Vitreal delivery of AAV vectored Cnga3 restores cone function in CNGA3−/−/Nrl−/− mice, an all-cone model of CNGA3 achromatopsia†

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Abstract

The CNGA3−/−/Nrl−/− mouse is a cone-dominant model with Cnga3 channel deficiency, which partially mimics the all cone foveal structure of human achromatopsia 2 with CNGA3 mutations. Although subretinal (SR) AAV vector administration can transfect retinal cells efficiently, the injection-induced retinal detachment can cause retinal damage, particularly when SR vector bleb includes the fovea. We therefore explored whether cone function-structure could be rescued in CNGA3−/−/Nrl−/− mice by intravitreal (IVit) delivery of tyrosine to phenylalanine (Y-F) capsid mutant AAV8. We find that AAV-mediated CNGA3 expression can restore cone function and rescue structure following IVit delivery of AAV8 (Y447, 733F) vector. Rescue was assessed by restoration of the cone-mediated electroretinogram (ERG), optomotor responses, and cone opsin immunohistochemistry. Demonstration of gene therapy in a cone-dominant mouse model by IVit delivery provides a potential alternative vector delivery mode for safely transducing foveal cones in achromatopsia patients and in other human retinal diseases affecting foveal function.

† The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

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Introduction

The human retina has ~6 million cone photoreceptors and 100 million rod photoreceptors. Cones are primarily responsible for central high resolution and color vision while operating in moderate-to-bright light. They are primarily concentrated in the macula with the central foveola being nearly 100% cones. Achromatopsia, or rod monochromatism, is a recessive genetic condition characterized by cone dysfunction, with an incidence of ~1/30,000 in western populations (1). Achromats exhibit total color vision loss, poor central vision, visual acuity of 20/200 or worse (2) and daylight blindness (photosensitivity), making them legally blind. Clinically, the first signs of achromatopsia in infants are nystagmus and photosensitivity as evidenced by squinting in bright light (2). Both rods and cones rely on cyclic nucleotide-gated (CNG) channels for photoreceptor plasma membrane hyperpolarization and signal transduction. Cone CNG channels consist of two CNGB3 subunits and two CNGA3 subunits. In European populations, ~25% of patients with achromatopsia have CNGB3 mutations, ~50% have CNGB3 mutations, and smaller fractions have mutations in the cone transducin or phosphodiesterase genes (3). Moreover, in the Middle East (4) and China (5), the fraction of achromatopsia due to mutations in the Nrl gene. Interestingly, CNGB3−/− mice lack cone-mediated ERG response, and rod-mediated scotopic light response due to its Nrl deficiency. Expression levels of M-opsin, S-opsin, Gnat2, and arrestin4 were all reduced in CNGB3−/− Nrl−/− mice. Furthermore, cone photoreceptors start to degenerate as early as postnatal Day 15 (P15), similar to single CNGB3−/− Nrl−/− mice following either SR or IVit injection (6). Therefore, this model permits testing of the retina-penetrating properties of AA8 (Y447, 733F) from the vitreous for functional and structural cone rescue with reference.

Results

AA8 (Y447, 733F) vector restores cone-mediated ERGs in CNGB3−/− Nrl−/− mice following either SR or IVit injection

Photopic ERGs at stimulus intensity of 1.4 log cd·s/m² were recorded at 2 months and were maintained for at least 6 months following either IVit or SR injection of AA8 (Y447, 733F)-IRBP/GNAT2-mCNGB3 to CNGB3−/− Nrl−/− eyes (sample ERG traces are shown in Fig. 1A). The photopic b-wave ERG amplitudes for all animals tested are shown in Figure 1B. Following IVit injection, amplitudes ranged from 5.1 to 206.5 μV at 2 months and 42.5–103.5 μV at 6 months after treatment. These values are somewhat lower than recorded for SR vector which ranged from 100 to 290.1 μV at 2 months and 110–150 μV at 6 months.

