SHORT COMMUNICATION

Axonal transport of adeno-associated viral vectors is serotype-dependent

EA Salegio1,3, L Samaranch1,3, AP Kells1, G Mittermeyer1, W San Sebastian1, S Zhou2, J Beyer1, J Forsayeth1 and KS Bankiewicz1

We have previously shown that adeno-associated virus type 2 (AAV2) undergoes anterograde axonal transport in rat and non-human primate brain. We screened other AAV serotypes for axonal transport and found that AAV6 is transported almost exclusively in a retrograde direction and, in the same way as AAV2, it is also neuron-specific in rat brain. Our findings show that axonal transport of AAV is serotype dependent and this has implications for gene therapy of neurological diseases such as Huntington’s disease.

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INTRODUCTION

Previously, we found that adeno-associated virus type-2 (AAV2) undergoes robust axonal anterograde transport when injected into either rat striatum1 or non-human primate thalamus.2 Injections of AAV2-glial cell line-derived neurotrophic factor (GDNF) into rat striatum resulted in GDNF expression in multiple basal ganglia nuclei, including globus pallidus, entopeduncular nucleus and substantia nigra pars reticulata. Similarly, injection of AAV2-GDNF or AAV2-green fluorescent protein (GFP) into primate thalamus resulted in broad GDNF and GFP expression in the cerebral cortex, including cingulate cortex, pre-frontal, pre-motor, primary and secondary somatosensory and motor areas of the cortex. In addition to the intense staining of individual neuronal cell bodies and cellular processes, GDNF staining was observed across multiple layers of the cortex with an intensity gradient that was highest in cortical layers III and IV after thalamic delivery of AAV2-GDNF. Although weak retrograde transport of AAV2 up the sciatic nerve from neuromuscular terminals has been reported,3 this phenomenon does not seem to be so in the brain. For example, GDNF is not observed in the cortex after delivery of AAV2-GDNF to the striatum even though cortical neurons project abundantly to the striatum.4 This suggests that AAV2 is not retrogradely transported by corticostriatal neurons in the brain, but exhibits a very strong bias towards anterograde transport.

In assessing axonal transport of vectors, it is important to recognize that transgenic proteins are often present at high levels in the cytoplasm of transduced neurons and, hence, neuronal fibers in distally innervated brain nuclei can be immunostained for the expressed protein. This phenomenon does not per se describe axonal transport; rather it is the presence of transduced cell bodies in non-injected areas of the brain that is indicative of potential axonal transport of the vector. Anterograde transport of intact virions is, therefore, characterized by the presence of transduced cell bodies in areas known to receive axonal projections from the site of vector delivery, but do not themselves have reciprocal projections to site of vector delivery. Thus, intact virions must be anterogradely transported along axons originating within the site of vector delivery, be released at the axon terminal, and then transduce neighboring cells.5 Retrograde transport, on the other hand, requires uptake of vector by axonal projections within the site of delivery and transport to the distally located soma where it transduces the host cell nucleus and results in the presence of the transgenic protein throughout the neuron soma and fibers (Figure 1).

As part of our investigation into axonal transport of AAV vectors, we conducted a survey of a number of AAV serotypes. In order to avoid confounding effects, we eliminated AAV vectors that were not exclusively neuron-specific. Of an initial screen of six AAV serotypes (1, 5, 6, 7, 8 and 9) in rat brain, only AAV6 was observed to be as neuron-specific as AAV2. Accordingly, we asked whether AAV6 underwent axonal transport similar to AAV2. To test this, we examined the transport of AAV6 and AAV2 along efferent and afferent projections by infusing them into different regions of the rat brain. We found that AAV6 not only transduced rat neurons several-fold better than AAV2, but was also transported almost exclusively in a retrograde direction. We suggest that AAV6 may find particular utility in diseases, such as Huntington’s disease, where cortico-striatal pathways could be targeted. Identification of the molecular determinants of anterograde and retrograde axonal transport is likely to aid our understanding of mechanisms of axonal transport of AAV and assist in the engineering of novel AAV vectors with altered function.

