Synthetic Adeno-Associated Viral Vector Efficiently Targets Mouse and Nonhuman Primate Retina *In Vivo*

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Gene therapy is a promising approach in the treatment of inherited and common complex disorders of the retina. Preclinical and clinical studies have validated the use of adeno-associated viral vectors (AAV) as a safe and efficient delivery vehicle for gene transfer. Retinal pigment epithelium and rods—and to a lesser extent, cone photoreceptors—can be efficiently targeted with AAV. Other retinal cell types however are more challenging targets. The aim of this study was to characterize the transduction profile and efficiency of *in silico* designed, synthetic Anc80 AAVs for retinal gene transfer. Three Anc80 variants were evaluated for retinal targeting in mice and primates following subretinal delivery. In the murine retina Anc80L65 demonstrated high level of retinal pigment epithelium and photoreceptor targeting with comparable cone photoreceptor affinity compared to other AAVs. Remarkably, Anc80L65 enhanced transduction kinetics with visible expression as early as day 1 and steady state mRNA levels at day 3. Inner retinal tropism of Anc80 variants demonstrated distinct transduction patterns of Müller glia, retinal ganglion cells and inner nuclear layer neurons. Finally, murine findings with Anc80L65 qualitatively translated to the Rhesus macaque in terms of cell targets, levels and onset of expression. Our findings support the use of Anc80L65 for therapeutic subretinal gene delivery.

Keywords: retinal transduction, gene therapy, adeno-associated virus, cone photoreceptors, *in silico* reconstruction, retina

INTRODUCTION

OVER THE PAST DECADE, approaches of gene augmentation in inherited retinal degeneration has yielded significant therapeutic results which culminated in several successful phase 1/2 clinical trials for Leber Congenital Amaurosis,¹⁻⁷ Chorioideremia,⁸ *MERTK*,⁹ and age-related macular degeneration.¹⁰⁻¹² Furthermore, preclinical studies from several research groups illustrate the ability to rescue disease phenotypes in animal models of retinal degeneration including various small and large species (reviewed by Carvalho and Vandenberghe¹³). A common denominator in these studies is the use of adeno-associated viral vectors (AAVs)

and the subretinal route of injection. The high affinity to the retinal pigment epithelium (RPE) and photoreceptor cells, the overall safety of the vector and injection routes have justified the selection of this approach for multiple ongoing programs for clinical translation.¹⁴⁻¹⁹

AAV is a small, nonpathogenic, nonenveloped, icosahedral, replication-deficient virus that encodes up to 4.7 kb of the transgene cassette of choice that can then be delivered to multiple types of cells and tissues. More than 100 natural isolates of AAVs have been described so far, many of which result in highly distinct levels and patterns of transduction and cell specificity after subretinal injection.^{13,20,21}

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For example, in the adult mouse retina, most serotypes will result in retinal pigment epithelium (RPE) and more limited photoreceptor transduction, a limitation which was described to be overcome by AAV5, 8, and 9.^{22–24} Inner retina cell types have proven a more challenging target however. Several approaches to optimize current AAV vectors seek to improve transduction of inner and outer retinal neurons and glia cells via different routes of administration. These studies have been met with relative success, but none of these efforts has provided a single vector with an overall more ubiquitously improved response.^{25–32}

Cone photoreceptor targeting and transduction has also been less documented, especially in a quantitative manner. Despite successful application of AAV-based gene replacement therapy in different animal models of cone or cone-rod dystrophies,^{33–48} some studies have indicated that there might be a discrepancy in cone targeting between serotypes with high photoreceptor tropism in the primate retina.^{49–54} Cone photoreceptors are responsible for color vision and acuity, so it is not surprising that most patients undergoing visual degeneration only report symptoms when cone-mediated vision starts to deteriorate. Therefore, efficient cone transduction is of the utmost importance when designing future therapeutic vectors, not only to directly target cone degeneration and functional loss in primary cone dystrophies, but also for use in neuroprotective treatments for secondary cone loss in rod-specific disorders. The majority of AAV-based ocular clinical trials have used AAV2 so far, and in at least one of these trials, the lack of functional improvement has been attributed to low levels of transgene expression,⁶ an effect that could be countered by using a different serotype with higher capacity for photoreceptors targeting and/or transduction.

In an effort to develop antigenically distinct AAVs and to study the structure–function relationship of AAV, we previously reported on an effort that reconstructed viral capsids along the putative evolutionary lineage of AAV using *in silico* phylogenetic and statistical modeling.⁵⁵ Anc80 is the most distal evolutionary node for which we inferred the sequence using ancestral sequence reconstruction (ASR) and is the predicted common ancestor of most known primate AAVs that are considered for gene therapy applications, including serotypes 1–3 and 6–9, but excludes AAV4 and AAV5. The uncertainty provided by the maximum likelihood ASR method was captured in an Anc80 variant library. Members of this library were evaluated for their ability to yield high titer infectious AAV-like particles. The 65th clone from that

screen, Anc80L65, underwent extensive characterization and revealed a potent gene therapy vehicle with broad and unique properties in murine and nonhuman primate (NHP) liver as well as muscle and retina targeting in mouse.⁵⁵ The aim of this study was therefore to further characterize the retinal tropism of Anc80L65 and two additional Anc80 variants alongside standard AAV serotypes widely used for retinal gene delivery.

MATERIALS AND METHODS

Ancestral lineage reconstruction, library, and vector selection and production

Ancestral AAV capsid sequences and lineage reconstruction was done using maximum-likelihood methods and is described in full in.⁵⁵ Prior *in vitro* characterization of the functional variants tested in this study are described by Zinn and colleagues⁵⁵ Briefly, a preliminary clonal screen of 776 clones from the 2,048-member Anc80 library was performed. These clones were tested in a functional assay for genome containing particle yield and HEK293 transduction. Anc80 vectors with superior performance on the combination of those two vector functional tests were progressed for further testing. For this study, three top-performing vectors were selected for further testing. Vector constructs used in this study were packaged as single-stranded genomes and expressed the enhanced green fluorescent protein (*eGFP*) driven by a ubiquitous promoter (cytomegalovirus [CMV]) and further regulated by a woodchuck hepatitis virus post-transcriptional response element (WPRE) to enhance transgene expression.

