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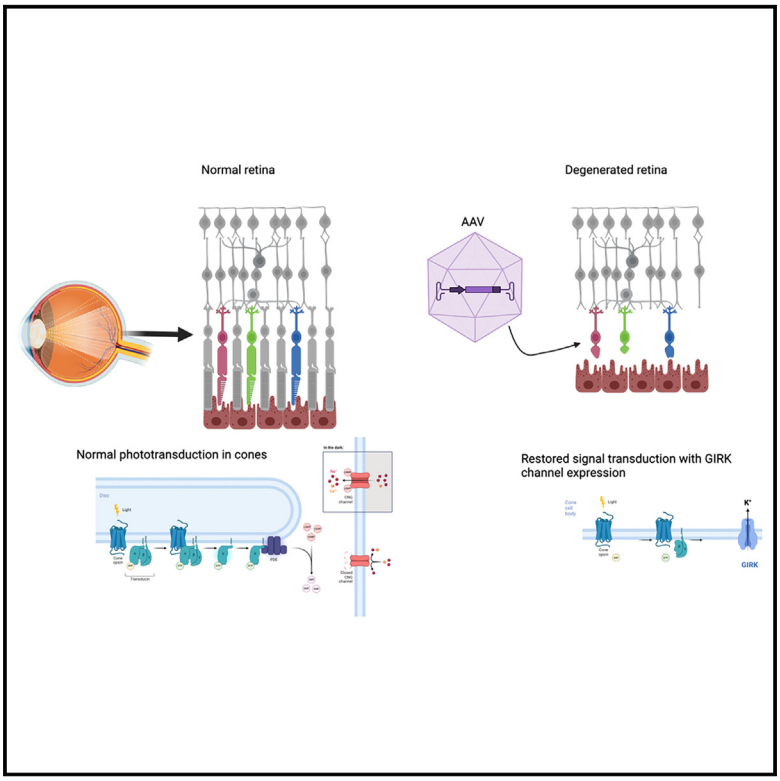
Reactivating the phototransduction cascade with a mutation agnostic gene therapy preserves vision in rod-cone dystrophies

Graphical abstract



Highlights

- GIRK channel expression reactivates cones in two RCD mouse models
- Opsin and arrestin expression persists in dormant cones of RCD patients
- GIRK therapy may restore light sensitivity, acuity, and color vision
- GIRK therapy offers mutation-independent cone reactivation

Authors

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In brief

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Reactivating the phototransduction cascade with a mutation agnostic gene therapy preserves vision in rod-cone dystrophies

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SUMMARY

Rod-cone dystrophy (RCD) comprises genetic conditions where rod photoreceptor degeneration leads to cone loss, causing progressive vision loss. We investigated the phototransduction cascade in degenerating cones using two RCD mouse models and found that opsin and arrestin expression continues in the cell body during outer segment degeneration. Based on this observation, we explored reactivating cones through G-protein-coupled inwardly rectifying K (GIRK) channel expression. Using adeno-associated viral delivery of GIRK channels, we achieved improved visual function in both mouse models. Additionally, we examined human tissue from late-stage RCD patients and confirmed the presence of cone opsin and cone arrestin expression, supporting the potential therapeutic application of this approach. This GIRK-channel-based strategy offers a promising method to preserve high-quality vision in RCD patients, regardless of their specific genetic mutation.

INTRODUCTION

Most genetic retinal degenerations result from mutations in photoreceptor or retinal pigmented epithelial (RPE) cells. Rod-cone dystrophy (RCD) is the most common type within these degenerations, marked by initial rod photoreceptor degeneration followed by the disappearance of cone photoreceptor outer segments, eventually causing blindness.[1] RCD is highly genetically heterogeneous. More than 60 different genes expressed in rods or the retinal pigment epithelium are involved.[2] The initial gene linked to RCD is the rhodopsin gene RHO, responsible for 25% of autosomal dominant RCD cases. Numerous other genes, such as those encoding the cGMP-phosphodiesterase (PDE) β or α subunit and the cyclic GMP-gated channel protein α or β subunit, have also been associated with RCD. Despite the variety of involved genes, the resulting RCD phenotype remains consistent among affected patients.[3] This condition first manifests with rod degeneration, resulting in night blindness. However, cones persist until the advanced stages of the disease, especially within the high acuity region of the fovea, resulting in tunnel vision.[4]

In later stages of the disease, the cone outer segment structures break down, resulting in total blindness. Some patients experience a complete loss of the cone soma and pedicle.[4] Recent experimental gene therapy strategies have focused on preserving the high-acuity central vision in patients with the goal of intervening in RCD.[5–9]

Most gene therapy efforts aim to supplement or correct defective genes, but these approaches are only viable for patients in the early stages of the disease with intact photoreceptors and a known causal mutation. Unfortunately, neither gene addition nor gene editing is feasible for the majority of retinal degeneration patients diagnosed late or with dominant negative mutations.[10] As of today, in 30% of cases, the causative mutation is not elucidated[11] or the rod photoreceptors bearing the most frequent mutations are already lost. To address these

challenges, we need mutation-independent gene therapies that extend beyond rod loss, catering to a broader range of patients without knowledge of the specific mutant gene. Optogenetics has emerged as a promising mutation-independent therapeutic strategy for restoring vision in blind patients.[12] Expressing microbial opsins, such as channelrhodopsin in retinal ganglion cells or the pump halorhodopsin in remaining cone photoreceptor cell bodies, allows light-dependent depolarization or hyperpolarization, respectively.[12–19] This strategy, although proven successful in the clinic, is constrained by the high expression levels and light intensity needed to activate microbial opsins at one specific wavelength. As a result, signal-amplifying goggles are necessary in conjunction with relatively high vector doses for efficacy.[12,16]

Microbial opsins have a lower signal transduction capability compared to vertebrate rhodopsins or cone opsins, which amplify the signal by activating G-protein-coupled cascades, like the phototransduction cascade found in healthy human photoreceptors.[20] To overcome this limitation of microbial opsins, previous studies explored the expression of G-protein-coupled opsins.[21–26] These opsins were expressed in inner retinal neurons. Although achieving some restoration of visual function at lower light intensities was possible with this approach, several questions persisted when considering the translation of these pre-clinical studies toward the treatment of human blindness: first, from a physiological perspective, vertebrate opsins are known to be promiscuous G-protein-coupled receptors. However, their effectiveness in signal transduction within inner retinal neurons relies on the recruitment of G proteins that typically do not couple to opsins in those cell types. Second, the mechanism by which the vertebrate opsin photocycle occurs in cell types distal from the retinal pigment epithelium (RPE) and Müller glial cells remains unclear.[27] Lastly, the restoration of function in a rod-dominant retina, as seen in mice, may differ significantly from that in a cone-dominant human retina, especially in the macula. For instance, if color-specific ganglion cells are uniformly stimulated through upstream bipolar cells expressing one type of opsin, it raises the question of what color(s) an individual would perceive in the world. Same question holds for direct insertion of opsins into ganglion cells.

In the current study, we have taken a conceptually similar approach in leveraging GPCR signaling via opsins but this time targeting degenerating cone cells. We have previously shown that light activation of cone opsins expressed in human kidney cells and brain neurons can stimulate the $G_{i/o}$ signaling pathways. Those pathways can then be linked to G-protein-gated inwardly rectifying potassium channel activity, coupling the change in the membrane potential to light exposure.[28] The ability to achieve light-mediated signaling in these cells was due to the endogenous presence or co-expression of G-protein-gated inwardly rectifying potassium (GIRK) channels. GIRK channels thus act as a general target molecule to the G proteins recruited by the opsins in multiple cell types.[21–26] Indeed, it is well known that GIRK channels are activated in excitable cells by GPCRs coupling to the $G_{i/o}$ pathway. After their activation by the $\beta\gamma$ subunit of a $G_{i/o}$ protein, potassium ions flow out of the cell, thus hyperpolarizing the membrane. It has therefore been possible to use vertebrate cone opsins for repetitive $G_{i/o}$ activation upon illumination *in vivo* within the anxiety circuitry, and the combination of cone opsins with GIRK has proven more efficient than microbial opsins at low light intensities. Indeed, for vertebrate opsins, light-induced GIRK currents were largest around 0.02 mW/mm^2, whereas microbial opsins were maximally activated at 2 mW/mm^2.[28] These findings strongly suggest that expressing GIRK channels in cones with shortened outer segments could enhance light responses.

To test the feasibility of GIRK-mediated sensitization of degenerating cones, we first investigated the state of the endogenous phototransduction cascade through the progression of the disease. We found that endogenous cone opsin transcripts are still present, and opsins are still expressed on the membrane of cone cell bodies in mouse models as well as in RCD patients' maculae postmortem. These findings suggest the possibility of linking opsin activity to GIRK channels despite reduction in transducin alpha subunits and phosphodiesterase occurring in later stages of degeneration.[29] Upon AAV-mediated GIRK2 gene transfer, significant visual improvements are seen in two mouse models of RCD with different underlying mutations. Our results point toward enhanced light-sensitivity in cone photoreceptors of RCD mouse models during degeneration of cone outer segments. Although the improvement in light sensitivity is still far from the wild-type level, this is the first time a light insensitive mammalian ion channel has been linked to intrinsic opsins opening new avenues in vision restoration. This concept can be implemented to increase light sensitivity even before complete outer segment degeneration as GIRK channel expression does not impact wild-type retinal function. Since our system makes use of intrinsic opsins expressed in degenerating cones, it would also enable, for the first time, color vision restoration, which has not been feasible in previous studies using optogenetics. Lastly, our preliminary retrospective clinical study shows a significant proportion of retinitis pigmentosa patients with minimal or no light perception maintain a detectable outer nuclear layer (ONL) composed of cones with diminished outer segments. Since cones in the macular region provide up to 50% of the input to the visual cortex,[30] targeting these cones with our new approach promises vision restoration respecting the normal retinotopy and high visual acuity distinctive of this area.

