Improvised gene delivery to adult mouse spinal cord through the use of engineered hybrid adeno-associated viral serotypes

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Adeno-associated viral (AAV) vectors are often used in gene therapy for neurological disorders because of its safety profile and promising results in clinical trials. One challenge to AAV gene therapy is effective transduction of large numbers of the appropriate cell type, which can be overcome by modulating the viral capsid through DNA shuffling. Our previous study demonstrates that Rec2, among a family of novel engineered hybrid capsid serotypes (Rec1 ~ 4) transduces adipose tissue with far superior efficiency than naturally occurring AAV serotypes. Here we assessed the transduction of adult spinal cord at two different doses of AAV vectors expressing green fluorescent protein (2 × 109 or 4 × 109 viral particles) via intraparenchymal injection at the thoracic vertebral level T9. In comparison with an equal dose of the currently preferable AAV9 serotype, Rec3 serotype transduced a broader region of the spinal cord up to ~ 1.5 cm longitudinally and displayed higher transgene expression and increased maximal transduction rates of astrocytes at either dose and neurons at the lower dose. These novel engineered hybrid vectors could provide powerful tools at lower production costs to manipulate gene expression in the spinal cord for mechanistic studies or provide potent vehicles for gene therapy delivery, such as neurotrophins, to the spinal cord.

INTRODUCTION

Recombinant adeno-associated viral (rAAV) vectors are not only powerful vehicles of gene delivery for basic research but also have been widely used for gene therapy in genetic and acquired diseases.1 rAAV vectors have become the preferred in vivo gene delivery system because of their characteristics including broad tropism of both dividing and postmitotic tissues, high efficiency of gene transfer, long-lasting transgene expression, low immunogenicity and minimal toxicity.2 For application in the central nervous system, extensive investigations have been made to characterize an optimal rAAV serotype for specific needs: global versus focal transduction, tropism for different cell types within the central nervous system (neurons, astrocytes and oligoden-drocytes) and route of administration, such as intraparenchymal stereotactic injection, intracerebroventricular injection to allow circulation throughout the cerebrospinal fluid and systemic delivery by intravenous injection.2 Furthermore, engineered serotypes via rational and combinatorial approaches are generated to overcome limitations of naturally occurring serotypes, improve gene transfer efficacy and avoid immune reactions.3 For example, novel hybrid AAV capsid serotypes Rec1, 2, 3 and 4 were generated by shuffling the fragments of capsid sequences that matched in all three non-human primate AAV serotypes cy5, rh20 and rh39, with AAV8.4 We recently evaluated the transduction efficacy of these engineered serotypes in adipose tissues that are difficult to be transduced by naturally occurring AAV serotypes.5–7 Rec2 vector leads to high transduction of adipose tissue, superior to naturally occurring serotypes (AAV1, AAV8 and AAV9), as well as other engineered serotypes (Rec1, Rec3 and Rec4).6 Rec2 vector is particularly efficient for gene delivery to brown adipose tissue, even at a dose that is at least one to two orders lower than the naturally occurring serotypes.8 Here we further study the tropisms of these engineered serotypes and explore their application in the spinal cord. Up to date, AAV9 has become the most preferable serotype for spinal cord gene transfer.9–14 Therefore, we characterized the gene delivery of the series of engineered serotypes (Rec2, Rec3 and Rec4) and compared with AAV9 by intraparenchymal injection to the spinal cord of adult mice.

RESULTS

Vector diffusion within the spinal cord

Adult mice were randomized to receive a single dose of Rec2, Rec3, Rec4 or AAV9 vectors carrying green fluorescent protein (GFP) at the T9 vertebral level (2 × 109 viral genomic particles (vg) AAV per mouse). GFP fluorescence was examined 3 weeks post injection (Figure 1a). The longitudinal transduction range was defined as the observance of GFP+ cell bodies (Figure 1c). Among the serotypes tested, Rec3 showed the most diffusion with its transduction as far as 1.45 ± 0.07 cm of the spinal cord, whereas Rec2 showed more focal transduction (0.59 ± 0.11 cm) (Figure 1b). On the other hand, AAV9 transduced 1.21 ± 0.18 cm, similar to the transduction range of Rec4 serotype (1.09 ± 0.12 cm).

Intensity of transgene expression

We next determined whether transgene expression was greater with the Rec vectors through quantification of fluorescence intensity on the ipsilateral, as well as contralateral, non-injected side of the spinal cord relative to injection site (Figures 2a and b). The section with the most intense GFP fluorescence from each spinal cord was selected and the fluorescence was measured. The average fluorescence intensity of Rec3 was significantly greater on

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both ipsilateral (1.7-fold) and contralateral (2.0-fold) spinal cord than AAV9 (Figures 2c and d). Assessing the sum fluorescence intensity of both sides of the spinal cord, Rec3 was still significantly 1.8-fold higher than AAV9 (Figure 2c), whereas no significant difference in GFP intensity was observed between AAV9, Rec2 and Rec4.

