Effects of Altering Heparan Sulfate Proteoglycan Binding and Capsid Hydrophilicity on Retinal Transduction by Adeno-associated Virus

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ABSTRACT Adeno-associated viruses (AAVs) have recently emerged as the leading vector for retinal gene therapy. However, AAV vectors that are capable of achieving clinically relevant levels of transgene expression and widespread retinal transduction are still an unmet need. Using rationally designed AAV2-based capsid variants, we investigate the role of capsid hydrophilicity and hydrophobicity as it relates to retinal transduction. We show that hydrophilic, single-amino-acid mutations (V387R, W502H, E530K, L583R) in AAV2 negatively impact retinal transduction when heparan sulfate proteoglycan (HSPG) binding remains intact. Conversely, addition of hydrophobic point mutations to an HSPG binding-deficient capsid (AAV2ΔHS) leads to increased retinal transduction in both mouse and macaque. Our top performing vector, AAV2 (4pMut)ΔHS, achieved robust rod and cone photoreceptor (PR) transduction in macaque, especially in the fovea, and demonstrates the ability to spread laterally beyond the borders of the subretinal injection (SRI) bleb. This study both evaluates biophysical properties of AAV capsids that influence retinal transduction and assesses the transduction and tropism of a novel capsid variant in a clinically relevant animal model.

IMPORTANCE Rationally guided engineering of AAV capsids aims to create new generations of vectors with enhanced potential for human gene therapy. By applying rational design principles to AAV2-based capsids, we evaluated the influence of hydrophilic and hydrophobic amino acid mutations on retinal transduction as it relates to vector administration route. Through this approach, we identified a largely deleterious relationship between hydrophilic amino acid mutations and canonical HSPG binding by AAV2-based capsids. Conversely, the inclusion of hydrophobic amino acid substitutions on an HSPG binding-deficient capsid (AAV2ΔHS) generated a vector capable of robust rod and cone photoreceptor (PR) transduction. This vector AAV2(4pMut)ΔHS also demonstrates a remarkable ability to spread laterally beyond the initial subretinal injection (SRI) bleb, making it an ideal candidate for the treatment of retinal diseases that require a large area of transduction.

KEYWORDS adeno-associated virus, gene therapy, retina

Gene therapy has emerged as a promising treatment approach for a variety of genetic diseases of the retina. Numerous vectors for gene delivery are under investigation; however, recombinant adeno-associated virus (rAAV)-based therapies have become the established leader, having been used successfully in a large number of proof-of-concept studies utilizing animal models of retinal disease (1). This has progressed to clinical trials with several in the later phases, including X-linked retinitis

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pigmentosa, choroideremia, and wet age-related macular degeneration, among others (2–4). FDA approval of rAAV-based therapies for spinal muscular atrophy (5) and Leber congenital amaurosis type 2 (LCA2) (6) solidified AAV as a safe and effective vector for retinal gene therapy. Despite these developments, vectors capable of achieving clinically relevant transgene expression in specific cell types and areas of the retina, simultaneously avoiding dose-related toxicity and iatrogenic pathologies, are still an unmet need. Unlike LCA2, many inherited retinal diseases (IRDs) are caused by mutations in photoreceptor (PR)-specific genes, making this cell type a primary target for therapy. Transduction of PRs, especially cones in the central retina, is critically important in humans, as macular cones are responsible for high-acuity daylight vision.

When targeting the retina for gene therapy, there are two common routes of administration, intravitreal injection (IVI) and subretinal injection (SRI). IVI can be performed in a clinic and is overall a less invasive/complex procedure. In cases where the retina is fragile, IVI would be a preferred administration route, as it does not result in a retinal detachment and is less likely to result in further degeneration. A major drawback of IVI, however, is that it requires AAV vectors capable of traversing the inner limiting membrane (ILM) from the vitreous, as the ILM is the major barrier to retinal transduction (7). Because of this, transduction is often limited to the layers of retinal cells most proximal to the ILM, such as retinal ganglion cells (RGCs), Müller glia, or nonretinal cells located anteriorly in the globe (8).

Currently, the best vectors for IVI are AAV2- or AAV6-based vectors, as these bind to heparan sulfate proteoglycan (HSPG), a major component of the ILM (7). The interaction between the AAVs and HSPG is mediated by positively charged amino acid residues on the capsid's surface. For AAV2, there are 2 critical arginine residues, R585 and R588, responsible for this interaction (9, 10), while AAV6's HSPG interaction is mediated by K531 (11). Recent studies have demonstrated that the ability to interact with HSPG is required for retinal transduction via IVI (12, 13). Multiple studies have reported changes in tropism and transduction of canonically non-HSPG binding AAVs (i.e., AAV5-, AAV8-, AAVrh.8R-, and AAV44.9-based vectors), with novel HSPG binding added via mutagenesis (9, 10, 14). These studies suggest that interactions between AAVs and HSPG, or perhaps other components of the extracellular matrix (ECM), can be modified by the addition of charged amino acid residues to the capsid surface. In this study, we evaluate the effects of single hydrophilic (charged) amino acid mutations to an AAV2-based capsid both *in vitro* and *in vivo* in mice, in the presence of intact or disrupted canonical HSPG binding. Our results suggest that increasing the hydrophilicity of AAV2-based capsids disrupts the ability of these vectors to productively infect cells when canonical HSPG binding is intact. We also investigated AAV2-based variants harboring both disrupted HSPG binding properties and hydrophobic surface mutations, with the goal of developing better vectors for SRI.

SRI is superior to IVI with regard to its ability to achieve robust PR transduction. However, until recently, transduction by SRI vectors was largely limited to the area of the injection bleb (14). In patients with LCA2, SRI of vector under the fovea led to central retinal thinning and loss of visual acuity in some patients (15). This could pose a risk to the treatment of patients with extensive retinal degeneration but that still retain central vision (e.g., retinitis pigmentosa). Development of AAVs that are capable of migrating outside of the initial SRI bleb would be ideal for treating IRDs without detaching the fovea. In pursuit of this goal, we evaluated one of our top-performing mouse vectors, rAAV2(4pMut) Δ HS, for its ability to transduce PRs in a clinically relevant model, cynomolgus macaque. The macaque retina is morphologically very similar to the human retina, with a cone-rich macula and cone-exclusive fovea. Importantly, rAAV2(4pMut) Δ HS exhibited lateral spread outside of the initial injection bleb, allowing for highly efficient transduction of foveal cones (94%) in the absence of detaching this region. These results suggest that this potent capsid will be useful in addressing IRDs by increasing the area of treatment, as well as allowing efficient targeting of the central retina while avoiding the need to physically detach it.

RESULTS

rAAV2 variants harboring hydrophilic surface amino acid substitutions display altered heparin binding and capsid stability. We hypothesized that, in addition to the canonical AAV2 HSPG binding footprint, overall capsid hydrophilicity and stability may influence the transduction efficiency of AAV2-based variants. To investigate this, we identified five surface-exposed amino acids spanning multiple capsid variable regions as candidates for mutation (Fig. 1A and B). Residues V387, W502, E530, M558, and L583 were selected for mutation because they were not associated with any previously described AAV2 capsid phenotype. Furthermore, mutation of these residues was predicted to lower capsid stability and/or increase HSPG affinity based on structural modeling. Using rAAV2(Y444F) as the parental vector, we incorporated each of the following point mutations to create a panel of “hydrophilic” rAAV2(Y444F) variants as follows: V387R, W502H, E530K, M558R, and L583R. rAAV2(Y444F) was used as the parent vector, as it is well characterized and highly efficient at transducing the inner retina via the vitreous, and the trabecular meshwork (TM), two tissues where HSPG binding is required for efficient transduction (12, 16–18).