RESULTS

Changes in the cone phototransduction cascade in the rd10 mouse retinas

To provide an understanding of the changes in the phototransduction cascade in RCD, retinal samples were collected from homozygous *rd10* mice between postnatal days 14 (p14) and 150 (p150). Cross-sections from the histology group were stained for cone phototransduction cascade proteins in comparison with wild-type retinal cross-sections (Figures 1 and S1). At day 150, we found that only the cone opsin and cone arrestin proteins were still expressed at detectable levels and localized to the cone cell body, whereas Gnat2 and Pde6c were no longer detectable by immunohistochemistry (Figure 1). To get more insight into the decline of cone function with respect to the declining expression of cone phototransduction transcripts, we further examined the phototransduction cascade gene

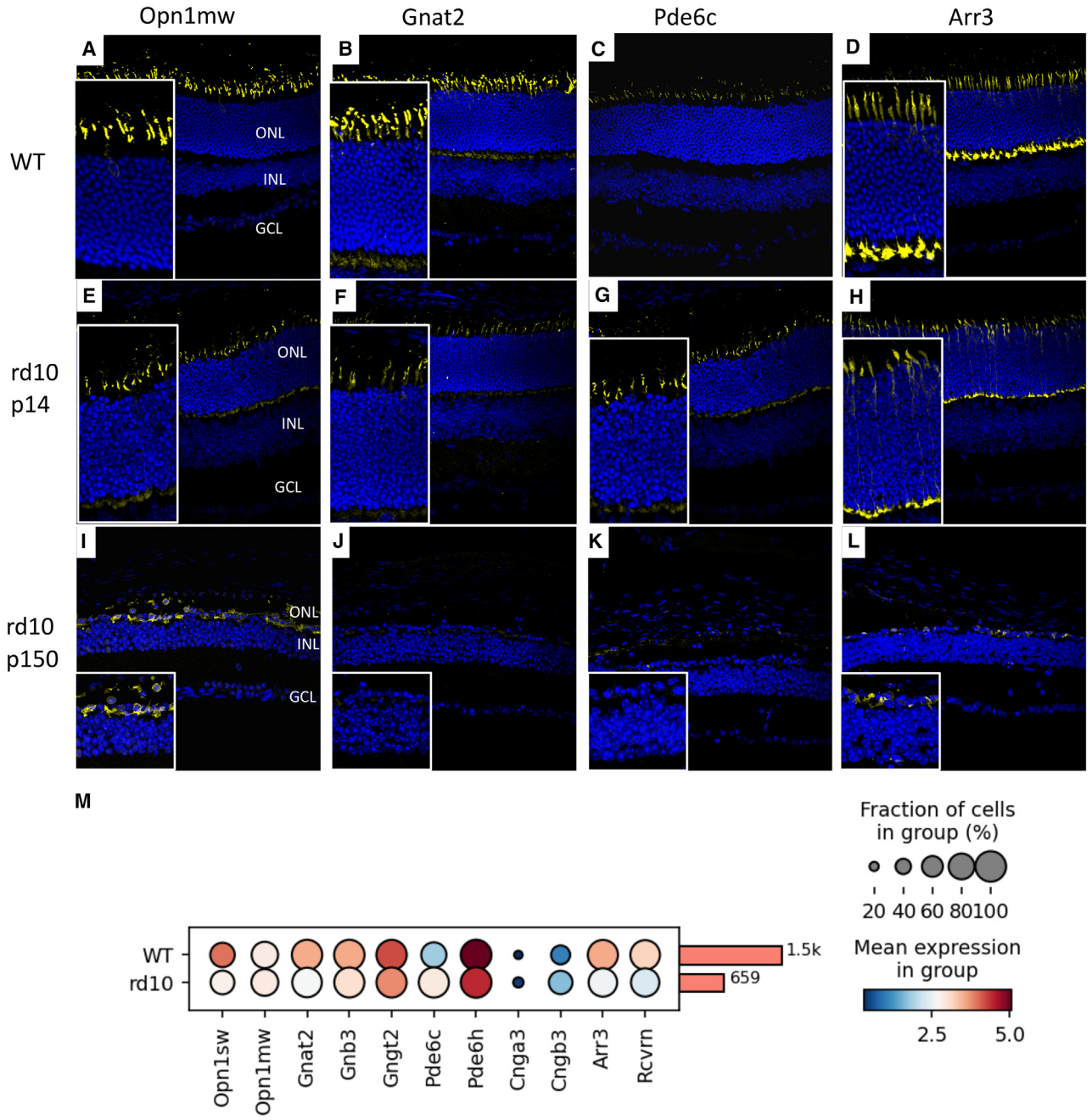


Figure 1. Phototransduction cascade proteins and transcripts in wild-type versus rd10 cones

(A–M) Immunohistochemistry against mid-wavelength-sensitive cone opsin (Opn1mw), transducin (Gnat2), phosphodiesterase6c (Pde6c), and cone arrestin (Arr3) in wild-type c57bl6j mouse retinas (A–D), in 14-day-old rd10 mouse retinas (E–H), and in 150 days old rd10 mouse retinas (I–L). Mean gene expression values from scRNAseq data obtained from 126-day-old rd10 mice compared to wild-type c57bl6 mice. The highest value represents the maximum expression of the highest gene (dark blue); lowest value is the expression of the lowest gene (dark red). The mean expression in group represents the number of reads for each gene out of the total number of reads (M).

transcripts in the cones of wild-type and *rd10/rd10* mice at postnatal days 70–112 and 126 respectively, by single-cell RNA sequencing (scRNAseq).[31–36] In addition to the genes *Opn1mw*, *Gnat2*, *Pde6c*, and *Arr3*, examined with immunohistochemistry, *Opn1sw*, *Gnb3*, *Gngt2*, *Pde6h*, *Cnga3*, *Cngb3*, and *Rcvrn*, belonging to the phototransduction cascade, were analyzed in rd10 mice at P126 using scRNAseq. scRNAseq revealed that there are no significant changes in *Opn1sw*, *Gnat2*, *Gnb3*, *Pde6h*, *Gngt2*, *Rcvrn*, and *Arr3* transcript levels in the *rd10* retinas compared to the similarly aged wild-type retinas; a

decreasing trend can be observed in rd10 retinas (Figure 1). An overall decrease in the level of membrane bound opsin is coherent with the loss of the outer segment that contains most of the membrane surface in normal cones, though it is interesting to note that short wavelength opsin transcripts were lower compared to mid-wavelength opsin transcripts that remained unchanged at the mRNA level. In absence of outer segment, there is less transcript; yet, translation of opsin and arrestin mRNA is still ensured at levels detectable by histology. On the contrary, at p126, rd10 transcript levels are all similar to wild-type levels although Gnat2 and Pde6c protein are not detected by immunohistochemistry at p150. This result can be attributed to a dysfunction in the translation pathway of *Pde6c and Gnat2* mRNA into proteins at later stages of cone degeneration.

GIRK2 expression leads to light-induced current density changes in HEK293 cells stably expressing mOpn4L

We have previously shown that cone opsins expressed in HEK cells or in serotonergic neurons expressing GIRK1/2 channels lead to light-mediated modulation of membrane potential *in vitro* and *in vivo*.[28] Based on the observation that cone opsins are still expressed in cones with diminished outer segments, we reasoned that delivering an AAV vector encoding GIRK channels can enhance these cells' response to light via the recruitment of other G proteins or via the activity of the beta/gamma subunits of remaining transducin. We hypothesized that expressing a truncated rat GIRK2 as a target channel in HEK cells stably expressing a mouse opsin can behave in the same way, as GIRK2 channels are capable of forming functional homotetramers.[37] We first tested the channel activity in HEK cells stably expressing Opn4 (see key resources table). Light-induced current density per capacitance of HEK293 cells expressing GIRK2.eGFP and mOpn4 were significantly different than those without GIRK2 expression. Light stimulation led to a reliable activation of mOpn4 by blue light (470 nm) mediating a GIRK current that concluded upon stimulation with green light (560 nm) (green trace in Figure S2). Untransfected cells did not show a current response when stimulated, allowing us to conclude that truncated rat GIRK2 can be operably linked to mouse opsins.

Ex vivo single-cell recordings show GIRK2 expression in dormant cones elicits light responses

We produced AAV vectors encoding GIRK2 under the strong cone-cell-specific PR1.7 promoter.[19,38] We performed subretinal injections in 30-day-old rd1 mice that are known to have very fast rod degeneration and long-term dormant cone preservation (ONL is reduced to a single layer of cones without outer segments that migrate into the INL and remain until days exceeding 266[17]). We waited until mice were 55–60 days old. This led to high-level GIRK2-GFP expression in rd1 cones as seen in fixed cryosections in cells expressing cone arrestin (Figure 2A). Live fluorescent images of retinas acquired prior to patch-clamp experiments (Figures 2A–2C) showed GFP positive cones in this outer part of the retina. Patch-clamp recordings were then performed in control and GIRK2-expressing cones. Resting membrane potential (RMP) of GIRK-GFP-expressing photoreceptors and control cells from the same retina not expressing GIRK was shown to be similar in the dark (at 0 current) with mean RMP values of −49, 21 ± 5.7 mV in cells expressing GIRK-GFP ($n = 7$) and −47.18 ± 2.14 mV in non-fluorescent control cells ($n = 10$). Next, light responses were measured from 7 GIRK-GFP+ cells after stimulation with two consecutive flashes at 380 and 500 nm (2 s flashes), at a light intensity of 1×10^{16} photons cm^2/s (Figure 2E). Cells were recorded in current-clamp zero configuration, and each trace in Figure 2E represents the average of at least two repetitions. Note that n6 and n7 neurons displayed a more robust response to the green stimulation. Seven out of thirty patched fluorescent cells were found to be light sensitive in this fast-degenerating rd1 mouse model. Mean response amplitude values measured at the end of the stimulation periods are annotated with vertical blue or green dotted lines in Figure 2F. Altogether these findings showed good membrane-bound expression of GIRK2 in rd1 mice cones at a stage where they have lost their outer segments and ability to respond to light. They further indicated that GIRK expression does not alter resting membrane potential in the dark, but using green and blue illumination it is possible to generate GIRK currents in cones without remaining outer segments. In our experimental conditions, we do not force the cell's membrane potential, yet we do observe a significant hyperpolarization, which is promising for *in vivo* potential of GIRK-mediated vision improvement tested subsequently in a slower model of retinal degeneration. Of note, the amplitude, time to peak, and rate of response decay for the responding cells are variable and show slow decay compared to *in vivo* flicker responses (see Figure S4 for quantification of the amplitude, time to peak, rate of response of responding cells represented in Figure 2).

