AAV Mediated GDNF Secretion From Retinal Glia Slows Down Retinal Degeneration in a Rat Model of Retinitis Pigmentosa

Deniz Dalkara[1–3], Kathleen D Kolstad[1], Karen I Guerin[1], Natalie V Hoffmann[1], Meike Visel[1], Ryan R Klimczak[1], David V Schaffer[1–3] and John G Flannery[1]

[1]Department of Molecular and Cell Biology and the Helen Wills Neuroscience Institute, University of California, Berkeley, California, USA; [2]Department of Chemical Engineering, University of California, Berkeley, California, USA; [3]Department of Bioengineering, University of California, Berkeley, California, USA

Mutations in over 80 identified genes can induce apoptosis in photoreceptors, resulting in blindness with a prevalence of 1 in 3,000 individuals. This broad genetic heterogeneity of disease impacting a wide range of photoreceptor functions renders the design of gene-specific therapies for photoreceptor degeneration impractical and necessitates the development of mutation-independent treatments to slow photoreceptor cell death. One promising strategy for photoreceptor neuroprotection is neurotrophin secretion from Müller cells, the primary retinal glia. Müller glia are excellent targets for secreting neurotrophins as they span the entire tissue, ensheath all neuronal populations, are numerous, and persist through retinal degeneration. We previously engineered an adeno-associated virus (AAV) variant (ShH10) capable of efficient and selective glial cell transduction through intravitreal injection. ShH10-mediated glial-derived neurotrophic factor (GDNF) secretion from glia, generates high GDNF levels in treated retinas, leading to sustained functional rescue for over 5 months. This GDNF secretion from glia following intravitreal vector administration is a safe and effective means to slow the progression of retinal degeneration in a rat model of retinitis pigmentosa (RP) and shows significant promise as a gene therapy to treat human retinal degenerations. These findings also demonstrate for the first time that glia-mediated secretion of neurotrophins is a promising treatment that may be applicable to other neurodegenerative conditions.

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INTRODUCTION

Remarkable success in the clinical application of adeno-associated virus (AAV)-mediated gene replacement therapy for Leber's congenital amaurosis, a monogenic inherited blinding disease, established the proof-of-concept for this class of gene therapy.[1–3] This approach is very effective for treating diseases resulting from recessive null mutations yet inapplicable to dominantly inherited retinal dystrophies, which affect the majority of visually impaired patients. The genetic and mechanistic diversity of the latter diseases presents an enormous obstacle for the development of gene therapy strategies. In particular, over 80 gene loci are involved in retinal diseases that result in photoreceptor cell death,[4] with the most common subtype being retinitis pigmentosa (RP).[5–7]

Because numerous mutations converge on photoreceptor cell death, a prosurvival therapeutic strategy to mitigate the neurodegenerative disease process should provide a general treatment for RP and other degenerative conditions. Neurotrophic and growth factors, such as glial-derived neurotrophic factor (GDNF) and basic-fibroblast growth factor, are known to promote photoreceptor survival in RP.[8,9] Notably, AAV-mediated secretion of GDNF from photoreceptors in a rat model of RP slowed disease progression as demonstrated by electroretinography (ERG) and histopathology.[10] Unfortunately, the treatment effect was lost after 60 days, presumably due to the progressive loss of photoreceptors secreting GDNF.

In addition to judicious choice of the therapeutic gene, targeting its expression to the most favorable cell type and restricting its expression in other cells are critical to the success of a gene therapy. Retinal Müller glial cells were chosen as the most favorable cells for neurotrophin secretion in our study since they radially transverse the retina ensheathing all retinal neurons[11] and naturally participate in mitigating retinal degeneration by releasing neurotrophins.[11,12] Furthermore from the standpoint of viral delivery, the accessibility of Müller glia from the vitreous face (inner aspect of the retina) enables intravitreal administration of the vector. This is a substantially safer and simpler procedure than subretinal injection and yields broader expression across the entire retina. Finally, glial cells survive until the latest stages of degeneration and can thus continue to supply neurotrophins as retinal neurons are lost to apoptosis.