With the exception of rAAV2(Y444F+M558R), which did not form DNA-containing viral capsids and was thus no longer pursued, each hydrophilic variant was capable of producing genome-containing viral capsids as observed by negative stain electron microscopy (EM) (Fig. 1C) and quantitative PCR (qPCR) (data not shown). Variants had capsid viral protein (VP) ratios (VP1/VP2/VP3) comparable to that of rAAV2(Y444F) as determined by SDS-PAGE (Fig. 1C). The HSPG affinity of each variant was inferred from a heparin binding assay (Fig. 1E), which confirmed that each variant indeed bound heparin. rAAV2(Y444F) Δ HS, containing R585S and R588T substitutions, was used as a non-heparin binding control. Two variants, rAAV2(Y444F+V387R) and rAAV2(Y444F+W502H), had a heparin binding profile similar to that of rAAV2(Y444F), with peak elution at 200 mM NaCl (Fig. 1E). Interestingly, both rAAV2(Y444F+E530K) and rAAV2(Y444F+L583R) demonstrated a higher affinity, eluting at 400 to 500 mM NaCl (Fig. 1E). Each of these hydrophilic variants, with the exception of rAAV2(Y444F+E530K), displayed an expected decrease in capsid stability as measured by differential scanning fluorometry (DSF) (Fig. 1D).

Hydrophilic rAAV2-based variants show differential transduction of retina cells *in vitro* and *in vivo*. Next, the ability of the hydrophilic variants to transduce cells *in vitro*, using the well-characterized ARPE-19 retinal cell line, was assessed. Surprisingly, none of the variants effectively transduced ARPE-19 cells at either a multiplicity of infection (MOI) of 5,000 or 10,000 (Fig. 2A). This observation was unexpected given that rAAV2-based vectors with intact HSPG binding properties generally transduce these cells efficiently (19).

The transduction profile of the hydrophilic rAAV2-based variants was investigated in mouse retina following either IVI or SRI of 1 μ l vector, at a concentration of 2×10^{12} viral genomes (vgs)/ml. Injections were performed in *Nrl*-GFP mice, which constitutively express green fluorescent protein (GFP) in rod PR cells. Fundoscopy for mCherry fluorescence at 4 weeks postinjection (p.i.) revealed that none of the hydrophilic variants transduced retina following IVI (Fig. 2B). This result was expected because *in vivo* transduction via IVI and *in vitro* transduction (which these variants did not display) are generally influenced by similar biophysical properties of AAV (i.e., HSPG receptor-mediated infection) (12, 13, 17, 20). Quantification of transduction in rod PRs (GFP and mCherry positive) and nonrod (mCherry positive) retinal cells by flow cytometry (Fig. 2C and D) confirmed qualitative observations in fundus images. Interestingly, only rAAV2(Y444F+L583R), with increased HSPG binding (Fig. 1C), was capable of transducing the retina via SRI (Fig. 2B), and the magnitude of this signal was $\sim$ 5-fold lower than for control vector rAAV2(Y444F) (unpaired 2-tailed Student's *t* test; $P < 0.05$; $n = 4$ to 6) (Fig. 2D). These results point to the hydrophobic residues substituted as having a functional phenotype in rAAV2 retinal transduction via SRI (13, 14, 21, 22), and these variants presumably still utilize HSPG binding as their primary receptor for cellular entry.

FIG 1 Biophysical properties of hydrophilic AAV2-based variants. Surface-exposed amino acids mutated to create the hydrophilic panel of rAAV2-based vectors shown on a single VP3 subunit (A) and the asymmetric unit of an AAV2 capsid (B). V387, blue; Y444, red; W502, violet purple; E530, cyan; L583, slate. (C) Hydrophilic rAAV2-based vectors have expected VP1:VP2:VP3 ratio (protein gels) and produce intact capsids as visualized by EM; scale bar 50 nm. (D) DSF shows an inverse correlation between the addition of hydrophilic mutants and capsid stability for all variants except rAAV2(Y444F+E530K) (yellow), which has a capsid stability comparable to rAAV2(Y444F) (blue). (E) Heparin binding affinity of hydrophilic AAV variants demonstrates that rAAV2(Y444F), rAAV2(Y444F+V387R), and rAAV2(Y444F+W502H) have heparin affinities comparable to rAAV2 (range, 150 to 250 mM NaCl). rAAV2(Y444F+E530K) and rAAV2(Y444F+L583R) have increased heparin affinity (range, 450 to 500 mM NaCl) relative to rAAV2. rAAV2(Y444F)ΔHS was used as a negative control for the heparin binding assay, as it does not bind heparin and is only detectable in the flow through (FT) and wash (W).

FIG 2 *In vitro* and *in vivo* transduction of hydrophilic variants. (A) Evaluation of hydrophilic vectors in ARPE-19 cells revealed that these rAAV2-based capsid variants are not infectious *in vitro* ($n=3$). (B) Representative fundus images from mice that received either IVI or SRI of hydrophilic (Continued on next page)

Hydrophilic variants bind to HEK293 cells but are not infectious. Since all four hydrophilic variants retain the ability to bind heparin yet demonstrate negligible transduction both *in vitro* and *in vivo*, we asked whether they maintained their ability to bind to other cell surfaces. An *in vitro* qPCR-based binding assay in HEK293 showed all variants capable of binding to HEK293 cells, with the following three variants exhibiting significantly increased binding relative to rAAV2(Y444F) [$F_{(5,42)} = 102.976$; $P < 0.001$]: rAAV2(Y444F+V387R) ($P = 0.007$), rAAV2(Y444F+W502H) ($P = 0.014$), and rAAV2(Y444F+E530K) ($P = 0.012$) (Fig. 2E). rAAV2(Y444F+L583R) had binding comparable to rAAV2(Y444F) ($P = 0.142$) (Fig. 2E). rAAV2(Y444F) Δ H5, which does not bind to HEK293 cells, was used as a negative control (Fig. 2E).

Disruption of canonical HSPG binding in hydrophilic rAAV2-based variants had differential effect on heparin binding and ablated cell binding properties. Because ablation of canonical HSPG binding drastically modifies both the transduction and biophysical properties of AAV2, we wanted to assess our panel of hydrophilic mutations on an HSPG binding-deficient (referred to as Δ H5) rAAV2-based variant background. We hypothesized that Δ H5 would fundamentally change the transduction of these variants and potentially allow productive transduction of PRs. Canonical HSPG binding was ablated by mutating the two critical arginine residues (R585S and R588T) controlling this property on the capsid surface (Fig. 3A and B). Interestingly, the combination of L583R with Δ H5 proved to be deleterious, inhibiting the production of genome-containing capsids. This variant was thus excluded from downstream experiments. We then characterized the remaining Δ H5 hydrophilic mutants in the same manner as described above.

