# Activation of Sigma 1 Receptor Extends Survival of Cones and Improves Visual Acuity in a Murine Model of Retinitis Pigmentosa

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**Purpose.** Retinitis pigmentosa (RP), a retinal photoreceptor degeneration, typically affects rod function and subsequently cones. Activation of sigma 1 receptor (Sig1R) has been shown to preserve cone function through 6 weeks in the *rd10* mouse model of RP, when mice were treated systemically with the Sig1R ligand (+)-pentazocine (PTZ). This study determined the extent to which cone function is preserved in *rd10* mice when Sig1R is activated.

**Methods.** *Rd10* mice were administered (+)-PTZ (alternate days beginning at postnatal day [P]14) over a period of 180 days. Mouse visual function and structure were measured in vivo using optokinetic tracking response, scotopic and photopic electroretinography plus photopic assessment using "natural" noise stimuli, and optical coherence tomography (OCT). Immunofluorescent methods were used to detect cones in retinal cryosections.

**Results.** Visual acuity was maintained in *rd10*(+)-PTZ-treated mice through P56, whereas *rd10* nontreated mice showed marked decline by P28. Cone responses were detected in (+)-PTZ-treated mice through P60, which were more robust when tested with natural noise stimuli; cone responses were minimal in nontreated *rd10* mice. OCT revealed significantly thicker retinas in (+)-PTZ-treated *rd10* mice through P60 compared to nontreated mice. Cones were detected by immunofluorescence in (+)-PTZ-treated *rd10* retinas through P120.

**Conclusions.** The extent to which cone rescue could be sustained in (+)-PTZ-treated *rd10* mice was evaluated comprehensively, showing that activation of Sig1R is associated with prolonged visual acuity, extended detection of cone function, and detection of cones in retinal histologic sections. The data reflect promising long-term neuroprotection when Sig1R is activated.

Keywords: mouse, retina, pentazocine, retinal degeneration, visual acuity, ERG, OCT

The major cause of untreatable blindness worldwide is retinal degenerative disease, often caused by dysfunction of photoreceptor cells (PRCs).[1,2] The retinal degenerative disease retinitis pigmentosa (RP) is caused by numerous mutations in a number of genes and is characterized by progressive loss of PRCs.[3] This disease affects ~1:3000 to ~1:5000 humans. Typically in RP, rod PRCs are lost initially, compromising vision in dim light. The more debilitating aspect of this disease is the subsequent loss of cone PRCs, which mediate best vision, compromising many activities of daily living. Development of strategies to preserve cone function, even when rods are lost, would have a tremendous therapeutic impact on RP and other retinopathies.

A novel target for treatment of retinal disease is sigma 1 receptor (Sig1R).[4,5] This unusual single-pass transmembrane protein, an evolutionary isolate with no homology to any other protein, crystallizes as a trimer.[6] It is considered a pluripotent modulator of cell survival with roles in normal and disease processes and is a novel target for treatment of neurodegenerations.[7,8] Sig1R is localized to the ER and the nuclear membrane in neuronal and glial cells of the retina.[9,10] Activation of Sig1R attenuates oxidative stress in retinal cells,[11–13] and oxidative stress has been proposed as a major pathogenic factor underlying RP.[14,15]

In a recent study, we observed dramatic rescue of cone PRCs in the *Pde6b*$^{rd10}$*/J* (*rd10*) mouse model of RP when mice were treated systemically with (+)-pentazocine (PTZ), a high-affinity Sig1R ligand.[16] The *rd10* mouse is considered a valuable model of RP and useful for testing rescue approaches.[17] The mouse initially loses rods due to a genetic defect in a rod-specific gene (β-subunit of rod phosphodiesterase) and then subsequently cones. The PRC loss is rapid and nearly complete within the first 35 days of life.[17] By postnatal day 25 (P25), the outer nuclear layer in which PRC nuclei reside is reduced to two or three rows of PRCs compared to 10 to 12 rows in wild-type (WT) mice. Only a few aberrant cones remain by P45. Owing to the very rapid progression of the retinal phenotype in *rd10* mice, our initial study of the effects of activating Sig1R as a neuroprotectant was conducted in mice through P42.[16] We performed retinal functional and architectural assessments including evaluation of cone electrophysiological function at P35 and immunohistochemical analysis to detect cones at P42.[16] Our electrophysiological assessment of PRC responses to natural stimuli revealed cone function that was significantly

better in (+)-PTZ-treated *rd10* mice than in nontreated littermates, and importantly, was similar to that in WT mice. Cones were significantly more abundant in (+)-PTZ-treated *rd10* mice than in nontreated *rd10* mice. The protective effects of (+)-PTZ treatment are attributable to Sig1R activation as there is no cone preservation in (+)-PTZ-treated *rd10/Sig1R*$^{-/-}$ mice.[16]

The data showing cone rescue in *rd10* mice treated with (+)-PTZ are encouraging, but they reflect only short-term preservation of function and do not address the duration of cone rescue. Thus, we do not know how long cone function can be preserved in this model if Sig1R is activated. We hypothesized that (+)-PTZ treatment can rescue cones beyond P35. In the current study, we evaluated visual function and retinal architecture comprehensively over a 180-day period in (+)-PTZ-treated *rd10* mice compared to nontreated mutants. We chose this extended time period anticipating that in this severe retinopathy we would reach the limit of rescue. (+)-PTZ treatment of *rd10* mice improved visual acuity through at least P56 and enhanced cone function beyond P60. Indeed, cone function was slightly detectable at P90, though not at P120 or beyond. Cone PRCs continued to be detectable through P90. Thus, activation of Sig1R holds promise as a longer-term neuroprotectant for retinal disease.

## Materials and Methods

### Animals

Breeding pairs of B6.CXBI-*Pde6βrd10/J* (*rd10*) mice were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). Confirmation of genotype was performed as described.[16] The *Crb1*$^{rd8/rd8}$ mutation responsible for focal retinal disruption in certain mouse strains[18] was not detected in any mice used in the study. Mice were fed Teklad Irradiated Rodent Diet 8904 for breeding or Diet 2918 for maintenance (Teklad, Madison, WI, USA). Animals were subjected to a standard 12-hour light/12-hour dark cycle. We adhered to institutional guidelines for humane treatment of animals and to the ARVO Statement for Use of Animals in Ophthalmic and Vision Research. Two groups of mice were evaluated over a period of 180 days: *rd10* mice (nontreated) and *rd10*+PTZ mice. The *rd10*+PTZ mice received an intraperitoneal injection of (+)-PTZ (0.5 mg/kg) (Sigma-Aldrich Corp., St. Louis, MO, USA) on alternate days beginning at P14. The dosage was based on studies showing efficacy in rescuing cone PRCs in *rd10* mice.[16] The Table indicates the numbers of mice analyzed at four time points (P60, P90, P120, and P180) following extended (+)-PTZ treatment.