Vector production, purification, and titrating are described in detail in other publications.^{55,56} AAV preparations were titrated by TaqMan qPCR amplification (Applied Biosystems 7500, Life Technologies) on DNase I-resistant vector genomes copies. Primers and probes detecting promoter, transgene, or polyadenylation signal coding regions of the transgene cassette were evaluated. Yields for the different vectors preparations used were as follows (in genome capsids per mL): AAV8, between 5E12 and 9E12; AAV2, between 1.7E12 and 2E12; AAV9, 7.2E12; Anc80L65, between 2E12 and 7E12; Anc80L27, between 1E12 and 6E12 and Anc80L121, 1E12. The purity of AAV preparations was evaluated by SDS-PAGE gel electrophoresis. For the NHP studies, vector preparations were titrated 3 times and tested for endotoxin levels (only preparations below 10 EU/mL were injected).

Capsid modelling of Anc80 variants

The SWISS-MODEL structure modelling server⁵⁷ (Swiss Institute of Bioinformatics) was used to

generate pseudoatomic models of Anc80L27, Anc80L65, and Anc80L121 VP3 using AAV8 crystal structure (PDB 2QA0) as a template. Sixtymer PDBs were further reconstructed using the UCSF Chimera package,⁵⁸ (Resource for Bio-computing, Visualization and Informatics), and prepared for electrostatic calculations using the PDB2PQR tool⁵⁹ with the PARSE forcefield. Protonation states were assigned at pH7 using PROPKA⁶⁰ (Jensen Research Group). Electrostatic calculations were run using the Adaptive Poisson–Boltzmann Solver software.⁶¹ Briefly, the linear Poisson–Boltzmann equation was solved on a $400 \times 400 \times 400$ Å grid to a resolution of 1 Å, using the single Debye–Hückel boundary condition, with the following parameters: dielectric constant of solute, 2; dielectric constant of solvent, 78.5; 0.150M monocations/anions with an exclusion radius of 2 Å.⁶² Calculated electrostatic potentials were further projected on the solvent accessible surface of viral capsids and visualized between ± 5 kT/e using the PyMOL Molecular Graphics System, Version 1.6.0.0 (Schrödinger, LLC).

Animals and *in vivo* vector administration

Mouse. Wild-type C57BL/6J mice (6–8 weeks old) were purchased from Charles River Laboratories and kept at the Schepens Eye Research Institute (SERI) Animal Facility. All animal procedures were performed in accordance with protocols approved by the institutional animal care and use committees at SERI and conformed to the guidelines on the care and use of animals adopted by the Association for Research in Vision and Ophthalmology (Rockville, MD). Animals were anaesthetized with ketamine/xylazine and subretinal injections were performed with 2 μ L of AAV vectors for a 2×10^9 total genome capsids injection dose per eye.

Nonhuman primate. Experiments with rhesus monkeys were performed at New England Primate Research Center. All experimental procedures were approved by the Office for Research Subject Protection, Harvard Medical Area Standing Committee on Animals, the Harvard Medical School Institutional Animal Care and Use Committee. Animals were sedated with ketamine or telazol in combination with dexdomitor. No vitrectomy was performed on these animals, but pressure was relieved by performing an anterior chamber tap of roughly the equivalent volume as the injected vector volume (150 μ L). Using a 39-gauge polyimide-tipped cannula, 150 μ L of AAV was administered subretinally near the superior vascular arcade at a dose of 1×10^{10} total genome capsids. There was no steroid regimen

for these animals initially except for a single perioperative subconjunctival dose of dexamethasone, which is short-acting. However, one animal (358-08) received an intravitreal injection in the contralateral eye as part of a separate study (and thought not to affect the results presented here) and was treated with one dose of intravitreal steroids (triamcinolone acetonide, 40 mg/mL) at 3 weeks postsurgery. After recovering from anesthesia, the animals were monitored weekly/fortnightly until the end of the study for general well-being and with eye exams including indirect ophthalmoscopy. At around 8–12 weeks postsurgery monkeys were euthanized and eyes were harvested.

Fundus imaging

Mouse fundus imaging was performed using a Micron III Retinal Imaging Microscope (Phenix Research Labs, Pleasanton, CA). Animals were anaesthetized with ketamine/xylazine. One percent Tropicamide Ophthalmic Solution (Akorn) was used to dilate pupils and 0.3% Gental (Novartis) was applied on the corneal surface prior to imaging. Images were taken at the maximum light intensity with a gain setup of 10. Primate fundus imaging was performed on five animals in the week prior to the end of the study (see Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/hum). Animals were sedated as described above and pupils dilated using 1% tropicamide and 2.5% phenylephrine hydrochloride (Paragorn). Imaging was done using a Topcon fundus camera and a standard color charge couple device camera, and fluorescein filters were used to take fluorescence images of green fluorescent protein (GFP) expression. *In vivo* optical coherence tomography images were acquired using a Zeiss Cirrus spectral domain optical coherence tomography system using standard settings.

Histology

Sample processing. Mouse eyes were collected and fixed in 4% paraformaldehyde (PFA, Sigma-Aldrich) on ice for 1 hour. Corneas and lenses were dissected out and eye cups were fixed for an extra 30 min in 4% PFA. Dissected eye cups were then submerged and frozen in optimal cutting temperature compound (Tissue-Tek O.C.T, Sakura) and kept at -20°C prior to cryosectioning. Nonhuman primate eyes were dissected after euthanasia and an incision was made on the sclera to allow the fixative to enter the eye. Eyes were incubated in 4% PFA for 24 h at 4°C . On the following day, cornea, lens, and iris were dissected and eye cups were then incubated for another 24 h in 4% PFA at 4°C .

followed by a 24 h incubation in 20% sucrose for 24 h at 4°C. Eye cups were then dissected into five pieces (superior, inferior, temporal, nasal, and central) and each was frozen in tissue freezing medium (TBS) and kept at -20°C prior to cryo-sectioning. Sections were done at 12 μ m for mouse eyes and 15–18 μ m for NHP using a Leica CM1950 cryostat. Flat-mounts for mouse retinas was done following the same protocol for fixation as described above with additional steps of dissecting the retina out and incubating it in 20% sucrose for 30 min at room temperature (RT) before proceeding with immunohistochemistry.