We recently engineered an AAV capsid variant, ShH10, that efficiently and specifically transduces Müller cells from the vitreous.[13] Here, we employ this variant to overexpress GDNF from Müller cells and thereby significantly slow the rate of retinal degeneration in a rat model of autosomal dominant RP. Importantly,

Correspondence: *John G Flannery, Department of Molecular and Cell Biology and the Helen Wills Neuroscience Institute, University of California, Berkeley, Berkeley, California 94720-3190, USA. E-mail: flannery@berkeley.edu*

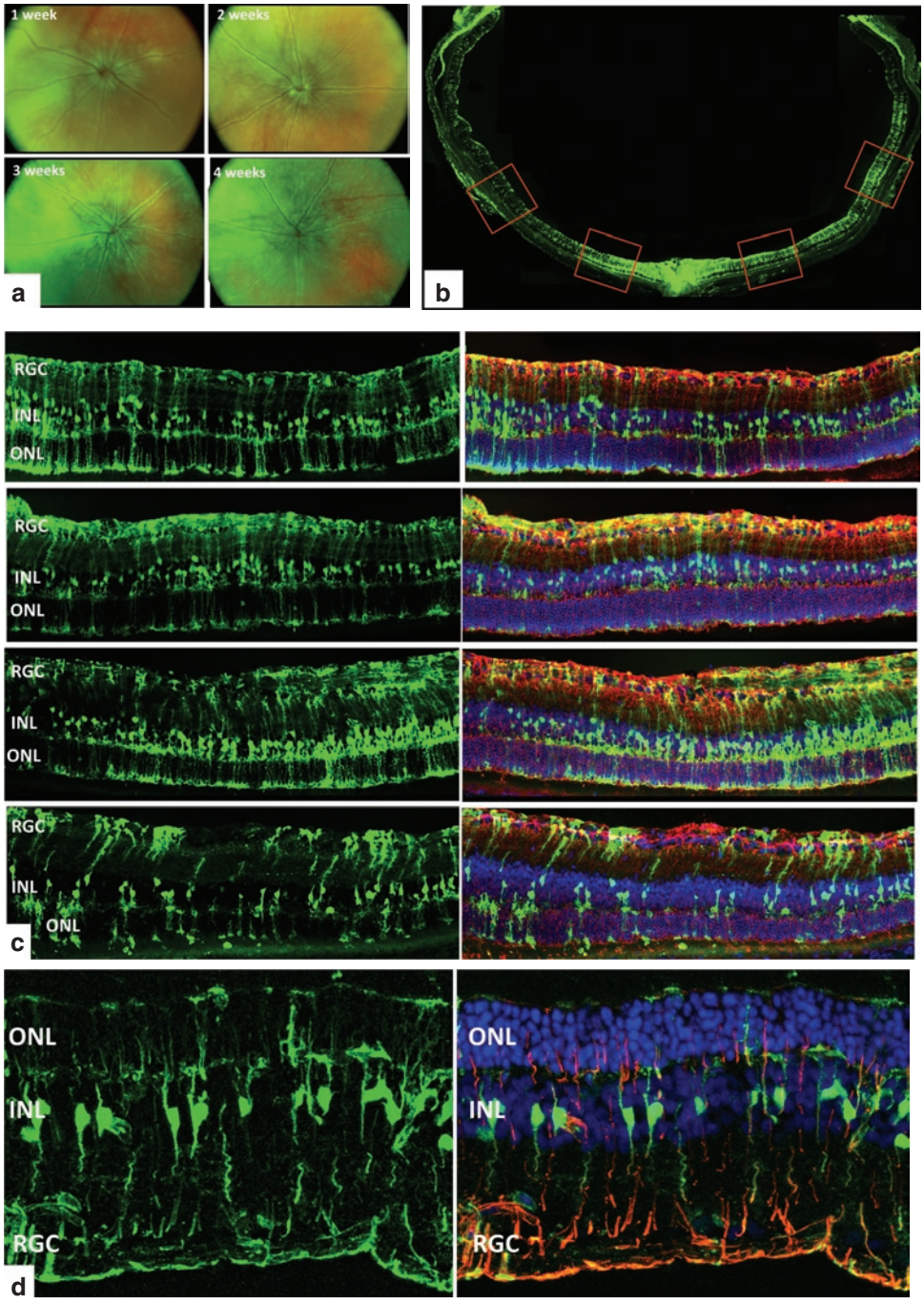

Figure 1 ShH10.Y445F.scCAG-GFP drives strong pan-retinal expression in Müller cells when intravitreally injected into S334-4ter rat eyes. (**a**) Representative fundus images at 1–4 weeks postinjection into p15 S334-4ter rat eyes show rapid onset of expression at 1 week postinjection before peaking and stabilizing at 3–4 weeks. (**b**) High-resolution montage of a retinal cryosection through the optic nerve head (ONH) showing the extent of green fluorescent protein (GFP) expression in Müller cells throughout the retina. (**c**) Low magnification (×10) images at four representative regions of the retinal cryosection. Left hand panels show GFP expression in Müller cells whereas right hand panels also show glutamine synthase-staining (in red) and nuclei stained with DAPI in blue. (**d**) High magnification image (×40) showing selective GFP expression in Müller cells in green alongside an overlay of GFP with glutamine synthase-staining (red) and DAPI-staining (blue).

the utilization of Müller glia results in secretion of higher levels of GDNF throughout the retina and slows the progression of retinal degeneration much longer than previous reports using GDNF.[10,14,15] Our findings therefore demonstrate that intravitreal transduction of Müller cells to secrete neurotrophins is an effective and sustainable treatment for retinal degeneration.

RESULTS

ShH10 leads to selective and efficient targeting of Müller glia in a rat model of RP

Previous characterization of our engineered AAV variant ShH10 had revealed efficient and specific transduction of rat Müller glia in wild-type animals.[13] Since high-level GDNF expression is