### Visual Acuity Assessment

Visual acuity was evaluated in *rd10* nontreated and *rd10*+PTZ mice ages P21 to P70; a cohort of WT C57Bl/6J mice (Jackson Laboratories) were also included in the assessment for comparison. The visual acuity was measured as described previously.[19] Briefly, spatial thresholds for optokinetic tracking of sine-wave gratings were measured using the OptoMotry system (CerebralMechanics, Medicine Hat, Alberta, Canada).[20] Mice were placed unrestrained on a pedestal and were presented vertical sine-wave gratings moving at 12°/s or gray of the same mean luminance within the OptoMotry device, which functions as a virtual cylinder. The cylinder hub was continually centered between the mouse's eyes to establish the spatial frequency of the grating at the mouse's viewing position as it shifted its position. Gray color was projected while the mouse was moving, but when movement ceased, the gray was replaced with the grating. Grating rotation under these circumstances elicited reflexive tracking, which was scored via live video using a method of limits procedure with a yes/no criterion as recommended by the manufacturer. A measure of spatial resolution was taken as the asymptote of a staircase procedure. The two eyes were tested in an interleaved fashion.

**Table.** Numbers and Ages of Mice Used in the Study

| Mouse Group | *n* | Age, Postnatal Days |
|---|---|---|
| ERG analysis of retinal function | | |
| Rd10 (no (+)-PTZ) | 4 | 60 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 6 | 60 |
| Rd10 (no (+)-PTZ) | 4 | 90 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 6 | 90 |
| Rd10 (no (+)-PTZ) | 7 | 120 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 4 | 120 |
| Rd10 (no (+)-PTZ) | 6 | 180 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 4 | 180 |
| OCT analysis of retinal structure | | |
| Rd10 (no (+)-PTZ) | 5 | 60 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 6 | 60 |
| Rd10 (no (+)-PTZ) | 4 | 90 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 6 | 90 |
| Rd10 (no (+)-PTZ) | 11 | 120 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 4 | 120 |
| Rd10 (no (+)-PTZ) | 6 | 180 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 4 | 180 |
| OptoMotry analysis of vision acuity | | |
| Wild type | 4 | 30 |
| Wild type | 6 | 60 |
| Rd10 (no (+)-PTZ) | 9 | 21 |
| Rd10 (no (+)-PTZ) | 9 | 28 |
| Rd10 (no (+)-PTZ) | 9 | 35 |
| Rd10 (no (+)-PTZ) | 9 | 42 |
| Rd10 (no (+)-PTZ) | 9 | 63 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 9 | 21 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 9 | 28 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 9 | 35 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 9 | 42 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 9 | 49 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 9 | 56 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 9 | 63 |
| Rd10 (0.5 mg kg$^{-1}$ (+)-PTZ) | 9 | 70 |

### Electroretinography (ERG)

Dark-adapted *rd10* nontreated and *rd10*+PTZ mice were anesthetized using isoflurane and electrophysiological function was assessed as described.[16] Briefly, dark-adapted ERGs were performed using silver-coated nylon fibers joined to flexible wires that were placed on the cornea. The electrical contact was enhanced by placing a drop of hypromellose on the cornea. Optic fibers (1-mm diameter) were positioned in front of the pupil through which highly controllable illumination was delivered to eyes using a 5500° white light-emitting diode. Rod function was assessed using a series of tests with 5-ms flashes of increasing luminance, followed by assessment of cone function using photopic testing with 5-ms flashes above a pedestal. Additionally, a photopic "natural noise" stimulus was presented. This stimulus changes luminance pseudorandomly over time, with the amplitudes of those changes inversely proportional to temporal frequency (as has been described for human subjects[21]), and phase being random. This produces relatively slow, continuous changes in luminance, rather than flashes, and is natural in the sense that real-world visual stimuli

similarly change slowly. Responses to noise stimuli are also random, but responses are correlated with stimuli to generate kernels that describe how the retina transforms arbitrary stimuli into ERG responses. Mice were evaluated at ages P60, P90, P120, and P180.

### Spectral-Domain Optical Coherence Tomography (SD-OCT)

*Rd10* nontreated and *rd10*+PTZ mice were anesthetized with ketamine/xylazine as described.[16] Retinal structure was evaluated in vivo using a Bioptigen Spectral Domain Ophthalmic Imaging System (SDOIS; Bioptigen Envisu R2200, Morrisville, NC, USA) in mice at ages P60, P90, P120, and P180. The imaging protocol included averaged single B-scan and volume intensity scans with images centered on the optic nerve head. Because of the considerable outer retinal disruption in *rd10* retina, it is preferable to use the manual caliper feature to measure retinal layers versus autosegmentation postimaging analysis (a feature of the InVivoVue Diver 2.4 software). We used the manual caliper feature to acquire inner, outer, and total retinal thickness measurements. Inner retina was measured from the superior boundary of inner limiting membrane (ILM) to the lower edge of the inner nuclear layer (INL). Outer retina was measured from the lower edge of the INL to the inferior boundary of the retinal pigment epithelium layer (RPE); if there was separation of photoreceptors from RPE, we obtained outer retinal thickness by adding the RPE layer thickness and the distance from the lower edge of INL to the edge of neuronal retina as described previously.[22]

### Intraocular Pressure

Intraocular pressure (IOP) was measured in *rd10* nontreated and *rd10*+PTZ mice at P60, P90, P120, and P180 using a handheld tonometer (Tonolab; Icare Laboratory, Finland) as described.[19] The procedure was conducted under typical laboratory lighting conditions (e.g., 46.50 foot-candles). The tonometer was positioned at the center of the cornea of mice anesthetized by isoflurane inhalation. All IOP measurements were made between 10:00 and 11:00 AM. Three repeated measurements were taken from each animal for quantification.

### Immunodetection of Cone Photoreceptor Cells and Retinal Gliosis

Eyes, enucleated from euthanized mice, were flash-frozen in liquid nitrogen, embedded in Tissue-Tek optimal cutting temperature compound (Electron Microscopy Sciences, Hatfield, PA, USA), and cryosections were prepared for immunohistochemistry as previously described.[12] Sections were incubated with fluorescein isothiocyanate–conjugated peanut agglutinin (FITC-PNA, catalog number L7381; Sigma-Aldrich Corp.) or cone arrestin (catalog number AB15282; Millipore, Temecula, CA, USA) to detect cone PRCs. Additional cryosections were incubated with rabbit anti-glial fibrillary acidic protein (GFAP) (catalog number Z0334; Dako Corp., Carpintaria, CA, USA) to detect gliosis. Alexa Fluor 555 and Alexa Fluor 488 anti-rabbit IgG (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) were used as the secondary antibody for cone arrestin and GFAP, respectively. Coverslips were mounted using Fluoroshield with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich Corp.). Retinas were examined using a Zeiss (Carl Zeiss, Göttingen, Germany) Axio Imager D2 microscope equipped with a high-resolution camera and processed using Zeiss Zen23pro software.