Immunohistochemistry. Antibodies used are listed in Supplementary Table S2. The basic protocol for immunohistochemistry for retinal sections was as follows: 1 h incubation with block solution (1% BSA; 0.1% Triton-X; 5% NGS/NDS) at RT followed by overnight incubation with primary antibody at 4°C. On the next day, slides were washed with 1 $\times$ phosphate-buffered saline (PBS) and then incubated with Alexa Fluor conjugated secondary antibody at 1:500 dilution for 2 h at RT followed by 1 $\times$ PBS washes and incubation with 4',6-diamidino-2-phenylindole (DAPI) solution for 30 min at RT. Slides were then mounted using fluorescent mounting media (DAKO), allowed to dry, and imaged. For labelling of retinal flat-mounts, dissected retinas were blocked in 1% BSA, 3% Triton-X and 5% NGS for 1 h at RT then incubated for 12–16 h at 4°C. The following day, retinas were washed in 1 $\times$ PBS and incubated for 12–16 h at 4°C in a mix of DAPI solution and Alexa Fluor conjugated secondary antibody. Retinas were then washed in 1 $\times$ PBS several times and mounted using Prolong Gold antifade reagent (Life Technologies) and allowed to dry at RT prior to imaging. All imaging was done using a Leica TCS-SP5 Upright Confocal Laser-Scanning Microscope. Confocal laser intensity for the green and red channels were consistent across all images (30% and 55% respectively). Gain settings in the green channel varied between panels. All GFP-positive images showed native GFP expression (no antibody used) except for the image shown in Fig. 3E, panel An80L121, where anti-GFP antibody was used.

Cone count protocol

Cone counts were performed on flat-mount of retinas dissected from injected eyes at 4 weeks postinjection (PI). Immunohistochemistry labelling for the cone-specific marker cone arrestin was performed as described above. For each group confocal images were taken from the injected area

as a single image (not z-stack) focused at the cone cell body level with settings kept consistent across each channel for all images. The merge image of the blue, green, and red channels (DAPI, GFP, and cone arrestin, respectively) was then used for processing. Image processing and automated cone counts were done using a script programed and run by FiJi ImageJ. A step-by-step diagram of the process is shown in Supplementary Fig. S1.

Qualitative real-time PCR

For comparing relative *GFP* transcript levels after subretinal delivery using Anc80 variants and contemporary AAV serotypes, eyes were collected from different timepoints after injections ($n = 4$ –6 per group/timepoint) and mRNA was extracted from whole eye cups using the RNeasy kit (Qiagen). Real-time PCR was performed in triplicates using the TaqMan® gene expression master mix (Thermo Fisher Scientific) on an ABI 7500 Real Time PCR System (Applied Biosystems). Relative transcript levels were assessed using the $2^{-\Delta\Delta CT}$ method with GAPDH as the reference gene (Taqman Assay Mm99999915_g1; catalog No. 4331182; Life Technologies). The following primers were used for GFP detection (5'–3'): agcaagacccaaccagaa (forward) and ggcggcggtacagaa (reverse) and probe was from Life Technologies.

RESULTS

In vivo retinal transduction by ancestral AAV variants

Anc80 was previously predicted to be the closest common ancestor of AAV serotypes 1–3 and 6–9 (Fig. 1A). The closest known AAV is rh.10 which diverges in over 8% in terms of sequence. To account for the uncertainty of the maximum likelihood ASR model, a library of Anc80 variants that permuted 11 dimorphic residues at fixed positions was constructed.⁵⁵ Based on particle yield and HEK293 *in vitro* infectivity, three Anc80 capsid variants, Anc80L27, Anc80L65, and Anc80L121, were selected for *in vivo* evaluation of retinal transduction⁵⁵ (data not shown). In Fig. 1B the amino acid present in each of these 11 sites is compared against AAV8 and AAV2 capsid sequences. The three variants differ from each other in only nine positions and have varying degrees of homology to AAV8 and AAV2 at these positions. Structural mapping of the variant sites predicts four to be exposed on the external side of the capsid, which results in a unique electrostatic profile for each variant, as shown in Fig. 1C (iii–v). More specifically, R/K 169 and T/S 206 are located within the VP1-2 domains. Based on the models of the Anc80 variants, R/

Figure 1. Molecular characterization of Anc80 library variants. **(A)** Collapsed ancestral sequence reconstruction phylogenetic reconstruction (not drawn to scale—see Supplementary Figure S2 for time-scaled version) of adeno-associated virus (AAV) evolutionary lineage showing the library of probabilistic space around the Anc80 node (blue circle). **(B)** Amino acid sequence alignment for the 11 dimorphic sites that compose the Anc80 library showing sequence difference between AAV8, AAV2, and three Anc80 variants, L27, L65, and L121. **(C)** (i) Structural mapping of amino acid changes as compared with AAV8 in the threefold symmetry axis region using the AAV8 crystal structure PDB 2QA0 as a template. Green residues are divergent in Anc80. Black residues are dimorphic in Anc80Lib and vary between Anc80L27, Anc80L65, and Anc80L121. (ii–v) Calculated electrostatic potential projected on the solvent accessible surface of AAV8 (ii), Anc80L27 (iii), Anc80L65 (iv), and Anc80L121 (v). The negative and positive electrostatic potentials are represented in red and blue, respectively, and the color legend below shows the surface values of the electrostatic potential between $-5 k_B T/e_o$ (red) and $5 k_B T/e_o$ (blue). Anc80Lib dimorphic residues are outlined in black (T/E 460; A/T 493; S/N 562; Q/E 576). The AAV8 residues aligned with these dimorphic sites are also represented in panels (i) and (ii). The approximate position of the threefold symmetry axis is represented with an orange triangle on each image. (vi) Dimorphic residue variability between Anc80L27, Anc80L65, and Anc80L121. Numbers represent residue positions within the sequence alignment shown in (B). A, alanine; E, glutamate; N, asparagine; Q, glutamine; S, serine; T, threonine.