RESULTS

The central aim of this project was to understand differences in axonal trafficking of different AAV serotypes. We have previously documented that AAV2 is axonally transported in an anterograde direction in rodent and non-human primate brain.1,2 In an initial screen, we looked for serotypes that transduced only neurons. We have previously made the observation that neuronal specificity offers protection against the problem of cell-mediated immunity arising from expression of non-native proteins in

1Department of Neurosurgery, University of California San Francisco, San Francisco, CA, USA and 3Research Vector Core, Center for Cellular and Molecular Therapeutics at The Children’s Hospital of Philadelphia, Philadelphia, PA, USA. Correspondence: Dr KS Bankiewicz, Department of Neurosurgery, University of California San Francisco, 1855 Folsom Street, MCB, Room 226, San Francisco, CA 94103-0555, USA.
E-mail: Krystof.Bankiewicz@ucsf.edu

2These authors contributed equally to this work.

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antigen-presenting cells in the brain.\(^4\) Infusion of AAV6-GFP into rat thalamus demonstrated this serotype's neuronal specificity (Figure 2). The tropism of other serotypes generated a mixed pattern of cellular transduction with GFP\(^+\) glia and neurons found in regions proximal and distal to the site of injection (data not shown).

In previous experiments, we found that the double-stranded nature of the AAV6-GFP vector evinces more powerful transduction than the single-stranded AAV2-GFP (data not shown). Therefore, to roughly equalize GFP expression, we decided to compare a 15-\(\mu\)l AAV2-GFP injection into the rat brain to a 3-\(\mu\)l injection of AAV6-GFP.

At 3 weeks after unilateral injection of these two vectors, we found robust GFP expression at the site of injection in all animals that were infused either in thalamus or in striatum (Supplementary Figure S1).

**Thalamic infusion**

To determine whether there were any differences in axonal transport, we injected AAV6-GFP and AAV2-GFP into the thalamus and analyzed transduction patterns 3 and 6 weeks after delivery. After 3 weeks, GFP\(^+\) neurons were only observed in the cortex of AAV6-GFP-injected rats (Figure 3a) but not in the cortex of those that received AAV2-GFP (Figure 3b). In contrast, 6 weeks after thalamic infusion, GFP\(^+\) cells were observed after both AAV6-GFP and AAV2-GFP injection (Figures 3c and d, respectively). These results suggest that transduction and axonal transport occur at a slower rate for AAV2 than AAV6. This difference in the timecourse of distal but not proximal transduction is, in our view, consistent with the idea that transduction of distal neurons by anterograde transport should take considerably longer than retrograde transport, and is in agreement with previous data showing slow changes in transduction pattern in non-human primates after delivery of AAV2-human L-amino acid decarboxylase to the putamen.\(^5\)

Clearly, transduction of thalamus is complicated by the bidirectional neuronal connections between thalamus and cortex (cortico-thalamic and thalamo-cortical projections, Figure 4a). Therefore, to determine any differences in transport between these vectors we looked for simpler connections between thalamus and other brain regions such as substantia nigra and striatum. Nigro-thalamic projections are attractive because the substantia nigra pars reticulata (SNr) sends projections to the thalamus (Figure 4a).\(^6\) Injection of AAV6-GFP into thalamus resulted in significant cell body GFP\(^+\) staining in SNr (Figure 4b) at 3 weeks after infusion. In contrast, thalamic AAV2-GFP gave no staining in SNr (Figure 4c), indicating absence of retrograde transport of AAV2 but robust retrograde transport of AAV6. In addition, thalamo-striatal projections are also attractive to determine whether or not AAV6 undergoes anterograde transport (Figure 4a). We found no GFP\(^+\) cells in the striatum of 6-week-old animals that received AAV6-GFP (Figure 5a). On the other hand, in accord with the previous data observed in the thalamo-cortical projections, we found GFP\(^+\) cells in the striatum of the animals that received AAV2-GFP in the thalamus (Figure 5b), indicating absence of anterograde transport of AAV6 and a delayed anterograde trans-synaptic transport of AAV2.

A few isolated cell bodies were found in the substantia nigra pars compacta (SNc) after thalamic AAV2, suggesting perhaps that there maybe some anterograde connections from thalamus to SNc, but the presence of such afferents remains to be elucidated. Thus, the provenance of this minimal expression is unclear.

**Figure 1.** Anterograde and retrograde transport after AAV infusion. Diagram illustrates anterograde and retrograde transport of AAV vectors from the site of delivery to a second distally located brain region. (a) Axonal anterograde transport requires the transport of viral particles via an axon projecting from the site of vector injection to a distal area with subsequent transduction of cells located within the brain region where the axon ends. The presence of fibers only in the distal area is not classified as anterograde transportation of the AAV vector. (b) Retrograde transport of AAV vectors occurs when viral particles are taken up by axonal terminals in the injection site and are then transported back to the neuronal cell soma where they subsequently transduce the neuron.