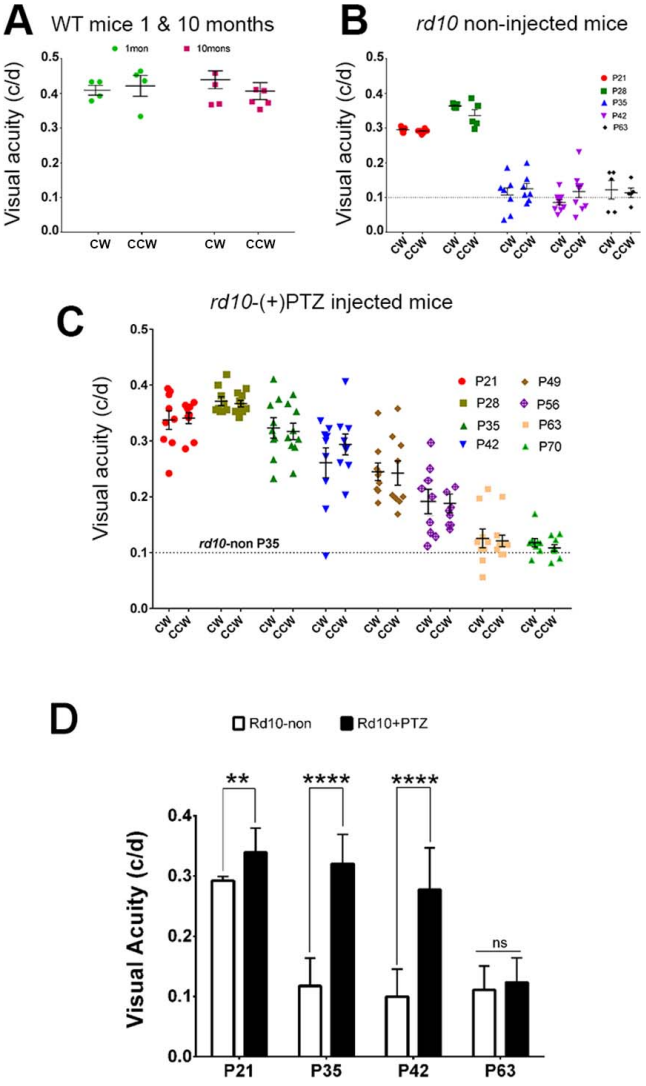


**Figure 1.** Visual acuity of *Pde6β*$^{rd10}$ (*rd10*) mice administered (+)-PTZ versus *rd10* nontreated mice. Visual acuity, expressed in cycles/degree, was measured using the OptoMotry system in (**A**) C57Bl/6J WT mice at 1 and 10 months of age; (**B**) *rd10* (nontreated) mice at ages P21, P28, P35, P42, and P63; and (**C**) *rd10* mice (administered (+)-PTZ every other day beginning at P14) at ages P21, P28, P35, P42, P49, P56, P63, and P70. (**D**) Data from the *rd10* nontreated and *rd10*(+)-PTZ-injected mice were compared at P28, P35, P42, and P63. Significantly different from nontreated animals: $^{**}P < 0.0761$, $^{****}P < 0.0001$. ns, not significant.

### Data Analysis

Statistical analysis used GraphPad Prism analytical program (La Jolla, CA, USA). Data were analyzed by 2-way ANOVA (factors: mouse group and age). Šidák's multiple comparison test was used for post hoc testing. For the ERG results, we used Igor Pro (WaveMetrics, Lake Oswego, OR, USA). Significance for all analyses was $P < 0.05$.

## Results

### Activation of Sig1R Enhances Visual Acuity in *rd10* Mice

Prior to conducting the electrophysiological and retinal architectural analyses in mice over the 180-day time period,

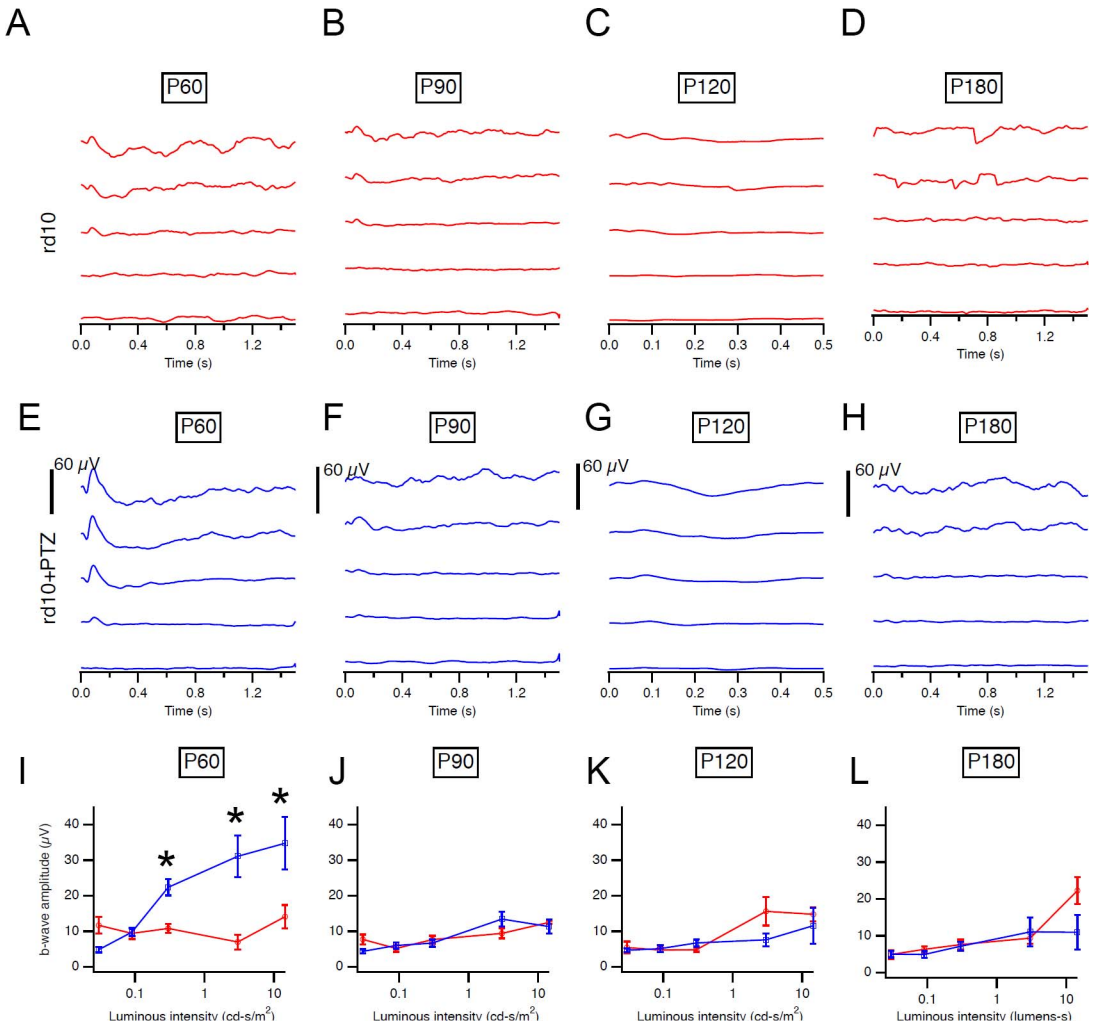


**Figure 2.** Representative scotopic ERG responses for *rd10* mice administered (+)-PTZ versus *rd10* nontreated mice assessed over 180 days. Averaged scotopic ERG responses to 5-ms flashes at five stimulus contrasts in *rd10* nontreated (**A–D**, at P60, P90, P120, and P180, respectively) and *rd10*+PTZ mice (**E–H**, at P60, P90, P120, and P180, respectively). The *rd10*+PTZ mice were treated every other day with (+)-PTZ beginning at P14. Amplitudes of the scotopic b-waves for the tests shown in *rd10* (**A–D**) and *rd10*+PTZ (**E–H**) are plotted against luminous intensity (**I–L**, at P60, P90, P120, and P180, respectively). *Significantly different from nontreated animals: $P < 0.05$.