K 313 and Q/E 413 are located on the luminal surface of the capsids, whereas N/D 612 is predicted to be buried and located at the threefold interface. Finally, T/E 463, A/T 496, N/S 565, and Q/E 579 are expected to be exposed on the external side of the capsid, in the vicinity of the threefold interface, resulting in the unique surface charge profile for each variant.

Next, high titer preparation of the selected Anc80 variants, alongside AAV2 and AAV8 controls, were produced in order to interrogate the impact of this structural variation on retinal targeting. Since in the majority of ocular AAV-based gene replacement clinical trials the treatment agent is delivered via the subretinal route, the aim of this study was to evaluate the tropism of the novel Anc80 vectors only after subretinal delivery. All vectors were injected subretinally into adult wild-type male C57Bl/6 mice (6–8 weeks old) at equal doses. Transgene expression was monitored through fundus imaging of the retina at 1, 2, 3,

and 4 weeks PI. Representative images for the 4 weeks PI timepoint for all serotypes injected is shown in Fig. 2A. Fundus expression patterns for AAV2 and AAV8 were concordant with the expected performance for these serotypes, in that AAV8 demonstrated higher level of retinal transduction. On average, Anc80L27 and Anc80L65 resulted in strong transgene expression, whereas Anc80L121 generated a less intense expression pattern. Next, eyes were collected for histological analysis at 4 weeks PI (Fig. 2B). All three Anc80 variants were capable of targeting RPE cells and photoreceptors, albeit at differing levels, while AAV2 led to near-exclusive RPE and AAV8 potent RPE and photoreceptor targeting, in line with published results.^{22,50} Anc80 variants, and to a lesser extent AAV8, also targeted a substantial number of inner nuclear layer (INL) cells as illustrated in Fig. 2C. Results show Muller glia transduction for all serotypes except AAV2, and

Figure 2. Comparison of transgene expression after subretinal delivery using Anc80 variants, AAV8 and AAV2. **(A)** Representative fundus imaging of adult mice at 4 weeks postinjection (PI) showing GFP fluorescence levels in eyes injected with Anc80L27, Anc80L65 and Anc80L121 alongside AAV8 and AAV2. **(B)** Representative confocal images of retinal cryosections at 4 weeks PI showing transduction comparison of RPE and photoreceptors cells (native GFP expression shown in *green*) in eyes injected with AAV2, AAV8, Anc80L27, Anc80L65, and Anc80L121. Cell nuclei is stained with DAPI (*blue*). Scale bar = 50 μ m. **(C)** Higher magnification of the INL area from images shown in the middle panel (B) with overexposed *green* channel to show transduction of inner nuclear neurons and glia. Scale bar = 25 μ m. GC, ganglion cells; GFP, green fluorescent protein; INL, inner nuclear layer; ONL, outer nuclear layer; RPE, retinal pigment epithelium.

Figure 3. Transduction of INL neurons, glia, and ganglion cells in eyes injected subretinally with Anc80 variants expressing a GFP transgene. The different panels show confocal images of retinal sections after staining for retinal neurons and cell markers. GFP intensity in the ONL was overexposed to reveal the weaker GFP signal observed in the other retinal layers. **(A)** Confocal images showing colocalization (white asterisks) of calbindin staining (red) for horizontal cells with GFP positive (green) cells in Anc80L27 and Anc80L121 variants. Scale bar = 25 μ m. **(B)** Confocal images of PKC α staining for rod bipolar cells (red) in retinas injected with Anc80 variants. No colocalization was observed with any of the variants. Scale bar = 25 μ m. **(C)** Amacrine cell transduction was only observed in eyes injected with the Anc80L27 variant (white asterisks). Anti-ChaT staining for amacrine cells is shown in red and GFP expression shown in green. Scale bar = 50 μ m. **(D)** Muller glia targeting in eyes injected with Anc80 variants. GFP positive (green) Muller glia cells were seen in all three variants (white asterisks). Anti-GS staining for Muller glia is shown in red. Scale bar = 25 μ m. **(E)** Colocalization of GFP expressing cells (green) in the ganglion cell layer with ganglion cell marker RBPMS (red) was seen mainly in eyes injected with Anc80L27 but a few GFP positive cells were also observed in Anc80L65 and Anc80L121 injected retinas (white asterisks). Anti-GFP antibody was only used for Anc80L121 in order to visualize weak GFP expression in the retinal ganglion cell layer. Scale bar = 25 μ m. **(F)** Images of optic nerve sections of eyes injected with Anc80 variants showing GFP positive fibers (green). Cell nuclei stained with DAPI (blue). Scale bar = 100 μ m. IPL, inner plexiform layer.

for Anc80 variants, a number of additional neuronal retinal cell types were clearly GFP positive.

