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PKM2 ablation enhanced retinal function and survival in a preclinical model of retinitis pigmentosa

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Conflicts of Interest

There are no conflicts of interest to disclose for any author.

Data Availability

All data in this manuscript are available for share.

ABSTRACT

Retinitis pigmentosa (RP) is a neurodegenerative disorder that causes irreversible vision loss in over 1.5 million individuals world-wide. In this study, we demonstrate that a metabolic reprogramming can treat degeneration in a *Pde6 β* preclinical model of RP. Pyruvate kinase M2 (PKM2) is a glycolytic enzyme that transfers phosphate from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP), promoting glucose catabolism. Ablation of PKM2 resulted in enhanced photoreceptor survival and function in *Pde6 β* -mutated mice compared with those without ablation. Electroretinogram (ERG) analyses revealed that the maximum b-wave is on average greater in *Pkm2* knockout mice than in mice with *Pkm2* intact. These rescue phenotypes from *Pkm2* ablation in a preclinical model of RP indicate that a metabolome reprogramming may be useful in treating RP.

INTRODUCTION

Retinal degenerative diseases affect nine million Americans.^{1,2} Among these conditions, retinitis pigmentosa (RP) is the most devastating in terms of severity and prevalence, making it one of the most common cause of irreversible vision loss. Associated with more than 80 genes, RP is a heterogeneous condition that is characterized by rod photoreceptor degeneration followed by secondary cone death,³⁻⁵ leading to night blindness, tunnel vision, and eventually, complete blindness. Current treatment options include the use of retinal prostheses^{6,7} or *RPE65* gene therapy.⁸⁻¹⁰ However, these strategies are only applicable for patients with end-stage RP or those carrying *RPE65* mutations,¹¹ respectively. At the same time, clinical gene therapy trials for RP primarily involve augmentation or repair of a single gene, which even if successful, would only be applicable to patients carrying those specific gene mutations.¹² More recently, insight into the genetic etiologies of retinal degeneration has found that photoreceptor death may be linked to apoptotic factors,^{13,14} metabolic derregulation,^{3,15} and oxidative stress.^{16,17} Targeting any one of these pathways, in turn, may offer an alternative gene non-specific therapy. As of yet, however, there is no effective therapeutic option for RP and other retinal degenerative diseases.

Punzo *et al.* studied gene expression changes in RP mouse models by linking mutations in rod-specific genes to their corresponding cellular processes.³ Microarray analysis of samples

collected at the onset of cone death revealed that 35% of annotated genes were involved in cellular metabolism, implicating metabolic processes in disease pathogenesis. To follow-up, Punzo *et al.* and Zhang *et al.* focused on the insulin/mTOR pathway and found that enhanced anabolic flux and lactate levels indeed slowed rod-cone degeneration.^{18,19} Consistently, rod-specific knockout of sirtuin 6, a transcriptional repressor of glycolytic enzymes, enhanced glycolysis, rod preservation, and photoreceptor function.²⁰ Augmenting anabolism in rod photoreceptors could therefore serve as a potential treatment for RP.

One identified key regulator of cellular metabolism is pyruvate kinase M2 (PKM2), a critical catalyst for the final step of glycolysis that aids in the transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP). PKM2 is allosterically regulated so that it exists in two forms: an inactive dimer with low binding affinity to PEP and an active tetramer form with high affinity to PEP.²¹ When phosphorylated PKM2 is in its inactive form, less consumption of PEP occurs, resulting in higher PEP levels. This has been found to activate the pentose phosphate pathway (PPP), a process that produces glycolytic intermediates for nucleotide and lipid synthesis. As a result, when PKM2 is inhibited, subsequently, PEP accumulates, promoting the PPP.²² PKM2's ability to regulate the production of glycolytic intermediates, which is essential for photoreceptor cells, makes it an attractive target to elucidate the role of glycolysis in rod and cone health. Previous studies have already found that PKM2 ablation enhances long-term photoreceptor survival in retinal detachment models, suggesting that PKM2 ablation may offer photoreceptor protection against acute external retinal stress.²³

More recently, PKM2 has been implicated in the regulation of PDE6 β ,²⁴ a phototransduction enzyme complex that blocks Ca²⁺ influx by closing cyclic nucleotide-gated (CNG) channels through cGMP hydrolysis. PDE6 is a heterotetramer with two catalytic subunits (PDE6A and PDE6B) and mutations in either can disrupt function similarly and yield identical downstream effects.²⁵⁻³⁷ One in 10 Americans carries a recessive RP allele,^{27,38} and 10% of those alleles confer mutations in one of the two subunits of phosphodiesterase-6 (*Pde6*), accounting for 5-8% of all RP cases.^{27,28} As such, PKM2 may very well also play a role in preserving photoreceptors from inherited retinal diseases, which represent internal sources of retinal stress.