Cellular tropism
We characterized the cell types transduced by the series of AAV vectors through immunohistochemistry colocalization with neuronal marker NeuN (Figure 3a). On the ipsilateral injection side, a trend towards higher transduction of NeuN+ neurons was observed in both Rec2 and Rec3 (88.07% and 87.96%, respectively) compared with that by AAV9 (73.20%) (Figure 3b). In addition, Rec3 and Rec4 also led to a trend of greater NeuN+ neuronal transduction than AAV9 (Rec3: 87.80%, Rec4: 72.02% and AAV9: 63.84%) on the contralateral non-injected side (Figure 3b).

Summating the total number of GFP+ neurons, all three Rec vectors resulted in higher—although not significant—transduction rates of neurons than AAV9 (Figure 3c).

Astrocyte transduction was quantified using the marker glial fibrillary acidic protein (GFAP, Figure 4a). Both Rec3 and Rec4 showed increased transduction rates of GFAP+ astrocytes compared with AAV9 (86.66% and 80.93% versus 63.43%, respectively; Figure 4b) on the ipsilateral injection side. Furthermore, Rec3 also transduced more GFAP+ astrocytes than AAV9 on the contralateral non-injected side (86.10% versus 54.85%, respectively, Figure 4b). Rec3 and Rec4 vectors resulted in higher astrocyte transduction rates than AAV9 when totaling the GFP+GFAP+ astrocytes bilaterally (Figure 4c).

Diffusion of transgene product
Lastly, we investigated the distribution of transgene product throughout the central nervous system when the AAV vectors
were injected intraparenchymally at the T9 level of the spinal cord (Figure 5a). GFP fluorescence was observed as far as the rostral spinal cord (Figures 5a and b) and caudal or lumbosacral spinal cord (Figures 5a and c), essentially the entire spinal cord. Through qualitative observations, Rec3 showed the highest number of GFP+ fibers throughout the spinal cord than the other serotypes (Figures 5b and c). No substantial GFP+ cell bodies were found in the brain. However, GFP+ axonal fibers were observed in both the cerebellum and brainstem for all AAV serotypes (Figure 6).

Vector properties at a lower dose
With the Rec3 serotype displaying the most diffusion, greatest fluorescence intensity and a trend towards transducing more neurons and astrocytes than AAV9, we next wondered whether this pattern would hold at a different viral dose. Thus, the same intraparenchymal injections were performed on adult mice, who received either Rec3 or AAV9 vectors carrying GFP at a dose of $4 \times 10^8$ vg. At this fivefold lower dose, GFP+ cell bodies were observed across three sections for Rec3 versus two for AAV9 (Figure 7a). Rec3 diffusion across the spinal cord was $1.22 \pm 0.07$ cm, while AAV9 transduced $0.98 \pm 0.03$ cm (Figure 7b). Regarding average fluorescence intensity, Rec3 was significantly greater than AAV9 by 1.5-fold, whether on ipsilateral (1.46-fold), contralateral (1.48-fold) or the entire spinal cord (1.47-fold) (Figures 7c and d). Next, quantification of NeuN+ neurons and GFAP+ astrocytes transduced (Figures 8a and d) revealed that ipsilaterally, a significantly larger percentage of NeuN+ neurons was transduced by Rec3 compared with AAV9 (92.37% vs 79.80%, respectively) (Figure 8b). In addition, Rec3 significantly transduced higher rates of NeuN+ neurons than AAV9 (81.15% vs 72.04%, respectively) on the contralateral non-injected side. Summating the total number of GFP+ neurons, Rec3 resulted in significantly higher transduction rates of neurons than AAV9 (Figure 8c). Similarly, astrocyte transduction for Rec3 was significantly greater on the ipsilateral injection side compared with AAV9 (82.28% vs 75.17%, respectively, Figure 8e). Contralaterally, however, GFAP+ astrocyte transduction was higher for Rec3, albeit insignificantly.