GIRK2 expression in cones increases photopic light responses in rd10 retinas but not in normal mice

Following *ex vivo* single-cell recordings from rd1 mice, we decided to use a mouse model with a slower pace of degeneration more accurately mimicking the degenerative process that occurs in most RP patients. Indeed, it has recently been shown that cones and cone pathways remain functional in advanced retinal degeneration in rd10 mice.[39] We thus reasoned that *in vivo* visual assessments of the effect of GIRK expression on retinal physiology and acuity can be performed in this model and compared to effects of GIRK expression in wild-type mice.[40] To this aim, we performed bilateral subretinal injections of AAV8 encoding GIRK2-GFP under the control of PR1.7 promoter on wild-type and rd10 mice at P15 as detailed in Table 1. One to three weeks after injection, *in vivo* and *ex vivo* retinal imaging showed localized GIRK2-GFP expression in cones of injected retinas (Figures 3A, 3B, and S4). Immunohistochemistry confirmed colocalization of GIRK expression with cone arrestin in both wild-type and rd10 cones (Figure 3C). We counted the red channel of this image showing cells positive for mouse cone arrestin and found 293 cells. In the green channel we counted 286 cells. This indicates that of all cone cells showing mCAR staining, 97.6% were also GFP positive (yellow) in this injected area. Cryosections further confirmed high-level, membrane-bound expression of GIRK2 throughout the outer retinal layer of rd10 retinas.

We next evaluated functional effect obtained by ectopic GIRK2 expression in cones, by performing photopic ERG

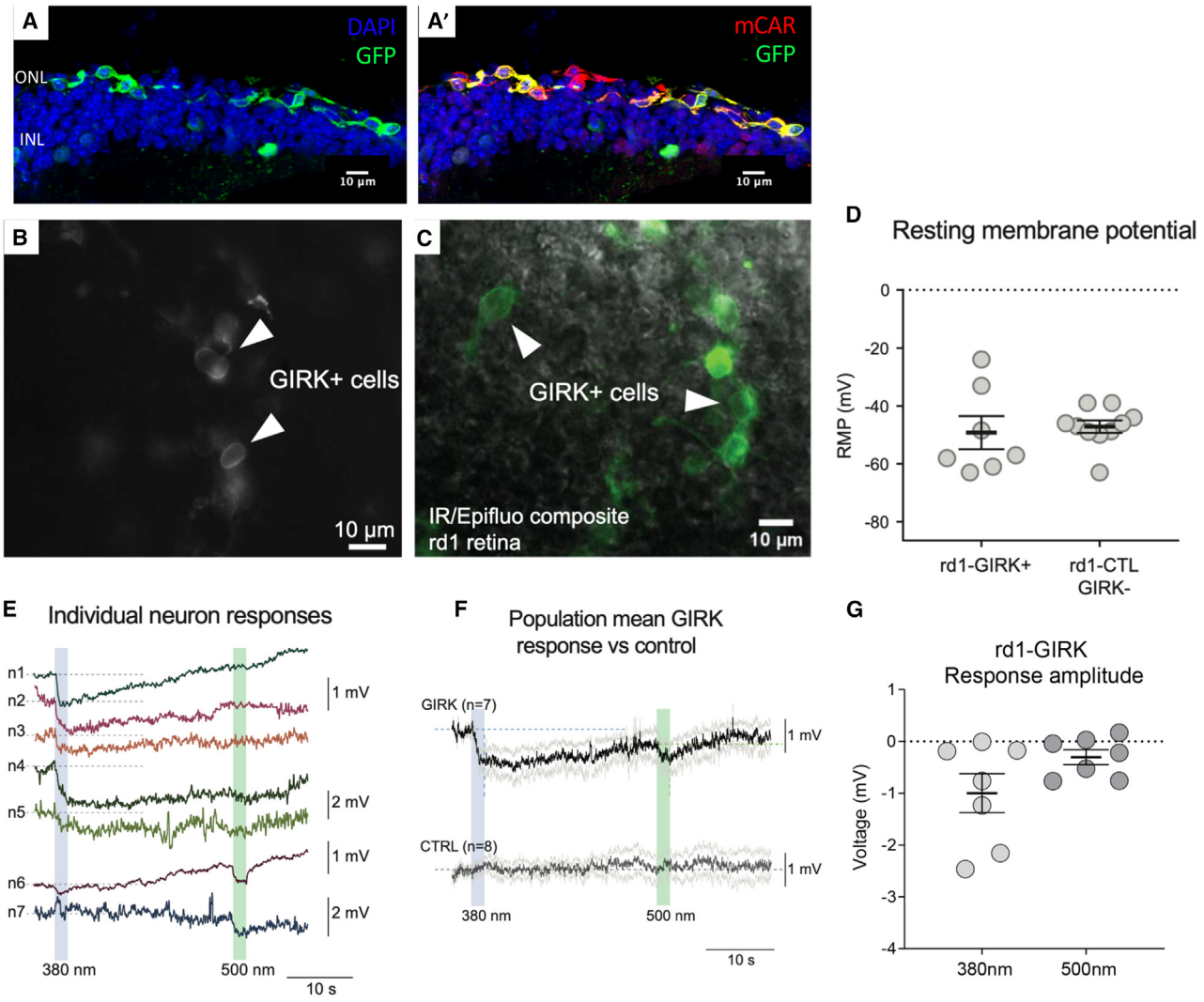


Figure 2. Representative images and single-cell recordings from dormant mouse cones expressing GIRK2-GFP

(A and A′) Immunohistochemistry against GIRK2-GFP (green) and mCAR (red) in cryosections of a transduced rd1 retina.

(B–C′) Composite of infrared and epifluorescence images showing transduced cells expressing GIRK-GFP in rd1 mouse retina prior to experiments in the patch-clamp rig.

(D) Resting membrane potential (RMP) of GIRK-GFP-expressing photoreceptors and control cells, in the dark (at 0 current). Mean ± SEM RMP values were −49, 21 ± 5.7 mV in cells expressing GIRK-GFP (n = 7) and −47.18 ± 2.14 mV in non-fluorescent control cells (n = 10).

(E) Light responses (hyperpolarization) from 7 GIRK-GFP+ cells after stimulation with two consecutive flashes at 380 and 500 nm (2 s flashes), at a light intensity of 1×10^{16} photons cm^{-2} s^{-1}. Cells were recorded in current-clamp zero configuration. Each trace represents the average of at least two repetitions.

(F) Average population response of all responsive cells (n = 7) and control cells (n = 8) at two different wavelengths, represented as mean ± SEM curves. Mean response amplitude values, measured at the end of the stimulation periods (vertical blue or green dotted lines) and compared to the pre-stimulus baseline (horizontal dotted lines) were −0.99 ± 0.37 mV at 380 nm and −0.30 ± 0.14 mV at 500 nm (n = 7).

(G) Response amplitudes shown in (E) plotted as a function of wavelength. The rd1 mice retinas we imaged and recorded from were 55–60 days old.

recordings at different time points after injections in wild-type and rd10 mice. We recorded photopic ERGs under two conditions: (1) photopic with white light flashes applied every second during 60 s at increasing light intensities (Figure 4A) and (2) 10Hz flicker stimulation with repetitive flashes at the same intensity for 60 s (Figures 4B and S4). There were no differences in the amplitudes of the photopic ERG B wave in light intensities ranging from 0.1 to 50 cd*s/m^2 in wild-type animals having received GIRK2 injections compared to PBS-injected eyes. Flicker amplitude and time were also unchanged for wild-type eyes recorded at P60 (Figure S4). On the contrary, there were statistically significant differences in photopic ERG amplitudes of rd10 mice eyes injected with GIRK2 in comparison to PBS-injected or naive eyes, already at P50 (Figures 4A and 4B). Data were collected on a weekly basis until P50 and then every 10 to 20 days until 20 weeks of age. Treated eyes showed a significant increase in ERG amplitudes compared to control eyes until P120 (Figure 4C). At 5 months of age, the difference

Table 1. Experimental groups, viral vector doses, and the injection volume

Injection table

Eyes	Viral vector injected	Viral vector dose	Volume injected
Right	PBS-pluronic 0.001%		1 μL for all conditions
Left	AAV8-PR1.7-GIRK2-GFP	5.10^8 AAV particles	

between treated and untreated eyes was no longer detectable under these conditions. This is likely due to the detection threshold of the ERG that requires a minimum number of active cones in order to have a recordable response to light stimuli. As a more sensitive readout, we evaluated behavioral responses of treated mice by optokinetic test (OKT). Significant differences in visual acuity of treated and untreated eyes were visible even at the latest time point (5 months old) (Figure 4D). The implicit time was stable for time points P35 through P50 with no significant differences between PBS-injected and GIRK2-injected eyes (Figure 4E). Nevertheless, a gradual decline in both ERG amplitudes and OKT responses was observed, with or without treatment. This decline can be attributed to decrease in cone numbers over time as GIRK2 treatment does not increase survival of cones (Figures S4H and S4I).

GIRK2 expression in cones increases photopic light responses in degenerating huP347S$^{+/-}$ retinas

To test whether GIRK2 can be useful to increase cone-mediated light responses in a mutation-independent manner, we tested our approach in another RCD mouse model. mRho$^{+/-}$ huRhoP347S$^{+/+}$ (huP347S$^{+/-}$) carrying a knock-in P347S mutant human rhodopsin[41] was used to perform an identical set of experiments. First, we analyzed the phototransduction cascade proteins interacting with cone opsin at different time points (Figures 5 and S4). Similarly to the *rd10* model, cone opsin and cone arrestin expression persisted in cone cell bodies at P150 but the phosphodiesterase and the transducin proteins were undetectable at this time point.