**Figure 2.** Cellular specificity of AAV6-GFP. Neuron-specific transduction after parenchymal infusion of AAV6-GFP (a, c and e) with no transgene expression detected in glia within regions of the cortex (a, b), striatum (c, d) and/or thalamus (e, f). Insets show high-power magnification images of GFP\(^+\) cells. Scale bar, 20\(\mu\)m.
To further investigate the distribution pattern of AAV6 transduction, we injected AAV6-GFP into the striatum, a nucleus known to receive projections from many regions such as cortex, SNc or thalamus (Figure 6a). At 3 weeks after striatal injection of AAV6-GFP, GFP⁺ neurons were found within the cortex, thalamus and SNc, indicative of transduction via retrograde transport of AAV6 (Figures 6b-d, respectively). We found GFP⁺ cells along the anterior-posterior axis of the brain from pre-frontal to occipital cortex, which excludes cannula reflux as an explanation (Supplementary Figure S2). To examine the possible anterograde transport of AAV6, we analyzed the SNr, which receives projections from the striatum. After 3 weeks, no GFP⁺ cell bodies in the SNr were found (Figure 6d). The complete absence of any positive cells, also suggests a lack of anterograde transport of AAV6-GFP, although the presence of GFP⁺ fibers shows that striatal GABAergic neurons were transduced.

DISCUSSION
Anterograde transport of AAV vectors is characterized by the transduction of cells in a region of the brain that is not directly injected but receives axonal projections from neurons in the primary injection target (Figure 1a). Inefficient internalization of AAV2 into the nucleus and the slow uncoating of the viral particles⁷ may facilitate anterograde transport of intact AAV2 particles to the terminals with subsequent release from axon terminals and/or trans-synaptic transport.

In contrast, retrograde transport of AAV is characterized by the transduction of neurons located in a brain region away from the site of injection, but that directly innervate the site of delivery (Figure 1b). Uptake of AAV particles at the axon terminal may result in retrograde transport to the nucleus and transduction of the entire neuron.
In differentiating between anterograde and retrograde transport of AAV, we limited our analysis to GFPþ cell bodies in nuclei with well-characterized axonal connections (e.g., thalamus and striatum). Evidence of retrograde transport of AAV6 was demonstrable and contrasted strikingly with the anterograde pattern of AAV2. GFPþ cortical neurons observed in rats 6 weeks after thalamic infusion, but not after merely 3 weeks, would be the result of this anterograde transport of AAV2-GFP, as described above. As the rat brain is a small structure, it is formally possible that infusing 15 m l of AAV2-GFP in the thalamus could result in some vector diffusion to the striatum. If so, the vector would enter some cortico-striatal terminals, retrogradely transport to the soma and transduce some cortical neurons. However, this is an unlikely possibility as thalamic infusion in our animals was well contained within the boundaries of the thalamus (Supplementary Figure 2b).

On the other hand, both striatal and thalamic infusions of AAV6-GFP resulted in the presence of GFPþ neurons in SNC, cortex and thalamus or SNr and cortex, respectively, indicating the retrograde transport of AAV6-GFP vector and subsequent transduction of distal structures that innervate these nuclei. In other words, examination of axonal nigro-thalamic and cortico-striatal projections provided a convincing argument for the retrograde transport of AAV6 along either striatal or thalamic connections. However, examination of thalamo-striatal and striato-nigral projections corroborated the absence of anterograde transport of AAV6 along the thalamus. AAV6 was not anterogradely transported either 3 or 6 weeks after infusion, as demonstrated by the lack of GFPþ cell bodies in brain nuclei innervated by either striatum or thalamus. These experiments permit the construction of a wiring diagram that describes axonal trafficking for each serotype after infusion into the thalamus (Figure 4a) or striatum (Figure 5a).

Little is known about the molecular mechanism of axonal transport of AAV although other viral vectors, such as herpes simplex 1, display both anterograde and retrograde axonal trafficking that seems to be dependent on specific protein motifs. Similarly, retrograde transport of adenovirus has been described in rat brain. It is unclear what drives the directionality of axonal AAV transport. One hypothesis is that differential engagement with molecular machinery that specifies anterograde or retrograde transport underlies this phenomenon. Herpes simplex virus 1 exhibits interactions with both kinesin and dynein to catalyze anterograde and retrograde axonal transport, respectively. We speculate that these two capacities may be separately defined in structural differences in AAV2 and AAV6 capsids. The present study defines distinct functional specificities for AAV2 and AAV6 that may be amenable to mutational analysis. In addition, we reiterate the importance of vector production, purification method, overall quality and animal species-specificity as major determinants in AAV serotype tropism.