In this report, we expand on previous findings by investigating the functional and morphological contributions of rod-specific *Pkm2* ablation in inherited retinal degeneration with a tamoxifen-inducible *Pkm2* knockout *Pde6 β _{H620Q/H620Q}* RP model. We hypothesize that *Pkm2* knockout in the rods will promote photoreceptor anabolism and, in doing so, preserve rod and cone survival and function, delaying retinal degeneration.

RESULTS

Deletion of *Pkm2* in *Pde6 β _{H620Q/H620Q}* mice

To evaluate whether and, if so, how PKM2 plays a role in rod metabolome reprogramming, we used the tamoxifen inducible Cre recombinase system to ablate *Pkm2* in a slow progressing *Pde6 β* RP model: *Pde6 β _{H620Q/H620Q}*.²⁹ Rod selective ablation of *Pkm2* significantly reduced total PKM2 levels in *Pde6 β _{H620Q/H620Q} Pkm2^{-/-}* mice compared to wild-type mice (Fig. 1).

Accordingly, there was also a reduction in phosphorylated PKM2 in *Pde6 β _{H620Q/H620Q} Pkm2^{-/-}* mice, suggesting a decrease in the catalytic activity of PKM2. It is important to note, however, that experimental mice retained detectable levels of PKM2 and P-PKM2, shown in Figure 1, due to the conditional nature of the rod-specific *Pkm2* knockout.

Deletion of *Pkm2* in *Pde6 β _{H620Q/H620Q}* mice improved retinal morphology

Subsequently, we gauged the survival impact of *Pkm2* ablation on retinal morphology using histology to compare the morphology of the outer nuclear layer (ONL), inner segment (IS), and outer segment (OS). *Pde6 β _{H620Q/H620Q} Pkm2^{+/+}* mice exhibited degenerated photoreceptors within ONL while thin ONLs while *Pde6 β _{H620Q/H620Q} Pkm2^{-/-}* mice had comparatively more cells (Fig. 2A). Similar trends were observed for IS/OS layers—compared to their isogenic controls, treated mice exhibited greater IS/OS thickness throughout the retina (Fig. 2A, B). Strikingly, all the layers in the experimental mice were still noticeably thinner than those in wild-type mice. Overall, ablation of *Pkm2* may be able to preserve but not completely rescue photoreceptor survival and preserved photoreceptors.

Deletion of *Pkm2* in *Pde6 β _{H620Q/H620Q}* mice improved retinal function

At four weeks post-treatment, *Pde6 β _{H620Q/H620Q} Pkm2^{-/-}* mice exhibited an enhanced scotopic b-wave amplitude compared to *Pde6 β _{H620Q/H620Q} Pkm2^{+/+}* (Fig. 3A), suggesting greater rod

preservation. In contrast, there was no significant difference in the mixed and photopic b-wave amplitudes between the two groups, indicating that cone preservation remained approximately the same. At eight weeks post injection, all b-wave amplitudes in both groups declined considerably. Notably, *Pde6 β ^{H620Q/H620Q} Pkm2^{-/-}* mice maintained significantly higher scotopic, maximal, and photopic b-waves ($p < 0.05$) compared to *Pde6 β ^{H620Q/H620Q} Pkm2^{+/+}* mice, indicating a more robust preservation in cone and rod function. Consistently, statistical analysis revealed a significant correlation ($p < 0.05$) between the value of the maximal b-wave and presence of tamoxifen treatment. These results reveal that rod-specific *Pkm2* ablation improved photoreceptor vitality in *Pde6 β* -mutated mice.