we measured visual acuity in *rd10* mice that had or had not received (+)-PTZ treatment and compared the findings with WT mice. The optokinetic tracking response performed using the OptoMotry system in the left and right eye of each animal measured the reflex response for clockwise and counterclockwise movement of gratings drifting at 12°/s. Data were recorded as the asymptotic convergence of a staircase procedure that estimates spatial resolution, or acuity, in units of cycles per degree (c/d). WT mice had an average visual acuity of 0.4 c/d (Fig. 1A). The visual acuity of *rd10* nontreated mice was ~0.30 c/d at P21 and ~0.35 c/d at P28, but decreased rapidly such that by P35 it was reduced to 0.1 c/d. Beyond P35, the visual acuity of *rd10* nontreated mice remained minimal (note responses at P42 and P63, Fig. 1B). In contrast, the *rd10* mice treated with (+)-PTZ sustained visual acuity through P56 (median visual acuity was ~0.32 c/d at P35, ~0.3 c/d at P42, ~0.25 c/d at P49, ~0.2 c/d at P56) (Fig. 1C). By P63 visual acuity was reduced to ~0.1 c/d. The data indicate that (+)-PTZ treatment postpones the precipitous decline in visual acuity observed in nontreated *rd10* mice. The averaged values for the visual acuity data are presented for comparisons between *rd10* nontreated and *rd10*-PTZ mice at P21, P35, P42, and P63 (Fig. 1D).

## Activation of Sig1R Extends Scotopic and Photopic ERG Responses in *rd10* Mice

Given that visual acuity was extended significantly in *rd10* mice administered (+)-PTZ through ~60 days compared to nontreated *rd10* mice (Fig. 1), we were interested in determining the extent to which electrical impulses, indicative of retinal cellular activity, could be detected in *rd10* mice versus (+)-PTZ-treated *rd10* mice. Mice, administered (+)-PTZ on alternate days beginning at P14, were subjected to comprehensive ERG analysis at P60, P90, P120, and P180 to assess rod (scotopic) and cone (photopic) function (Figs. 2–5). Responses were compared with age-matched *rd10* nontreated mice. Representative scotopic tracings are shown for *rd10* mice, and responses were minimal (Figs. 2A–D). There was significant rod function detected at higher luminous intensities in *rd10*+PTZ mice at P60 (Fig. 2E), although this response was no longer detectable at P90, P120, and P180 (Figs. 2F–H). At P60, these responses were typical of scotopic threshold responses seen in WT mice at much lower luminous intensities.[23] The b-wave amplitudes of these data are shown in Figures 2I through 2L. The data indicate that PRC function is discernible in the (+)-PTZ-treated *rd10* mice through P60,

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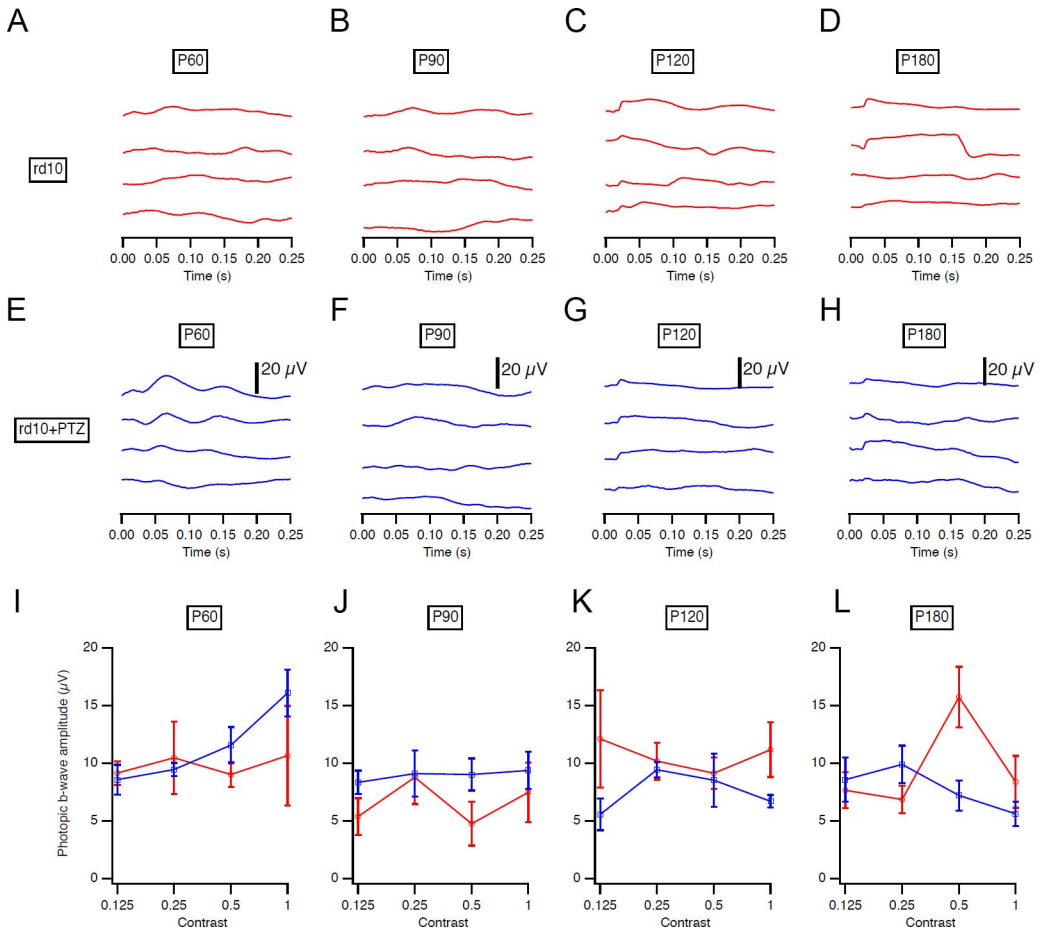