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**Figure 2.** Transgene expression of Rec serotypes vs AAV9 as measured by GFP intensity. (a) Representative images of sections quantified. (b) Example of methodology applied to quantify GFP intensity in a random, unbiased manner. (c) Quantification of total GFP intensity across whole slice. (d) Quantification of GFP intensity ipsilateral and contralateral to side of injection. $n = 5$ mice per vector. $n = 12$ random quadrants per side. Error bars = s.e.m. $^* P < 0.05$ and $^{***} P < 0.001$. Scale bar = 100 μm.
when compared with AAV9 (73.10% versus 70.93%, respectively). Lastly, Rec3 vector resulted in higher astrocyte transduction rates than AAV9 when totaling the GFP+GFAP+ astrocytes bilaterally (Figure 8f).

**DISCUSSION**

The goal of this study was to determine whether the newly engineered hybrid serotypes of rAAV harbored desirable properties for gene transfer to the spinal cord. Among the naturally occurring serotypes that have been examined for intraparenchymal injection to the spinal cord of adult rodents, AAV9 displays the highest transduction efficiency and widest distribution.10,14 Thus, we compared the efficacy of a single thoracic intraparenchymal injection of Rec2, Rec3 and Rec4 serotypes with AAV9. Overall, Rec3 showed the highest transduction efficiency among the serotypes tested, including AAV9. The longitudinal diffusion of GFP-expressing cell bodies in Rec3 vector injected mice reached ~1.5 cm, covering a large portion of the thoracic and lumbar spinal cord. Snyder et al.10 reports that intraparenchymal injection of AAV9 (6.25 × 10^8 vg per injection) results in <5 mm longitudinal diffusion, which is better than that of AAV1, AAV6 and AAV8. Although the doses we used in this study were higher (2 × 10^9 vg) and lower (4 × 10^8 vg) than that of Synder et al.,10 the longitudinal range of transgene-expressing cell bodies of Rec3 serotype was still significantly increased compared with AAV9 at both high and low dosages tested. Furthermore, the longitudinal transduction range of intraparenchymal Rec3 (2 × 10^9 vg) was at least twofold farther than the intrathecal injection of AAV9 (2.5 × 10^9 vg) using the same promoter, the chicken β-actin promoter with cytomegalovirus enhancer.10 Moreover, Rec3 demonstrated higher transduction on the contralateral non-injected side of the spinal cord than all the serotypes tested. Overall, Rec3 provided the most widespread transduction, whereas Rec2 displayed the most focal transduction.

Quantification of the GFP fluorescence intensity showed that Rec3 led to the highest maximal transgene protein on both the ipsilateral injected and contralateral non-injected side of the spinal cord. Interestingly, Rec2 serotype is highly efficient at transducing adipose tissues, whereas Rec3, Rec4 and AAV9 perform poorly.8,15 The ability of these recombinant serotypes to

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**Figure 3.** Comparison of neuronal transduction by Rec vectors vs AAV9. (a) Transduction (GFP, green) of neurons assessed by NeuN (red) colocalization. 4',6-Diamidino-2-phenylindole (DAPI, blue) = nuclear counterstain. Example colocalized cell indicated by arrowheads. (b) Quantification of GFP+/NeuN+ neurons ipsilateral and contralateral to injection side. (c) Quantification of total GFP+/NeuN+ neurons across entire slice. n = 5 mice per vector. n = 12 random quadrants per side. Error bars = s.e.m. *P < 0.05. Scale bar = 20 μm.
transduce different tissues may be concerning when tissue specificity is preferred. However, focal, localized transduction within a tissue or specific tissue type is achievable by a combination of approaches including limiting dose, direct injection to the targeting tissue, using a tissue-specific promoter and incorporating microRNA targeting sequences (for example, miR-122 targeting sequence to restrict transgene expression in the liver). The distinct tissue tropism of these engineered serotypes provides an example that engineering naturally occurring serotypes is a useful approach to expand the current AAV vector toolkit for both basic and translational applications. In diseases such as spinal cord injury, where injury may be focal, utilizing a vector with localized transduction such as Rec2 could be ideal. On the other hand, for diseases requiring widespread transduction, Rec3 vector, which leads to broader transduction and higher transgene expression, may be economically advantageous for the clinic, whereas lower doses, and therefore less viral vector production, would be required to produce an effect equivalent to AAV9.