To confirm experimentally that IVit-AA88 (Y447, 733F) vector was accurately delivered to the vitreous and was not delivered accidentally to the SR space, we mixed cone-specific therapeutic AA8 (Y447, 733F)-IRBP/GNAT2-mCNGB3 vector with an AA2 (Y444, 500, 730F)-smCBA-GFP vector that transduces distinct populations of retinal cells depending on whether it is delivered to the vitreous or SR space (20). One mouse had one eye subretinally injected and the other eye intravitreally injected with this vector mixture. Photopic ERG rescue was seen in both eyes at 2 months post-injection. These ERG b-wave responses are circled in

AA8 (Y447, 733F) capsid can penetrate the inner retina and rescue cone function in CNGB3−/− Nrl−/− mice after IVit injection. The CNGB3−/− Nrl−/− mouse serves as a unique mammalian cone-dominant model with a CNGB3 channel deficiency. This double knock-out mouse model possesses genetically and phenotypically well-characterized cone degeneration (25) and partially mimics the foveal structure-function of human achromatopsia 2 with CNGB3 mutations, i.e., impaired cone function and cone degeneration, hence serving as a useful model to explore cone gene therapy for human achromatopsia. CNGB3−/− Nrl−/− mice lack cone-mediated ERG response, and rod-mediated scotopic light response due to its Nrl deficiency. Expression levels of M-opsin, S-opsin, Gnat2, and arrestin4 were all reduced in CNGB3−/− Nrl−/− mice. Furthermore, cone photoreceptors start to degenerate as early as postnatal Day 15 (P15), similar to single CNGB3−/− Nrl−/− mice (26). Therefore, this model permits testing of the retina-penetrating properties of AA8 (Y447, 733F) from the vitreous for functional and structural cone rescue with reference.
Figure 1. Photopic electroretinograms (ERGs) and statistical analysis. (A) Representative photopic ERG tracings elicited at 1.4 log cd-s/m² from CNGA3−/−/Nrl−/− and control eyes. (B) Distribution of photopic b-wave amplitudes from each treated and untreated eyes elicited at 1.4 log cd-s/m² (mouse eyes which had one eye subretinally injected and the other eye intravitreally injected with a mixture of AAV8 (Y447, 733F)-IRBP/GNAT2-mCNGA3 and AAV2 (Y444, 500, 730F)-smCBA-GFP vectors are marked with circles). (C) Statistical analysis of photopic b-wave amplitudes elicited at 1.4 log cd-s/m². (D) Statistical analysis of photopic b-wave implicit times at 1.4 log cd-s/m². All of photopic ERG scale bars are the same (y-axis: 50 μV/Div, x-axis: 50 ms/Div) except for NRL−/− mice at 2 months of age (y-axis: 100 μV/Div, x-axis: 50 ms/Div). NS, no statistical difference; *P < 0.05; **P < 0.01; ***P < 0.001. A1 = 2.5-month-old CNGA3−/−/Nrl−/− untreated eyes, A2 = 2.5-month-old CNGA3−/−/Nrl−/− eyes 2 months after AAV5 IVit injection at P14, A3 = CNGA3−/−/Nrl−/− eyes 2 months after AAV8 (Y447, 733F) IVit injection at P14, A4 = CNGA3−/−/Nrl−/− eyes 2 months after AAV8 (Y447, 733F) SR injection at P14, A5 = 2.5-month-old NRL−/− eyes, A6 = 2.5-month-old C57 BL/6J eyes, B1–B6 groups are similar as those of A1–A6, but the time is 6/6.5 months instead of 2/2.5 months.
Age-matched μ amplitudes (Fig. 1C) were 63.76 ± 24.05 IVit-AAV8 (Y447, 733F) treatment. The average photopic b-wave mediated ERGs were also performed at 6 months following those from age-matched untreated eyes (105.6 ± 20.06 ms, P = 0.011). The motor performance improved only after IVit-AAV8 (Y447, 733F), whereas the contralateral uninjected eyes lacked motor performance improved only after IVit-AAV8 (Y447, 733F), whereas the contralateral uninjected eyes lacked