important to our present study, we further enhanced the infectivity of ShH10 by mutating a surface exposed tyrosine residue to phenylalanine for potentially more efficient trafficking to the nucleus.[16,17] Intravitreal injection of this recombinant ShH10.Y445F variant with a scCAG.GFP transgene in S334-4ter rats revealed strong, selective expression in Müller cells throughout the retina, peaking 3 weeks postinjection (**Figure 1a–d**) with no expression in untreated eyes (**Supplementary Figure S1**). Furthermore, 53% of all Müller cells showed green fluorescent protein (GFP) expression in TgS334-ter retinas infected with ShH10.Y445F. This indicates a significant increase compared to the number of Müller cells infected in wild-type retinas after ShH10 vector introduction.[13] This is likely due to an increased transduction efficiency afforded by the additional tyrosine mutation, alongside the more permissive nature of degenerating retinas to AAV-mediated transduction.[18]

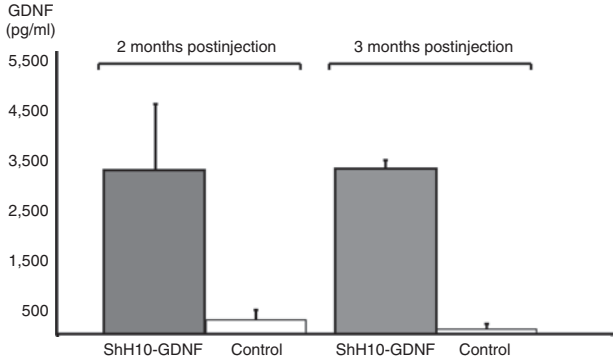

Figure 2 Enzyme-linked immunosorbent assay (ELISA) measurements of human glial-derived neurotrophic factor (hGDNF) protein in retinal homogenates 2 (*n* = 7) and 3 months (*n* = 6) following intravitreal delivery of ShH10.Y444F.scCAG.hGDNF. All animals received hGDNF vector treatment in the right eye and no injection in the left eye.

Given the high specificity of our vector (see **Supplementary Figure S5**), we next inserted a self-complementary human GDNF transgene driven from the same promoter.[19,20] Following intravitreal delivery of ShH10.Y444F scCAG.hGDNF, enzyme-linked immunosorbent assay (ELISA) measurements revealed robust secretion of hGDNF from Müller cells both 2 and 3 months postinjection (**Figure 2**). At >2.5 ng/ml, these hGDNF levels are nearly tenfold higher than those produced in previous studies that have achieved photoreceptor degeneration rescue through GDNF overexpression from retinal neurons after subretinal injection[9,10] or from intraocularly placed mouse embryonic stem cells.[15] Importantly, the expression is sustained, as ELISA measurements