**Figure 3.** Representative photopic ERG responses for *rd10* mice administered (+)-PTZ versus *rd10* nontreated mice assessed over 180 days. Averaged photopic ERG responses to 5-ms flashes at four stimulus contrasts in *rd10* nontreated (**A–D**, at P60, P90, P120, and P180, respectively) and *rd10*+PTZ mice (**E–H**, at P60, P90, P120, and P180, respectively). The *rd10*+PTZ mice were treated every other day with (+)-PTZ beginning at P14. Amplitudes of the photopic b-waves for the tests shown in *rd10* (**A–D**) and *rd10*+PTZ (**E–H**) are plotted against stimulus contrast (**I–L**) at P60, P90, P120, and P180, respectively). Data for the highest-contrast stimulus are shown in Figure 4.

which is significantly longer than our original observation of improved scotopic response through P35.[16]

Regarding cone responses, the b-wave amplitude measured in the photopic ERG response was nearly the same in *rd10*+PTZ mice compared to nontreated *rd10* mice (Figs. 3A–L). There was improvement in cone response at the highest contrast (Fig. 4A) in *rd10*+PTZ mice at P60, although not in *rd10*+PTZ mice at P90, P120, and P180 (Figs. 4B–D). Stronger rescue of cone function was observed in *rd10*+PTZ mice at P60 and P90 when they were tested with a stimulus that changed more slowly in time (Figs. 5A, 5B, 5E). This natural stimulus ("green noise") detected responses in *rd10*+PTZ mice that were significantly greater than in nontreated *rd10* mice at P60 (Fig. 5A) and P90 (Fig. 5B). This improved response was no longer observed in (+)-PTZ-treated *rd10* mice at P120 or P180. The quantification of the green noise amplitude in *rd10*+PTZ versus *rd10* nontreated mice is shown in Figure 5E.

## Activation of Sig1R Sustains Retinal Architecture Measured In Vivo by SD-OCT in *rd10* Mice

We visualized the structure of retinas in situ using SD-OCT. Representative images are shown (Figs. 6A–D). At P60, the *rd10* mouse retina was ~90 to 100 μm thick (Fig. 6A). There is often significant separation of the neural retina from the RPE in *rd10* mice by this age as the inner and outer segments are markedly diminished. In (+)-PTZ-treated *rd10* mice the retinas at P60 were significantly thicker (~110 μm) than in nontreated (Figs. 6A, 6E), and the neural retina–RPE separation was attenuated. The outer retina (Fig. 6F) and inner retina (Fig. 6G) of (+)-PTZ-treated *rd10* mice were significantly thicker than in nontreated mice at P60 as well. Interestingly, while the differences between (+)-PTZ-treated and nontreated *rd10* mice were only slightly significant or nonsignificant at P90 and P120 (Figs. 6E–G), by P120 the differences were highly significant for total retinal thickness (Fig. 6E) as well as inner retinal thickness (Fig. 6G).

In addition to the aforementioned analyses (Figs. 1–6), we also assessed the IOP of the mice as part of a comprehensive ophthalmologic evaluation. Altered IOP has not been reported in *rd10* mice, and our data indicate that pressure was within normal limits for all mice examined (Supplementary Fig. S1).

## Activation of Sig1R Prolongs the Immunodetection of Cones in *rd10* Mice

Following functional visual assessments, animals were euthanized and eyes were processed to determine whether cones were retained in *rd10*+PTZ retinas. In earlier studies, cone PRCs were detected in (+)-PTZ-treated *rd10* retinas through P42. In the present study, we were interested in determining whether cones persisted over a longer treatment regimen. We

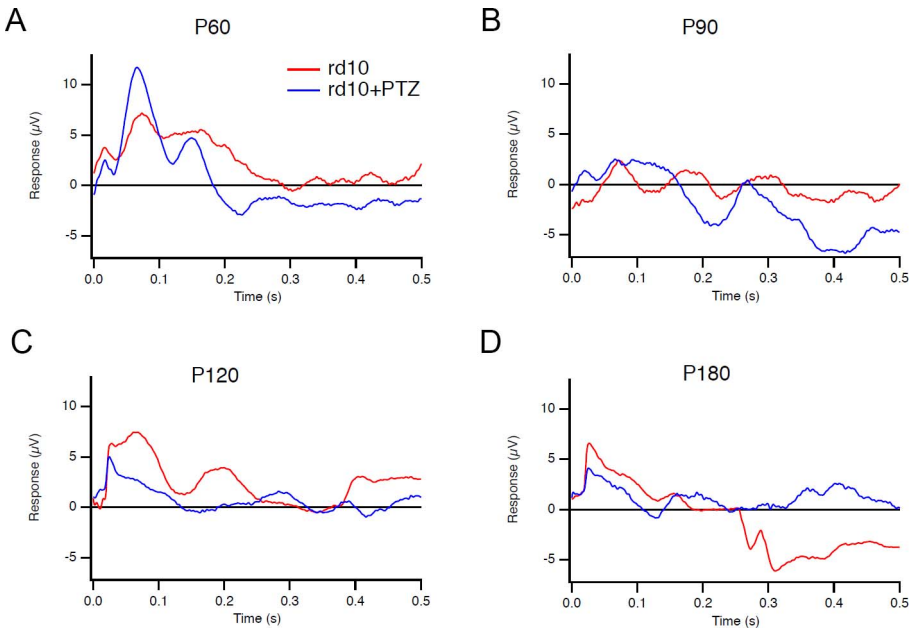


**Figure 4.** High-contrast photopic ERG averaged responses in *Pde6β*$^{rd10}$ (*rd10*) mice administered (+)-PTZ versus *rd10* nontreated mice assessed over 180 days. The highest-intensity data shown in Figure 3 were replotted to illustrate differences in photopic responses in the *rd10* nontreated versus (+)-PTZ-treated *rd10* mice at P60 (**A**), P90 (**B**), P120 (**C**), and P180 (**D**).

detected cone cells using FITC-PNA. We observed some labeling in *rd10* nontreated retinas at P60 as well as in *rd10*+PTZ mice at P60 (Fig. 7A, arrowheads). FITC-PNA labeling in the *rd10* nontreated mice was minimal at P90, P120, and P180 but was discernible in *rd10*+PTZ retinas at P90 and P120 (Figs. 7B–D). The data suggest that some cones are retained in the (+)-PTZ-treated *rd10* retinas, at least through P120. Thus, activation of Sig1R via the treatment with (+)-PTZ contributes to cone rescue beyond P60. These data were confirmed using a second antibody to detect cones (cone arrestin) as shown (Supplementary Fig. S2).

In healthy retinas, GFAP is present primarily in retinal astrocytes, which reside in the retinal ganglion cell layer; its expression is minimal in the remainder of the retina. Under stress, however, GFAP is detected at much higher levels in retina, particularly in the radially oriented Müller cells.[24] This was reported in *rd10* mice and attenuated with (+)-PTZ treatment through P42.[16] Here, we investigated whether long-term (+)-PTZ treatment alters gliosis in *rd10* retinas; however, we did not observe quantitative differences in GFAP immunofluorescence at ages P60 and beyond (Supplementary Fig. S3). Our data suggest that gliosis is considerable as the severe *rd10* retinopathy progresses regardless of (+)-PTZ treatment.