Parenchymal retrograde axonal transport of AAV6 has some potential in neurological diseases with cortical involvement, such as Huntington’s disease, as it could address gene therapy strategies designed to suppress mutant htt gene expression because both medium spiny neurons in striatum and cortico-striatal projections are affected in the disease. Delivery of an AAV6 vector with the appropriate transgene to the striatum would be expected to ameliorate the disease more effectively than would an AAV2 vector. We have recently developed an advanced MRI-guided delivery system for striatal gene therapy for Parkinson’s disease, and the present study supports integration of AAV6 vectors with this delivery system for the treatment of Huntington’s disease and perhaps other diseases in which axonal transport may be a consideration.
MATERIALS AND METHODS

Animals
A total of 24 Sprague-Dawley rats (~250–350 g) were randomly allocated into groups that received injections of AAV2-GFP (N = 12) or AAV6-GFP (N = 12) into either thalamus or striatum by convection-enhanced delivery as previously described. All procedures were performed in accordance with the UCSF Institutional Animal Care and Use Committee.

Adeno-associated virus (AAV) vector
AAV2-GFP (single-stranded) and AAV6-GFP (double-stranded) were formulated at a concentration of 1.2 × 10^12 vg ml⁻¹ (AAV2) or 2.5 × 10^13 vg ml⁻¹ (AAV6) by the Vector Core at Children’s Hospital of Philadelphia. AAV6 titer was adjusted to ~1.2 × 10^11 vg ml⁻¹ before infusion with phosphate-buffered saline (PBS) containing 0.001% v/v Pluronic F-68.

Vector infusion
Animals were anesthetized with isoflurane (Baxter, Deerfield, IL, USA) and placed in a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA). A 1 cm-long incision was made in the skin overlying the skull and a burr-hole was drilled at the following coordinates (thalamus, AP: –2.8, ML: +1.6, DV: –5.5 mm; striatum, AP: +1.2, ML: +2.4, DV: –5.0 mm). A cannula made from fused silica (Polymeric Technologies, Phoenix, AZ, USA) with a 1-mm step was used to deliver infusate unilaterally into the right thalamus or striatum by convection-enhanced delivery at a rate of 0.5 μl min⁻¹. All animals were infused with either 3 μl (AAV6-GFP) or 15 μl (AAV2-GFP) per target site (Supplementary Figure S1).

Tissue processing
At 3 or 6 weeks after vector infusion, animals were transcardially perfused with PBS followed by 4% paraformaldehyde/PBS, their brains harvested and cryoprotected in 30% w/w sucrose. A sliding microtome (HM 450, Thermo Scientific, Waltham, MA, USA) was used to cut 40-μm serial sections that were then processed for immunohistochemistry.

Immunoperoxidase staining
A polyclonal antibody against GFP (rabbit anti-GFP, Millipore, Billerica, MA, USA) was used for immunodetection of the transgene. Briefly, sections were washed with PBS (3 × 5 min), endogenous peroxidase activity quenched in 1% H₂O₂/30% ethanol for 30 min, and sections were then washed briefly in 1% PBST, blocked (1:200, Jackson ImmunoResearch) and anti-mouse-TRITC (1:200, Jackson ImmunoResearch) in PBST for 1 h at room temperature, washed in PBS and wet mounted on frosted slides.