Disruption of canonical HSPG binding for rAAV2(Y444F+W502H) Δ H5 ablated its ability to bind heparin (Fig. 3C). Unexpectedly, rAAV2(Y444F+V387R) Δ H5 maintained its ability to bind heparin as well as rAAV2(Y444F+E530K) Δ H5, albeit to a much lesser extent (Fig. 3C). Both rAAV2(Y444F+W502H) Δ H5 and rAAV2(Y444F+E530K) Δ H5 were unable to effectively bind cells in the cell binding assay (Fig. 3D). rAAV2(Y444F+V387R) Δ H5 had significantly higher binding than control vector rAAV2(Y444F) Δ H5 ($P < 0.001$), though it was negligible considering rAAV2(Y444F) binding was approximately 80-fold higher than rAAV2(Y444F) Δ H5 ($P < 0.001$) [$F_{(4,15)} = 282.256$; $P < 0.001$] (Fig. 3D).

Transduction of mouse retina by IVI rAAV2-based hydrophilic Δ H5 variants depends on more than HSPG binding. To assess the transduction of the hydrophilic Δ H5 vectors *in vivo*, vectors were administered by both IVI (1 μ l at 2×10^{12} vg/ml) and SRI (1 μ l at 2×10^{11} vg/ml) to *Nrl*-GFP mice as above. Vectors delivered by SRI were administered at a 10-fold lower dose than IVI vectors because rAAV2-based Δ H5 vectors are generally more potent in the subretinal space than traditional rAAV2-based vectors (12, 23, 24). rAAV2(Y444F) was used as a positive control for the IVI vector panel. None of the Δ H5 vectors transduced the retina by IVI (Fig. 4A), which in the case of rAAV2(Y444F+W502H) Δ H5 and rAAV2(Y444F+E530K) Δ H5 is unsurprising given their absent or low heparin binding, respectively. However, rAAV2(Y444F+V387R) Δ H5 also failed to transduce retina following IVI (Fig. 4A) despite the fact that it binds heparin, indicating that there are additional factors influencing the potency of this vector that are independent of HSPG binding. Fundus images and flow cytometry showed no retina transduction by the hydrophilic Δ H5 vectors following IVI (Fig. 4B).

Some rAAV2-based hydrophilic Δ H5 variants can transduce retina following SRI. When delivered by SRI, both rAAV2(Y444F+W502H) Δ H5 and rAAV2(Y444F+E530K) Δ H5 efficiently transduced the retina (Fig. 4A). In contrast, rAAV2(Y444F+V387R) Δ H5 failed to

FIG 2 Legend (Continued)

mutants show that retinal transduction was only achieved following SRI of rAAV2(Y444F+L583R) and rAAV2(Y444F+W502H) to a very low extent. Quantification of *in vivo* transduction was done by flow cytometry for IVI (C) and SRI (D) eyes ($n = 4$ to 6). Of all of the variants, only rAAV2(Y444F+L583R) (magenta) achieved rod transduction levels detectable by flow. (E) Analysis of cell surface binding by hydrophilic mutants in HEK293 cells indicated that rAAV2(Y444F+V387R) (green), rAAV2(Y444F+W502H) (yellow), and rAAV2(Y444F+E530K) (magenta) each had significantly increased binding to HEK293 cells relative to control vector rAAV2(Y444F) (red) by 1-way ANOVA ($P < 0.001$) and *post hoc* Holm-Sidak test ($P < 0.05$). rAAV2(Y444F+L583R) had binding comparable to rAAV2(Y444F) ($P = 0.142$). rAAV2(Y444F) Δ H5 (which does not bind to HEK293 cells) had negligible binding.

FIG 3 Biophysical properties of hydrophilic Δ HS variants. Surface-exposed amino acids mutated to create the Δ HS hydrophilic panel of rAAV2-based vectors shown on a single VP3 subunit (A) and the asymmetric unit of an AAV2 capsid (B). V387, blue; Y444, red; W502, violet purple; E530, cyan; L583, slate. All Δ HS variants harbor a Ser mutation at R585 (green) and a Thr mutation at R588 (lime green). (C) Heparin binding affinity of hydrophilic Δ HS rAAV variants shows that rAAV2(Y444F+V387R) Δ HS retains moderate heparin binding even with the Δ HS mutations (row 2). Additionally, rAAV2(Y444F+E530K) Δ HS (row 4) has a low affinity for heparin. All other Δ HS vectors failed to bind heparin and are present only in the flow through (FT) and wash (W). (D) Binding of Δ HS-containing vectors to HEK293 cells was low for all variants. rAAV2(Y444F+V387R) Δ HS had significantly increased binding relative to rAAV2(Y444F) Δ HS; however, this binding was about 40-fold less than benchmark rAAV2(Y444F) alone (determined by 1-way ANOVA, $P < 0.001$, and *post hoc* Holm-Sidak test, $P < 0.001$).

transduce the retina by SRI. Transduction of rod PRs by rAAV2(Y444F+W502H) Δ HS ($P = 0.355$) or rAAV2(Y444F+E530K) Δ HS ($P = 0.393$) did not differ significantly from parent vector rAAV2(Y444F) Δ HS [$F_{(3,18)} = 5.428$; $P = 0.008$] (Fig. 4C). Notably, both rAAV2(Y444F+W502H) Δ HS ($P = 0.005$) and rAAV2(Y444F+E530K) Δ HS ($P < 0.001$) had significantly lower transduction than rAAV2(Y444F) Δ HS in nonrod retinal cells [$F_{(3,18)} = 13.572$; $P < 0.001$], perhaps indicating decreased spread through the retinal layers (Fig. 4C).

Addition of hydrophobic mutations to Δ HS rAAV2-based variants enhances retinal transduction in mice following SRI. Though addition of the Δ HS mutations in conjunction with hydrophilic W502H or E530K mutations yielded infectious vectors, their performance *in vivo* was, at best, comparable to that of their parent vector rAAV2(Y444F). This suggests that hydrophilic mutations, or at least the specific residues investigated here, do little to enhance transduction for rAAV2-based vectors and, moreover, appear to be detrimental to transduction. Conversely, numerous studies have demonstrated an

FIG 4 Transduction of mouse retina by hydrophilic ΔHS variants. (A) Fundoscopy and IHC of *Nrl*-GFP mouse retinas following injection with hydrophilic ΔHS variants. Removal of the canonical HSPG binding footprint on both rAAV2(Y444F+W502H)ΔHS and rAAV2(Y444F+E530K)ΔHS promoted their transduction of retina via SRI. None of the vectors were capable of retinal transduction via IVI, and rAAV2(Y444F+V387R)ΔHS was not infectious by any administration route. Transduction of IVI (B) and SRI (C) retinas was quantified by flow cytometry. rAAV2(Y444F+W502H)ΔHS and rAAV2(Y444F+E530K)ΔHS transduced rods with comparable efficiency to rAAV2(Y444F)ΔHS (1-way ANOVA, $P < 0.01$, and *post hoc* Holm-Sidak test, $P > 0.05$; $n = 7$ to 8). Both variants transduced significantly less nonrod retinal cells than rAAV2(Y444F)ΔHS (1-way ANOVA, $P < 0.001$, and *post hoc* Holm-Sidak test, $P < 0.01$; $n = 7$ to 8). RPE, retinal pigmented epithelium; IS, inner segments; OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GC, ganglion cell layer. Scale bar (white) is 50 μm .