The engineered serotypes as well as AAV9 transduced both neurons and astrocytes in the adult spinal cord. When analyzing regions around the dorsal and ventral horns, all Rec vectors significantly enhanced the maximal transduction rate of astrocytes compared to AAV9 at the dose of $2 \times 10^9$ vg per mouse. Rec3 was particularly efficient, leading to ~86% transduction of the total GFAP$^+$ astrocytes, whereas AAV9 showed ~63%. At the fivefold lower dose, the transduction of neurons and astrocytes by Rec3 is significantly more pronounced relative to AAV9. This could quite possibly be due to the larger transduction volume and/or better detection of colocalized cells due to increased transgene fluorescence. Future three-dimensional imaging studies of whole spinal cord, beyond the regions near the dorsal and motor horns, would confirm or improve the characterization of these results. A recent study reports that a single intrathecal injection of rhesus-10 AAV serotype (rAAVrh10) into the lumbar cistern can transduce 60–90% of the cells in the spinal cord at a dose of $4 \times 10^{10}$ vg per mouse. In addition, the transgene is expressed in all cell types including neurons, glial, ependymal cells and endothelial cells in the spinal cord. Furthermore, transgene expression is detected in some brain areas as far as the frontal cortex and olfactory bulbs. In this study, no GFP$^+$ cell bodies were observed in the brain when the Rec vectors were injected intraparenchymally into the spinal cord at a dose of $2 \times 10^9$ vg per mouse, although GFP fluorescence was found in brainstem and cerebellum, suggesting retrograde transport of transgene protein. It will be interesting to examine the transduction efficiency, cell tropism and potential distribution.
to the broad central nervous system of these engineered serotypes—in particular Rec3—via intrathecal or other systemic delivery methods such as intravenous injection.\textsuperscript{17–21} In summary, the novel engineered Rec serotypes—and in particular Rec3—were highly efficient in transducing the mature mouse spinal cord via intraparenchymal injection. The Rec3

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**Figure 5.** Anterograde and retrograde transport of GFP transgene protein in the spinal cord. (a) Representative locations of rostral and caudal spinal cord images (b, c) relative to injection site at vertebral level T9. (b) Representative images of rostral spinal cord axons transduced with virus. (c) Representative images of caudal spinal cord axons transduced with vector. Scale bar = 100 μm.

**Figure 6.** GFP distribution in brain after direct spinal cord gene transfer of AAV9 and Rec vectors. Representative images of central nervous system transduction in the cerebellum (a) and brainstem (b), assessed by GFP fluorescence in axonal fibers. Scale bar = 100 μm.
serotype specifically led to the most widespread transduction, highest transgene expression and the most maximal transduction rate of both neurons and astrocytes among all the serotypes examined, including the widely used and clinically approved AAV9 (Gene Transfer Clinical Trial for Spinal Muscular Atrophy Type 1: https://clinicaltrials.gov/ct2/show/NCT02122952). These engineered serotypes provide vehicles for genetic manipulations of the spinal cord for basic research, disease modeling and potential gene therapy for diseases affecting entire the spinal cord, such as spinal muscular atrophy and amyotrophic lateral sclerosis.

MATERIALS AND METHODS

rAAV vector construction and packaging

The rAAV capsid serotypes (Rec1-4) were generated by shuffling the fragments of capsid sequences that matched in all three nonhuman primate AAV serotypes (cy5, rh20 and rh39) and AAV8. The three new recombinant serotypes previously identified, with greater transduction efficiency than rAAV8, cy5, rh20 and rh39 were originally supplied by Guangping Gao and the Gene Therapy Program Vector Core, Department of Medicine, University of Pennsylvania, where further details of the identification of these sequences is available. The details of Rec1-4 serotypes are described before. To generate hybrid AAV vectors, GFP was cloned into an AAV expression plasmid under the control of the chicken β-actin promoter plus cytomegalovirus enhancer and containing woodchuck hepatitis virus posttranscriptional regulatory element, and bovine growth hormone polyadenylation signal flanked by AAV2 inverted terminal repeats. Human embryonic kidney 293 cells were co-transfected with three plasmids—AAV plasmid, appropriate helper plasmid encoding rep and cap (Rec1-4) genes or AAV9 and adenoviral helper pΔ6—using standard calcium phosphate transfection. rAAV vectors were purified from the cell lysate by ultracentrifugation through an iodixanol density gradient. Vectors were titrated using real-time quantitative PCR (ABI StepOnePlus; Applied Biosystems, Foster City, CA, USA) and adjusted to 2 × 1013 vg per ml 0.01% pluronic F-68 in phosphate-buffered saline (PBS) for stereotaxic injections. Two different batches of vector per serotype were used in this study.

Mice

Male C57BL/6 mice, 9 weeks of age were purchased from Charles River Laboratories, Wilmington, MA, USA. Animals were housed in groups of no more than five per cage in a 12:12 light:dark cycle with ad libitum access to standard rodent chow and water in a temperature- and humidity-controlled environment. All mice experiments were carried out in compliance with the regulations of the Ohio State University Institutional Animal Care and Use Committee.