Immunofluorescent staining
Double immunolabeling was performed to determine whether GFP⁺ cells were located within the SNC (tyrosine hydroxylase, TH⁺). Briefly, brain sections were immunostained for GFP (rabbit polyclonal, 1:100, Millipore) and TH (mouse monoclonal, 1:100, Millipore), washed with PBST, blocked for 60 min in 20% normal horse serum (Jackson Immunoresearch, West Grove, PA, USA) and incubated with primary antibodies in Biocare DaVinci Green diluent (#PD900, Biocare Medical) for 24 h at 4°C. After incubation with primary antibodies, sections were washed in PBST, incubated with a cocktail of secondary antibodies anti-rabbit-FITC (1:200, Jackson Immunoresearch) and anti-mouse-TRITC (1:200, Jackson ImmunoResearch) in PBST for 1 h at room temperature, washed in PBS and wet mounted on frosted slides.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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REFERENCES
1 Ciesielska A, Mittermeyer G, Hadaczek P, Kells AP, Forsayeth J, Bankiewicz KS. Anterograde axonal transport of AAV2-GDNF in rat basal ganglia. Mol Ther 2011; 19: 922 - 927.
2 Kells AP, Hadaczek P, Yin D, Bringas J, Varenika V, Forsayeth J et al. Efficient gene therapy-based method for the delivery of therapeutics to primate cortex. Proc Natl Acad Sci USA 2009; 106: 2407 - 2411.
3 Kaspar BK, Llado J, Scherkt N, Rothstein JD, Gage FH. Retrograde viral delivery of IGF-I prolongs survival in a mouse ALS model. Science 2003; 301: 839 - 842.
4 Hadaczek P, Forsayeth J, Mirek H, Munson K, Bringas J, Pirvovito P et al. Transduction of nonhuman primate brain with adeno-associated virus serotype 1: vector trafficking and immune response. Hum Gene Ther 2009; 20: 225 - 237.
5 Daadi MM, Pirvovito P, Bringas J, Cunningham J, Forsayeth J, Eberling J et al. Distribution of AAV2-hAADC-transduced cells after 3 years in Parkinsonian monkeys. Neuroreport 2006; 17: 201 - 204.
6 Kha HT, Finkelstein DL, Tomas D, Drago J, Pow DV, Horne MK. Projections from the substantia nigra pars reticulata to the motor thalamus of the rat: single axon reconstructions and immunohistochemical study. J Comp Neurol 2001; 440: 20 - 30.
7 Thomas CE, Storm TA, Huang Z, Kay MA. Rapid uncoating of vector genomes is the key to efficient liver transduction with pseudotyped adeno-associated virus vectors. J Virol 2004; 78: 3110 - 3122.
8 Frantom Jr AR, Goins WP, Nakano K, Burton EA, Glorioso JC. HSV trafficking and development of gene therapy vectors with applications in the nervous system. Gene Therapy 2005; 12: 891 - 901.
9 McGraw HM, Awasthi S, Wojcieszowskij, JA, Friedman HM. Anterograde spread of herpes simplex virus type 1 requires glycoprotein E and glycoprotein I but not Usq. J Virol 2009; 83: 8315 - 8326.
10 Kuo H, Ingram DK, Crystal RG, Mastrangeli A. Retrograde transfer of replication deficient recombinant adeno-virus vector in the central nervous system for tracing studies. Brain Res 1995; 705: 31 - 38.
11 Diefenbach RJ, Miranda-Saksena M, Douglas MW, Cunningham AL. Transport and egress of herpes simplex virus in neurons. Rev Med Virol 2008; 18: 35 - 51.
12 Gao G, Vandenberghen LH, Alivra MR, Lu Y, Caldecro R, Zhou X et al. Clades of Adeno-associated viruses are widely disseminated in human tissues. J Virol 2004; 78: 6381 - 6388.
13 Burger C, Gorbatsyuk O, Velardo M, Peden C, Williams P, Zolotukhin S et al. Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1 and 2 and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. Mol Ther 2004; 10: 302 - 317.
14 Choi VW, McCarty DM, Samulski RJ. AAV hybrid serotypes: improved vectors for gene delivery. Curr Gene Ther 2005; 5: 299 - 310.
15 Fitzsimons HL, Bland RJ, During MJ. Promoters and regulatory elements that improve adeno-associated virus transgene expression in the brain. Methods 2002; 28: 227 - 236.
16 Green AP, Huang JJ, Scott MO, Kierstead TD, Beapure I, Gao GP et al. A new scalable method for the purification of recombinant adenovirus vectors. Hum Gene Ther 2002; 13: 1921 - 1934.
17 Klein RL, Dayton RD, Tatom JB, Henderson KM, Henning PP, AAV8, 9, Rh10, Rh43 vector gene transfer in the rat brain: effects of serotype, promoter and purification method. Mol Ther 2008; 16: 89 - 96.
18 Berardelli A, Noth J, Thompson PD, Bollen EL, Curra A, Deuschl G et al. Pathophysiology of chorea and Bradykinin in Huntington’s disease. Mov Disord 1999; 14: 398 - 403.
19 Richardson RM, Kells AP, Rosenbluth KH, Salegio EA, Fiandra MS, Larson PS et al. Interventional MRI-guided putaminal delivery of AAV2-GDNF for a planned clinical trial in Parkinson’s disease. Mol Ther 2011; 19: 1048 - 1057.

Supplementary Information accompanies the paper on Gene Therapy website (http://www.nature.com/gt)