AAV5 vector did not restore cone-mediated ERG in CNGA3−/− Nrl−/− mice 2 months following IVit injection with photopic ERG responses almost undetectable (8.33 ± 2.89 μV, n = 3). Photopic b-wave ERG amplitudes for IVit-AAV8 (Y447, 733F) were significantly larger (98.96 ± 58.43 μV, n = 14, P = 0.02) than that of untreated eyes (16.05 ± 8.861, n = 4, P = 0.01), being ~28% of that of the age-matched Nrl−/− controls (356.9 ± 55.15 μV, n = 5) and 55% of that of SR AAV8 (Y447, 733F)-treated eyes (179.8 ± 58.19 μV, n = 10). Photopic b-wave amplitudes for untreated eyes (118.8 ± 14.29 ms, n = 4) were somewhat delayed compared with age-matched Nrl−/− mice (63.21 ± 4.78 ms, n = 7). IVit AAV5-treated CNGA3−/−/Nrl−/− eyes showed an average visual acuity of 8.205 ± 0.442 (Fig. 2B), significantly better than those of untreated CNGA3−/−/Nrl−/− eyes (visual acuity: 8.589 ± 0.250; P = 0.0141 and 0.0016, respectively). In contrast, IVit AAV8 (Y447, 733F) treated eyes showed an average visual acuity of 7.176 ± 1.470 (Fig. 2B), significantly better than those of untreated CNGA3−/−/Nrl−/− eyes (visual acuity: 0.437 ± 0.045 cyc/deg; contrast sensitivity: 8.589 ± 0.250; P = 0.0141 and 0.0016, respectively). In all cases, SR treatment with either vector yielded significantly improved visual acuity and contrast sensitivity, as expected (Fig. 2).

AAV8 (Y447, 733F), but not AAV5-mediated CNGA3 expression restores optomotor responses in CNGA3−/−/Nrl−/− mice following vitreal vector treatment

We then test whether restoration of cone ERG function translated into improvement in cone-mediated visual behavior by measuring optomotor responses to rotating sine-wave gratings (27). Under photopic conditions, untreated CNGA3−/−/Nrl−/− eyes performed poorly (Fig. 2A). Consistent with ERG responses, optomotor performance improved only after IVit-AAV8 (Y447, 733F), not after IVit-AAV5 at 2 months post-treatment (Fig. 2). For IVit AAV5-treated eyes, visual acuities (0.077 ± 0.016 cyc/deg, n = 4, Fig. 2A) and contrast sensitivities (1.066 ± 0.032, Fig. 2B) were nearly identical to those of the untreated CNGA3−/−/Nrl−/− eyes (visual acuity: 0.057 ± 0.017 cyc/deg; contrast sensitivity: 1.037 ± 0.021, n = 4, P = 0.1198 and 0.1404, respectively). In contrast, IVit-AAV8 (Y447, 733F) treated eyes showed an average visual acuity of 0.447 ± 0.032 cyc/deg (n = 4, Fig. 2A) and a contrast sensitivity of 8.205 ± 0.442 (Fig. 2B), significantly better than those from untreated CNGA3−/−/Nrl−/− eyes (P < 0.0001), but not as good as those from age-matched Nrl−/− eyes (visual acuity: 0.510 ± 0.018 cyc/deg; contrast sensitivity: 9.473 ± 0.599; P = 0.0016 and 0.0019, respectively). At 6 months following injection, IVit-AAV8 (Y447, 733F)-treated eyes showed an average visual acuity of 0.346 ± 0.041 cyc/deg (n = 3, Fig. 2A) and contrast sensitivity of 7.176 ± 1.470 (Fig. 2B), significantly better than those of untreated CNGA3−/−/Nrl−/− eyes (P < 0.0001), but again less than those from age-matched Nrl−/− eyes (visual acuity: 0.437 ± 0.045 cyc/deg; contrast sensitivity: 8.589 ± 0.250; P = 0.0141 and 0.0016, respectively). In all cases, SR treatment with either vector yielded significantly improved visual acuity and contrast sensitivity, as expected (Fig. 2).

AAV8 (Y447, 733F), but not AAV5-mediated CNGA3 expression was detected in cones following IVit injection

Six months after SR or IVit vector treatment, CNGA3 expression was assayed by immunohistochemistry (Fig. 3). Age-matched wild-type C57BL/6j mice showed robust outer segment (OS) CNGA3 expression (Fig. 3A). CNGA3 expression was also detected in CNGA3−/−/Nrl−/− eyes following SR-AAV5 or IVit-AAV8 (Y447, 733F) treatment, whereas the contralateral uninjected eyes lacked...
detectible CNGA3 labeling. Co-localization of mouse CNGA3 and cone-specific lectin peanut agglutinin (PNA) (Fig. 3A) confirmed that CNGA3 expression was localized to cone OSs.