EFFICIENT AND UNIQUE CELL-SPECIFIC ANC80 INL TRANSDUCTION PATTERN

To further characterize the INL transduction pattern, cell-specific staining was performed. Retinal sections were subjected to immunohistochemistry using antibodies against specific markers for horizontal, bipolar, amacrine, ganglion and Müller glia cells (Fig. 1; Supplementary Table S2 for antibodies). Different INL targeting profiles were observed with each of the three variants. Variant Anc80L27, and to a much lesser extent Anc80L121, were capable of targeting horizontal cells (Fig. 3A); while all three variants transduced Müller glia cells (Fig. 3D). Strong amacrine cell targeting was seen with the Anc80L27 variant only (Fig. 3C), while none of the three variants was capable of transducing bipolar cells (Fig. 3B). Ganglion cell transduction was seen in two variants but to differing efficiencies; Anc80L27 appears to have the strongest affinity to ganglion cells, while only weak and sparse targeting is seen by Anc80L65 and Anc80L121. The ganglion cell transduction pattern for Anc80L27 and Anc80L65 seen in Fig. 3E was also confirmed by GFP positive ganglion cell fibers in the optic nerve of

injected eyes (Fig. 3F), where a much stronger GFP expression was seen in the Anc80L27 variant compared to Anc80L65. Variant Anc80L121 did not show any GFP positive ganglion cells on retinal sections but surprisingly weak GFP expression was observed in the optic nerve, whether from ganglion cell or astrocytes fibers remains to be confirmed.

Targeting of cone photoreceptors

Given that all three Anc80 variants showed high transduction capacity for outer nuclear layer (ONL) cells, which are dominated in number by rod photoreceptors, we sought to quantify the extent of cone photoreceptor transduction among the AAV serotypes tested. Previous studies have shown that both AAV8 and AAV9 are capable of targeting cone photoreceptors at similar levels in the murine retina²² but AAV9 is substantially better than AAV8 at transducing cones in the NHP retina.⁵⁰ Figure 4A shows the colocalization of GFP positive injected areas of the retina with the mammalian cone-specific marker cone arrestin after subretinal delivery of the three Anc80 variants. Despite colocalization being observed in all three variants, it was also noted that cone transduction was not uniform and that not all cone cells were being targeted. Therefore a more quantitative analysis was performed, comparing the three ancestral variants against conventional

Figure 4. Comparison of cone transduction efficiency between Anc80 variants and contemporary AAV serotypes. **(A)** Single stack confocal images of ONL area of 4 weeks PI retinal sections of eyes injected subretinally with Anc80 variants Anc80L27, Anc80L65, and Anc80L121 stained with cone arrestin antibody (red). **(i–iii)** Higher magnification images showing colocalization of GFP positive photoreceptors (green) and cone-specific marker for cone arrestin (red). Panels shown indicate overlay image with colabelled cones indicated by white arrows **(i)**, GFP only **(ii)**, and cone arrestin only **(iii)**. Scale bar = 25 μ m. **(B)** Quantification of cone transduction efficiency for each serotype counted from flat-mount retinal images. Efficiencies are shown in percentage of total cones that were also GFP positive (values shown above each bar). Error bars show SEM. Total of cones counted for each group are as follows: AAV8, $n=2781$; AAV9, $n=2565$; Anc80L27, $n=1294$; Anc80L65, $n=6453$; and Anc80L121, $n=2946$. **(C)** Histogram of the GFP intensity for all cones counted in each group showing intensity distribution and mean.

serotypes that have high photoreceptor transduction. The selected control serotypes were AAV8, due its high affinity to photoreceptors, and AAV9, which has been shown to have improved cone tropism.⁵⁰ This quantitative analysis was done using an automated ImageJ script that was run on single layer confocal images of AAV injected retinal flat-mounts labelled with cone arrestin. Images were taken with the same gain settings, using a 20 $\times$ lens plus a 2.5 $\times$ optical zoom, and focus was aimed at the cone cell body layer. Due to the irregular nature of retinal flat-mounts, areas imaged that were focused on the outer/inner segment area, cell axon and synapse terminals were eliminated from the analysis (Supplementary Fig. S1). The ImageJ script would then split the red and green channels and convert them to binary images. Processing steps to isolate the cone cells were applied to the red channel, creating a mask of numbered cone cells in the given image. This mask was then applied to the green channel, and the mode intensity for each cone cell was recorded (Supplementary Fig. S1). The same process was applied to an uninjected control image. The highest mode intensity for the uninjected background control (bckg – max mode intensity = 20) was multiplied by three and that value (60) was set as the cutoff for cone cells considered GFP positive.

Figure 4B shows a graph of the percentage of the total number of cones counted for each AAV serotype that were also considered GFP positive. Both Anc80 variants Anc80L65 (67%) and Anc80L121 (53%) had comparable levels of cone targeting compared with AAV9 (48%) and AAV8 (41%) with Anc80L65 showing the highest percentage of transduced cones. Variant Anc80L27 showed the lowest level of cone targeting at around less than half of what was seen for AAV8 at 18%. Figure 4C shows the histogram distribution of all cone cells counted in each group. Despite the almost 20% increase on cone targeting seen when comparing AAV9 to Anc80L65, the natural variation of subretinal injections within the groups meant that Anc80L65 and Anc80L121 were not statistically significant compared to AAV9 and AAV8. The cutoff mark (60) for cones considered GFP positive based on mode intensity is shown on the graph, as is the overall mean mode intensity for all counted cones (Fig. 4C).

Faster onset of expression of Anc80 variants compared with contemporary AAVs

During the course of the above experiments it was noted through fundus imaging that animals injected with two out of the three Anc80 variants (Anc80L27 and Anc80L65) seemed to have a faster onset of GFP expression compared with AAV8 and AAV2 (Fig. 5A).

Fundus imaging at the initial PI timepoints showed that already at 1 day PI, considerably higher levels of GFP were being expressed in eyes injected with Anc80L65 and Anc80L27 compared with AAV8. This was maintained at 3 days and 1 week PI (Fig. 5A), even though at 1 week PI, eyes injected with AAV2 and AAV8 were starting to show more sustained and visible levels of GFP expression. This pattern was also confirmed in histological sections of GFP fluorescence, as shown in Fig. 5B. At 3 days PI, strong RPE expression is already present in both eye injected with the Anc80 variants, while very weak expression is seen in AAV8-injected eyes and almost none was observed in AAV2-injected eyes.