FIG 5 Transduction of mouse retina by hydrophobic Δ HS variants. Surface-exposed amino acids mutated to create the Δ HS hydrophobic panel of rAAV2-based vectors shown on a single VP3 subunit (A) and the asymmetric unit of an AAV2 capsid (B). Y444, red; R487, forest; T491, raspberry; Y500, firebrick; R585, green; R588, lime green; Y704, orange. The Δ HS set of mutations for this vector panel includes R487G, R585S, and R588T. (C) Representative fundus and IHC images show retinal transduction by these capsids following SRI. (D) Quantification of transduction by flow cytometry indicates that rAAV2(4pMut) Δ HS has significantly higher rod transduction than rAAV2 Δ HS and rAAV2(tripleY-F) Δ HS (1-way ANOVA, $P < 0.01$, and *post hoc* Holm-Sidak test, $P < 0.05$; $n = 4$ to 7). rAAV2(tripleY-F) Δ HS rod transduction was not significantly different than rAAV2 Δ HS ($P > 0.05$). rAAV2(4pMut) Δ HS also transduced nonrod retinal cells significantly more than rAAV2 Δ HS (1-way ANOVA, $P < 0.05$, and *post hoc* Holm-Sidak test, $P < 0.01$). RPE, retinal pigmented epithelium; IS, inner segments; OS, outer segments; ONL, outer nuclear layer; INL, inner nuclear layer; GC, ganglion cell layer. Scale bar (white) is 50 μ m.

enhancement of transduction with rAAV2-based vectors by incorporating hydrophobic tyrosine to phenylalanine (Y-F) and/or threonine to valine (T-V) surface mutations. However, it is unknown whether or not hydrophobic Y-F and T-V mutations confer the same transduction enhancement in a Δ HS vector. To test this, we generated three Δ HS rAAV2-based variants (Fig. 5A and B), rAAV2 Δ HS, rAAV2(tripleY-F) Δ HS, and rAAV2(4pMut) Δ HS, and compared their ability to transduce mouse retina. In addition to the baseline rAAV2 Δ HS vector, rAAV2(tripleY-F) Δ HS and rAAV2(4pMut) Δ HS were chosen because both sets of mutations have

been shown to enhance retinal transduction in the context of intact canonical HSPG binding (25–27).

rAAV2(tripleY-F) Δ HS and rAAV2(4pMut) Δ HS were SRI in mice at a dose of 2×10^8 vg using rAAV2 Δ HS as a benchmark. Transduction at 4 weeks p.i. was assessed by funduscopy (Fig. 5C) and quantified by flow cytometry (Fig. 5D). rAAV2(4pMut) Δ HS, the capsid with the most hydrophobic substitutions, was significantly more potent at transducing rod PRs than rAAV2 Δ HS ($P = 0.035$) and rAAV2(tripleY-F) Δ HS ($P = 0.041$) following SRI [$F_{(2,13)} = 5.181$; $P = 0.022$], whereas rAAV2(tripleY-F) Δ HS ($P = 0.938$) was not significantly different than rAAV2 Δ HS. rAAV2(4pMut) Δ HS was also more potent in non-rod retinal cells ($P = 0.004$) than rAAV2 Δ HS [$F_{(2,13)} = 8.232$; $P = 0.005$]. The enhancement observed with rAAV2(4pMut) Δ HS may be due to the T491V mutation itself or an additive effect of having an additional hydrophobic capsid residue with respect to rAAV2(tripleY-F) Δ HS. These results suggest that even in the context of a Δ HS rAAV2-based vector, the quantity and specificity of hydrophobic Y-F and T-V mutations can influence vector transduction.

rAAV2(4pMut) Δ HS efficiently transduces rods and cones via SRI in macaques.

We observed here, and in our previous study (12), exceptional transduction of mouse retina following SRI of rAAV2(4pMut) Δ HS. Because of the enhanced potency observed in mice, we next wanted to characterize transduction by this vector in a more clinically relevant, foveated species, *Macaca fascicularis*. Our first goal was to evaluate the tropism of AAV2(4pMut) Δ HS in the central and peripheral retina following submacular and peripheral SRI. AAV2(4pMut) Δ HS containing a GFP cassette driven by either the ubiquitous chimeric cytomegalovirus (CMV)/chicken β -actin (CBA) or PR-specific human rhodopsin kinase-1 (hGRK1) promoter were SRI into two 60- μ l blebs at a concentration of 1×10^{12} vg/ml. Representative fundus (Fig. 6A, day 0) and confocal scanning laser ophthalmoscopy (cSLO) (Fig. 6A, weeks 1 to 4) images of the eye demonstrate the location of the submacular and peripheral injection blebs and show expression of GFP spreading laterally outside the margin of the injection blebs in weeks 2 and 4 p.i. Retinal sections obtained 6 weeks p.i. exhibit robust GFP expression in foveal (Fig. 6B) and peripheral cones (Fig. 6C) by both CBA- and hGRK1-containing vectors. Quantification of immunohistochemical (IHC) sections indicates that both CBA and hGRK1 vectors expressed transgene in a high percentage of foveal cones ($\sim 89\%$ and 93% , respectively) (Fig. 6D) and peripheral cones (92% and 100% , respectively) (Fig. 6E). Both CBA- and hGRK1-containing vectors expressed transgene in central rods ($\sim 58\%$ and $\sim 57\%$, respectively) (Fig. 6D) and peripheral rods (56% and 95% , respectively) (Fig. 6E) as well. rAAV2(4pMut) Δ HS-hGRK1-mediated GFP expression was higher in peripheral rods than that observed with rAAV2(4pMut) Δ HS-CBA. Interestingly, we observed substantial GFP expression in the retinal pigmented epithelium (RPE) of eyes that received the rAAV2(4pMut) Δ HS-hGRK1 vector. While the hGRK1 promoter has been shown to drive transgene expression exclusively in PRs of mice and macaques in the context of other capsids (i.e., rAAV5, rAAV44.9 [14, 21]) following SRI, it has also been shown to promote leaky expression in mouse RGCs following IVI (27). We propose that the rAAV2(4pMut) Δ HS capsid has relatively efficient tropism for RPE, and, when delivered at this concentration to the subretinal space, the hGRK1 promoter has activity in those cells.

rAAV2(4pMut) Δ HS transduces foveal cones via extrafoveal SRI. Our second goal was to determine if lateral spread of rAAV2(4pMut) Δ HS outside of the injection bleb margins would allow transduction of foveal cones in the absence of a foveal detachment. To test this, three SRI blebs were administered to the superior, temporal, and inferior retina at a distance of approximately 25 degrees of eccentricity from the fovea. Two eyes total were injected, each with rAAV2(4pMut) Δ HS-hGRK1-GFP at a concentration of 1×10^{12} vg/ml with about 30 μ l of viral suspension per bleb. The location of the injection blebs relative to the macula and GFP fluorescence over time was captured by cSLO (Fig. 7A). To observe lateral spread of the vector outside of the injection bleb, the retina was divided into 5 zones (Fig. 7B, denoted in orange) and sectioned for microscopy/quantification of GFP expression. Sixty-five percent of foveal cones (zone 3) and