DISCUSSION

In this report, we demonstrated that rod-specific ablation of *Pkm2* can rescue both cone and rod photoreceptor degeneration. Using both structural and functional assays, we are the first to use a preclinical RP model to study the effects of *Pkm2* knockout. These findings further advance the work done by Chinchore *et al.* who first discovered that PKM2 mediates Warburg glycolysis in healthy photoreceptors, ³⁹ implicating its role in photoreceptor wellbeing. Since then, various studies have sought to elucidate a direct cause-and-effect relationship. Notably, Wubben *et al.* observed that photoreceptor-specific ablation of *Pkm2* impaired photoreceptor survival and function under baseline conditions, but improved long-term photoreceptor survival under acute outer retinal stress. To account for the rescue in the retinal detachment model, Wubben *et al.* noted the upregulation of *Pkm1* and other genes involved in aerobic metabolism, proposing that accumulation of PK substrate 2-phosphoenolpyruvate was sufficient to counteract the insult from iatrogenic cell damage.

Here, we advance the current understanding of PKM2 by uncovering its genetic contributions in inherited retinal degeneration. Notably, rod-specific *Pkm2* knockout noticeably increases the thickness of the ONL and IS/OS layers, indicating better photoreceptor survival. ERG functional analysis was consistent with anatomical findings and revealed that *Pkm2* ablation also improved both cone and rod function significantly. These results suggest that PKM2 deficiency was indeed able to rescue retinal degeneration. As proposed by Wubben *et al.*, the rescue phenotypes may be a direct result of enhancing catabolism and therefore ATP output in photoreceptors. This

explanation, however, handles photoreceptors as isolated systems whose metabolism works in a cell autonomous manner. In this paper, we propose an alternative yet complementary framework that aims to consider PKM's role in photoreceptors in relation to the RPE, offering insight into how it may regulate the delicate metabolic dynamic between the two.

One of the first evidences of a metabolic coupling was described by Punzo, *et al.*, who found that the disruptions in the insulin/mTOR signaling pathway coincided with cone death in four mouse models for RP.³ Strikingly, systemic supplementation of insulin was able to rescue cone death, suggesting that photoreceptor death in RP progresses in a cell non-autonomous manner.

Subsequent studies characterizing the nature of this relationship have led to the emergence of the “metabolic coupling hypothesis”, which proposes that metabolic imbalances leads to cone photoreceptor death via glucose deprivation.^{3,19,40} Normally, photoreceptors take up glucose from the retinal pigment epithelium (RPE) and convert it into fat and lactate,⁴¹ which are subsequently used to drive essential processes throughout the retina. In photoreceptors, the steady production of protein and lipids drives the daily disposal and regeneration of the OS.^{42,43} In contrast, the RPE consumes the generated lactate as the carbon source for the tricarboxylic acid (TCA) cycle,^{39,44,45} saving glucose for photoreceptors. In RP, however, impaired photoexcitation prevents diseased rods from generating sufficient amounts of lipid and lactate. This not only stagnates OS biogenesis but also prompts the RPE to use glucose through anabolism. The depletion of glucose by the RPE, in turn, deprives photoreceptors of nourishment and leads to prevalent rod and subsequent cone death – the hallmark of late-stage RP.

In light of this framework, the rescued phenotypes indicate a rejuvenation of the disrupted metabolic dynamic within the diseased retina. Previous studies have shown that phosphorylated PKM2 leads to the accumulation of 2-phosphoenolpyruvate and the subsequent activation of the anabolic PPP pathway.²⁴ As such, PKM2 deficiency may elevate anabolism and the synthesis of proteins and lipids, fueling the regeneration of the OS and promoting oxidative phosphorylation in the RPE. This metabolic reprogramming, in turn, restores a steady supply of glucose to the diseased photoreceptors, precluding the need to optimize metabolism. As a result, the photoreceptors return to a preferred anabolic state and employ aerobic glycolysis to satisfy their ATP needs, promoting photoreceptor health and function. It is important to note that this model

is not necessarily incompatible with the notion of catabolism-mediated photoreceptor survival as described by Wubben *et al.* Roughly 80%-90% of the glucose in photoreceptors is metabolized through aerobic glycolysis, indicating that a small, but significant, fraction still undergoes catabolism. While we highlight that different anabolic processes can provide photoreceptors with both the raw materials and energy to survive and function, it is possible that a certain level of catabolism is needed to sufficiently meet the high energy demands of a healthy photoreceptor and counteract the insult from metabolic imbalance.