Because CNGA3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice exhibit a progressive loss of cone photoreceptors, most opsins are absent at 7 months of age (25). As for Nrl<sup>−/−</sup> mice, cone OSs of CNGA3<sup>−/−</sup>/Nrl<sup>−/−</sup> mice are
never well developed and very short around this age (Fig. 3). Cone opsin in CNGA3−/−/Nrl−/− mice were maintained 6 months after P14 SR injection of either AAV8 (Y447, 733) or AAV5 vector with therapeutic gene (Fig. 3B). The positive M- and S-opsin staining reported here confirms that cone degeneration was prevented by IVit-AAV8 (Y447, 733) treatment (Fig. 3B). Furthermore, the accuracy of our vitreal injections is additionally confirmed because in no case did IVit AAV5 vector lead to functional or structural rescue, whereas SR AAV5 yielded measurable rescue by all functional and structural parameters (see above).

**Discussion**

CNG channels play a central role in phototransduction. Mutations in the cone CNG channel subunits CNGA3 and CNGB3 account for >70% of all known cases of achromatopsia (28,29). CNGA3−/− mice undergo a progressive loss of cone photoreceptors, with cones in the ventral retina nearly absent by 3 months of age (26). A subsequent study showed that CNGA3−/−/Nrl−/− mice had a retinal phenotype similar to the sum of their respective single knock-out counterparts, i.e. impaired cone function and cone degeneration (25). Cone OS in Nrl−/− retinas are shorter than wild type, with occasional abnormalities of the outer nuclear layer at an early age, referred to as rosettes (30,31). Nrl−/− cones undergo a slow degeneration so that by 31 weeks of age many cone opsins-positive cells have degenerated and nearly all rosettes have disappeared (32). Although only 28% of the age-matched Nrl−/− photopic ERG amplitudes were restored in CNGA3−/−/Nrl−/− eyes at 2 months following IVit-AAV8 (Y447, 733F) treatment, this fraction increased to 46% by 6 months. One reason for this is that cone ERGs at 6 months are at <2 months in Nrl−/− mice due to the loss of cone rosettes and the slow cone loss as noted above. Since IVit vector must transit the inner retina before reaching cones, vector titer is reduced before reaching their intended cone target, and this likely accounts for the less-than-complete rescue of cone function by IVit vector.

To distinguish between whether IVit-AAV8 (Y447, 733F) vectors were reaching cones through inner retinal layers as intended or through some vector being delivered inadvertently to the SR space, AAV8 (Y447, 733)-IRBP/GNAT2-mCNGA3 vector and a nontherapeutic AAV2 (Y444, 500, 730F)-smCBA-GFP tracing vector, which have distinct transfection patterns whether delivered intravitreally or subretinally, were injected together into CNGA3−/−/Nrl−/− eyes. Intravitreally delivered vector mixture clearly had a GFP expression pattern diagnostic for vitreal vector while the other eye, with the mixture subretinally injected, had a GFP expression pattern expected of SR vector. Critically, following injection at either site resulted in cone ERG rescue and, as expected, both now exhibited positive CNGA3 expression in cones (data not shown). This confirms that vitreal AAV8 (Y447, 733) vector can indeed penetrate inner retinal layers before reaching cones and mediate a therapeutic response in CNGA3−/− cones.

The restoration of cone-mediated retinal function observed in IVit-AAV8 (Y447, 733)-IRBP/GNAT2-mCNGA3 treated eyes was also supported by behavioral responses collected by optokinetic testing. AAV5 vector, quite therapeutic subretinally, restores neither visual acuity nor contrast sensitivity following IVit treatment in CNGA3−/−/Nrl−/− mice (Fig. 2), further confirming the accuracy of our IVit vector delivery.

Validation here of the retina-penetrating property of AAV8 (Y447, 733F) by the IVit injection route in CNGA3−/−/Nrl−/− mice has implications for current human gene therapy paradigms, where damage caused by SR delivery is a concern (9-11). However, there are potentially critical differences between mouse and human retinal anatomies that may be relevant. First, the ILM that separates the vitreous from the retina is much thicker in primates than in rodents (33); hence, the barrier for a penetrating vector may be higher in primates. Second, rodents have no macula or fovea and have fully intact inner retinal layers spanning the central retina, whereas in the primate fovea the inner retinal cells have been displaced concentrically outward during development (34), leaving few inner retinal cells for a vitreal vector to transit before contacting foveal cones. Moreover, the thick primate ILM outside the central fovea thins considerably over the central fovea (33). This likely accounts for reports of foveal cone transduction by even unmodified vitreal AAV vectors but no transduction of more peripheral photoreceptors (35). In short, these data suggest that if central foveal cones are the retinal disease target, AAV vectors may be quite useful. As such, the vector technology reported here could be potentially very useful in treating foveal cones in a variety of cone maladies, including multiple genetic forms of achromatopsia, in which both foveal and extrafoveal cones would be therapeutic targets, and other cone dystrophies such as blue cone monochromatism, red-green color blindness, early macular degeneration or late stage retinitis pigmentosa. In addition, mutagenesis of surface exposed threonine (T) residues to valine (V) or alanine (A) may also increase transduction efficiency by decreasing phosphorylation of capsid and subsequent ubiquitination as part of the proteasomal degradation pathway (18,36,37). Retina-penetrating AAV variants, like the AAV8 (Y447,733F) vector studied here or the other similar novel AAV vectors (38), may also have application for gene delivery to retinal pigmented epithelial (RPE) cells located behind the fovea and for more peripheral rods and cones as well. This would greatly expand the therapeutic usefulness of retinal gene therapy. Full validation of whether this new class of AAV vectors has application to human retinal disease will now require its careful evaluation in nonhuman primates.