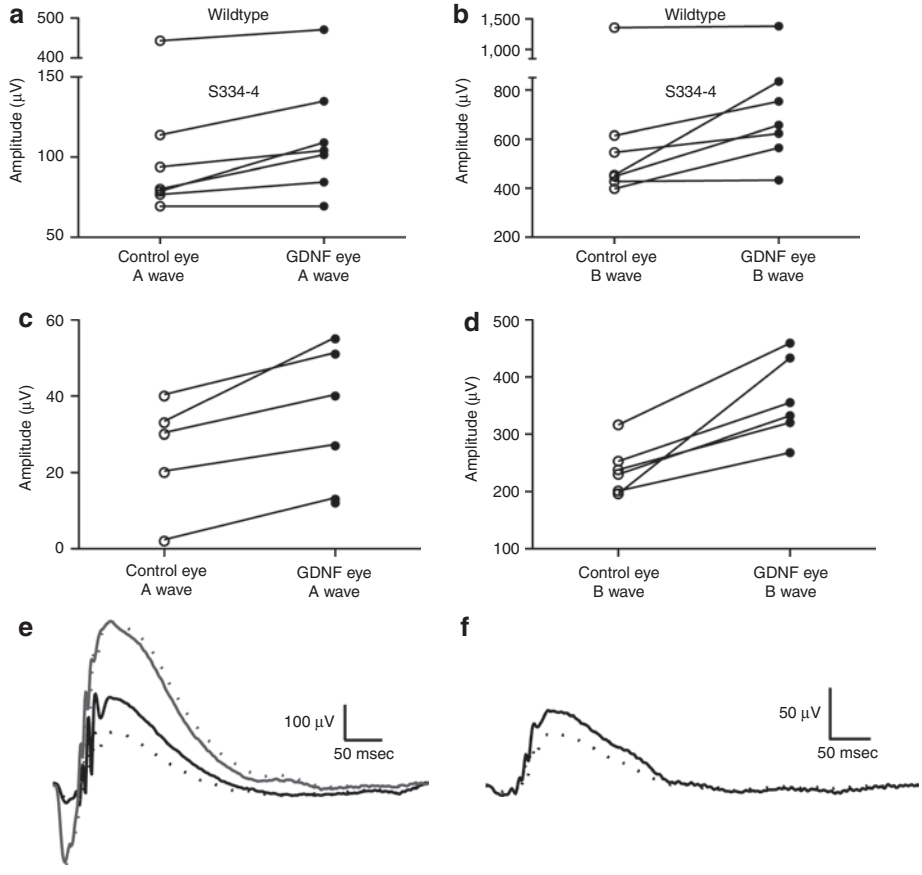

Figure 3 Scatter plots of electroretinography (ERG) (a) a- and (b) b-wave amplitudes in response to 1 log cd × s/m² in glial-derived neurotrophic factor (GDNF)-treated and contralateral control eyes 1 month postinjection and at 5 months (c,d). All animals (*n* = 6) were injected at p15. Representative ERG traces at 1 log cd × s/m² from a wild-type animals eyes (gray solid and dotted traces) and an animal with GDNF-treated (solid black line) versus control eye (dotted black line) at (**e**) 1 month postinjection. Representative ERG trace at 1 log cd × s/m² from a GDNF-treated animal (solid black line) versus contralateral control eye (dotted black line) at (**f**) 5 months.