Next, we injected mice at P15 with the same AAV vector encoding GIRK2 fused with GFP (Table 1) and recorded ERGs to monitor cone response to light stimuli at various time points (Figures 6A and 6B). The amplitude responses of treated eyes were significantly higher than that of control eyes until P100 (Figure 6C). This confirmed that our approach is a mutation-independent therapy that preserves vision at late stages regardless of the causal RCD mutation. Moreover, we recorded similar ameliorations in flicker ERG responses in this mouse model (*data not shown*). As for *rd10* mice, no significant difference in light responses was observed in treated eyes compared to control eyes at 5 months likely due to progressive loss of cones and the increased variability of ERG responses at late stages. However, treated eyes showed an improved visual acuity compared to control eyes until P150 (Figure 6D). Similar to *rd10* mice, a gradual decline in ERG amplitudes and behavioral responses can be observed in treated eyes as well as control eyes, but treated eyes displayed improved function compared to controls until the latest time points. The decline in number of cones is once again consistent with the fact that our approach does not stop the degeneration but allows for enhanced light sensitivity of cones through GIRK2.

GIRK2-mediated vision restoration: Feasibility in RP patients

Is GIRK gene therapy translatable to RCD patients? To check the applicability of this approach at the cellular level in the human retina, we used postmortem retinal specimen to analyze phototransduction cascade proteins in the macular region in four RP patients aged between 73 and 92 years (see key resources table) who were in intermediate and advanced stages of the disease (Postmortem eyes obtained from the Cole Eye Institute Eye Tissue Repository through the Foundation Fighting Blindness [FFB] Eye Donor Program [Columbia, MD]). We also analyzed the expression of phototransduction cascade proteins in a healthy control retina from a 91-year-old patient (tissue was obtained from the Surgery School of Paris). Importantly, we found that similar to RCD mouse models, cone opsin and cone arrestin remain in cone cell bodies of RP patients (Figure 7). Indeed, three of four donors (donor 2 to 4) had cones with diminished outer segments. In all three patients, cones were found co-expressing opsin and arrestin (Figures 7B–7D). The donor 5, who was blind, had only a few sparse cones remaining (Figure 7E). Altogether these data corroborate the applicability of GIRK-mediated gene therapy in human cones. Our short phototransduction cascade strategy can potentially reactivate cone function in RCD patients. The activation of remaining cone opsin by a light stimulus would therefore trigger the short GIRK2-mediated phototransduction cascade and lead to enhanced light sensitivity in RP patients.

To address more closely the question of applicability in patients, we investigated the proportion of potential eligible patients in our RCD cohort from the center of rare disease of the Quinze-Vingts Eye Hospital (Paris). We sorted out patients based on two criteria: (1) visual acuity <2/10 with low to no light perception and (2) presence of a detectable ONL filled with cone photoreceptor cells displaying shortened or absent outer segments (referred to as "dormant cones") in optical coherence tomography (OCT) B-scans (Spectralis, Heidelberg Engineering, Germany). As a preliminary starting point, we screened a database of 350 eyes with genetically confirmed Retinitis Pigmentosa (RP) diagnosis, and we sorted eyes having a visual acuity below 2/10 (Figure 8A). We focused on those patients because our approach using GIRK2 would increase light sensitivity in degenerating cone photoreceptor cells and allow the patient to regain light perception and high acuity vision in the fovea. Based on these criteria, we eliminated 235 eyes, and we focused on 115 eyes with very reduced or lost light perception (Figure 8A). Then, we eliminated eyes with no discernible ONL by examining the fine structure of the foveal region containing the cone cells last to degenerate in RCD (also referred to in the literature as remnant cones or dysflective cones[42]), targets of our gene therapy approach. Among the 115 eyes with low to no light perception, 29 eyes had a distinguishable ONL composed of cones with shortened or absent outer segments

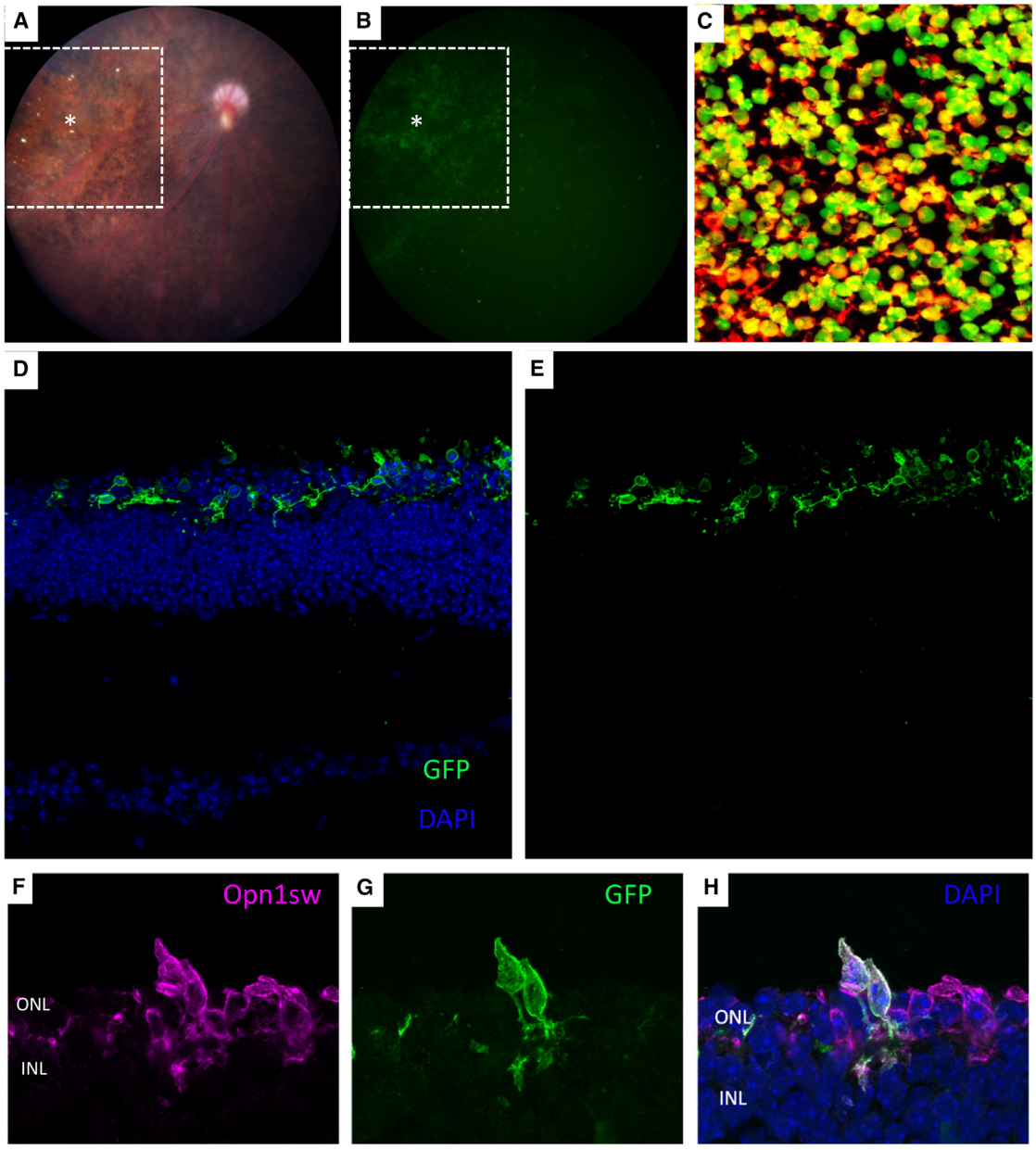


Figure 3. Representative images from rd10 mouse cones expressing GIRK2-GFP

(A–H) Live fundus imaging in rd10 mice after subretinal injection in the upper temporal retina. (A) Brightfield image of the eye fundus. (B) Fluorescence mode showing expression around the injection site marked with asterisk. (C) Immunohistochemistry against GIRK2-GFP (green) and cone arrestin (red) in retinal flatmounts and in cryosections (D and E) of GIRK2-GFP-expressing rd10 retinas. (F–H) Zoomed confocal images of a transduced rd10 retina with immunostaining for GIRK2-GFP (green) and Opnsw (magenta) showing membrane localization of GIRK and cone opsin.

(Figures 8A–8E). This indicates that roughly one-quarter of RP patients with low to no light perception can be eligible for GIRK2 therapy. In order to deeply characterize the dormant cone phenotype and examine the inner segment structures overlying the diminished outer segments, we analyzed the cone cells of a patient with a very high-quality OCT scan, using adaptive optics scanning laser ophthalmoscopy (AOSLO) (Physical Sciences Inc., Andover MA, USA) (Figure 7C). Intact cones were captured in the confocal channel and intact inner segments in the split detection channel. Refractive changes in the inner and outer segments of the foveal cones suggested it is possible to identify the presence of light insensitive ''dormant'' cone population using this technique (Figure 8G). The combination of the above-mentioned imaging techniques along with more recently described indicators of cone cell function (such as fundus autofluorescence imaging),[43] makes it feasible to select patient populations that would most benefit from GIRK2 gene therapy.