## Discussion

Blindness exacts an extremely burdensome personal, emotional, and financial toll and represents an urgent unmet health care need demanding novel therapeutic strategies.[1,2] Sig1R represents a novel target for delaying neuronal loss in retinal disease. While earlier reports indicated impressive rescue of cone PRCs in the *rd10* mouse model of RP,[16] the extent to which this rescue could be sustained was not explored, but is clinically significant. In the current study we evaluated, over an extended time period, the effects of activating Sig1R in *rd10* mice by assessing visual acuity, retinal electrophysiological function, retinal architecture, and immunodetection of cones. The most profound observations in mice treated with the Sig1R ligand were prolonged visual acuity, extended detection of cone function using a natural noise stimulus in electrophysiological analysis, and detection of cones in retinal histologic sections.

Regarding visual acuity, we observed a dramatic improvement in visual acuity in *rd10*+PTZ mice compared with nontreated animals. The nontreated mice had an average acuity of ~0.35 c/d at P28 and by age P35 the average acuity was less than 0.1 c/d, which is markedly decreased compared to the normal (WT) value of ~0.4 c/d. In contrast, the (+)-PTZ-treated *rd10* mice had visual acuity >0.3 c/d at P35 and ~0.3 c/d at P42 and continued to demonstrate a robust, albeit decreased, response through P56. Only when tested at P63 and beyond did *rd10*+PTZ mouse responses compare similarly to the nontreated *rd10* mice. Other investigations of therapeutic intervention strategies in *rd10* mice have also evaluated visual acuity, although generally for a limited time period. For example, in a study evaluating the benefit of rasagiline, an anti-Parkinsonism compound that inhibits monoamine oxidase B, the *rd10*-treated mice showed improvement in visual acuity. However, the study was terminated at P30[25]; hence it is unknown whether the benefits of this compound extended beyond the period of greatest PRC death in the mutant model. Similarly, the benefit of exercise (active running wheel) on visual acuity has been evaluated in *rd10* mice.[26,27] One of these studies evaluated optokinetic tracking in exercised mice at P35 and P42 and found improved responses at both ages compared to nonexercised mice, although the spatial frequency threshold at P42 was only ~0.175 c/d in the exercised group.[26] The other exercise-related study spanned a longer time frame and examined exercised and standard conditioned mice at 1 year. By that advanced age, the visual acuity in the exercised mice was quite low (~0.1 c/d), although it was greater than in those reared under standard conditions.[27] An intriguing study, conducted through P60, demonstrated promising effects on visual acuity in *rd10* mice treated with myriocin, an inhibitor of serine-palmitoyl transferase, the rate-limiting factor in de novo ceramide synthesis.[28] The assessment of visual acuity is an important measure of the efficacy of treatment strategies, and it appears that activation of Sig1R

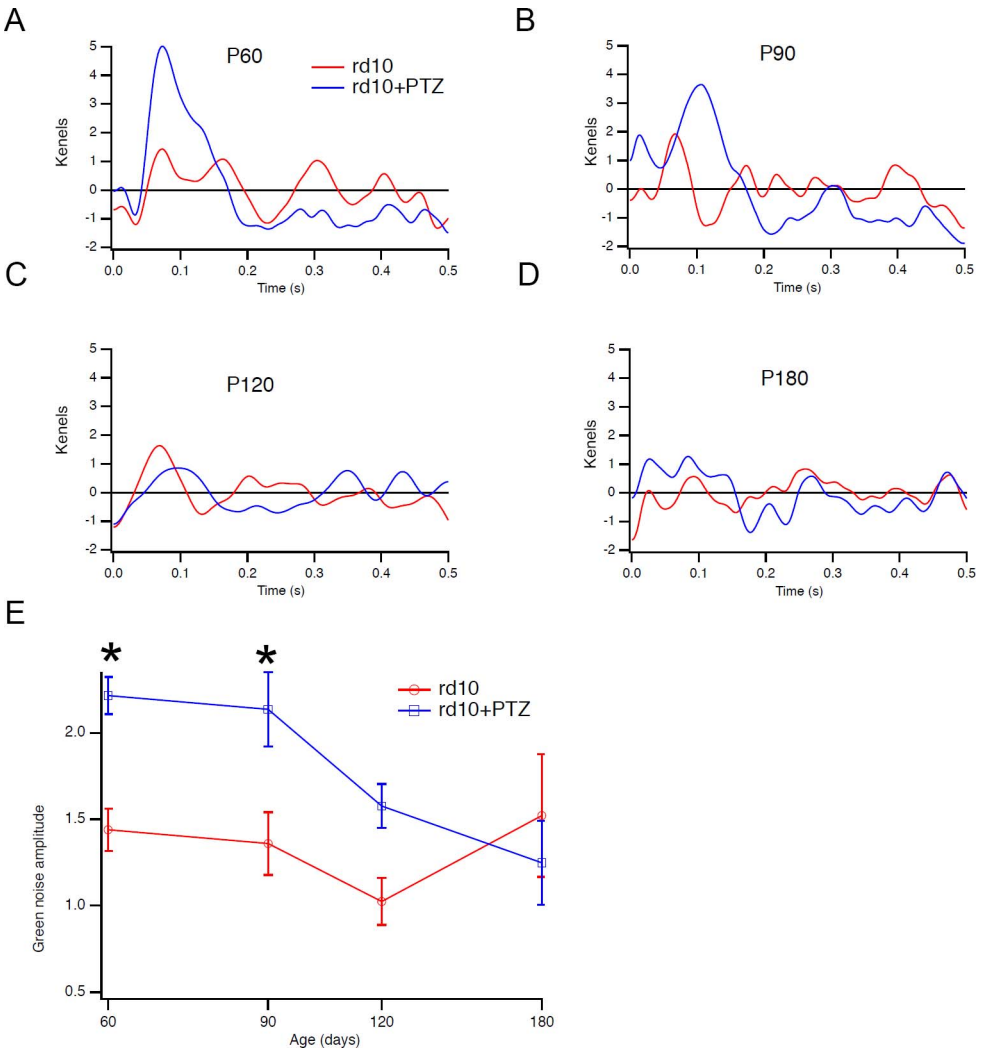


**Figure 5.** Kernels derived from natural noise stimulation in *Pde6β*$^{rd10}$ (*rd10*) mice administered (+)-PTZ versus *rd10* nontreated mice assessed over 180 days. The *rd10* nontreated and (+)-PTZ-treated *rd10* mice were tested with photopic "natural" noise stimuli, which is a slowly varying luminance time series with amplitude inversely proportional to temporal frequency. Kernels were computed from responses to natural noise stimuli. This assessment reflects how the retina transforms luminance modulations that include relatively slower changes than can be seen with the brief flashes. (**A**–**D**) Averaged kernels from natural noise stimulation for (+)-PTZ-injected *rd10* versus *rd10* nontreated mice at P60, P90, P120, and P180, respectively. The *rd10*+PTZ mice were treated every other day with (+)-PTZ beginning at P14. (**E**) Kernel (RMS) amplitude plotted versus mouse age (P60, P90, P120, P180 days) for *rd10* and *rd10*+PTZ groups. *Significantly different from nontreated animals: $P < 0.05$.

affords robust improvement compared to nontreatment and to several other strategies as well.

