with PND 5, and (2) on both PNDs 5 and 21, the two AAV2/9 vectors showed higher diffusion in the CNS compared with the AAV2/5-based vector.

Discussion

The results presented here demonstrate that intracerebroventricular administration of AAV2/9-CMV-eGFP on PND 5 resulted in broad distribution of the transgene throughout

the brain with preferential transduction of astrocytes, whereas intracerebroventricular administration of AAV2/9-CMV-eGFP on PND 21 resulted in preferential transduction of neurons. Treatment with the same AAV2/9 vector construct, but swapping the CMV promoter for the synapsin 1 promoter (AAV2/9-SYN-eGFP), resulted in extensive neuron-specific transduction regardless of the time of administration. In contrast to AAV2/9 vectors, low transduction efficiency was observed after intracerebroventricular delivery of titer-matched AAV2/5-eGFP, administered either on postnatal day 5 or 21. These results highlight the important effects of AAV serotype, the promoter, and the age at administration on the intensity, distribution, and cell type specificity of AAV transductions.

We observed age-related differences in cell type specificity after intracerebroventricular administration of AAV2/9-CMV-eGFP in neonatal versus juvenile mice. Previous reports from fetal and neonatal intravenous administration of AAV2/9-GFP showed preferential transduction of neurons and astrocytes, respectively (Rahim *et al.*, 2011). Similarly, transduction in neonatal neurons and adult astrocytes after

FIG. 4. Transgene expression in the CA1 region of the hippocampus after administration of AAV2/9-CMV-eGFP (A–F) or AAV2/9-SYN-eGFP (G–L) vector on PND 5 or PND 21. Brain sections from the hippocampus of AAV-injected mice were double labeled with anti-GFP and anti-NeuN. *Insets:* Cells of interest shown at higher magnification. Scale bars: 50 μ m. Color images available online at www.liebertpub.com/hgtb

intravenous injections has been reported in another study using AAV2/9-GFP vectors (Foust *et al.*, 2009). Differences in cell-type-specific transduction profiles at different stages in development are important considerations when planning gene therapy studies for monogenic neurological disorders, where the aim of the study is to introduce the missing protein specifically to either neurons or glia. On the basis of our results, administration of the AAV2/9 vector driven by the CMV promoter on PND 21 would result in the therapeutic protein being produced predominantly in neurons, where it is most critically required in many neurological disorders. Examples include Angelman syndrome and Rett syndrome, in which the disease pathology is caused primarily by the absence or near absence of a missing protein in neurons (Hampson *et al.*, 2012).

Compared with AAV2/9 vectors, we observed low transduction efficiency after intracerebroventricular delivery of titer-matched AAV2/5 when administered either on PND 5 or PND 21. This is in line with other reports in the literature regarding the low efficiency of transduction after intracerebroventricular delivery of AAV5-based vectors in

neonates (Passini *et al.*, 2003) and adults (Davidson *et al.*, 2000). Among the various recombinant AAV serotypes used for global gene delivery to the brain, AAV9 vectors appear particularly appealing for their transduction robustness, ability to cross the blood–brain barrier (Foust *et al.*, 2009; Rahim *et al.*, 2011), and ability to undergo axonal transport (Cearley and Wolfe, 2006).

A goal of this study was to compare the cell type specificity of virus transduction after treatment with AAV2/9-eGFP directed by the CMV versus the synapsin-1 promoter. In contrast to the AAV2/9-CMV-eGFP vector, which preferentially targeted astrocytes in neonatal mice and neurons in juvenile mice, the AAV2/9-SYN-eGFP vector resulted in extensive neuron-specific transduction regardless of age at the time of injection. Therefore, intracerebroventricular delivery of AAV2/9-CMV vectors can be of potential interest for the development of gene therapy strategies in neurological disorders, for which therapeutic success relies on preferential expression of the transgene in neurons. Although not investigated here, it is worth noting that apart from changing

FIG. 5. Transgene expression in the cerebellum. eGFP expression was seen in the cerebellum after injection of mice with AAV2/9-CMV-eGFP or AAV2/9-SYN-eGFP on PND 5 (A–L) but not in mice injected on PND 21 (data not shown). AAV2/9-CMV-eGFP resulted in widely distributed transduction of Purkinje neuron cell bodies and dendrites in the molecular layer of the cerebellum (A–C, D–F). With AAV2/9-SYN-eGFP, GFP transgene expression was seen in both Purkinje neurons and in some granule neurons (G–I, J–L). Co-staining with anti-calbindin was done to confirm the presence of GFP-positive cells in Purkinje neurons. M, molecular layer; G, granular layer; P, Purkinje cell layer. *Insets:* Selected Purkinje neurons shown at higher magnification. Scale bars: (A–C, G–I) 200 μ m; (D–F, J–L) 50 μ m. Color images available online at www.liebertpub.com/hgtb

the promoter to provide cell-specific or ubiquitous expression, the choice of gene enhancer, 5' untranslated region (UTR) sequences, and 3' UTR polyadenylation signal can also have strong effects on transgene expression.