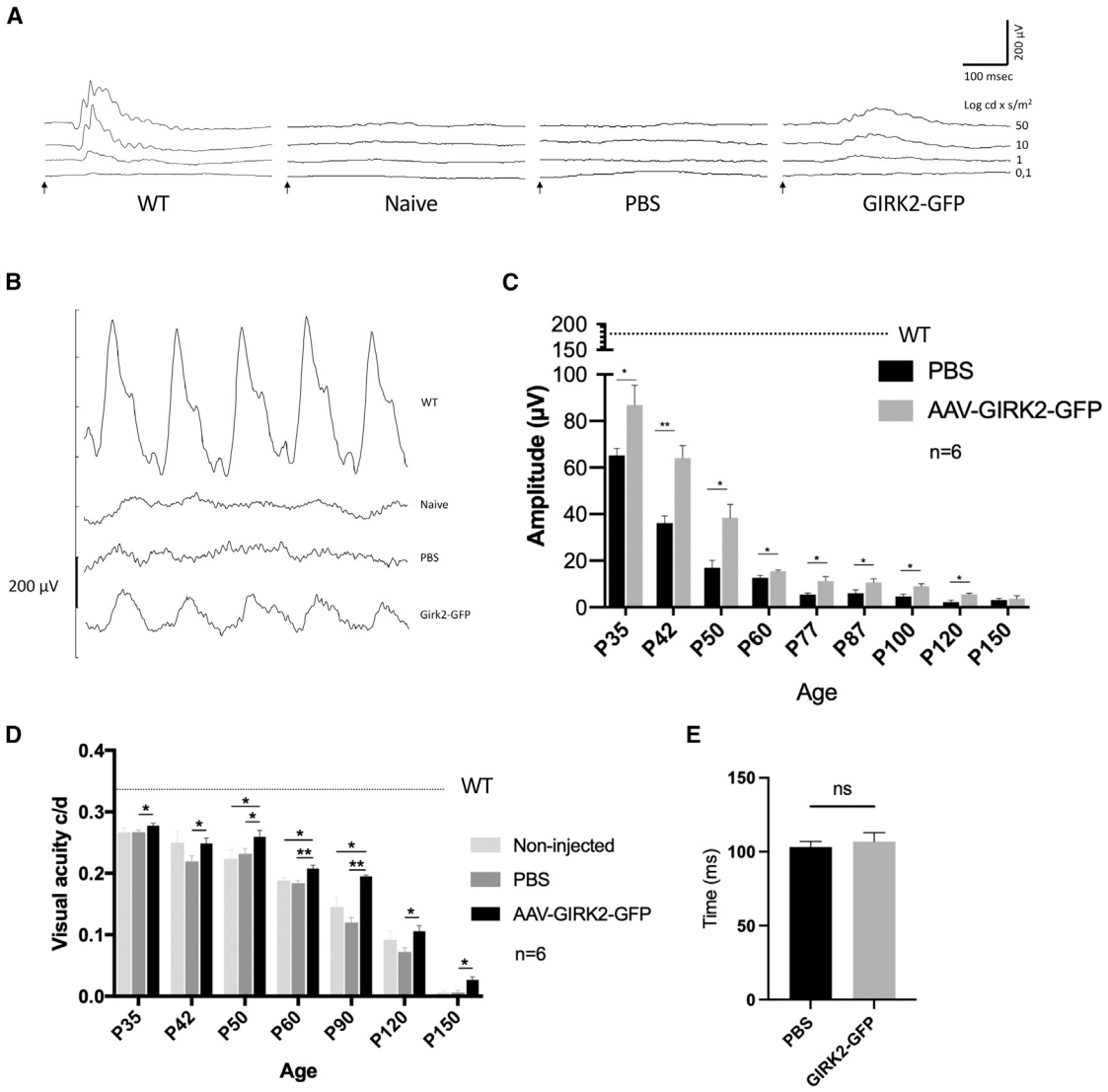


Figure 4. Long-term effect of GIRK2 gene transfer on visual responses of mice

(A) Representative photopic B-wave traces recorded from *rd10* mice injected with AAV-GIRK2-GFP compared with those injected with PBS-pluronic 0.001% or non-injected naive *rd10* mice at P50.

(B) Representative flicker ERGs recorded from wild-type (WT) mice and *rd10* mice injected with AAV-GIRK2-GFP, PBS-pluronic 0.001%, or non-injected naive *rd10* mice at P50.

(C) Photopic ERG amplitudes (50 cd s/m^2) in *rd10* mice injected with AAV-Girk2-GFP. Control eyes were injected with PBS-pluronic 0.001% (n = 6). Wild-type ERG mean ± SEM (dotted line) is for P60.

(D) Measure of the visual acuity by optokinetic reflex in *rd10* mice injected with AAV-GIRK2-GFP. Control eyes were injected with PBS-pluronic 0.001% (n = 6).

(E) Flicker latency of GIRK2-GFP-injected and sham-injected eyes plotted for P50. Comparisons between values used unpaired two-tailed non-parametric Mann-Whitney's test. Significance levels were set at $^*p < 0.05$, $^{**}p < 0.01$.

DISCUSSION

Previous work had shown the long-term presence of degenerating cones in RP patients and mouse models.[17–19] These cones are often referred to as "dormant cones" once their outer segment is significantly diminished. Such cones retaining the nucleus and inner segment and perhaps remnants of outer segments are also referred to as dysflective cones or more generally as remnant cones.[42] These cones may be the cells most likely to respond to therapies designed to prevent cell death and restore visual function.[39,44] Cone photoreceptor reactivation studies using optogenetics showed the feasibility of restoring vision in high light conditions using microbial opsin-based optogenetics.[17–19] However, the high light requirements and the potential immunogenicity of using an opsin from prokaryotic origin are inherent disadvantages of such an approach. An interesting alternative is the use of mammalian opsins for vision restoration; however, all work in this field has so far been focused on inner retinal

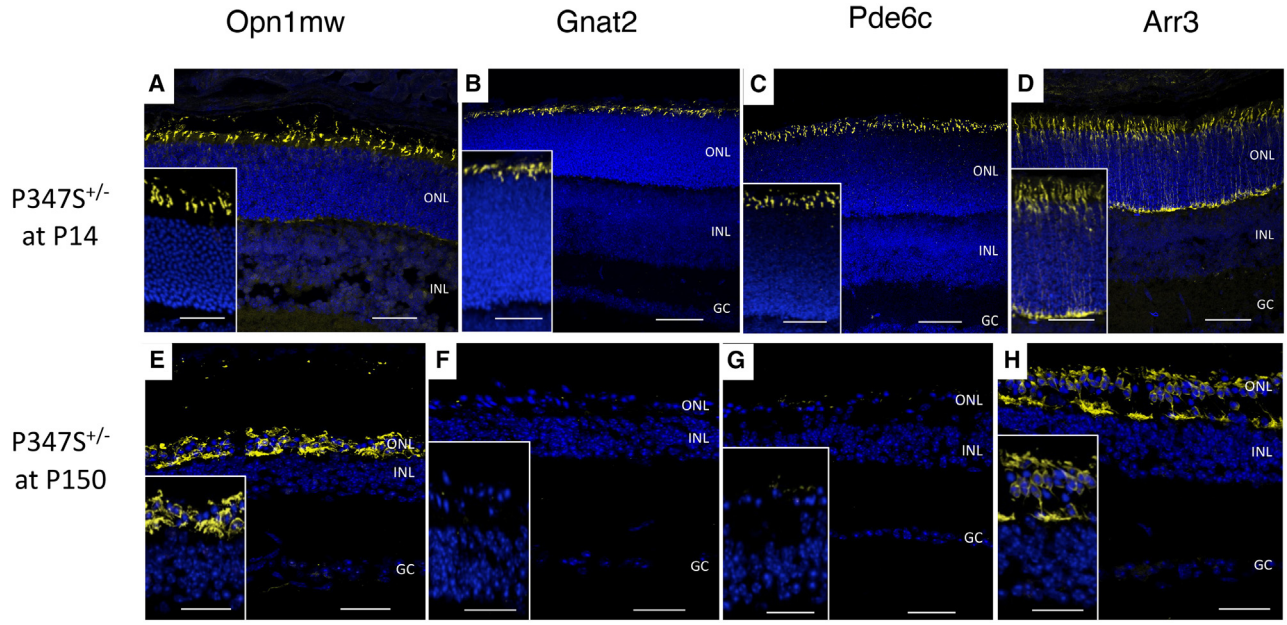


Figure 5. Immunohistochemistry against phototransduction cascade proteins in huP347S$^{+/-}$ mice at P150

(A–D) Retinal cross-section of a huP347S$^{+/-}$ mouse at P14 stained with DAPI (*blue*), (E) opsin (Opn1mw) (*yellow*), (F) transducin (Gnat2) (*yellow*), (G) phosphodiesterase (Pde6c) (*yellow*), and (H) cone arrestin (Arr3) (*yellow*).

(E–H) Retinal cross-section of a huP347S$^{+/-}$ mouse at P150 stained with DAPI (*blue*), (E) opsin (Opn1mw) (*yellow*), (F) transducin (Gnat2) (*yellow*), (G) phosphodiesterase (Pde6c), and (H) cone arrestin (Arr3) (*yellow*). ONL, outer nuclear layer; INL, inner nuclear layer; GC, ganglion cell. Scale bar, 50 μm. Inset scale bar, 25 μm.

neurons.[22,23,25,45–47] After exploring the phototransduction cascade in two RCD mouse models, we propose a new gene therapy strategy based on remaining endogenous cone opsins (Scheme 1). Indeed, we revealed that cone opsin and cone arrestin remain expressed in cone cell bodies at late disease stages both in mouse models of RP and in human RP patients. Based on this information, it is plausible to insert a channel acting via $G_{i/o}$ proteins recruited by the activation of the remaining opsin and thereby creating an alternative "short phototransduction cascade" within the cone photoreceptor (Scheme 1B). Such phototransduction cascade provides light responses as long as endogenous cone opsins are still present in viable cones. It may be possible to further increase these light responses by implementing other types of GIRK channels or mutants with higher K^+ conductance in follow-up development leading to clinical studies.[48] As for the mechanism of action, we propose that in early stages, although transducin is still present, the activation of the opsin by a light stimulus recruits the α subunit of transducin leaving the $\beta\gamma$ subunits available for activation of GIRK2 channels, generating additional hyperpolarization (Scheme 1B). Alternatively, or additionally in later stages when transducin is no longer present, the opsin can recruit other G proteins present in degenerating cones targeting GIRK2 channels, subsequently allowing the efflux of potassium ions at the resting membrane potential of degenerating cones.[17] K^+ efflux via GIRK2 channel hyperpolarizes cone photoreceptors in response to light modulating glutamate release and light responses in two RCD mouse models by ERG and OKT. Since remaining opsin in cone cell bodies is still functional within its regular spectrum, the insertion of GIRK2 in all cones with PR1.7 promoter leads to light responses following the spectral properties of each of the opsins, therefore allowing the preservation of color vision. We thus anticipate that our approach will provide, for the first time, color-vision restoration with both high acuity and acceptable light sensitivity.