In addition to extended visual acuity observed in *rd10*+PTZ mice, we also observed extended electrophysiological function in this cohort of mice compared to nontreated animals. There was discernible PRC function in *rd10*+PTZ mice at P60, which was not observed in *rd10* nontreated mice at this age. There was no rod function observed at P90 or beyond regardless of (+)-PTZ treatment. Regarding cone function, the (+)-PTZ-treated mice had more robust responses at P60 (when measured using the standard photopic ERG), which could be appreciated even more when cone function was interrogated using a pseudorandom luminance noise test that has power at low temporal frequencies. Indeed, there were detectable responses at P60 and P90. This is quite encouraging for treatment of retinal disease. As with the assessment of visual acuity mentioned above, electrophysiological function assessments have frequently been performed during the acute phase of rod loss through ~P42 in *rd10* mice.[25,26] The studies limiting ceramide synthesis using myriocin were quite promising because they preserved cone function for a longer time period.[28] Indeed, those studies and our own are noteworthy because in neither case is the treatment strategy directly targeting the genetic mutation; rather these studies are addressing disruption of a biological function. In the case of the myriocin studies, rod function was likely preserved because the compound inhibited ceramide, which can be toxic to retinal cells.[29]

In the field of Sig1R biology, the mechanism of neuronal protection may involve attenuating oxidative stress, perhaps by modulation of NRF2.[11,12] Other studies have targeted oxidative stress in the *rd10* mouse, especially the Campochiaro laboratory.[30] They administered the antioxidant compound N-acetylcysteine via the drinking water to *rd10* mice and observed improved photopic responses through P50.[30] There are other modalities of neuronal protection in the visual system ascribed to activation of Sig1R. For example, Sig1R is able to differentially modulate the extracellular signal–regulated protein kinase (ERK1/2) in a cell type–specific manner.[31–33] This is relevant to retinal neuronal protection because of the

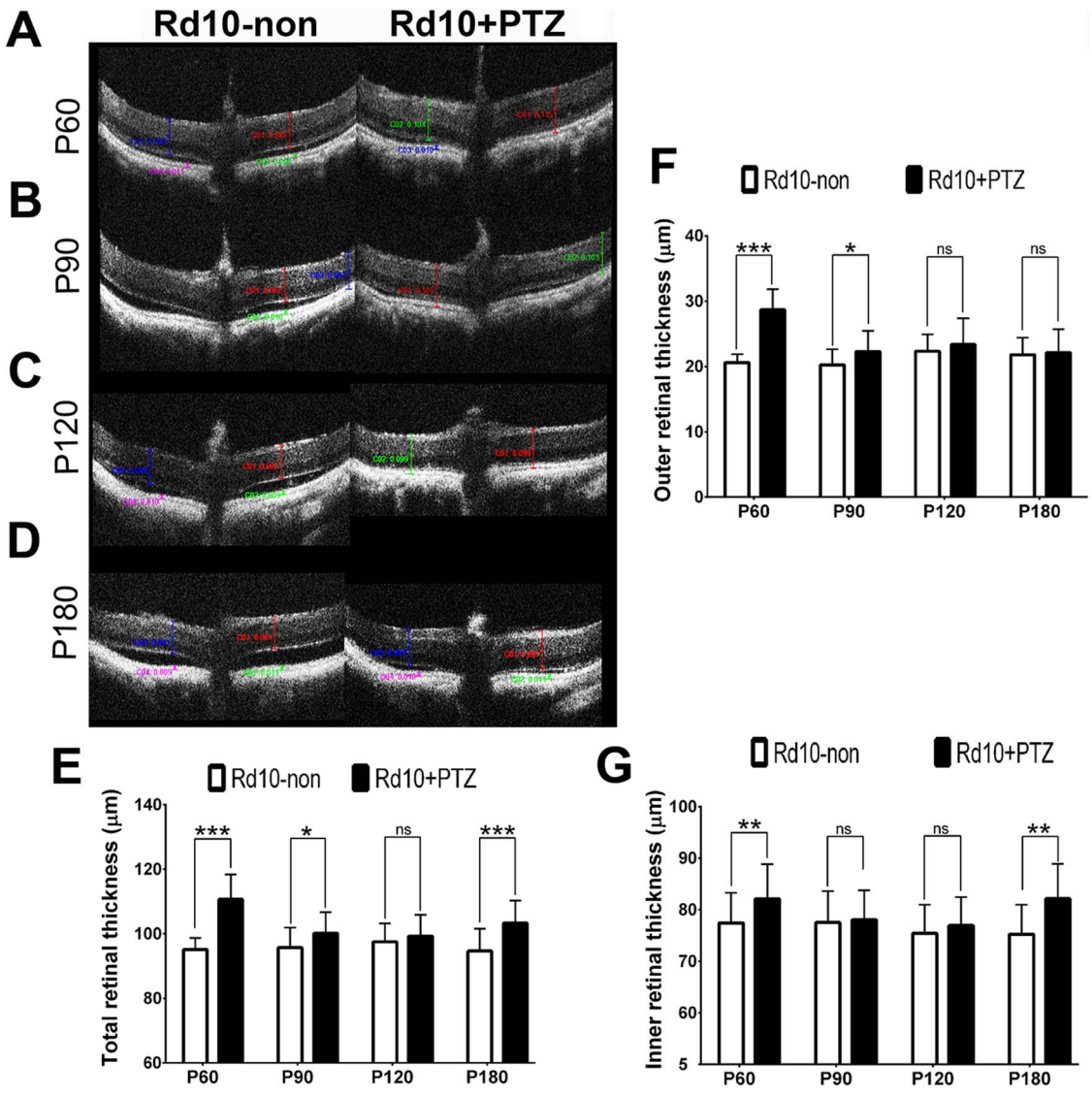


**Figure 6.** SD-OCT assessment of *rd10* mice administered (+)-PTZ versus *rd10* nontreated over 180 days. The *rd10*+PTZ mice were treated every other day with (+)-PTZ beginning at P14 and retinal structure was evaluated in situ and compared to *rd10* that did not receive (+)-PTZ treatment (*rd10* non). Representative SD-OCT data obtained from *rd10* nontreated mice and *rd10*+PTZ mice at (**A**) P60, (**B**) P90, (**C**) P120, (**D**) P180. The data were quantified and presented as (**E**) total retinal thickness, (**F**) outer retinal thickness, and (**G**) inner retinal thickness from *rd10* nontreated and *rd10*(+)-PTZ-injected mice compared at P60, P90, P120, and P180. Significantly different from nontreated animals: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. ns, not significant.

regulatory role played by ERK1/2 in fundamental cellular processes including survival, proliferation, and differentiation. There is evidence also that Sig1R regulates mitochondrial function, including restoring mitochondrial membrane potential and cytochrome c oxidase activity in retinal cells.[34] That study showed that depriving cells of oxygen and glucose deprivation impacted mitochondrial function negatively, but overexpression of Sig1R improved mitochondrial function. There is considerable evidence that activation of Sig1R modulates retinal cell survival and attenuates apoptosis by modulating L-type voltage-gated calcium channels.[35,36] Finally, studies show that activation of Sig1R in models of cone and rod degeneration may mediate neuroprotection by influencing autophagy.[37]

The (+)-PTZ-treated *rd10* mice used in the present study demonstrated improved retinal architecture when examined by OCT over the extended time period. Moreover, the separation of outer retina from RPE, frequently observed in this mutant mouse, was diminished when mice were treated for the extended time period. Additionally, the immunohistochemical studies detected many more cones in the (+)-PTZ-treated animals than in nontreated animals through P90 and P120.