FIG 6 Transduction of macaque retina by rAAV2(4pMut)ΔHS vectors following SRI. (A) Representative cSLO images show location of submacular and peripheral injection blebs and GFP expression at 1, 2, and 4 weeks postinjection. Dotted green line defines the boundaries of the initial injection bleb (day 0) and the extent of GFP for each injection bleb (1, 2, and 4 weeks p.i.). Retinal vasculature is highlighted in red for orientation. By 4 weeks p.i., GFP expression was clearly detectable outside the boundaries of the injection blebs. At 6 weeks p.i., vector transduction (GFP, green) was observed in the fovea (B) and in the peripheral retina (C). Sections were costained for cone arrestin (red) and counterstained with DAPI (blue). Robust rod and cone transduction by both vectors were observed in both the central and peripheral retina. GFP expression in RPE was also observed (even mediated by the hGRK1-containing vector). rAAV2(4pMut)ΔHS-CBA and rAAV2(4pMut)ΔHS-hGRK1 transduced 89% and 93% foveal cones, respectively (D), and 92% and 100% peripheral cones, respectively (E). rAAV2(4pMut)ΔHS-CBA and rAAV2(4pMut)ΔHS-hGRK1 transduced 58% and 57% of central rods, respectively (D), and 56% and 95% of peripheral rods, respectively (E). GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

22% of central rods expressed GFP even though the fovea was not detached during surgery. Similar to findings in Fig. 6, GFP expression within rods and cones was observed in all 5 zones (Fig. 7B), even those most distant from the injection bleb. In zones 1 and 2, which are immediately adjacent to or within the injection bleb, >99%

FIG 7 Extrafoveal SRI of rAAV2(4pMut) Δ HS-hGRK1 in macaque. (A) Representative cSLO images show vector-mediated GFP expression at 1, 2, and 4 weeks p.i. Vascular landmarks are highlighted in red for orientation. GFP fluorescence expands with time from weeks 1 to 4 p.i. cSLO (B, top left) and OCT images (B, top right) show GFP fluorescence at week 4 p.i., location of the injection blebs, and confirm that the fovea was not detached during SRI, respectively. The numbers in orange represent 5 discrete zones of the retina that were sectioned for IHC analysis (horizontal orange line shows plane of sections). Vector-mediated GFP expression was detected in all zones, even in zone 3 (the fovea), which was not detached during surgery and is outside of the initial injection blebs. Expression was observed in zones 4 and 5 as well, which are also outside of the margins of the initial injection blebs, highlighting the lateral spread of this vector. (B) Bar graph shows quantification of rod and cone transduction throughout these zones, with corresponding IHC sections below. RPE transduction was observed throughout the retina, even with the use of the PR-specific hGRK1 promoter. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

of cones and >94% of rods were GFP positive, respectively (Fig. 7B, zones 1 and 2). Transgene expression in zones 4 and 5, peripheral retina external to the injection blebs, was lower but still present at 32% and 34% GFP positive for cones and 24% and 24% GFP positive for rods, respectively. As with the previous rAAV2(4pMut) Δ HS-hGRK1 injections, transgene expression in RPE was observed throughout the retina especially within the macula (Fig. 7B, zone 3).

DISCUSSION

Here, we investigate the transduction capabilities of novel AAV2-based variants containing hydrophilic, single amino acid substitutions spanning multiple variable regions within the capsid as follows: V387R, W502H, E530K, M558R, and L583R. V387 is partially buried in the capsid body at the outer base of the 3-fold protrusion- and 3-fold symmetry-related interface, in an environment composed of noninteracting residues L380, R389, and Y704 and interacting residue N382. Mutation of the valine to a basic residue V387R causes several charged residues to come into closer proximity, namely, D528, R566, and N511. These interacting and noninteracting residues in the vicinity of residue 387 are important for different steps in the AAV life cycle; for example, R389 and Y704 are important for pH-mediated endolysosomal trafficking (28, 29), and N511 binds the integrin coreceptor $\alpha v\beta 1$ (30). Ultimately, the mutation of V387 to R387 converts a largely hydrophobic pocket to a hydrophilic environment. Similarly, W502 is partially surface exposed with surrounding residues D269, D472, D507, D514, L516, K507, and R471. This residue is highly hydrophobic in a predominantly hydrophilic environment. Mutation to a basic residue W502H maintains the aromatic nature of the side chain but converts the hydrophobic side chain to a basic residue. This mutation converts this environment to a totally hydrophilic surrounding. E530 is surface exposed and interacts with R487, K527, and F533. Mutation of E530 to K530 creates a predominantly positive and hydrophilic environment with K527 and R487. Residue L583 is located on the ascending arm of the 3-fold protrusion on the external surface of the virus capsid and is located within the 3-fold symmetry-related interface. L583 is in interacting distance with R484 and R487 and noninteracting distance with E574. Mutation of L583 to R583 brings the residue into interacting distance with E530 and K527. L583R is similar to W502H, V389R, and E530K, as the mutations creates a totally hydrophilic and predominantly positively charged environment. The generation of patches of positively charged residues at these locations of the virus capsid was predicted to play a role in the HSPG binding affinity of the four variants. From a capsid assembly standpoint, with the exception of M558R, these mutations were well tolerated. However, none of the variants were capable of transducing cells *in vitro* or mouse retina *in vivo* via either IVI or SRI, with the exception of rAAV2(Y444F+L583R), which had significantly reduced transduction via SRI. Similar findings were observed by Ogden et al. who report that positively charged amino acid substitutions more often result in decreased capsid fitness for AAV2 (31). What is unclear, however, is how these hydrophilic mutations negatively impact vector transduction.

AAV2, alongside AAV3 and AAV6, binds HSPG (32–35). Unlike AAV6, which utilizes sialic acid (SIA) as its primary receptor (35–37), AAV2 and AAV3 use HSPG as their primary cellular receptor (34). The interaction between the AAV2 capsid and HSPG is mediated by positively charged amino acid residues located on the wall of the protrusion surrounding the 3-fold axis of symmetry (38). Using a heparin binding assay as well as an *in vitro* cell binding assay, we demonstrate that all hydrophilic variants retain their ability to bind heparin as well as attach to the cell surface. Both rAAV2(Y444F+E530K) and rAAV2(Y444F+L583R) have an increased affinity for heparin relative to rAAV2(Y444F) alone. Previous studies have demonstrated reduced transduction or tropism shifts associated with increased heparin affinity. This has been observed in canonical HSPG binding rAAV6-based variants and non-HSPG binding variants (i.e., rAAV5 and rAAV8), suggesting that this increased heparin affinity may contribute to the low transduction observed with rAAV2(Y444F+E530K) and rAAV2(Y444F+L583R).

(9, 12, 23, 39). Interestingly, variants containing V387R, W502H, and E530K had significantly increased cell binding. This may indicate an enhanced interaction of the capsid with the cell surface and/or ECM mediated by these hydrophilic, positively charged residues. A similar theory has been postulated with regard to AAV3B S586R/T589R variants, where increased HSPG affinity may cause increased ECM interactions and be less advantageous for cellular infection (40). Likewise, with AAV1, substitution of E531K (equivalent to E530K substitution on AAV2) allows the capsid to interact with HSPG, leading to a shift in tropism similar to AAV6, yet AAV1(E531K) still maintains the ability to productively transduce cells through its SIA cellular receptor (35, 41).