By 1 week PI, all serotypes started to show GFP fluorescence in the RPE, but both Anc80 variants still remained at considerably higher levels compared with AAV8 and AAV2. By 4 weeks PI, GFP expression in AAV8 injected eyes has finally picked up and reached a similar level of expression compared with Anc80L27 and Anc80L65. This is then maintained at a later timepoint of 12 weeks PI. However, fundus or histology images are not reliable methods for *GFP* quantification; therefore, to quantify this fast onset and higher level of transgene expression observed in eyes injected with these two Anc80 variants, eyes were collected for real-time quantitative PCR (qPCR) analysis at two of the early timepoints (3 days and 1 week PI) and at the last timepoint of 12 weeks PI. Results for the qPCR are shown in Fig. 5C and are normalized for AAV2 expression at the 3 days PI timepoint ($\Delta\Delta Ct$). Interestingly, these results demonstrate that at 3 days PI, AAV8 expression levels are 10-fold higher compared with AAV2, but Anc80L27 and Anc80L65 are an impressive 60- and 148-fold higher, respectively. At 1 week PI, Anc80L27 has increased its expression levels considerably, with a 211-fold increase. Anc80L65 has increased to 188-fold higher levels, while AAV8 and AAV2 remain around 50-fold. At the final timepoint of 12 weeks, where expression levels are believed to have been stabilized, we still observe higher expression of the Anc80 variants which now show a 159- and 155-fold increase for Anc80L27 and Anc80L65, respectively, compared with AAV2 at 3 days PI. AAV8 has stabilized at an 89-fold increase, while AAV2 showed an unexpected decrease from 1 week PI, which was not reflected in the fundus or histology data (17-fold, possibly due to injection quality in this group).

Analogous tropism of Anc80 variant in primate retina

Next we evaluated if the retinal tropism of the Anc80L65 variant observed in the mouse could be replicated in a NHP retina. Rhesus macaque ani-

Figure 5. Rapid onset of expression of Anc80 variants. **(A)** Representative fundus images of adult mouse eyes after subretinal injections using AAV2, AAV8, Anc80L27, and Anc80L65 showing early onset of GFP expression of the Anc80 variants. **(B)** Representative confocal images of retinal cryosections of injected eyes comparing the progression of GFP expression over time from 3 days to 12 weeks PI. GFP is shown in *green*, cell nuclei are shown in *blue* (DAPI). Scale bar=100 μ m. Rightmost panels show higher magnification images from 12 weeks post injection focusing on the RPE/outer segment/ONL borders. Scale bar=25 μ m. **(C)** Quantitative analysis of transgene mRNA levels for AAV2, AAV8, Anc80L27, and Anc80L65 over time relative to AAV2 3 days PI. Errors are SEM; $n=4$ eyes per group per timepoint. Student's t -test analysis ($p<0.01$) against AAV2 expression at each timepoint shows that at 3 days only Anc80L65 is significantly increased, while at 1 week no serotype is statistically significant, and at 12 weeks AAV8, Anc80L65, and Anc80L27 all show significant expression increase. Anc80L65 was also shown to have statistically significant increased expression at 3 days compared to AAV8 ($p<0.01$).

mals were injected subretinally at a dose of 1×10^{10} total genome copies of the same CMV.eGFP.WPRE transgene construct used in the mouse experiments packaged in AAV5, AAV9, or Anc80L65, all vectors that our mouse studies, as well as previous reports, have indicated to transduce cone photoreceptor.^{49,50} Supplementary Table S1 shows the outline of the primate study where a total of 7 eyes were evaluated. Out of the 7 eyes, 2 were injected with AAV5, 2 with AAV9 and 3 with Anc80L65. Unfortunately, in the 2 eyes that were injected with AAV8 (data not shown), one received a failed injection and the second eye was in an animal that

had to be sacrificed earlier due to complications in the contralateral eye unrelated to this study. Animals were assessed by clinical eye examinations after following surgery to the extent possible at 1, 3, and 7 days in the first week and weekly afterward. These examinations demonstrated that all eyes responded well to the surgery, with good recovery and no surgical complications observed. Also noted at these weekly exams was the first timepoint at which GFP fluorescence was observed (Supplementary Table S1). This was done using indirect ophthalmoscopy with built-in blue light excitation that allows for green fluorescence visu-

alization. As seen in Supplementary Table S1, the fast onset of Anc80L65 observed in the mouse retina was also seen in the NHP eyes injected with Anc80L65 as compared with AAV9 and AAV5 (data unavailable for AAV8). Out of the three Anc80L65-injected eyes, one eye (411-01 OS) already showed GFP expression at 1 week, whereas the remaining two eyes first recorded strong GFP fluorescence at 2 weeks PI (338-04 and 431-99). AAV9-injected eyes had a slower transgene expression onset compared to Anc80L65 with one eye showing very weak GFP fluorescence at 1 week that only increased around 4 weeks PI, while the second eye only had visible expression at 3 weeks PI. Both AAV5-injected eyes had the slowest onset, with GFP fluorescence observed only at 4 weeks PI.

Furthermore, fundus imaging of a cohort of the eyes are shown in Fig. 6A (i–v), with white light images on the left panel and green fluorescent images on the right. Images 6A (i) and 6A-ii are from AAV9-injected eyes taken at 5 weeks PI (358-08 OD and 411-11 OD, respectively); image 6A-iii is from an AAV5-injected eye at 8 weeks PI (338-04 OD); and images 6A-iv and 6A-v are from Anc80L65-injected eyes taken at 5 weeks PI (411-01 OS) and 8 weeks PI (338-04 OS), respectively. Consistent with previous studies, we observed that in AAV5-injected retinas transgene expres-

sion was restricted to the bleb region, while in AAV9- and Anc80L65-injected retinas expression was seen beyond the bleb.^{50,54} However, the small number of NHP animals used here does not allow for an in-depth and quantitative characterization of bleb × lateral spread of transgene. The difference in expression levels of the GFP transgene in the NHP retina was then confirmed in retinal sections (Fig. 6B) where comparison of AAV9-, AAV5-, and Anc80L65-injected eyes indicates a much stronger GFP fluorescence in the ONL and RPE after transduction with Anc80L65.