Stereotaxic surgery

Surgicals were performed in collaboration with The Ohio State Neuroscience Center Injury and Behavior Core. Adult male mice were randomized to receive AAV serotype vectors. Mice anesthetized with a single dose of 100 mg per kg ketamine and 20 mg per kg xylazine. Laminctomy was then performed to expose the spinal cord at the T9 vertebral level. Mice then received one unilateral right-side injection of high (2 × 109 vg) or low (4 × 108 vg) dose AAV in 1.0 μl (ML: 0.5, DV: −0.8) at the gray–white matter junction with a pulled glass micropipette and air-pressurized nanoinjector. Afterwards, muscles were sutured overlying the surgical clips. All animals were administered 2 ml saline in heated recovery cages post surgery and, after waking and ambulating, were returned to standard cages and monitored weekly until killing 3 weeks later.

Tissue preparation

Mice were intracardially perfused with 4% paraformaldehyde (Sigma, St Louis, MO, USA) in PBS and fixed spinal cords were placed in 4% paraformaldehyde overnight. The following day, spinal cords were washed in PBS three times before being immersed in 30% sucrose in PBS for at least 3 days. Spinal cords were then divided into eight 0.5 cm segments and frozen in O.C.T. (Sakura Finetek, Torrance, CA, USA) before being sectioned into 30 μm slices on a Leica cryostat (Buffalo Grove, IL, USA).

Immunohistochemistry and imaging

Slides with cryosections were dried, washed in PBS and blocked in 5% normal goat serum, 1% bovine serum albumin, 0.3% Triton TX-100, 0.3 M glycine and 0.03% sodium azide in PBS for 1 h. Also in blocking buffer, primary antibodies included mouse anti-NeuN (Chemicon, catalog number
Figure 8. Comparison of neuronal and astrocyte transduction by Rec3 vs AAV9 serotypes at a lower viral dose. (a) Transduction (GFP, green) of neurons assessed by NeuN (red) colocalization. 4',6-Diamidino-2-phenylindole (DAPI, blue) = nuclear counterstain. Example colocalized cells indicated by arrowheads. (b) Quantification of GFP⁺/NeuN⁺ neurons ipsilateral and contralateral to injection side. (c) Quantification of total GFP⁺/NeuN⁺ neurons across entire slice. (d) Transduction of astrocytes assessed by GFAP (red) colocalization. Example colocalized cells indicated by arrowheads. (e) Quantification of GFP⁺/GFAP⁺ neurons ipsilateral and contralateral to injection side. (f) Quantification of total GFP⁺/GFAP⁺ neurons across entire slice. *P < 0.05. Scale bar = 20 μm.
MAB377, 1:500, Darmstadt, Germany) or mouse anti-GFAP (Millipore, catalog number MAB3402, 1:500, Darmstadt, Germany) and were applied onto tissue overnight at 4 °C. The following day, slides were washed three times in PBS before incubation with appropriate AlexaFluor secondary antibodies (Invitrogen, Carlsbad, CA, USA) and nuclear counterstain 4',6-diamidino-2-phenylindole in blocking buffer at room temperature for 1 h. After three washes in PBS, slides were mounted using Aqua-Poly/Mount (Polysciences, Warrington, PA, USA) and coverslipped. Confocal microscopy was performed on a FluoView FV1000 microscope (Zeiss, Oberkochen, Germany).

Quantification of images
Regarding diffusion, images were captured at ×2.5 or ×10 magnification. The longitudinal diffusion of GFP was determined by counting the number of sections (in one of each series of eight 30 μm sections) that had GFP expression in cell bodies and then multiplying them by 0.5 cm, the approximate distance between consecutive adjacent sections (that is, 3 sections × 0.5 cm between sections in each series). Each mouse spinal cord was sectioned into 15 series/rows on 1 slide. From all five mice injected per serotype, the values of all series were summed and averaged. This estimate was calculated for each serotype and used to compare diffusion distance between serotypes. GFP fluorescence intensity was quantified using ImageJ/Fiji (NIH, Bethesda, MD, USA). The most fluorescent coronal section from each serotype (n = 5 mice per serotype) was acquired. Random rectangular selections of equal height and width (or pixel area) were drawn for both ipsilateral and contralateral spinal cord across all samples. Six random areas were selected in the dorsal and ventral sections of both the ipsilateral and contralateral sides of injection. Quantification of colocalized cells was also analyzed with the same method and measurements from the ipsilateral or contralateral side were summed and analyzed with statistical software (below).

Statistical analysis
Values are expressed as mean ± s.e.m. Normal distribution was confirmed and f-test (low dose comparisons, two samples) or one-way analysis of variance with Dunnett’s multiple comparisons test (high dose comparisons, four samples) was used to assess for significant differences with Prism Mac version 6.0f software (GraphPad, La Jolla, CA, USA).

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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