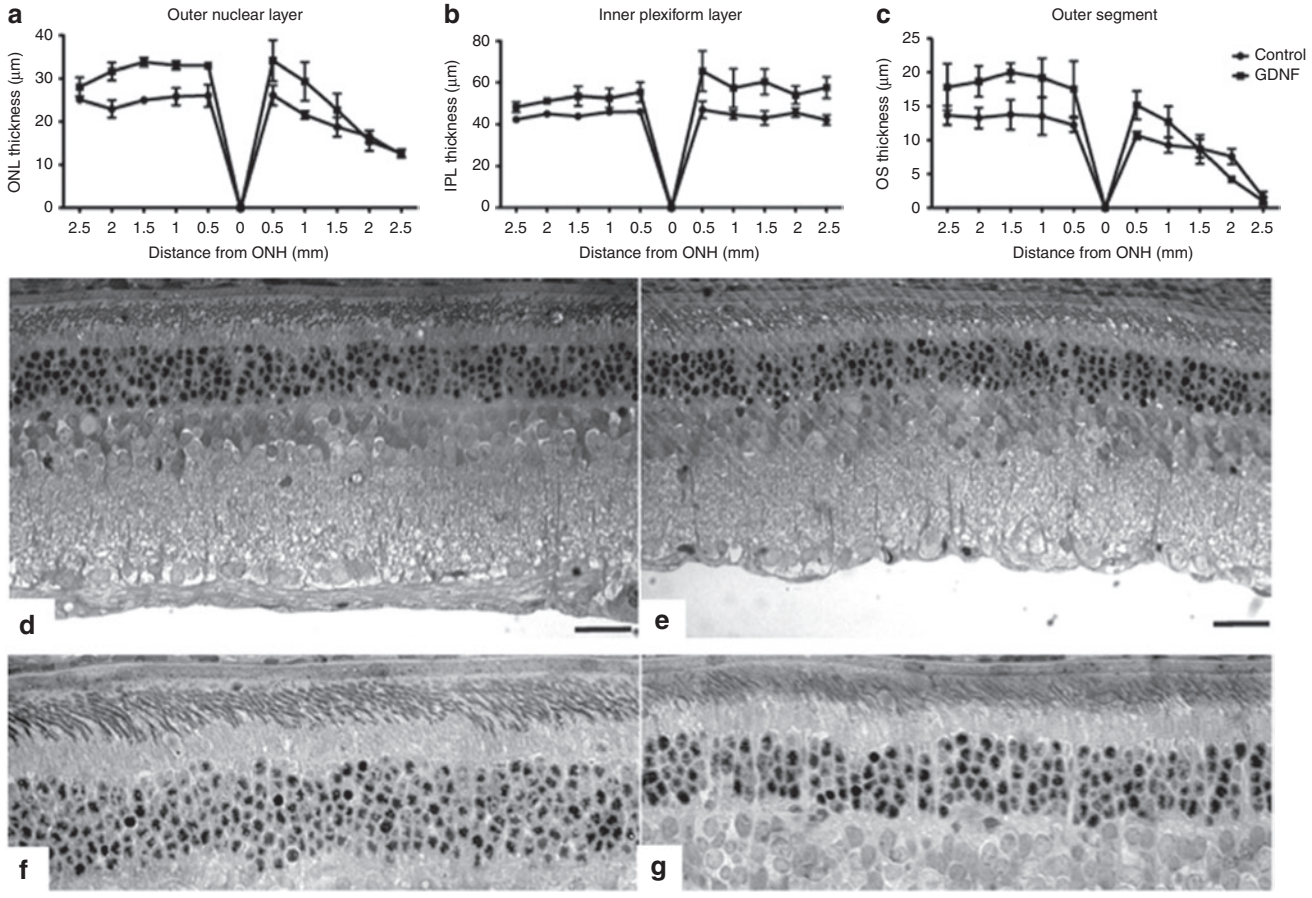

Figure 4 Measurements of (a) outer nuclear layer thickness (b) inner plexiform layer thickness and (c) photoreceptor outer segment length along the vertical meridian of the eye from the optic nerve head (ONH) to the ora serrata in rats at 3 months postinjection (p105). Rats were either uninjected (filled circles) or injected with ShH10.Y445F.scCAG.GDNF (filled squares) at p15. Light micrographs from inferior retinas of (**d**) ShH10.Y445F.scCAG.GDNF injected of (**e**) uninjected rats at ×20 magnification, Bars = 25 µm. High magnification micrographs from inferior retinas of (**e**) ShH10.Y445F.scCAG.GDNF injected or (**f**) uninjected rats at ×40 magnification, showing differences in outer segments length in the central part of inferior retinas.

of GDNF in the vitreous of rats 5 months postinjection show elevated levels of the therapeutic protein (**Supplementary Figure S2**), which are both safe[21] and necessary for sustained rescue.

Müller cell secretion of GDNF slows down retinal degeneration in S334-4ter rats

We next used ERG to assess the visual function of S334-4ter animals injected intravitreally at p15 with ShH10.Y445F.scCAG-GDNF. At 1 month postinjection, we see small increases in both a- and b-wave amplitudes of the treated eyes relative to untreated eyes (either uninjected or injected with ShH10.Y444F.scCAG.GFP) (**Figure 3a,b,e**). Although the ERG amplitudes are heterogeneous among animals, rescue is consistent between the control and contralateral GDNF-injected eyes, with an average b-wave value of 644 µV (±142 µV) in treated eyes versus 481 µV (±82 µV) for the control eyes (**Supplementary Figure S3a**). Wild-type animals demonstrate a-wave amplitudes of ~455 µV (**Figure 3a**) and b-wave amplitudes of 1,370 µV at the same age (**Figure 3b**). Remarkably, from 3 to 5 months after the injection, the physiological rescue becomes more pronounced, with an average amelioration of 50% in b-wave amplitude among all animals at 5 months and a nearly twofold increase observed in one animal (**Figure 3d**), with similar improvements in a-wave amplitudes (**Figure 3c**). Representative ERG traces corresponding to the average values at 1 (**Figure 3e**) and 5 (**Figure 3f**) months postinjection are shown below.