For the rAAV2-based vectors in this study, inclusion of these hydrophilic mutations ablates or severely reduces vector transduction. This observation is not consistent across serotypes, however, as our group and others have demonstrated tolerance of the E530K (or equivalent amino acid substitution) on AAV44.9 and AAV1 (14, 35). This prompts the following question: what sets the AAV2 capsid apart from other serotypes? The most glaring answer is the strong hydrophilic patch of positively charged amino acid residues responsible for HSPG binding. Recently, however, numerous studies have investigated infectious rAAV2-based variants that are deficient in HSPG binding (12, 13, 42, 43). These studies demonstrate that although AAV2 uses HSPG as its primary cellular receptor, it is capable of infecting cells by an alternate, uncharacterized pathway when this canonical receptor binding footprint is disrupted. We hypothesize that the hydrophilic mutations examined in this study negatively impact the ability of AAV2 to productively transduce cells by either competing with the canonical HSPG binding patch for receptor binding or increasing the HSPG affinity of the capsid to a deleterious degree. To test this hypothesis, we disrupted the canonical HSPG footprint on our hydrophilic mutants by mutating R585 and R588 to serine and threonine, respectively, creating a Δ HS panel of hydrophilic variants.

Unsurprisingly, the Δ HS mutations ablated or reduced heparin binding for all capsids except rAAV2(Y444F+V387R) Δ HS. Likewise, cell binding *in vitro* was severely reduced for all vectors, which was expected as rAAV2-based Δ HS vectors traditionally do not infect cells *in vitro*, presumably because of a deficiency in binding (9, 12). Interestingly, inclusion of the Δ HS mutations into rAAV2(Y444F+W502H) Δ HS and rAAV2(Y444F+E530K) Δ HS conferred transduction *in vivo* via SRI comparable to that observed with rAAV2(Y444F) Δ HS. This could indicate that, in an HSPG binding intact capsid, these hydrophilic mutations interfere with HSPG-associated internalization or release from the HSPG molecule once internalized. Alternatively, the deleterious effects of hydrophilic mutations may be completely independent from HSPG receptor binding and entry. For example, the V387R mutation yielded noninfectious vector independent of canonical HSPG binding. It is likely that this V387R mutation disrupts a key step of the AAV infection cycle downstream of viral attachment. In general, hydrophilic mutations may affect aspects of the AAV2 infection cycle downstream of binding and entry (i.e., nuclear trafficking, uncoating, expression, etc.), and these postentry interactions may be disparate for wild-type (WT) and Δ HS AAV2. In fact, it is feasible that viral entry and cellular trafficking of these Δ HS AAV2 vectors differ from those of WT AAV2 and may behave more closely to those of other non-heparan binding serotypes (44, 45).

In a juxtaposing role to hydrophilic amino acid residues, surface-exposed hydrophobic mutations (namely, Y-F and T-V) on rAAV2 have been shown in numerous studies to confer an enhancement of transduction (16, 25, 46, 47). This transduction enhancement has largely been attributed to a decrease in proteasomal degradation of the capsid due to a decreased number of surface-exposed amino acid residues, which can be targeted for phosphorylation and associated subsequent ubiquitination (46, 48–50). Interestingly, mutation of surface tyrosine and threonine residues in other serotypes, like AAV5 and AAV8, does not result in a concordant enhancement of transduction as seen with AAV2 (42). This may be due to differences in the receptors utilized by these vectors, HSPG for AAV2 and SIA for AAV5 (38, 51, 52), or possibly differences in postentry trafficking of the viruses (44). Since various combinations of surface Y-F and T-V

TABLE 1 Summary of specific substitutions to the AAV2 *cap* gene

Capsid nomenclature	Mutation(s) ^a
AAV2	None
AAV2(Y444F)	Y444F
AAV2(Y444F+V387R)	Y444F, V387R
AAV2(Y444F+W502H)	Y444F, W502H
AAV2(Y444F+E530K)	Y444F, E530K
AAV2(Y444F+L583R)	Y444F, L583R
AAV2ΔHS	R487G, R585S, R588T
AAV2(Y444F)ΔHS	Y444F, R585S, R588T
AAV2(Y444F+V387R)ΔHS	Y444F, V387R, R585S, R588T
AAV2(Y444F+W502H)ΔHS	Y444F, W502H, R585S, R588T
AAV2(Y444F+E530K)ΔHS	Y444F, E530K, R585S, R588T
AAV2(tripleY-F)ΔHS	Y444F, Y500F, Y730F, R487G, R585S, R588T
AAV2(4pMut)ΔHS	Y444F, Y500F, Y730F, T491V, R487G, R585S, R588T

^aBold residues indicate substitutions in the canonical HSPG binding footprint.

mutations have been shown to enhance AAV2 transduction (25, 53, 54), we wanted to determine if the same enhancement effect would be observed using ΔHS AAV2-based capsids. Specifically, we compared rAAV2(tripleY-F)ΔHS, which contains three surface Y-F mutations, and rAAV2(4pMut)ΔHS, which contains the same three Y-F mutations plus an additional T-V mutation at amino acid residue 491 (see Table 1 for amino acid residue information). While rAAV2(tripleY-F)ΔHS did not result in an enhancement of transduction relative to rAAV2ΔHS, rAAV2(4pMut)ΔHS did. These results suggest that, though some combinations of hydrophobic residues may not enhance transduction in ΔHS AAV2 vectors, other combinations or specific amino acid residues may. Further studies are required to determine if this enhancement is primarily due to the T491V mutation alone or a combinatorial effect of T491V with specific Y-F mutations.

Because of the enhanced retinal transduction observed in mice using rAAV2(4pMut)ΔHS, both in this and a previous study (12), we wanted to evaluate the qualities of this capsid in a clinically relevant animal model, i.e., a model with similar retinal anatomy to humans, the cynomolgus macaque. Initial SRI of both rAAV2(4pMut)ΔHS CBA-GFP and hGRK1-GFP vectors yielded robust central (~90%) and peripheral (~92%) cone transduction, as well as substantial central (~57%) and peripheral (~57% to 95%) rod transduction. These observations indicate a preferential tropism toward cones over rods, though rod transduction is still considerable. Notable expression of GFP in RPE was observed for both CBA- and hGRK1-driven vectors, indicating that transduction levels in RPE are high enough to elicit leaky expression from the hGRK1 promoter. Leaky expression from the hGRK1 promoter has been previously reported in mouse RGCs following IVI (27). This off-target expression is likely the result of excessive transduction and high copy number of vgs present in the off-target cells. GFP expression was detected well outside the margins of the initial injection blebs, indicating that this vector has the ability to laterally spread through the retina, similar to AAV9 (55) and the recently discovered AAV44.9 (14).

The ability of vectors to laterally spread is crucial in patients with degenerative retinas since the fovea is often a critical target for therapy, and submacular injections in these patients may be iatrogenic. Because of its observed lateral spread, we tested the ability of rAAV2(4pMut)ΔHS to transduce foveal PRs when it is placed subretinally into the peripheral retina. Importantly, rAAV2(4pMut)ΔHS vectors were able to transduce ~62% of foveal cones and 22% of central rods in the absence of a submacular detachment (see optical coherence tomography [OCT] in Fig. 7B), with >94% transduction of rods and cones located within the margins of the injection bleb. Cone and rod transduction were also detected in the peripheral retina far outside of the injection blebs, approximately 1,600 μm nasal to the optic nerve head, albeit at lower levels (24% to 34% range for both cones and rods). Once again, transgene expression in RPE was observed for both CBA and hGRK1 vectors, the latter indicating high RPE tropism by

this capsid. This lateral spreading attribute of rAAV2(4pMut) Δ HS makes it an ideal candidate for diseases where macular transduction is required for therapy, but macular detachment is contraindicated. This vector may also be preferred for treatment of diseases that require large areas of retinal transduction, such as retinitis pigmentosa and choroideremia. Additionally, rAAV2(4pMut) Δ HS when administered by intraparenchymal injection was found to promote increased volumetric spread of brain transduction (42). The conventional wisdom has been that HSPG-deficient binding rAAV2 vectors lead to little or no transduction when delivered systemically. Consistent with this, we observed no transduction when systemically administering rAAV2(4pMut) Δ HS to mice (12). However, recent work by Cabanes-Creus et al. has found that similar AAV2 variants deficient in HSPG binding are capable of strong transduction of liver in a human liver xenograft mouse model (19). It remains to be seen if rAAV2(4pMut) Δ HS also has this ability and whether modulation of the hydrophobicity via Y-F and T-V impacts such liver transduction.