Since the mouse data showed that Anc80L65 was capable of transducing cone photoreceptors, we next wanted to evaluate whether this was also true in the NHP retina. Colocalization of GFP-positive photoreceptors with two different cone markers in eyes injected subretinally with Anc80L65 is shown in Fig. 6C–E. Panel C shows GFP colocalization with peanut-agglutinin (PNA; red) labelling of cone outer segments of eye 431-99 OS, while panels D and E show cone arrestin labelling (red, eye 338-04 OS) colocalizing with GFP in the cone cell bodies. However, despite a number of GFP-positive cones observed, the GFP signal in cones appear on average weaker than in rods and the small number of injected eyes in the NHP study invalidated any quantitative analysis.

Figure 6. Retinal tropism of Anc80L65 in the nonhuman primate (NHP) retina. **(A)** Fundus imaging of NHP eyes injected with AAV9 (*i*, *ii*), AAV5 (*iii*), and Anc80L65 (*iv*, *v*). Left panel shows normal light images and right panel shows green fluorescent images. Images shown correspond to the following animal and eye ID: (*i*) 358-08 OD; (*ii*) 411-01 OD; (*iii*) 338-04 OD; (*iv*) 338-04 OS; (*v*) 431-99 OS. **(B)** Representative retinal cryosections showing GFP expression (green) after subretinal injection with AAV5 (*i*, 338-04 OD); AAV9 (*ii*, 358-08 OD); and Anc80L65 (*iii*, 431-99 OS). Cell nuclei are stained with DAPI (blue). Scale bar = 100 μ m. **(C–D)** Confocal z-stack images showing cone-specific targeting in NHP retinas after injection with Anc80L65. In panel (C), GFP positive (green) cone outer segments in the peripheral retina colocalize with peanut-agglutinin (red) (from animal 431-99 OS), while in (D) GFP-positive cone cell bodies in the outer nuclear layer of the central retina are shown colocalizing with cone-specific cone arrestin staining in red (yellow colocalization; animal 338-04 OS). (*i*) Merged images, (*ii*) green channel only, and (*iii*) red channel only. White dashed lines mark the upper and lower borders of cone cell bodies. **(E)** Selected amplified view from (D) of a single stack confocal image showing colocalization of GFP expressing cones (green) and cone arrestin marker (red). Scale bar = 20 μ m (C and D) and 10 μ m (E). White dashed lines delimitate the upper and lower borders of cone cell bodies.

DISCUSSION

The recent successful applications of gene therapy for inherited eye disorders have provided valuable information on the safety and efficiency of the currently approved AAV2 vector.^{16,63–65} AAV-based vectors are emerging as attractive vehicles for gene replacement therapies due to its safety and transduction profiles and a range of targeting properties to the various cell type and therapeutic targets in the retina.^{13,17,20} However, despite the current extent of information and studies regarding characterization of different AAV serotypes, there are very few vectors currently available that can be used effectively in a wider set of applications. An AAV vector with a broad tropism that could be used in different applications provides a significant advantage on which to develop a platform. For those indications requiring specific targeting, modalities of transcriptional regulation can be then combined with a vector system for which safety and efficacy data is available from other programs.

The distinct tropisms observed in different AAV serotypes is thought to be mediated by the presence of unique elements on the capsid surface generated by sequence variation of the capsid proteins.^{66,67} However, modifications on capsid protein sequences have to always take into account structural constraints imposed by the secondary, tertiary, and quaternary interactions of the 60 monomers that form the icosahedral AAV capsid.^{66,68} Keeping these constraints in mind, the reconstruction of the AAV lineage and characterization of the furthestmost ancestral node recently published by Zinn and colleagues⁵⁵ has provided a set of viable libraries of ancestral AAVs from which these structure–function relationships can be investigated. From this effort, one variant—Anc80L65—was described as an infectious and highly stable viral particle with a broad *in vivo* transduction profile. This preliminary *in vivo* transduction data indicated a particularly high affinity of Anc80L65 for targeting retinal cells, implying that other variants from within this library might also share similar retinal affinity. Indeed, the results presented here confirm this phenotype for two other variants from the Anc80 library, Anc80L27 and Anc80L121. All three variants—Anc80L27, Anc80L65, and Anc80L121—are capable of efficiently targeting retinal cells via subretinal delivery, and not only did the three variants show extremely high affinity to RPE and photoreceptor cells, but they were also capable of transducing neurons from the INL, Muller glia, and ganglion cells. These results demonstrate a broader potential for Anc80 to target INL neurons

and glia via subretinal administration when compared with AAV8, which has only been shown to occasionally transduce ganglion cells and Muller glia.^{22,23} Furthermore, Anc80L65 and Anc80L27 variants were shown to have a much faster onset of expression compared with AAV8 and AAV2, with Anc80L65 already showing transgene expression at one day post injection, a property that is relevant experimentally in aggressive retinal degeneration models and possibly in clinical settings where acute intervention is desirable. In addition, our data illustrates that Anc80L65 expression is kept at sustained higher levels compared with AAV8 for up to 12 weeks. Faster onset of expression was also seen in the NHP retina, where Anc80L65 expression onset was, on average, 1 week ahead of AAV9 and 2 weeks ahead of AAV5 (Supplementary Table S1).