For delivery of therapeutic macromolecules such as AAV-expressed transgenes to the CNS, either mode of administration (intracerebroventricular or intravenous) has advantages and disadvantages. Intravenous delivery is relatively noninvasive and can result in homogeneous vector distribution in the CNS. Several groups have reported on the ability of AAV9 vectors to cross the blood–brain barrier and transduce neurons and astrocytes after intravenous injection into neonatal mice, adult mice, cats, and nonhuman primates (Duque *et al.*, 2009; Foust *et al.*, 2009; Rahim *et al.*, 2011; Mattar *et al.*, 2013). However, a disadvantage of this approach is the high biodistribution of the vector to off-target peripheral tissues, thus potentially compromising the level of expression and treatment efficacy. This problem can be circumvented by intracerebroventricular administration. Our results demonstrate that intracerebroventricular treatment with AAV2/9 vector results in excellent diffusion properties

in the brain, especially when administered during the early postnatal period. Finally, another advantage of delivering AAV vectors into the cerebrospinal fluid is the possibility of targeting ependymal cells that line the inside perimeter of the ventricles and form the interface between the ventricles and brain parenchyma. For transgenes that produce soluble proteins, uptake into ependymal cells provides the potential to secrete the transgene into the cerebrospinal fluid, where it can diffuse into the brain; this strategy was successfully employed to treat mice with mucopolysaccharidosis VII, using AAV4 vectors (Ghodsi *et al.*, 1999; Fu *et al.*, 2007).

In summary, our results demonstrate that intracerebroventricular injection of AAV2/9 vectors can be used to induce extensive neuronal or astrocytic transduction in the neonatal and juvenile mouse brain, and that the distribution and cell type specificity of viral vector transduction is influenced by the age at the time of vector administration, and by the gene promoter and AAV serotype used. These findings are potentially applicable to the formulation of preclinical gene therapy strategies for the treatment of both neurodevelopmental disorders and neurodegenerative diseases.

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Author Disclosure Statement

No competing financial interests exist.