Another notable finding is that despite the significant improvements in both retinal morphology and function, PKM2 ablation was not able to induce a complete rescue in either case.

Pde6 β ^{H620Q/H62Q} Pkm2^{-/-} mice exhibited thinner ONLs than that of wildtype mice, indicating increased photoreceptor death. Moreover, the retinal function of *Pde6 β ^{H620Q/H62Q} Pkm2^{-/-}* mice decreased after the initial improvement. Both these findings are likely a result of the short-lived increase in anabolism from *Pkm2* ablation. It has been suggested that PKM1 mediates ATP synthesis by promoting catabolism in the absence of PKM2.^{23,24} However, Rajala *et al.* recently demonstrated that the upregulation of PKM1 from *Pkm2* deletion is unable to match the catalytic activity of endogenous PKM2, suggesting that PKM1 cannot compensate for PKM2 deficiency.²⁴ Bringing these findings together, it is possible that PKM1 upregulation interferes with the anabolic influx from PKM2 inhibition and promotes catabolism. The resultant inhibition of anabolism deprives photoreceptors of essential building blocks, leading to the observed decrease in ONL thickness. At the same time, PKM1 is unable to generate sufficient energy to sustain photoreceptor function, resulting in the decrease in ERG response over time. Alternatively, it is likely that retinal degeneration impacts a wide range of independent metabolic pathways. As such, PKM2 ablation is only able to affect PKM2-dependent processes, leading to a partial but not complete rescue of the deleterious phenotypes.

Although this study identifies PKM2 as one of the major players in slowing down retinal degeneration, future studies identifying specific molecules and pathways would be beneficial in understanding the mechanism of this relationship. Wubben *et al.* offered critical insight into the specific genes impacted by PKM2 ablation in the retina.²³ Expanding their analysis to include gene expression changes in the RPE offers a quantitative way to not only support PKM2's role in

regulating metabolic coupling, but also track the metabolic changes over time. Another promising direction for research is to characterize PKM1's role and whether its interaction with PKM2 is conserved in inherited retinal disease. This question is important as it would clarify whether PKM1 indeed acts as an antagonistic regulator of anabolic influx that may limit the impact of metabolic reprogramming. Other potential directions include tuberous sclerosis complex 1 (TSC1), an mTOR inhibitor, and hypoxia inducible factor-1 α (HIF-1 α), a transcription factor responsible for oxygen consumption regulation. In our earlier studies, we showed that both of these molecules are linked to photoreceptor rescue from metabolic reprogramming.^{19,46} Moreover, separate groups have found that PKM2 interacts directly with mTOR and HIF-1 α , hinting towards possible mechanistic pathways.⁴⁷⁻⁴⁹

One important consideration is that Rajala *et al.* have shown *in vitro* evidence that PKM2 may regulate transcriptional activity through the PDE6 β promoter.²⁴ This finding suggests that PKM2 knockout may have promoted photoreceptor health by working directly with Pde6 β , which would indicate that the beneficial effects are specific to Pde6 β deficiency. As such, we highlight the need to use a different RP model to evaluate whether the rescue phenotypes from PKM2 ablation are in fact a result of its role in a gene non-specific metabolic pathway.

Current CRISPR/Cas9-based gene therapy relies on a gene- and mutation-specific approach. Given that over 80 genes have been implicated in the development of RP, this strategy is impractical as it is not amenable to large-scale production or treatment. Here, we have demonstrated that PKM2 ablation offers 6 equivalent years of improved eyesight in our RP model,⁵⁰ suggesting that elevations in rod-specific anabolism can offer protection against degeneration in a non-gene-specific manner. Importantly, the metabolic pathways highlighted in this study are common to not only retinal degeneration, but also neurodegenerative conditions including Alzheimer's Disease.^{51,52} As such, reprogramming the metabolism of a terminally differentiated neuron towards a more anabolic state may offer a universal, gene non-specific treatment for multiple neurodegenerative conditions. The classical function of PKM2 as a catalyst for the final step in glycolysis lies at the heart of metabolic regulation. Therefore, a deeper understanding of its role in coupling the dynamic metabolisms of the retina and RPE may offer critical insight into and advancement towards the development of gene non-specific

therapeutics. Such a strategy can then be used synergistically with gene supplementation therapy to optimize efficacy.