Despite the high sequence homology between the variants, it is interesting that a distinct phenotype was observed for each variant. For example, the fast onset reported in variant Anc80L65 was not seen in variant Anc80L121 and was seen to a lesser extent in Anc80L27. Horizontal cell targeting was not observed with Anc80L65 but was present using variants Anc80L27 and Anc80L121. Anc80L27 was the only variant capable of transducing amacrine cells and showed the highest affinity for ganglion cells. These three variants only differ in nine positions in the capsid sequence, which offers a unique opportunity to study the effect of structure–function relationships on viral tropism and helps define how these associations are formed (Fig. 3). Indeed, despite the high homology between the variants, structural mapping of viral capsid proteins shows a diverse electrostatic potential for each variant that could account for these functional differences. Modeling these variants can offer us some insight but comes with limitations. The crystal structure has only been defined for the AAV VP3 capsid protein; therefore, information provided by this approach is based on VP3 only structure and not the native VP1, VP2, and VP3 capsid.

Of the three variants tested here, Anc80L65 has emerged as a strong candidate for retinal gene replacement capable of targeting multiple retinal cell types of therapeutic relevance in mouse and NHP. Of particular interest is the finding that Anc80L65 is at least as good as AAV8 and AAV9 in targeting cone photoreceptors in the murine retina. In this study we were able to, for the first time, fully quantify cone targeting using AAV vectors in the mouse retina. We show that both AAV8 and 9 are capable of targeting only around 40–50% of mouse cone photoreceptors, while Anc80L65 reached 65% of cells in an equal dose comparison. Although not statistically signifi-

cant, it remains to be seen whether using a cone-specific promoter could drive cone transduction by Anc80L65 to be significantly higher than AAV8 or AAV9. Despite the low target rate observed in the mouse retina, it has been reported that efficiency of cone targeting in the retina of NHPs is substantially increased when using AAV9. Previous studies have shown that, within the injected area, AAV9 at a dose of 1×10^{10} vg is capable of transducing 40% of cones,⁵⁰ while AAV5 at a dose of 1×10^{13} vg targets 5–12% of cones.⁶⁹ Although the delivery of a therapeutic transgene to primate cones was achieved using AAV5 in only one study,⁶⁹ the ability of AAV5 to target primate cones efficiently after subretinal delivery remains unclear, with studies showing no cone targeting at all,⁴⁸ weak or locally restricted transgene expression,^{53,69} or robust cone targeting across a variety of retinal areas.⁵⁴ Other serotypes that have also been shown to efficiently target cone photoreceptors include AAV7m8,⁵² AAV8BP2,⁵² and AAV2tYF,⁷⁰ although proper quantification of cone targeting by these capsids is still needed. Different experimental approaches like choice of promoter, serotype, and postoperative analysis could explain these discrepancies between studies. However, it is most likely that vector dose has the strongest effect on cone targeting, with only concentrations above 1×10^{10} vg capable of efficiently transducing cones.^{49,50,52,54}

Due to the small number of NHPs used in this study, it was unfortunately not possible to quantify cone transduction in eyes injected with Anc80L65 compared with AAV5 and AAV9. The preliminary results shown here indicate that Anc80L65 is capable of targeting cones in the primate retina, but a more quantifiable and in-depth evaluation of this novel vector is needed to confirm whether it performs in a similar, or indeed better (as shown here for the mouse retina), range as AAV9 and/or AAV5. It would also be interesting to evaluate whether there is differential cone subtype/retinal location targeting by Anc80. Many applications for retinal gene therapy rely on efficient transduction of cone photoreceptors. Since these specialized sensory cells are responsible for high acuity and color vision, their loss is usually felt early and more acutely by patients with inherited retinal degeneration. Even in cases of rod-specific genetic lesions where cone cell dismissal is a secondary effect to a rod cell death, efficient cone targeting would therefore be essential in efforts to protect and preserve vision.

In summary, this study presents an in-depth evaluation of the efficiency and tropism of conventional and designer AAVs for broad retinal gene

therapy applications in a small and large animal model. Our work illustrates the impact of minimal changes in capsid composition on aspects relevant to experimental and clinical gene transfer applications. Primarily, we identified a novel AAV with broad potential applications for retinal gene therapy following subretinal injection that can serve as an alternative to the current AAV technologies with in addition phenotypes of fast onset of expression, cone photoreceptor targeting, and access to the majority of other retinal cell types.

ACKNOWLEDGMENTS

We would like to thank Dr. Petr Baranov for his help with ImageJ scripting and Dr. Tatjana Jakobs for the kind gift of the ChaT antibody. This work was supported by a donation from Giving/Grousebeck, the Ush2A consortium, the Candyce Henwood Fund, and grants through the Foundation Fighting Blindness, Research to Prevent Blindness, and NIH 5DP1EY023177 (L.H.V.), the Australian Research Council (L.S.C.), and the Harvard Medical School Department of Ophthalmology Age-Related Macular Degeneration Center of Excellence (L.S.C.).

L.S.C. and L.H.V. were responsible for the study outline and experimental design, with L.H.V. responsible for overall supervision of the project. L.S.C. directly conducted and/or supervised all experiments. R.X. and S.S. assisted in murine injections and animal monitoring. E.Z. was responsible for Anc80 variants selection. S.P. conducted the computational analysis and modelling of AAV variants. S.J.W. was responsible for immunohistochemistry and image analysis. A.L. was responsible for NHP sample preparation and analysis. J.I.C., L.A.K., and L.L. conducted the nonhuman primate surgeries and ocular evaluations. L.S.C. and L.H.V. wrote the manuscript.

AUTHOR DISCLOSURE

L.H.V. is an inventor on several technologies licensed to pharmaceutical and biotechnology companies including Anc80 technology, which was licensed to Astellas, Vivet Therapeutics, Lonza Houston, and Selecta Biosciences. L.H.V. receives research funding from and consults for Lonza Houston and Selecta Biosciences. L.H.V. is co-founder and equity holder of GenSight Biologics, a retinal gene therapy company. L.H.V. is also a member of the Scientific Advisory Board of GenSight Biologics and NightStarX. For authors L.S.C., R.X., S.J.W., A.L., E.Z., S.P., S.S., J.I.C., L.A.K., and L.L., no competing financial interests exist.

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Received for publication August 11, 2017;
accepted after revision January 9, 2018.

Published online: January 11, 2018.