Taken collectively, our data indicate that activation of Sig1R is beneficial in attenuating cone death in this mutant mouse model. It is encouraging for the field of neuroprotection that benefits were observed at time points significantly exceeding the known loss of rods and cones. There are, however, limitations to the study, one of which is the extremely rapid loss of rods and cone cells in r*d10* mice.[17] While not as rapid a degeneration perhaps as in the *rd1* mouse,[38] the phenotype of the *rd10* mouse has been described as a "catastrophic" retinal dystrophy. Thus while it serves the critical purpose of identifying promising targets, it may not be as informative about long-term benefits. This may not be because a particular treatment strategy is inherently flawed, but simply because the

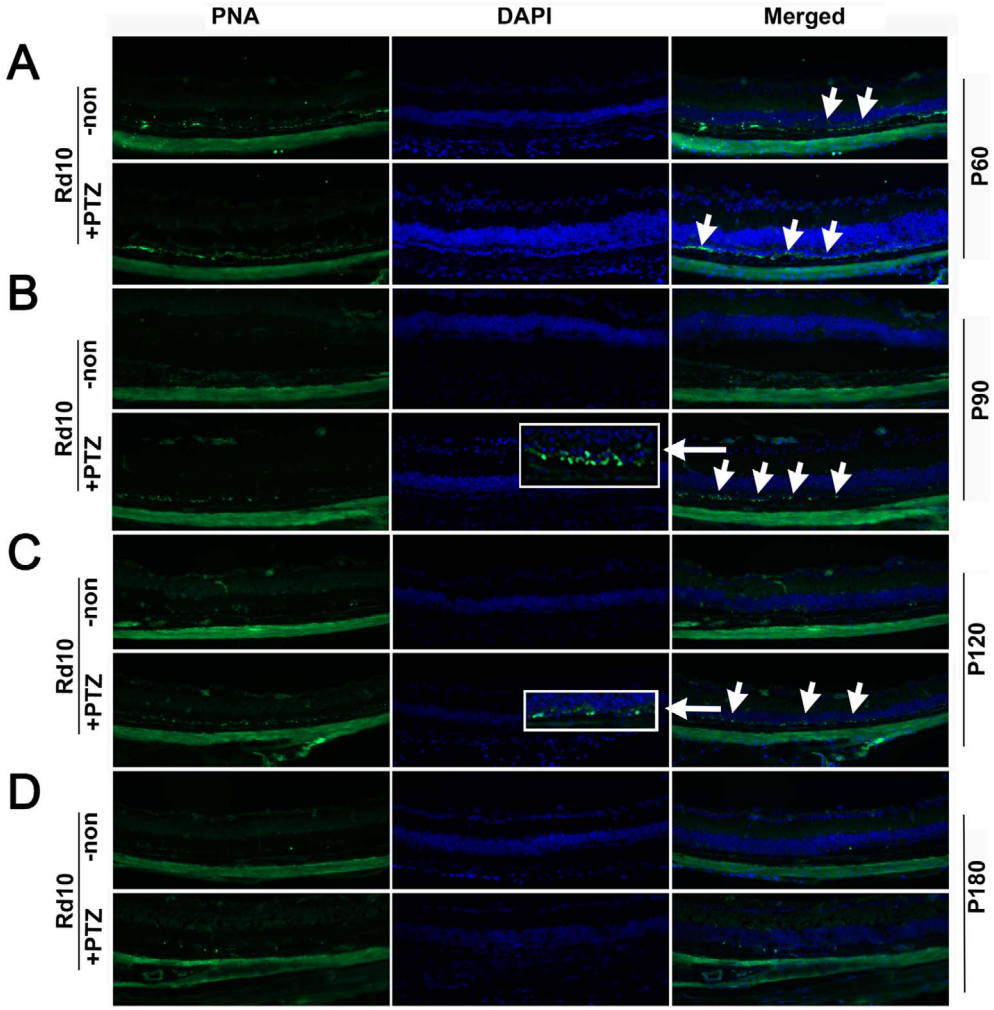


**Figure 7.** Cone photoreceptor cell labeling in retinas of *rd10* mice administered (+)-PTZ versus *rd10* nontreated mice over 180 days. The *rd10* mice were treated every other day with/without (+)-PTZ beginning at P14. Eyes were harvested and processed for cryosectioning and immunodetection of FITC-PNA to detect cone photoreceptor cells. Representative retinal cross sections of PNA labeling in *rd10* nontreated and *rd10*+PTZ mice for (**A**) P60, (**B**) P90, (**C**) P120, and (**D**) P180. *Arrows* point to cells that label positively for PNA (*green* fluorescence). Note that the insets associated with (**B**, **C**) show enlarged images of PNA-positive cells at P90 and P120, respectively, for the *rd10*+PTZ retina. DAPI-stained nuclei are labeled *blue*.

model system has limitations. It is extremely important that promising interventions take advantage of models that are more similar to human disease. A new mouse model exhibiting the P23H mutation of opsin has gained attention because it reflects the most common form of autosomal dominant RP.[39] What is noteworthy about the model is that heterozygous mice (*P23H/+*) have photoreceptor degeneration that spans several months, mimicking more closely the human condition and allowing an extended period of time to assess therapeutic efficacy. Indeed, in this comprehensive study from Sakami et al.,[39] data are provided for the retinal function and architecture in human patients afflicted with RP, allowing comparison to the murine model. Given that we have had such promising findings in attenuating retinal disease when Sig1R is activated in short-term studies[16] as well as in longer-term studies (described here), it will be critically important to validate the benefits of Sig1R activation on retinal structure and function (especially long-term benefits) in a highly clinically relevant model, such as the P23H mouse.

A consideration regarding administration of (+)-PTZ is that its elimination half-life is ~3.6 hours (range, 1.5–10 hours); ~60% of the total dosage is eliminated within 24 hours.[40] In our administration scheme (injecting every other day), we provide a booster at a time when the available compound will have reached a very low level. Whether administration of (+)-PTZ in a sustained-release route would be prove more efficacious than our present dosing regimen is not known, but could be explored in future studies. Future studies are also necessary to establish whether cone rescue observed as a consequence of (+)-PTZ treatment is through a direct effect on PRCs or whether the effect is mediated by actions on supportive cells, such as the Müller glial cell or the RPE.

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