MATERIALS AND METHODS

Animals

All mice were maintained in the Columbia University Pathogen-free Eye Institute Annex Animal Care Services Facility under a 12-hour light/12-hour dark cycle. *PKM2^{flox/flox}* mice (B6;129S-*Pkm^{tm1.1Mgvlh}/J*) were purchased from The Jackson Laboratory while *Pde6b^{H620Q/H620Q}* mice were re-derived via oviduct transfer from morulae provided by the European Mouse Mutant Archive (EMMA). Our lab generated *Pde6g^{CreERT2}* mice.⁵³

The inducible *Pde6g^{CreERT2}* driver was used to exert both temporal and spatial control over *PKM2* ablation in *Pde6b^{H620Q/H620Q}* mice. *PKM2* ablation was activated during the mid-stage of degeneration for clinical relevance and limited to the rod photoreceptors to prevent embryonic lethality. To generate this conditional *PKM2* knockout in a *Pde6b^{H620Q/H620Q}* mouse model, *Pde6b^{H620Q/+}* mice were crossed first with *Pde6g^{CreERT2}* mice and then *Pkm2^{flox/flox}* mice. The resultant progeny were then backcrossed four generations to generate heterozygous *Pde6b^{H620Q/+} Pkm2^{flox/flox} Pde6g^{CreERT2}*. A total of six generations of backcrosses were performed to produce the desired homozygous genotype of *Pde6b^{H620Q/H620Q} Pkm2^{flox/flox} Pde6g^{CreERT2}*.

The experimental mice were injected intraperitoneally with tamoxifen (amount: 100 µg/g body weight; concentration: 100 mg/ml in ethanol; dilution: 10 mg/ml in corn oil at 42 °C; catalog T5648; Sigma-Aldrich) at P8-11 to generate a genotype of *Pde6b^{H620Q/H620Q} Pkm2^{-/-}*. Isogenic control and wild-type mice were injected with ethanol (amount: 100 µg/g body weight; dilution: 10% in corn oil) to generate a genotype of *Pde6b^{H620Q/H620Q} Pkm2^{+/+}*. The mice were not discriminated based on sex.

All experiments were approved by the Columbia University Institutional Animal Care and Use Committee (IACUC) prior to the start of the study. All mice were used and maintained in accordance with the Statement for the Use of Animals in Ophthalmic and Vision Research of the

Association for Research in Vision and Ophthalmology and the Policy for the Use of Animals in Neuroscience Research of the Society for Neuroscience.

Immunoblotting

Following the protocol described by Tsang, *et al.*,^{54,55} retina samples were collected from mice two weeks after injection. To evaluate PKM2 expression levels, the samples were homogenized using a solution consisting of M-PER Mammalian Protein Extraction Reagent (Prod #78501 Thermo Scientific), phosphatase inhibitor cocktail (catalog P2850-5 mL; Sigma), and protease inhibitor cocktail (catalog P8340-1 mL; Sigma). After transfer onto nitrocellulose membrane (BioRad), samples were blocked in 5% skim milk 902887 MP Biomedicals, LLC). The membrane was then immersed overnight at 4 °C in the following primary antibodies: rabbit Anti-PKM2 (1:1000; #3198S; Cell Signaling Technology), mouse anti- β -actin (1:1000; ab125248; Abcam), and rabbit anti-phospho-PKM2 (1:1000; #3827S; Cell Signaling Technology). The following day, the membrane was washed in 0.5% PBST (500 μ l Tween-20 in 1,000 ml PBS), and subsequently incubated in mouse monoclonal anti-rabbit IgG-HRP antibody (1:2000; ab99697; Abcam) and rabbit anti-mouse IgG-HRP antibody (1:2,000; sc-358914; Santa Cruz Biotechnology Inc.). Chemiluminescence (EMD Millipore) was detected using Biomax film (Kodak), and PKM2, P-PKM2, and actin levels were then quantified.