A clear advantage of microbial opsins is their robustness and millisecond scale kinetics.[12,49] For systems using other opsins, it should be considered that in order to respond to another light stimulus, the cascade has to be deactivated to recover light sensitivity. In absence of this, cones may stay hyperpolarized after GIRK2 channel activation, limiting their ability to modulate synaptic transmission at a movie rate compatible with motion vision. In our case, signal termination in the cones was made possible, thanks to the cone arrestin that is still maintained at advanced stages of the disease in both RCD models and patient retinas. This is readily visible in the 10-Hz flicker ERG traces showing responses of the retina during repetitive light stimuli and also by the improved optokinetic reflex of treated mice. It is interesting to note that we observed a significant difference in response kinetics between our *in vivo* flicker ERG recordings and single-cell patch-clamp recordings from rd1 cones. The *in vitro* recordings displayed small amplitude and slow decay. This discrepancy may stem from several factors. For one, *in vivo* and *in vitro* environments differ in physiological conditions, neural network integrity, and adaptation states. *In vivo* conditions expose the retina to light patterns and intensities that maintain cones in a state suitable for rapid responses, whereas *in vitro* preparations are first screened for fluorescently labeled cells using high-intensity light prior to recordings. Furthermore, the rd1

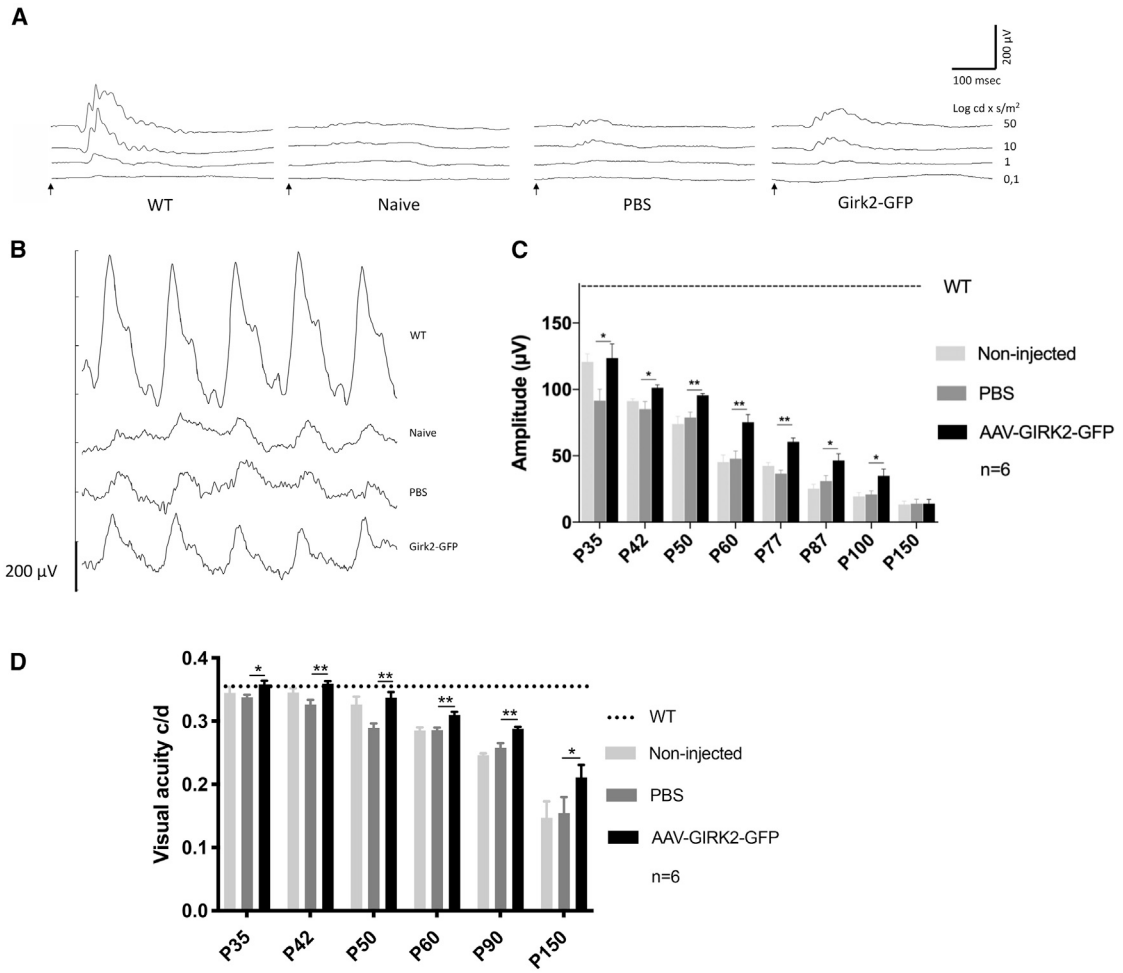


Figure 6. Effect of GIRK2 expression on retinal function of huP347Smice

(A) Photopic B-wave amplitude traces were significantly higher in *huP347S*$^{+/-}$ mice injected with AAV-GIRK2-GFP compared with those injected with PBS-pluronic 0.001% or non-injected naive *huP347S* mice at P60.

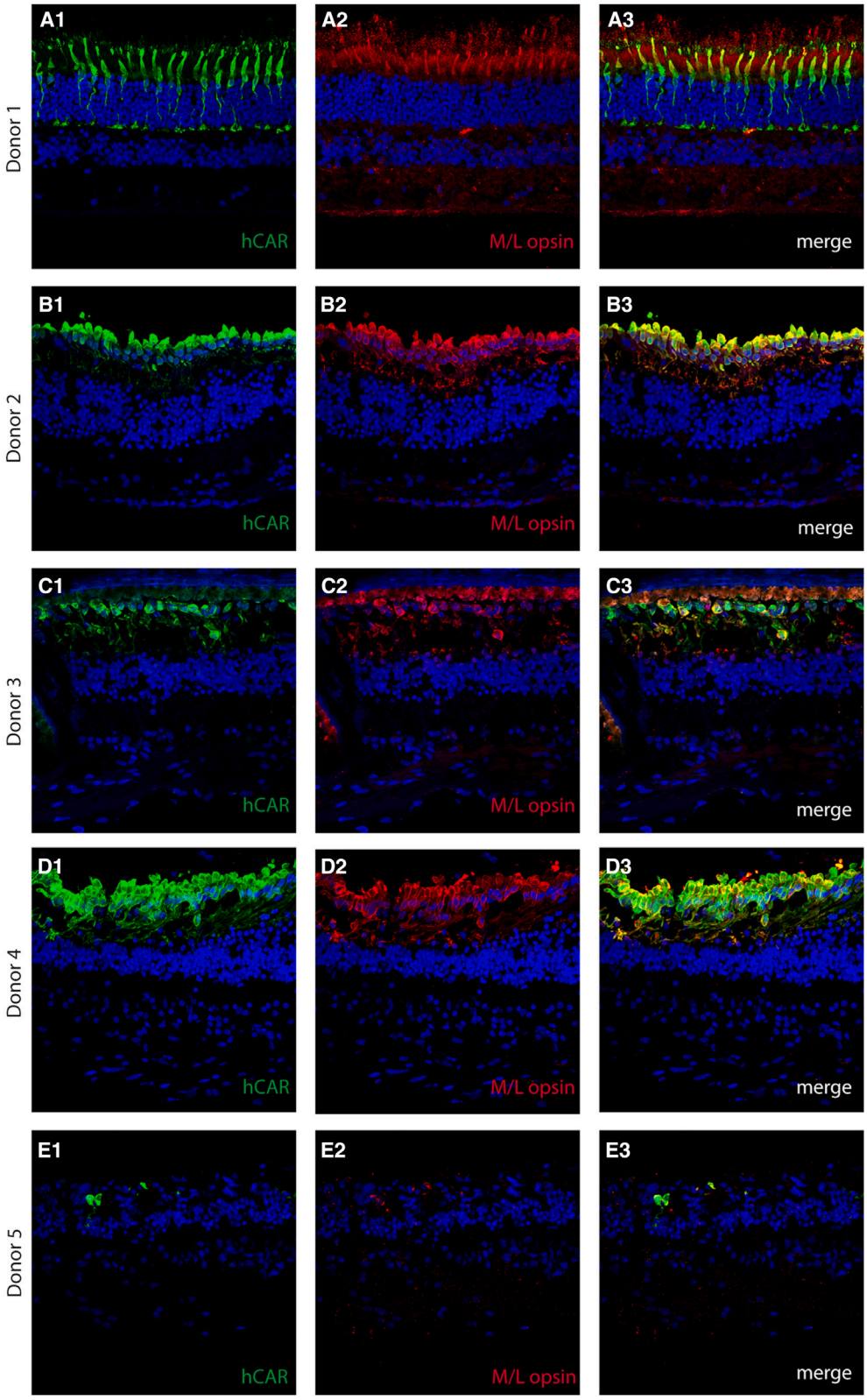
(B) Representative flicker ERGs recorded from wild-type (WT) mice and *huP347S*$^{+/-}$ mice injected with AAV-GIRK2-GFP, PBS-pluronic 0.001% or non-injected naive *huP347S*$^{+/-}$ mice at P60.

(C) Photopic ERG amplitudes (50 cd s/m^2) in *huP347S*$^{+/-}$ mice injected with AAV-Girk2-GFP. Control eyes were injected with PBS-pluronic 0.001% (n = 6).