In summary, using an intravitreally delivered AAV8 (Y447,733)-IRBP/GNAT2-mCNGA3 vector, we have shown for the first time that gene therapy effectively restores cone function in a mouse without the potentially retina damaging effects of SR vector delivery. Our results suggest that intravitreally delivered AAV-mediated gene replacement therapy has the potential to become a safer therapeutic option than currently possible for human achromatopsia and other cone maladies.

**Materials and Methods**

**Animals**

C57 BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA); the CNGA3−/− (39), Nrl−/− (31) (kindly provided by Dr Anand Swaroop at NEI/NIH), and CNGA3−/−/Nrl−/− (25) mice were generated as described previously. Eighty mice with both sexes from 2 weeks to 6.5 months were used in this study with non-blind designation. All mice were maintained in the University of Florida Health Science Center Animal Care Service Facilities on a 12 h/12 h light–dark cycle with <15 cd·s/m² environmental illumination. All animals were maintained under standard laboratory conditions (18–23°C, 40–65% humidity) with food and water available ad libitum. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Florida and conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and National Institutes of Health regulations.
Construction of AAV vectors

Mouse Cnga3 cDNA (8) was cloned under the cone-specific IRBP/GNAT2 promoter (40) to make AAV-IRBP/GNAT2-mCNGA3 construct. The construct was packaged into serotypes 5 and 8 (Y447, 733F), which was originally made in Boye’s lab (38). AAV2 (Y444, 500, 730F)-smCBA-GFP vector was also packaged as previously described (20). Vectors were purified and titered according to previously published methods (41).

Subretinal and intravitreal injections

Prior to injection, each mouse was given 1% atropine eye drops to dilate the pupil starting 1 day before and the injecting day starting 5 h before anesthesia by ketamine (75 mg/kg)/xylazine (4 mg/kg) intramuscular injection. Administration of 2.5% phenylephrine hydrochloride eye drops for quick mydriasis was used every hour starting from 4 h before the anesthesia. At P14, 1 μl of AAV5-IRBP/GNAT2-mCNGA3 or AAV8 (Y447, 733F)-IRBP/GNAT2-mCNGA3 vector (both at a titer of 1 × 10^{13} vector genomes/ml) was injected subretinally or intravitreally into one eye of each CNGA3−/−/Nrl−/− mouse. The other eye remained uninjected as a control. SR injections were performed as previously described (24,42), and only eyes with no apparent surgical complications were used for further evaluation. Trans-corneal IVit injections were performed (43). This approach avoids the possibility of vitreal vector reaching the SR space through the injection hole as may happen following trans-sclera IVit injection.

For injection of mixtures of two vectors, one microliter of an AAV8 (Y447, 733F)-IRBP/GNAT2-mCNGA3 and AAV2 (Y444,500, 730F)-smCBA-GFP mixture (both at a titer of 2 × 10^{13} vector genome/ml) was intravitreally or subretinally injected into both the right and left eyes of five CNGA3−/−/Nrl−/− mice at P14. Following the injection, one drop of 1% atropine (H-Tech Pharmacal Co. Inc., Amityville, NY, USA) and a small amount of Ophthalmic Ointment with Neomycin, Polymyxin B Sulfates & Dexamethasone (E. Fougera and Co., Melville, NY, USA) were applied to the eye to reduce injection-related inflammation and prevent possible infection (24,42).