References

- Bockstael, O., Foust, K., Kaspar, B., and Tenenbaum, L. (2011). Recombinant AAV delivery to the central nervous system. *Methods Mol. Biol.* 807, 159–177.
- Cearley, C.N., and Wolfe, J.H. (2006). Transduction characteristics of adeno-associated virus vectors expressing Cap serotypes 7, 8, 9, and Rh10 in the mouse brain. *Mol. Ther.* 13, 528–537.
- Cederfjäll, E., Sahin, G., Kirik, D., and Björklund, T. (2012). Design of a single AAV vector for coexpression of TH and GCH1 to establish continuous DOPA synthesis in a rat model of Parkinson's disease. *Mol. Ther.* 20, 1315–1326.
- Davidson, B.L., Stein, C.S., Heth, J.A., *et al.* (2000). Recombinant adeno-associated virus type 2, 4, and 5 vectors: Transduction of variant cell types and regions in the mammalian central nervous system. *Proc. Natl. Acad. Sci. U.S.A.* 97, 3428–3432.
- Duque, S., Joussemet, B., Riviere, C., *et al.* (2009). Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons. *Mol. Ther.* 17, 1187–1196.
- Foust, K.D., Nurre, E., Montgomery, C.L., *et al.* (2009). Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat. Biotechnol.* 27, 59–65.
- Fu, H., Kang, L., Jennings, J.S., *et al.* (2007). Significantly increased lifespan and improved behavioral performances by rAAV gene delivery in adult mucopolysaccharidosis IIIB mice. *Gene Ther.* 14, 1065–1077.
- Gadalla, K., Bailey, M., Spike, R., *et al.* (2013). Improved survival and reduced phenotypic severity following AAV9/*MECP2* gene transfer to neonatal and juvenile male *Mecp2* knockout mice. *Mol. Ther.* 21, 18–30.
- Ghods, A., Stein, C., Derksen, T., *et al.* (1999). Systemic hyperosmolality improves β -glucuronidase distribution and pathology in murine MPS VII brain following intraventricular gene transfer. *Exp. Neurol.* 160, 109–116.
- Goncalves, M.A. (2005). Adeno-associated virus: From defective virus to effective vector. *Virol. J.* 2, 43.
- Gray, S., Foti, S., Schwartz, J., *et al.* (2011). Optimizing promoters for recombinant adeno-associated virus-mediated gene expression in the peripheral and central nervous system using self-complementary vectors. *Hum. Gene Ther.* 22, 1143–1153.
- Gray, S.J., Blake, B.L., Criswell, H.E., *et al.* (2010). Directed evolution of a novel adeno-associated virus (AAV) vector that crosses the seizure-compromised blood–brain barrier (BBB). *Mol. Ther.* 18, 570–578.
- Hampson, D.R., Gholizadeh, S., and Pacey, L.K. (2012). Pathways to drug development for autism spectrum disorders. *Clin. Pharmacol. Ther.* 91, 189–200.
- LeWitt, P.A., Rezai, A.R., Leehey, M.A., *et al.* (2011). AAV2-GAD gene therapy for advanced Parkinson's disease: A double-blind, sham-surgery controlled, randomised trial. *Lancet Neurol.* 10, 309–319.
- Loeb, J.E., Cordier, W.S., Harris, M.E., *et al.* (1999). Enhanced expression of transgenes from adeno-associated virus vectors with the woodchuck hepatitis virus posttranscriptional regulatory element: Implications for gene therapy. *Hum. Gene Ther.* 10, 2295–2305.
- Marks, W.J., Jr., Bartus, R.T., Siffert, J., *et al.* (2010). Gene delivery of AAV2-neurturin for Parkinson's disease: A double-blind, randomised, controlled trial. *Lancet Neurol.* 9, 1164–1172.
- Mattar, C., Waddington, S., Biswas, A., *et al.* (2013). Systemic delivery of scAAV9 in fetal macaques facilitates neuronal transduction of the central and peripheral nervous systems. *Gene Ther.* 20, 69–83.
- McCown, T.J. (2011). Adeno-associated virus (AAV) vectors in the CNS. *Curr. Gene Ther.* 11, 181–188.
- Mittermeyer, G., Christine, C.W., Rosenbluth, K.H., *et al.* (2012). Long-term evaluation of a phase 1 study of AADC gene therapy for Parkinson's disease. *Hum. Gene Ther.* 23, 377–381.
- Passini, M.A., Watson, D.J., Vite, C.H., *et al.* (2003). Intraventricular brain injection of adeno-associated virus type 1 (AAV1) in neonatal mice results in complementary patterns of neuronal transduction to AAV2 and total long-term correction of storage lesions in the brains of β -glucuronidase-deficient mice. *J. Virol.* 77, 7034–7040.
- Paxinos, G., and Franklin, K. (2013). *The Mouse Brain in Stereotaxic Coordinates*, 4th ed. (Elsevier, San Diego, CA).
- Rahim, A., Wong, A., Hofer, K., *et al.* (2011). 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. *FASEB J.* 25, 3505–3518.
- Ramaswamy, S., and Kordower, J.H. (2012). Gene therapy for Huntington's disease. *Neurobiol. Dis.* 48, 243–254.
- Weinberg, M., Samulski, R., and McCown, T. (2013). Adeno-associated virus (AAV) gene therapy for neurological disease. *Neuropharmacology* 69, 82–88.
- Wu, Z., Asokan, A., and Samulski, R.J. (2006). Adeno-associated virus serotypes: Vector toolkit for human gene therapy. *Mol. Ther.* 14, 316–327.
- Zeier, Z., Kumar, A., Bodhinathan, K., *et al.* (2009). Fragile X mental retardation protein replacement restores hippocampal synaptic function in a mouse model of fragile X syndrome. *Gene Ther.* 16, 1122–1129.
- Zhang, H., Yang, B., Mu, X., *et al.* (2011). Several rAAV vectors efficiently cross the blood–brain barrier and transduce neurons and astrocytes in the neonatal mouse central nervous system. *Mol. Ther.* 19, 1440–1448.
- Zuleta, A., Vidal, R.L., Armentano, D., *et al.* (2012). AAV-mediated delivery of the transcription factor XBP1s into the striatum reduces mutant Huntingtin aggregation in a mouse model of Huntington's disease. *Biochem. Biophys. Res. Commun.* 420, 558–563.

Address correspondence to:
Dr. David R. Hampson
University of Toronto
144 College Street
Toronto, ON M5S 3M2
Canada

E-mail: d.hampson@utoronto.ca

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