(D) Measure of the mean ± SEM visual acuity by optokinetic reflex in *huP347S*$^{+/-}$ mice injected with AAV- GIRK2-GFP. Control eyes were injected with PBS-pluronic 0.001% (n = 6). Comparisons between values used unpaired two-tailed non-parametric Mann-Whitney's test. Significance levels were set at *$p < 0.05$, **$p < 0.01$.

cones used in single-cell recordings degenerate more rapidly than the rd10/hRHO cones used in *in vivo* studies. For example, in isolated retina preparations, chromophore replenishment is slower due to the absence of RPE and reduced Müller cell functionality. This effect may be more pronounced in rd1 cones due to reduced RDH12 expression. The rd1 and rd10 mouse models also exhibit different timelines of retinal degeneration, which introduces age as a variable when comparing their single-cell transcriptomic and electrophysiological profiles. In rd1 mice, retinal degeneration begins very early, with rod photoreceptor death starting around postnatal day 8 (P8) and progressing rapidly to complete degermation in the periphery at P18, leading to almost complete loss of rods by P21. Cones start to degenerate at P25.[50] In contrast, rd10 mice show a slower progression, with rod degeneration beginning around P18 and continuing over several weeks with central photoreceptor loss by P45.[51] This difference in degeneration timelines means that when performing single-cell transcriptomics or electrophysiology, the retinal cells in rd1 and rd10 mice are at different stages of the degenerative process at any given age. For example, at P21, rd1 retinas would be in an advanced stage of degeneration, whereas rd10 retinas would be in early stages. This temporal disparity can lead to differences in gene expression profiles and electrophysiological responses that are not solely due to the genetic mutation but also reflect the different stages of degeneration and cellular stress responses.

Our observations of "dormant cones," i.e., degenerating cones with diminished outer segments and light sensitivity, in



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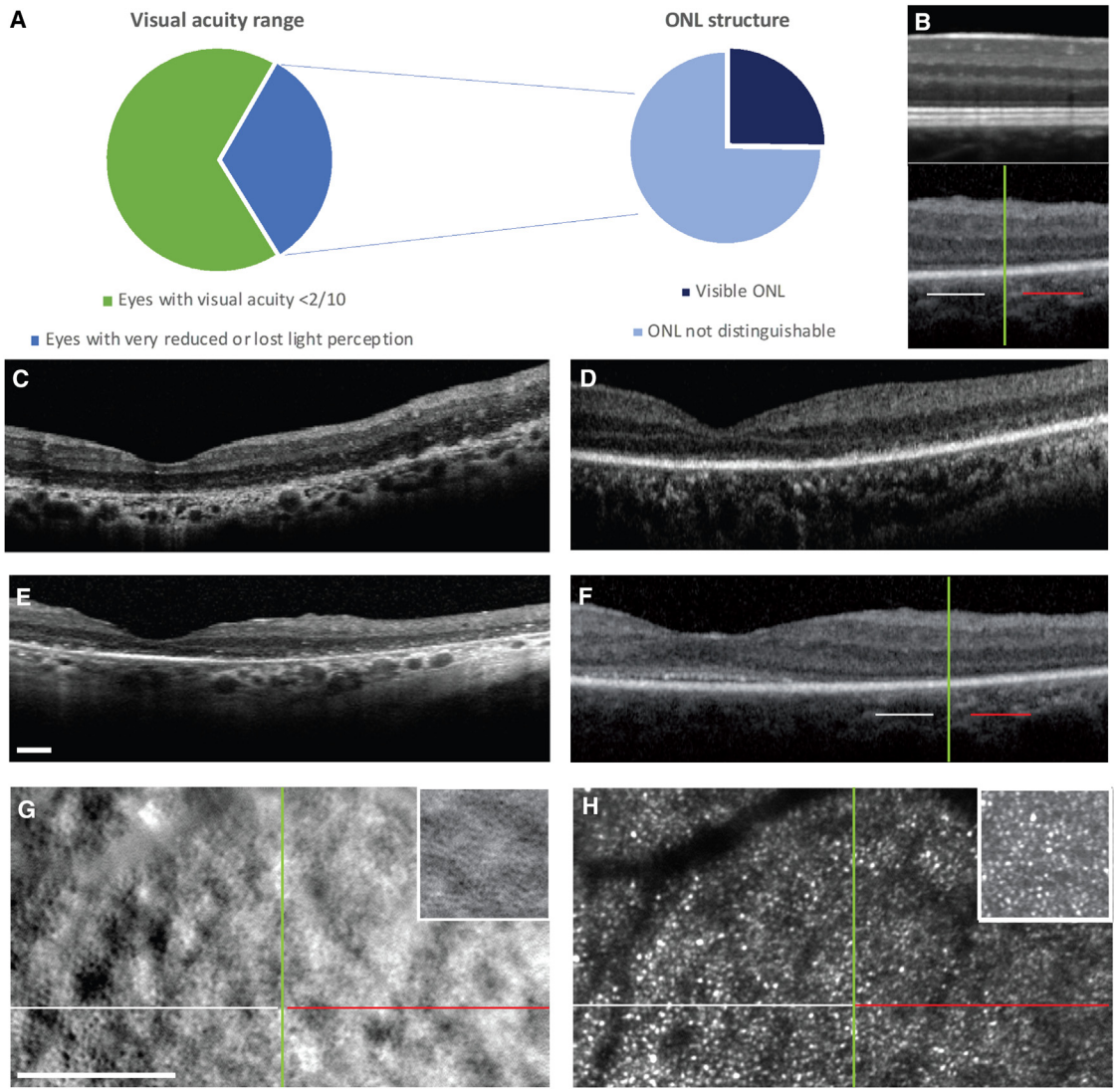


Figure 8. Phenotyping RP patients to define an eligible population for GIRK2 gene therapy

(A) Proportion of retinitis pigmentosa patients with very reduced or lost light perception and have a detectable ONL filled with diminished outer segment cone photoreceptor cells.

(B) Zooms on OCT scans of a healthy retina (top) versus the patient shown in (F–H) suffering from retinitis pigmentosa (bottom). The green line on the OCT cross-section marks the transition between a zone with external limiting membrane, implying some residual outer segment structure (white bar) and a zone with absent external limiting membrane (red bar).

(C–F) Representative OCT scans from the left eye of four RP patients (aged 32–77 years) suffering from retinitis pigmentosa.

(G and H) AOSLO images over the same zone shown in (B) on a retinitis pigmentosa patient, with inserts from a healthy subject for comparison. Split detection (G) and confocal (H) modalities show transition from clear cone mosaics (white bar) suggesting cones with some residual structure maintaining them in a mosaic packing to presumed damaged cones with no clear mosaic visible (red bar) over this region. Scale bar, 200 μm.

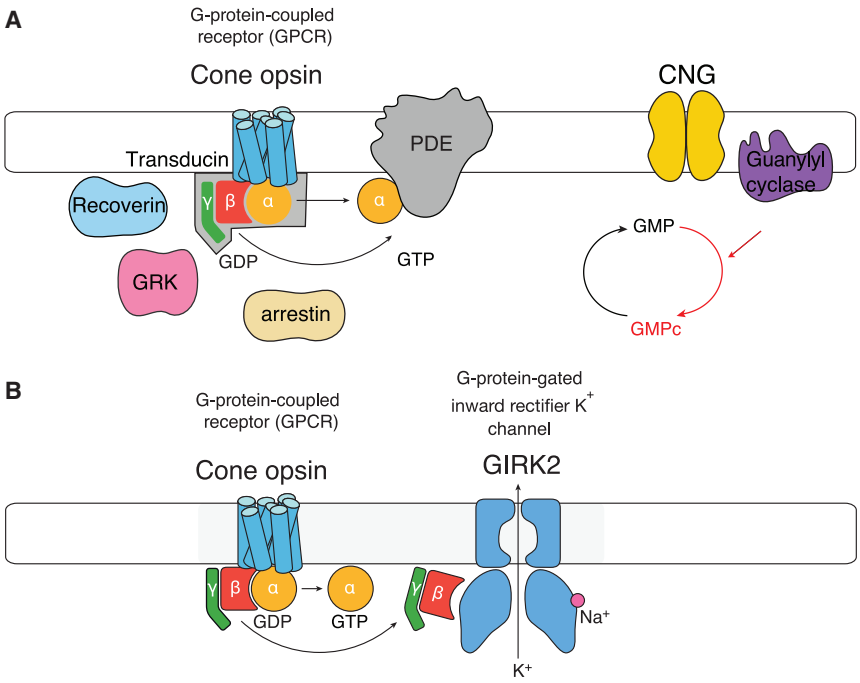
human RP retinas is consistent with previous reports.[4,17,29,52,53] Li et al.[4] reported somata with very short or absent outer segment (OS) reactive for cone opsins, recoverin, and transducin-∝ cones in several RCD patients, whereas Lin et al.[29] also found cones with abnormal, diminished OS that were positive for recoverin immunostaining in one RCD patient. Busskamp et al.[17] showed the presence of such cones using OCT in RCD patients. We corroborated these findings in our patient cohort.

Figure 7. Cone opsin and arrestin expression in normal and RCD human retinal tissue

Human cone arrestin (Arr3) and M/L opsin immunostainings are shown in green and red, respectively. Nuclei are stained with DAPI, in blue.

(A) Retinal cross-section of a 91-year- old individual with no visual impairment (40x).

(B–E) Retinal cross-sections of human RCD maculae from four different donors (40x).



Scheme 1. Mechanism of action of GIRK mediated cone light response

(A) Normal photranduscton cascade elements in cones.

(B) Proposed mechanism of action of GIRK mediated reactivation of cone opsin signaling.

In postmortem retinas from human patients with RCD, due to the limited number of samples we were not able to stain for the additional cascade elements (PDE, Gnat2, and CNG channel expression) showing similar reduction to mouse studies. Despite this limitation, we clearly showed the presence of dormant cones in the maculae of three of four RP patients studied. Importantly, we found co-expression of both cone opsins and cone arrestin in the same cells, which supports our GIRK2 gene therapy approach as these two phototransduction cascade proteins are required for our cone reactivation strategy using GIRK2. Last, the fact that incorporation of GIRK2 enhances existing light responses in cones even prior to complete outer segment loss offers the possibility to implement this gene therapy in mid stages of the disease.