What are the biophysical properties of AAV2(4pMut) Δ HS that confer the ability to laterally spread? We hypothesize that this phenotype is primarily due to the disruption of the canonical HSPG binding site (Δ HS), which ultimately reduces interactions between the vector and the ECM, allowing the vector to spread more effectively. This is supported by studies that have documented decreases in retinal transduction of rAAV44.9 and rAAVhr.8R vectors once HSPG binding was grafted onto the vectors by targeted mutagenesis (14, 56). Moreover, AAV serotypes that interact with abundant glycans like HSPG or SIA, namely, AAV2 and AAV5, do not display the same lateral spreading observed in non-SIA or HSPG binding vectors (14, 15). However, empirical studies are required to determine the exact interactions between AAV vectors and the ECM.

Our study assessed a number of AAV2-derived variants for their ability to transduce the retina following both IVI and SRI. Inclusion of hydrophilic variants in an attempt to increase ECM interactions, particularly within the ILM, and promote transduction via IVI was counterproductive and ablated transduction. Transduction via SRI was rescued for vectors containing W502H and E530K mutations when canonical HSPG binding was disrupted, suggesting that the canonical HSPG receptor binding activity of rAAV2 is not compatible with other surface hydrophilic mutations. Conversely, the inclusion of hydrophobic Y-F and T-V mutation on an AAV2 Δ HS backbone yielded vectors with enhanced SRI transduction in the mouse retina. Our best performing vector, rAAV2(4pMut) Δ HS, efficiently transduced PRs in the nonhuman primate (NHP) retina and is capable of laterally spreading into the macula when injected in peripheral SRI blebs. These results strongly suggest that rAAV2(4pMut) Δ HS will be useful in treating diseases of the central retina or diseases that require a large area of transduction.

MATERIALS AND METHODS

AAV structural representations. AAV2 capsid structural images were constructed in PyMol using the AAV2 capsid model published by Xie et al. (Protein Data Bank accession number [1LP3](#)) (57).

rAAV vector production and purification. AAV2-based capsid mutants were generated by site-directed mutagenesis of the AAV2 *rep-cap* plasmid pACG2 (58) using the QuikChange Multi site-directed mutagenesis kit (Agilent). In some cases, synthetic DNA fragments were used to modify pACG2 using standard molecular cloning techniques. When possible, silent mutations were introduced to add or remove restriction endonuclease sites, aiding in the screening of clones. Mutated *cap* genes were fully sequenced prior to vector production. All substitutions to the AAV2 *cap* gene are detailed in Table 1. For all *in vitro* and *in vivo* mouse studies, self-complementary rAAV vectors contained the mCherry reporter cDNA under expression of the small chimeric cytomegalovirus/chicken β -actin promoter (smCBA) (59). rAAV constructs used in primate studies contained a GFP reporter under the expression of either the CBA promoter or the PR-specific hGRK1 promoter (60).

rAAV vector production has been previously described in detail (61). In short, rAAVs were generated using a polyethyleneimine-mediated triple transfection of adherent HEK293 cells (passages 25 to 50). Viral capsids were recovered from cells using freeze-thaw lysis, Benzonase (Thermo Fisher) treated, and purified via iodixanol gradient centrifugation. After iodixanol purification, viral vectors used in DSF or negative stain electron microscopy were subjected to additional purification by affinity capture chromatography using AVB resin (GE Healthcare). All vectors were then buffer exchanged and concentrated into balanced salt solution buffer (Alcon) supplemented with 0.001% Pluronic (BSS-P). The titers of the vectors were determined by qPCR using an in-house reference vector (primers TP-CB2-F and TP-CB2-R

TABLE 2 Primers used for qPCR experiments and determining the titers of vectors

Oligonucleotide (name)	Target	Sequence
TP-CB2-F	CMV enhancer (transgene)	TCCCATAGTAACGCCAATAGG
TP-CB2-R	CMV enhancer (transgene)	CTTGGCATATGATACACTTGATG
18S-F	Human <i>RNA18S</i> gene	GTAACCCGTTGAACCCATT
18S-R	Human <i>RNA18S</i> gene	CCATCCAATCGGTAGTAGCG

listed in Table 2). Vectors used for NHP studies (i.e., rAAV2(4pMut) Δ HS) were purified as above with the addition of ion-exchange chromatography using a HiTrap Q column (GE Healthcare) as previously described (62). Undiluted NHP vectors were then tested for the presence of endotoxin, all of which registered less than 5 endotoxin units (EU)/ml.

EM and protein gels. To determine the morphology of the purified rAAV vectors, 5 μ l of each sample was loaded onto glow-discharged carbon-coated copper EM grids (Ted Pella Inc.; catalog number 01754-f) for 2 min and negatively stained with 5 μ l of 2% uranyl acetate for 20 s. The grids were visualized on a Tecnai G2 Spirit TWIN equipped with a charge-coupled device (CCD) camera and at a magnification of 40,000. To determine the purity of the rAAV vectors, 10 μ l of each purified vector was denatured at 100°C for 10 min and used to run SDS-PAGE. The SDS gel was stained with Coomassie blue to visualize the VP bands.

Heparin binding assay. Heparin binding assays were conducted as previously described (12). Briefly, approximately 300 ng of each rAAV vector was dissolved in Low PBS-MK buffer (2/3 $\times$ phosphate-buffered saline [PBS], 1 mM MgCl₂, 2.5 mM KCl) and loaded onto 200 μ l heparin-agarose type I resin (Sigma) in Micro Bio-Spin columns. The flowthrough was collected by gravity flow. Columns were washed with 5 column volumes of Low PBS-MK, and viruses were eluted with increasing 50 mM increments of NaCl-supplemented Low PBS-MK buffer. Samples were denatured by boiling at 100°C for 10 min and then dotted onto a polyvinylidene fluoride (PVDF) membrane for dot blot analysis. Membranes were immunoblotted with the mouse monoclonal B1 primary antibody (catalog number 03-65158; 1:3,000; American Research Products, Inc.), which recognizes a linear epitope of the AAV viral proteins and a fluorescently linked goat anti-mouse secondary (1:10,000; catalog number 926-68070; LI-COR). Fluorescence was detected using an Odyssey CLx imager (LI-COR).

Differential scanning fluorometry. DSF was used to determine the stability of the rAAV vectors. This method monitors binding of the dye SYPRO orange to exposed hydrophobic regions of the protein during denaturation and unfolding. For the analysis, 2.5 μ l of 1% SYPRO orange (Invitrogen) was added to 22.5 μ l of rAAV vectors at $\sim$ 0.1 mg/ml to make a total reaction volume of 25 μ l. The assay was run using a Bio-Rad MyiQ2 thermocycler instrument, and the experimental temperature ranged from 30°C to 99°C with temperature ramping of 0.5°C per step. The rate of change of fluorescence (dRFU) with temperature (dT) was recorded as an inverse thermal profile, namely, $-dRFU/dT$ versus temperature. The $-dRFU/dT$ values were multiplied by -1 and normalized to 1 by dividing the raw values with the peak value for evaluation. The peak value recorded on the thermogram is the T_m . All experiments were conducted in triplicate.