Histological rescue of photoreceptors

We next determined histological rescue by measuring the thickness of the outer nuclear layer, a well-established indicator of photoreceptor survival. Histological examination of GDNF-injected and control retinas corroborate the preservation of function observed in the electroretinograms. The outer nuclear layers of superior and inferior GDNF-treated retinas are thicker up to 4 mm from the optic nerve head at the inferior retina and up to 2 mm from the optic nerve at the superior retinas at 3 months postinjection (**Figure 4a**). Additionally, the inner plexiform layer and the photoreceptor outer segments are shorter in most of the inferior and a fraction of the superior control retinas (**Figure 4**).

DISCUSSION

Our findings suggest that targeting the retinal glial cells for overexpression of survival factors is a robust strategy for achieving and sustaining high levels of neurotrophin secretion in the retina. Application of the AAV variant ShH10, which was previously

created via the directed evolution of AAV to better infect glial cells,[13] results in selective targeting of glia and minimizes ectopic expression in neurons (see **Supplementary Figure S4**), which contrasts to the tropism of most natural AAV serotypes that preferentially transduce neurons with minimal glial transduction.[22–24] In most previous work, restricting gene expression to a given cell type was achieved primarily via incorporation of cell-specific promoters,[25,26] leading in most cases to weaker expression than can be achieved with strong, ubiquitous promoters. In contrast, transductional targeting at the initial step of viral interaction with receptors on the cell surface minimizes the loss of vector genomes to uptake by off-target cells that do not express the transgene. This can allow lower dosages of viral particles, reducing the possibility of immune responses to the viral capsid.[27] Previous studies in our lab have also indicated that some Müller cell-specific promoters may be cytotoxic, as subretinal injection of AAV9 carrying *GFAP.GFP* transgene lead to strong autofluorescence indicative of toxicity in fundus images of treated retinas, whereas a *CBA.GFP* gene delivered by the same AAV9 virus displayed no discernable toxicity. Furthermore, even a GFAP promoter followed by a noncoding sequence led to reduced ERG a- and b-waves 1 week postinjection,[28] a result that may be attributed to the toxicity of the promoter. Although the exact mechanism by which this damage occurs is unknown, it may be that the saturation of Müller cells by these cell-specific promoters hijacks essential transcriptional machinery for the cell to continue its normal functions. Thus, coupling the use of an engineered capsid to direct expression to the desired cell type with a strong promoter is clearly advantageous and is responsible for the high levels of neurotrophin we observe (**Figure 2**).

To our knowledge, our study is the first to demonstrate slowing of retinal degeneration via a sustained trophic factor genetic therapy that does not require subretinal injection. The intravitreal route has advantages in that it causes less retinal trauma and pathologic gliosis than the retinal detachment that accompanies subretinal injections, as well as generates pan-retinal gene expression (**Figure 1**). We found the onset of trophic factor mediated rescue is slower than in previous studies employing subretinal injection, which may be due to the lack of retinal detachment and its associated neuronal injury responses. One month following vector injection, our ERG results show a small, but significant improvement in the injected versus uninjected eye; however, this difference increases significantly at later time points. Similarly the differences in anatomical preservation of the photoreceptor layer in treated versus untreated eyes are less substantial than in some previous studies using subretinal delivery of vectors encoding GDNF,[10] fibroblast growth factor,[29] or ciliary neurotrophic factor.[30] Furthermore, the histological ameliorations in the inferior and superior retinas are different despite the pan-retinal nature of the Müller cell transduction leading to GDNF secretion. This is likely related to the degeneration being more severe in the superior retinas of this transgenic model[31] and the trophic effect of GDNF being less pronounced in these areas. Some of these differences must also be considered in the context of the very rapid time course of retinal degenerations in rodent models in comparison to human patients, such that in humans it may be possible to tolerate a slower therapeutic onset in order to achieve a larger and more sustained beneficial result.