Based on our investigation of the proportion of potential eligible patients in our RCD cohort from the Quinze-Vingts hospital, we found that roughly one-quarter of RP patients with low to no light perception can be eligible for GIRK2 therapy to restore light sensitivity in their dormant cone population. Examination of the inner and outer segment structures in RCD patients using AO imaging revealed refractive changes in the inner and outer segments of the foveal cones, suggesting it is possible to distinguish between cones with diminished outer segments, inner segments, and light insensitive "dormant" cone populations using this technique. The combination of the above-mentioned imaging techniques along with more recently described indicators of cone cell function (such as fundus autofluorescence imaging[43]) can be used to select patient populations for this type of gene therapy.

Despite functional improvements in GIRK2 expressing cones, our treatment does not stop the degeneration of cones. GIRK2 expression might have a protective effect on overall retinal function in earlier stages of RP, possibly through network-level effects[39] and/or by preserving the function of cones with diminished outer segments. However, in more advanced stages of degeneration, the direct effects of GIRK2 on highly compromised cones might not be as beneficial at the single-cell level as seen by the slower response kinetics of late-stage rd1 cones. However, retinal degeneration in mice is much faster than in humans, thus a few months of therapeutic efficiency in mice may be equal to several years in humans. We recorded a decrease in the response of treated cones to light stimuli, which was consistent with decrease in cone numbers and the fact that we did not transduce all cones due to subretinal injection further limiting the beneficial effect. AAV vectors showing better lateral spread can be used to increase transduced cone numbers beyond the bleb.[19] In order to increase the therapeutic window, neurotrophic factors can be implemented alongside our approach. Indeed, AAV-mediated secretion of neurotrophic factors such as the rod-derived cone viability factor (RdCVF) has been shown to delay cone cell death and may be combined with GIRK2-mediated sensitization.[5]

Guanine-nucleotide-binding proteins act as molecular switches inside a multitude of cells.[54] They have a seven-transmembrane domain receptor, and their activity is regulated by factors that control their ability to bind and hydrolyze GTP to GDP. The most commonly identified G proteins in the retina are heterotrimeric and are composed of three subunits (α, β, γ). They are activated by light-sensitive G-protein-coupled receptors (GPCRs) such as rhodopsin or cone opsin and transmit the message by activating other proteins in the cascade. In photoreceptors, the G protein that activates the cascade (transducin) belongs to the $G_{i/o}$ family, inhibiting the production of cyclic guanosine monophosphate (cGMP) from GTP and therefore inducing hyperpolarization of photoreceptor membrane and a subsequent decrease of glutamate release.[55] Recently, the expression of a medium-wavelength-sensitive cone opsin in the retinal ganglion cells has been shown to restore high light-sensitive vision with adaptation.[21] Even though the G-protein-coupled mechanism used by cone opsin in retinal ganglion cells remains unknown at this stage, such results further show the potential of vertebrate cone opsins in vision restoration. They also suggest that cone opsins are not specific to a single type of G protein with the ability to activate target channels in different neurons.[56] Various ion channels can be activated via a G protein starting with the light-stimulated opsin. These findings altogether lead us to believe that our combined approach with GIRK2 or a similar dual approach with a target channel and opsin could be

implemented in other subtypes of neurons broadening the field of applications toward other targets.[28]

Limitations of the study

Although the proposed GIRK2-based gene therapy demonstrates significant potential for restoring light sensitivity and color vision in degenerating cones, several limitations should be considered. First, although the study highlights the retention of cone opsins and cone arrestin in dormant cones, it does not fully address whether all key components of the phototransduction cascade required for sustained cone function are preserved in advanced stages of retinal degeneration, particularly in human RP patients. The slower kinetics observed in *in vitro* single-cell recordings compared to *in vivo* ERG responses underscore the challenges of replicating physiological conditions *ex vivo*, where factors such as chromophore replenishment and neural network integrity are diminished. Additionally, the limited transduction efficiency of the AAV vector due to the subretinal injection and incomplete cone coverage may restrict the therapeutic efficacy, particularly in advanced degeneration. The study also does not halt the underlying degenerative process, which suggests that combining GIRK2 gene therapy with neuroprotective approaches, such as neurotrophic factors, may be necessary to prolong therapeutic benefits. Lastly, the applicability of these findings to humans remains uncertain due to differences in the timeline and progression of retinal degeneration between mouse models and human patients, necessitating further validation in preclinical and clinical studies to confirm long-term safety and efficacy.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to Deniz Dalkara (deniz.dalkara@gmail.com).

Materials availability

This study did not generate unique reagents or mouse lines. The HEK cell line stably expressing mOpn4L can be obtained by contacting Pr Stefan Herlitze (Stefan Herlitze sxh106@googlemail.com). All other materials used were commercially available or from established research repositories.

Data and code availability

- Data: our RNAseq data are publicly available here: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE286529.
- Code: no codes were generated for this study.
- Other resources: all relevant accession codes can be found in the key resources table.

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AUTHOR CONTRIBUTIONS

C.J.S. performed literature review, conducted experiments including mouse injections and *in vivo* recordings, cone counting on mouse retinas, histology on huP347S+/− retinas, acquired data, analyzed data, and contributed to write the manuscript. M.F. performed histology on rd10 retinas. H.K. performed histological analysis of postmortem human retinas and contributed to RT-PCR experiments, figures, and text to the manuscript. A.C. performed electrophysiological recordings. K.G. and N.N. performed Adaptive Optics experiments on patients and contributed to figures and text to the manuscript. A.G. and I.A. analyzed the cohort of patients with a visual acuity below 2/10. D.E. modeled the GIRK2 channel. M.R.—a former master student—acquired ERG recordings. M.D. designed cloning constructs. A.G. and S.S. acquired OCT and visual acuity data from RP patients in the Quinze-Vingts hospital (Paris). V.L.B. sampled human retinas. S.M.S. and I.A. provided and cared for selected study patients. I.A. and A.G. critically reviewed the patient study proposal and collected patient data. M.P., S.P., J.A.S., J.D., and S.H. provided scientific input and advice on the project and manuscript. D.D. designed and supervised the experiments and wrote the manuscript.

DECLARATION OF INTERESTS

D.D. and H.K. are inventors on patent applications on noninvasive methods to target cone photoreceptors (EP17306429.6 and EP17306430.4) licensed to Gamut Tx (now SparingVision). D.D., H.K., I.A., and S.P. are founders of Gamut Tx (now SparingVision). D.D. is a consultant for SparingVision. D.D., C.J.S., and S.H. are inventors on pending patent application (EP20315148.5) on G-protein-gated K-channel-mediated enhancements in light sensitivity in rod-cone dystrophy. J.A.S. is a founder of SparingVision, Prophesee, Tilak, Chronolife, Gensight, SharpEye, Cilensee, and GenSight Biologics and is a founder and consultant for Tenpoint, Avista. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti opsin red/green	Millipore	AB5405
Rabbit anti mouse cone arrestin (Arr3)	Millipore	AB15282
Mouse anti Human Cone arrestin (ARR3)	Gift from Dr. Peter McLeish	N/A
Rabbit anti Pde6c	Antibodies online	ABIN954067
Rabbit anti Gnat2	SantaCruz Biotechnology	sc-390
PNA-Lectin with FITC	Vector Laboratories	FL-1071
Biological samples		
Donor ID	age	Eye disease
1	91	None
2	92	Recessive RP
3	73	RP
4	74	RP
5	77	RP with blindness
Deposited data		
Single Cell RNA sequencing	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE286529	GSE286529
Experimental models: Cell lines		
HEK293 cells stably expressing mouse melanopsin (mOpn4L) fused to mCherry	VectorBuilder	N/A

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mouse models

Two mouse models were used in this study: C57BL/6j, rd10/rd10 (rd10) mice carrying a mutation in Pde6b gene expressed by rods, leading to a dysfunctional phototransduction cascade and rod-cone dystrophy, and huP347S+/− mice *(45)*, obtained by crossing homozygous males (KO of mouse rhodopsin Rho gene and KI of mutated human rhodopsin RHO gene with P347S mutation) with C57BL/6j (wild type) females.

Cell lines

HEK293 cells stably expressing mouse melanopsin (mOpn4L) fused to mCherry were obtained from VectorBuilder (https://en.vectorbuilder.com/). They were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, and 1% geneticin at 37°C and 5% CO2 in a humidified incubator.

Human subjects

The human retinal tissue specimens described in this study were originally collected through funding from the Foundation Fighting Blindness (FFB). While FFB no longer supports this collection, the specimens continue to be maintained, characterized, and processed at the Cole Eye Institute under the supervision of Dr. Vera Bonilha. Due to the significant costs associated with maintaining these valuable specimens, access to these materials is available through scientific collaboration. Researchers interested in accessing these specimens should contact Dr. Bonilha (BONILHAV@ccf.org).

Written informed consent was received from all participants prior to their inclusion in the eye imaging study.

METHOD DETAILS

AAV administration

Mice were first anesthetised with isoflurane inhalation (5% induction and 2% sustain). Eyes were dilated with 8% Neosynephrine (Neosynephrine Faure 10%, Europhta) and 42% Mydriaticum (Mydriaticum 0,5%, Thea) diluted in 0.9% NaCl. Then eyes were

protected with Lubrithal eye gel (VetXX). A total volume of 1μl of vector solution was injected subretinally with a Hamilton syringe, in the dorsal-temporal area of the retina. Different mice were used for ERG recordings (n=6) and OKT recordings (n=6). Exclusion criteria for subretinal injections were hemorrhages, absence of retinal detachment and lens damage. Fradexam, an ophthalmic ointment, was applied after injection.

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QUANTIFICATION AND STATISTICAL ANALYSIS

Cell counting

Flat mount retinas were analyzed for DAPI+ Arr3+ cells using FIJI software. Counts were performed manually using z-stack reconstructions covering the entire outer nuclear layer thickness.

Statistical methods

Data was analyzed using GraphPad Prism and expressed as mean ± SEM. Comparisons between values used unpaired two-tailed non-parametric Mann-Whitney's test. Significance levels were set at $p < 0.05$, $p < 0.01$, and $p < 0.001$.

Study approval

The human and animal studies described in this work was approved by the appropriate institutional review boards. The animal studies were conducted in accordance with ARVO guidelines and approved by the local ethics committee Charles Darwin. For human studies, written informed consent was received prior to participation.