Electroretinography

Electroretinogram (ERG) was performed every 2 months following SR or IVit injections. A UTAS Visual Diagnostic System with a Big Shot Ganzfeld (LKC Technologies, Gaithersburg, MD, USA) was employed using methods previously described with minor modifications (7,24,44). Age-matched C57 BL/6j and Nrl−/− mice were used as controls. Briefly, all mice were dark-adapted overnight and 1% atropine eye drops were given 1 h before anesthesia by ketamine (75 mg/kg)/xylazine (4 mg/kg) intramuscular injection, followed by topical administration of 2.5% phenylephrine hydrochloride eye drops. Dark-adapted ERGs were assessed at a stimulus intensity of 0.4 log cd·s/m² and inter-stimulus intervals of 30 s, with 10 recordings averaged. Then mice were light adapted for 10 min at an intensity of 30 cd·s/m² before photopic ERG measurements were recorded at stimulus intensity of 1.4 log cd·s/m² in the presence of continuous 30 Ganzfeld cd·s/m² background light with inter-stimulus intervals of 0.4 s. Fifty recordings were averaged for each light-adapted ERGs measurement. B-wave amplitudes were defined as the difference between the trough and peak of each waveform. Scotopic and photopic b-wave amplitudes were averaged and used to generate average responses and standard deviations (SD). ERG data are presented as mean ± SD. Statistical analysis was performed with unpaired t-test and significance defined as a P-value of <0.05.

Optokinetic testing

Photopic visual acuities and contrast sensitivities of treated and untreated eyes of CNGA3−/−/Nrl−/− mice were measured using a two-alternative forced choice paradigm as described previously with minor modifications (24,27,42,45). Thresholds for each eye were determined simultaneously via stepwise functions for correct responses in both the clockwise and counter-clockwise directions. Acuity was defined as the highest spatial frequency (100% contrast) yielding a threshold response, and contrast sensitivity was defined as 100 divided by the lowest percent contrast yielding a threshold response (sinusoidal pattern at 0.256 cyc/deg). For acuity measurement, the initial stimulus was a 0.200 cyc/deg sinusoidal pattern with a fixed 100% contrast. For contrast sensitivity measurements, the initial pattern was presented at 100% contrast, with a fixed spatial frequency of 0.128 cyc/deg. Photopic visual acuity was measured at a mean luminance of 70 cd·s/m². Visual acuities and contrast sensitivities were measured for both eyes of each mouse four to six times over a period of 1–2 weeks. Treated CNGA3−/−/Nrl−/− mice following either SR or IVit injection (n = 4), together with the age-matched Nrl−/− mice (n = 4) as reference responses, were used. Unpaired t-tests were carried out on acuity and contrast measurements to determine significance, defined as a P-value of <0.05.

Tissue preparation and immunohistochemistry

Eyes were enucleated at 2 or 6 months after injection. Retinal sections were prepared according to previously described methods (24,46). Briefly, immediately following sacrifice, the eyes were enucleated and fixed in 4% paraformaldehyde overnight at 4°C. The cornea and lens were then removed from eyes without disturbing the retinas to prepare the eyecups, which were rinsed with PBS and then cryoprotected by placing them in 30% sucrose/PBS for 4 h at 4°C. Eyecups were then embedded in cryostat compound (Tissue TEK OCT, Sakura Finetek USA, Inc., Torrance, CA, USA) and frozen at −80°C. Retinal cryosections were cut at 12 μm thickness, then rinsed in PBS and blocked in 2% normal goat serum, 0.3% Triton X-100 in 1% BSA in PBS at 4°C for 1 h. The sections were then covered with 3% H2O2 diluted in methanol (99%) for 10 min to block endogenous peroxidase activity. Sections were rinsed in PBS and blocked in 2% normal goat serum, 0.3% Triton X-100 and 1% BSA in PBS at 4°C. Sections were then incubated with the primary antibody overnight at 4°C. The sections were then washed three times with PBS, and incubated with Alexa Fluor 488-conjugated donkey anti-goat secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:200 in PBS at 4°C for 2 h. Sections were then mounted with Vectorshield (H-1400, Vector Labs, Inc., Burlingame, CA, USA) and cover slipped. Sections were analyzed with a Zeiss LSM 780 microscope fitted with Axiovision Rel. 4.6 software. All fluorescent images were acquired using identical exposure settings.

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Conflict of Interest Statement: W.W.H. and the University of Florida have a financial interest in the use of AAV therapies, and own equity in a company (AGTC Inc.) that might, in the future, commercialize some aspects of this work.
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