Histology

As described by Velez, *et al.*, Eyes were enucleated from euthanized mice, by following along the limbus to create a puncture at the 12 o'clock position.⁵⁶ Each eye was then placed in a solution of 1:2x Karnovsky fixative for 24 hours. Following fixation, eyes underwent a saline wash and then dehydration to be embedded in paraffin. Afterwards, hematoxylin and eosin were used to stain paraffin sections, which were then examined by light microscopy.

Rod OS Measurement

Following euthanasia, the eye was enucleated followed by cornea and lens dissection and vitreous removal. At every 100 microns from the optic nerve, Rod OS was measured using a Image J (NIH, Bethesda)e. Measurements were taken for segments 500 microns away from the

optic nerve on both eyes. All procedures were done in accordance with previously established IACUC guidelines.

ERG

we obtained scotopic, mixed maximum, and photopic electroretinogram (ERG) recordings for experimental and control mice four and eight weeks after injection. Both eyes of each mouse (20 eyes) were analyzed and averaged due to the correlation between mouse eyes. Following a 12 hour overnight dark adaptation, Mice were anesthetized using the protocol described by Sancho-Pelluz, *et al.*³³ All procedures performed on the mice were completed under dim lighting. Mice were dilated using a solution composed of phenylephrine hydrochloride (2.5%, Paragon) and tropicamide (1%, Akorn) that was administered topically. Mouse body temperature was maintained during all experiments using a heating pad. Three different experiments were performed – scotopic recording (pulse intensity: 0.001 Cd/m²), mixed maximal recording (pulse intensity: 3 Cd/m²), and photopic recording (pulse intensity: 30 Cd/m²). An ERG was conducted using Espion E2 Electroretinography System (Diagnosys LLC)]. All ERG data was collected using Espion E2 Electroretinography System (Diagnosys LLC).

Statistical Analysis

Using the ERG results, the maximal b-wave wave of each eye was taken and used to fit a linear model using generalized least squares. This method of regression was chosen because of the correlation between the eyes of each mouse and to account for unequal variance between treated and non-treated mice.

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FIGURES

Figure 1. Deletion of *Pkm2* in *Pde6β*^{H620Q/H620Q} mice.

Immunoblot analysis was performed two weeks after tamoxifen and oil injections. Selective *Pkm2* deletion in photoreceptors reduced Pkm2 and P-Pkm2 expression (n=3 for both groups). Actin served as loading control.

Figure 2. Deletion of *Pkm2* in *Pde6β*^{H620Q/H620Q} mice improved retinal morphology.

(A) H&E-stained retinal sections were analyzed by light microscopy and compared at P60. Selective deletion of *Pkm2* enhanced but did not completely rescue ONL thickness in *Pde6β*^{H620Q/H620Q} *Pkm2*^{-/-} mice (n=3), but not in *Pkm2*^{+/+} *Pde6β*^{H620Q/H620Q} controls. At P9, experimental mice were injected tamoxifen while wildtype and control mice were injected with oil. (Purple bar indicates INL, blue bar indicates ONL)

(B) The difference in Rod OS length was quantified. Selective deletion of *PKM2* increases Rod OS thickness at every distance from the ON (n=3). Red represents experimental mice, and black represents control mice. Highlighted line shows average Rod OS while faded dots represent individual measurements for mice.

Figure 3 Deletion of *Pkm2* in *Pde6β^{H620Q/H620Q}* mice preserves rod and cone function.

ERG b-wave magnitudes for scotopic, mixed maximal, and photopic response were taken and compared in control (*Pkm2*^{+/+} *Pde6β^{H620Q/H620Q}*, blue bar) and experimental (*Pkm2*^{-/-} *Pde6β^{H620Q/H620Q}*, green bar) mice.

(A) ERG recordings were obtained at 4 weeks post injection. Experimental mice exhibited enhanced scotopic b-wave amplitudes compared to controls. Differences in the magnitudes of mixed maximal and photopic b-waves were not statistically significant. (n=12 for both groups; (**P* < 0.05)

(B) ERG recordings were obtained at 8 weeks post injection. Experimental mice exhibited enhanced b-wave magnitudes for all groups compared to controls. (n=6 for control group, n=8 for experimental group; **P* < 0.05)

(C) Representative ERG tracings for scotopic, mixed maximal, and photopic response at 4 weeks and 8 weeks after injection.