Evaluation of the relative efficacy of neurotrophic factor secretion in retinal disease is complicated by the different surgical methods used. Studies indicate that retinal detachment induces substantial upregulation of endogenous neurotrophin genes and gliotic factors[32,33] on a timescale that correlates with the significant "rescue effect" seen early in gene therapy studies using subretinal injection of vector.[10,31,34] In most of the published studies, the surgical "control" retinas show an upregulation of neurotrophins in response to the trauma that persists for 1–2 weeks. In contrast, we report a rescue with a time course corresponding to the expression profile of our viral vector,[13] starting weeks after injection, and we see no functional or histological differences between untreated and ShH10.Y444F-GFP-injected retinas (data not shown). We conclude that in many of the published reports employing subretinal injection of viral vector, the initial, short-term therapeutic effect is primarily due to this retinal injury response. This "injury response" contributes substantially to the outcome in rodent models that go blind over the course of weeks, but it is likely to be insignificant in the context of a decade-long course of photoreceptor loss in human disease. However, it is important to note that in our study, both treated and untreated animals still lost ~50% of their 1 month postinjection ERG amplitudes at 5 months. Therefore, while the GDNF-treated eyes show an average of 50% better b-wave amplitudes at this time point, the degeneration progresses at a similar pace to the untreated animals between 1 and 5 months.

Finally, even in cases where the underlying genetic and mechanistic causes of the retinal degeneration are well-elucidated, trophic factor therapy could further enhance gene replacement therapies. For example, it has previously been shown that GDNF secretion enhances gene replacement therapy in Prph2Rd2/rd2 mouse and Royal College of Surgeons rat models of inherited retinal degeneration.[14] In this context, the value of targeting Müller cells for the secretion of GDNF would be further emphasized because two vectors could be applied simultaneously, one delivering the healthy copy of the defective gene to the photoreceptors and the other targeting Müller cells for GDNF overexpression, without competing for the receptors and transcription factors available in photoreceptors.

Additionally, the novel ShH10.Y444F AAV vector coupled with intravitreal delivery may be readily adapted to secrete other neurotrophins to promote survival of retinal ganglion cells in glaucoma, or antiangiogenic factors to suppress angiogenesis in neovascular "wet" forms of macular degeneration and diabetic retinopathy.[25] We propose that targeting retinal glia, rather than retinal neurons or epithelia, can be a broad strategy for delivering secreted compounds to treat human retinal disease. Lastly, secretion of neurotrophins from glia can be a promising strategy to treat neurodegenerations outside of the retina (*i.e.*, Parkinson's or Huntingtons disease).

In summary, targeting therapeutic genes to appropriate cell types in a minimally invasive manner is important to the success of gene therapies and their translation to the clinic. Transductional targeting via viral capsid engineering can route vector specifically to the target cells and subsequently drive high-level gene expression. In our study, the viral transduction capabilities of our ShH10 variant[13] were further strengthened by rationally mutating

a surface exposed tyrosine residue to enhance its intracellular trafficking and using a self-complimentary AAV genome to accelerate GDNF protein expression. Therefore, engineering the capsid via directed evolution and rational tyrosine mutations[13,16] and utilizing optimal expression constructs can yield highly elevated levels of GDNF in the rodent retina. Moreover, the use of viral vector engineering, as employed in this study, potentially coupled with inner limiting membrane thinning enzyme treatment,[35] may enable sufficient gains in intravitreal retinal transduction to promote rescue through neurotrophin secretion in more challenging larger animal models[36] as these strategies progress toward the clinic. Importantly, it is worth noting that though the rescue window we report in rats is ostensibly modest relative to human chronology, it may translate into several years of functional rescue in humans where the time-line of degeneration is significantly longer. We are currently in the process of examining the efficacy of this approach in canines and primates.

MATERIALS AND METHODS

Generation of recombinant AAV vectors. AAV vectors were produced by the plasmid co-transfection method.[37] Recombinant AAV was purified via iodixanol gradient ultracentrifugation[35] and heparin column chromatography (GE Healthcare, Chalfont St Giles, UK). The viral eluent was desalted and concentrated with Amicon Ultra-15 Centrifugal Filter Units to a final volume of 200 µl and titered by quantitative PCR relative to standards.