In vitro transduction assay. ARPE-19 cells (ATCC), seeded at a density of 1e4 cells/well in a 96-well plate, were infected with rAAV at an MOI of 10,000. Three days postinfection, cells were collected and subjected to flow cytometry in order to quantify reporter (mCherry) fluorescence. mCherry expression was measured by multiplying the mean mCherry fluorescence by the number of positive cells as previously described (12, 63). Transduction assays were conducted in triplicate.

Animal ethics statement. All mice were bred and housed at the University of Florida's Health Science Center animal facility with *ad libitum* access to food and water under a 12 h light/dark cycle. Experiments were approved by the University of Florida's Institutional Animal Care and Use Committee (IACUC) in accordance with the Association for Research in Vision and Ophthalmology (ARVO). All procedures performed on macaques were approved by the Charles River Laboratories' IACUC and performed in accordance with the Association for Research in Vision and Ophthalmology's Statement for the Use of Animals in Ophthalmic and Vision Research.

Mouse injections. One microliter of vector at a concentration of either 2×10^{12} vg/ml or 2×10^{11} vg/ml was delivered either via IVI or SRI to 6- to 8-week-old *Nrl*-GFP mice (64). A small amount of fluorescein was added to the vector to help visualize the injection bleb. Injections were performed under a Leica M80 stereomicroscope as previously reported (27, 65). Immediately after injection, blebs were observed for injection accuracy. Animals that received comparable successful injections ($\geq$ 60% retinal detachment and minimal complications) were used for downstream analysis.

Quantification of rAAV transduction in mouse retina. Four weeks after vector administration in *Nrl*-GFP mice, either by SRI or IVI, mCherry fluorescence was documented in life using a Micron III fundoscope (Phoenix Research Laboratories) with a red fluorescence filter. Image exposure settings remained consistent for all IVI or SRI mice. In cases where negligible mCherry fluorescence was observed, image brightness was enhanced for emphasis (Fig. 2 and 4). The next day, neural retinas were collected from mice ($n=3$ to 8) and dissociated with papain (catalog number 3150; Worthington Biochemical) as previously described (12). The dissociated neural retinas from injected mice and untreated controls were then subjected to flow cytometry (BD LSR II equipped with FACSDiva; BD Biosciences) to quantify the

percentage of cells that were positive for GFP (i.e., rod PRs), mCherry (nonrod retinal neurons or Müller glia transduced by rAAV), or both (rod PRs transduced by rAAV).

rAAV binding assay. The binding assay protocol was modified based on a previously described method (66). Briefly, passage 30 to 40 HEK293 cells (ATCC) were seeded in 24-well plates at approximately 25% confluence. Two days later, when cells had reached confluence, the medium was replaced with fresh, ice-cold medium (high-glucose Dulbecco modified Eagle medium [DMEM]; Fisher) supplemented with 5% fetal bovine serum (FBS) (VWR), and cells were incubated at 4°C for 5 min. After the cells had cooled, rAAV variants were added to the cells at an MOI of 10,000, and cells were incubated at 4°C for an additional 30 min to allow for virus binding but not internalization. Cells were thoroughly washed 3 × with ice-cold PBS to remove unbound virus, lysed by proteinase K digestion for 1 h, and then processed using the DNeasy blood and tissue kit (Qiagen). Vector genomes for each sample were quantified by qPCR using primers targeting the cytomegalovirus (CMV) element in the rAAV vector cassette (Table 2). The housekeeping rRNA 18S gene was used as an internal calibrator for each sample (Table 2) (67). Both primer sets had an efficiency greater than 95%. Fold change in rAAV genome levels relative to baseline vector rAAV2(Y444F) were calculated using the $\Delta\Delta CT$ method (68).

Tissue preparation and immunohistochemistry. A detailed account of the IHC procedures performed on mouse and macaque retinal sections has been previously described (12, 14). Briefly, processed mouse retinal sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) prior to imaging native GFP and mCherry fluorescence via confocal microscopy (Leica TCS SP8). Macaque tissues were stained overnight at 4°C with a rabbit polyclonal cone arrestin antibody (1:30,000; generously provided by Cheryl Craft) and detected by secondary fluorescence using a goat anti-rabbit fluorescent secondary (1:500, Alexa Fluor 555; Invitrogen). IHC image of rAAV2 Δ HIS expression in mouse retina (Fig. 5C) was digitally edited to remove a large gap where the RPE cell layer had separated from the rest of the retina.

Subretinal injections in macaque. rAAV2(4pMut) Δ HIS vectors containing either the ubiquitous CBA- or PR-specific hGRK1 promoter driving GFP were SRI into three cynomolgus macaques at a concentration of 1×10^{12} vg/ml. The first animal received two 60- μ l injections, one intentionally placed submacular and one placed peripherally. The other two animals received three 30- μ l extrafoveal injections placed at approximately 25 degrees eccentricity from the fovea. Temporal injections were made along the horizontal meridian either immediately superior or inferior to the temporal nerve fiber raphe. Superior and inferior blebs were made along the vertical meridian immediately adjacent to the venule within the vascular arcades when possible. In-life optical coherence tomography (OCT) and funduscopy imaging were performed on injected macaques before injection, immediately postinjection (p.i.), and at 1, 2, 4, and 6 weeks p.i. Confocal scanning laser ophthalmoscopy (cSLO) was used in conjunction with OCT and funduscopy to observe in-life vector-mediated GFP expression.

Quantification of GFP-positive cones and rods in SRI macaque retinas. A detailed description of how transduction of rods and cones in macaque was quantified has been previously described (14). Briefly, three blinded observers counted the total number of GFP-positive and GFP-negative rods and cones in macaque retinal sections. Sections were stained with DAPI to identify individual PRs in the outer nuclear layer and cone arrestin to specifically identify cone PRs.

Statistical analysis. For both qPCR- and flow cytometry-based quantification methods, a 1-way analysis of variance (ANOVA) was used to assess the statistical differences between the rAAV variants being tested. A *P* value of <0.05 was considered a significant interaction for the 1-way ANOVA. If the ANOVA was in fact significant, a *post hoc* Holm-Sidak test with an *a priori* α of 0.05 was applied to make pairwise comparisons between samples or a benchmark vector. In the case where only two samples were compared, a 2-tailed Student's *t* test with an *a priori* α of 0.05 was applied.

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S.E.B. and S.L.B. are scientific founders of and equity holders in Atsena Therapeutics, Inc., and are patent holders on the use of AAV vectors for the treatment of ocular disease. M.A.-M. is an SAB member for AGTC, has a sponsored research agreement with Adverum Therapeutics, Inc., Voyager Therapeutics, Inc., and Intima Biosciences, Inc., and is a consultant for Voyager Therapeutics and Intima Biosciences. M.A.-M. is a cofounder of StrideBio, Inc. This is a biopharmaceutical company with interest in developing AAV vectors for gene delivery application. M.A.-M. is an inventor in several AAV patents. S.M.C. is an inventor of an AAV-based gene therapy patent.

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