Intraocular injections. TgS334-4ter rats were used for all studies, and all animal procedures were conducted according to the ARVO Statement for the Use of Animals and the guidelines of the Office of Laboratory Animal Care at the University of California, Berkeley, CA. Rats were first anesthetized with ketamine (72 mg/kg) and xylazine (64 mg/kg) by intraperitoneal injection. An ultrafine 30 1/2-gauge disposable needle was then passed through the sclera, at the equator and next to the limbus, into the vitreous cavity. Five micro liter containing $1–5 \times 10^{12}$ vg/ml of AAV were injected with direct observation of the needle in the center of the vitreous cavity.

Cryosections. Animals were humanely euthanized by CO_2 overdose and cervical dislocation. Eyes were enucleated and immersion fixed in 10% formalin. The cornea and lens were removed and the resulting eye-cups were cryoprotected in 30% sucrose before embedding in optimal cutting temperature compound (Miles Diagnostics, Elkhart, IN). 5–10-µm thick transverse retinal sections were cut.

Immunolabeling. Tissue sections were blocked in 1% bovine serum albumin, 0.5% Triton X-100, and 2% normal donkey serum for 2–3 hours and treated with a rabbit anti-glutamine synthetase monoclonal antibody (Sigma G2781) at a 1:3,000 dilution in blocking solution overnight at 4 °C. After three phosphate-buffered saline washes, Cy3-conjugated anti-rabbit secondary (GE Healthcare) was applied at a 1:1,000 dilution in blocking solution for 2 hours at room temperature. The results were examined by confocal microscopy (LSM5; Carl Zeiss Microimaging, Peabody, MA).

ERG. Rats were dark-adapted for minimum of 2 hours and then anesthetized, followed by pupil dilation. Contact lenses were positioned on the cornea of both eyes. Reference electrodes were inserted subcutaneously in the cheeks and a ground electrode was inserted in the tail. Electroretinograms were recorded (Espion ERG system; Diagnosys LLC, Littleton, MA) in response to seven light flash intensities ranging from −4 to 1 log cd × s/m^2. Each stimulus was presented in series of three. Light flash intensity and timing were computer controlled. Data were analyzed with MatLab (v7.7; Mathworks, Natick, MA). ERG a and b waves from control and treated eyes were compared using Mann–Whitney paired *t*-test.

Histology. Rats were killed by CO_2 overdose. The superior cornea was marked, and enucleated eyes were immersion fixed in formalin followed by removal of cornea and lens. Eye-cups were then fixed in 1% osmium tetroxide, dehydrated by incubation in increasing ethanol concentrations and a final incubation in 100% propylene oxide. The samples were then embedded in an epon-araldite resin and hardened overnight at 65 °C. One-micrometer thin plastic sections were cut along the vertical meridian, through the optic nerve with a sapphire blade. Measurements of outer nuclear layer, IPL and OS thickness from the optic nerve head to ora serrata in three rats 3 months post-treatment were made on high-resolution montages of the retinas imaged at ×40 using ImageJ software. Fifty-four measurements of the outer nuclear layer, inner plexiform layer and outer segment were made at 18 contiguous fields around the entire retinal section (three measurements per field). These measurements were plotted as a distribution of thickness across the central retina.

ELISA. Brief sonication was used to homogenize treated and control retinas. ELISA was performed using the DuoSet Kit for human GDNF (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

SUPPLEMENTARY MATERIAL

Figure S1. Contralateral GDNF-injected eye immunolabeled with anti-glutamine synthetase antibodies and stained with DAPI.

Figure S2. ELISA measurements of hGDNF protein in vitreous samples 5 months following intravitreal delivery of ShH10.Y444F.scCAG. hGDNF ($n = 6$) and one uninjected animal ($n = 1$).

Figure S3. Average ERG b-wave amplitudes at 1 log cd × s/m^2 from GDNF-treated and control eyes (**a**) 1 month postinjection and at (**b**) 5 months.

Figure S4. Confocal stacks through the entire depth of flat-mounted retinas showing GFP expression in S334-ter rats at 2 months postinjection.

Figure S5. Confocal images taken around the optic nerve head in eyes infected with (**a**) AAV2-cx36-GFP and (**b**) ShH10.Y445F.scCAG-GFP.

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